Title

A Minor Subset of Super Elongation Complexes Plays a Predominant Role in Reversing HIV-1 Latency

Journal

Molecular and Cellular Biology, 36(7)

ISSN

0270-7306

Authors

Li, Zichong
Lu, Huasong
Zhou, Qiang

Publication Date

2016-04-01

DOI

10.1128/mcb.00994-15

Peer reviewed

AMERICAN SOCIETY FOR MICROBIOLOGY | Molecular and Cellular Biology

A Minor Subset of Super Elongation Complexes Plays a Predominant Role in Reversing HIV-1 Latency

Zichong Li,[a] Huasong Lu,[a,b] Qiang Zhou[a]

Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, California, USA[a]; Innovation Center of Cell Signaling Network, School of Pharmaceutical Sciences, Xiamen University, Xiamen, Fujian, China[b]

Promoter-proximal pausing by RNA polymerase II (Pol II) is a key rate-limiting step in HIV-1 transcription and latency reversal. The viral Tat protein recruits human super elongation complexes (SECs) to paused Pol II to overcome this restriction. Despite the recent progress in understanding the functions of different subsets of SECs in controlling cellular and Tat-activated HIV transcription, little is known about the SEC subtypes that help reverse viral latency in CD4$^+$ T cells. Here, we used the CRISPR-Cas9 genome-editing tool to knock out the gene encoding the SEC subunit ELL2, AFF1, or AFF4 in Jurkat/2D10 cells, a well-characterized HIV-1 latency model. Depletion of these proteins drastically reduced spontaneous and drug-induced latency reversal by suppressing HIV-1 transcriptional elongation. Surprisingly, a low-abundance subset of SECs containing ELL2 and AFF1 was found to play a predominant role in cooperating with Tat to reverse latency. By increasing the cellular level/activity of these Tat-friendly SECs, we could potently activate latent HIV-1 without using any drugs. These results implicate the ELL2/AFF1-SECs as an important target in the future design of a combinatorial therapeutic approach to purge latent HIV-1.

HIV-1 latency, which is characterized by transcriptional silence of the integrated proviruses, is the principal impediment to eradication of viral infection. Although antiretroviral therapy (ART) has been used successfully to drive HIV-1 into this silent state, thereby decreasing the plasma viremia to undetectable levels, the proviruses can quickly resume transcription and active replication once ART is interrupted (1). To obtain a real cure for HIV/AIDS, one strategy nicknamed "shock and kill" has been proposed to eliminate the latent viral reservoirs by first activating the proviruses in infected cells. This is followed by the next phase, where spread of the activated viruses can be suppressed by ART and the virus-producing cells are eliminated simultaneously (2).

A number of cytokines and small-molecule drugs that include histone deacetylase inhibitors (HDACi), protein kinase C (PKC) agonists, BET bromodomain inhibitors, and others have been tested for their latency-reversing potentials (3, 4). However, virtually all of them have been found to display low efficacy and/or unacceptable side effects, which have limited their clinical use (3). Thus, better and more-specific means to activate the latent proviruses are urgently needed, which can be achieved only through in-depth characterization of the molecular mechanism and factors that control viral latency.

Without stimulation, RNA polymerase II (Pol II), which transcribes the integrated proviral DNA, has a strong tendency to pause and then terminate near the transcription start site, resulting in the production of only short transcripts (5). This abortive transcription presents a major hurdle to efficient escape of HIV-1 from latency (2). To overcome this hurdle, a multicomponent complex containing the virally encoded Tat protein and its specific host cofactors must form on the nascent 5′ end of the HIV-1 transcript, which folds into a stem-loop structure called the TAR (transactivation response) RNA. This Tat/TAR-containing complex converts the paused Pol II into a highly processive form capable of generating the full-length HIV-1 transcripts (5). In 2010, a set of human transcription factor complexes, called the super elongation complexes (SECs), was identified as the specific Tat cofactor (6, 7). A typical SEC contains CDK9 and cyclin T (CycT; either CycT1 or T2), collectively referred to as P-TEFb, as well as one of each of the three pairs of homologous proteins: ELL1/ELL2, AFF1/AFF4, and ENL/AF9 (7–9). Owing to the ability of these proteins to create multiple different combinations among them, a fairly large family of related SEC complexes exists *in vivo* (10, 11).

The P-TEFb component of a SEC stimulates transcriptional elongation through phosphorylating the Pol II carboxyl-terminal domain (CTD) and negative elongation factors (5). The ELL1/2 subunit, on the other hand, can directly increase the catalytic rate of Pol II by suppressing transient pausing (12). As these two elongation stimulatory factors act simultaneously on a single polymerase complex at the HIV-1 promoter, they synergistically boost viral transcription (9, 11). In addition to P-TEFb and ELL1/2, AFF1/AFF4 is another essential SEC component due to its ability to serve as a flexible scaffold to recruit all the other subunits into a complete complex (6, 13).

Our recent structural and biochemical analyses indicate that AFF1/4 and Tat bind right next to each other to the surface of CycT1 and that this arrangement significantly enhances the interaction between Tat and P-TEFb (14). Compared to AFF1, AFF4 displays a greatly diminished ability to promote the Tat–P-TEFb binding because of a critical amino acid variation between the two AFF proteins (11). While this functional difference was observed mostly in HeLa and HEK293 cells, it remains to be determined whether it also exists in HIV-1's natural host, CD4$^+$ T cells. Unlike AFF1, which stimulates the Tat–P-TEFb interaction, the human

Received 2 November 2015 **Returned for modification** 15 December 2015
Accepted 21 January 2016

Accepted manuscript posted online 1 February 2016

Citation Li Z, Lu H, Zhou Q. 2016. A minor subset of super elongation complexes plays a predominant role in reversing HIV-1 latency. Mol Cell Biol 36:1194–1205. doi:10.1128/MCB.00994-15.

Address correspondence to Qiang Zhou, qzhou@berkeley.edu.

TABLE 1 CRISPR-Cas9 genome-targeting statistics

Gene	Exon targeted[a]	Genotyping primer sequences[b]	sgRNA sequence	On-target score[c]	No. of cell clones sequenced	No. of KO clones[d]
ELL2	1	5′-GAGCGCCCGGATCGCCGTCT, 5′-CGTCGGAAAGTCCCGCAG	5′-GAGCGCCCGGATCGCCGTCT	91	2	2
AFF4	1	5′-AAGTGTTTTGTTGGGGTGGGTT, 5′-GGTGAAGTACCCGCCGATG	5′-AGAACGGGAAAGGCGGAATC 5′-TGCGTATGAAAGAACGGGAA	83 81	9 6	5 2
AFF1	2	5′-CCTGCAGATGAAAAGCTTCCAC, 5′-GTGCCATTTTAACTCAATTCCCCTG	5′-CCTTCTCTCTAATTCGAAGC 5′-AGAGAAGGAAAGACGCAACC	89 73	5 2	2 1
ELL1	1	5′-ATATGCAACAACTTGGGGCG, 5′-CAGCTTCCCCCTATCACGGT	5′-TCGTGCGGGCGGGTTAGCGA 5′-TCTGGCGCGGTAGCTCTCGA	98 93	10 24	0 0

[a] Position of the sgRNA-targeted exon within the gene.
[b] Sequences of primer pairs used to amplify targeted genomic regions for Surveyor Assay and Sanger sequencing.
[c] Faithful on-target score predicted based on http://crispr.mit.edu/.
[d] Number of cell clones containing homozygous frameshift mutations as confirmed by Sanger sequencing.

BET bromodomain protein BRD4 competes with Tat for binding to P-TEFb, leading to the inhibition of Tat transactivation (15, 16). Importantly, a small-molecule inhibitor termed JQ1 has been shown to antagonize BRD4's inhibitory effect through occupying its bromodomains. This in turn promotes the Tat–P-TEFb/SEC binding, Tat transactivation, and the exit of HIV-1 from latency (17–19).

Despite the recognition of the key role that the SECs play in HIV-1 transcription, little is known about how they may control viral latency. Specifically, it remains to be seen to what extent the reversal of latency depends on the SECs and whether any particular members of the SEC family may exert a predominant role in this process. Furthermore, it is unclear whether the cellular level/activity of a SEC can be manipulated in $CD4^+$ T cells to efficiently reverse latency in the absence of any chemical inducers that have serious side effects.

Previously, it has been difficult to answer these questions due to the frequently encountered partial effectiveness of RNA interference (RNAi) in silencing gene expression. In the present study, we used the state-of-the-art CRISPR-Cas9 genome-editing tool to knock out (KO) the gene encoding the SEC subunit ELL2, AFF1, or AFF4 in 2D10 cells, a well-characterized Jurkat T cell-based HIV-1 latency model (20). Depletion of these SEC subunits significantly reduced both spontaneous and drug-induced latency reversal by suppressing viral transcription at the stage of elongation. Surprisingly, despite its very low abundance, a minor subset of SECs was found to play a predominant role in facilitating the Tat-dependent latency exit. Furthermore, by simply elevating the cellular level/activity of these Tat-friendly SECs, we were able to efficiently reverse latency without using any drugs.

MATERIALS AND METHODS

Generation of knockout and rescue cell lines. The procedures for disrupting the genes encoding the SEC subunits ELL2, AFF1, and AFF4 using the CRISPR-Cas9 system in the Jurkat-based 2D10 cell line (20) have been described previously (21). The plasmid vector pSpCas9(BB)-2A-Puro (PX459), which expresses Cas9 and single guide RNA (sgRNA), was from Addgene (plasmid 48139). The sgRNA and genotyping primer sequences used in the procedures are listed in Table 1. The positive KO clones were identified by Sanger sequencing of the genomic amplicons obtained with the TA Cloning kit (Life Technologies), and the loss of expression of the disrupted genes was verified by immunoblotting of the target proteins.

