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Review

Transcriptional control of HIV latency: Cellular signaling pathways, epigenetics, happenstance and the hope for a cure



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ABSTRACT

Replication-competent latent HIV-1 proviruses that persist in the genomes of a very small subset of resting memory T cells in infected individuals under life-long antiretroviral therapy present a major barrier towards viral eradication. Multiple molecular mechanisms are required to repress the viral transactivating factor Tat and disrupt the regulatory Tat feedback circuit leading to the establishment of the latent viral reservoir. In particular, latency is due to a combination of transcriptional silencing of proviruses via host epigenetic mechanisms and restrictions on the expression of P-TEFb, an essential co-factor for Tat. Induction of latent proviruses in the presence of antiretroviral therapy is expected to enable clearance of latently infected cells by viral cytopathic effects and host antiviral immune responses. An in-depth comprehensive understanding of the molecular control of HIV-1 transcription should inform the development of optimal combinatorial reactivation strategies that are intended to purge the latent viral reservoir.

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Introduction

Potent combination antiretroviral therapy (HAART) can effectively block viral replication in the host and reduce the circulating virus to undetectable levels. Although patients adhering to the HAART regimen have minimal viremia, HIV persists due to the existence of latent but replication-competent proviruses in a very small population of resting memory CD4⁺ T cells (~1 in 10⁶ cells) in peripheral blood (Finzi et al., 1999) and tissues (Yukl et al., 2010). Viral persistence during antiretroviral therapy has also been attributed to ongoing low-level viral replication in anatomical sanctuary sites (e.g. gut-associated lymphoid tissues, other lymphoid tissues, genital tract and the central nervous system) that may be comparatively inaccessible to drugs (Cory et al., 2013; Palmer et al., 2011).

In virtually all patients, when HAART is interrupted circulating HIV levels rebound within 2 to 8 weeks and uncontrolled replication resumes (Chun et al., 1999; Stöhr et al., 2013) although a few rare patients are able to spontaneously control their infections (Sáez-Cirión et al., 2013). However, residual virus recovered from treated patients (Brennan et al., 2009), and the rebounding virus recovered during the short treatment interruptions (Joos et al., 2008), have much less sequence heterogeneity than seen in actively replicating viral populations, suggesting that replenishment of the viral population arises from reactivation of a very restricted set of latently infected cells. HIV-1 subsets in the GALT of patients under HAART initiated at the acute phase have low diversity, suggesting highly restricted viral replication in this anatomical compartment (Evering et al., 2012).

HIV-1 infects CD4⁺ T lymphocytes, and to a lesser extent, myeloid cells including perivascular macrophages, microglia, astrocytes and dendritic cells in the brain. Both T-cells and myeloid cells can establish latent infections (Marban et al., 2007; Wires et al., 2012). Latently infected T-cells are usually established during the acute phase (Chun et al., 1998), are undetectable by host immune surveillance, are extremely stable ($t_{1/2}$ ~44 months) (Siliciano et al., 2003, 2007), and can be replenished through homeostatic proliferation (Chomont et al., 2009).

The latent viral reservoir has therefore proven to be the major obstacle towards finding a sterilizing cure or even a functional cure for HIV-1 infection. As part of current efforts to identify a broad-based cure for HIV infection, research has focused on identifying drugs capable of inducing latent HIV reservoirs without causing deleterious host systemic inflammatory responses (“shock”). Reactivation of proviruses in the presence of HAART is expected to make them visible to the immune system and lead to the clearance of latently infected cells (“kill”). For instance, a recent promising study by Shan et al. demonstrated that latently infected resting CD4⁺ T cells treated with the histone deacetylase inhibitor SAHA to reactivate proviral gene expression were susceptible to efficient killing *in vitro* by HIV antigen-specific CD8⁺ T cells (Shan et al., 2012).

This article will review our knowledge of these intricate mechanisms involved in the establishment and maintenance of latent HIV infections and how these insights are informing the development of therapies that are intended to purge latent HIV reservoirs. Over the last 20 years numerous factors involved in the

regulation of HIV transcription have been identified. To help the reader, Table 1 provides a glossary and overview of the key factors referred to in this review.

Regulation of HIV transcription by Tat

Control of latency by the Tat feedback mechanism

There are no specific repressors of proviral transcription encoded by HIV. Instead, the entry into latency is a consequence of multiple cellular restrictions at the promoter and elongation phase of viral transcription that ultimately repress expression of the viral trans-activating factor Tat and disrupt the positive regulatory Tat feedback circuit (Fig. 1) (Karn, 2011). In particular, transcriptional silencing of proviruses via epigenetic and non-epigenetic mechanisms, and regulation of the essential Tat-cofactor P-TEFb, are critical for the establishment and maintenance of latent infections.

The vast majority of productive HIV infections occur in activated, or at least partially activated, T-cells. Activated effector CD4⁺ T cells have an intracellular environment that makes them highly permissive for productive infection by HIV (Siliciano and Greene, 2011), however, the transition of infected effector cells to a resting memory phenotype substantially diminishes transcription initiation at the HIV LTR which enables the establishment of transcriptionally silent proviruses. Following re-stimulation, latently infected resting memory CD4⁺ T-cells convert to a permissive intracellular environment with high levels of P-TEFb and transcription initiation factors in the nucleus. The central role of the Tat feedback regulatory mechanism to reactivate HIV from latency is clearly demonstrated by two observations: First, expression of Tat *in trans* from an ectopic promoter prevents viral entry into latency (Pearson et al., 2008); and second, latent proviruses encoding a functionally defective Tat mutant (C22G) can initiate transcription following T-cell activation but are absolutely defective for viral gene expression (Kim et al., 2011).

Structure and function of the proviral long terminal repeat

The U3 and R regions of the proviral 5' long terminal repeat (LTR) act as the viral promoter. Although both the 5' LTR and 3' LTR have identical sequences, concomitant transcription from the 3' LTR is highly inefficient due to interference of its U3 region by positive-sense transcribing complexes that terminate RNA synthesis at the R/U5 junction (Cullen et al., 1984; Gallastegui et al., 2011).

The viral core promoter comprises three tandem Sp1 binding sites, a TATA box, and an initiator element at the transcription start site. The enhancer and modulatory regions of the LTR contain *cis*-acting recognition elements for regulatory cellular transcription factors. Specifically, NF-κB p65/p50 heterodimers, NFAT, STAT5 and AP-1 have been identified as signal-dependent positive regulators of HIV transcription initiation (Kinoshita et al., 1997; Nabel and Baltimore, 1987; Selliah et al., 2006; Yang et al., 1999).

