REGULATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 LATENCY BY HISTONE DEACETYLASES

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ABSTRACT

KARA KEEDY: Regulation of Human Immunodeficiency Virus Type 1 Latency by Histone Deacetylases
(Under the direction of David Margolis)

Despite highly effective antiretroviral therapies capable of suppressing plasma viremia, human immunodeficiency virus type 1 (HIV-1) persists in latent reservoirs in the millions of infected individuals worldwide. A significant contributor to viral persistence is the ability of the HIV-1 genome to stably integrate into the DNA of resting CD4+ T cells and adopt a state of latency, evading both immune detection and pharmaceutical attack. Once integrated, HIV-1 resides in the host chromatin environment where DNA is packaged around histones. Histone deacetylases (HDACs) are a family of 11 enzymes that can deacetylate histone tails, creating a repressive chromatin environment unfavorable to transcription. Enzymatic inhibition of HDACs with pan-HDAC inhibitors can reactivate latent HIV-1. However, the specific HDAC isoforms that regulate transcription from the HIV-1 5' long-terminal repeat (LTR) promoter during latency have not been completely defined.

In this dissertation, I hypothesized that specific HDACs are recruited to the HIV-1 LTR during latency to maintain transcriptional repression. Using chromatin immunoprecipitation assays, I showed that the class I HDACs HDAC1, -2, and -3 are recruited to the HIV-1 LTR in the J89 cell line model of HIV-1 latency. These HDACs were highly expressed in the nuclei of resting CD4+ T cells, the primary latent HIV-1 reservoir. Targeted depletion of HDAC2 or HDAC3 using siRNA led to induction of HIV-1 expression

in latency cell line models. However, simultaneous knockdown of HDAC1, -2, and -3 abolished this effect. In contrast, HDAC inhibitors that target HDAC1, -2, and -3 in tandem were potent inducers of latent HIV-1, suggesting a mechanistic difference between HDAC knockdown and enzymatic inhibition. When HDAC1, -2, or -3 were knocked down in resting CD4+ T cells isolated from aviremic, HIV-1-infected patients, we observed outgrowth of a selected number of proviral integrants. Collectively, these findings suggest that HDAC inhibitors that target a limited number of class I HDACs, specifically some combination of HDAC1, -2, and -3, may be effective antilatency therapies.

This dissertation is dedicated to the Christians in my life, I love you guys.

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5-aza-dC	5-aza-2’ deoxycytidine
AcH3	Acetylated histone 3
AIDS	Acquired immunodeficiency syndrome
AP-4	Activator protein 4
ART	Antiretroviral therapy
BRG1	Brahma-related gene 1
BSO	Buthionine sulfoximine
CBF-1	C-promoter binding factor-1
CCR5	C-C chemokine receptor 5
ChIP	Chromatin immunoprecipitation assays
CoREST	Co-repressor of repressor element 1-silencing transcription factor
CpG	Cytosine-phosphate-guanine
CTIP2	Chicken ovalbumin upstream promoter transcription factor-interacting protein 2
CXCR4	CXC chemokine receptor 4
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EZH2	Enhancer of zeste homolog 2
FBS	Fetal bovine serum
FSC	Forward scatter

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
H3K9	Histone 3 lysine 9
H3K27	Histone 3 lysine 27
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HEXIM	HMBA-induced protein 1
HIV-1	Human immunodeficiency virus type 1
HMBA	Hexamethylene biscacetamide
hu	Humanized
IgG	Immunoglobulin G
IL	Interleukin
IUPB	Infected units per billion
LSF	Late SV40 factor
LTR	Long terminal repeat
MEF2	Myocyte enhancer factor 2
MFI	Mean fluorescent intensity
miRNA	MicroRNA
ml	Milliliter
MRK	Merck
N-CoR	Nuclear hormone receptor co-repressor
NFAT	Nuclear factor of activated T cells

NF-κB	Nuclear factor-kappa B
NS	Non-specific
nuc	Nucleosome
NURD	Nucleosome remodeling deacetylase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin A
PCR	Polymerase chain reaction
PTB	Polypyrimidine tract binding protein
P-TEFb	Positive transcription elongation factor b
RNAPII	RNA polymerase II
RT-PCR	Reverse-transcriptase PCR
SAHA	Suberoylanilide hydroxamic acid
SEM	Standard error of the mean
shRNA	Short hairpin RNA
Sin3A	SWI-independent 3A
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
Sp1	Specificity protein 1
SSC	Side scatter
SUV39h1	Suppressor of variegation 3-9 homolog 1
SWI/SNF	SWItch/sucrose non-fermentable

T_{CM}	Central memory CD4+ T cell
T_{TM}	Transitional memory CD4+ T cell
TAR	Transactivation response region
TF	Transcription factor
TNF-α	Tumor necrosis factor alpha
TSA	Trichostatin A
VPA	Valproic acid

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

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ABSTRACT

Given the scope of the human immunodeficiency virus type 1 (HIV-1) pandemic, millions of people will be in need of chronic antiretroviral therapy (ART) for decades into the future. It is hoped that progress in the prevention of HIV-1 infection can be made, but there have been few successes in this effort thus far. At the same time, lifelong ART presents formidable problems. Therefore, while research must continue on improvements in prevention and treatment, future HIV-1 research should now also seek to develop temporally contained therapies capable of eradicating HIV-1 infection. This introduction will explore what is known about the mechanisms that restrain HIV-1 expression and result in persistent, latent proviral infection, and what these mechanisms tell us about potential approaches towards eradication of HIV-1 infection. I will focus in detail on histone deacetylases (HDACs), a family of enzymes that negatively regulate HIV-1 expression during latency and the prospect that inhibitors of these enzymes have as antilatency therapeutics.

INTRODUCTION

With more than 7,000 new infections each day, the human immunodeficiency virus type 1 (HIV-1) pandemic remains an important public health issue (125). Access to antiretroviral therapy (ART) is increasing, but more people are infected every day than initiate ART. Further, despite highly effective antiretroviral agents capable of reducing plasma viremia to less than 50 copies of HIV-1 per milliliter (ml) in the millions of treated individuals (56), there is only a single report of an HIV-1-infected individual in whom HIV-1 infection might have been cleared (62). Virus quickly rebounds upon treatment interruption, thus life-long access and adherence to antiretrovirals are necessary to control viremia (69). Over the long term, the burden of life-long ART in millions of patients across the world may not be sustainable.

HIV-1 persistence is primarily due to the twin phenomena of the ability of HIV-1 to latently infect long-lived cells of the immune system and continued virus release from undefined reservoirs. For the past two decades, most therapeutic research in this field has focused on developing vaccines and designing antiretrovirals to block specific steps in the virus life cycle. Only of late has there been a reawakening of interest in strategies to purge the latent reservoir of HIV-1 with the goals of a drug-free remission of viremia and, ultimately, virus eradication. Recently, several researchers called for a broad collaboration between governments, institutional donors, academia, and the pharmaceutical industry to pursue antilatency research similar to current initiatives for HIV-1 vaccine research (110). This chapter summarizes recent discoveries into the mechanisms that regulate HIV-1 latency as well as proposed strategies to eradicate HIV-1.