A pcDNA3-based vector expressing FLAG-tagged ELL2 (ELL2-F) was stably introduced into the ELL2 KO cell line ΔELL2 by nucleofection. Rescue clones expressing various levels of ELL2-F were obtained by sorting of single cells, selection in G418 at 400 μg/ml for 16 days, and finally anti-Flag immunoblotting.

Generation of stable AFF1 or AFF4 knockdown (KD) HeLa cells. The short hairpin RNA (shRNA) sequences that target AFF1 (shAFF1, 5′-CCGGGCCTCAAGTGAAGTTTGACAACTCGAGTTGTCAAACTTCACTTGAGGCTTTTTG-3′) and AFF4 (shAFF4, 5′-CCGGGCACCAGTCTAAATCTATGTTCTCGAGAACATAGATTTAGACTGGTGCTTTTTG-3′), respectively, were cloned into the lentiviral vector pLKO.1. shRNA targeting green fluorescent protein (GFP) was used as a nontarget control. Lentivirus production and infection of HeLa cells were conducted as previously described (22).

Detection of HIV-1 latency reversal. To test the effects of SEC subunit KO and the rescues on spontaneous and drug-induced latency reversal, 1×10^6 wild-type (WT) or the various KO cells were treated with 1 nM phorbol myristate acetate (PMA), 2 μM prostratin, 1 μM JQ1, or 0.1% dimethyl sulfoxide (DMSO) as a negative control. After treatment for 16 h, the cells were subjected to fluorescence-activated cell sorter (FACS) analysis to detect the GFP fluorescence. Data were analyzed with Flowjo (Tree Star) software by first selecting living cells using the forward scatter/side scatter (FS/SS) gates and then reading the GFP-positive percentage for each sample under the same threshold throughout the experiment. The percentage of GFP^+ cells and the standard deviation for each sample were calculated based on the triplicate treatments.

Reverse transcription followed by quantitative real-time PCR (RT-qPCR) analysis. Total RNA was extracted by TRIzol reagent (Life Technologies) from drug-treated or untreated WT Jurkat/2D10 cells or the various KO cell lines and reverse transcribed using random hexamer primers (Life Technologies). The cDNA was amplified with the DyNAmo HS SYBR green qPCR kit (Fisher F-410L) on a CFX96 system (Bio-Rad) using the HIV-1 long terminal repeat (LTR) forward primer 5′-GGGTCTCTCTGGTTAGACCAG-3′ in combination with either HIV-1 LTR reverse primer-1 (5′-GGGTTCCCTAGTTAGCCAGAG-3′) or reverse primer-2 (5′-CTGCTAGAGATTTTCCACACTGAC-3′) to examine the levels of the short, just-initiated and longer, elongated HIV-1 transcripts, respectively (23). All reactions were performed in triplicate with melting curves to ensure specificity. The PCR signals were normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and displayed.

Coimmunoprecipitation. Approximately 5×10^8 WT or KO cells were swollen in hypotonic buffer A (10 mM HEPES-KOH [pH 7.9], 1.5 mM $MgCl_2$, and 10 mM KCl) for 10 min and then centrifuged at $362 \times g$ for 10 min. The nuclei were extracted with Dounce tissue homogenizer in buffer C (20 mM HEPES-KOH [pH 7.9], 0.4 M NaCl, 25% glycerol, 0.2

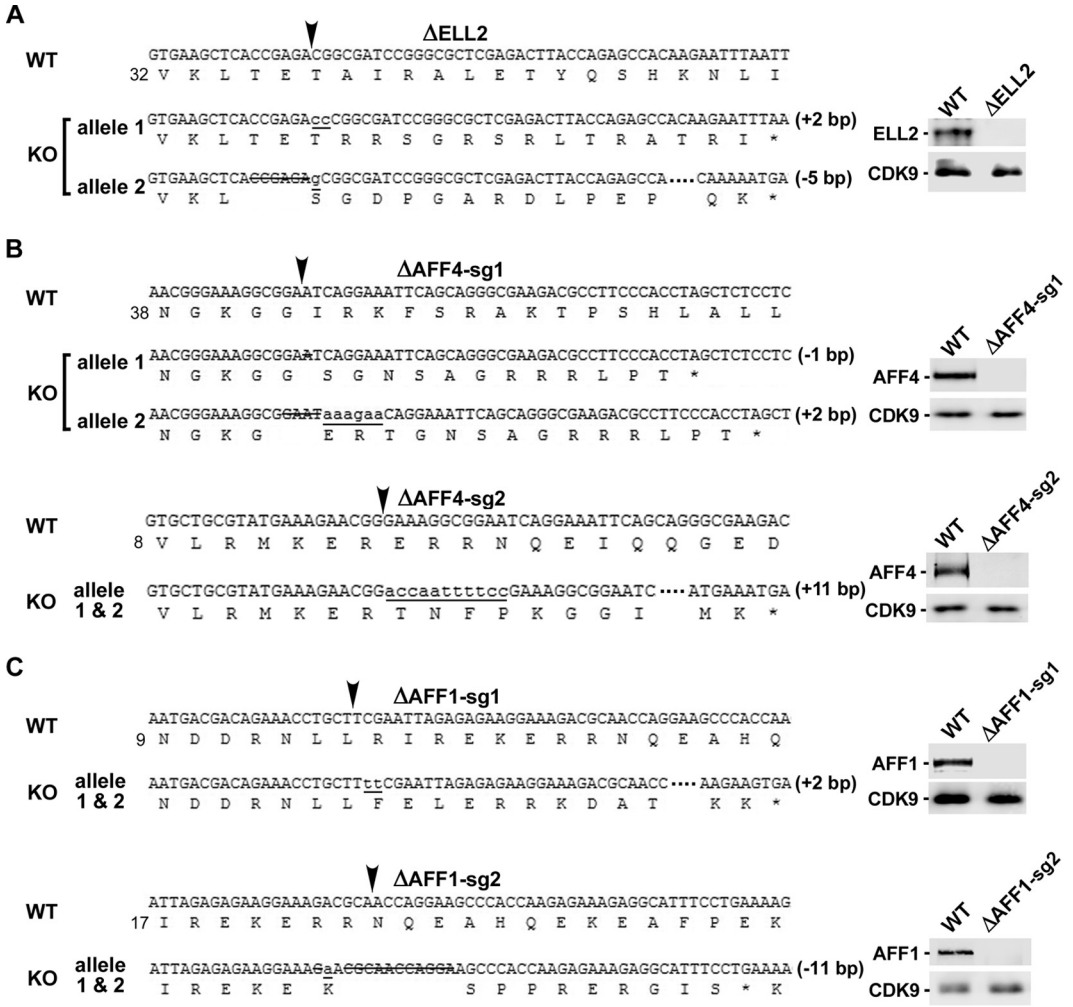

FIG 1 Verification of Jurkat/2D10-based knockout cell lines in which the genes encoding three SEC subunits are disrupted by CRISPR-Cas9. Nucleotide and predicted amino acid sequences surrounding the intended Cas9 cleavage sites (arrowheads) in wild-type ELL2 (A), AFF4 (B), and AFF1 (C) genes as well as their mutant alleles generated by CRISPR-Cas9 are shown. Insertions of extra nucleotides are indicated by underlined lowercase letters, deletions are indicated by capital letters containing strike-throughs, and the omitted nucleotides are marked by four consecutive dots. In the names of the AFF1 and AFF4 KO (ΔAFF1/4) clones, sg1 and sg2 refer to two independent clones obtained by using two separate sgRNA sequences targeting different regions of each gene locus. Premature stop codons as a result of frameshift mutations are indicated by a star. The loss of protein expression from the disrupted genes in the various KO clones was confirmed by immunoblotting with the indicated antibodies.

mM EDTA, 1.5 mM $MgCl_2$, 0.2% NP-40, and 1× protease inhibitor cocktail). Nuclear extracts (NE) were mixed with 4 μg specific antibodies or control normal IgG and incubated at 4°C for 3 h. Subsequently, 15 μl protein A beads (Life Technologies) was added into each reaction volume, and the mixtures were rotated at 4°C overnight. The beads were then washed extensively with buffer D (20 mM HEPES-KOH [pH 7.9], 0.3 M KCl, 15% glycerol, 0.2 mM EDTA, 0.2% NP-40, 1× protease inhibitor cocktail) and eluted with 30 μl 0.1 M glycine (pH 2.0). For Western blotting, 2% of the total input for immunoprecipitation (IP) and 25% of the IP eluate were loaded into each NE and IP lane, respectively.

RESULTS

Generation of Jurkat/2D10-based AFF1, AFF4, or ELL2 KO cell lines using the CRISPR-Cas9 system. Using the CRISPR-Cas9 system (21), we introduced double-strand breaks into the first exon of the ELL2 and AFF4 genes as well as the second exon of the AFF1 gene in the Jurkat-based 2D10 cells, a postintegrative latency model developed by the Karn laboratory (20). The HIV-1 sequence contained in this cell line encodes a partially attenuated Tat variant H13L as well as the short-lived d2EGFP reporter protein in place of the *nef* gene. The positive knockout (KO) clones were identified by Sanger sequencing, which detected frameshift mutations resulting from nonhomologous end-joining repair in both alleles of the target genes (Fig. 1 and Table 1). The complete loss of protein expression by the target genes was also verified by Western analysis of the KO cell lysates (Fig. 1).