Immediately downstream of the transcription start site is the TAR sequence which encodes an RNA stem-loop structure that is directly bound by Tat in order to mediate recruitment of a cellular

Table 1
Host factors involved in the regulation of HIV proviral transcription.

Factor or complex	Description	Reference
Initiation		
Sp1	Required for assembly of the pre- initiation complex at the LTR TATA box. Co-operatively interacts with NF-κB p65/p50	Perkins et al. (1993)
TFIIH	Component of the pre-initiation complex whose recruitment to the HIV LTR is NF-κB-dependent. Stimulates RNAPII to clear the promoter and initiate transcription	Kim et al. (2006)
NF-κB (p65/p50)	Central to the signal-dependent activation of HIV transcription initiation. Enables recruitment of histone acetyltransferases (HATs) to the LTR	Nabel and Baltimore (1987) Gerritsen et al. (1997)
NFAT	Calcium-dependent activator of HIV transcription initiation. NFAT recognition sequences on the viral promoter overlap with NF-κB elements	Chan et al. (2013) Giffin et al. (2003)
AP-1	Nuclear complex of c-fos and c-jun that is formed upon T-cell activation and can directly bind the HIV LTR and enhance NF-κB activity	Yang et al. (1999)
Repression		
LSF/YY1	Cooperative recruitment of LSF and YY1 to the LTR enables repression via recruitment of HDAC1	He and Margolis (2002)
CBF-1	CBF1 interacts with the LTR and represses proviral transcription by facilitating recruitment of HDAC1 and its co-repressor subunits CIR and mSin3A	Tyagi and Karn (2007) Tyagi et al. (2010)
p50/p50	Binding of p50 homodimers to the κB elements permits recruitment of HDAC1	Williams et al. (2006)
E2F1	Can partner with p50 to bind κB elements and suppress transcription initiation, probably by recruiting BRD2	Kundu et al. (1997) Karn (2013)
CTIP2	Suppresses HIV transcription initiation in microglial cells in cooperation with COUP-TF and Sp1. Recently identified as a regulatory component of 7SK snRNP in T-cells	Marban et al. (2007) Cherrier et al. (2013)
BRD2	Present at the promoter of latent proviruses. Suppresses proviral transcription initiation, possibly through an interaction with E2F1	Boehm et al. (2013) Karn (2013)
Chromatin modification		
HDACs	Histone deacetylases HDAC1, HDAC2, and HDAC3 are each involved in establishment and maintenance of HIV latency	Keedy et al. (2009)
HATs	The histone acetyltransferases p300/CBP and P/CAF reverse chromatin blocks and are recruited to the viral promoter by NF-κB and NFAT	Gerritsen et al. (1997)
SUV39H1	Trimethylates histone H3 Lys9 (H3K9me3), an epigenetic marker of HIV latency	du Chene et al. (2007)
G9a	Dimethylates histone H3 Lys9 (H3K9me2)	Imai et al. (2010)
EZH2	Histone methyltransferase component of PRC2. Responsible for histone H3 Lys27 trimethylation (H3K27me3) found on silenced proviral templates	Friedman et al. (2011)
DNMT1	Methylates CpG islands at the HIV LTR	Blazkova et al. (2012)
Chromatin remodeling		
BAF	SWI/SNF chromatin remodeling complex which positions proviral Nuc-1 to repress transcription	Rafati et al. (2011)
PBAF	Replaces BAF at the viral LTR to remodel Nuc-1 and enable transcription	Rafati et al. (2011)
Promoter-proximal pausing		
DSIF	Heterodimer of hSpt4 and hSpt5 which interacts with RNAPII, mRNA capping enzyme, and nascent RNA. Stimulates 5' RNA capping and recruitment of NELF. Phosphorylation of hSpt5 by P-TEFb transforms DSIF into a positive elongation factor	Yamaguchi et al. (2002)
NELF	Heterotetramer that binds TAR RNA and a preformed DSIF/RNAPII complex. Induces premature termination and formation of a stable RNAPII paused complex	Pagano et al. (2014) Yamaguchi et al. (2002)
Microprocessor	Processes TAR RNA leading to recruitment of Setx, Xrn2, and Rrp6. These factors cooperatively induce RNAPII pausing and abortive transcription	Wagschal et al. (2012)
Elongation		
P-TEFb	Heterodimer of CDK9 and cyclin T1. Recruited by Tat to paused RNAPII at TAR RNA. Stimulates processive transcription by phosphorylating RNAPII, E subunit of NELF and hSpt5 subunit of DSIF	Yamada et al. (2006) Fujinaga et al. (2004) Czudnochowski et al. (2012)
Super elongation complex (SEC)	Complex comprising the factors shown in Fig. 3A. AFF4 and AFF1 organize the assembly of Tat/P-TEFb into the SEC. ELL proteins stimulate efficient elongation by preventing RNAPII pausing and backtracking. AF9 and ENL link the SEC to RNAPII	He et al. (2011) Chou et al. (2013)
Tat modifiers	Histone modifying enzymes that regulate the trans-activating function of Tat by modifying its P-TEFb or Arg-rich RNA binding regions. These include P/CAF, SIRT1 and LSD1/CoREST	Ott et al. (2011), Lu et al. (2013)
Co-transcriptional processing		
SKIP	Splicing factor that is recruited by Tat/P-TEFb to the HIV LTR. Associates with activated spliceosomes within the transcribed region of the proviral gene	Bres et al. (2005)
P-TEFb regulation		
7SK snRNP	Provides an exchangeable pool of P-TEFb to support transcription of proviral and cellular genes. Contains 7SK snRNA, HEXIM, LARP7 and MEPCE	Nguyen et al. (2001) Yang et al. (2001)
CDK7	Kinase component of TFIIH. Recently identified as the CDK9-activating kinase	Larochelle et al. (2012)
PPM1A	Inactivates CDK9 via dephosphorylation of its activation loop	Budhiraja et al. (2012b)
BRD4	Extracts P-TEFb from 7SK snRNP and directs it to cellular genes. Competes with Tat for P-TEFb binding	Jang et al. (2005) Bisgrove et al. (2007)

elongation factor complex that stimulates efficient transcription elongation (Dingwall et al., 1990; Kao et al., 1987).