HIV-1 REPLICATION

Overview of the HIV-1 lifecycle

HIV-1 is a member of the family *Retroviridae*, of the lentivirus genus, with the Latin prefix lenti- referring to the slow nature of disease progression caused by these viruses. HIV-1 is the etiological agent of acquired immunodeficiency syndrome (AIDS), a devastating disease that slowly destroys the immune system, leaving its victims susceptible to opportunistic infections (6). HIV-1 largely infects and replicates in immune cells that express the HIV-1 primary receptor CD4, including activated CD4+ T cells, macrophages, and some dendritic cells (36). Preceding viral entry to the cell, the HIV-1 envelope glycoproteins gp120 and gp41 bind to CD4 and a co-receptor, either C-C chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4) (25, 31). This leads to fusion between the viral and cellular membranes and results in partial uncoating of the viral particle. A hallmark of retroviruses is the process of reverse transcription, where the viral enzyme reverse transcriptase synthesizes a double-stranded DNA copy of the RNA genome. Reverse transcriptase has a high error rate with 3×10^{-5} mutations per nucleotide per replication cycle (91). This high mutation rate contributes to the dramatic intra- and interpatient sequence diversity seen between HIV-1 isolates and aids the virus in escaping immune system defenses (7, 95).

After DNA synthesis, the viral preintegration complex translocates to the nucleus. The HIV-1 integrase protein, along with other viral and cellular factors, mediates integration of the proviral genome into cellular DNA (123). Studies of the integration sites of HIV-1 proviral DNA in resting CD4+ T cells have revealed that HIV-1 integrates into the introns of actively expressed cellular genes more than 90% of the time (48). There does not appear to

be a directional preference for integration of the proviral genome with respect to the transcriptional orientation of the cellular gene.

Following integration, HIV-1 utilizes cellular transcription machinery to transcribe viral proteins and the viral genome. The HIV-1 genome contains three genes that encode structural proteins—*gag, pol,* and *env*—and six genes that encode regulatory proteins—*vif, vpr, tat, rev, vpu,* and *nef* (36). Transcription initiates at the HIV-1 promoter within the 5' long terminal repeat (LTR) of the proviral genome (106). The first transcripts exported to the cytoplasm are the multiply spliced mRNAs encoding Tat, Rev, and Nef (111). After translation, Tat and Rev translocate to the nucleus where Tat increases transcription more than several hundred-fold through the recruitment of activating transcriptional machinery. Rev exports both partially spliced and unspliced HIV-1 mRNAs that encode additional viral proteins and the RNA genome to the cytoplasm (30). The viral genome is incorporated into virions and released from the cell through viral budding (42). Proteolytic processing of encapsidated viral polyproteins results in mature, infectious virions, completing the viral lifecycle (71, 73).

Transcription of the HIV-1 proviral genome from the LTR

Efficient transcription of the HIV-1 proviral genome can influence whether an infected cell produces virus or becomes latently infected. HIV-1 transcription is driven by the viral promoter located in U3 region of the 5' LTR (106). Numerous consensus binding sites for both activating and repressive transcription factors are present in the LTR sequence (see Fig. 1.1). Of particular importance are two nuclear factor-kappa B (NF-κB) sites in the enhancer region and three specificity protein 1 (Sp1) sites and a TATA element in the core

promoter region of the LTR. Deletion or mutation of the latter two sequence elements dramatically reduces HIV-1 transcription (51, 67, 68). Consistent with their role as an enhancer, mutations in the NF-κB binding sites can reduce HIV-1 replication rates (16). However, these sites are not essential for HIV-1 expression (85).

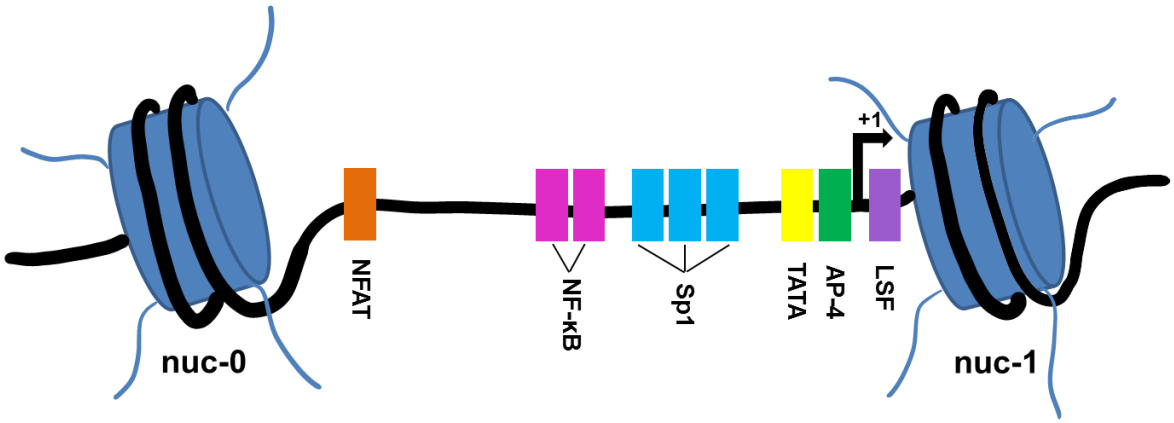


Figure 1.1 Organization of the HIV-1 5' long terminal repeat (LTR). A large nucleosome (nuc)-free region exists in the HIV-1 5' LTR where numerous activating and repressive transcription factor binding sites are located. Nuc-0 and nuc-1 flank this region. The enhancer region of the LTR possesses two nuclear factor-kappa B (NF-κB) binding sites. The core promoter contains three specificity protein 1 (SP1) binding sites and a TATA element. Other consensus binding sites shown include nuclear factor of activated T cells (NFAT), activator protein 4 (AP-4), and late SV40 factor (LSF), the latter of which is located immediately downstream of the transcription start site (+1).

LTR-driven transcription is regulated in part through the association of proviral DNA with nucleosomes at discrete sequences throughout the HIV-1 genome. Two of these nucleosomes, nuc-0 and nuc-1, flank the enhancer and core-promoter regions of the 5' LTR (Fig. 1.1) (131). The positioning of nuc-1 immediately downstream of the HIV-1 transcription start site sterically hinders mRNA synthesis, leading to a low basal level of HIV-1 transcription following integration. Initially, RNA polymerase II (RNAPII) associates with the LTR and catalyzes the production of short transcripts that terminate at the

transactivation response region (TAR), a secondary RNA structure produced during HIV-1 mRNA synthesis (5). However, some full-length transcripts are produced. The first of these transcripts serve as a template for translation of the HIV-1 Tat, Rev, and Nef proteins. Once Tat is synthesized, it translocates to the nucleus where it recruits the positive transcription elongation factor b (P-TEFb) complex to TAR (145). This action leads to hyperphosphorylation of the C-terminal domain of RNAPII, which permits RNAPII elongation past TAR resulting in processive, full-length HIV-1 transcripts (100).

Tat and activating transcription factors, such as nuclear factor of activated T cells (NFAT), can recruit histone acetyl transferases to the LTR (40, 93). This leads to acetylation of specific lysine residues on the amino-terminal tails of histones 3 and 4 at the HIV-1 LTR. Acetylation of histone tails neutralizes the positive charge on lysine residues, which results in a decreased affinity between proviral DNA and histones. This unwinding facilitates binding of activating transcription factors to the enhancer region of the LTR as well as RNAPII loading and processivity. Additionally, acetylated lysine residues on histone tails serve as docking sites for bromodomain-containing chromatin remodeling and modifying complexes that recognize and bind to the acetyl-lysine motifs (52, 140). Association of the ATP-dependent chromatin remodeling complex SWItch/sucrose non-fermentable (SWI/SNF) with the HIV-1 LTR, for example, is stabilized by the binding of bromodomain-containing brahma-related gene 1 (BRG1) to acetylated histones at nuc-1 (54). Transcriptional expression of HIV-1 as described here occurs readily in activated CD4+ T cells, where activating factors like NFAT and NF-κB p50-p65 are highly expressed in the nucleus. Infected resting CD4+ T cells, however, do not express HIV-1 and are a source of persistent infection.