Two different clones each of the AFF1- and AFF4-KO were obtained by using two separate single guide RNA sequences that target distinct regions of the AFF1 or 4 gene (Fig. 1 and Table 1). These twin AFF1/4-KO clones were analyzed in key experiments (see below) to ensure that the results obtained can indeed be attributed to the loss of AFF1 or -4 and not some unintended off-target effects of the sgRNAs and the CRISPR-Cas9 system. For the single ELL2 KO clone, we performed rescue experiments by reintroduction of wild-type (WT) or mutant ELL2-

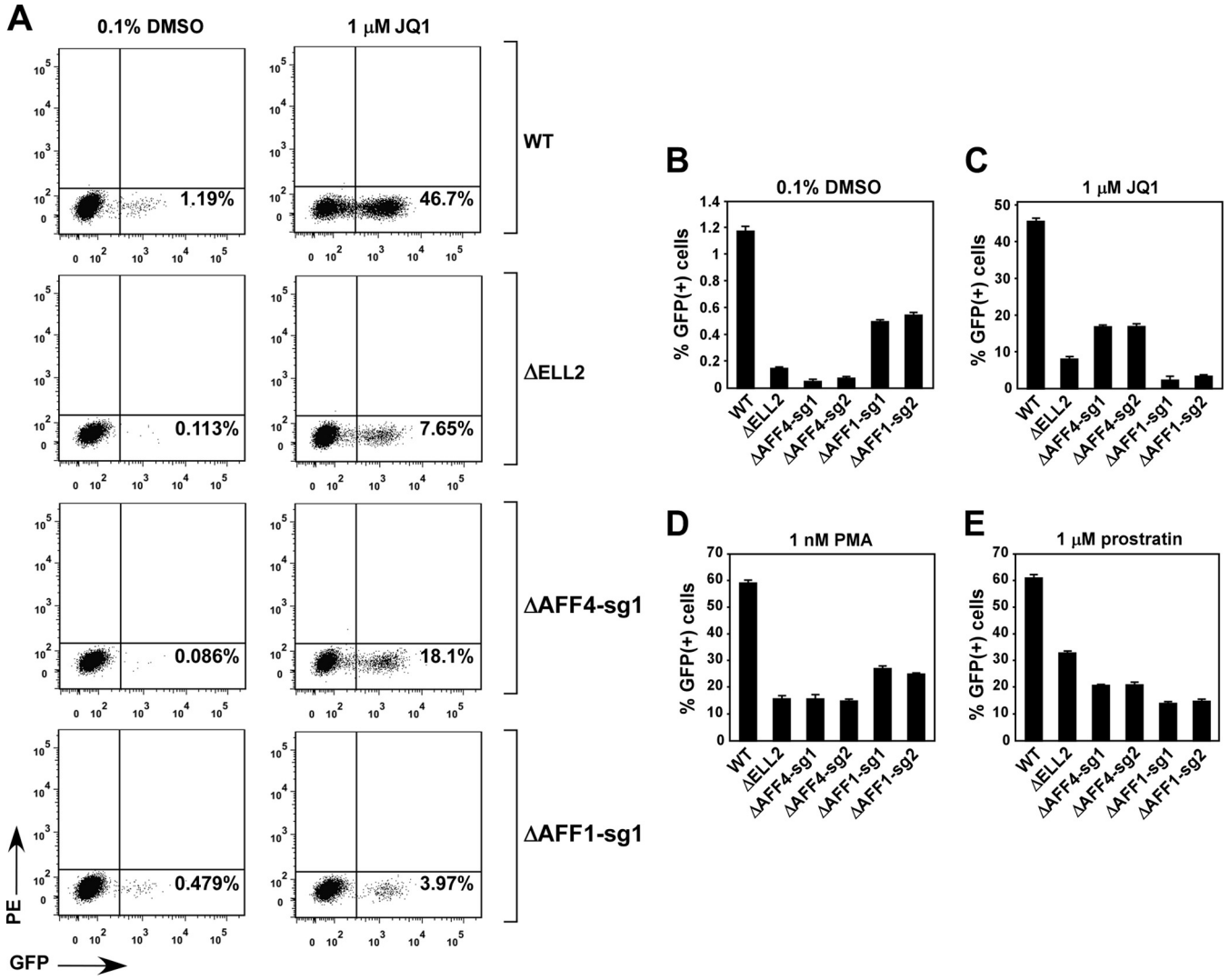

FIG 2 AFF1, AFF4, and ELL2 are differentially required by the various HIV-1 latency-reversing small molecules. The indicated cell lines were exposed to the various agents labeled on top and then subjected to FACS analysis to determine the percentage of GFP$^+$ cells present in each cell population. WT, wild-type Jurkat/2D10 cells. Representative FACS plots (A) and the bar graphs (B to E) based on these and other plots are shown. Each column represents the average from three independent experiments, with the error bars indicating means ± standard deviations.

expressing constructs as a way to rule out any off-target effects (see below).

It is important to point out that our efforts to obtain homozygous ELL1 KO were unsuccessful despite the use of two distinct sgRNA sequences and the sequencing of at least 34 different clones (Table 1). Only heterozygous KO cells in which one allele was found to contain a frameshift mutation while the other had an in-frame deletion or insertion that restored the ELL1 open reading frame were obtained. These observations suggest that ELL1 is an essential gene, a notion that is supported by a recent genome-wide study showing that the sgRNA sequences for ELL1 were significantly more depleted than those for ELL2, AFF1, and AFF4 in a negative-selection screen (24).

AFF1, AFF4, and ELL2 are differentially required by various agents to reverse HIV-1 latency. We next performed FACS analysis to examine the effects of the ELL2, AFF1, or AFF4 KO on the abilities of several well-known latency-reversing small molecules to promote the HIV-1 LTR-dependent GFP expression. Representative FACS plots are shown in Fig. 2A, and based on these and others, the processed data are summarized in bar graphs in Fig. 2B to E. First of all, when treated with the vehicle control DMSO, only a small percentage (~1.2%) of WT cells became GFP positive, and the loss of any of the three SEC components was found to further reduce this spontaneous, basal-level exit from latency (Fig. 2B). Notably, between the two AFF proteins, AFF4 KO produced a more pronounced effect (<0.1% GFP$^+$ cells) than did AFF1 KO (~0.5%) in suppressing this process.

As for the drug-induced latency reversal, all three SEC subunits were shown to be important in this process, although the responses to their KO varied from compound to compound. The BET bromodomain inhibitor JQ1 is known to activate HIV-1 transcription by antagonizing BRD4's inhibition of the Tat-P-TEFb/SEC interaction, thereby stimulating the Tat/SEC-dependent viral transcription (17, 18). We have recently demonstrated

that the AFF1-containing SECs are preferentially employed by Tat to transactivate the HIV-1 LTR (11). Consistent with these observations, the JQ1-induced latency reversal was strongly dependent on AFF1 (from 47% in WT cells to ~2 to 3% GFP^+ cells remaining in the KO population [Fig. 2C]) but much less so on AFF4 (47% to 18%). PMA and prostratin, on the other hand, function primarily by activating protein kinase C (PKC) and hence NF-κB, which then binds to and stimulates transcription from the viral LTR. Compared to JQ1's strong reliance on AFF1, latency reversal by these two compounds was not so much decreased by the AFF4 or AFF1 KO (at least 14% GFP-positive cells still remaining), and moreover, it was affected somewhat similarly by the loss of AFF1 or AFF4 (Fig. 2D and E).

Among the three latency-reversing compounds tested, JQ1 displayed the highest sensitivity to the KO of ELL2 (Fig. 2C), whereas prostratin was the least sensitive (Fig. 2E), and PMA was in the middle (Fig. 2D). The strong dependence on ELL2 by JQ1 is consistent with the earlier demonstrations that the Tat-SEC complex plays an especially important role in allowing JQ1 to reverse HIV-1 latency (17). In a subsequent assay, we investigated whether the homologous ELL1 protein can rescue the ELL2 KO to enable JQ1 to regain this ability (see below).

KO of SEC subunits suppresses latency reversal by decreasing HIV-1 transcriptional elongation. In the above latency reversal assay, the two independent AFF1 or AFF4 KO clones, which were generated with sgRNAs targeting two different regions of AFF1 or AFF4, produced nearly identical responses under all treatment conditions (Fig. 2B to E). The twin AFF1/4 KO clones also behaved the same in many other experiments described below, thus ruling out their displayed phenotypes as potential off-target effects of CRISPR-Cas9. Thus, for simplicity of presentation, only the results obtained with just one clone each of the AFF1/4 KO (ΔAFF1-sg1 and ΔAFF4-sg1) will be discussed henceforth.

In light of the above GFP data showing the KO-induced decrease in HIV-1 LTR activity, we wanted to further investigate whether the decrease occurred at the transcription initiation or elongation level. To this end, RT-qPCRs using mixtures containing two distinct pairs of LTR primers (23) that can distinguish between a just-initiated short (59-nucleotide [nt]) HIV-1 transcript and an elongated longer form (190 nt) were performed (Fig. 3A). Consistent with the notion that the SEC is involved exclusively in the elongation control, production of the short HIV-1 RNA, i.e., transcription initiation, was mostly unaffected by the loss of any of the three SEC subunits (Fig. 3B). This was true not only for JQ1, the BRD4 antagonist and Tat/SEC activator, which elevated production of the 59-nt transcript, i.e., initiation, only ~2-fold compared to DMSO, but also for PMA and prostratin, the two PKC/NF-κB activators, which stimulated this process nearly 80-fold.