Transcriptional pausing of RNAPII at the proviral LTR

One of the most insightful recent advances in studies of transcriptional control is that the majority of mammalian protein-encoding genes carry poised RNAP II in the promoter proximal region (Core and Lis, 2008). The paused RNAP II permits rapid responses to transcription

signals. Not surprisingly, recent studies from our laboratory have shown that latent HIV proviruses is also always primed for rapid transcriptional responses due to promoter proximal pausing of RNAP II (Jadlowsky et al., 2014) (Fig. 2A). Promoter proximal pausing accounts for the characteristic formation of short transcripts of ~60 nucleotides that accumulate following processing of the nascent transcripts produced by abortive RNAP II escape (Laspia et al., 1989) and is seen in both actively transcribing and latently infected cells. Recently, Wagschal et al. (2012) have proposed that the short transcripts are

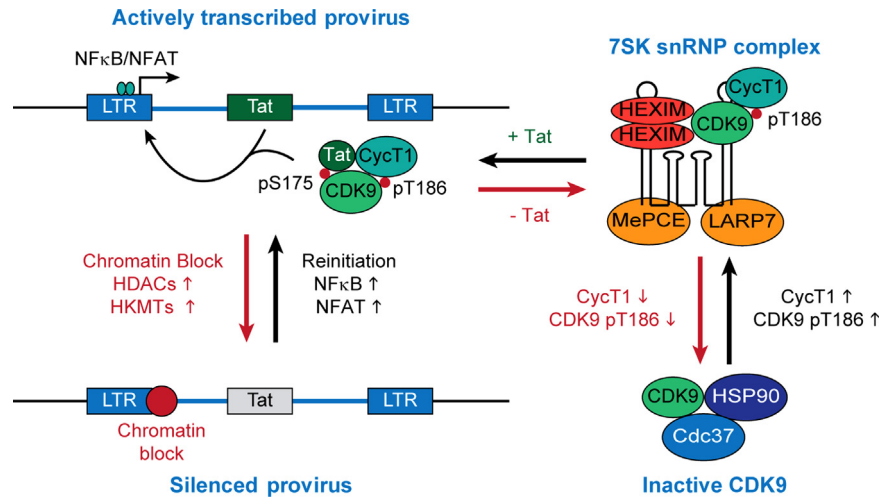


Fig. 1. Autoregulation of HIV transcription by Tat via recruitment of P-TEFb from 7SK snRNP. Inactivation of the HIV promoter, mainly due to epigenetic silencing, drives down Tat to below its threshold levels leading to entry of the provirus into latency, whereas stimulation of transcription initiation induces Tat production. Tat is able to extract the P-TEFb enzyme complex from the inhibitory 7SK snRNP. Competitive binding of Tat to P-TEFb is favored by signal-dependent modification of the activation loop of the CDK9 subunit.

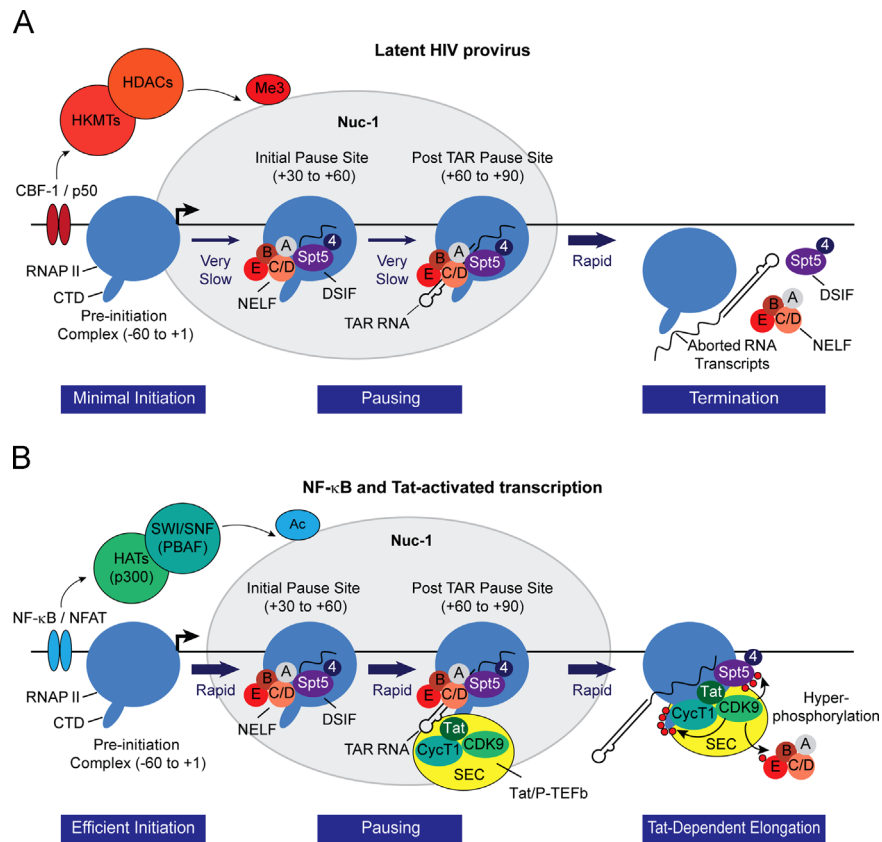


Fig. 2. Epigenetic regulation and Tat transactivation of proviral transcription. (A) Latent proviruses are characterized by the presence of histone deacetylases (HDACs), histone methyltransferases (HMTs), and their associated modifications at Nuc-1 situated around the transcription start site. The deacetylated proviral chromatin becomes a target for additional silencing via recruitment of polycomb repressive complex-2 (PRC2) which mediates histone methylation. Viral transcripts are inefficiently synthesized but over time there is a build-up of transcriptionally paused polymerase complexes due to the absence of Tat. Escaping RNAP II complexes are forced into abortive transcription due to NELF. (B) Productive transcription. Efficient assembly of pre-initiation complexes due to chromatin remodeling at the HIV LTR is a signal-dependent process that is driven by the activity of NF- κ B/NFAT and associated co-activators such as p300. The transitioning of paused polymerase into productive elongation requires kinase activity of P-TEFb. Tat efficiently transactivates HIV transcription elongation by recruiting P-TEFb to the paused complex at the TAR hairpin.

generated by processing of TAR RNA by microprocessor, the termination factors Setx and Xrn2, and the 3' to 5' exonuclease, Rrp6.