PERSISTENT HIV-1 INFECTION

ART has been extremely effective at controlling viral replication in HIV-1-infected individuals. However, the persistent presence of HIV-1 RNA (without evidence of full rounds of viral replication) can be detected in HIV-1-infected patients on durably successful ART using sensitive assays for viral RNA in the plasma (99, 102). Recent studies have demonstrated that intensifying standard ART with an additional potent drug such as the non-nucleoside reverse transcriptase inhibitor efavirenz, the fusion inhibitor enfuvirtide, protease inhibitors lopinavir/ritonavir or atazanavir/ritonavir, or the HIV-1 integrase inhibitor raltegravir, does not reduce residual viremia in patients (27, 66, 83). These studies suggest that eradication of HIV-1 may not be achieved by antiretroviral drugs that block HIV-1 replication and that additional efforts should be focused on purging the persistent latent viral reservoirs.

Early in infection, HIV-1 primarily infects activated CD4+ T cells. Infection of these cells is almost always productive and quickly leads to cell death. Once ART is initiated, studies of the kinetics of the decay of viremia illustrate multiple phases of decay (Fig. 1.2). The initial two phases of decay have been long assumed to originate first from activated CD4+ T cells, and then from long-lived cells such as macrophages (107). However, in ART that includes an HIV-1 integrase inhibitor, only a single initial phase of decay is observed. This led to the suggestion that the second, slower phase of decay originates from cells with slow rates of cycling in which the kinetics of replication progress at slower rates (97).

Nevertheless, after the initial decay, which occurs over a period of a few months, there is a slower decay thought to represent depletion of virus in cells with a half-life of approximately 39 weeks. The final phase consists of a stable, low level of plasma viremia of

approximately 1 to 5 copies per ml, observable in most patients with the use of highly sensitive assays, for which there is no measurable rate of decay (102).

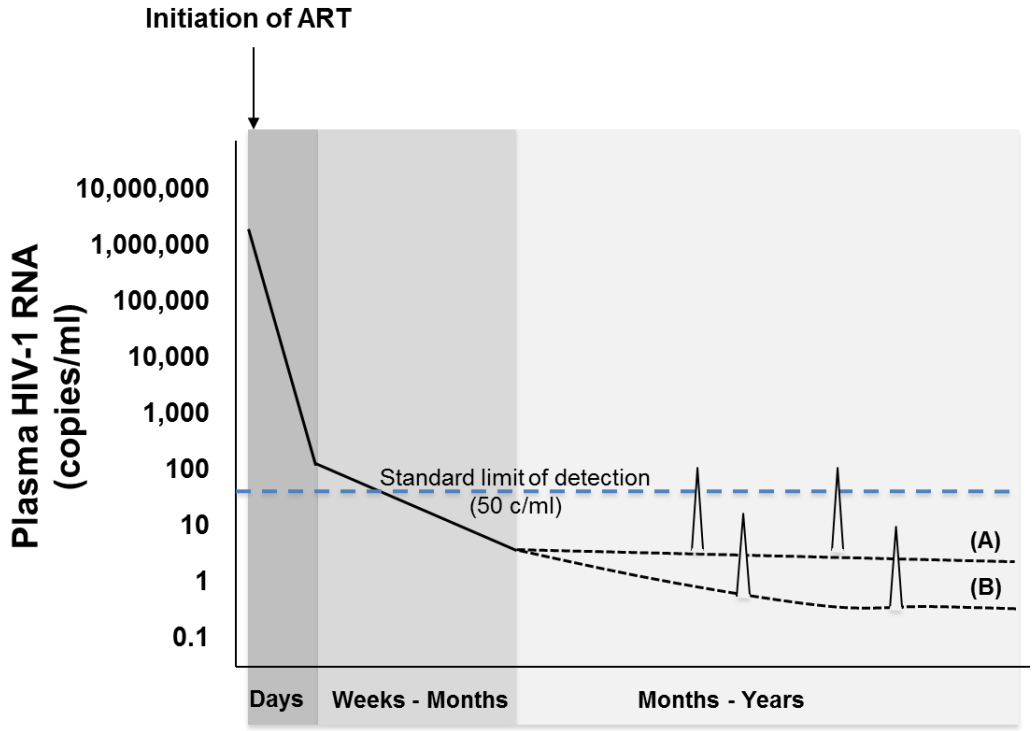


Figure 1.2 Decay of plasma viremia induced by current antiretroviral therapy (ART). Initiation of potent ART results in a precipitous decline in measurable plasma viremia. The level of viremia drops below the level of detection of commonly available clinical assays (~50 copies of HIV-1 RNA/ml) within weeks, but sometimes takes up to 6 months to decline below the limit of detection. This initial phase of decay often occurs in a biphasic pattern, as illustrated here, but a monophasic decline can be seen with regimens that contain an integrase inhibitor. After this initial decline, the slope(s) of further declines in levels of plasma viremia appear to gradually approach zero. Stable production of low levels of viremia is detectable in a minority of patients with more sensitive assays (A), and even lower levels may be established in most patients at levels that cannot be detected with current assays (B). Intermittent viremia (blips) may occur due to stochastic surges in viral production and/or variation in assay performance near its limit of detection, and may be detected in both patient populations.

Resting memory CD4+ T cells are relatively resistant to HIV-1 infection due to the intrinsic resting phenotype of the cell, which results in a lower efficiency of entry, reverse transcription, and integration in these cells (23). Although the number of latently infected resting CD4+ T cells is very low (less than 1 per million cells contain a replication competent

integrated HIV-1genome), the reservoir is rapidly generated early in infection (120). Seeding of this reservoir with virus is thought to occur when an activated CD4+ T cell is infected as it is transitioning to the resting, memory T cell state. Once the cell has obtained a resting phenotype, virus replication is further impeded due to multiple blocks to the HIV-1 life cycle that occur in resting CD4+ T cells. If HIV-1 has already integrated into the DNA of the T cell, then the virus could remain latent for the life of the cell. It has been proposed that this population of resting CD4+ memory T cells containing latent, replication-competent viruses might be expanded if the infected memory cell can undergo mitosis, a natural homeostatic process that preserves immunological memory, and enter the cell cycle without inducing viral replication and the destruction of the infected cell (17). If this is so, then the stable frequency of resting cell infection in patients on prolonged ART must reflect a balance between the extremely rare entry of a new proviral genome into the resting pool during ART, the activation of a resting memory cell by an immune stimulus, and the homeostatic proliferation of infected memory CD4+ T cells.

Sequence analysis of HIV-1 proviral DNA in resting CD4+ T cells has not uncovered evidence of viral evolution in this reservoir in patients who are stably suppressed with antiretroviral therapy (69, 99). Phylogenetic analyses of proviral DNA from resting CD4+ T cells and plasma HIV-1 RNA indicate that the persistent, low-level viremia in patients on ART might be released from some other cell type, as plasma sequences sometimes do not match proviral sequences from resting CD4+ T cells (14, 113). Nevertheless, patients who are stably suppressed on ART rarely develop resistance to antiretroviral drugs (75), suggesting that there may not be full rounds of ongoing viral replication in patients on ART

and that plasma viremia stems solely from virus that has been released from an unidentified cell or a clonal population of cells.

However, studies such as the ones cited above have limitations, including only sampling virus from the periphery and the current sensitivity of virus detection assays (approximately 1 copy per ml (103)). One recent report found evidence of viral evolution in such patients (119). Thus, we are still not able to confidently settle the ongoing debate about whether there is continued viral replication on ART or if plasma viremia only represents virus released from stable reservoirs.

