CHEMICAL REVIEWS

Review

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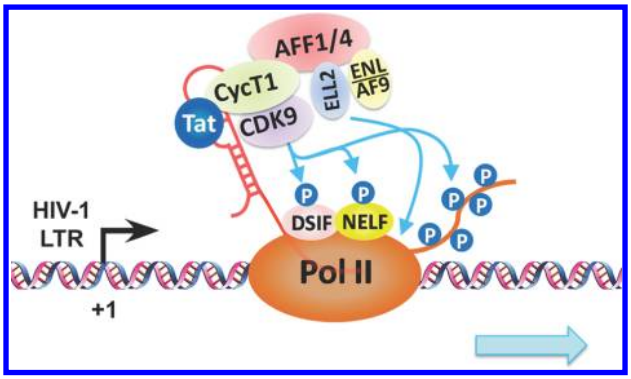
Viral–Host Interactions That Control HIV-1 Transcriptional Elongation

Huasong Lu,[†,‡,§] Zichong Li,[‡,§] Yuhua Xue,[†] and Qiang Zhou*[,‡]

[†]School of Pharmaceutical Sciences, Xiamen University, Xiamen, Fujian 361005, China

[‡]Department of Molecular and Cell Biology, University of California–Berkeley, Berkeley, California 94720, United States



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1. SUMMARY

Regulation of the pause and elongation by ribonucleic acid (RNA) polymerase II (Pol II) is used widely by metazoans to attain the pattern of gene expression that is essential for optimal cell growth/renewal, differentiation, and stress response. Currently, much of what we know about Pol II elongation control comes from pioneering studies of the HIV-1-encoded transactivating (Tat) protein and its host cellular cofactors. The interaction between the two fuels a powerful feedback circuit that activates human immunodeficiency virus (HIV) transcription and prevents the virus from entering latency. One of the key Tat cofactors is the human positive transcription elongation factor b (P-TEFb), which exists in a family of complexes with distinct functions during Tat transactivation. This article reviews recent progress in HIV transcription research with an emphasis on the intricate control of the various P-TEFb complexes, structural and functional insights into their interactions with Tat, the multifaceted roles of posttranslational modifications of Tat, and epigenetic control of HIV chromatin in modulating Tat activity and HIV latency. The knowledge from these studies will not only help the design of better strategies to fight HIV infection and transcriptional latency, but also advance the overall understanding of the mechanism controlling transcriptional elongation in general.

2. INTRODUCTION

Transcription of the HIV-1 proviral deoxyribonucleic acid (DNA) into messenger RNA (mRNA) is a critical step in the viral life cycle, as the mRNA serves not only as the template for the synthesis of all viral structural and accessory proteins but also as the genome for the next generation of viral particles. Upon reverse transcription of the viral RNA in CD4+ T cells or macrophages, the resulting HIV proviral DNA must be integrated into the human chromatin before it can be transcribed by host RNA polymerase II (Pol II). For all simple retroviruses, transcription is mediated exclusively by the host Pol II transcriptional apparatus. However, for a complicated retrovirus such as HIV, this process is additionally controlled by the virus-encoded transcriptional transactivator Tat protein (Figure 1). Without Tat, Pol II initiates transcription from the HIV promoter efficiently but travels only a short distance on the viral template, producing short abortive transcripts that do not support viral replication.[1] To overcome this restriction, Tat

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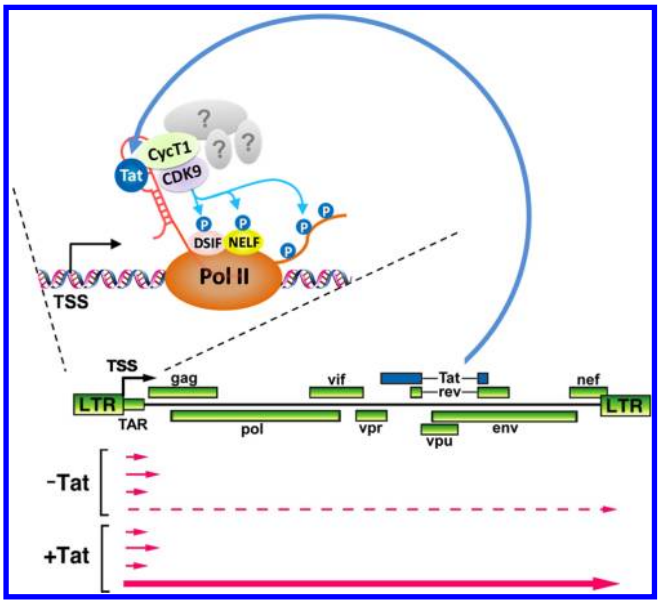

Figure 1. HIV-encoded Tat protein stimulates the production of full-length viral transcripts through binding to the HIV TAR RNA stem-loop structure. The genomic structure of the HIV-1 virus is shown with the coding region for the two-exon form of Tat highlighted in blue. Tat, in conjunction with other cellular cofactors (indicated by question marks), binds to the TAR RNA structure that is formed at the 5′ end of the nascent HIV transcript to stimulate the production of the full-length HIV mRNA (i.e., transcriptional elongation) by Pol II. TSS indicates the transcription start site.

is employed by HIV to dramatically increase the processivity of Pol II to produce the full-length viral transcripts (Figure 1).

Unlike most other transcriptional activators that target specific DNA sequences located in promoters or enhancer regions, HIV Tat stimulates Pol II elongation through interaction with the transactivation response (TAR) RNA element, a stem-loop structure located at the 5′ end of all nascent viral transcripts and synthesized by Pol II right before pausing (Figure 1). Tat binds mainly to the 3-nucleotide bulge and its immediate surrounding sequences right below the 6-nucleotide TAR apical loop (Figure 1). Although the loop sequence contributes minimally to the binding of Tat to TAR in vitro, it is absolutely required for Tat to transactivate HIV in vivo,[2] suggesting that the loop sequence might have other important functions (see section 3, last paragraph).

Because Tat is produced from the same HIV mRNA whose production it stimulates, it fuels a powerful feedback circuit that can lead to rapid and robust HIV gene expression under optimal conditions.[3] However, when HIV transcription initiation is restricted by epigenetic silencing or low activity/levels of key transcriptional activators (e.g., NF-κB or Sp1) on the viral long terminal repeat (LTR), Tat concentrations can fall below threshold levels, leading to the establishment of HIV latency.[4] Conversely, even a small increase in Tat expression can sometimes be enough to drive a provirus from its latent state into productive replication.[5] It is worth noting that the origin of Tat could even be extracellular, as Tat is able to traverse lipid membranes through its arginine-rich transduction motif.[6] As such, Tat secreted from productively infected cells can act as a viral growth factor to target neighboring cells to stimulate HIV replication and latency reactivation. All in all, the highly sensitive and signal-amplification nature of the Tat-based feedback circuit establishes this small HIV protein as a molecular switch that determines the "on" and "off" states of HIV replication.

It is worth noting that HIV Tat has long been used as a model system for studying the factors and molecular mechanism that govern Pol II transcriptional elongation in general. Exactly 20 years after elongation was first recognized as a major check point that controls HIV gene expression,[1] analyses conducted in both *Drosophila* and human stem cells revealed that a great number of cellular genes also employ the same mechanism to control their transcription.[7] These genes, which are mostly involved in stress response, cell growth/renewal, and cell differentiation, contain paused Pol II at their promoter-proximal regions. It is generally believed that the de novo recruitment of Pol II and assembly of a functional preinitiation complex (PIC) are very time-consuming. Thus, the paused Pol II at these gene promoters under resting, unstimulated conditions enables highly sensitive, rapid, and synchronous induction of transcription that is essential for stress response, cell growth/renewal, and development.[8]

In this article, we review recent progress in our understanding of HIV transcriptional control with an emphasis on the effects exerted by macromolecular assemblies containing HIV Tat/TAR and the host cellular cofactors. This progress is critical not only to ongoing efforts to eradicate HIV/AIDS through reactivating and then eliminating latent HIV reservoirs that are in a transcriptionally silent state, but also to the elucidation of the general mechanism that controls transcriptional elongation of numerous cellular genes.

3. P-TEFB IS A KEY HOST CELLULAR COFACTOR FOR TAT ACTIVATION OF HIV TRANSCRIPTION

Although elongation is a major rate-limiting step during HIV transcription and HIV employs its own Tat and TAR to overcome this restriction, mounting evidence in the 1990s suggested that the mere binding of Tat to TAR is not sufficient to induce HIV transactivation and that specific host cellular cofactors are required in this process.[9] This notion was supported by many observations, and an especially important one in 1990 showed that mutations in the apical loop of TAR strongly block Tat activation of HIV transcription but have no obvious effect on Tat binding in vitro.[10]

The ensuing years saw many unsuccessful attempts to isolate and identify the specific Tat cofactors. After numerous trials and errors, a major breakthrough finally arrived in the late 1990s with the identification of the human positive transcription elongation factor b (P-TEFb) as such a cofactor.[11] These studies demonstrated unequivocally that the Tat−TAR−P-TEFb interaction at the HIV promoter is absolutely essential for efficient activation of viral transcription.