A striking feature of elongation control of the HIV promoter is that both the restriction of elongation in the absence of Tat and the efficiency of elongation in the presence of Tat is much stronger

than typically seen in cellular promoters (Kim et al., 2011). Combined surveillance by the negative elongation factor (NELF) and the DRB sensitivity-inducing factor (DSIF) is used to block productive elongation in the absence of Tat (Fig. 2). Human DSIF, a two-subunit complex of hSpt4 and hSpt5, is recruited to newly

initiated transcription complexes and stimulates capping (Wen and Shatkin, 1999). Spt5 forms direct contact with nascent RNA as it emerges from the RNAPII exit site (Cheng and Price, 2008; Missra and Gilmour, 2010) and facilitates the recruitment of NELF (Yamaguchi et al., 2002). NELF is able to force premature termination over a range of several hundred nucleotides (Renner et al., 2001), limiting the escape of transcription complexes from the promoter proximal pause site. Pagano et al. (2014) have recently characterized a consensus NELF subunit E binding element (CUGAGGA) on nascent cellular RNAs associated with transcriptionally paused genes, which is homologous to a NBE-like element (CUGGGA) present within the loop region of TAR RNA, and may lead to enhanced NELF recruitment to the HIV LTR. Thus, knock-down of NELF by shRNA leads to enhanced basal HIV transcription and escape from latency (Jadlowsky et al., 2014; Natarajan et al., 2013; Zhang et al., 2007).

Stimulation of proviral transcription by Tat/P-TEFb

As summarized diagrammatically in Fig. 2B, the switch from promoter proximal pausing to productive elongation is mediated by Tat and the positive transcription elongation factor (P-TEFb) (Herrmann and Rice, 1995; Wei et al., 1998). P-TEFb contains the CDK9 serine/threonine kinase and cyclin T. Human cyclin T1 is the only cyclin T that can interact with Tat due to the formation of Zn^{2+} -coordinated interactions with the N-terminal cysteine-rich region of Tat (Cys22–Cys37) (Bieniasz et al., 1998; Fujinaga et al., 1998; Garber et al., 1998). Crystallographic studies (Tahirov et al., 2010) have shown that the binding of Tat to CDK9/cyclin T1 (P-TEFb) allows the viral factor to establish partial helical secondary structure and induces significant conformational changes in P-TEFb, allowing co-operative recognition and stable binding of Tat and the cyclin T1 subunit of P-TEFb to TAR (Wei et al., 1998).

Recruitment of P-TEFb at TAR enables CDK9 to phosphorylate NELF-E and force its dissociation from TAR RNA and the paused polymerase complex (Fujinaga et al., 2004; Jadlowsky et al., 2014; Yamaguchi et al., 2002; Zhang et al., 2007). CDK9 also extensively phosphorylates the CTD of RNAPII mainly at Ser2 and Ser 5 residues of the heptad repeats (Czudnochowski et al., 2012), and homologous heptapeptide repeats (G-S-Q/R-T-P) of hSpt5 at Thr4 residues which converts it into a stimulatory elongation factor (Bourgeois et al., 2002; Yamada et al., 2006). Therefore, the overall effect Tat and P-TEFb is to remove the blocks to elongation imposed by NELF

and DSIF and to stimulate efficient elongation and co-transcriptional processing of proviral transcripts. However, because promoter proximal RNAP II is continuously replaced, there is no loss of RNAP II from the promoter proximal region (Jadlowsky et al., 2014).

Tat/P-TEFb is known to remain associated with the elongating RNAPII machinery throughout transcription (Bourgeois et al., 2002; Keen et al., 1997; Sobhian et al., 2010) raising the possibility that it exerts an influence throughout the transcription cycle. The temporal and spatial recruitment of transcriptional and RNA processing factors to actively transcribed genes is guided by the dynamic changes in the phosphorylation marks on the CTD of RNAPII that are observed to occur over the course of a transcriptional cycle (Drogat and Hermand, 2012; Perales and Bentley, 2009). Since CDK9 kinase activity is crucial for controlling regular and alternative RNA splicing and for the recruitment of polyadenylation factors at the 3' ends of RNAPII-transcribed genes (Ahn et al., 2004; Lenasi et al., 2011) it seems likely that Tat can also influence these processes. For example, the splicing-associated c-Ski-interacting protein (SKIP) which had previously been shown to be a stable component of activated mammalian spliceosome complexes that are essential for global RNA splicing is recruited by Tat/P-TEFb to the HIV LTR (Bres et al., 2005). SKIP recruitment dramatically enhances HIV transcription elongation, alternative splicing of viral RNA, and Tat-dependent LTR-driven proviral gene expression. How splicing and transcription termination is modified by Tat remains one of the unsolved problems in HIV transcription control.

The Tat/P-TEFb super elongation complex

Although P-TEFb was regarded for many years to be a discrete elongation factor, recent work has shown that P-TEFb is an integral component of larger “super elongation complex” (SEC) (Chou et al., 2013; He et al., 2010; Sobhian et al., 2010) (Fig. 3A). siRNA knockdowns of individual SEC components strongly inhibits Tat-dependent LTR-driven reporter gene expression, with knockdown of the elongation factor ELL2 producing the greatest effect (He et al., 2010; Sobhian et al., 2010). In actively replicating cells ELL2 is present at limiting levels due to rapid protein degradation by the polyubiquitination-proteasomal pathway (He et al., 2010; Liu et al., 2012). Both Tat and the SEC scaffold protein AFF4 prevent the degradation of ELL2 leading to higher levels of SEC associated with

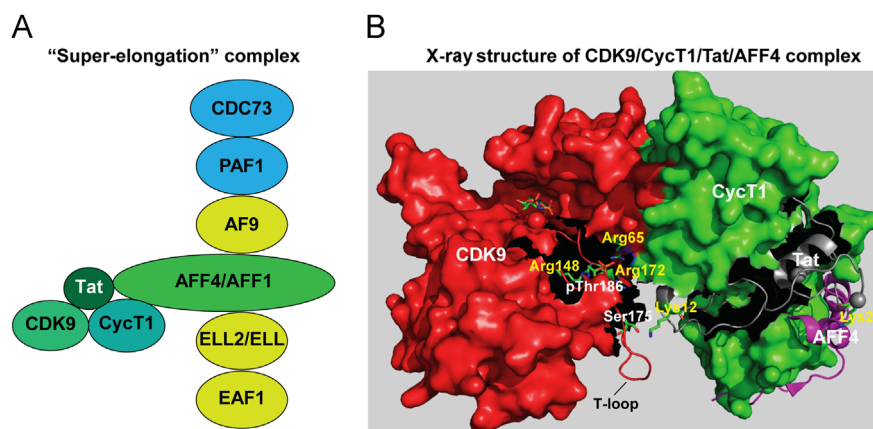


Fig. 3. The super elongation complex (SEC). (A) Schematic diagram of the SEC. AFF4 (or AFF1) provides a molecular scaffold that organizes the assembly of the SEC. By preventing the rapid degradation of ELL2, Tat also facilitates SEC assembly at the HIV LTR. The SEC is linked to the paused polymerase complex via an interaction between ENL or AF9 and the PAF1 subunit. (B) Cartoon and surface renderings of the Tat/P-TEFb 3D structure (Tahirov et al., 2010) superposed onto AFF4/P-TEFb (Schulze-Gahmen et al., 2013) showing CDK9 T-loop contacts with Tat and the close proximity of Tat to AFF4. The T-loop of CDK9 can potentially make contacts with the N-terminal region of Tat as reported by Tahirov et al. (2010) and these interactions would be strengthened by the signal-dependent phosphorylation of Ser175. Hydrogen bonding coordination of the CDK9 Thr186 phosphate moiety by three highly conserved arginine residues reduces the flexibility of the upper T-loop region and may be essential for stabilizing the CDK9/hCycT1 heterodimer complex.