MECHANISMS OF PROVIRAL LATENCY

Transcriptional blocks to HIV-1 expression in resting CD4+ T cells

Numerous transcriptional and post-transcriptional blocks to HIV-1 replication have been identified in resting CD4+ T cells. A major transcriptional block is the lack or sequestering of activating transcription factors and co-factors in resting CD4+ T cells. Cytoplasmic localization of NFAT and NF-κB p50-p65, sequestration of the P-TEFb by hexamethylene bisacetamide (HMBA)-induced protein 1 (HEXIM), and low levels of P-TEFb components cyclin T1 and cyclin-dependent kinase 9 in resting CD4+ T cells all contribute to HIV-1 latency and have been described in detail (20, 21, 38, 41, 101, 137). The site of virus integration can also have profound effects upon HIV-1 transcriptional expression. Although HIV-1 primarily integrates into genomic regions of euchromatin characterized by active transcription (48), occasionally the virus may integrate into heterochromatic regions of limited transcriptional activity. Furthermore, virus that integrates

into euchromatin can be subject to transcriptional interference by upstream or downstream cellular promoters (49, 84).

Once integrated, HIV-1 is subject to the same epigenetic mechanisms of transcriptional regulation as host DNA. Certain histone-modifying enzymes have been shown to maintain HIV-1 latency. These include histone deacetylases (HDACs), a family of enzymes that catalyze removal of acetyl-groups from Ɛ-amino-lysine residues on both histone tails and non-histone substrates. Deacetylation of lysine residues on histone tails leads to an increase in the net positive charge of histones, resulting in tighter association of histones with the negatively charged phosphate backbone of DNA. This creates a condensed, repressive chromatin environment and also removes important acetyl-lysine motifs that serve as docking sites for transcriptional activators (52, 112). The histone methyltransferases enhancer of zeste homolog 2 (EZH2) and suppressor of variegation 3-9 homolog 1 (SUV39h1) have also been reported to regulate HIV-1 latency in cell-line models (37, 92). EZH2 catalyzes the transfer of mono-, di-, or tri-methyl groups to lysine 27 of histone 3 (H3K27), marks of increasing transcriptional repression (117). SUV39h1 is a H3K9-specific methyltransferase (109). H3K9 tri-methylation serves as a docking signal for heterochromatin protein 1, which has been shown to associate with the HIV-1 LTR during latency in model cell lines (92). As H3K9 and H3K27 are also substrates for histone acetylation, HDACs work cooperatively with methyltransferases to silence transcription.

In addition to histone modifications, some integrated proviral genomes have been shown to be methylated on cytosine residues of DNA at the LTR in regions referred to as ‘CpG islands’ (9, 74). DNA methylation of promoters is associated with transcriptional silencing and a general resistance to activating signals. In the case of HIV-1, cytosine-

methylation at a CpG island located between nuc-1 and nuc-2 leads to recruitment of the HDAC-containing corepressor complex nucleosome remodeling deacetylase (NuRD) (74).

Post-transcriptional blocks to HIV-1 expression

Post-transcriptional mechanisms also regulate HIV-1 latency. Resting cells have low expression of polypyrimidine tract binding protein (PTB), a factor involved in export of HIV-1 RNA to the cytoplasm. Overexpression of PTB in resting CD4+ T cells from HIV-1-infected patients leads to increased transcription of latent HIV-1 *ex vivo* (82). This induction of HIV-1 gene expression is presumably due to an increase in the export and eventual translation of the viral proteins Tat and Rev, which are important regulators of HIV-1 RNA transcription and nuclear export, respectively.

Five cellular microRNAs (miRNAs) enriched in resting CD4+ T cells were demonstrated to inhibit translation of HIV-1 proteins (60). Like PTB, these miRNAs prevent nuclear accumulation of the HIV-1 proteins Tat and Rev, which inhibits HIV-1 gene expression and RNA export. Thus, resting cells provide a particularly unfavorable environment for HIV-1 replication and this can result in silencing of integrated HIV-1 proviral genomes in this population of cells. Figure 1.3 summarizes several blocks to HIV-1 expression in resting CD4+ T cells that have potential as antilatency therapy targets.

Stability and proliferation of the latent reservoir

In addition to possessing numerous blocks to replication, resting CD4+ T cells contribute to the persistence of HIV-1 due to their intrinsic stability and ability to undergo antigen-driven and homeostatic proliferation. Resting CD4+ T cells include both naïve and

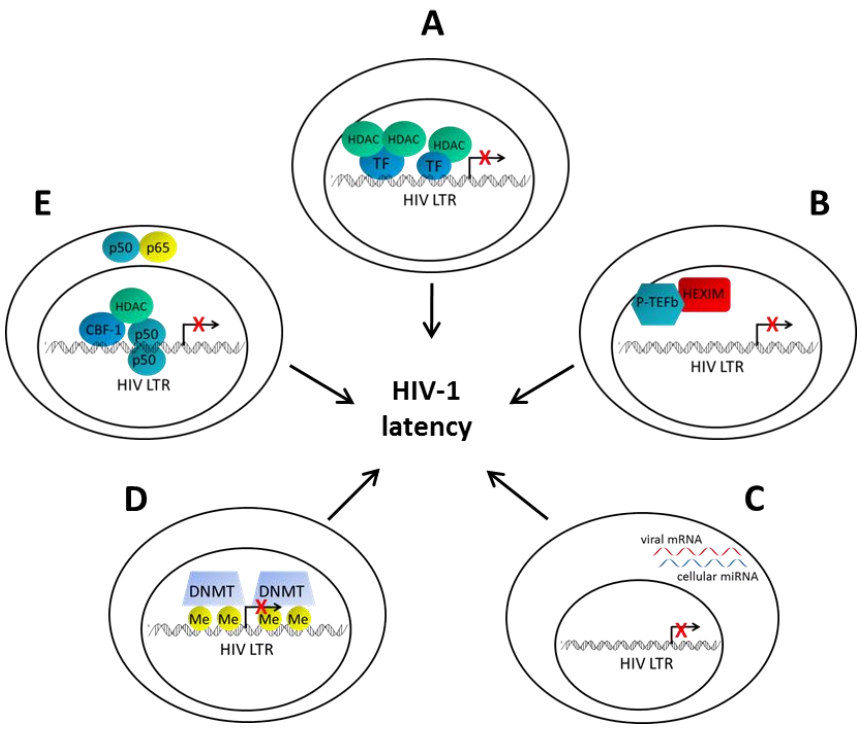


Figure 1.3 Blocks to HIV-1 expression in resting CD4+ T cells. (A) Histone deacetylases (HDAC) are recruited to the HIV-1 5' long terminal repeat (LTR) by multiple transcription factors (TF) to maintain histone deacetylation and repress transcription during latency. (B) Positive transcription elongation factor b (P-TEFb) is sequestered by hexamethylene bisacetamide-induced protein 1 (HEXIM), preventing RNA polymerase II phosphorylation and elongation. (C) Cellular microRNAs (miRNA) enriched in resting CD4+ T cells prevent HIV-1 mRNA translation. (D) Cytosine residues at CpG dinucleotides in the LTR can be methylated by DNA methyltransferases (DNMT) leading to transcriptional silencing. (E) In resting CD4+ T cells, the activating nuclear factor kappa B (NF-κB) heterodimer p50-p65 is sequestered in the cytoplasm. In its absence, TFs such as C-promoter binding factor-1 (CBF-1) and the repressive NF-κB p50 homodimer recruit HDACs to the NF-κB binding sites at the HIV-1 LTR.

memory cell populations. Naïve CD4+ T cells have not been exposed to antigen and rarely contain integrated HIV-1 proviral DNA (13). When a naïve CD4+ T cell comes into contact with its corresponding antigen, it will become an activated CD4+ T cell and proliferate. These activated CD4+ T cells are permissive to HIV-1 and quickly die following viral

replication. Some activated CD4+ T cells will revert to a resting state and become memory CD4+ T cells that are ready to respond should they encounter the same antigen again. Activated CD4+ T cells that become infected as they are transitioning to a resting memory phenotype are likely to be the source of latent HIV-1 in this population of cells (50).