P-TEFb is composed of the cyclin-dependent kinase 9 (CDK9) and its regulatory partner cyclin T (CycT). Whereas the 42-kDa isoform of CDK9, denoted as $CDK9_{(42)}$, is the predominant form of CDK9 in many cell types, there is also a 55-kDa isoform, denoted as $CDK9_{(55)}$, which has a 117-residue amino terminal extension missing in $CDK9_{(42)}$.[12] Similarly, in addition to CycT1, minor CDK9-associated CycT2a and T2b molecules also exist in many cell types.[13] However, Tat does not recognize CycT2a and T2b and can bind to CycT1 only through a Tat:TAR recognition motif (TRM) located at the carboxy-terminal edge of the cyclin domain in T1.[14]

Although P-TEFb is ubiquitously present in all cell types, in CD4+ T lymphocytes and monocytes, which are highly relevant for HIV infection and replication, the expression of CycT1 is

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normally low and repressed at the level of protein synthesis.[15] In fact, the limited supply of P-TEFb could be an important contributing factor to viral latency in primary T cells.[16] However, the induced activation of T cells or the differentiation of monocyte into macrophages, which markedly enhances permissiveness of these cells to HIV infection, has been shown to increase the protein but not mRNA level of CycT1.[15] Although the posttranscriptional mechanism restricting CycT1 production in resting CD4+ T cells is still unknown, a microRNA-dependent mechanism involving miR-198 was reported to repress CycT1 mRNA translation and HIV replication in monocytes.[17]

P-TEFb functions by phosphorylating serine residues located at the second position (Ser2) within the heptapeptide repeats that constitute the C-terminal domain (CTD) of the largest subunit of Pol II. The phosphorylated CTD functions as a scaffold upon which various transcription and RNA processing factors meet and operate. These factors collectively control the elongation and termination phases of Pol II transcription and also facilitate cotranscriptional processing of pre-mRNAs.[18] In addition to the Pol II CTD, two negative transcription elongation factors, DSIF and NELF, are also phosphorylated by P-TEFb. The phosphorylation antagonizes their inhibitory actions, leading to the release of Pol II from promoter-proximal pausing and transition into productive elongation.[18]

The identification of P-TEFb as a key host cofactor for Tat transactivation has provided satisfactory explanations for a number of long-standing observations in the HIV transcription field. For example, compared to many other cellular and viral gene promoters, transcription from the HIV LTR had long been indicated as particularly sensitive to kinase inhibitors such as DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) and flavopiridol.[19] The strong correlation detected between the ability of these two drugs to inhibit CDK9's kinase activity and their suppression of Tat transactivation immediately reveals the reason behind this high sensitivity.

Moreover, it has long been known that Tat acts in a species-specific manner to efficiently transactivate the viral LTR in many human and primate cell types but not in cells from other species (e.g., yeast, *Drosophila*, and murine cells).[20] After the identification of a direct interaction between Tat and human CycT1, it was revealed that the interaction requires zinc as well as essential cysteine residues in both proteins.[21] However, murine CycT1 lacks a critical cysteine (C261 in human CycT1) that is required for a stable interaction. The substitution of a cysteine for tyrosine at this position in murine CycT1 restores its zinc-dependent binding to Tat, as well as its ability to support Tat transactivation.[14,22]

Finally, earlier studies showed that the defect in Tat transactivation in murine cells is also at the level of TAR RNA recognition[23] and that a hypothetical cellular Tat cofactor encoded by a gene on human chromosome 12 confers on Tat the ability to activate HIV transcription in a TAR loop-dependent manner.[24] This is despite the observation that no specific loop sequence is required for the Tat–TAR interaction in vitro. After the identification of human CycT1 as a direct binding partner of Tat, the discovery that the CycT1 gene indeed maps to human chromosome 12 and that CycT1 touches the TAR loop and forms a stable ternary complex with Tat and TAR with a stringent requirement for the wild-type TAR loop sequence[11b,20,25] provides the long-sought validation of the earlier observations.

4. SEQUESTRATION OF P-TEFB IN CATALYTICALLY INACTIVE 7SK SNRNP

As a general transcription factor, P-TEFb is required not only for Tat activation of HIV transcription but also for efficient expression of a vast array of cellular genes. As such, the availability and activity of P-TEFb must be carefully controlled to respond to changes in the global transcriptional demand.[26] For most CDKs, which belong to an extensive family of protein kinases that are studied mainly for their roles in regulating the cell cycle, it is well-known that specific inhibitors are used to regulate their kinase activities.[27] Because CDK9 is a member of this superfamily, it is not surprising that the CDK9–CycT1 heterodimer, which constitutes the core of P-TEFb, can also exist in a form that lacks catalytic activity. However, the way P-TEFb is inhibited involves a mechanism that is quite different from those used for other CDKs.

What sets P-TEFb apart from other CDKs is the involvement of a small noncoding RNA molecule, termed the 7SK small nuclear ribonucleic acid (snRNA), in inhibiting CDK9's kinase activity. Affinity-purified as an associated factor of CDK9,[28] 7SK is an abundant small nuclear RNA of 331 nucleotides that is transcribed by RNA Pol III. When P-TEFb is present in a 7SK snRNA-containing large complex (Figure 2), it displays

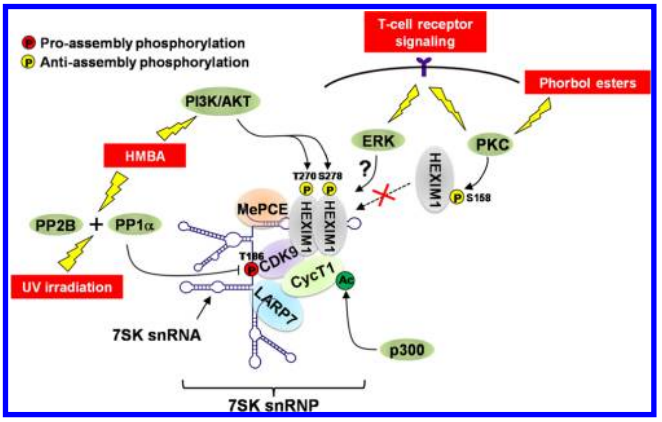

Figure 2. P-TEFb is sequestered in 7SK snRNP and released in response to various signaling events. Under normal conditions, the majority of nuclear P-TEFb is sequestered in 7SK snRNP, where P-TEFb's kinase activity is inhibited by HEXIM1 in a 7SK snRNA-dependent fashion. The stability of 7SK RNA is maintained by MePCE and LARP7, which bind to the 5′ and 3′ ends, respectively, of 7SK. When cells are subjected to the indicated treatments (highlighted in red), various signal transduction pathways are turned on, leading to changes in posttranslational modifications that include phosphorylation and acetylation on the indicated 7SK snRNP subunits and release of P-TEFb from 7SK snRNP.

little kinase activity toward the CTD heptapeptide repeats of RNA Pol II.[28a] Although the 7SK–P-TEFb interaction was found to be necessary for CDK9's inhibition, it was soon discovered that 7SK snRNA alone is insufficient for this task and that additional factor(s) existing in the large 7SK/P-TEFb-containing complex, termed the 7SK small nuclear ribonucleic particle (snRNP; Figure 2), must work together with the snRNA to suppress CDK9's catalytic activity.[29]

Indeed, nuclear protein HEXIM1 was subsequently identified through affinity purification of P-TEFb-associated factors and found to reside in 7SK snRNP.[29] It efficiently inhibits P-TEFb's phosphorylation of the Pol II CTD on Ser2 only in the presence of 7SK snRNA. This is because 7SK snRNA is

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required to bridge the interaction of P-TEFb with the central region in HEXIM1 that contains positively charged residues reminiscent of the arginine-rich TAR RNA-binding motif in HIV Tat.[30] In addition to HEXIM1, a close homologue denoted as HEXIM2 was also found in the inactive P-TEFb-containing 7SK snRNP.[31] Although these two HEXIM proteins exhibit distinct expression patterns in many human tissues and established cell lines, they have the potential to form stable homo- and heterodimers in a small set of 7SK snRNPs that display similar or highly related functions.[31a,b] Consistent with this notion, in HEXIM1-knocked-down cells, HEXIM2 was shown to functionally and quantitatively compensate for the loss of HEXIM1 to maintain a constant level of 7SK snRNPs in vivo.[29a] Despite the functional similarity between HEXIM1 and HEXIM2, HEXIM1 has a unique N-terminal region that exhibits self-regulatory activity,[29a] suggesting that these two homologous proteins might be regulated differently through their regions of unique sequences.

7SK snRNA is known to serve as a scaffold in maintaining the overall stability of 7SK snRNP.[32] Because of the stringent demand on its integrity, the stability of this snRNA is ensured by two additional 7SK snRNP components that bind to the 5′ and 3′ terminal regions of 7SK RNA (Figure 2). At the 3′ end, the poly(U) sequence of the nascent 7SK snRNA is initially protected by the Lupus antigen (La) protein during transcription and then replaced by the more specific La-related protein 7 (LARP7) after synthesis.[32,33] LARP7 is a highly specific and abundant partner of 7SK snRNA, as approximately 90% of the snRNA in cells is bound to and relies on LARP7 for stability.[33] Notably, LARP7 is frequently mutated in human gastric, breast, and cervical cancers, largely due to microsatellite instability-associated frame-shift mutations that result in C-terminally truncated proteins with no ability to bind to 7SK snRNA and sequester P-TEFb into 7SK snRNP.[32a,34] Consistent with the tumor suppressor role of a *Drosophila* homologue of LARP7, loss of LARP7 function in human breast epithelial cells has been shown to disrupt epithelial differentiation and cause P-TEFb-dependent malignant transformation.[32a]

On the opposite end of 7SK snRNA, MePCE, an S-adenosyl methionine-dependent methylphosphate capping enzyme, is responsible for monomethylation of the 5′ triphosphate of 7SK snRNA, which helps protect against cleavage by exonucleases (Figure 2).[32b,35] However, it appears that 7SK is capped by only the LARP7-free MePCE, probably in a cotranscriptional manner prior to its sequestration into 7SK snRNP.[32b] Once inside the snRNP, MePCE exerts a capping-independent function to promote the LARP7−7SK interaction. Thus, MePCE and LARP7 act cooperatively to stabilize 7SK RNA and maintain the integrity of 7SK snRNP, which sequesters P-TEFb in an inactive form.[32b]