P-TEFb (He et al., 2010; Liu et al., 2012). Thus Tat is not only able to recruit the SEC to the HIV LTR but it is also able to significantly enhance the intracellular fraction of P-TEFb that becomes associated with the SEC (He et al., 2010; Sobhian et al., 2010), providing a complementary mechanism leading to enhanced proviral reactivation.

AFF4 and its structural homolog AFF1 are large flexible proteins (1218 and 1163 residues, respectively) that are believed to act as scaffolds to organize the assembly of the SEC (Chou et al., 2013; He et al., 2011). Recently a 2.9 Å resolution 3D structure of the N-terminal CycT1 interacting region of AFF4 (AFF4₂₋₇₃) in complex with P-TEFb was solved (Schulze-Gahmen et al., 2013), which we have modeled on the earlier P-TEFb structure (Tahirov et al., 2010), as shown in Fig. 3B. Tat and AFF4 bind to adjacent sites on hCycT1 and AFF4 can potentially form direct contacts with three Tat residues (Met1, Lys28, and Phe32). These structural observations help to explain why acetylation of Tat Lys28 by P/CAF (Bres et al., 2002; Kiernan et al., 1999) is able to stabilize the Tat/P-TEFb/TAR ternary complex while mutation of Lys28 (K28A) significantly diminishes the ability of Tat and P-TEFb to trans-activate HIV in vivo (D'Orso and Frankel, 2009; Kiernan et al., 1999). Thus, Tat association with AFF4 is highly regulated by post-translational modifications and these biochemical pathways help orchestrate the recruitment of the SEC to the proviral transcription unit.

Establishment of HIV latency

Epigenetic silencing of HIV transcription

HIV preferentially integrates within intragenic regions of actively transcribed regions of the host genome (Lewinski et al., 2006). Thus, somewhat surprisingly, the majority of repressed, but inducible, proviruses are found within the introns of expressed cellular genes (Lewinski et al., 2005; Shan et al., 2011; Sherrill-Mix et al., 2013). The implication is that establishment of HIV latency is regulated independently of the control of the expression of the host gene into which the provirus has integrated.

Indeed, control of the chromatin structure formed specifically on the proviral LTR appears to be a key mechanism leading to proviral silencing. Independent of the proviral integration site, the viral 5' LTR is invariably occupied by two precisely positioned nucleosomes namely, Nuc-0 and Nuc-1 (Verdin et al., 1993). Nuc-1 is situated around the transcription start site and therefore provides a potential block to the release of promoter proximal transcription complexes.

A wide array of epigenetic modifications contribute to the silencing of latent proviruses. In particular nucleosomes on the 5' LTR of latent proviruses typically display deacetylated histones and trimethylated histones, a pattern of histone modifications that is associated with formation of repressive heterochromatic structures (Friedman et al., 2011; Keedy et al., 2009). In addition the histone methyltransferases (HMTs) themselves (SUV39H1, G9a, and EZH2) have been found to be associated with the latent proviral LTR (du Chene et al., 2007; Friedman et al., 2011; Imai et al., 2010; Marban et al., 2007). The dominant histone methyltransferase controlling HIV latency in T-cells is EZH2 (Friedman et al., 2011), which in addition to its H3K27 methyltransferase activity, is an important structural component of polycomb repressive complex 2 (PRC2). During heterochromatin formation PRC2 offers a binding platform for multiple chromatin-modifying enzymes including HDACs and DNA methyltransferase-1 (DNMT1) (Cheng et al., 2011; Tae et al., 2011; Vire et al., 2006). Methylation of histone H3K9 due to SUV39H1 and G9a activity additionally stabilizes heterochromatin structures by tethering the chromodomain-containing adaptor proteins HP1 α , β , and γ (du Chene et al., 2007).

Numerous trans-acting factors have been found that facilitate the recruitment of HDACs, PRC2 and other repressor complexes to the core promoter, suggesting that repression mechanisms may be both redundant and utilized in different cell types. For example, the co-operative binding of LSF and YY1 to the LTR allows YY1 to exert its repressive effect on proviral transcription by interacting with and recruiting HDAC1 to Nuc-1 (He and Margolis, 2002). Occupancy of the NF- κ B element at the LTR enhancer by p50 homodimers has also been shown to mediate the recruitment of HDAC1 (Williams et al., 2006). Similarly CBF-1, a key effector of the Notch signaling pathway, recruits HDACs to LTR of latent proviruses in both infected primary CD4⁺ T cells and a Jurkat T-cell model of HIV latency (Tyagi and Karn, 2007; Tyagi et al., 2010). Finally, the bromodomain protein BRD2 has recently been reported to mediate repression of the HIV LTR (Boehm et al., 2013). It is possible that potential BRD2 recruitment to the proviral LTR, mediated by an interaction with the transcriptional repressor E2F1, may in turn promote the recruitment of repressor complexes carrying acetylated lysine residues (Karn, 2013).

DNA methylation at two CpG islands flanking the transcription start site has also been associated with silencing of the HIV promoter in latently infected Jurkat T cells and primary CD4⁺ T cells (Blazkova et al., 2009; Kauder et al., 2009). However, two recent studies analyzing the latent viral reservoir of HAART-treated aviremic patients found very low or no methylation of the LTR of their HIV-1 DNA suggesting that extensive proviral DNA methylation is unlikely to be a marker of latent proviruses (Blazkova et al., 2012; Palacios et al., 2012).

Control of HIV latency by transcriptional interference

An alternative explanation for proviral silencing is that transcriptional interference, characterized by occlusion or dislodgement of transcription initiation or elongation complexes from the provirus by readthrough transcription of the viral-containing host gene or termination of host-initiated transcripts at the polyadenylation site in the 5' LTR inactivates the provirus (Han et al., 2008; Lenasi et al., 2008). While there are specific cases where transcriptional interference has been documented, there is only a slight preference for proviral integration in the same orientation as the host gene (63.8% vs 36.2%) in latently infected primary CD4⁺ T cells (Shan et al., 2011). Therefore, it seems likely that epigenetic repression of the provirus always makes a contribution to maintenance and establishment of latency.