Memory CD4+ T cells can be further divided into various functional subsets based on surface expression markers. These subsets include central memory (T_{CM}), transitional memory (T_{TM}), effector memory, and terminally differentiated memory cells, the latter of which do not persist for long periods of time and do not enter the persistent memory pool. A recent study that examined the prevalence of HIV-1 proviral DNA within these subpopulations observed that T_{CM} cells possess the highest rate of latent HIV-1 in patients with high CD4 counts (17). In addition to being extremely long-lived cells, this population can propagate the latent reservoir via antigen-driven proliferation. However, patients with low CD4 counts possess a larger latent reservoir in T_{TM} cells, which persist through homeostatic proliferation driven by interleukin-7 (IL-7) (12). Thus, multiple mechanisms of immune cell stability and proliferation contribute the persistence of the latent HIV-1 reservoir in resting memory CD4+ T cells.

REGULATION OF HIV-1 LATENCY BY HDACS

As mentioned in the previous section, because HIV-1 integrates into cellular DNA, HIV-1 transcription is regulated in part by the association of proviral DNA with cellular histones and the enzymes that modify these histones. The processes of histone acetylation and deacetylation can have dramatic effects on HIV-1 expression. Acetylation of histones 3 and 4 at nuc-1 of the HIV-1 LTR is associated with active HIV-1 expression (89).

Conversely, deacetylation of histones at the LTR is correlated with repression of HIV-1 transcription (53).

Human HDACs are divided into four different classes based on domain organization and amino acid sequence similarity (Table 1.1) (43). The "classical" HDACs that comprise classes I, II, and IV include HDAC1 to -11. Their deacetylase activity is zinc-dependent and the "canonical" HDAC inhibitors that target these enzymes induce latent HIV-1 expression. Class I HDACs include HDAC isoforms 1, 2, 3 and 8 and class II HDACs include isoforms 4, 5, 6, 7, 9, and 10. HDAC11 is similar to both class I and II HDACs and is the sole member of class IV. The class III HDACs are also known as the sirtuins due to their similarity to the yeast protein Sir2. These nicotinamide adenine dinucleotide-dependent deacetylases are not sensitive to canonical HDAC inhibitors and are not known to play a role in HIV-1 latency (108). The remaining discussion will focus on the classical, zinc-dependent HDACs.

Table 1.1 Classification of human histone deacetylases (HDACs).

Class I	***Class II***	***Class III***	***Class IV***
HDAC 1-3, 8	HDAC 4-7, 9, 10	Sirtuin 1-7	HDAC11

Class I HDACs

The predominantly nuclear class I HDACs HDAC1 and HDAC2 are widely expressed throughout tissues and have been reported to regulate transcription of numerous genes (24, 139). As is the case with all of the classical HDACs, HDAC1 and HDAC2 do not bind directly to DNA and are instead recruited to promoters through association with

multiprotein corepressor complexes that often include other histone remodeling and modifying enzymes (141). HDAC1 and HDAC2 frequently co-localize in the same corepressor complexes including SWI-independent 3A (Sin3A), corepressor of repressor element 1-silencing transcription factor (CoREST), and NuRD. Because HDAC1 and HDAC2 are often recruited together to promoters, some of their target genes are likely to overlap. However, this is not always the case. Deletion of HDAC1 is embryonic lethal in mice. Thus, HDAC2 cannot fully compensate for its absence (80). Furthermore, HDAC2 knockout mice survive gestation, but are born with severe heart defects (96).

Like HDAC1 and HDAC2, HDAC3 regulates transcription of numerous genes (139). HDAC3 is an essential member of the corepressor complexes SMRT (silencing mediator of retinoid and thyroid hormone receptors) and N-CoR (nuclear hormone receptor corepressor) (46, 86, 134). As the names of these complexes imply, HDAC3 has frequently been linked to control of nuclear hormone receptor-regulated genes (72). Unlike HDAC1 and HDAC2, HDAC3 possesses a nuclear export signal and can shuttle between the nucleus and the cytoplasm (138). Additionally, HDAC3 can associate with the class II HDACs HDAC4, -5, -7, and -10 and may regulate their deacetylase activity (33, 34).

The final class I HDAC, HDAC8, is the only isoform known to possess deacetylase activity independently of its association with other proteins. It has been reported to be both exclusively nuclear and exclusively cytoplasmic (59, 127, 133). This discrepancy is probably attributable to the different cell types studied and variations in how HDAC8 is regulated in these cells.

Class II HDACs

Phylogenetic analyses have revealed that the class II HDACs can be further subdivided into two groups (43). The class IIA HDACs include HDAC4, -5, -7, and -9. These HDACs have been shown to associate with each other as well as with the class I HDAC3. Although their enzymatic activity increases upon association with HDAC3, the class IIA HDACs are relatively weak acetyl-lysine deacetylases, suggesting that they may have additional substrates or functions that have yet to be identified (81). These HDACs are capable of nucleo-cytoplasmic shuttling mediated by association with the chaperone protein 14-3-3 (44, 70, 143). This property leads to signal-dependent regulation of class IIA target genes. For example, HDAC4, -5, -7, and -9 associate with myocyte enhancer factor 2 (MEF2) at the promoters of MEF2-target genes and represses transcription. Calcium/calmodulin-dependent protein kinsase signaling leads to phosphorylation of the class IIA HDACs. These phosphorylation sites serve as a docking platform for 14-3-3 proteins, which then shuttle the HDACs to the cytoplasm. In the absence of the class IIA HDACs, histone acetyltransferases (HATs) associate with MEF2 leading to histone acetylation and transcription of MEF2-dependent genes (94).

HDAC6 and HDAC10 are classified as IIB HDACs. At 1215 amino acids, HDAC6 is the largest HDAC. Furthermore, it is the only HDAC to contain two active catalytic domains. Although frequently classified as exclusively cytoplasmic in the literature, HDAC6 has been reported to localize to both the nucleus and cytoplasm (24, 61, 130). The cytoskeletal protein alpha-tubulin is a well characterized substrate for HDAC6 (61). Enzymatic inhibition or siRNA-mediated knockdown of HDAC6 enhances the ability of HIV-1 to infect cells and induce syncytia formation (126). Conversely, overexpression of HDAC6 inhibits HIV-1

fusion and infection of cells. These findings have been attributed to the ability of HDAC6 to deacetylate alpha-tubulin, leading to altered cell membrane dynamics.

In contrast to HDAC6, HDAC10 is only 669 amino acids in length (43). It possesses a second vestigial catalytic domain, but only one functional domain. Like the class IIA HDACs, HDAC10 has also been reported to interact with HDAC3 (122). Very little is known about the targets of HDAC10, and there is a discrepancy in the literature about whether it is predominantly nuclear, cytoplasmic, or capable of shuttling between the two (32, 45, 122).

Class IV HDACs

HDAC11 is the sole member of class IV and at 347 amino acids, it is the smallest HDAC (39). HDAC11 possesses sequence similarity to both class I and class II HDACs and is highly conserved in all free-living eukaryotic organisms with the exception of fungi (43). A recent report showed that HDAC11 is recruited to and regulates the expression of the IL-10 promoter in antigen presenting cells (132). There is only one report of HDAC11 localization, which found overexpression of epitope-tagged HDAC11 to be predominantly nuclear (39). However, for the most part, cellular localization of HDAC isoforms has been poorly characterized, with several reports and reviews claiming specific isoforms as exclusively nuclear or cytoplasmic and other reports clearly demonstrating localization to both compartments. The precise location of HDACs within a cell is likely to be specific to the cell state and type, with HDAC activity regulated in part by changes in cellular localization.