Recently, in vivo RNA−protein interaction assays provided more details about the sequence and structural elements of human 7SK snRNA that direct the assembly of the 7SK−LARP7−MePCE core snRNP.[36] Whereas MePCE was shown to interact with the basal part of the 7SK 5′-hairpin (the so-called 5′-terminal G1-U4/U106-G111 helix-tail motif), LARP7 was found to bind to the 3′-terminal hairpin and the following poly(U) tail of 7SK. Furthermore, the direct 7SK−LARP7 binding was demonstrated to be a prerequisite for recruiting P-TEFb into 7SK snRNP, indicating that, in addition to providing stability for 7SK snRNA, LARP7 plays a more complex role in 7SK-mediated P-TEFb regulation than previously anticipated.[36]

5. SIGNAL-INDUCED DISRUPTION OF 7SK SNRNP TO RELEASE P-TEFB FOR STIMULATION OF HIV AND CELLULAR GENE TRANSCRIPTION

Depending on the cell types, from 50% to 95% of cellular P-TEFb has been reported to reside in 7SK snRNP.[28a,37] Because of the sequestration of such high levels of P-TEFb, this complex is believed to serve as the primary cellular reservoir of unused P-TEFb.[26c] Although the kinase activity of CDK9 is suppressed by HEXIM1/2 within 7SK snRNP, CDK9 becomes fully active once released because it already has the phosphorylated Thr186 at the tip of the so-called T-loop (Figure 2) and is thus suspended in a preactivation state even before the release.[38]

A variety of conditions that globally affect cell growth and differentiation have been shown to cause P-TEFb to reversibly associate with 7SK snRNP (Figure 2), and thus the level of the complex is dynamically controlled in cells.[39] First, exposure of cells to various stress-inducing agents such as transcriptional inhibitors and DNA-damaging agents (e.g., flavopiridol, DRB, actinomycin D, staurosporine, camptothecin and UV irradiation) leads to the rapid dissociation of 7SK snRNP and an increase in the pool of active P-TEFb.[38b,40] This process is presumably responsible for promoting the expression of many stress-responsive genes as part of the natural cellular response to stress. In fact, several of these agents (e.g., DRB and UV) are known to activate HIV transcription in a P-TEFb-dependent manner.[41]

In addition to playing a key role in stress response, the signal-induced release of active P-TEFb from 7SK snRNP also contributes to activation of HIV and cellular gene transcription under certain growth-promoting conditions (Figure 2). For example, the engagement of the T cell antigen receptor (TCR) or activation of T cells by phorbol esters has been shown to cause the disruption of 7SK snRNP and liberation of P-TEFb to increase HIV transcription and terminate viral latency.[42] Moreover, conditions that induce cardiac hypertrophy also dissociate 7SK snRNP to release P-TEFb, leading to a global increase in cellular RNA and protein contents and the enlargement of heart cells.[43] Finally, the differentiation inducer hexamethylene bisacetamide (HMBA) has been found to induce a biphasic response in the cellular 7SK snRNP level in murine erythroleukemia cells, with an initial and transient disruption of 7SK snRNP that is followed by a permanent increase in the levels of HEXIM1 and 7SK snRNP after a prolonged treatment to induce terminal differentiation.[39] Taken together, these observations reveal a strong correlation between signal-induced disruption of 7SK snRNP and the induction of HIV and cellular gene transcription during stress responses and cell proliferation.

As increasing numbers of agents and conditions that cause the release of P-TEFb from 7SK snRNP have been identified, the mechanisms and signaling pathways responsible for the release have also gradually been revealed (Figure 2). For example, in HeLa cells, because the phosphorylation of Thr186 in the CDK9 T-loop is important for P-TEFb's sequestration into 7SK snRNP,[38b] one model proposes that protein phosphatases such as PP1α and PP2B are activated by HMBA or UV irradiation through a calcium-dependent signaling pathway and then act cooperatively to dephosphorylate Thr186 to release P-TEFb, which is subsequently rephosphorylated through a still-unknown mechanism to regain activity.[38a] The CDK9 T-loop can also be dephosphorylated by phosphatase PPM1A and, to a lesser extent, PPM1B, leading to

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repression of HIV transcription.[44] However, it is unclear whether the PPM1A-mediated dephosphorylation can occur in the context of 7SK snRNP to cause the disruption.

In experiments conducted in Jurkat T cells, HMBA was shown to activate the PI3K/Akt pathway, which, in turn, induces phosphorylation of HEXIM1 on the conserved Thr270 and Ser278 in the CycT1-binding domain and concomitant disruption of 7SK snRNP.[45] In a separate study also performed in Jurkat T cells, the ERK kinase but not the PI3K/Akt pathway was indicated as important in the disruption of 7SK snRNP by activated TCR.[37] Reaching a different conclusion, a recent report showed that protein kinase C (PKC) can phosphorylate Ser158 in HEXIM1 in response to the engagement of TCR or activation by phorbol esters.[42a] Once Ser158 is phosphorylated, HEXIM1 neither binds to 7SK snRNA nor inhibits P-TEFb. Finally, adding yet another twist, acetylation of CycT1 by the acetyltransferase p300 was reported to liberate P-TEFb from 7SK snRNP, although this modification is apparently not required for the release of P-TEFb as induced by DRB, actinomycin D, or HMBA.[46] Taken together, these observations suggest that multiple signaling pathways and mechanisms exist to facilitate the dissociation of P-TEFb from 7SK snRNP and that different agents, conditions, or cell types can employ different pathways to achieve this goal. It will be interesting to test whether any of these pathways can communicate and coordinate their actions to increase the overall efficiency of 7SK snRNP disruption.

6. TAT EXTRACTS P-TEFB FROM 7SK SNRNP

Extensive structural and functional analyses have so far revealed several interesting similarities between the Tat–TAR–P-TEFb complex and 7SK snRNP. First, the arginine-rich TAR-binding motif in Tat is highly homologous to and functionally interchangeable with a portion of the 7SK-binding motif in HEXIM1.[30a] Second, the Tat-binding site in the TAR RNA is structurally and functionally similar to a region in 7SK snRNA that is recognized by HEXIM1.[30b] Third, a small region near the cyclin box of CycT1 can be bound by either HEXIM1 or Tat.[29b,47] These observations raise the possibility that Tat can take advantage of these similarities to directly extract P-TEFb from 7SK snRNP.

Indeed, a number of in vitro and in vivo studies have shown that Tat does exhibit such a capability (Figure 3).[48] Likely owing to this capability, a significant reduction in the level of 7SK snRNP and an increase in the Tat–P-TEFb interaction have been observed in both human primary blood lymphocytes and cultured cell lines that are infected with HIV.[48b,49] From the perspective of HIV, it makes perfect sense for Tat to directly target 7SK snRNP to obtain P-TEFb for HIV transactivation. This is because the snRNP not only sequesters up to 95% of all P-TEFb in the cell and is thus the principal source of unobligated P-TEFb, but also keeps P-TEFb in a preactivated state that is marked by the phosphorylated CDK9 T-loop.[38a]

Although the ability of Tat to extract P-TEFb from 7SK snRNP has been clearly demonstrated, the underlying mechanism(s) for this extraction is less clear. It is possible that Tat might combine several different methods to maximize its chance for success. Based on the observations that the cysteine-rich domain of Tat binds to CycT1 with an affinity higher than that displayed by HEXIM1,[48a,50] Tat has been proposed to use this domain to outcompete HEXIM1 for binding to CycT1. After P-TEFb is captured by Tat, a concomitant conformational change in 7SK snRNA might permit the ejection of HEXIM1 from the snRNP.[51] Another model proposes that the RNA-binding motif within the arginine-rich domain of Tat is responsible for P-TEFb's extraction.[30b] The idea is that Tat can use this motif to interact with the region of 7SK snRNA that is normally contacted by HEXIM1, thus displacing HEXIM1 and forming a transitional HEXIM1-free 7SK snRNP (Figure 3).[30b] After the synthesis of HIV TAR RNA, Tat and P-TEFb could be transferred out of this complex to form the Tat/TAR/P-TEFb-containing complex on the viral LTR. Recently, Tatcom2, a novel Tat/P-TEFb-containing complex that lacks HEXIM1 but contains all of the other 7SK snRNP subunits, has been identified.[52] Detected in cells lacking the TAR RNA, this complex appears to fit the descriptions of a transitional complex that exists between 7SK snRNP and the eventual Tat/TAR/P-TEFb-containing complex (Figure 3).

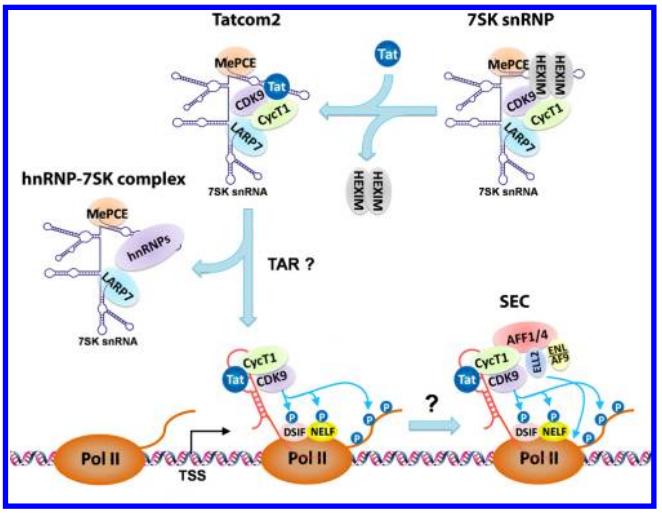

Figure 3. Tat induces transfer of P-TEFb from 7SK snRNP possibly through Tatcom2 to the SEC, where P-TEFb cooperates with ELL2 to synergistically activate HIV LTR transcription. Tat is known to target 7SK snRNP to capture P-TEFb and release HEXIM1. The Tatcom2 complex, whose composition is similar to that of 7SK snRNP except for the substitution of HEXIM1 by Tat, could be a reaction intermediate before the emergence of HIV TAR RNA. Once TAR is produced, P-TEFb and Tat are transferred onto the TAR structure, and through a still unknown mechanism, they nucleate the formation of the multisubunit SEC. In addition to P-TEFb, which phosphorylates the Pol II CTD and negative elongation factors NELF and DSIF to antagonize their inhibitory effects, SEC also contains another well-characterized elongation stimulatory factor ELL2, which directly enhances the catalytic activity of Pol II. By acting on the same Pol II enzyme, P-TEFb and ELL2 synergistically activate HIV transcription.

