

Identification of novel CDK9 and Cyclin T1-associated protein complexes (CCAPs) whose siRNA depletion enhances HIV-1 Tat function

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Identification of novel CDK9 and Cyclin T1-associated protein complexes (CCAPs) whose siRNA depletion enhances HIV-1 Tat function

Rajesh Ramakrishnan¹, Hongbing Liu¹, Hart Donahue¹, Anna Malovannaya², Jun Qin² and Andrew P Rice^{1*}

Abstract

Background: HIV-1 Tat activates RNA Polymerase II (RNAP II) elongation of the integrated provirus by recruiting a protein kinase known as P-TEFb to TAR RNA at the 5' end of nascent viral transcripts. The catalytic core of P-TEFb contains CDK9 and Cyclin T1 (CCNT1). A human endogenous complexome has recently been described – the set of multi-protein complexes in HeLa cell nuclei. We mined this complexome data set and identified 12 distinct multi-protein complexes that contain both CDK9 and CCNT1. We have termed these complexes CCAPs for CDK9/CCNT1-associated protein complexes. Nine CCAPs are novel, while three were previously identified as Core P-TEFb, the 7SK snRNP, and the Super-Elongation Complex. We have investigated the role of five newly identified CCAPs in Tat function and viral gene expression.

Results: We examined five CCAPs that contain: 1) PPP1R10/TOX3/WDR82; 2) TTF2; 3) TPR; 4) WRNIP1; 5) FBXO11/CUL1/SKP1. siRNA depletions of protein subunits of the five CCAPs enhanced Tat activation of an integrated HIV-1 LTR-Luciferase reporter in TZM-bl cells. Using plasmid transfection assays in HeLa cells, we also found that siRNA depletions of TTF2, FBXO11, PPP1R10, WDR82, and TOX3 enhanced Tat activation of an HIV-1 LTR-luciferase reporter, but the depletions did not enhance expression of an NF- κ B reporter plasmid with the exception of PPP1R10. We found no evidence that depletion of CCAPs perturbed the level of CDK9/CCNT1 in the 7SK snRNP. We also found that the combination of siRNA depletions of both TTF2 and FBXO11 sensitized a latent provirus in Jurkat cells to reactivation by sub-optimal amounts of α CD3/CD28 antibodies.

Conclusions: Our results identified five novel CDK9/CCNT1 complexes that are capable of negative regulation of HIV-1 Tat function and viral gene expression. Because siRNA depletions of CCAPs enhance Tat function, it is possible that these complexes reduce the level of CDK9 and CCNT1 available for Tat, similar to the negative regulation of Tat by the 7SK snRNP. Our results highlight the complexity in the biological functions of CDK9 and CCNT1.

Background

A critical step in the HIV-1 replication cycle is transcription of the integrated provirus by RNA polymerase II (RNAP II). Although RNAP II initiates transcription from the HIV-1 LTR at a relatively high basal rate, the polymerase stalls after synthesis of a short transcript due to the action of two negative elongation factors, NELF and DSIF, which associate with the RNAP II complex and inhibit elongation [reviewed in [1-4]]. Pausing of RNAP II elongation is also seen in cellular genes and serves as a quality

control mechanism to enable efficient capping of the 5' end of the mRNA [5,6]. In the case of HIV-1, the viral Tat protein recruits a cellular protein kinase complex termed P-TEFb to the TAR RNA element that forms at the 5' ends of the nascent viral transcript. The catalytic component of P-TEFb is composed of CDK9 as the catalytic subunit and a Cyclin regulatory subunit, either Cyclin T1 (CCNT1) or Cyclin T2 (CCNT2). P-TEFb activates transcriptional elongation by phosphorylating the carboxyl terminal domain (CTD) of the large subunit of RNAP II; P-TEFb also phosphorylates specific protein subunits of NELF and DSIF, and this negates their inhibition of elongation. For cellular genes, P-TEFb can be recruited to the RNAP II complex by different mechanisms to stimulate elongation.

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The bromodomain protein BRD4 is bound to a portion of P-TEFb and directs P-TEFb to actively transcribed genes that are marked by acetylated histones [7-9]. Transcription factors that bind directly to DNA elements in cellular promoters, such as NF- κ B, cMyc, and MEF2, can also recruit P-TEFb to active genes [10-12].

P-TEFb is known to exist in three distinct complexes: 1) the "Core" complex composed of CDK9/CCNT1 or CCNT2/BRD4; 2) the "7SK snRNP" complex composed of CDK9/CCNT1 or CCNT2/7SK snRNA/ HEXIM1 or HEXIM2/LARP7/ MEPCE; 3) "Super Elongation Complex" (SEC) composed of CDK9/CCNT1 or CCNT2/ ELL2/AFF4/ENL/AF9 [4]. Gel filtration analyses of purified Flag-tagged CDK9 have confirmed that these three complexes exist as distinct biochemical entities [13]. CDK9 kinase activity is repressed in the 7SK snRNP, and this complex is not thought to directly activate transcriptional elongation. Rather, the 7SK snRNP may be a catalytically inactive pool of P-TEFb from which active P-TEFb can be recruited to function in either the Core or SEC P-TEFb complex to activate elongation [14]. Consistent with this idea, disruption of the 7SK snRNP by siRNA depletions of HEXIM1 results in increased levels of Tat activation of the viral LTR [15].

The HIV Tat protein binds directly to CCNT1 and thereby targets only CCNT1-containing P-TEFb complexes [16]. Tat is capable of utilizing the Core P-TEFb complex by displacing Brd4 from CDK9/CCNT1 [17]. Tat can trigger the release of CDK9/CCNT1 from the 7SK snRNP and utilize this P-TEFb complex [18-20]. Tat also associates with the SEC and stabilizes this P-TEFb complex to activate viral gene expression [21,22].

Nearly all cellular processes are carried out by multi-protein complexes [23,24]. A recent study using a high-throughput integrative mass spectrometry-based analysis described the human endogenous complexome – the set of multi-protein complexes in HeLa cells [25]. In this landmark study, 1,796 primary antibodies were used in 3,390 immunoprecipitations of HeLa cell nuclear extracts. Conditions of immunoprecipitations were developed to preserve weak protein interactions and high levels of reciprocity in affinity purifications were used to identify distinct multi-protein complexes. We have mined this data set to identify multi-protein complexes that contain CDK9 and CCNT1. The three known P-TEFb complexes – Core, 7SK snRNP and SEC – as well as nine additional CDK9/CCNT1 complexes were identified in this analysis. In this study, we focused on five of these novel complexes and found that siRNA depletion of protein subunits of these novel complexes enhanced HIV-1 Tat function and viral gene expression. Although the mechanisms whereby siRNA disruption of these novel CDK9/CCNT1 complexes stimulate Tat function remain to be elucidated, our data suggest that their disruption may increase the level of

P-TEFb available for Tat to utilize, similar to disruption of the 7SK snRNP. Our study indicates that the complexity of P-TEFb is considerably greater than previously appreciated.