In contrast to HIV-1 initiation, the production of the longer, 190-nt transcript, i.e., transcription elongation, was sensitive to KO of SEC subunits, although the degree of sensitivity varied under different treatment conditions (Fig. 3C). Just like the situations encountered above in the GFP reporter assay, between AFF1 and AFF4, the low, basal-level elongation in DMSO-treated cells was extremely sensitive to the loss of AFF4 but not to that of AFF1. In contrast, the activated elongation in JQ1-treated cells was completely gone only after the KO of AFF1 but not AFF4 (Fig. 3C), reinforcing the notion that JQ1 acts primarily by stimulating the Tat/SEC-dependent HIV-1 elongation and that the AFF1-SEC is preferentially utilized in this process (11, 17, 18). As for HIV-1 elongation in cells treated with either PMA or prostratin, it was similarly sensitive to the loss of AFF1 or AFF4, although the dependence of the two compounds on AFF1/4 was a few orders of magnitude lower than that of JQ1 on AFF1 (Fig. 3C).

Finally, just like the KO of AFF1/4, the KO of ELL2 did not much affect the HIV-1 initiation but uniformly decreased basal as well as activated HIV-1 elongation under all treatment conditions (Fig. 3B and C), confirming this protein as a key contributor to the SEC-mediated elongation stimulation. Together, the above data indicate that even though the three latency-reversing agents can preferentially target different stages of HIV-1 transcription (PMA and prostratin primarily activate the NF-κB-mediated initiation, whereas JQ1 activates mostly the Tat-stimulated elongation), the SEC-dependent HIV-1 elongation is an indispensable step that all three compounds must activate, either directly or indirectly, in order to efficiently promote the viral exit from latency.

Latency reversal in KO cell lines is restored by reintroduction of the missing proteins or in some cases their functional homologues. To investigate whether the homologous ELL (ELL1 and ELL2) and AFF (AFF1 and AFF4) proteins may possess redundant functions in promoting the exit from HIV-1 latency, we expressed them individually from nucleofected cDNAs in WT Jurkat/2D10 and the three SEC subunit KO cell lines. With the exception of AFF4-F (Flag-tagged AFF4), this resulted in an increase in basal-level GFP production in WT cells (Fig. 4A). However, compared to ELL2-F, ELL1-F was significantly less effective in this process. Upon close examination, we suspected that even this relatively weak stimulatory effect was likely caused by ELL1-F's much higher accumulation than ELL2-F in nucleofected cells (Fig. 4A, bottom panel). Indeed, when the expressions of the two ELL proteins were adjusted to about the same level, ELL1-F completely lost the ability to activate GFP expression (Fig. 4B). Thus, between the two homologous pairs of SEC subunits, only the introduction of extra ELL2 or, to a smaller extent, AFF1 into WT cells was able to promote the non-drug-induced latency reversal, suggesting that these two SEC subunits are normally present at levels or in states that are suboptimal for the reversal.

In the various KO lines, the spontaneous latency reversal was restored or even elevated to levels higher than that seen in WT cells upon reintroduction of the cDNAs encoding the missing proteins. For example, significantly elevated GFP production was observed when ELL2-F but not ELL1-F was reintroduced into the ELL2 KO cells (Fig. 4B). A smaller increase was seen only after ELL1-F was expressed at a much higher level than ELL2-F or when extra AFF1-F was introduced (Fig. 4A). The specificity of the rescue has effectively ruled out the phenotypes displayed by the ELL2 KO cells as off-target effects.

Similar to the situation found in the ELL2 KO cells, ectopic expression of AFF1-F but not AFF4-F in the AFF1 KO cells was able to reverse latency beyond the level detected in WT cells (Fig. 4A). In contrast, introduction of not only AFF4-F but also AFF1-F into the AFF4 KO cells was able to elevate the level of GFP production to that of WT cells, although the introduction of ELL1-F or ELL2-F completely failed to produce such an effect (Fig. 4A). The observation that AFF1 effectively complemented the loss of AFF4 but AFF4 was unable to replace AFF1 suggests that during

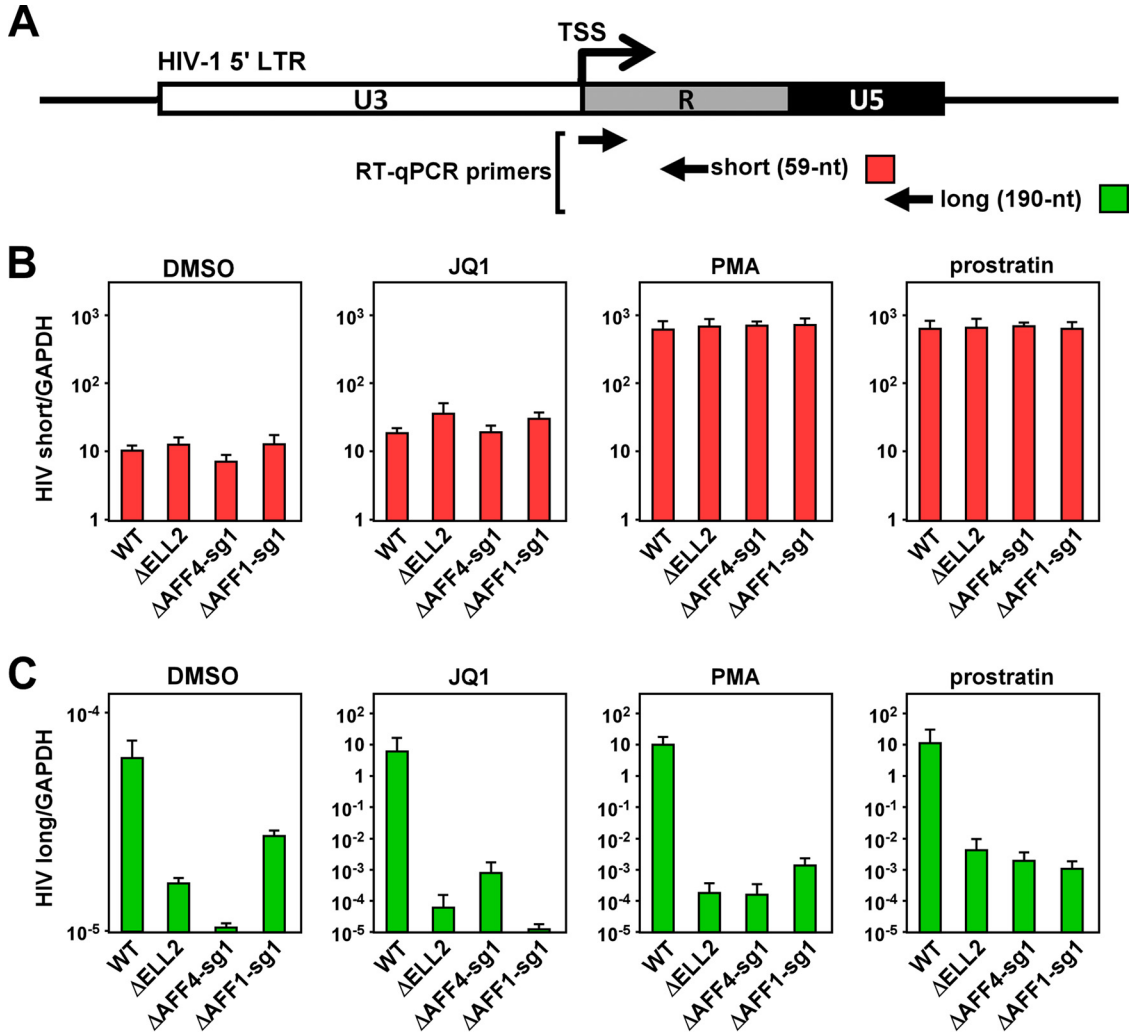

FIG 3 KO of SEC subunits suppresses latency reversal by inhibiting HIV-1 transcriptional elongation. (A) A schematic diagram showing the various regions that make up the HIV-1 5′ LTR and the positions of transcription start site (TSS) as well as the primer sets that were used in RT-qPCRs to quantify the short (59-nt) and long (190-nt) HIV-1 transcripts. (B and C) RT-qPCR quantification of the short (B) and long (C) HIV-1 transcripts using the specific primers shown in panel A. The qPCR signals were normalized to that of GAPDH. Each column represents the average of three independent RT-qPCR measurements, with the error bars indicating means ± standard deviations.

spontaneous latency reversal, AFF1 possesses all the essential functions that AFF4 does but not vice versa.

Next, we repeated the above-described rescue experiment under the JQ1 treatment conditions. The data in Fig. 4C indicate that although the overall percentages of GFP^+ cells upon exposure to JQ1 were much higher than those obtained under basal, non-drug-induced conditions, the JQ1-induced latency reversal responded very similarly to the introduction of ELL1/2 and AFF1/4 into WT and the KO cell lines (compare Fig. 4C with Fig. 4A). Despite this overall similarity, there was a noticeable difference between the two with regard to the responses displayed by the AFF4 KO cells. While the introduction of extra ELL2 into this line failed to enhance basal GFP production (Fig. 4A), it was almost as effective as the introduction of either AFF1 or AFF4 in rescuing the JQ1-induced latency reversal (Fig. 4C). Previously, it has been shown that JQ1 activates HIV-1 transcription and reverses viral latency through mostly the Tat/SEC-dependent pathway (17, 18) and that the SEC subunits ELL2 and AFF1 play an especially important role in this process (9, 11). Given these revelations, it is likely that the extra ELL2 or AFF1 introduced into the AFF4 KO cells was used to assemble the Tat-friendly, ELL2/AFF1-containing SECs that efficiently promoted latency reversal in the absence of AFF4.