The exact subnuclear location where Tat extracts P-TEFb from 7SK snRNP has yet to be determined. Although the vast majority of the snRNP appears to exist off the chromatin and can be easily extracted from the nucleus,[53] a small amount was recently detected at the HIV promoter.[54] This latter observation led to the proposition that the Tat–P-TEFb elongation complex is assembled de novo on the HIV template through the Tat–TAR interaction with the concomitant release of P-TEFb from 7SK snRNP.[55] Finally, the process through which Tat extracts P-TEFb from 7SK snRNP might also be assisted by other cellular cofactors. For example, the interaction of Tat with protein phosphatase PP1,[56] which contributes to stress-induced 7SK snRNP disruption through dephosphorylation of Thr186 at the CDK9 T-loop,[56] could conceivably

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help Tat in the extraction process. In agreement with this notion, PP1 has been shown to play a positive role in Tat transactivation and HIV replication.[57]

7. TAT ASSEMBLES SUPER ELONGATION COMPLEX (SEC) TO ACTIVATE HIV TRANSCRIPTION

As discussed previously, Tat can cause the release of P-TEFb from 7SK snRNP. However, it was unclear whether Tat delivers P-TEFb alone or in combination with additional factors to the HIV LTR to stimulate viral transcription. To answer this question, sequential affinity purifications that target HA-tagged Tat, Flag-tagged CDK9, and any protein(s) associated with these two factors in a single complex were performed.[58] This procedure led to the identification of a set of chromosomal translocation partners of the mixed lineage leukemia (MLL) protein, including ELL2, AFF4, ENL, and AF9, all of which are demonstrated transcription factors/cofactors, as novel components of the Tat−P-TEFb complex (Figure 3). Notably, the same set of factors plus several others (e.g., the AFF4 homologue AFF1, the ELL2 homologue ELL1, and components of the polymerase-associated factor complex PAFc) were also independently isolated through a similar affinity-purification scheme that targets HIV Tat alone.[52]

Because the form of CDK9 present in 7SK snRNP has the phosphorylated Thr186 at the tip of the T-loop,[38] it is expected to be catalytically active once captured by Tat from 7SK snRNP. As such, it had long been thought that the Tat−P-TEFb interaction alone is sufficient to activate HIV transcription. The great surprise coming from the identification of several novel partners of the Tat−P-TEFb complex is that Tat recruits not only P-TEFb but also another well-characterized elongation factor, ELL2 or -1 to the viral LTR (Figure 3). (Data obtained in our laboratory suggest that Tat strongly prefers ELL2 over ELL1; N. He and Q. Zhou, unpublished observations.)

Unlike P-TEFb, which stimulates transcriptional elongation by phosphorylating key target proteins (the Pol II CTD, DSIF, and NELF), ELL2/1 directly increases the catalytic rate of Pol II by keeping the 3′ end of the nascent mRNA properly aligned with the catalytic center within the polymerase enzyme to prevent Pol II backtracking.[59] Thus, Tat is able to deliver P-TEFb and ELL2/1, which belong to different classes of elongation factors, within a single complex to the viral LTR, where these two factors can act on the same paused Pol II to synergistically stimulate elongation (Figure 3). This observation has significantly expanded the conventional view of the mechanism of Tat transactivation and also explains why Tat is such a powerful transcriptional activator. Because of the existence of at least two well-established elongation stimulatory factors in a single complex, this novel multisubunit complex is now called the super elongation complex (SEC; Figure 3).

Recent structural and functional analyses of the SEC reveal that AFF1 and AFF4, which are likely present in separate but closely related SECs,[60] function as a central scaffold to mediate the formation of the complexes.[26b,58,61] Detailed mapping studies indicate that AFF4 (likely also the homologous AFF1) employs short hydrophobic regions along its structurally disordered axis to directly bind to and recruit other factors into an SEC.[61] Direct binding partners CycT1, ELL1/2, and ENL or AF9 act as bridging components that link this complex to two major elongation factors, P-TEFb and PAFc. The unique scaffolding properties of AFF4 thus allow dynamic and flexible assembly of multiple elongation factors while connecting the components not only to each other but also to a larger network of transcriptional regulators.

For a powerful elongation factor complex such as an SEC, it would be hard to imagine that it is used only by Tat to stimulate HIV transcription. The fact that all SEC subunits except P-TEFb have previously been reported as MLL fusion partners implies an intimate relationship between the SEC and leukemic pathogenesis. Indeed, at about the same time as the SEC was identified as a cofactor and binding partner of HIV Tat, independent biochemical purifications of several frequently occurring MLL chimaeras such as MLL-AFF1 and MLL-ENL and their interacting molecules have led to the isolation of the same SEC that turns out to be essential for MLL-mediated leukemogenesis.[62] It is believed that this high-order P-TEFb-containing complex, which is biochemically distinct from the MLL histone methyltransferase complex, promotes uncontrolled transcriptional elongation of MLL target genes to induce leukemic transformation.[62]

8. ELL2 STABILIZATION PROMOTES SEC FORMATION AND HIV TRANSCRIPTION

The Tat−SEC interaction, which provides the basis for SEC's original identification, not only allows Tat to recruit the complex to the HIV LTR to activate viral transcription, but also promotes SEC formation by stabilizing ELL2, which is otherwise a highly labile protein.[58] Unlike all of the other SEC subunits, including its close homologue ELL1, ELL2 is stoichiometrically limiting and uniquely regulated at the level of protein stability. Recently, the RING domain protein Siah1 was identified as the specific E3 ubiquitin ligase for ELL2 polyubiquitination and proteasomal degradation.[63] Depletion of Siah1 was shown to promote the formation of SECs and enhance SEC-dependent HIV transcription, whereas overexpression of Siah1 resulted in the degradation of ELL2 and, to a lesser degree, AFF1 and AFF4.[63]

Consistent with an earlier observation that the interaction of ELL2 with the scaffolding protein AFF4 (likely also AFF1) dramatically increases the former's half-life,[58] Siah1 cannot access any ubiquitinate ELL2 that is bound to AFF4, although at high concentrations, it also degrades AFF1/4 to destroy existing SECs.[63] Unlike AFF4, Tat does not appear to act by inhibiting ELL2 polyubiquitination, and the exact mechanism by which it stabilizes ELL2 remains elusive at this moment. There exists a possibility that Tat affects a step downstream of the Siah1-mediated ELL2 polyubiquitination to directly suppress proteasomal degradation of ELL2. In agreement with this idea, Tat has been shown to directly bind to the β subunits of the constitutive 20S proteasome, thereby inhibiting the proteolytic activity of the proteasome in cells.[64]

9. BRD4−P-TEFB INTERACTION AND ITS CONTROL OF HIV TRANSCRIPTION AND LATENCY

Many cellular genes, especially those that function as primary response genes in stimulus-responsive pathways, often contain paused Pol II and negative elongation factors at their promoter-proximal regions before full induction.[65] It has been shown that these genes employ the nuclear protein Brd4 as an adaptor to recruit P-TEFb to their chromatin loci.[65b,66] To these genes, Brd4 serves as the cellular equivalent of HIV Tat to antagonize the inhibitory actions of negative elongation factors and promote Pol II transcriptional elongation in a P-TEFb-dependent manner.

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As a member of the bromodomain and extraterminal domain (BET) protein family, Brd4 has two N-terminal bromodomains that bind to acetylated histones H3 and H4,[67] a P-TEFb-interaction domain (PID) at its C-terminus that interacts with P-TEFb,[68] and a central extraterminal (ET) domain that has been implicated in the interaction with a number of other proteins.[69] Notably, Brd4 remains bound to the chromatin throughout the mitosis.[67a,70] As an epigenetic reader, it is thus able to transmit epigenetic memory across cell divisions by recruiting and depositing P-TEFb onto promoters of key growth-promoting genes prior to the onset of G1.[70,71] In addition to exerting its gene-activating effect through interaction with acetylated chromatin, Brd4 is also known to bind to the mediator complex within the preinitiation complex (PIC), which might further enhance its overall transcriptional activity.[66a,72]

Just as Brd4 plays an important role in recruiting P-TEFb to many cellular gene promoters to release Pol II from pausing, it also produces a positive effect on basal, Tat-independent HIV transcription, although the effect obtained with the stably integrated proviral DNA is generally smaller than that obtained with the transiently transfected HIV LTR reporter construct,[66a,72,73] which cannot assemble into a chromatin template. Thus, the interaction of Brd4 with the mediator complex but not acetyl histones might play an even more prominent role in mediating basal HIV transcription.

