

Mechanisms of HIV Latency: an Emerging Picture of Complexity

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Abstract Rarely HIV type 1 establishes proviral latency within the host genome, maintained with little or no viral gene expression. This state has been quantitated in peripheral blood and lymphoid tissues of HIV-infected patients, appearing in the earliest days of infection. These rare cellular reservoirs are unaffected by current antiretroviral therapy and unrecognized by the host immune response, and can regenerate disseminated viremia if therapy is interrupted. Proviral latency may be established when a newly HIV-infected cell exits the cell cycle and returns to the resting state. Rarely, direct infection of resting cells may also occur. Multiple molecular mechanisms appear to underlie the establishment and maintenance of persistent, latent HIV infection, most frequent in the resting central memory CD4⁺ T cell. Interrupting processes that maintain latency may allow therapeutic attack of this primary form of persistent HIV infection, but a better understanding of relevant mechanisms *in vivo* is needed.

Keywords Latency · Resting CD4⁺ T cell · Chromatin · Histone · HDAC · Methylation · IL-7

Introduction

Antiretroviral therapy (ART) has markedly decreased morbidity and mortality in HIV-1-infected individuals in the developed world. Successful therapy often results in stable plasma levels of HIV-1 RNA below the limits of

detection of clinical assays. Nevertheless, HIV-1 infection has not been cured by ART. The causes of persistence of HIV infection in the face of current therapy appear to be multifactorial: latent but replication-competent provirus in resting CD4⁺ T lymphocytes, cryptic viral expression below the limits of detection of clinical assays, and viral sanctuary sites might all contribute to persistence.

Evidence that cellular factors are required to maintain quiescence implies that proviral latency is an unstable state of HIV infection, amenable to therapeutic attack. An understanding of how latency is maintained may lead to novel therapies directed at the viral reservoir within resting cells. Other obstacles to the therapeutic clearance of established HIV infection exist—therapies to attack proviral genomes must be developed before eradication of HIV infection can be considered.

Clearance of HIV infection will almost certainly require a multimodality approach that includes potent suppression of HIV replication, therapies that reach all compartments of residual HIV replication, and depletion of any reservoirs of persistent, quiescent proviral infection. Here we highlight the basic mechanisms for the establishment and maintenance of viral reservoirs, suggesting approaches toward their elimination.

The persistence of virus in HIV-infected patients receiving potent ART was conclusively demonstrated when rare, integrated, replication-competent HIV was recovered from patients' resting CD4 memory T cells [1–3]. To date, this reservoir remains the most widely studied and best understood cause of viral persistence. Evidence suggests that the resting T-cell reservoir is established early after infection and is extremely stable. Current estimates based on long-term clinical studies suggest that the half-life of these stably infected cells is approximately 44 months in successfully treated individuals [4], and that without ART

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viremia reemerges from these reservoirs [5]. The eradication of infection by current ART is thus impractical.

It cannot be overemphasized that the persistence of HIV infection despite ART is not a unidimensional problem. Low-level replication of HIV may persist in other cell types or distinct sequestered compartments, such as the gut-associated lymphoid tissue or central nervous system. Persistent expression of HIV RNA (without evidence of full rounds of replication) can be detected in HIV-infected patients on durably successful ART by research assays in the plasma [6]. It is incompletely understood how expression may persist on ART without the development of drug resistance; one explanation is that persistent expression of HIV RNA originates exclusively in cells that were infected prior to ART initiation.

Nevertheless, as antiretroviral drugs improve, allowing increasingly potent inhibition of active HIV-1 replication, a role for therapeutic tools to attack the persistent proviral state is emerging. This is illustrated by the significant increase in the level of effort directed around the world to understanding the mechanisms that underlie HIV proviral latency, and the number of significant insights gained recently. These advances give hope that rational therapeutic approaches to the persistent proviral state might reduce the frequency of the emergence of drug resistance, improve the ability of an augmented immune response to contain HIV replication, or ultimately allow the clearance of HIV infection.