Results

Identification of novel CDK9/CCNT1-associated proteins (CCAPs)

We mined the recently described human complexome dataset [25] to identify distinct protein complexes that contain both CDK9 and CCNT1. As shown in Figure 1, this analysis identified previously reported and novel CDK9/CCNT1-containing complexes. We refer to these complexes as CCAPs, for CDK9/CCNT1-associated protein complexes. Two CCAPs are mutually exclusive in our data set – HEXIM1/LARP7/MEPCE (complex B) and BRD4 (complex C). The identification of these mutually exclusive CCAPs agrees precisely with traditional experimental approaches that identified these two distinct CDK9/CCNT1 complexes [26]. Additionally, the recently identified Super-elongation complex (SEC) complex (AFF4/AFF1/MLLT1/ELL2/MLLT3; complex D [21,22,27]) was identified in our analysis, further validating this experimental approach to identify CCAPs of likely relevance to cellular and HIV-1 gene expression.

A prominent CCAP not shown in Figure 1 due to size constraints contains CDK9/CCNT1 in a very large multi-protein complex that regulates transcription initiation and chromatin remodeling [CBP/p300; the 15 subunit INO80 complex; 20 subunit SWI/SNF complex; 30 subunit Mediator (Mediator has been implicated in HIV-1 replication in siRNA screens [28]). Two CCAPs involved in transcription elongation are prominent in Figure 1: AFF4/AFF1/MLLT1/ELL2/MLLT3 (Super-Elongation complex D) and WDR82/PPP1R10/TOX3 (complex E) that interacts with the LEO1/PAF complex (histone methyltransferase activity) and is implicated in transcriptional elongation [25]. TTF2 (complex G) is a CCAP involved in RNA Polymerase II termination. Two CCAPs associate with nuclear pores, RANGAP1 (complex H) and TPR (complex I). Other CCAPs included the checkpoint module ATR/ATRIP (complex J), ubiquitin binding protein WRNIP1 (complex K), and SCF^{FBXO11} ubiquitin E3 ligase (complex L). The proteins PRPF8 (complex F) and RANBP2 (complex H) have been implicated in HIV-1 replication in siRNA screens [28]. The identification of novel CCAPs involved in RNA elongation, RNA splicing, transcription termination, and nuclear pores suggest that the role of CDK9/CCNT1 in cellular and HIV-1 gene expression is considerably more intricate than previously appreciated.

SiRNA depletion of CCAPs

To begin to investigate if CCAPs are involved in HIV-1 Tat function, we used siRNAs to deplete protein

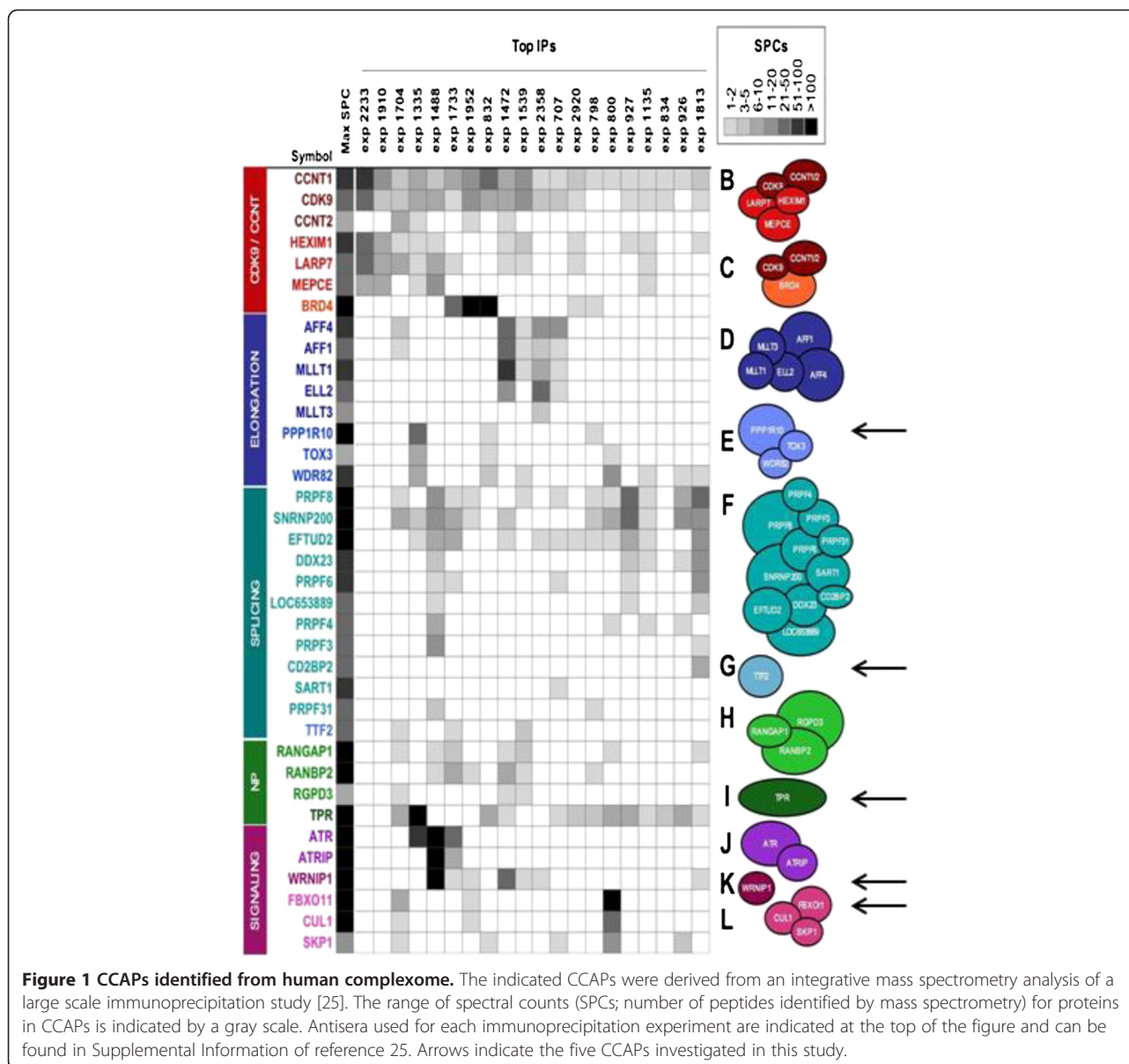


Figure 1 CCAPs identified from human complexome. The indicated CCAPs were derived from an integrative mass spectrometry analysis of a large scale immunoprecipitation study [25]. The range of spectral counts (SPCs; number of peptides identified by mass spectrometry) for proteins in CCAPs is indicated by a gray scale. Antisera used for each immunoprecipitation experiment are indicated at the top of the figure and can be found in Supplemental Information of reference 25. Arrows indicate the five CCAPs investigated in this study.

subunits of various CCAPs. Our preliminary functional assays for the effects of depletions of protein subunits of the five CCAPs indicated by arrows in Figure 1 suggested that these CCAPs negatively regulate Tat function. We therefore focused on these five CCAPs in this study. The properties of protein subunits of these five CCAPs are summarized in Table 1.