In contrast to the stimulatory effect of Brd4 on basal, Tat-independent HIV transcription, Brd4 has been demonstrated to be a potent inhibitor of Tat transactivation.[66a,68] This is because Tat and Brd4 can directly compete with each other for binding to P-TEFb and the Brd4−P-TEFb interaction precludes the more efficient and highly specific recruitment of P-TEFb/SEC to the viral LTR by Tat and TAR (Figure 4A).[66a] As a result of this competition, overexpression of just the PID domain of Brd4 has been shown to antagonize Tat-dependent HIV transcription and latency reactivation.[68]

10. REACTIVATION OF HIV TRANSCRIPTION AND LATENCY BY BET BROMODOMAIN INHIBITORS

Latent reservoirs of HIV are the principal impediment to eradication of infection, as they harbor transcriptionally silent proviruses that can evade immune surveillance and resume replication once the Highly Active Antiretroviral Therapy (HAART) is disrupted.[74] Strategies, generally referred to as "shock and kill", are currently being developed to reactivate latent HIV, which can then be cleared by HAART and the immune system.[75] In devising such strategies, focus has been placed on finding specific and effective ways to reactivate latent HIV without causing generalized T-cell activation. To this end, several laboratories have recently reported that the BET bromodomain inhibitors JQ1 and iBet151, which can competitively bind to the Brd4 bromodomains and displace it from chromatin, efficiently reactivate latent HIV in a variety of cell-line-based latency models.[76]

In-depth analyses reveal that the mechanism of JQ1-inducted HIV latency reactivation is mainly through antagonization of Brd4's inhibition of Tat transactivation (Figure 4B).[73a,77] By displacing Brd4 from the LTR region of HIV chromatin and thereby decreasing Brd4's local concentration at the promoter, JQ1 significantly increases the association of P-TEFb/SEC with Tat.[73a,77] As a result of this enhanced association, more SECs are recruited by Tat/TAR onto the viral LTR to promote HIV transcriptional elongation (Figure 4B).[73a] Further confirming

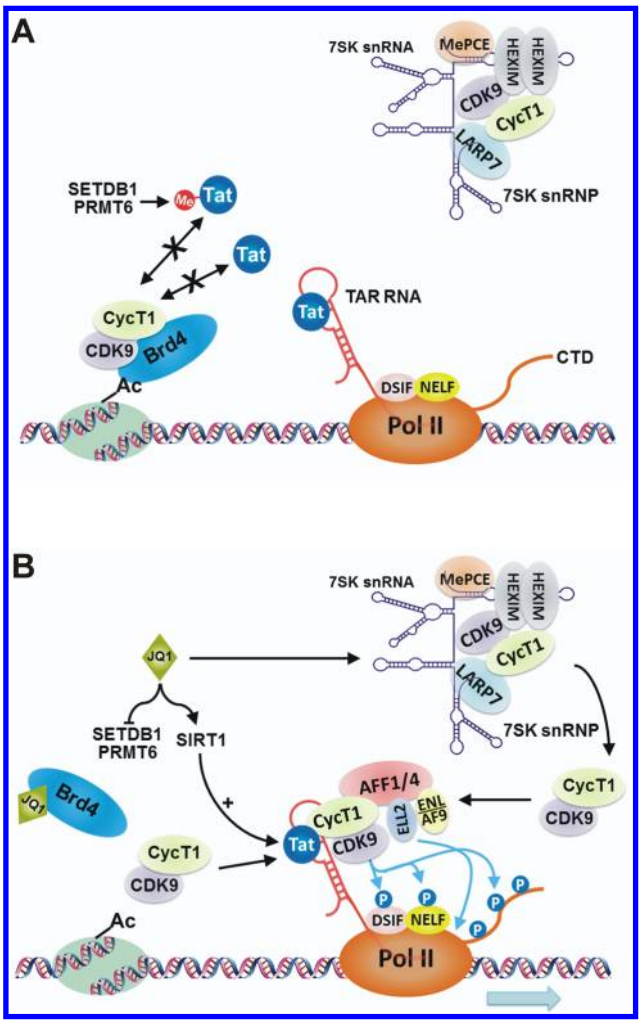

Figure 4. Brd4 is a potent suppressor of Tat transactivation, and BET bromodomain inhibitor JQ1 efficiently antagonizes this suppressive effect. (A) In the absence of JQ1, the promoter-bound Brd4 (by interacting with acetylated histones or Ac) competitively blocks the interaction between P-TEFb and Tat. Likewise, methylation of Tat by SETDB1 and PRMT6 also prevents this interaction. Meanwhile, most cellular P-TEFb is sequestered in the inactive 7SK snRNP. All of these features inhibit the ability of Tat to form on the HIV TAR RNA a functional SEC that is essential for activated viral transcription. (B) JQ1 dissociates Brd4 from the HIV promoter and increases the local concentration of active P-TEFb for Tat to assemble into the SEC for efficient phosphorylation of the Pol II CTD, DSIF, and NELF and activation of productive elongation. Additionally, JQ1 inhibits the expressions of SETDB1 and PRMT6 while promoting the production of SIRT1, which deacetylates Tat to enhance the Tat−P-TEFb interaction. Finally, JQ1 also disrupts 7SK snRNP to release P-TEFb, providing another source of P-TEFb for SEC assembly at the HIV promoter.

the functional significance of the SEC in HIV latency reactivation, the siRNA-mediated knockdown of the key SEC component ELL2 significantly reduces JQ1's activation of the LTR.[73a]

Similarly to JQ1, stable ectopic expression of a CDK9 mutant carrying the Ser175 to Ala mutation (S175A) in latently infected cells has also been shown to induce a robust Tat-dependent reactivation of the provirus.[78] Although S175A destroys CDK9 phosphorylation on Ser175 that is induced by TCR or phorbol ester (PMA) signaling, it only slightly reduced

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the Tat−P-TEFb interaction. In contrast, the binding of Brd4 to P-TEFb is completely blocked by this mutation.[66a,78] Because Brd4 is unable to compete with Tat for binding to CDK9 carrying S175A, just like JQ1, the mutation effectively removes the inhibition by Brd4, thus allowing the Tat-mediated HIV transactivation and latency activation to occur.

In addition to JQ1's direct and prominent enhancement of Tat transactivation, additional activities displayed by this compound might also contribute to its stimulation of HIV transcription. For example, JQ1 has been shown to partially and transiently release P-TEFb from 7SK snRNP (Figure 4B),[73a,79] which could be responsible for its weak activation of basal HIV transcription in the absence of Tat.[73a,77] Although this effect of JQ1 is weaker than that on Tat transactivation, it might help trigger the first few rounds of productive elongation to help build up the cellular Tat level and establish a positive feedback circuit. Another potentially beneficial effect caused by JQ1 is the induction of posttranslational modifications of Tat (Figure 4B).[76] This may be achieved through JQ1's induction of the expression of SIRT1, a deacetylase and positive regulator of Tat, and down-regulation of the expression of methyltransferases SETDB1 and PRMT6, which are two negative modifiers/regulators of Tat (see section 12, seventh paragraph). However, it remains to be determined whether Tat indeed undergoes the implicated changes in the modifications in JQ1-treated cells and, if yes, whether these changes directly contribute to the JQ1-induced HIV latency activation.

It should be pointed out that, in primary CD4+ T cells derived from patients on long-term HAART, the JQ1-induced latency reactivation is relatively inefficient and highly heterogeneous.[76,77] This is likely because JQ1 targets primarily the Tat transactivation step, which depends exquisitely on the availability of the Tat cofactor P-TEFb/SEC, and because the level of the key SEC component P-TEFb is very low in resting CD4+ T cells.[17,80] However, because full latency activation must rely on Tat and its interaction with the SEC, which is strongly promoted by JQ1, future studies will be very informative to reveal whether a cocktail of drugs that contains JQ1 as a key component plus other classes of latency activators can be used to efficiently reactivate HIV latency through overcoming multiple restrictions in resting CD4+ T cells. As a proof of concept of this idea, JQ1 was shown to synergize with prostratin, another well-known latency activator that works by enhancing loading of RNA Pol II onto the HIV promoter, thereby promoting transcriptional initiation, to reactivate latent HIV.[73a] This synergism is detected in both Jurkat T cell-based HIV latency models as well as in pools of resting CD4+ T cells isolated from HIV-infected, HAART-treated patients.[73a,77]

JQ1 has been proposed as an anticancer drug that works by reducing the Brd4-dependent c-myc expression, leading to the induction of differentiation and growth arrest of cancer cells that are addicted to the c-myc oncogene.[81] Consistent with its general antigrowth property, JQ1 has been shown to potently suppress T-cell proliferation with minimal cytotoxic effect.[76] This inability to cause generalized T-cell activation, together with its use of a largely Tat-specific mechanism in reactivating latent HIV, make JQ1 and its derivatives attractive candidates for implementing the "shock" phase of the shock-and-kill strategy to reactivate latent HIV reservoirs for subsequent eradication.[75b]

11. STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF TAT INTERACTIONS WITH ITS PARTNERS

The Tat gene has two exons, and the first one encoding amino acid residues 1−72 is both necessary and sufficient for HIV transactivation. Among the five conserved regions within the first exon, the first three (acidic/proline-rich, cystein-rich/Zn finger, and core) constitute the minimal transactivation domain that is vital for the Tat−P-TEFb interaction. According to the crystal structure of the Tat−P-TEFb complex solved by Tahirov et al.,[55] this 49-amino-acid (49-aa) minimal transactivation domain, which is the only well-resolved portion of the 86-aa recombinant Tat protein in the crystal structure, interacts extensively with residues located mainly in the cleft between the two cyclin box repeats of CycT1 and also extends to partially touch the CDK9 T-loop. Whereas an intramolecular zinc finger coordinated by Cys22, Cys34, Cys37, and His33 is clearly visible within the Tat structure, a proposed intermolecular zinc finger involving Cys261 of CycT1 and Cys25, Cys27, and Cys30 of Tat, which are not conserved in Tat proteins from other species, has been questioned for its physiological relevance.[73b] Nevertheless, as the interaction surface between CDK9 and CycT1 is ∼40% smaller than those in other CDK−cyclin pairs,[82] the extensive Tat−P-TEFb interaction with a surface area twice as large as the average value for stable protein−protein interactions[55,83] explains how Tat stabilizes the P-TEFb complex.[84]

It is interesting to note that, when existing alone in solution without any binding partners, Tat is highly flexible and has no defined secondary structure along most of its sequence.[85] Only upon binding to P-TEFb does Tat obtain its defined structure.[55] In fact, Tat adopts a conformation that is highly complementary to the surface of P-TEFb. In return, Tat also induces significant conformational changes in P-TEFb,[55,82] leading to the establishment of a more active kinase complex.[84]