Mechanisms That Allow Persistent, Latent HIV Infection

The prevailing hypothesis for the establishment of latently infected CD4⁺ T cells in patients is that the virus infects T cells just prior to their natural reversion to a quiescent state, as with memory T cells, or in the case of the naïve T-cell population, infects cells that are undergoing differentiation during thymopoiesis. Given the potency of the viral activator Tat, and the responsiveness of the HIV promoter to many cellular activating signals, counterregulatory mechanisms that repress transcription appear to be required to allow HIV to establish or maintain a persistent, nonproductive infection. Although expression of the HIV-1 long terminal repeat (LTR) promoter is augmented by numerous cellular factors, relatively few factors have been identified that downregulate the LTR [7].

Deficiency of Host Factors Required for HIV Expression

Several features of the metabolism of resting CD4 cells are critical for the establishment of latency [8]. First, resting cells lack the coactivating factors nuclear factor (NF)- κ B or nuclear factor of activated T cells. Induction of either transcription factor by drugs, or by T-cell receptor activa-

tion, provides a powerful signal leading to the resumption of transcription by latent HIV proviruses. Critically, the viral transactivator, Tat, recruits a transcription complex that contains novel components, including the transcription elongation factor P-TEFb (positive transcription elongation factor b). Reductions in the level of the P-TEFb component CycT1 and sequestration of the P-TEFb complex by the HEXIM/7SK RNA complex also appear to restrict HIV transcription in resting lymphocytes. In addition to these mechanisms, it has also been suggested that HIV mRNA export is impaired in resting T cells, posing a further barrier to expression of provirus in resting cells [9]. There is evidence that host microRNAs can impede HIV mRNA expression or translation [10, 11].

Transcriptional Interference

Recent data have demonstrated that transcriptional interference mediated by nearby host gene promoters contributes to the quiescence of some HIV proviral genomes [12, 13]. This concept arose following the observation that most viral integrants reside in introns of actively transcribed genes [14]. Therefore, host gene “readthrough” transcription could prevent transcription initiation within the HIV LTR viral promoter. This effect could be negative if host and viral genes are convergently transcribed, but could prevent latency and upregulate proviral expression if the provirus is integrated in the same orientation as the host gene. Using two different model systems, Han et al. [12] made observations consistent with these expectations, but Lenasi et al. [13] discovered that convergent transcription inhibited downstream HIV expression, presumably by promoter occlusion, and that inhibiting upstream expression in this situation allowed HIV expression. The findings illustrate the complexity of eukaryotic transcriptional systems. Indeed, both situations could exist if the effect of transcriptional interference differs depending on the distance between the interacting promoters. Nevertheless, if an integrating provirus lands in a genomic location and orientation favorable for negative transcriptional interference, this integrant may be much more likely to establish latency. However, given the multiplicity of integration sites, each of potentially different orientation and spacing in a spectrum of host promoters, it is difficult to envision a translational strategy to deplete persistent infection that takes advantage of this variegated regulatory mechanism.

Chromatin Restrictions

Histone Acetylation

Transcription factors have long been known to affect chromatin structure, regulating gene expression, and may

influence proviral latency of HIV (Fig. 1). Multiple signaling pathways result in enzymatic covalent modifications (eg, acetylation, phosphorylation, and methylation) of specific amino acids in histone tail domains. The “histone code” hypothesis holds that combinations of distinct modifications occurring at particular sites on the histone tail direct which proteins are capable of interacting with histone–DNA complexes and determine gene activity [15]. Already more than 50 enzymes are known that selectively modify the histone tail, thus providing the means to make a combinatorial histone code. One canonical example is that of the histone acetylases, acting to allow the transcriptional machinery access to the DNA template, compete with histone deacetylases (HDACs) that blunt transcription by reducing accessibility of DNA templates. These modifications do not simply make chromatin more or less accessible, but inscribe biophysical marks on gene regions, signals for the ordered recruitment of complexes of regulatory factors that up- or down-regulate gene expression. There is a significant body of evidence suggesting that these mechanisms regulate HIV expression as well.