Host and viral control of P-TEFb activation

Control of Cyclin T1 expression and P-TEFb assembly

Activation of P-TEFb in latently infected T cells is a sequential process that involves de-repression of hCycT1 expression, phosphorylation of CDK9 at Thr186, assembly of P-TEFb into 7SK snRNP, signal-dependent and Tat-dependent dissociation of 7SK snRNP, and eventually, Tat-dependent recruitment of P-TEFb and the SEC to the HIV provirus (Fig. 1). Expression of hCycT1 in resting CD4⁺ T cells is highly restricted at the post-transcriptional level, and this is likely to be mediated by several micro-RNAs (Budhiraja et al., 2012a; Chiang et al., 2012) and direct translational control mechanisms (Hoque et al., 2011). Levels of hCycT1 protein are elevated very rapidly (within 1 h) upon stimulation of resting T cells with cytokines, protein kinase C agonists, or ligation of the T cell receptor complex (Mbonye et al., 2013; Ramakrishnan et al., 2009; Sung and Rice, 2006). Undifferentiated monocytes which, unlike monocyte-derived macrophages and dendritic cells, are known to be non-permissive to HIV replication also

show micro-RNA dependent CycT1 restriction (Chiang et al., 2012; Sung and Rice, 2009).

In contrast to hCycT1, CDK9 is constitutively expressed in resting T cells and its levels are only modestly increased after prolonged periods (> 24 h) of T-cell activation (Ghose et al., 2001; Mbonye et al., 2013; Ramakrishnan et al., 2009). However, in resting cells the CDK9 kinase is inactive as a consequence of dephosphorylation of its regulatory activation loop (T-loop) at the highly conserved CDK residue Thr186 (Budhiraja et al., 2012a). Activation of T cells rapidly induces Thr186 phosphorylation of CDK9 concomitant with the elevation in hCycT1 expression (Mbonye et al., 2013; Ramakrishnan et al., 2009). Using an elegant chemical genetic approach Laroche et al. (2012) have recently identified the CDK7 subunit of TFIIF to be the nuclear CDK9-activating kinase. Similarly, siRNA knockdown studies have identified PPM1A as the inactivating phosphatase responsible for maintaining Thr186 dephosphorylation of CDK9 in resting CD4⁺ T cells (Budhiraja et al., 2012b; Wang et al., 2008).

Like other CDKs, CDK9 is activated by binding of the cyclin subunit which relieves steric blockade at the catalytic cleft by inducing large conformational changes to the T-loop (Jeffrey et al., 1995; Russo et al., 1996). The 3D structures of the T-loop phosphorylated CDK9/hCycT1 and CDK2/cyclin A reveal that a hydrogen bonding coordination of the phosphate moiety by three conserved arginine residues appear to be essential for stabilizing intermolecular hydrogen bonding interactions at the CDK/cyclin dimer interface (Fig. 3) (Russo et al., 1996; Tahirov et al., 2010). Since the phosphorylation of CDK9 at Thr186 is required to stabilize its heterodimer interactions with hCycT1 (Mbonye et al., 2013; Russo et al., 1996), the majority of CDK9 in resting T cells is likely to be present within Hsp90/Cdc37 kinase-specific chaperone complexes (O'Keefe et al., 2000).

Regulation of P-TEFb pools by the 7SK RNP complex

The majority of P-TEFb in activated T-cells is found in the 7SK small nuclear ribonucleoprotein (snRNP) complex (Kim et al., 2011; Nguyen et al., 2001; Yang et al., 2001; Yik et al., 2003). The 7SK snRNP provides an exchangeable pool from which the catalytically competent and transcriptionally active form of P-TEFb can be readily extracted and directed to genes. The sequestration of P-TEFb by 7SK snRNP in T-cells occurs shortly after its signal-dependent biogenesis and requires Thr186 phosphorylation of CDK9 (Budhiraja et al., 2012a; Chen et al., 2004; Mbonye et al., 2013).

7SK snRNA provides a scaffold for binding two molecules of P-TEFb in complex with homo- or heterodimers of the inhibitory proteins HEXIM1 or HEXIM2 (Li et al., 2005; Peterlin et al., 2012) (Fig. 1). Within the 7SK snRNP the RNA is protected from nuclease degradation by the 5' methylphosphate capping enzyme MEPCE which remains bound at the 5' end, and the La-related protein LARP7 which binds the polyuridine sequence at the 3' end (Jerónimo et al., 2007; Krueger et al., 2008; Markert et al., 2008).

Recruitment of P-TEFb by Tat to the provirus and competition with BRD4

As described above, Tat is able to induce release of P-TEFb from 7SK snRNP (Fig. 1). The Tat-dependent release mechanism is thought to involve RNA binding by Tat and induction of an RNA conformational change that poises the viral factor to competitively displace HEXIM1 from association with P-TEFb (Krueger et al., 2010; Muniz et al., 2010; Sedore et al., 2007). Therefore, in addition its classical role as a trans-activator of HIV transcription these studies have established an important role for Tat as a viral activator of P-TEFb.

Formation of the Tat/P-TEFb complex is in competition with P-TEFb binding by the chromatin-binding bromodomain protein BRD4

(Jang et al., 2005; Yang et al., 2005). Like Tat, BRD4 has a well-defined C-terminal P-TEFb interacting domain (Bisgrove et al., 2007) and can induce dissociation of P-TEFb from 7SK snRNP (Krueger et al., 2010; Schroder et al., 2012). Knockdown of BRD4 in the background of a latent HIV infection, or treatment of latently infected cell lines and primary T cells with the bromodomain inhibitor JQ1, results in a robust reactivation of Tat-dependent proviral gene expression because in the absence of competing BRD4 Tat binding to P-TEFb is strongly enhanced (Bartholomeeusen et al., 2012; Boehm et al., 2013; Li et al., 2013; Mbonye et al., 2013; Zhu et al., 2012).

In addition to BRD4, other factors may help recruit P-TEFb to transcription complexes. Both the DNA-binding transcriptional repressor CTIP2 and the SR-splicing factor SRSF2 have been identified as additional regulatory components within 7SK snRNP. CTIP2 mediates the recruitment of 7SK snRNP to promoters (Cherrier et al., 2013) while SRSF2 has been proposed to aid transfer of P-TEFb to promoter-proximally paused RNAPII on cellular genes by binding nascent RNA transcripts, employing a mechanism analogous to the recruitment of P-TEFb to TAR by Tat (Ji et al., 2013).