Given the demonstration that ELL1 and ELL2 possess similar activities in stimulating transcriptional elongation *in vitro* (25), it is intriguing that the two proteins displayed nonredundant functions during HIV-1 latency reversal. To determine the underlying mechanism, we replaced the N-terminal transactivation domain of ELL1 (amino acids [aa] 1 to 291) with the corresponding region in ELL2 (aa 1 to 290) and determined the abilities of the overexpressed WT and engineered ELL1 protein (E2N-E1C-F) to promote the exit of HIV provirus from latency in the ELL2 KO-2D10 cells (Fig. 4D). While WT ELL1 was largely inactive in this assay, the presence of the N-terminal domain of ELL2 dramatically enhanced the ability of the engineered ELL1 to reverse viral latency to a level similar to that caused by WT ELL2 (compare Fig. 4A and

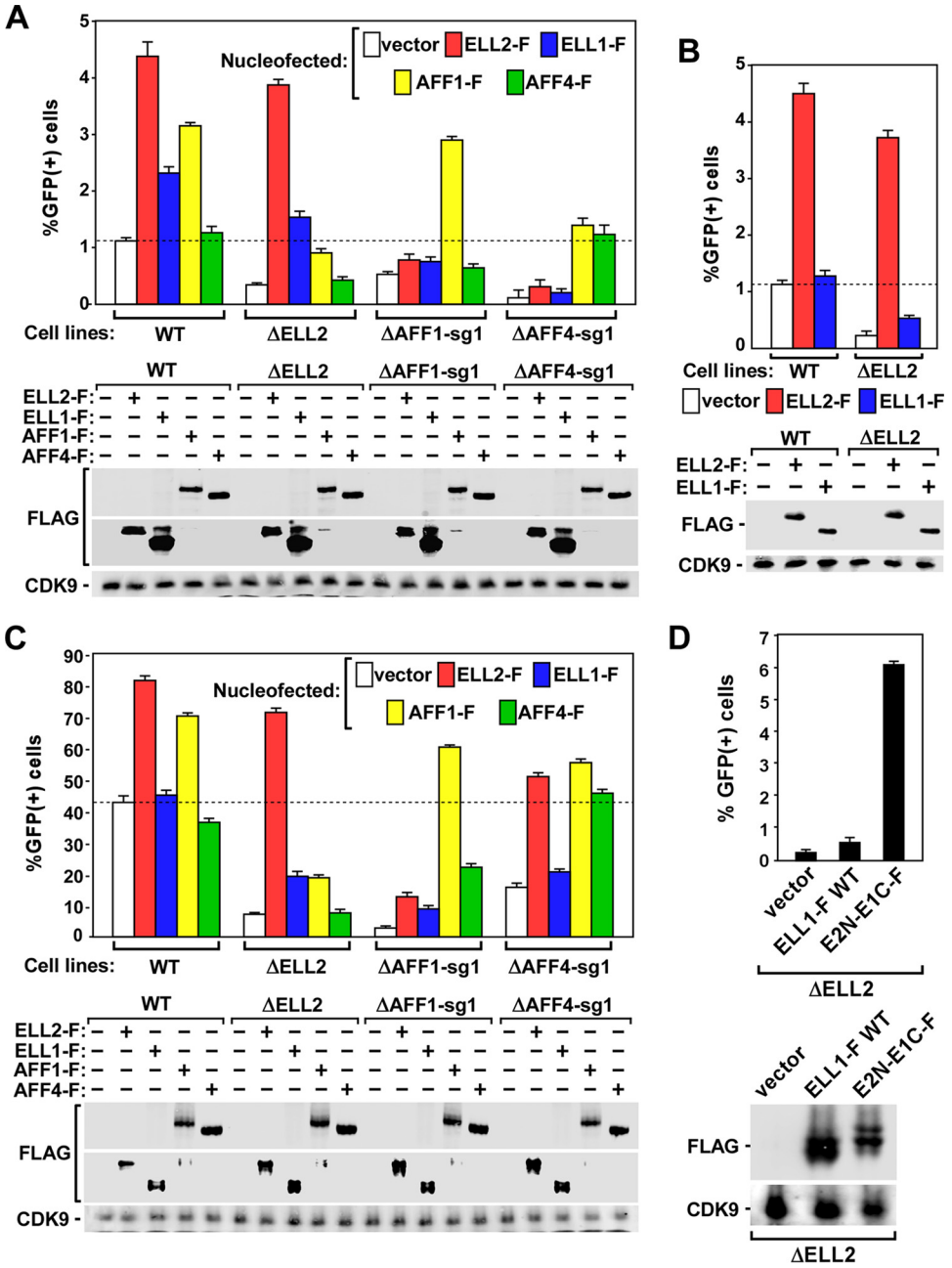

FIG 4 Restoration of HIV-1 latency reversal in SEC subunit-KO cell lines by reintroduction of the missing proteins or in some cases their functional homologues or an ELL2-ELL1 chimeric protein. Vectors expressing the indicated Flag-tagged SEC subunits (A to C) or the ELL2-ELL1 chimeric protein E2N-E1C-F (D) were nucleofected into WT Jurkat/2D10 cells or the various SEC subunit-KO cells derived from this cell line. Forty-eight hours later, the cells were either untreated (A, B, and D) or treated with JQ1 for an additional 16 h (C). The percentages of GFP^+ cells among the various cell populations were then determined by FACS and plotted in bar graphs, with the error bars representing means ± standard deviations from three independent measurements. The dashed lines mark the levels of latency activation achieved in WT cells containing an empty vector and are used as a reference. An aliquot of cells from each group was checked for levels of the indicated proteins by immunoblotting.

D). This result suggests that the different abilities of the two ELL proteins to reverse latency can be attributed to their distinct N-terminal Pol II-binding domains.

ELL2 synergizes with AFF1 overexpression or BRD4 knockdown to reverse HIV-1 latency in the absence of any chemical inducers. Having observed that ELL2 played an exceptionally important role in promoting the exit of HIV from latency, we further explored the potential of using ELL2 overexpression either alone or in combination with other manipulations to reverse latency in the absence of any chemical inducers. Toward this goal, the ELL2 KO cell line was used as the basis to generate a series of stable lines expressing various levels of ELL2-F as indicated by anti-ELL2 immunoblotting (Fig. 5A, bottom panel). While no ELL2 was detected in ΔE2-R1, the ELL2 levels in ΔE2-R2 and ΔE2-R3 cells