Important work from the Verdin laboratory has shown that transcription is restricted by the presence of a strictly positioned nucleosome (Nuc-1) that is very close to the viral RNA start site (+10 to +155). In models of chronic HIV infection, increased accessibility of chromatin about the LTR has been associated with transcriptional activation [16]. More recently, it has been shown that reactivation of HIV transcription requires remodeling of the critical Nuc-1 by SWI/SNF [7, 8].

It was originally believed that integration into the quiescent or centromeric region of the human genome, or acquired mutations of the virus itself [17], were responsible for the creation of these chromatin structures. However, evidence from both laboratory studies and more recent studies of resting CD4+ T-cell populations obtained from HIV-infected patients shows that HIV integrates preferentially into active regions of the human genome [18]. It therefore seems more likely that epigenetic silencing contributes to the entry of HIV into latency, given what is known about the suppression of inappropriate initiation sites mediated by transcription-directed histone modifications.

The first example of a host silencing mechanism that downregulates HIV expression was that of the cooperative recruitment of HDAC1 to the HIV LTR by the host transcription factors YY1 and LSF [19]. Synthetic small molecules designed to specifically inhibit LSF binding to the LTR and HDAC1 recruitment induced the outgrowth of HIV from resting CD4+ T cells obtained from HIV-infected donors, demonstrating that specific and targeted inhibition of HDAC activity at the HIV LTR disrupts proviral latency in HIV-infected patients' cells [20]. Consistent with a major

role for HDACs in establishing HIV latency, many drugs that inhibit HDAC activity, such as trichostatin A (TSA) and valproic acid (VPA) [21], are effective inducers of HIV transcription in latently infected cells.

HDACs are unable to directly associate with proviral DNA but are recruited through cellular DNA-binding proteins that recognize sequences in the viral LTR, including YY1, NF- κ B p50, and AP-4. A fourth complex containing c-Myc and Sp1 was found to recruit HDAC1 to the LTR. Recently, CBF-1, a CSL (CBF1/RBP-Jkappa/suppressor of hairless/LAG-1) type transcription factor and key effector of the Notch signaling pathway, was shown to be a remarkably potent and specific inhibitor of the HIV-1 LTR promoter, which acts by recruiting HDACs [22]. These findings echo the ability of multiple coactivator complexes to recruit histone acetylases to promoters, including the HIV LTR [23].

To identify all the potential HDACs involved in HIV transcription regulation, we recently performed a systematic survey for the presence of HDACs, in addition to HDAC1, at the LTR [24•]. We found RNA expression for all 11 isoforms of HDACs (including four splice variants of HDAC9) in the resting CD4+ T cells of HIV-infected, aviremic, ART-treated patients. The highest expression levels were seen for HDACs 1, 3, and 7, intermediate expression was seen for HDACs 2, 4, and 5, and HDACs 6, 8, 9, 10, and 11 showed low expression levels. Similarly, HDAC protein was detected by Western blot in whole cell extracts of these cells, except for HDACs 9 and 10. Overall, the class I HDACs 1, 2, and 3 and the class II HDACs 4, 6, and 7 could be detected by Western blot in the nucleus of resting CD4+ T cells and are therefore candidate enzymes for direct regulation of integrated HIV genomes.

Chromatin immunoprecipitation (ChIP) can directly document the association of factors at specific promoter regions and therefore could provide definitive proof of local regulation of the HIV LTR by HDACs. However, it is not possible to perform the ChIP assay in patients' cells, as the target viral DNA is too rare. Therefore, we used a Jurkat CD4+ T-cell model system to study HDAC occupancy at the HIV LTR. In cells verified to encode a single HIV LTR provirus, RNA and protein for HDACs 1 to 11 are easily detectable in the cytoplasm and/or nucleus. However, occupancy of only the class I HDACs 1, 2, and 3 is detectable by ChIP at the HIV LTR using the same antibodies that detect these proteins by Western blot in J89s and primary cells [24•]. The expected loss of HDAC occupancy after TSA exposure provides further validation of the specificity of these ChIP results. Changes in ChIP occupancy seen for HDAC1 and 2 are statistically significant ($P < 0.05$) by real-time DNA quantitation. Occupancy of the LTR by class II HDACs (ie, HDACs 4–7, 9, and 10) was not observed.