Recruitment of HDACs to the HIV-1 LTR

Multiple labs have identified numerous transcription factors that recruit HDAC1 to the HIV-1 LTR including Yin Yang 1 (YY1) and late SV40 factor (LSF) (22), NF-κB (136), C-promoter binding factor-1 (CBF-1) (124), activator protein-4 (AP-4) (63), c-Myc and Sp1 (65), and chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (CTIP2) (92). YY1 binds to HDAC1 and together these factors are recruited to the LTR by LSF. Williams et al. reported that NF-κB p50 homodimers recruit HDAC1 to the LTR enhancer region in the J-Lat latency cell line model. In contrast, Tyagi and Karn reported that CBF-1, a transcription factor enriched in resting CD4+ T cells, is responsible for the recruitment of HDAC1 to the NF-κB binding sites in the LTR enhancer region. AP-4 recruits HDAC1 to a site on the HIV-1 LTR adjacent to the TATA-element and this association blocks the recruitment of the activator transcription factor IID (TFIID). c-myc represses HIV-1 expression and in conjunction with Sp1, recruits HDAC1 to the HIV-1 LTR. In addition to recruiting HDAC1, CTIP2 was shown to recruit HDAC2 to the Sp1-binding sites of the HIV-1 LTR in a microglial cell line (92).

There is limited evidence suggesting that HDAC3 may also associate with the LTR. HDAC3 was found to associate with the LTR in a Jurkat-based LTR-luciferase reporter cell line (90). Additionally, the HDAC3-containing corepressor complex SMRT/N-CoR was shown to be recruited to the LTR by the unliganded form of thyroid hormone receptor in a *Xenopus laevis* oocyte model of chromatin assembly (58). Although HDACs clearly associate with the HIV-1 LTR, the importance of these specific class I HDACs to maintaining HIV-1 transcriptional repression and the question of whether or not additional HDACs contribute to HIV-1 latency has not been explored. Identification of the specific

HDAC isoforms that regulate HIV-1 latency will pave the way for research into more selective eradication strategies.

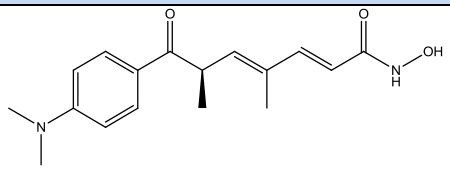
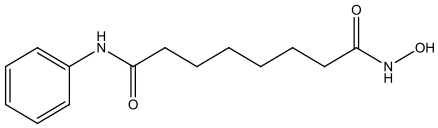
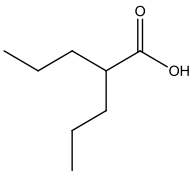
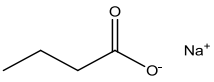
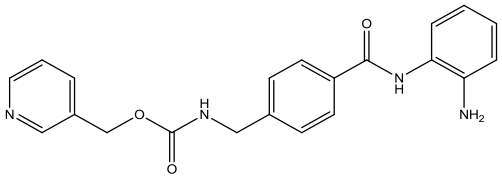
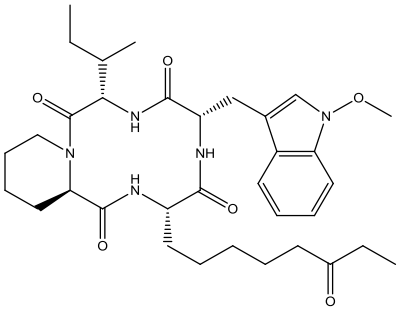
APPROACHES TO TARGET HIV-1 LATENCY IN RESTING CD4+ T CELLS

Even though other reservoirs may exist, targeting latent virus in resting CD4+ T cells is an important step in eradicating HIV-1. Once this population of infected cells is eliminated, it may permit easier identification and study of other reservoirs. Studies of the mechanisms of HIV-1 latency have pointed to multiple targets with the potential for therapeutic intervention.

HDAC inhibitors as antilatency therapeutics

Global HDAC inhibitors have emerged as potential antilatency therapies due to their ability to induce latent HIV-1 expression in cell line models and *ex vivo* outgrowth assays from resting CD4+ T cells isolated from HIV-1-positive patients (118, 128, 142). Known classes of small molecule HDAC inhibitors include hydroxamates, benzamides, cyclic tetrapeptides, short chain fatty acids, and keto derivatives (8). Table 1.2 summarizes the classes and structures of various common HDAC inhibitors. All of these compounds induce HIV-1 expression to varying degrees *in vitro* (1, 9, 108). Suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) also induce *ex vivo* outgrowth of latent proviruses from resting CD4+ T cells obtained from aviremic patients (1, 142). Important to their use as potential antilatency therapies, HDAC inhibitors do not induce T cell activation or increase *de novo* HIV-1 infection (1, 142).

Table 1.2 Chemical classes of common histone deacetylase inhibitors.

Compound	*Structure*	*Chemical class*
TSA		Hydroxamic acid
SAHA		Hydroxamic acid
VPA		Short-chain fatty acid
Sodium butyrate		Short-chain fatty acid
MS-275		Benzamide
Apicidin		Cyclic tetrapeptide

TSA: trichostatin A, SAHA: suberoylanilide hydroxamic acid, VPA: valproic acid

Some HDAC inhibitors have been approved for treatment of certain conditions, and numerous HDAC inhibitors are currently in clinical trials (4). VPA, a short-chain fatty acid, has been a successful therapeutic for the prevention of seizures and treatment of bipolar

mood disorders and migraines (55). In 2006, the hydroxamic acid SAHA, trade name vorinostat, was approved by the United States Food and Drug Administration for the treatment of cutaneous T cell lymphoma (29). The approval of these drugs for the clinic makes them prime candidates for translational studies of antilatency therapeutics in humans.

In a proof-of-concept study, our laboratory reported a reduction of resting cell infection in the latent reservoir of three out of four patients following a regimen of enhanced ART with the fusion inhibitor enfurvitide and HDAC inhibitor VPA (83). However, in a later study of patients receiving intensified ART and VPA, only some of the patients exhibited a decline in resting cell infection levels, while the majority of patients did not show a sustained decline (2). VPA, however, is a relatively weak HDAC inhibitor. Translational studies with more potent HDAC inhibitors, like SAHA, are required to more fully evaluate the ability of HDAC inhibitors to deplete the latent reservoir.

The identification and development of isoform-selective HDAC inhibitors are an active area of research. Although some of these inhibitors are truly specific to particular HDACs, most are selective for specific classes or subclasses of HDACs in a concentration-dependent manner. HDAC inhibitors selective for the class I HDACs HDAC1 and HDAC2, or HDAC1, -2, and -3, the class IIA HDACs, and even specific HDAC6 and HDAC8 inhibitors have been developed (4). As the crystal structures of some HDACs, including HDAC4, -7, and -8, have recently been solved, more selective inhibitors are anticipated in the near future (10, 28, 47, 115, 129). Determining the minimal HDAC inhibitory requirement for induction of the HIV-1 LTR may permit a more selective antilatency strategy with fewer toxicities and off-target effects on cellular pathways and genes.

Theoretically, HIV-1 expression in infected cells will lead to cell death either by viral cytolysis or immune surveillance. It has been proposed that the addition of the glutathione-synthesis inhibitor buthionine sulfoximine (BSO) to HDAC inhibitors could accelerate the apoptosis of cells that are producing virus (114). HIV-1 expression decreases levels of glutathione, creating a pro-oxidant cellular environment, which in turn stimulates HIV-1 transcription (64). The combination of BSO inhibitors and HDAC inhibitors permits a more potent induction of virus expression in latent cell line models at lower concentrations than required when HDAC inhibitors are used alone (114). Additionally, increased apoptosis of cells after virus induction is observed following the combined treatment, presumably due to the inability of infected cells to increase glutathione levels. These findings suggest one strategy of inducing virus expression and apoptosis of infected cells, while simultaneously decreasing the concentrations of HDAC inhibitors required to induce virus expression, thereby decreasing toxicity to uninfected cells.