D'Orso and Frankel (2010), D'Orso et al. (2012) have recently developed the provocative hypothesis that Tat is recruited to the DNA template before TAR is synthesized. As RNAP II transcribes through the TAR region there is an exchange that leads to displacement of P-TEFb from the promoter-associated 7SK snRNP to TAR RNA. Key experiments supporting this idea come from ChIP and ChIP-seq data showing HEXIM and other 7SK associated proteins bound to upstream sites on both HIV and cellular promoters (D'Orso and Frankel, 2010; D'Orso et al., 2012; Ji et al., 2013). However, other groups, including ourselves, have been unable to detect P-TEFb and 7SK snRNP components upstream of the transcription start site. One possible explanation for these discrepancies is that D'Orso and Frankel (2010) performed their experiments in HeLa cells which typically display a much higher level of basal HIV transcription and less restriction by heterochromatic structures compared to the latently infected Jurkat T-cell lines that we have used in our studies. It therefore possible that the P-TEFb recruitment they observed in the absence of Tat is due to residual transcription in the HeLa cell system, perhaps mediated by the high levels of BRD4 that they have found to be associated with the HIV provirus. It is also possible that the 7SK RNP complex is only loosely associated with promoters and can only be detected by ChIP after extensive crosslinking (Ji et al., 2013).

Emergence from latency

Activation of P-TEFb in resting memory T-cells

Activation of latently infected T cells through engagement of the T-cell receptor (TCR) or stimulation with PKC-agonists results in the immediate stimulation of processive proviral transcription (Brooks et al., 2003; Kim et al., 2011; Natarajan et al., 2010). The early rounds of proviral transcription coincide with the recruitment of P-TEFb to the proviral LTR and requires functional Tat, even though the short duration of T-cell stimulation is not sufficient to build-up Tat expression above its undetectable sub-threshold basal levels (Mbonye et al., 2013).

Recently, we identified pSer175 as an additional major phosphorylation on the CDK9 T-loop that is induced by TCR and PKC-agonist stimulation (Mbonye et al., 2013). Signal-dependent Ser175 phosphorylation of CDK9, although not required for Tat binding to P-TEFb, appears to favor binding of Tat over BRD4 thereby shifting the equilibrium in favor of Tat. The pSer175 modification also provides an extremely useful marker to identify the transcriptionally active forms of P-TEFb since it is absent from P-TEFb in the 7SK RNP complex (Mbonye et al., 2013). In a related study, Fujinaga et al. (2012) demonstrated that the TCR- or PMA-mediated disassembly of

7SK sRNP to release P-TEFb requires activation of the protein kinase C isoform PKC- θ which in turn phosphorylates HEXIM1 protein at Ser158, which is situated right in the middle of the protein's bipartite RNA binding sequence. Consequently, the Ser158 phosphorylated HEXIM1 is unable to bind to 7SK snRNA and is therefore unable to bind and inhibit P-TEFb. Thus, multiple PTMs on 7SK RNP components appear to weaken protein–protein and protein–RNA interactions and shift the equilibrium to favor P-TEFb/Tat complex formation instead of P-TEFb/BRD4 complex formation. With increasing cycles of HIV transcription and a progressive elevation in Tat expression, Tat-dependent dissociation of P-TEFb from 7SK snRNP is expected to amplify the signal-dependent release of P-TEFb.

Chromatin remodeling and activation of HIV transcription initiation

The absence of cellular transcription initiation factors in the nuclei of latently infected resting memory CD4⁺ T cells and non-activated macrophage lineage cells is another major contributor towards maintenance of HIV latency. In the Jurkat T-cell model, TCR stimulation induces an initial wave of HIV transcription initiation that is largely dependent on the nuclear localization of NF- κ B p65/p50 heterodimer complexes and their binding to the duplicate NF- κ B elements at the HIV LTR (Kim et al., 2011; Tyagi et al., 2010). Stimulation of latently infected T lymphocytes and macrophage lineage cells with PKC agonists or pro-inflammatory cytokines also activates HIV transcription initiation in a NF- κ B p65-dependent manner (Kim et al., 2006; Nabel and Baltimore, 1987; Williams et al., 2006, 2007).

Nuclear NF- κ B p65/p50 directs the recruitment of a co-activator complex of histone acetyltransferases (HATs) such as p300/CREB-binding protein (p300/CBP) and p300/CBP-associated factor (P/CAF) to the HIV LTR (Gerritsen et al., 1997; Perkins et al., 1997). Acetylation of Nuc-1 appears to be a signal allowing recruitment of the chromatin remodeling SWI/SNF complexes. Rafati et al. (2011) have observed that two functionally distinct subclasses of SWI/SNF, BAF and PBAF are exchanged at the LTR. Maintenance of HIV latency requires BAF, which helps position the repressive Nuc-1. Upon activation, BAF is lost from the HIV promoter, while PBAF is selectively recruited to reposition Nuc-1 and facilitate LTR transcription (Rafati et al., 2011).

The NF- κ B p65 subunit is itself subject to acetylation by p300/CBP which serves to maintain it in the nucleus by inhibiting interaction with I κ B α (Chen et al., 2001; Furiá et al., 2002). Deacetylation of p65 by HDAC3 switches off NF- κ B-dependent transcription by allowing I κ B α binding and subsequent nuclear export of p65/p50 (Chen et al., 2001). By contrast, lysine methylation of p65 stabilizes NF- κ B binding to the LTR (Lu et al., 2010).

In primary T-cells NF- κ B is typically replaced by NFAT (Bosque et al., 2011; Bosque and Planelles, 2009; Chan et al., 2013) which is activated by calcium/calmodulin signaling (Kim et al., 2011). Like NF- κ B p65/p50, NFAT proteins can bind CBP/p300 and recruit its HAT activity to gene promoters (García-Rodríguez and Rao, 1998). Surprisingly, NFAT is dispensable in most Jurkat T-cell models (Chan et al., 2013; Kim et al., 2011). Since NFAT and NF- κ B bind in a mutually exclusive manner to overlapping sites on the proviral enhancer (Giffin et al., 2003) it seems likely they are used sequentially during T-cell activation, with NF- κ B responsible for the initial responses and NFAT acting at later times.

Post-transcriptional modification of Tat during transcription

The complex interactions between Tat, histone modifying enzymes, and elongation factors are coordinated through a series of post-translational modifications of Tat. In addition to targeting Nuc-1, HATs are reported to directly modify Tat on lysine residues (Lys50 and Lys51) that are situated within its arginine-rich TAR RNA binding motif (ARM) (Col et al., 2001; Kiernan et al., 1999; Ott et al., 1999).