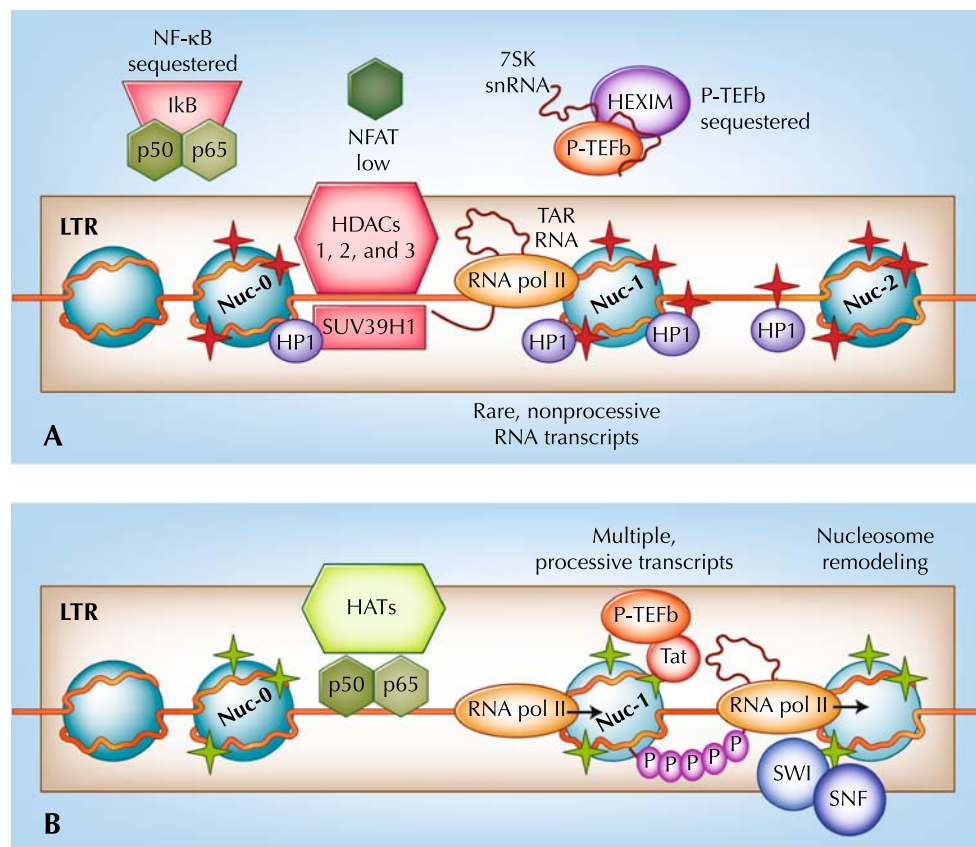


Fig. 1 Molecular mechanisms that allow or restrict HIV proviral expression in quiescent T cells. **a** At the HIV long terminal repeat (LTR), histone deacetylases (HDACs) 1, 2, and 3 lead to histone deacetylation (pink), and this allows recruitment of histone methyltransferases such as SUV39H1, and heterochromatin proteins such as HP1- α . Methyltransferases can ultimately methylate DNA (red). In this setting some polymerase complexes may initiate transcription, but infrequently due to low nuclear levels of co-activators such as nuclear factor (NF)- κ B and nuclear factor of activated T cells (NFAT). Transcription that is initiated is abortive and nonprocessive due to the lack of positive transcription elongation factor b (p-TEFb). **b**

When NF- κ B or NFAT are available, histone acetyltransferases (HATs) can be recruited. HATs acetylate chromatin, assisting in transcription complex recruitment. As transcription proceeds, nucleosome remodeling complexes such as SWI/SNF enhance the opportunity for subsequent waves of transcription. Early waves of transcription produce the HIV transactivator protein Tat, which then actively recruits the p-TEFb complex to the TAR RNA structure at the 5' end of the nascent HIV RNA. This allows for phosphorylation and activation of RNA polymerase (pol) II and other factors, leading to an explosive increase in transcriptional activation and the escape of provirus from latency