We used the TZM-bl cell line for our depletion experiments; this cell line is a HeLa cell derivative that expresses CD4, CXCR4, and CCR5 on its cell surface and therefore can be infected by HIV-1 [40]. TZM-bl cells also contain an integrated copy of the HIV-1 LTR with a Luciferase reporter protein. Tat transactivation assays can be readily performed in TZM-bl cells by transfection of a Tat expression plasmid and

measurement of Luciferase expression from the integrated provirus. As the human complexome data set was generated in HeLa cells [25], the CCAPs shown in Figure 1 are likely similar if not identical between TZM-bl and HeLa cells.

In the experiment shown in Figure 2A and 2B, TZM-bl cultures were transfected with siRNAs directed against either TTF2 (Figure 1, complex G), TPR (complex I), WRNIP1 (complex K), or FBXO11 (subunit of complex L). Cell lysates were prepared 72 hours after siRNA transfections, and the degrees of protein depletion were analysed by immunoblots. SiRNAs against TPR was effective in depleting TPR but had no effect on WRNIP1, TTF2, or FBXO11 (Figure 2A). SiRNAs against TTF2 effectively depleted TTF2 but had no

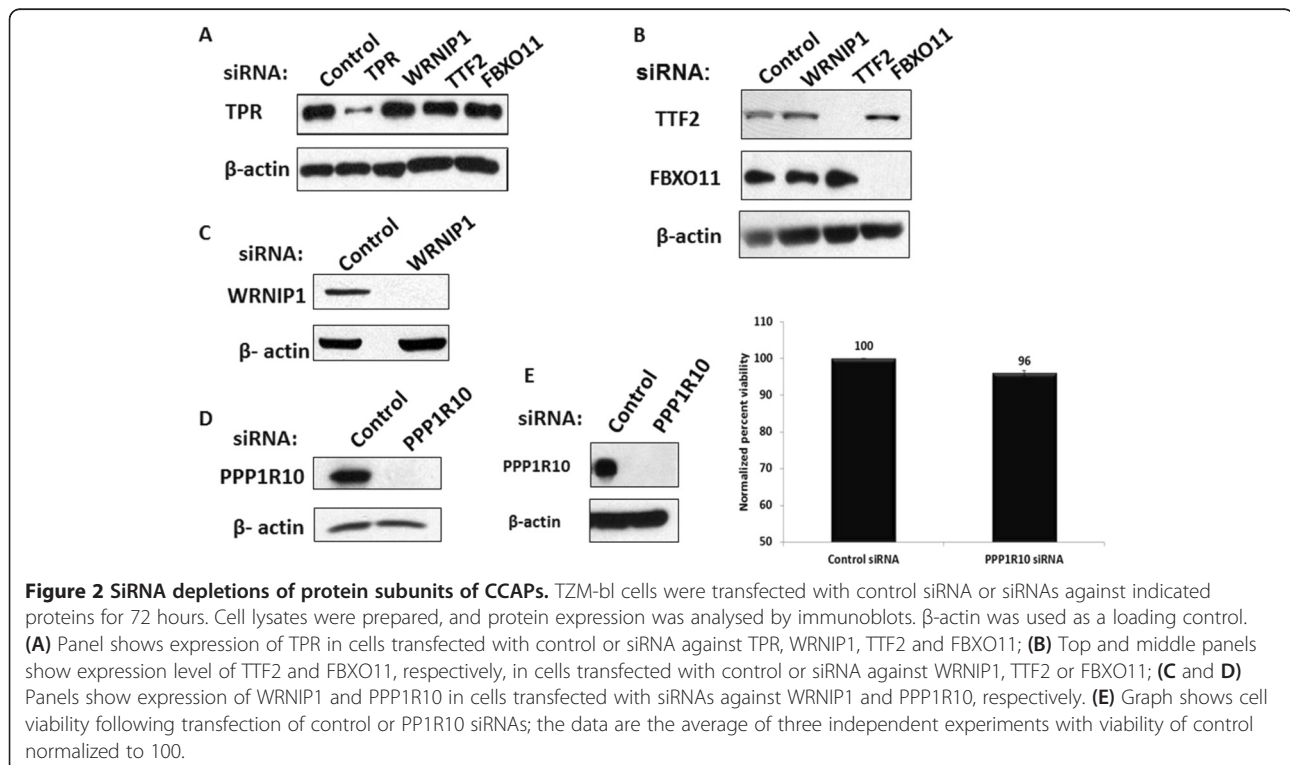
Table 1 Protein subunits of CCAPs depleted in this study

Protein depleted	CCAP	Name	Other names	Function of depleted protein	Reference
FBXO11	FBXO11	F-box protein 11	UBR6;VIT1; FBX11; PRMT9; UG063H01	Phosphorylation-dependent ubiquitination, regulates p53 and BCL6	[29,30]
	CUL1	Cullin 1	-		
	SKP1	S-phase kinase-associated protein 1	OCP2; p19A; EMC19; SKP1A; OCP-II; TCEB1L		
PPP1R10		protein phosphatase 1, regulatory subunit 10	FB19; CAT53; PNUTS; PP1R10	Targets protein phosphatase-1 (PP1) to the nucleus, in DNA damage response	[31]
WDR82	PNUTS	WD repeat domain 82	SWD2; MST107; WDR82A; MSTP107; PRO2730; TMEM113; PRO34047	Component of the mammalian SET1A /SET1B histone H3-Lys4-methyltransferase complexes	[32]
TOX3		TOX high mobility group box family member 3	CAGF9; TNRC9	Unwinding of DNA, chromatin structure alteration and neuronal transcription	[33]
TPR	TPR	Translocated promoter region (to activated MET oncogene)		Directly interacts with several components of nuclear pore complexes (NPCs). Nuclear export of mRNAs and some proteins.	[34]
TTF2	TTF2	Transcription termination factor, RNA polymerase II	HuF2	Critical role in altering protein-DNA interactions. Has dsDNA-dependent ATPase and RNAPII termination activity. Plays a role in pre-mRNA splicing.	[35,36]
WRNIP1	WRNIP1	Werner helicase interacting protein 1	WHIP; bA420G6.2; RP11-420G6.2	Interacts with the N-terminal portion of Werner protein containing the exonuclease domain and implicated in Werner's syndrome. It may influence aging process.	[37-39]

The proteins that were depleted using siRNA in this study are listed. The core complex these proteins exist in cells as presented in Figure 1 is included along with brief description of their cellular function.

effect on WRNIP1 or FBXO11 (Figure 2B); likewise, siRNAs against FBXO11 were effective against FBXO11 but had no effect on WRNIP1 or TTF2 (Figure 2B). SiRNAs

transfected in TZM-bl cells against WRNIP1 (complex K) and PPP1R10 (complex E) were also effective in depleting the target protein (Figure 2C, 2D). These data



indicate that siRNA depletions are effective for the targeted subunits of these CCAPs.