As a key regulatory protein encoded by a small virus, the flexibility of Tat and its dependence on host partners to adopt defined structures can be advantageous in several respects. First, they can allow Tat to interact with more than one host factor complex and thus facilitate the transfer of Tat from one complex (e.g., Tatcom2[52]) to another (e.g., the SEC[58]) at different stages of HIV transactivation. Second, they are economical to the relatively small HIV genome, which does not need to encode an exceptionally long polypeptide that might be necessary to stabilize the Tat structure.[86] Finally, they afford Tat a relatively high tolerance to sequence variations (up to 40%) without losing transactivation activity.[85]

As the Tat−P-TEFb complex is only a nonphysiological subcomplex of the larger functional Tat−SEC assembly recruited to the HIV promoter for transactivation, it is important to determine how the interactions between P-TEFb and other SEC subunits impact the Tat−P-TEFb interaction. As an important step toward this goal, the crystal structure of a tripartite complex containing the recognition regions of P-TEFb and AFF4, which functions as a scaffold in the SEC, was recently solved.[87] AFF4 was shown to use the Leu34−Ile66 segment to meander over the surface of CycT1 on the opposite side of the CDK9-binding site, thus making no direct contact with CDK9 (Figure 5). Interface mutations were found to reduce CycT1 binding and AFF4-dependent HIV transcription. Although Tat is not present in the structure, a unique intersubunit pocket created cooperatively by AFF4 and

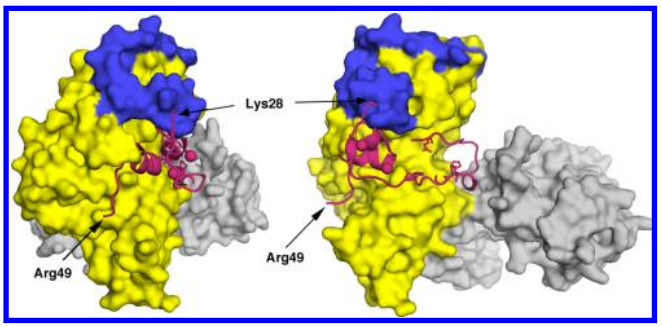

Figure 5. HIV Tat and SEC component AFF4 are predicted to make direct contacts on CycT1. This superposition of the crystal structures of the AFF4–P-TEFb complex and the Tat–P-TEFb complex using the CycT1 subunit (yellow) shows the close proximity of AFF4 (blue) and Tat (red). The resulting Tat–AFF4–P-TEFb complex model is shown in two different orientations that are rotated by about 90° (CDK9 is in gray). The side chains of Tat are from only those residues that are known to have an effect on transcription when mutated and that do not have any identified binding partner or structural function. Lys28, which is well-known for its influence on complex stability upon acetylation, is engaged in the AFF4 interaction in this model but exposed to solvent in the Tat–P-TEFb complex. Arg49, which is the last visible Tat residue in the structure, indicates where the RNA binding domain will be located. (Figure courtesy of Ursula Schulze-Gahmen and Tom Alber of UC Berkeley.)

CycT1 would provide an excellent fit for Tat (Figure 5). Consistent with this prediction, Tat was shown to significantly increase the affinity of AFF4 for P-TEFb by more than 10-fold, which could be the reason for Tat to recruit the whole SEC but not P-TEFb alone to the viral LTR. Interestingly, one particular residue in Tat that is predicted to directly contact AFF4 is Lys28 (Figure 5), which was previously reported to be important for the formation of the Tat–TAR–P-TEFb complex.[88] As Lys28 is strictly conserved and exquisitely regulated by reversible acetylation,[73b,89] it will be interesting to investigate whether this residue plays a key role in Tat-mediated recruitment of the SEC to TAR as well as termination of the TAR-dependent phase of Tat transactivation (reviewed in section 12, sixth paragraph).

At this point, none of the published structures of human P-TEFb and P-TEFb-containing complexes contains the HIV TAR RNA.[55,82,87] Although the arginine-rich motif (ARM) region of Tat was not visible in the solved Tat–P-TEFb structure,[55] it is safe to predict that this region will likely adopt a well-defined conformation once bound to the recognition region in TAR. Indeed, a recent crystal structure of the tripartite ribonucleoprotein complex formed by EIAV Tat and TAR and the corresponding equine CycT1 strongly supports this notion.[90] In this structure, both the C-terminal part of EIAV Tat (amino acids 41–69) that encompasses the ARM as well as the associated TAR RNA were resolved. Whereas the core and the C-terminal hydrophobic regions of EIAV Tat wrap around the surface of equine CycT1 at the first cyclin box repeat, the ARM, which lies between the two Tat regions, interacts with the major groove of TAR RNA. The interactions are largely electrostatic in nature and involve the basic residues in the Tat ARM and the negatively charged phosphate backbone of TAR. These electrostatic interactions, although not expected to afford high selectivity among the various TAR species of different base pair compositions, can still be modulated through reversible acetylation of specific lysine residues in the ARM to alter its charge properties (reviewed in section 12, third paragraph). Finally, in the tripartite structure formed by EIAV Tat/TAR and equine CycT1, the TAR displays a stable six-nucleotide hairpin-loop, with the fifth nucleotide directly contacting CycT1, which contributes to the specificity of the overall interaction.[90]

Although much progress has been made toward the structural and functional characterization of the interactions of Tat with a subset of its binding partners, the more challenging and also urgent task remaining is to solve the structures of several complete macromolecular assemblies, such as 7SK snRNP, Tatcom2, and the Brd4–P-TEFb and Tat–TAR–SEC complexes, that are essential for the proper function of Tat. Obtaining such structures will provide unprecedented insights into the molecular mechanisms by which Tat and its cofactors activate HIV transcription. It might also reveal novel druggable targets and assist the design of small-molecule inhibitors that can specifically target the Tat-associated transactivation machinery with minimal cytotoxic side effects.

12. POSTTRANSLATIONAL MODIFICATIONS OF TAT AND THEIR EFFECTS ON HIV TRANSCRIPTION

Given its central role in HIV transactivation, it is not surprising to see that the activity of Tat is elaborately controlled by posttranslational modifications that include methylation, acetylation, phosphorylation, and polyubiquitination (Figure 6). Most of these modifications are located in the ARM of Tat and can influence the formation or disruption of the Tat–TAR–SEC complex. Notably, many of these sites are targeted by both the "writing" and "erasing" enzymes, which permit the fine-tuning of Tat function during different phases of HIV transactivation. Interestingly, many of the Tat modification

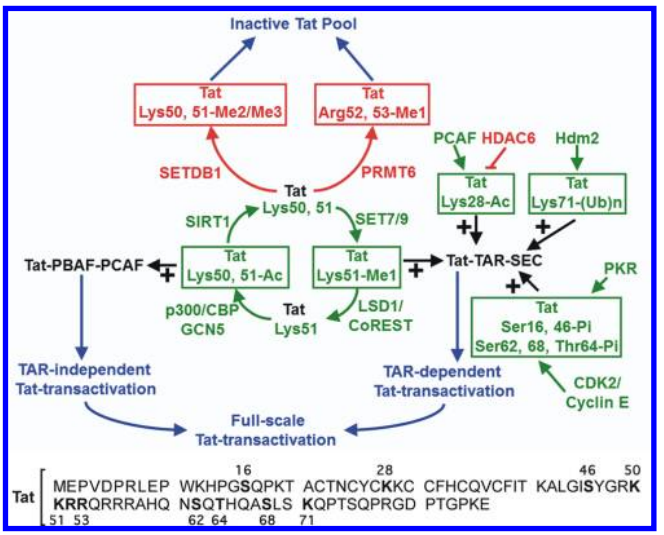

Figure 6. Posttranslational modifications of Tat and their effects on HIV transcription. The modifications (modified residues shown in boxes) can be classified into two types based on their impact on Tat's transactivation activity: positive (green) and negative (red). The physiological consequences of the modifications during different phases of the HIV and Tat transactivation cycles are displayed in blue. The black arrows accompanied by a plus (+) sign indicate that the modifications promote the formation of the indicated complexes. Me1, Me2, and Me3 denote mono-, di-, and trimethylation, respectively. Ac, acetylation; Pi, phosphorylation; (Ub)n, polyubiquitination. The amino acid sequence of the 86-aa form of HIV-1 Tat is shown at the bottom with the modified residues indicated in boldface type and their positions shown above and below the sequence.

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enzymes are also involved in histone modifications, thus linking the control of Tat activity to the chromatin status of an integrated HIV provirus.

The most heavily modified residue in Tat is Lys51 (Figure 6), which plays a central role in the interaction with HIV TAR RNA.[90] Monomethylation of Lys51 by SET7/9 (KMT7) enhances Tat activity likely by strengthening the Tat−TAR interaction.[90,91] Because SET7/9 itself has been shown to bind to TAR,[91] its proximity to Tat might allow for efficient methylation of Lys51 to facilitate the recruitment of the Tat−SEC complex to the TAR. Interestingly, whereas monomethylation of Lys51 is an activation marker for Tat, removing this methyl group by the lysine-specific demethylase LSD1 (KDM1) and its cofactor CoREST is apparently also required for the full transactivating function of Tat, as well as the reactivation of latent HIV.[92] To reconcile these seemingly contradictory observations, it has been proposed that Tat transactivation is actually composed of two distinctive phases, namely, the TAR-dependent early phase and the subsequent TAR-independent phase.[73b] In the TAR-dependent phase, Lys51 is monomethylated by SET7/9 to facilitate the recruitment of the Tat−SEC complex to the TAR RNA. Subsequently, LSD1 and CoREST demethylate the same lysine to facilitate the dissociation of the Tat−TAR−SEC complex and prepare Tat for acetylation at Lys51, which are important for the subsequent TAR-independent phase of Tat transactivation (Figure 6).[73b] During this second phase, Tat is proposed to directly interact with Pol II[93] and recruit the histone acetyltransferase (HAT) PCAF and chromatin remodeling complex PBAF to generate a further relaxed chromatin environment in a TAR-independent manner. Thus, Lys51 acts as a molecular switch, with its monomethylation promoting the Tat−TAR−SEC formation and acetylation enhancing the Tat−PBAF-PCAF assembly (Figure 6).