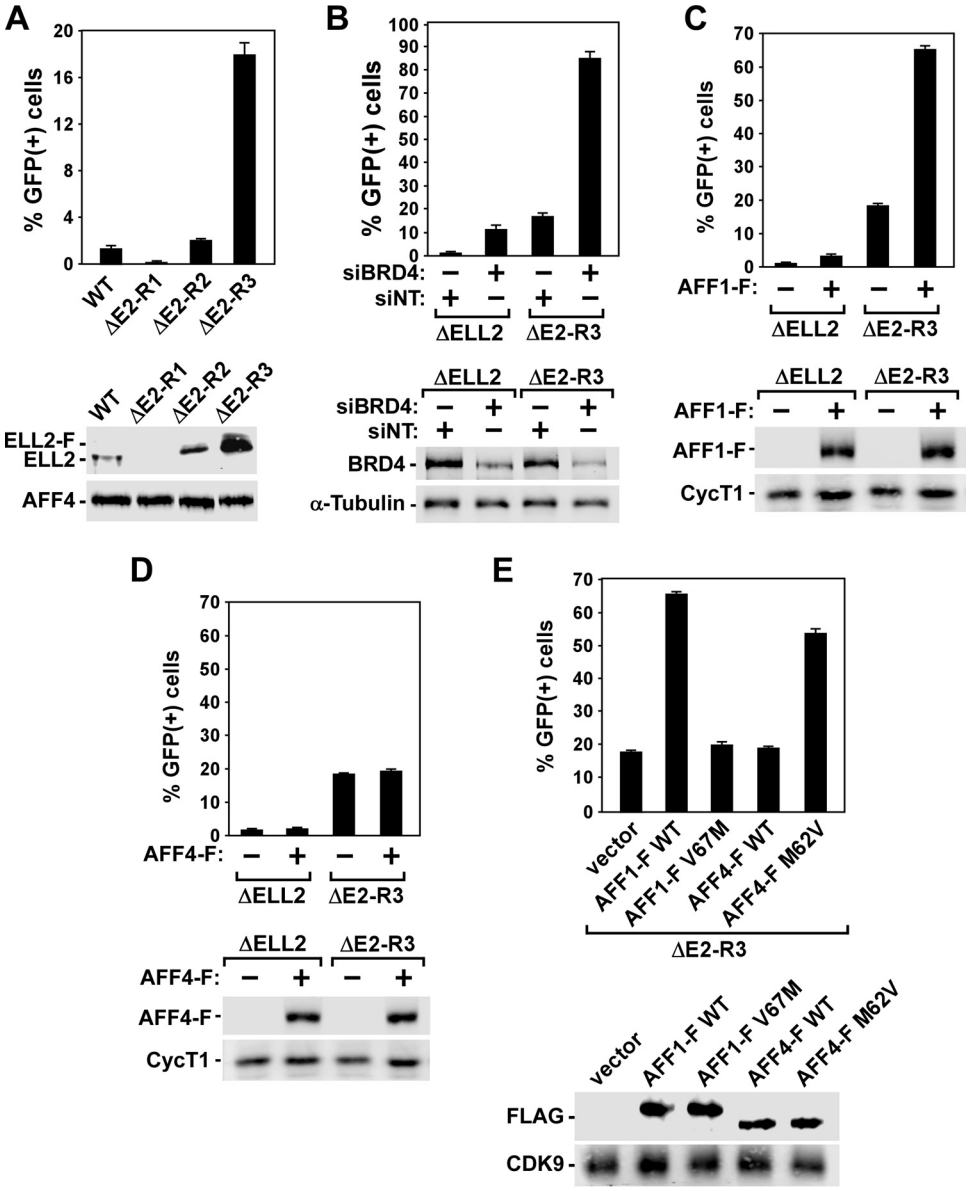

FIG 5 ELL2 synergizes with AFF1 overexpression or BRD4 knockdown to promote drug-free HIV-1 latency reversal. (A) An ELL2-F expression vector was stably introduced into the ELL2 KO (ΔELL2) cells. Three independent clones (ΔE2-R1, ΔE2-R2, and ΔE2-R3) expressing different amounts of ELL2-F were selected based on anti-ELL2 immunoblotting (bottom) and examined for the percentages of GFP$^+$ cells by FACS analysis (top). (B) ΔELL2 and ΔE2-R3 cells were nucleofected with either the BRD4-specific siRNA (siBRD4) or a control nontarget siRNA (siNT) and examined by immunoblotting for the presence in cell lysates of the indicated proteins (bottom) and by FACS for the percentages of GFP$^+$ cells (top). (C and D) ΔELL2 and ΔE2-R3 were nucleofected with plasmids expressing nothing (−), AFF1-F (C), or AFF4-F (D) and then analyzed as described for panel B. (E) E2-R3 cells were nucleofected with either an empty vector or plasmids expressing the indicated AFF1/4 proteins and then analyzed as described for panel B.

were 1.3 and 3.1 times higher than that of endogenous ELL2 in WT cells, respectively.

When these clones were analyzed by FACS, the expression of ELL2-F was found to increase the percentage of GFP$^+$ cells in each population in a dose-dependent manner (Fig. 5A). More importantly, the overexpressed ELL2-F alone in ΔE2-R3 cells was able to single-handedly produce 18% GFP$^+$ cells, again supporting the idea that ELL2 in WT Jurkat/2D10 cells is normally present in an amount or state that is inadequate for efficient HIV-1 transcription and latency reversal.

Previously, we and others have shown that the BET bromodomain protein BRD4 acts as a direct competitor of HIV-1 Tat for binding to P-TEFb/SEC and that the inactivation of BRD4 alleviates this inhibition to promote Tat-stimulation of HIV transcription and exit from latency (15–18). Consistent with these observations, the small interfering RNA (siRNA)-mediated knockdown (KD) of BRD4 expression was found to strongly synergize with ELL2 overexpression to reverse HIV-1 latency in ΔE2-R3 cells (11% and 17% GFP$^+$ cells were caused by siBRD4 and ELL2 overexpression alone, respectively, versus 85% caused by the combination of the two [Fig. 5B]).

Not only did the KD of BRD4, which resulted in more SECs

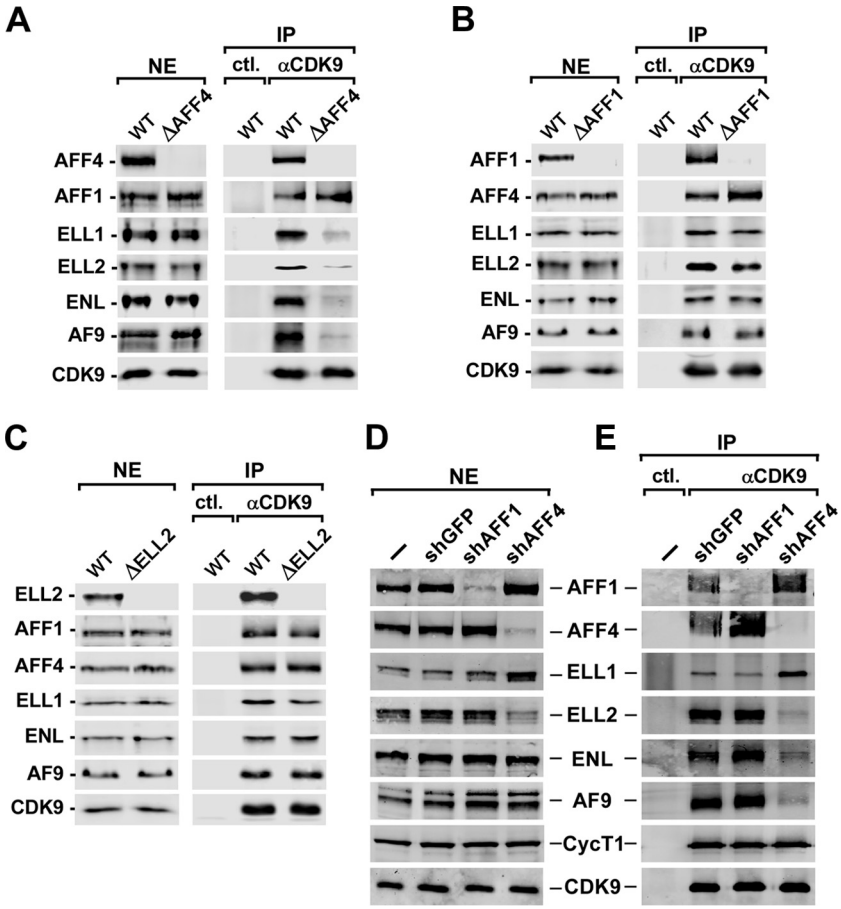

FIG 6 AFF1 is present in only a minor subset of SECs. Nuclear extracts (NE) were prepared from wild-type (WT) Jurkat/2D10 cells or the Jurkat/2D10-based AFF1 KO (ΔAFF1-sg1) (A), AFF4 KO (ΔAFF4-sg1) (B and C), and ELL2 KO (ΔELL2) (D) cells and subjected to immunoprecipitation (IP) with the specific anti-CDK9 (A, B, and D) or anti-AFF1 (C) antibody or nonspecific, normal IgG as a control (ctl.). The immunoprecipitates were examined by immunoblotting for the presence of the various proteins labeled on the left. (E) NE from HeLa cells stably expressing the indicated shRNAs or nothing (—) were subjected to the same IP-immunoblotting analysis as described for panel A.

that could be bound by Tat and thus amounted to an elevation of the effective SEC level in ΔE2-R3 cells, strongly promote the drug-free exit from latency, but also a similar effect was produced by the direct overexpression of the SEC subunit AFF1-F but not AFF4-F in these cells (Fig. 5C and D). The latter result once again highlights the functional difference between the two AFF proteins in promoting the Tat/SEC-dependent latency reversal. Further analyses have shown that this functional difference can be traced to a single amino acid variation between AFF1 and AFF4. Compared to WT AFF1, AFF1 V67M, in which Val67 was replaced with the bulkier Met found at a homologous position (amino acid 62) in AFF4, lost the ability to reverse latency in ΔE2-R3 cells (Fig. 5E). In contrast, AFF4 M62V, which contains Val at position 62 instead of the normal Met, gained the ability and became much more effective than WT AFF4 in the assay.

We have recently shown that in HeLa cells, the above-mentioned reciprocal exchange of amino acids between AFF1 and AFF4 alters the abilities to these two proteins to promote Tat binding to P-TEFb/SEC and activation of HIV-1 transcription (11). The current study has further extended these observations into the Jurkat/2D10-based latency model by showing that the single amino acid difference between the two AFF proteins allows AFF1 but not AFF4 to strongly synergize with ELL2 to reverse latency in a Tat/SEC-dependent manner.

AFF1 is present in only a minor subset of SECs. We have recently reported that as structural scaffold, AFF1 and AFF4 nucleate the formation of separate SECs with largely nonoverlapping functions in HeLa cells (11). However, the relative abundance of the AFF1 and AFF4 SECs inside a cell has not been determined, and it is unclear whether the two AFF proteins assemble their SECs with a similar or different efficiency. To address this issue, we took advantage of the AFF1 and AFF4 KO cell lines ΔAFF1-sg1 and ΔAFF4-sg1 and performed anti-CDK9 immunoprecipitations (IP) in nuclear extracts and examined the association of a panel of signature SEC subunits with CDK9 by immunoblotting.

Surprisingly, other than causing an ~50% increase in the level of AFF4 bound to CDK9, the AFF1 KO produced little noticeable effect on CDK9's interactions with ELL1, ELL2, ENL, and AF9 (Fig. 6A). In contrast, the AFF4 KO greatly reduced the amounts of these SEC components associated with CDK9 but had no significant effect on the AFF1-CDK9 interaction (Fig. 6B). Although the amount of CDK9 sequestered in the total SEC population in ΔAFF4-sg1 cells was significantly decreased, the AFF4 KO had little impact on the ability of AFF1 to nucleate the assembly of the

AFF1-SECs and thus selectively retain CDK9 in this subpopulation (Fig. 6C). Together, these results suggest that in Jurkat/2D10 cells, AFF4 is the main scaffolding protein responsible for formation of the vast majority of the SECs, whereas AFF1 is assembled into only a minor subset of the total SEC population. Importantly, it is the latter subset of SECs that has been shown above to play a predominant role in supporting HIV-1 transactivation and escape from latency. Unlike AFF1/4, ELL2 does not play a structural role during SEC assembly (9, 13). As expected, the ELL2 KO had no obvious effect on interactions of CDK9 with the remaining SEC subunits (Fig. 6D).