Because PPP1R10 is associated with Protein Phosphatase 1 and regulates the activity of a cellular enzyme that regulates many cellular processes, we monitored the effect of siRNA depletion of PPP1R10 on cell viability using a Trypan Blue exclusion assay. TZM-bl cells were transfected with siRNAs against PPP1R10 or control siRNAs. Cells were collected at 72 hours post-transfection and divided into two groups – one group was lysed, and depletion of PPP1R10 was verified by immunoblot (data not shown); and the other group was assayed for cell viability. As shown in Figure 2E, there was only a small reduction in the viability of the cells depleted of PPP1R10 compared to control siRNA-treated cells. This small reduction in cell viability by siRNA depletion of PPP1R10 is unlikely to explain the enhancement of Tat function by the depletion (see below).

siRNA depletion of CCAPs enhances Tat activation of HIV-1 LTR

To examine the functional effects of depletions of protein subunits of CCAPs, TZM-bl cells were transfected with siRNAs, followed by transfection of expression plasmids for either wild type Tat (wtTat) or the transactivation-defective mutant termed Pro18IS Tat (mTat) [41]. Cell lysates were prepared 24 hours after Tat plasmid transfections and analysed for Luciferase expression from the integrated HIV-1 LTR in TZM-bl cells. Additionally, siRNAs against CCNT1 were transfected as a control in which Tat function should be reduced. When compared with transfection of control siRNAs, the depletion of CCNT1 inhibited activation of the integrated HIV-1 LTR Luciferase reporter by the wtTat protein as expected (Figure 3A, C). Depletion of subunits of each CCAP examined – TPR, WRNIP1, TTF2, FBXO11, PPP1R10, WDR82, TOX3 – increased activation by the wtTat protein that was statistically significant in a Student's T-test (Figure 3A, C, E). Depletion of the individual subunits of the PPP1R10 complex – PPP1R10, WDR82 and TOX3 – also increased activation by wt Tat to a statistically significant level, from approximately 3- to 10-fold (Figure 3A). TTF2 and FBXO11 depletion activated the HIV-1 LTR Luciferase reporter by approximately 3- and 2-fold, respectively (Figure 3C), while depletion of TPR and WRNIP1 resulted in activation by approximately 3- and 2-fold, respectively, relative to control siRNAs (Figure 3E). We obtained similar results as those shown with wt Tat in Figure 3 with siRNA depletions of CCAPs in Jurkat T cells infected with an HIV-1 luciferase reporter virus, although the magnitude of Tat enhancement was less than that seen in TZM-bl cells (data not shown), likely due to inefficient siRNA transfections and depletions in Jurkat cells.

In cells transfected with transactivation-defective mTat plasmid, depletion of TPR, WRNIP1, TTF2, FBXO11, and WDR82 also resulted in an induction HIV-1 LTR-driven Luciferase expression, although the level of Luciferase expression was considerably less than cells transfected with wtTat (Figure 3B, D, F). These data suggest that disruption of the five newly identified CCAPs by siRNA depletions enhances Tat activation of the HIV-1 LTR, perhaps by increasing the pool of CDK9/CCNT1 that is available for Tat function.

Based on the results shown in Figure 3, it is conceivable that the five CCAPs examined could function additively to regulate Tat function. Therefore, we evaluated the effects of depletions of combinations of CCAPs on Tat transactivation in TZM-bl cells (Additional file 1: Figure S1). This analysis demonstrated that some combinations showed a strong additive effect on increasing Tat transactivation, especially the combination of TTF2 and FBXO11.

Activation of NF- κ B is not responsible for the increase of HIV-1 gene expression upon depletion of CCAPs

It is possible that the enhancement of viral gene expression by siRNA depletions of CCAPs may involve activation of NF- κ B arising from a stress response to the depletions. To investigate this possibility, we carried out plasmid transfection assays with HIV-1 LTR and NF- κ B Luciferase reporter plasmids in HeLa cells. Cultures were transfected with control or specific siRNAs, and 48 hours later cells were co-transfected a HIV-LTR Luciferase reporter plasmid plus a wtTat plasmid Tat or a NF- κ B Luciferase reporter plasmid plus the CMV parental vector. Cell lysates were prepared 24 hours post-plasmid transfection, and Luciferase expression was measured (Figure 4). As expected, depletion of CCNT1 reduced expression of the HIV-1 LTR, while depletion of CCAPs (TTF2, FBXO11, PPP1R10, and WDR82,) enhanced viral LTR expression. Depletion of CCNT1 reduced expression from the NF- κ B reporter, while depletion of all CCAPs other than PPP1R10 also reduced expression of the NF- κ B reporter. These data indicate that depletion of CCAPs does not increase HIV LTR-directed gene expression through activation of NF- κ B with the possible exception of PPP1R10.

Enhancement of Tat activation by CCAP depletions is not the result of increased Tat protein expression or increased release of CDK9/CCNT1 from 7SK snRNP

It is possible that siRNA depletions of CCAPs results in increased Tat protein levels from the Tat expression plasmid, and this might contribute to increased Tat function in TZM-bl cells. To investigate this possibility, we transfected HeLa cells with control siRNA or siRNA against CCNT1, HEXIM1, or PPP1R10, followed by transfection of the Flag-tagged wild type HIV-1 Tat expression