To explore the specific roles of individual class I HDACs, siRNA knockdown studies targeting single class I HDACs were performed in a reporter cell line. siRNAs targeting the class I HDACs 1, 2, 3, and 8 or nonspecific siRNAs were tested. siRNA-mediated knockdown of HDAC2 induced a significant ($P < 0.001$) increase in LTR-driven expression compared with the mock control. However, as siRNA is usually incomplete, even with multiple siRNAs (as in these experiments), we repeated these studies in the presence of global HDAC inhibition (TSA), increasing the basal level of HIV LTR expression approximately 20%. In the presence of submaximal HDAC inhibition, siRNA knockdown of HDAC3 upregulated LTR-driven expression. Similar studies utilizing primary cells rather than cell lines will be required to validate the specific roles of class I HDACs in HIV latency.

In companion studies [25•], we tested the effect of selective HDAC inhibitors (HDACi) at concentrations that induce HIV LTR expression in cell line models. Findings illustrate the mean frequency of viral recovery in the presence of maximal mitogen or HDACi. Nonselective (VPA) and class 1-selective HDACi allow the recovery of virus from patients' resting CD4⁺ T cells. Effects on cell viability do not account for this difference. Paradoxically, HDACi that are highly selective for the principal HDACs that are resident and active at the LTR, the class I HDACs 1, 2, and 3, are optimal inducers of viral outgrowth, although they are sufficient for proviral expression. Toxicity or inhibition of cell growth is not significantly different at the concentrations used in these studies. There is more to be learned about the mechanisms of HIV repression mediated by HDACs and the optimal use of HDACi.

Histone Methylation

In addition to regulation of histone acetylation at the latent provirus, two recent reports demonstrate that the histone methyltransferase Suv39H1, histone H3 methylated on K9, and the repressive HP1 proteins all accumulate on transcriptionally inactive proviruses [26, 27]. Finally, in an important and elegant study, Pearson et al. [28•] demonstrated that progressive, iterative histone modifications can drive a proviral promoter into latency within primary CD4+ T cells.

Methylation of DNA itself is an important and durable epigenetic mark, but the role of DNA methylation in HIV latency has been controversial. Recently, Blazkova et al. [29•] and Kauder et al. [30•] presented new evidence that CpG methylation of HIV promoter DNA can durably suppress HIV expression. Ironically, it was the potent HDACi suberoylanilide hydroxamic acid that was demonstrated to most efficiently reactivate densely methylated HIV-1 promoters.

Persistence Via Homeostasis

A totally different model of persistent infection within resting CD4+ T-cell infection has recently been put forward. It implies that proviral infection is not completely stable, and that in fact infected resting cells leave the quiescent memory pool, but the frequency of infection in this pool of cells is maintained by homeostatic proliferation of infected cells.

Chomont and colleagues [31••] recently reported that central (T_{CM}) and transitional memory (T_{TM}) CD4+ T cells are the major cellular reservoirs in which integrated HIV DNA persists in patients. Their data suggest that viral persistence is maintained through T-cell survival and low-level antigen-driven proliferation and is slowly depleted with time in T_{CM} cells. In contrast, in aviremic patients with lower CD4+ counts and higher levels of interleukin (IL)-7-mediated homeostatic proliferation, proviral DNA is preferentially detected in T_{TM} cells. This finding suggests that IL-7-driven proliferation may result in host-driven replication of proviral genomes without the death of these infected cells, ensuring the persistence of this reservoir. Although the numbers of cells available were too limited to perform robust quantitation of replication-competent virus with memory cell populations, recovery of virus in coculture assays was generally consistent with integration frequency by *Alu*-polymerase chain reaction. Patients with immune preservation (higher CD4 counts) had proportionally more central memory and naïve cells, and fewer proviral integrants, and patients with lower CD4 counts had proportionally more proviral integrants found in effector memory T cell populations.