To efficiently disrupt the Tat−TAR−SEC complex, upon the demethylation of Lys51, Lys51 and its neighboring Lys50 must be acetylated by acetyltransferases p300/CBP (KAT3B) or GCN5 (KAT2A) (Figure 6).[89,94] The acetyl groups neutralize the positive charges in the highly basic ARM to dissociate Tat from TAR. This step is critical to terminate the TAR-dependent phase and enable the transition into the TAR-independent phase.[95] Of note, Lys50 acetylation also facilitates the recruitment of chromatin-modification transcriptional coactivators such as PCAF (KAT2B) and the SWI/SNF complex PBAF onto Tat.[96]

Just as for the demethylation of Lys51, the deacetylation of Lys50 by sirtuin 1 (SIRT1) is also required for the full transactivating function of Tat.[91] A possible reason for this requirement is that the deacetylation happens toward the end of productive transcription to recycle Tat back to the unacetylated state so that it can bind to TAR/SEC again and start a new round of transactivation.[73b] Paradoxically, the overexpression of SIRT1 was found to significantly hinder Tat transactivation in latently infected cells.[97] This is probably because the Tat level is very low at the beginning of latency reactivation and too much SIRT1 activity will interfere with the TAR-independent Tat transactivation step and disrupt the establishment of the positive Tat feedback circuit that is critical for latency reactivation.[97]

These observations concerning reversible modifications at Lys50 and Lys51 indicate that the activity of Tat is elaborately regulated by both writers and erasers of posttranslational modification marks. The functions of individual modification enzymes cannot be simply classified as positive or negative with regard to the overall Tat transactivation and latency reactivation process. Rather, they should be viewed as indispensible components of a complete, interrelated regulatory network (Figure 6) that exerts proper control of HIV transcription in response to changing conditions and viral replication status within an infected cell.

Similarly to Lys50 and Lys51, Lys28, which is located in the cysteine-rich region of Tat, can also be acetylated. Catalyzed by PCAF (KAT2B), the acetylation of Lys28 enhances the binding of Tat to P-TEFb/SEC and TAR[89,96a] to promote Tat transactivation. Unlike the deacetylation on Lys50, deacetylation on Lys28 by HDAC6, a class II HDAC also responsible for deacetylating α-tubulin,[98] inhibits Tat transactivation as it destabilizes the Tat−TAR−P-TEFb/SEC complex.[99] The interaction between HDAC6 and Tat appears to depend on microtubules,[99] and the binding of Lys28-acetylated Tat to tubulin/microtubules perturbs microtubule dynamics, leading to apoptosis of T lymphocytes.[100] Because HDAC6 can suppress both Tat transactivation and Tat-induced apoptosis, it might serve as a potent host cell antagonist of HIV/AIDS.

In addition to the already mentioned reversible modifications, Tat is also subject to posttranslational modifications for which no erasers have been identified. These modifications span across the entire length of Tat and include di-/trimethylation, phosphorylation, and polyubiquitination. For example, Lys50 and Lys51 can be di- or trimethylated by SETDB1 (KMT1E).[101] Likewise, the neighboring Arg52 and Arg53 can also be methylated by another methyltransferase, PRMT6 (Figure 6).[102] There is currently no enzyme that can erase these modifications. Located in the Tat ARM region, these methylation events interfere with the formation of the Tat−TAR−P-TEFb/SEC complex probably through steric hindrance.[90] Of note, the methylations on Arg52 and Arg53 have an additional role in increasing the stability of Tat,[103] suggesting that they might serve to tag a stable pool of inactive Tat in latently infected cells.

HIV Tat can also be phosphorylated by the PKR kinase and the CDK2/cyclin E kinase complex.[104] PKR phosphorylates Ser62, Thr64, and Ser68, which results in faster and stronger binding of Tat to TAR and promotes Tat transactivation.[104a] CDK2/cyclin E phosphorylates two highly conserved Tat residues Ser16 and Ser46, and this event is reported to be important for the expression from a transiently transfected HIV LTR-LacZ reporter construct and viral replication in a HeLa-based proviral system.[104b]

Polyubiquitination is frequently used as a tag that marks a protein for degradation by the proteasome. However, in the case of Tat, this particular modification has been shown to promote the transactivating function of Tat without inducing Tat degradation.[105] The polyubiquitination appears to be catalyzed by the proto-oncoprotein Hdm2, which interacts with Tat and functions as a specific E3 ligase to induce ubiquitination on Lys71 (Figure 6). At this moment, the mechanism by which the polyubiquitinated Tat enhances HIV transactivation is unclear. By targeting and dissociating the 26S proteasome, Tat has been shown to activate HIV transcription in a manner that does not depend on the proteolytic activity of the proteasome.[106] It is tempting to speculate that the polyubiquitinated Tat in association with key proteasomal components might play a key role in this process.

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13. EPIGENETIC REGULATION OF HIV TRANSCRIPTION

In addition to the central role that the Tat−TAR−SEC axis plays in overcoming the restriction on Pol II elongation, HIV transcription is also subjected to epigenetic regulation. The types of regulation directly affect the compactness of the HIV chromatin, thus altering the accessibility of a provirus to general transcription factors/cofactors and transcriptional regulators, which in turn determines whether the provirus enters the repressive or active transcription state. In the context of HIV transcription, the epigenetic regulation can be divided into two categories: (1) covalent modifications that include histone acetylation, histone methylation, and DNA methylation and (2) chromatin remodeling that moves nucleosomes around relative to key binding sites in a ATP-dependent manner. One particular nucleosome called Nuc-1, which is located ∼50 bp downstream of the HIV transcription start site (TSS), plays a central role in blocking Pol II transcription of the provirus.[96c,107] Thus, most types of epigenetic regulation that affect the transcriptional state of the HIV provirus frequently target this nucleosome.

Histone deacetylation is known to induce the compact chromatin structure that represses the recruitment and assembly of active transcriptional machinery at the promoter. In the case of HIV, two transcriptional repressors, LSF and YY-1, have been reported to interact with the Nuc-1 region to recruit histone deacetylase 1 (HDAC-1) to the viral promoter and repress transcription.[108] In addition to LSF and YY-1, a number of other proteins can also recruit HDACs to different regions of the HIV LTR. For example, the homodimer formed by the NF-κB p50 subunit binds to the NF-κB binding sites located in the HIV enhancer region, leading to the recruitment of HDAC1 to the LTR and inhibition of viral transcription.[109] Consistently, the RNAi-mediated p50 knockdown reduces HDAC1 binding to the LTR, which in turn results in enhanced recruitment of Pol II and reactivation of latent HIV in J-Lat 6.3 cells.[109] Upon stimulation by the pro-inflammatory cytokine TNF-α, the NF-κB p50/p65 heterodimer displaces the p50 homodimer and activates HIV transcription.[110]

As a transcriptional repressor in the Notch signaling pathway, CBF-1 and its cofactors CIR and mSIN3A can also bind to the NF-κB binding sites in the viral LTR and recruit HDACs.[16,111] In a latency model based on primary CD4+ T cells, CBF-1, CIR, and mSin3A, together with HDAC1 and other markers of restrictive chromatin, were all detected on the proviral chromatin before induction.[16] The induction with the α-CD3/CD28 antibodies substantially decreased the levels of CBF-1, CIR, mSIN3A, and HDAC-1, but increased the level of Pol II at the promoter. Further expanding the list of sequence-specific transcription factors that are capable of recruiting HDACs to the HIV LTR, both HDAC1 and HDAC2 can also be recruited by the corepressor COUP-TF interacting protein (CTIP2) by binding to the Sp1 elements in the LTR.[108b,112] Finally, several other transcription factors such as AP-4, c-Myc, and Sp1 can also recruit HDAC1 to the HIV-1 promoter.[113] Taken together, these studies indicate the recruitment of HDACs to the viral promoter to repress transcription and establish latency as a common property shared by many transcription factors that interact with the enhancer or promoter-proximal region of the HIV LTR.

Whereas histone deacetylation contributes to the repressive chromatin structure, acetylation of histones produces the opposite effect. As for HIV, histone acetylation at the viral promoter is associated with induced latency reactivation. Various stimuli can induce the accumulation of the NF-κB p65 subunit in the nucleus, which results in the formation of the p50/p65 heterodimer, recruitment of cellular HATs such as p300/CBP, PCAF, and GCN5 to the viral enhancer region, acetylation of chromatin, improved accessibility for Pol II, and eventually activation of HIV transcription initiation.[114] The recruitment of HATs can, in turn, stabilize NF-κB on the LTR, as acetylation of p65 increases NF-κB's DNA-binding affinity.[115] In addition to NF-κB, several other transcription factors such as NFAT, GR, C/EBP, LEF-1, IRF, Ets-1, AP-1, c-Myb, and Sp1 can also recruit HATs onto the LTR to activate transcription initiation.[114c,116] Finally, in addition to these cellular transcription factors, HIV Tat has also been reported to recruit p300 and PCAF to the LTR through binding to the TAR RNA, which might further contribute to its ability to activate the integrated provirus.[114d]