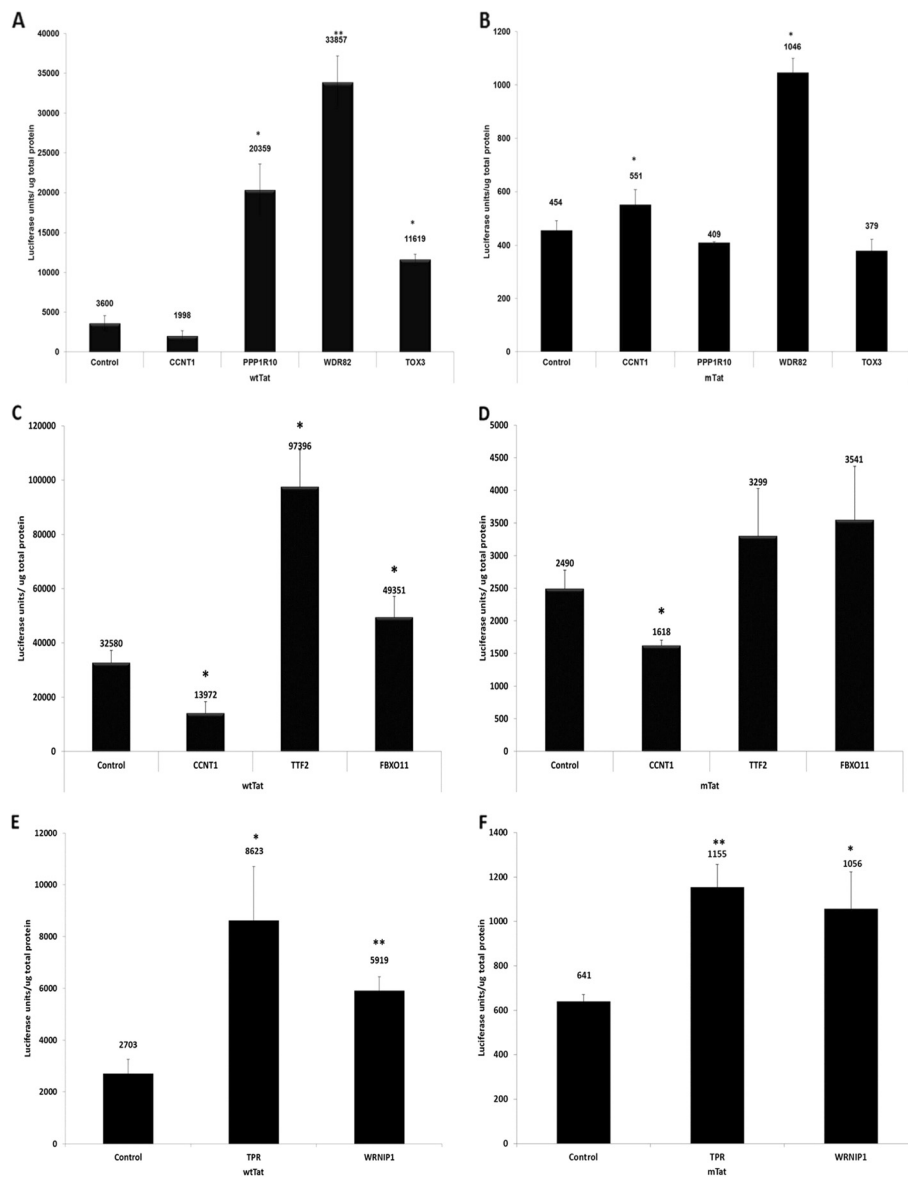


Figure 3 Effects of siRNA depletions of CCAPs on Tat transactivation. T2M-bl cells were transfected in triplicate with control siRNA or indicated siRNAs. 48 hours later, cells were transfected with expression plasmid for wild-type Tat (wtTat) (A, C and E) or transactivation-defective mutant Pro181S Tat (mTat) (B, D and F). Luciferase expression from the integrated HIV-1 LTR was measured 24 hours post-plasmid transfection and normalized to total cellular protein. A representative experiment is shown from three independent experiments. Statistical significance was estimated using Student's t-test. (* $p \leq 0.05$ and ** $p < 0.005$). Note the difference in scales between panels A, C, E and B, D, F.

plasmid. PPP1R10 was used in this control experiment as its depletion resulted in relatively high levels of enhancement of Tat activation (see Figure 3A and Figure 4). Cell lysates were prepared 24 hours post-plasmid transfection, and protein levels were analysed by immunoblot (Figure 5A). SiRNAs were effective in depletion of CCNT1 and PPP1R10, while depletion of HEXIM1 was somewhat less efficient. We did not observe a significant difference in the expression of Flag-Tat upon between the PPP1R10 depletion and control siRNA-treated cells (Figure 5A), although Tat activity was enhanced under these conditions

of PPP1R10 depletion (Figures 3A, 4). This result suggests that depletion of CCAPs enhances Tat function through mechanisms other than increasing Tat levels from the plasmid vector.

We also investigated whether depletion of CCAPS might affect the amount of CDK9/CCNT1 in the 7SK snRNP. Depletion of TTF2 and FBXO11 was used for this control experiment because this combination of CCAP depletions showed the greatest level of Tat enhancement observed in this study (see Additional file 1: Figure S1). HeLa cells were first transfected with a combination of

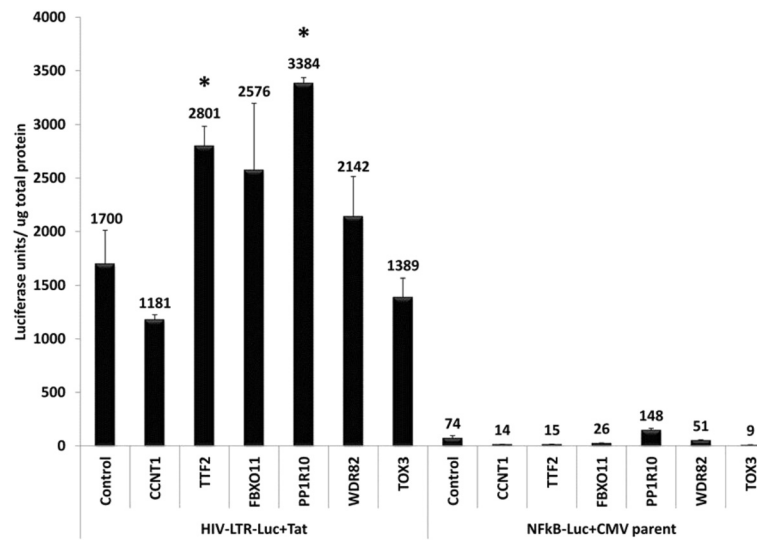


Figure 4 Enhancement of Tat activation by CCAP depletions is not due to activation of NF- κ B. HeLa cells were transfected for 48 hours with control siRNA or the indicated siRNAs. Cells were then co-transfected with 500 ng HIV-1 LTR-Luciferase reporter plasmid plus 50 ng wtTat plasmid or 500 ng NF- κ B-Luciferase reporter plasmid plus 50 ng pCMV parent vector. Cell extracts were prepared 24 hours post-plasmid transfection, and Luciferase expression was normalized to total cellular protein in cell extracts. Statistical significance was determined using a t-test. A representative experiment from three independent experiments is shown (* represents p value ≤ 0.05).

TTF2 and FBXO11 siRNAs or control siRNAs, and 48 hours later cells were transfected with expression plasmids for Flag-tagged wild type Tat or mutant Tat and HA-CCNT1; 48 hours later cell extracts were prepared for immunoprecipitations with an antiserum against CDK9. As shown in Figure 5B, no difference in the association of CDK9 with CCNT1 or HEXIM1 was observed in cells transfected with Flag-tagged wild type and mutant Tat

and treated with either control or TTF2+ FBXO11 siRNAs. This result suggests that depletion of CCAPs does not affect the level of CDK9/CCNT1 in the 7SK snRNP.

Combination of TTF2 and FBXO11 depletion sensitizes Jurkat CD4⁺ T cells to reactivation of latent HIV-1

The results presented above indicate that depletion of CCAPs increases Tat function and HIV-1 gene expression.