To investigate the contributions of AFF1 and AFF4 to SEC assembly in a different cell type, we repeated the above-described experiment in engineered HeLa cells, in which the expression of AFF1 or AFF4 had been stably knocked down by target-specific shRNAs (shAFF1 and shAFF4). The shRNA that targets GFP (shGFP) was used as a negative control. Very similar to the situations encountered in the Jurkat/2D10-based KO cells (Fig. 6A and B), the KD of AFF1 in HeLa cells had little effect on the interactions of ELL2, ENL, and AF9 with CDK9 compared to the shGFP control, whereas the AFF4 KD drastically reduced these interactions (Fig. 6E). Thus, in HeLa cells, AFF4 was also used as the predominant scaffolding protein to assemble most of the SECs.

Despite the similarity between the two cell types, there were also obvious differences. First, unlike the AFF4 KO in Jurkat/2D10 cells that has led to a dramatic decrease in the level of the CDK9-bound ELL1 (Fig. 6B), the KD in HeLa cells actually elevated the ELL1 level in both the anti-CDK9 immunoprecipitates and the nuclear extract (NE) through an unknown mechanism (Fig. 6E). Second, the ELL2 level in HeLa NE was decreased markedly upon AFF4 KD (Fig. 6E), but only very slightly in Jurkat/2D10 cells after the AFF4 KO (Fig. 6B). This decrease could be due to the loss of AFF4's protection of ELL2 from the E3 ubiquitin ligase Siah1 (26). Apparently, the degree of protection varied between the two cell types. Finally, the KD of AFF1 or AFF4 in HeLa cells significantly increased binding of CDK9 to the remaining nontargeted AFF paralog (Fig. 6E), suggesting the existence of a robust compensatory relationship between the two AFF proteins in this cell line. In comparison, a somewhat weaker compensation was detected in the Jurkat/2D10-based AFF1/4 KO cells (Fig. 6A and B). Since AFF4 is the predominant scaffolding protein for SEC assembly, its ability to compensate for the lost AFF1 through increased binding to CDK9 may also partially explain why the interactions of CDK9 with several signature SEC components remained largely unaffected by either AFF1 KD or KO.

DISCUSSION

P-TEFb is a component of the multisubunit SECs, and recent studies indicate that it is not the isolated P-TEFb but rather a complete SEC that is recruited by Tat to the HIV-1 promoter for efficient transcriptional activation (7, 9, 11). Although P-TEFb has been extensively studied and recognized as a key host factor required for HIV-1 to escape latency (27, 28), the contribution by the non-P-TEFb part of a SEC to this process has not been determined. Furthermore, since the SECs belong to a fairly large family of related complexes (10), it remains to be tested whether the SEC subtype that has been shown to be key for Tat activation of HIV-1 transcription in HeLa cells (11) is also important for the HIV provirus to escape latency in $CD4^+$ T cells. To address these questions, the present study employs the CRISPR-Cas9 system to knock out the genes encoding three key SEC subunits, AFF1, AFF4, and ELL2, in 2D10 cells, a well-characterized Jurkat T cell-based HIV-1 latency model (20). Through this loss-of-function approach that is much more rigorous than the RNAi-mediated knockdowns performed previously, we show that latency reversal, regardless of whether it was drug induced or occurred spontaneously, was unable to proceed in the absence of these non-P-TEFb SEC subunits.

Among the three SEC subunits analyzed in the current study, AFF1 and AFF4 are characterized as scaffolding proteins required for assembly of the AFF1- and AFF4-containing SECs, respectively (9, 22). Our data indicate that these two subsets of SECs are differentially required for HIV-1 latency reversal depending on how the process was induced. For example, spontaneous, basal level latency reversal depended more on the AFF4 SECs, whereas the JQ1-induced process was much more sensitive to the loss of the AFF1 SECs (Fig. 2B and C). Mirroring these different requirements, the JQ1-induced HIV-1 transcriptional elongation was strongly inhibited by the KO of AFF1 but much less so by KO of AFF4 (Fig. 3C). In contrast, basal HIV-1 elongation was extremely sensitive to the depletion of the AFF4 but not AFF1 SECs (Fig. 3C).

A likely explanation for the strong dependence on the AFF4 SECs for basal, spontaneous HIV-1 latency reversal and elongation is that a cellular recruitment factor such as the polymerase-associated factor complex (PAFc) (6) or/and the mediator complex (29) is likely responsible for attracting a SEC to the viral promoter under these Tat-free conditions. Between the AFF1 and AFF4 SECs, the latter have been shown in the current study to be strongly predominant *in vivo* (Fig. 6). Moreover, PAFc and mediator are not known to prefer one subtype over the other. For these reasons, the KO of AFF4 is expected to exert a bigger impact than the KO of AFF1 at this stage, when only an extremely low level of viral transcription is triggered. In contrast, when cells are treated with JQ1, which antagonizes the BRD4 inhibition of the Tat-CycT1 interaction, thereby boosting Tat's ability to recruit a SEC to the paused Pol II at the viral promoter (17), the AFF1 SECs are preferentially selected because the Tat-CycT1 binding is stronger in the presence of AFF1 than AFF4 (11, 17). Thus, even though the AFF1 SECs represent a minor subset of the total SEC population *in vivo*, they are preferentially used in JQ1-treated cells and play an especially critical role in Tat-dependent HIV-1 latency reversal and transcriptional elongation.

In addition to AFF1 and AFF4, the SEC subunit ELL2 has also been shown to occupy an important place in activating latent proviruses. Because of the failure to obtain an ELL1 KO line, it is difficult to directly assess the role of ELL1 in controlling this process. However, several pieces of evidence suggest that as a SEC subunit, it may not be as effective as ELL2 in supporting HIV-1 transcriptional elongation and escape from latency. First, except for the observations made in prostratin-treated cells, ELL2 KO caused a >50% reduction in both HIV-1 transcriptional elongation and latency reversal under basal as well as drug (JQ1 or PMA)-induced conditions (Fig. 2 and 3C). In other words, the remaining ELL1-containing SECs in the ELL2 KO cells have provided less than one-half of the total SEC activity under these conditions. In addition, ELL1 failed to rescue the loss of ELL2 when it was expressed at about the same level as that of ELL2, indicating that it was less active than ELL2 in supporting spontaneous and JQ1-induced latency reversal (Fig. 4B and C). A weak complementation was observed only after ELL1 was expressed to a much

higher level (Fig. 4A). Mechanistically, our domain-swapping experiment (Fig. 4D) reveals that differences between the N-terminal Pol II-binding domains of ELL1 and ELL2 underlie the different activities of the two ELL proteins in HIV-1 latency reversal. Future studies are needed to determine how the ELL2 N-terminal domain may establish a more productive interaction with Pol II at the HIV-1 promoter to activate viral transcription and reverse latency.

Another important finding of the present study is that by merely increasing the level/activity of the ELL2/AFF1-containing SECs in Jurkat/2D10 cells, we were able to efficiently reverse HIV-1 latency without using any drugs (Fig. 5), suggesting that this subset of SECs is normally a rate-limiting factor for Tat transactivation in these cells. Since the simple introduction of extra Tat protein alone without the matching host cofactors produces only limited effects in reversing latency (30, 31), an array of human proteins has been discovered to activate HIV-1 proviruses to various degrees in latently infected cells once their expression is enhanced or silenced (reviewed in reference 4). However, in most cases this has not been a very effective strategy except for the p65 (RelA) subunit of NF-κB. The Greene laboratory reported in 2006 that overexpression of p65 was able to activate over 80% of the latent proviruses in Jurkat-based J-Lat cells (32).

This level of activation is similar to what we have accomplished in the current study through overexpressing ELL2 and AFF1 together (65% latency reversal) or combining ELL2 overexpression with BRD4 KD (85%). Both strategies can increase the effective concentrations of the ELL2/AFF1-containing SECs that are targeted by Tat for HIV-1 transactivation. Given the pleiotropic functions of NF-κB, especially in the immune system, and the unacceptable side effects of its activating drugs PMA and prostratin, the NF-κB pathway may not be an ideal therapeutic target for latency reversal (33). In contrast, the SECs appear to only selectively regulate a minor fraction of genes (34). In our ongoing efforts to identify additional, more druggable targets to achieve robust latency reversal, the ELL2/AFF1-contaning SECs merit serious considerations because efficient and complete reversal cannot proceed without these low-abundance, Tat-specific SECs and their interactions with Tat.

It is important to point out that the present conclusion about the predominant effects of the ELL2/AFF1-contaning SECs in HIV latency reversal has been made on the basis of results in activated $CD4^+$ T cells. A vital difference between quiescent memory $CD4^+$ T cells and activated T cells is that the former have extremely low levels of active P-TEFb (27). Thus, the overexpression of ELL2 and AFF1, if it is to be used in a future therapeutic approach to reverse HIV latency, should most likely be combined with additional manipulations, e.g., treatment with a cocktail of cytokines (35) or prostratin (36), that can increase the nuclear concentration of active P-TEFb without completely activating T cells.

ACKNOWLEDGMENTS

We thank Kartoosh Heydari and Hector Nolla of the Flow Cytometry Facility at UC Berkeley for their help with single cell sorting.

This work is supported by Public Health Service grants R01AI041757 and R01AI095057 from the National Institutes of Health, a Tang Predoctoral Fellowship to Z.L., and a grant from the China Ocean Mineral Resources R & D Association (DY125-15-T-03) to H.L.

FUNDING INFORMATION

HHS | National Institutes of Health (NIH) provided funding to Qiang Zhou under grant number R01AI095057. HHS | U.S. Public Health Service (USPHS) provided funding to Qiang Zhou under grant number R01AI041757.