Acetylation of the ARM region is thought to dissociate the binding of Tat to TAR, and simultaneously reinforce its interactions with chromatin modifying transcriptional co-activators by providing acetyl binding sites for their bromodomains (Lu et al., 2013; Ott et al., 2011). By contrast, acetylation of Tat by P/CAF at Lys28 situated within the cysteine-rich region of its activation domain has also been reported to enhance the association of Tat with P-TEFb and stabilize the Tat/P-TEFb/TAR ternary complex (Bres et al., 2002; D'Orso and Frankel, 2009; Kiernan et al., 1999). At the end of the transcription cycle SIRT1 binds and deacetylates Tat at Lys50 which mediates the recycling of unacetylated Tat thought to be necessary for subsequent rounds of viral transcription (Pagans et al., 2005). Following deacetylation of Tat at the ARM region, monomethylation at Lys51 by Set7/9 bound to TAR enhances Tat-dependent HIV transcription by facilitating Tat interactions with TAR and recruitment of the host elongation factor P-TEFb to the RNA (Pagans et al., 2010). Subsequent demethylation of Lys51 by LSD1/CoREST allows for the re-acetylation of the ARM of Tat which triggers entry into the TAR-independent phase of proviral transcription elongation (Sakane et al., 2011). Detailed studies monitoring the kinetics of each of these Tat modifications will be needed to provide a fuller understanding of their roles in regulating HIV transcription.

Stochastic events

Only a very small subset of latent proviruses (<1%) in peripheral blood becomes reactivated following in vitro 'maximum' T-cell activation with the mitogen PHA and irradiated allogeneic PBMCs (Ho et al., 2013). Significant genomic sequence defects (e.g. large internal deletions and APOBEC3G-mediated hypermutations) account for the lack of reactivation of most of the non-induced proviruses (~88%) (Ho et al., 2013) but the remaining fraction of non-induced latent proviruses (~11%) have intact sequences, are integrated into actively transcribed regions, and have been demonstrated in viral reconstruction experiments to be replication-competent (Ho et al., 2013).

Why only a small fraction of proviruses can be reactivated is mysterious, and potentially creates a barrier to cure efforts. Within a clonal population of latently infected Jurkat T-cells, cell-to-cell stochastic fluctuations in the assembly of transcription factor and chromatin remodeling complexes at the HIV LTR produce occasional transient bursts of viral transcription in a sub-population of these cells (Pearson et al., 2008; Singh et al., 2012; Weinberger et al., 2005). Although the reactivation of latent HIV ex vivo is now deemed to be inherently stochastic (Ho et al., 2013; Weinberger and Weinberger, 2013), sustained cellular activation is clearly needed in order to maintain HIV transcription (Kim et al., 2011; Pearson et al., 2008; Williams et al., 2007). Thus the stochastic nature of proviral reactivation may contribute to inefficient proviral reactivation during a first round of T-cell activation (Ho et al., 2013).

An alternative, but not exclusive explanation for why certain subsets of silenced proviruses fail to get reactivated when cells are stimulated is that epigenetic silencing of HIV-1 results in complex and heterogeneous patterns of histone modifications and DNA methylation (Blazkova et al., 2009; Kauder et al., 2009; Pearson et al., 2008). Interestingly, latent proviruses that reactivate poorly in response to cellular activators are found to carry higher levels of H327me3 marks (Friedman et al., 2011).

Summary and perspectives

We believe that the most practical approach to HIV eradication therapy will be based on using drugs to induce the transcriptional activity of latent HIV-1 without inducing the polyclonal activation of non-infected cells (a "shock" phase) since this type of therapy can

ultimately be delivered in normal clinical settings. Once the virus is reactivated a “kill” phase will be used to eliminate the induced cells through existing immune responses, viral cytopathogenicity or cytotoxic drugs.

The detailed molecular studies described above strongly imply that effective activation of the entire latent viral pool may ultimately require a cocktail of drugs that stimulate both transcription initiation and P-TEFb mobilization. Protein kinase C agonists are a major class of drugs that are able to induce transcriptional activity of latent HIV-1 (Beans et al., 2013; Sanchez-Duffhues et al., 2011). All of these drugs are expected to activate signaling pathways leading to both P-TEFb and NF- κ B mobilization but they tend to lead to global T-cell activation and associated toxicity. Finding drugs that can selectively activate P-TEFb in resting T-cells is a major challenge for the field.

The second major class of activators that are under investigation are drugs that induce epigenetic modifications such as histone deacetylase (HDAC) inhibitors (e.g. SAHA, panobinostat, rombigedpsin) (Archin et al., 2012; Friedman et al., 2011; Van Lint et al., 1996) and histone methyltransferase inhibitors (DZNep, BIX01294) (Friedman et al., 2011; Imai et al., 2010). SAHA has been shown to induce transient HIV RNA production in patients and is thus the first potential inducer of latent proviruses that has been evaluated as part of the HIV Cure agenda (Archin et al., 2012). As expected from the mechanisms described above, transcriptional activation of latent HIV by HDAC inhibitors occurs independently of NF- κ B activity and is associated with significant remodeling of the proviral Nuc-1 region that includes displacement of Nuc-1, decreased HDAC occupancy and histone hyperacetylation (He and Margolis, 2002; Kim et al., 2011; Van Lint et al., 1996). Similarly, treatment of latently infected cells with selective HMT inhibitors can also reactivate silenced proviruses in various HIV latency cell line models (Bernhard et al., 2011; Friedman et al., 2011; Imai et al., 2010). Recently, Bouchat et al. (2012) reported that ex-vivo treatments of resting CD4⁺ T cells isolated from HIV-1-infected individuals with the histone methyltransferase inhibitors chaetocin or BIX01294 induced virus production to a comparable extent as treatment with either SAHA or the PKC agonist prostratin.

Although SAHA and other HDAC inhibitors are able to induce viral RNA transcription in clinical settings it does not typically lead to release of infectious virions. This may be because although it is able to allow release of the paused RNAP II it is unable to induce P-TEFb and ignite a full cycle of viral transcription. The big take home lesson from the molecular studies of HIV transcription is that efficient viral reactivation is only seen when there is a reversal of epigenetic blocks (which is normally accomplished by NFAT or NF- κ B activation) and activation of P-TEFb (which is normally induced through TCR activation of PKC). It follows that specific combinations of drugs hitting these two pathways will be needed. We are confident that new, highly potent, drug combinations will soon be identified. The hope for an HIV cure, only a pipedream a few years ago, remains high.

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