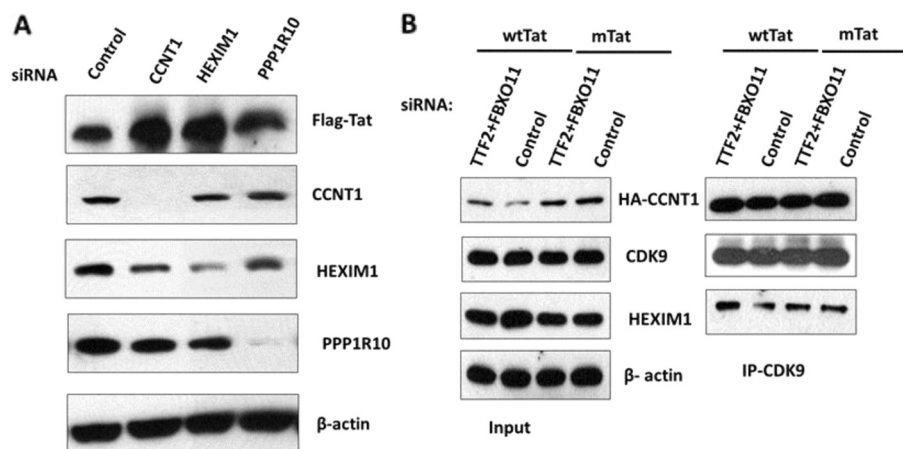


Figure 5 Enhancement of Tat activation by CCAP depletions is not due to increased Tat expression or differential association of CDK9/CCNT1 with 7SK/HEXIM1. (A) HeLa cells were transfected with indicated siRNAs for 48 hours followed by transfection of Flag-tagged wild type HIV-1 Tat expression plasmid. At 24 hours post-plasmid transfection, cell lysates were prepared and immunoblot analysis carried out for expression levels of Flag-Tat, CCNT1, HEXIM1 and PPP1R10; β -actin was probed as loading control. (B) HeLa cells were transfected with control or TTF2+ FBXO11 siRNAs and 48 hours later transfected with expression plasmids for HA-CCNT1 or Flag-wild type Tat (wtTat) or mutant Tat (mTat) for a further 48 hours. Cell lysates were prepared and immunoprecipitated with CDK9. Left part of the panel shows input levels, and right part shows the immunoprecipitated proteins.

We wished to determine if depletions of CCAPs would have an effect on reactivation of a latent HIV-1 provirus. We used the Jurkat 2D10 cell line for this experiment; 2D10 cells contain a latent provirus virus with a destabilized eGFP in place of Nef [42]. When treated with α CD3/CD28 beads, activation of the latent provirus can be examined by flow cytometry. We evaluated depletion of TTF2 + FBXO11 in this experiment, as this combination of depletion has the strongest enhancement of Tat

activation in T2M-bl cells (Additional file 1: Figure S1). 2D10 cells were transfected with control siRNA or siRNAs against TTF2 and FBXO11. Thirty six hours post-siRNA transfection, cells were treated with sub-optimal amounts of α CD3/CD28 beads, and GFP expression was monitored by flow cytometry 16 and 24 hours later. Depletion of both TTF2 and FBXO11 resulted in an increase in the number of GFP+ cells relative to the control siRNA (Figure 6). At 16 hours post-activation, the TTF2/FBXO11

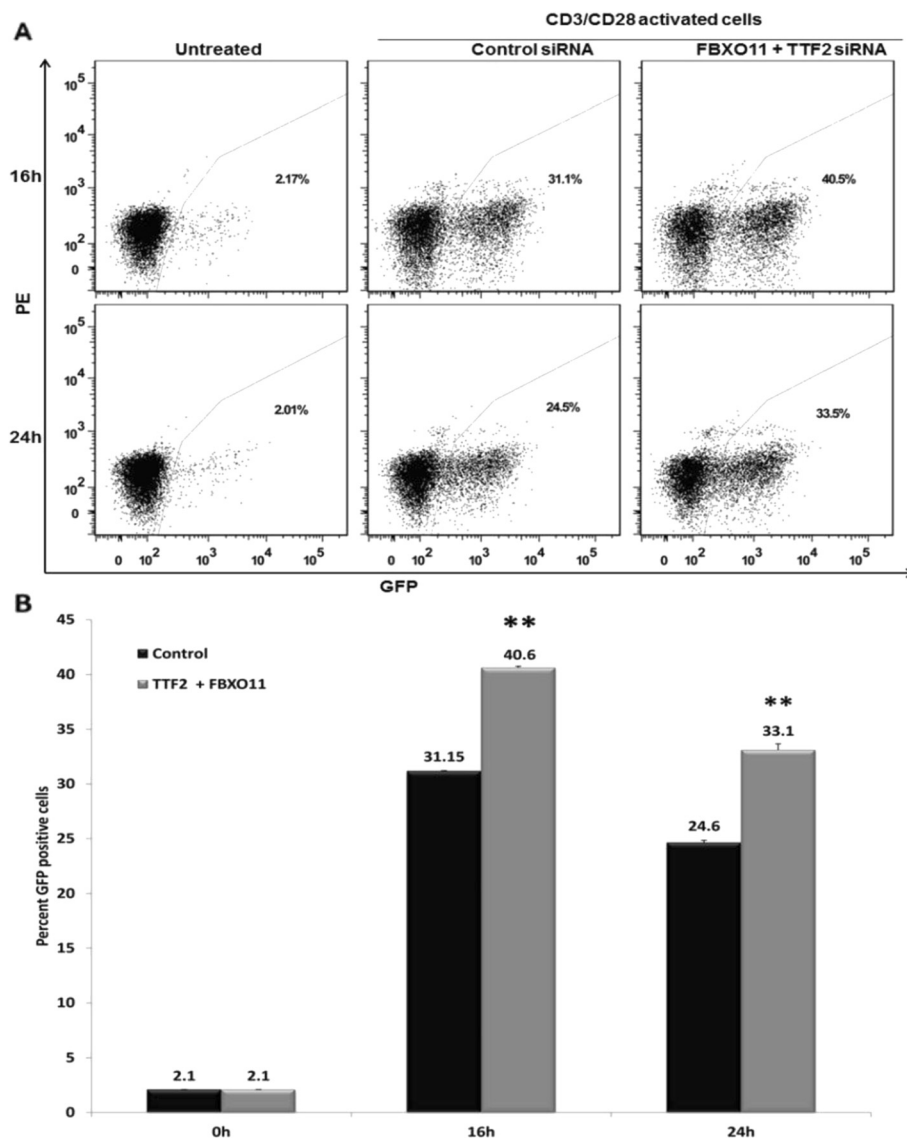


Figure 6 Depletions of TTF2 and FBXO11 sensitize latent provirus in Jurkat 2D10 cell line to re-activation. Duplicate Jurkat 2D10 cultures were transfected with control siRNA or siRNA against TTF2 and FBXO11 for 36 hours. Cells were then activated with suboptimal amounts (2 μ l) of CD3/CD28 beads and GFP expression from re-activated provirus was analysed by flow cytometry. A representative experiment from two independent experiments is shown. **(A)** The panel shows GFP expression in untreated cells (background GFP expression) and in activated cells treated with either control siRNA or TTF2 and FBXO11 siRNA at the indicated time points. The x-axis is GFP, and the y-axis is PE that is used as a placeholder fluorophore to enable visualization as a dot plot. A representative flow cytometric analysis of GFP expression from the duplicate samples is shown. **(B)** The graph represents average GFP expression in cells that were transfected with indicated siRNA. The untreated cells represent background GFP expression. Statistical significance was computed using the t-test (* represent $p \leq 0.05$ and ** represents $p < 0.005$).

depletion demonstrated 34% GFP+ cells, and the control depletions demonstrated 27%. At 24 hours post-activation, 27% of cells depleted for both TTF2 and FBXO11 remained GFP+, while only 21% of cells transfected with control siRNAs were GFP+. These differences at 16 and 24 hour post-activation between the TTF2/FBXO11 and control siRNA depletions were statistically significant as determined by a t-test (p values < 0.005 and 0.05, respectively). This result is consistent with the data presented in Figures 3, 4 and Additional file 1: Figure S1 that indicate that depletion of the five CCAPs evaluated in this study enhances HIV-1 gene expression. Similar to depletion of HEXIM1 in the 7SK snRNP, it is possible that depletion of these CCAPs enhances viral gene expression by increasing the levels of CDK9/CCNT1 available for Tat function.