REFERENCES

1. **Siliciano RF, Greene WC.** 2011. HIV latency. Cold Spring Harb Perspect Med **1:**a007096. http://dx.doi.org/10.1101/cshperspect.a007096.
2. **Karn J.** 2011. The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. Curr Opin HIV AIDS **6:**4–11. http://dx.doi.org/10.1097/COH.0b013e328340ffbb.
3. **Bullen CK, Laird GM, Durand CM, Siliciano JD, Siliciano RF.** 2014. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. Nat Med **20:**425–429. http://dx.doi.org/10.1038/nm.3489.
4. **Mbonye U, Karn J.** 2014. Transcriptional control of HIV latency: cellular signaling pathways, epigenetics, happenstance and the hope for a cure. Virology **454-455:**328–339. http://dx.doi.org/10.1016/j.virol.2014.02.008.
5. **Ott M, Geyer M, Zhou Q.** 2011. The control of HIV transcription: keeping RNA polymerase II on track. Cell Host Microbe **10:**426–435. http://dx.doi.org/10.1016/j.chom.2011.11.002.
6. **He N, Chan CK, Sobhian B, Chou S, Xue Y, Liu M, Alber T, Benkirane M, Zhou Q.** 2011. Human polymerase-associated factor complex (PAFc) connects the super elongation complex (SEC) to RNA polymerase II on chromatin. Proc Natl Acad Sci U S A **108:**E636–E645. http://dx.doi.org/10.1073/pnas.1107107108.
7. **Sobhian B, Laguette N, Yatim A, Nakamura M, Levy Y, Kiernan R, Benkirane M.** 2010. HIV-1 Tat assembles a multifunctional transcription elongation complex and stably associates with the 7SK snRNP. Mol Cell **38:**439–451. http://dx.doi.org/10.1016/j.molcel.2010.04.012.
8. **Lin CQ, Smith ER, Takahashi H, Lai KC, Martin-Brown S, Florens L, Washburn MP, Conaway JW, Conaway RC, Shilatifard A.** 2010. AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia. Mol Cell **37:**429–437. http://dx.doi.org/10.1016/j.molcel.2010.01.026.
9. **He N, Liu M, Hsu J, Xue Y, Chou S, Burlingame A, Krogan NJ, Alber T, Zhou Q.** 2010. HIV-1 Tat and host AFF4 recruit two transcription elongation factors into a bifunctional complex for coordinated activation of HIV-1 transcription. Mol Cell **38:**428–438. http://dx.doi.org/10.1016/j.molcel.2010.04.013.
10. **Luo Z, Lin C, Shilatifard A.** 2012. The super elongation complex (SEC) family in transcriptional control. Nat Rev Mol Cell Biol **13:**543–547. http://dx.doi.org/10.1038/nrm3417.
11. **Lu H, Li Z, Zhang W, Schulze-Gahmen U, Xue Y, Zhou Q.** 2015. Gene target specificity of the Super Elongation Complex (SEC) family: how HIV-1 Tat employs selected SEC members to activate viral transcription. Nucleic Acids Res **43:**5868–5879. http://dx.doi.org/10.1093/nar/gkv541.
12. **Shilatifard A.** 1998. Factors regulating the transcriptional elongation activity of RNA polymerase II. FASEB J **12:**1437–1446.
13. **Chou S, Upton H, Bao K, Schulze-Gahmen U, Samelson AJ, He NH, Nowak A, Lu HS, Krogan NJ, Zhou Q, Alber T.** 2013. HIV-1 Tat recruits transcription elongation factors dispersed along a flexible AFF4 scaffold. Proc Natl Acad Sci U S A **110:**E123–E131. http://dx.doi.org/10.1073/pnas.1216971110.
14. **Schulze-Gahmen U, Upton H, Birnberg A, Bao K, Chou S, Krogan NJ, Zhou Q, Alber T.** 2013. The AFF4 scaffold binds human P-TEFb adjacent to HIV Tat. Elife **2:**e00327. http://dx.doi.org/10.7554/eLife.00327.
15. **Bisgrove DA, Mahmoudi T, Henklein P, Verdin E.** 2007. Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. Proc Natl Acad Sci U S A **104:**13690–13695. http://dx.doi.org/10.1073/pnas.0705053104.
16. **Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, Zhou Q.** 2005. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Mol Cell **19:**535–545. http://dx.doi.org/10.1016/j.molcel.2005.06.029.
17. **Li Z, Guo J, Wu Y, Zhou Q.** 2013. The BET bromodomain inhibitor JQ1 activates HIV latency through antagonizing Brd4 inhibition of Tat-transactivation. Nucleic Acids Res **41:**277–287. http://dx.doi.org/10.1093/nar/gks976.
18. **Zhu J, Gaiha GD, John SP, Pertel T, Chin CR, Gao G, Qu HJ, Walker**

BD, Elledge SJ, Brass AL. 2012. Reactivation of latent HIV-1 by inhibition of BRD4. Cell Rep **2:**807–816. http://dx.doi.org/10.1016/j.celrep.2012.09.008.

19. **Banerjee C, Archin N, Michaels D, Belkina AC, Denis GV, Bradner J, Sebastiani P, Margolis DM, Montano M.** 2012. BET bromodomain inhibition as a novel strategy for reactivation of HIV-1. J Leukoc Biol **92:**1147–1154. http://dx.doi.org/10.1189/jlb.0312165.
20. **Pearson R, Kim YK, Hokello J, Lassen K, Friedman J, Tyagi M, Karn J.** 2008. Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. J Virol **82:**12291–12303. http://dx.doi.org/10.1128/JVI.01383-08.
21. **Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F.** 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc **8:**2281–2308. http://dx.doi.org/10.1038/nprot.2013.143.
22. **Lu H, Li Z, Xue Y, Schulze-Gahmen U, Johnson JR, Krogan NJ, Alber T, Zhou Q.** 2014. AFF1 is a ubiquitous P-TEFb partner to enable Tat extraction of P-TEFb from 7SK snRNP and formation of SECs for HIV transactivation. Proc Natl Acad Sci U S A **111:**E15–E24. http://dx.doi.org/10.1073/pnas.1318503111.
23. **Lassen KG, Bailey JR, Siliciano RF.** 2004. Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4(+) T cells in vivo. J Virol **78:**9105–9114. http://dx.doi.org/10.1128/JVI.78.17.9105-9114.2004.
24. **Wang T, Wei JJ, Sabatini DM, Lander ES.** 2014. Genetic screens in human cells using the CRISPR-Cas9 system. Science **343:**80–84. http://dx.doi.org/10.1126/science.1246981.
25. **Shilatifard A, Duan DR, Haque D, Florence C, Schubach WH, Conaway JW, Conaway RC.** 1997. ELL2, a new member of an ELL family of RNA polymerase II elongation factors. Proc Natl Acad Sci U S A **94:**3639–3643. http://dx.doi.org/10.1073/pnas.94.8.3639.
26. **Liu M, Hsu J, Chan C, Li ZC, Zhou Q.** 2012. The ubiquitin ligase Siah1 controls ELL2 stability and formation of super elongation complexes to modulate gene transcription. Mol Cell **46:**325–334. http://dx.doi.org/10.1016/j.molcel.2012.03.007.
27. **Tyagi M, Pearson RJ, Karn J.** 2010. Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. J Virol **84:**6425–6437. http://dx.doi.org/10.1128/JVI.01519-09.
28. **Kim YK, Mbonye U, Hokello J, Karn J.** 2011. T-cell receptor signaling enhances transcriptional elongation from latent HIV proviruses by activating P-TEFb through an ERK-dependent pathway. J Mol Biol **410:**896–916. http://dx.doi.org/10.1016/j.jmb.2011.03.054.
29. **Takahashi H, Parmely TJ, Sato S, Tomomori-Sato C, Banks CAS, Kong SE, Szutorisz H, Swanson SK, Martin-Brown S, Washburn MP, Florens L, Seidel CW, Lin CQ, Smith ER, Shilatifard A, Conaway RC, Conaway JW.** 2011. Human mediator subunit MED26 functions as a docking site for transcription elongation factors. Cell **146:**92–104. http://dx.doi.org/10.1016/j.cell.2011.06.005.
30. **Jordan A, Bisgrove D, Verdin E.** 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J **22:**1868–1877. http://dx.doi.org/10.1093/emboj/cdg188.
31. **Donahue DA, Kuhl BD, Sloan RD, Wainberg MA.** 2012. The viral protein Tat can inhibit the establishment of HIV-1 latency. J Virol **86:**3253–3263. http://dx.doi.org/10.1128/JVI.06648-11.
32. **Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC.** 2006. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J **25:**139–149. http://dx.doi.org/10.1038/sj.emboj.7600900.
33. **Baud V, Karin M.** 2009. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov **8:**33–40. http://dx.doi.org/10.1038/nrd2781.
34. **Lin C, Garrett AS, De Kumar B, Smith ER, Gogol M, Seidel C, Krumlauf R, Shilatifard A.** 2011. Dynamic transcriptional events in embryonic stem cells mediated by the super elongation complex (SEC). Genes Dev **25:**1486–1498. http://dx.doi.org/10.1101/gad.2059211.
35. **Ghose R, Liou LY, Herrmann CH, Rice AP.** 2001. Induction of TAK (cyclin T1/P-TEFb) in purified resting CD4(+) T lymphocytes by combination of cytokines. J Virol **75:**11336–11343. http://dx.doi.org/10.1128/JVI.75.23.11336-11343.2001.
36. **Sung TL, Rice AP.** 2006. Effects of prostratin on cyclin T1/P-TEFb function and the gene expression profile in primary resting CD4+ T cells. Retrovirology **3:**66. http://dx.doi.org/10.1186/1742-4690-3-66.