The initiation of HIV transcription is also repressed by methylation of histone H3 such as di- or trimethylation on Lys9 (H3K9me2 or H3K9me3) and trimethylation on Lys27 (H3K27me3).[4c,112b,117] Histone lysine methyltransferase (HKMT) SUV39H1 is responsible for H3K9me3, which is frequently associated with heterochromatin formation and serves as a platform to recruit the chromodomain protein HP1γ.[117a] Of note, SUV39H1 can also interact with CTIP2, which is able to recruit HDAC1 and HDAC2 to the HIV LTR as mentioned above. Thus, by enforcing two kinds of repressive histone modifications of the HIV chromatin, the SUV39H1-CTIP2 interaction cooperatively contributes to the establishment of a highly repressive chromatin structure.[112b] Whereas H3K9me3 is generated by SUV39H1, H3K9me2 is induced by another HKMT called G9A. Similarly to H3K9me3, H3K9me2 also contributes to the maintenance of HIV latency as demonstrated by the treatment with BIX01924, a specific inhibitor targeting G9A, which caused the virus to exit latency.[117b] Finally, the HKMT responsible for H3K27me3 is a nuclear protein called EZH2, which is a component of the polycomb repressive complex 2 (PRC2).[118] Knockdown and specific inhibition of EZH2 show that it appears to play an even more important role in maintaining HIV latency than do SUV39H1 and G9A and, thus, might serve as a central regulator of HIV epigenetic silencing.[118]

Similarly to the methylation of histone H3 mentioned previously, DNA methylation is also a repressive mark on the HIV promoter and detected on cytosine residues in the two CpG islands flanking the HIV transcription start site (TSS) in latently infected Jurkat and primary CD4+ T cells.[119] The methyl-CpG binding domain protein 2 (MBD2) binds to the second CpG island and recruits HDAC2 to help maintain the repressive chromatin structure during latency.[119] Inhibition of cytosine methylation by 5-aza-2′-deoxycytidine (aza-CdR) has been shown to abrogate the recruitment of MBD2 and HDAC2. As such, aza-CdR can synergize with prostratin or TNF-α to reactivate latent HIV.[119] Further supporting the role of CpG methylation in HIV latency, it has been found that, in the latent reservoirs of HIV-infected individuals with non-detectable plasma viremia, the HIV promoter and enhancer are hypermethylated and resistant to reactivation, as opposed to the hypomethylated 5′ LTR in viremic patients.[120] Finally, implicating a strong correlation between DNA methylation and histone methylation and deacetylation in maintaining HIV latency, a latently infected Jurkat cell line with densely

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methylated HIV promoter displays much higher level of H3K27me3 and is more efficiently reactivated by the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) than other cell lines containing latent proviruses with little or no 5′ LTR DNA methylation.[120]

SWI/SNF is an ATP-dependent multisubunit nucleosome-remodeling complex with two key subunits Brg-1 and Brm that possess ATP-dependent nucleosome remodeling activities. It can exist in two different forms: PBAF with the defining subunits Brd7, BAF180, and BAF200 and BAF with the defining subunit BAF250a (ARID1a). Both forms participate in HIV transcriptional control albeit with opposing functions: Whereas BAF represses HIV transcription initiation by positioning Nuc-1 downstream of the TSS,[121] PBAF enhances initiation by removing Nuc-1.[96c,122] Suggesting that these two SWI/SNF complexes of opposing activities might function at different stages of the HIV life cycle, upon activation of HIV transcription, BAF dissociates from the LTR region, and PBAF is then recruited by the Lys50-acetylated Tat to the promoter to stimulate transcription (Figure 6).[121,123] In addition to inducing a more relaxed chromatin conformation, the acetylated histones at the HIV promoter, especially those induced by the Tat-recruited PCAF,[114d] might also serve to stabilize PBAF complex on HIV promoter through binding to the bromodomain of PBAF's component Brg-1.[124]

14. FUTURE PERSPECTIVES

Over the past 15 years or so, remarkable advances have been made toward the elucidation of the mechanism and factors that regulate HIV transcriptional elongation. Among these, the identification of P-TEFb as a key host cellular cofactor for Tat transactivation, the isolation and in-depth analyses of several P-TEFb-containing complexes and their impact on HIV transcription, and the determination of crystal structures of Tat in complex with its key binding partners are some of the highlights that have provided unparalleled molecular insights into the intricate control of HIV transcription.

As expected, these advances have raised new questions even though some of the old ones still remain unanswered. For example, high-resolution structures of the various P-TEFb-containing complexes are yet to be determined to reveal how P-TEFb activity is regulated in these complexes and what triggers P-TEFb's transfer from one complex to the other. It is also important to investigate whether the assembly, stability and function of these complexes are regulated by posttranslational modifications, of which only a small number have been identified and studied. Moreover, structural and functional analyses are yet to be performed to determine how HIV Tat and TAR interact with the complete SEC and whether these interactions present any useful therapeutic targets for developing novel antiviral drugs. Finally, critical epigenetic controls of the HIV chromatin and their precise roles in contributing to the establishment, maintenance and termination of viral latency remain to be further elucidated. Judging by the accelerated rate of discovery in this field, answers to some of these questions will undoubtedly be obtained within the next few years, which will provide exciting opportunities to design novel strategies to eradicate latent HIV reservoirs and achieve a real cure for HIV/AIDS.

AUTHOR INFORMATION

Corresponding Author

*E-mail: qzhou@berkeley.edu.

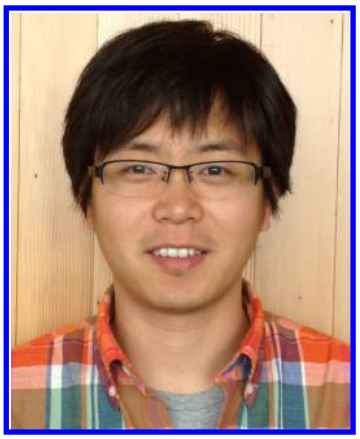
Author Contributions

[§]H.L. and Z.L. contributed equally to this article.

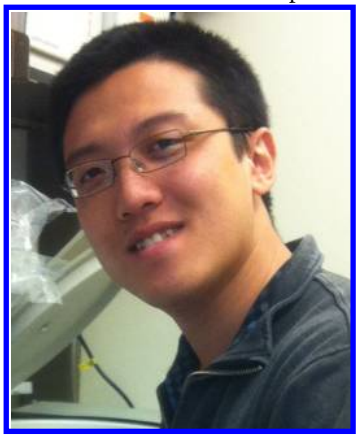
Notes

The authors declare no competing financial interest.

Biographies



Huasong Lu received his B.S. degree in Bioinformatics in 2009 from Zhejiang University, Zhejiang, China, and his M.S. degree in Marine Biology in 2012 from the Chinese Third Institute of Oceanography, Xiamen, China, where he studied the invertebrate innate immune response to pathogen infection. After that, he worked as a Research Specialist in the Department of Molecular and Cell Biology at UC Berkeley for 2 years. Huasong is currently pursuing his Ph.D. degree in the School of Pharmaceutical Sciences, Xiamen University, where his research focuses on the molecular mechanism utilized by the HIV-encoded Tat protein to stimulate viral transcriptional elongation.



Zichong Li obtained his B.S. degree from Laiyang Agricultural College in 2006 and his M.S. degree from Xiamen University in 2010. Initially joining Dr. Qiang Zhou's laboratory as a visiting scholar in 2010, he is presently a graduate student in the Department of Molecular and Cell Biology at UC Berkeley. His research interest lies in the regulation of super elongation complexes (SECs) and the transfer of elongation factors between different complexes. In 2012, he was among the first to identify the effect of a newly identified epigenetic drug, JQ1, on the reactivation of HIV latency. His data show that the host transcription factor Brd4 is a previously overlooked inhibitory factor of the HIV-encoded Tat protein and that JQ1 antagonizes Brd4's inhibitory action to reactivate HIV latency. This discovery might contribute to the

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design of "shock-and-kill" approaches to purge latent HIV reservoirs from infected patients.

Yuhua Xue obtained her B.S. degree from Langfang Normal University in 2004. She subsequently attended Xiamen University, Xiamen, China, to pursue graduate study. Since 2007, she has served first as a visiting student and then as a postdoctoral researcher at UC Berkeley, where she completed a project investigating how the two P-TEFb-associated factors MePCE and LARP7 control the formation of the human 7SK snRNP, which plays a key role in regulating HIV gene expression. In 2011, she joined the School of Pharmaceutical Sciences at Xiamen University as an assistant professor, where she is studying the interaction between HIV and its human host cells at the molecular level and identifying small-molecule drugs that can block this interaction.

Qiang Zhou obtained his B.S. degree from the University of Science and Technology of China in 1986. Supported by the prestigious CUSBEA fellowship, he then pursued graduate study at UCLA. He was among the first to purify and characterize the general transcription factor TFIID, a multisubunit complex required for RNA polymerase II transcription initiation. Supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research, he received postdoctoral training from 1993 to 1996 in Dr. Phillip Sharp's laboratory at MIT, where he studied the mechanism by which the HIV-encoded Tat protein stimulates viral transcriptional elongation and identified and characterized a nuclear protein called Tat-SF1 as a key contributor to this process. In 1997, he joined the Department of Molecular and Cell Biology (MCB) at UC Berkeley, where he is presently a full professor. Dr. Zhou's main research interest lies in the investigation of viral–host interactions at the molecular level, with a special emphasis on mechanisms and host factors that control HIV transcription and latency. His research has made important contributions to the elucidation of the mechanisms that control both cellular and HIV transcriptional elongation. His laboratory was the first to discover that most of P-TEFb, a key human transcription elongation factor and HIV Tat cofactor, is normally sequestered in a catalytically inactive state in 7SK snRNP. He identified 7SK snRNA as well as HEXIM1/2 and LARP7 as key subunits of 7SK snRNP and determined their roles in inhibiting CDK9 kinase activity, maintaining 7SK snRNP integrity and suppressing cellular transformation. In addition, he was also the first to identify the bromodomain protein Brd4 as a key factor in recruiting P-TEFb to many cellular promoters as well as the HIV LTR in the absence of Tat. Recently, his laboratory uncovered a connection between HIV Tat and super elongation complexes (SECs) and showed that Tat recruits SECs to the viral LTR, where SEC subunits P-TEFb and ELL2 synergistically activate HIV transcription. This result has expanded the conventional view of the mechanism of Tat transactivation.

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