Discussion

The production of cellular mRNA and HIV-1 RNA from the integrated provirus is a highly regulated process in which the individual steps are linked. RNA splicing is coupled to transcriptional elongation [43], and nuclear export of processed RNA can be affected by splicing [44]. Most, if not all proteins responsible for production of RNA exist in multi-protein complexes, and the understanding of mechanisms involved in HIV-1 RNA production will require the identification of protein complexes involved in the individual steps of viral RNA production. In this study, we mined the recently described HeLa cell nuclear complexome [25] to investigate multi-protein complexes involved in HIV-1 Tat function and viral gene expression. Our analysis of the complexome data set identified 12 multi-protein complexes which contain CDK9/CCNT1, the enzymatic core of the general RNAP II elongation factor known as P-TEFb that mediates Tat transactivation. Our analysis identified the three previously known CDK9/CCNT1 complexes –Core, 7SK snRNP, and SEC P-TEFb complexes [4], thereby validating our analytical approach. We identified nine additional multi-protein complexes that contain CDK9/CCNT1; eight of these novel CDK9/CCNT1 complexes are shown in Figure 1, while the largest complex identified (not shown in Figure 1) contains CBP/p300, the 15 subunit INO80 complex, 20 subunit SWI/SNF complex, and 30 subunit Mediator complex. We have termed these multi-protein complexes CCAPs for CDK9/CCNT1-associated protein complexes.

To begin to investigate the role of novel CCAPs in Tat function, we carried out siRNA depletions of protein subunits of five CCAPs. We chose to focus on these five CCAPs in the present study as our initial results indicated that depletions of their protein subunits enhanced Tat function. We found that disruption of these CCAPs by siRNA depletions resulted in enhanced Tat function in TZM-bl cells. Because previous work has shown that

depletion of HEXIM1 in the 7SK snRNP enhances Tat function by increasing the available pool of CDK9/CCNT1, we favour the hypothesis that depletion of these five CCAPs enhances Tat function by also increasing the available pool of CDK9/CCNT1. However, future studies will be required to determine the molecular mechanisms whereby disruption of these five CCAPs enhances Tat function. Our initial results with a CCAP containing ATR/ATRIP (Complex J, Figure 1) indicated that depletion of these proteins inhibits Tat function, consistent with a previous study [45]. Additionally, it is likely that depletion of subunits of the largest CCAP (CBP/p300, the 15 subunit INO80 complex, 20 subunit SWI/SNF complex, and 30 subunit Mediator complex) will inhibit Tat, as some of these protein subunits have been shown to play a positive role in Tat function and HIV-1 replication [28,46,47].

CCAPs examined in this study

The protein subunits of the CCAPs examined here have been previously shown to be involved in a number of cellular processes of interest to cellular and HIV-1 RNA metabolism. The finding that these proteins associate with CDK9/CCNT1 has a number of intriguing functional and mechanistic implications. Short descriptions of the known functions of the protein subunits of these CCAPs are presented below.

PPP1R10/TOX3/WDR82

Complex E (Figure 1) recruits the phosphatase PP1 into the nucleus and is involved in the DNA damage response [31,48]. Additionally, WDR82 is found in the SET1A histone H3-Lys4-methyltransferase complex [32] and this chromatin modification is associated with actively expressed genes.

TTF2

This CCAP subunit is involved in the termination of RNAP II transcription [35]. TTF2 has also been implicated in mitotic repression of transcriptional elongation [36]. A recent publication reported that TTF2 is associated with decapping factors and the Xrn2 exonuclease at transcriptional start sites and is involved in premature termination of RNAP II transcription [49].

TPR

This CCAP subunit is a large coiled-coiled protein and is localized within the nuclear basket of the nuclear pore complex; TPR plays a role in nuclear export of mRNA [34,50]. Fusion of the 5' end of the TPR gene with several different kinase genes is associated with cancer.

WRNIP1

This CCAP subunit interacts with the Werner protein and is implicated in Werner's syndrome [37]. The Werner protein is involved in RNAP II transcription and its dysfunction in Werner's syndrome results in an accelerated aging phenotype [38].

FBXO11/CUL1/SKP1

This CCAP is involved in phosphorylation-dependent ubiquitylation of target proteins and has been shown to regulate BCL6 and p53 [29,30].

Complexity of P-TEFb and CCAPs

The identification of 12 CCAPs in this study and the heterogeneity in the catalytic core of P-TEFb reveal an enormous potential complexity in the biological functions of CDK9 and CCNT1. The catalytic core is composed of a heterodimer of CDK9 and a Cyclin subunit, either CCNT1 or CCNT2 [3]. Additionally, two spliced variants of CCNT2 are expressed, CCNT2a and CCNT2b [51], as are two isoforms of CDK9, a major 42 kDa isoform and a minor 55 kDa isoform that arises from an upstream transcriptional start site [52]. It is possible that these two CDK9 isoforms may differentially associate with the CCAPs shown in Figure 1, and it is possible that one or both of the CCNT2 isoforms may replace CCNT1 in these multi-protein complexes. Complexes that differ by their CDK9 or Cyclin subunit may have distinct functional properties, as well as distinct expression patterns in different tissues. Transcriptional profiling of cells depleted for either CCNT1 or CCNT2 has identified mRNAs whose expression patterns appear dependent on either CCNT1 or CCNT2 [53,54]. The expression pattern of the 42 kDa and 55 kDa CDK9 proteins varies across different murine tissues, with liver having higher expression of the 55 kDa protein than the 42 kDa protein [55]. The 55 kDa, but not 42 kDa, CDK9 protein has also been shown to accumulate in the nucleolus of HeLa cells [56].

Regulation of in CCAPs in CD4⁺ T lymphocytes and monocytes/macrophages

The expression patterns of the protein subunits of CCAPs in CD4⁺ T cells and monocytes/macrophages have important implications for HIV-1 replication, as these are the two major cell types infected by HIV-1 *in vivo*. Resting CD4⁺ T cells do not support HIV-1 replication, in part because P-TEFb function is limiting [57,58]. In resting CD4⁺ T cells, CCNT1 levels are low, and this involves repression of translation of CCNT1 mRNA by miRNAs [59]. Upon T cell activation, this repression is released and there is a strong induction of CCNT1 protein levels [60-62]. In resting CD4⁺ T cells, the catalytic function of CDK9 is further repressed by dephosphorylation of T186 in the T-loop of the kinase; upon T cell activation there is a rapid induction

of T-loop phosphorylation and CDK9 kinase activity [63,64]. Also in resting CD4⁺ T cells, the level of the 7SK snRNP complex is low, and upon T cell activation there is a large increase the level of the 7SK snRNP [65,66]. In preliminary experiments, we found that the protein subunits of the five CCAPs investigated in this study are expressed at low level in resting CD4⁺ T-cells and are induced upon cellular activation, suggesting that the levels of these CCAPs are up-regulated following T cell activation. Thus, it appears that resting CD4⁺ T cells require only very low levels of CCAPs to support basal metabolism in quiescent T cells.