Additional druggable targets of HIV-1 latency

Although HDAC inhibitors show promise as antilatency therapies, the mechanisms that lead to latency are most assuredly multifactorial and thus a combined approach directed at both HDACs and another block to HIV-1 transcription may be more effective at targeting a diverse population of proviral integrants. Figure 1.4 summarizes potential antilatency therapies that have been proposed to target transcriptional and post-transcriptional blocks to HIV-1 expression in resting CD4+ T cells.

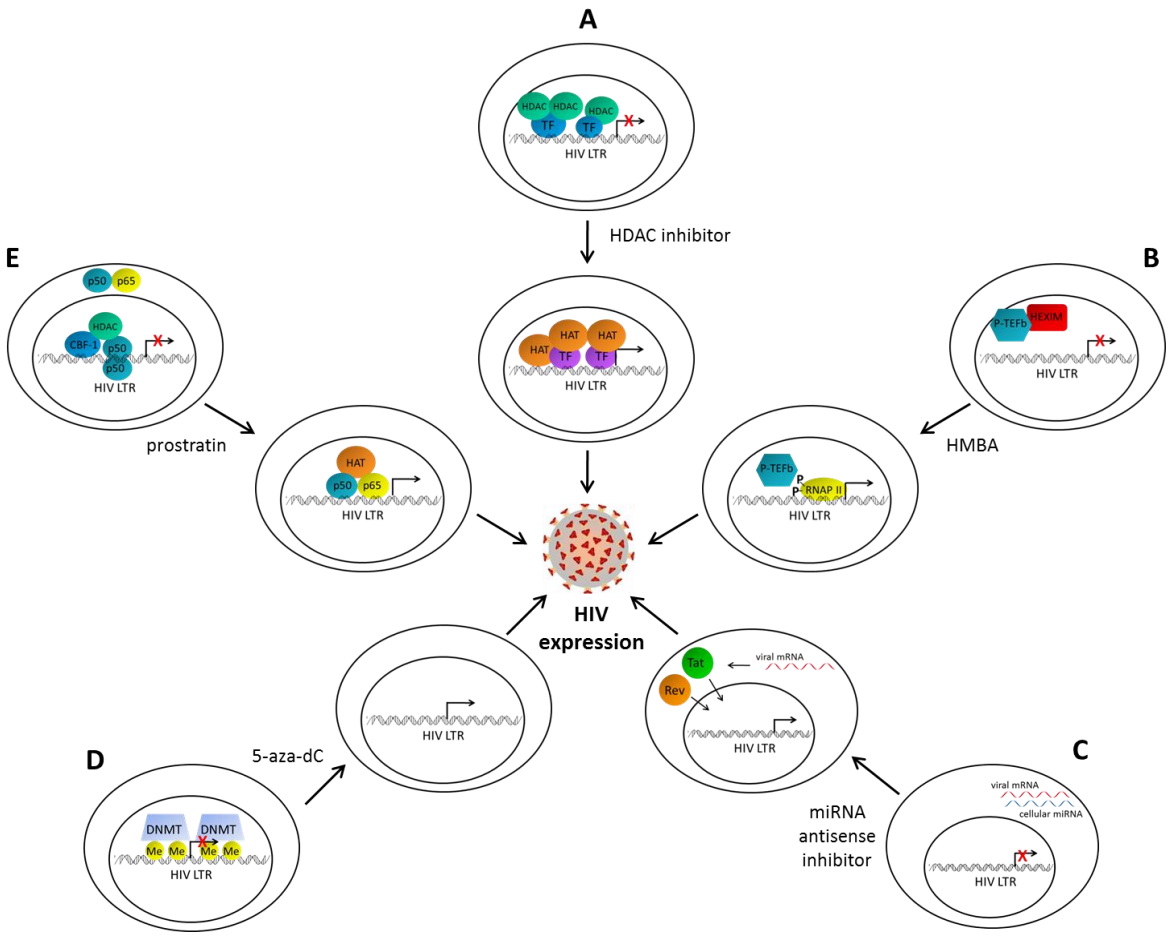


Figure 1.4 Strategies to purge the latent reservoir. (A) Histone deacetylase (HDAC) inhibitors inactivate HDACs at the HIV-1 5' long-terminal repeat (LTR) promoter. This allows unopposed activity of histone acetyltransferases (HAT) recruited to the LTR by transcription factors (TF), resulting in acetylation of histones at nucleosomes near the LTR transcription start site and HIV-1 expression. (B) Hexamethylene bisacetamide (HMBA) induces the release of positive transcription elongation factor b (P-TEFb) from HMBA-induced protein 1 (HEXIM), leading to phosphorylation of RNA polymerase II (RNAPII) at the LTR and subsequent processive HIV-1 transcription. (C) Cellular microRNAs (miRNA) in resting CD4+ T cells prevent the translation of HIV-1 mRNA. Inhibition of these miRNAs with antisense miRNA inhibitors permits translation of the HIV-1 proteins Tat and Rev, which translocate to the nucleus and facilitate HIV-1 expression. (D) Cytosine residues at the LTR can be methylated by DNA methyltransferases (DNMT), a phenomenon associated with transcriptional silencing. The DNA methylation inhibitor 5-aza-2' deoxycytidine (5-aza-dC) removes the methylation marks and can stimulate HIV-1 expression. (E) Complexes at the enhancer sites of the LTR, such as C-promoter binding factor-1 (CBF-1), recruit HDACs during latency. In resting CD4+ T cells, the activating NF-κB heterodimer p50-p65 is sequestered in the cytoplasm. Prostratin induces nuclear translocation of p50-p65 and recruitment of HATs to the NF-κB binding sites on the LTR, resulting in HIV-1 expression.

Compounds that target transcription factors and co-factors that are either limiting or sequestered in resting CD4+ T cells have been proposed as antilatency therapeutics (reviewed in (11)). These include drugs such as prostratin, which induces nuclear accumulation of the activating NF-kB heterodimer p50-p65, and HMBA, which promotes the release of p-TEFb from HEXIM, leading to transcriptional activation of the HIV-1 promoter (18, 21, 78, 135). Prostratin has been shown to induce the expression of latent HIV-1 *ex vivo*, but hurdles in pre-clinical safety and toxicity testing have prevented the approval of this compound for further testing in humans (57, 108).

Histone methyltransferases and DNA methyltransferases also contribute to HIV-1 transcriptional repression in cell line models of latency. In recent studies from two teams, exposure of latently infected cell lines to the DNA cytosine methylation inhibitor 5-aza-2' deoxycytidine (5-aza-dC) led to induction of HIV-1 expression (9, 74). However, it is clear that not all integrated HIV-1 genomes are heavily methylated at the LTR in resting CD4+ T cells obtained from patients (9). Therefore, additional strategies may need to be employed to purge all latent HIV-1 genomes in this population of cells. Strategies that target multiple transcriptional blocks, such as combining inhibitors to both HDACs and specific histone or DNA methyltransferases, may provide an even more effective induction of latent HIV-1. Alternatively, as HATs and HDACs regulate the recruitment and activity of methyltransferases (15, 35) it is also possible that sufficiently potent and appropriately targeted HDAC inhibitors may be sufficient to induce the expression of latent HIV-1. Indeed, even in heavily methylated promoters, the most effective inducing agent was found to be the potent HDAC inhibitor SAHA (9). It appears that studies in animal models (or even human clinical studies) will be required to definitively answer these questions.