Conclusions

Chronic, lifelong ART may be needed for decades into the future to prevent AIDS in the millions of HIV-infected people, and to control the spread of the HIV pandemic. But unraveling the relevant mechanisms of proviral latency may allow the development of therapeutic strategies that eradicate infection. Some strategies have already emerged from our current understanding, but because these approaches have thus far been impractical or unsuccessful, new discoveries and further translational effort are needed [32].

Activating Expression of Latent HIV

Several kinase agonists, including hexamethylbisacetamide, a compound previously tested in human cancer trials, activate intracellular signaling cascades that mobilize p-TEFb in the absence of Tat and can induce the expression of HIV in latently infected cells. Prostratin, a nontumorigenic phorbol ester isolated from the Samoan medicinal plant *Homalanthus nutans*, induces HIV expression in latently infected cell lines and cells isolated from HIV-infected, highly active ART-treated patients in the absence of cellular proliferation [7]. Recently, novel phorbol esters and molecules have been described that act like NF- κ B but do not induce the cell surface marker phenotype of T-cell activation [33–35]. These promising approaches must overcome the hurdles of potential cellular toxicities and undergo cautious testing to avoid the induction of more virus expression than can be contained by ART [36].

IL-7, a cytokine essential for maintenance of T-cell homeostasis, can induce HIV expression from quiescent resting cells without global T-cell activation, via the JAK/STAT5 signaling pathway. This cytokine has recently been studied for use in HIV-infected patients and thus might be tested for its ability to purge quiescent HIV genomes [37–39]. However, the findings of Chomont et al. [31••] raise the concern that IL-7 exposure could induce the homeostatic proliferation of latently infected resting memory cells.

HDACi as Potential Therapeutics

Because HDAC recruitment to the LTR is required to maintain latency, HDACi should disrupt proviral quiescence. ChIP assays performed in latently infected T-cell lines containing a single integrated HIV genome documented histone H4 acetylation at the nucleosome about the LTR transcriptional start site upon exposure to the HDACi VPA. VPA did not alter de novo HIV infection or the activation phenotype of primary cells, but it induced viral outgrowth from the resting CD4+ cells of patients without activation [21].

These preclinical studies led to a translational experiment, examining the effects of the administration of the HDACi VPA with intensified ART to HIV-infected patients. Initial findings suggest the potential of HDACi as anti-latency therapeutics, and the first targeted approach to this reservoir of persistent HIV infection [40]. A significant decline in resting cell infection (RCI) was measured in three of four patients (mean reduction 70%; range 58% to >84%) after intensification of ART and the addition of VPA. Because reductions observed in three of four patients exceeded the 50% threshold pre-established as being greater than any that would be expected by natural decay of this reservoir, intensified ART, or the variation of our assay, findings suggested that treatment with an HDACi and intensified highly active ART safely depleted RCI *in vivo*.

Subsequently, the effect on RCI of the addition of VPA to standard ART was tested. There was a significant decline in RCI in four of 11 patients receiving VPA therapy combined with standard ART, and a nonsignificant decline in two others. Patients without a decline in RCI were more likely to have low-level (1–50 copy/mL) viremia [41]. However, no significant change in RCI during periods of VPA treatment for neurologic and psychiatric disorders was reported, using an assay with a larger apparent intra-assay variation [42], and the average frequency of resting cells with detectable HIV RNA was no different in 10 patients taking valproate compared with patients taking only ART [43]. Thus, although VPA provides an important proof-of-concept that HDACi might provide a therapeutic approach to virus eradication, the preliminary clinical evidence available suggests that taken alone, the weak, nonselective HDACi VPA is insufficient to induce the required profound and reproducible depletion of resting CD4 cell infection in most clinical scenarios.

More potent HDACi such as suberoylanilide hydroxamic acid have been shown to be capable of inducing the expression of latent provirus [44, 45], but clinical testing of this approach has thus far not been possible. Combinatorial approaches have been modeled in the laboratory [46], and the field may find it challenging to translate such oncologic approaches in clinical testing within stable HIV-infected populations, in whom the acceptable level of risk is likely to be very low.

Thus, further study of the epigenetic regulators of HIV latency is needed, to allow the development of rational approaches to the problem of latent HIV infection and the identification of more effective and safe inducers of the latent proviral populations.

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