Similarly, CDK9 and CCNT1 are highly regulated in monocytes and macrophages. In monocytes, miR-198 represses expression of CCNT1 [67] and although the 42 kDa CDK9 protein is generally expressed at a high level, the CDK9 T-loop is not phosphorylated [68]. Upon macrophage differentiation, CCNT1 levels are strongly up-regulated and the CDK9 T-loop is phosphorylated [68-70]. The 55 kDa CDK9 protein is also up-regulated during macrophage differentiation [56]. Similar to resting and activated CD4⁺ T cells, the 7SK snRNP complex is expressed at only low levels in quiescent monocytes and it is induced following macrophage differentiation [71]. Upon extended time in culture, CCNT1 expression is shut-off by proteasome-mediated proteolysis [69]. However, CCNT1 but not CCNT2 expression can be up-regulated in late differentiated macrophage by either HIV-1 infection or activation with pathogen-associated molecular patterns (PAMPs). With the exception of Core P-TEFb and the 7SK snRNP, the expression patterns of CCAPs shown in Figure 1 have not been examined in monocytes and macrophages.

Future studies

Four of the novel CCAPs identified by our analysis remain to be investigated for potential roles in Tat function (Figure 1, complexes F, H, J, and the CBP/p300, the 15 subunit INO80 complex, 20 subunit SWI/SNF complex, and 30 subunit Mediator complex). Our preliminary results with the ATR/ATRIP CCAP suggest that it may act to positively regulate Tat function and viral gene expression, in agreement with a previous study [45]. ATR is a protein kinase involved in the DNA damage response, and it is activated by the HIV-1 Vpr protein [72]. The finding that ATR/ATRIP is found in a complex with CDK9/CCNT1 raises the intriguing possibility of a mechanistic link between Tat and Vpr functions. Complex H contains RNAGAP1/RANBP2/RFPD3 and is involved in nuclear pore function, suggesting that Tat activation of RNAP II elongation may be coupled to RNA export and perhaps HIV-1 Rev function via this complex. In summary, our identification of 12 multi-protein complexes which contain CDK9/CCNT1 reveals considerable

biological complexity in this cellular kinase and will serve as the basis for future research on molecular mechanisms involved in HIV-1 gene expression and replication.

Conclusions

Our results identified a very large potential complexity in the biological functions of CDK9 and CCNT1. Our results suggest that at least five newly identified multi-protein complexes contain both CDK9 and CCNT1 and may reduce the amount of these proteins available for Tat function.

Methods

Cell culture and activation

TZM-bl, HeLa, and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Jurkat CD4⁺ T cells and Jurkat 2D10 cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. 2D10 cells were activated using Dynabeads CD3/CD28 T cell expander beads (Invitrogen) according to the manufacturer's protocol.

SiRNA and plasmid transfections

Control and siRNAs against individual subunits of CCAPs were purchased from Santa Cruz Biotechnology, CA as pools of three target specific siRNAs. The pool of three siRNAs was specific for distinct target sites in the transcript of the protein to be depleted. This approach has an advantage over using individual siRNAs since depletion of target protein could be achieved with lower amount of siRNA reducing the risk of off-target effects. SiRNAs (10–30 pmol) was delivered into TZM-bl, HeLa, Jurkat or 2D10 cells by either a reverse or traditional transfection method. For reverse transfections, TZM-bl or HeLa cells were seeded in 10 cm plates at 24 hours before experiment. On the day of transfection, the cells were collected by trypsinization and suspended in DMEM with 10% FBS and no antibiotics (50,000 cells/ well/sample) in 24 well tissue culture plates. Transfection was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. SiRNAs and Lipofectamine RNAiMAX were diluted in Opti-MEM1 and mixed together. The siRNA-transfection reagent mixture was incubated at room temperature (RT) for 20 minutes and added to the cells. For traditional transfections, the protocol used was as described above except that TZM-bl or HeLa cells were seeded in 24 well tissue culture plates (50,000 cells/well/sample) at 18 hours before experiments. Jurkat cells and 2D10 cells were seeded the same day of experiment and transfected with siRNAs as described above.

In experiments with TZM-bl cells in 24 well tissue culture plates, cells were transfected for 48 hours with 50

ng of pFLAG- wild type HIV-1 Tat (wtTat) or pFLAG-pro18IS-HIV-1 Tat (mTat) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In other plasmid transfection experiments carried out in HeLa cells, 500 ng HIV-1 LTR reporter plasmid and 50 ng Tat expression plasmid were transfected as described for TZM-bl cells. In these experiments, cells were also transfected with 500 ng pNF-kB-Luc and 50 ng pcDNA empty vector (pCMV parent) ensuring equal amounts of DNA were transfected.

Immunoblot and immunoprecipitation analysis

Cells were lysed with EBCD buffer (50mM Tris-HCl, pH 8.0, 120mM NaCl, 0.5% NP-40, 5mM dithiothreitol) containing protease inhibitor cocktail (Sigma). Immunoblotting was performed as described previously [4]. TTF2, FBXO11, WRNIP1, TPR, PPP1R10, Hsp70 and β -actin were probed using antibodies against TTF2 (Abcam, 1:1000), FBXO11 (Bethyl Labs, 1:2000), WRNIP1 (Santa Cruz, 1:1000), TPR (Santa Cruz, 1:1000), PPP1R10 (Abcam, 1:1000), and β -actin (Sigma) (1:5000), respectively. Immunoblots were quantified using Image J software [73]. Immunoprecipitation was carried out as described previously [65,74].

Luciferase reporter assays

Cells were washed with sterile PBS and cell lysates prepared with Cell Culture Lysis Buffer (Promega). Cell lysates were analyzed for Luciferase activity using Luciferase Assay Kit (Promega) according to manufacturers' protocol. Luciferase assay products were measured using a luminometer (Turner). Total protein in the lysates was estimated using a Bradford assay (Bio-Rad) and used to normalize the Luciferase readings.

Flow cytometry analysis

2D10 cells were examined for GFP expression on a BD Fortessa flow cytometer. 10,000 events were collected and the data analysed using BD FACS Diva software. For visualization of the data as a dot plot, PE was used as a placeholder fluorophore.

Additional file

Additional file 1: Figure S1. Depletion of combinations of CCAPs has an additive effect on HIV-1 Tat function in TZM-bl cells. TZM-bl cells were transfected with control siRNA or combination of the indicated siRNAs for 48 hours. Cells were then transfected with HIV-1 Tat (wtTat) and 24 hours later cell lysates were prepared and examined for Luciferase expression. Luciferase expression values were normalized to amount of total cellular protein. A representative experiment from three independent experiments is shown. Statistical significance was estimated using t-test. (* $p \leq 0.05$ and ** $p < 0.005$).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RR, HL, HD carried out experiments and analysed the data; APR, AM and JQ analysed data; RR and APR wrote the manuscript. All authors read and approved the final manuscript.

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