Specific cellular miRNAs present a post-transcriptional block to HIV-1 expression in resting CD4+ T cells. *Ex vivo* exposure of resting CD4+ T cells from aviremic patients to 2'-O-methyl-oligoribonucleotide antisense inhibitors of these specific miRNAs leads to latent HIV-1 outgrowth without T cell activation (60). Delivery systems for anti-miRNA inhibitors in humans do not currently exist, but development of such delivery systems is an area of active research (77, 104, 105, 146). Consequently, anti-miRNA inhibitors may be a potential antilatency therapy in the future. Because a single miRNA can regulate hundreds of target RNA molecules (87), more research is warranted into the effects of inhibiting these specific miRNAs on the host.

The intrinsic properties of stability and proliferation in memory CD4+ T cells present major obstacles to the eradication of HIV-1 with current antiretroviral therapies. Patients who are treated late after initial HIV-1 infection typically have low CD4+ T cell counts and high levels of immune activation. In these patients, latent HIV-1 proviral DNA is primarily detected in T_{TM} cells (17). Depletion of CD4+ T cells correlates with plasma IL-7 levels and IL-7 promotes the survival and proliferation of T_{TM} cells (12, 88). Thus, the process of homeostatic proliferation is potentially a key mediator of persistent latent HIV-1 in the T_{TM} cells of patients with low CD4 counts. Anti-IL-7 therapies have been proposed as a potential means to purge this specific latent reservoir by preventing proliferation of infected T_{TM} cells (17).

MODELS FOR EVALUATING ANTILATENCY THERPEUTIC APPROACHES

Linked to the study of the causes of HIV-1 latency is the evaluation of strategies to target virus eradication. Theoretically, antilatency strategies would purge the latent reservoir

by inducing transcriptional activation of latent virus. Infected cells would undergo apoptosis following virus expression and antiretroviral therapy would prevent infection of new cells. Antiviral immune responses might also aid in the clearance of infected cells and prevent the spread of viral infection.

Until recently, latency research has primarily made use of numerous cell-line models where integrated HIV-1 proviral DNA is transcriptionally silent. Examples of such latency models used in the studies described in this dissertation include HeLa P4/R5 cells and J89GFP cells. P4/R5 cells are a MAGI-derived cell line that contain an integrated HIV-LTR driving the reporter gene *lacZ* (76). These cells were selected for a low basal level of β-galactosidase activity, and thus serve as a model of the transcriptionally silent HIV-1 LTR. Because P4/R5 cells do not express Tat, induction of HIV-1 expression is regulated entirely from the LTR promoter. The main advantage of this cell line is its suitability for high-throughput assays. J89 cells are perhaps a more relevant model system of latency (79). These cells are a Jurkat-based T cell line that possess a single, full-length HIV-1 proviral genome (HIV-1 strain 89.6) with an enhanced green fluorescent protein (GFP) reporter inserted between the *env* and *nef* genes. J89 cells were clonally selected for a low level of basal GFP expression that can be induced by specific stimuli, such as HDAC inhibitors.

The main disadvantage of cell line models of latency is that these cells are proliferating, a state that does not accurately reflect the quiescent phenotype of resting CD4+ T cells, the primary latent HIV-1 reservoir. A more physiologically relevant system for pre-clinical evaluation of antilatency therapeutics is to study the impact of such compounds on latent proviruses in resting CD4+ T cells. Outgrowth assays using resting CD4+ T cells harvested from HIV-1-infected patients on ART have been a useful tool for the evaluation of

antilatency approaches (1, 3). Resting cells are exposed to potential HIV-1-inducing compounds and maintained in limiting dilution cultures over a two and a half week period along with CCR5-expressing, stimulated peripheral blood mononuclear cells (PBMCs) to permit spread and amplification of reactivated latent virus. Virus outgrowth is measured at the end of the culture period by HIV-1 p24 antigen ELISA and the number of infected resting CD4+ T cells from the initial cell pool is calculated using a maximum likelihood method (98).

Resting CD4+ T cell infection also occurs in rhesus macaques infected with simian immunodeficiency virus (SIV), suggesting that this model system may be one means of exploring antilatency therapies (116). However, studies of proviral latency mechanisms have not been done in this model. Thus, it remains to be determined whether the blocks to SIV expression in the latent reservoir of macaques are the same as those in the resting CD4+ T cells of humans. Importantly, humanized mouse models have been established that show promise as a model for antilatency research. These models include bone marrow-liver-thymus mice (26, 121) and humanized (hu-) $Rag2^{-/-}\gamma_c^{-/-}$ mice (144). We recently demonstrated that plasma viremia can be suppressed below the limit of detection following treatment of HIV-1-infected hu-$Rag2^{-/-}\gamma_c^{-/-}$ mice with combination antiretroviral therapy (19). When ART is interrupted, HIV-1 levels quickly rebound and depletion of CD4+ T cells resumes. These findings give hope that humanized mouse models may soon be utilized to study HIV-1 latency and investigate potential HIV-1 eradication strategies.

CONCLUDING REMARKS

Chronic, lifelong ART may be needed for decades into the future to prevent AIDS in millions of HIV-1-infected people and to control the spread of the HIV-1 pandemic. Several strategies, as described in this contribution, have already emerged from our current understanding of persistent HIV-1 infection, but no approach has yet been practical or successful. The evidence discussed here suggests that regulation of integrated HIV-1 proviral DNA by HDACs is an attractive antilatency therapeutic target. Pan-HDAC inhibitors are potent inducers of HIV-1 expression in cell line models and *ex vivo* outgrowth assays from resting CD4+ T cells isolated from aviremic patients. Identification and development of isoform-selective HDAC inhibitors are active areas of research. Defining the specific HDACs that regulate HIV-1 latency will guide development of antilatency strategies with fewer toxicities and off-target effects.

The primary objective of this dissertation was to identify the precise HDAC isoforms that regulate HIV-1 latency. Our central hypothesis is that specific HDACs are recruited to the HIV-1 LTR during latency to maintain transcriptional repression. In chapters 2 and 3, we describe studies that test this hypothesis using a combination of biochemical, genetic, and pharmaceutical approaches. Our findings suggest that a more selective induction of latent HIV-1 expression by HDAC inhibitors is a feasible strategy and point to the specific HDACs that should be targeted.

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CHAPTER 2

A LIMITED GROUP OF CLASS I HISTONE DEACETYLASES ACTS TO REPRESS HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 EXPRESSION

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ABSTRACT

Silencing of the integrated human immunodeficiency virus type 1 (HIV-1) genome in resting CD4+ T cells is a significant contributor to the persistence of infection, allowing the virus to evade both immune detection and pharmaceutical attack. Nonselective histone deacetylase (HDAC) inhibitors are capable of inducing expression of quiescent HIV-1 in latently infected cells. However, potent global HDAC inhibition can induce host toxicity. To determine the specific HDACs that regulate HIV-1 transcription, we evaluated HDAC1 to HDAC11 RNA expression and protein expression and compartmentalization in the resting CD4+ T cells of HIV-1-positive, aviremic patients. HDAC1, -3 and -7 had the highest mRNA expression levels in these cells. Although all HDACs were detected in resting CD4+ T cells by Western blot analysis, HDAC5, -8, and -11 were primarily sequestered in the cytoplasm. Using chromatin immunoprecipitation assays, we detected HDAC1, -2, and -3 at the HIV-1 promoter in Jurkat J89GFP cells. Targeted inhibition of HDACs by siRNA demonstrated that HDAC2 and HDAC3 contribute to repression of HIV-1 long terminal repeat expression in the HeLa P4/R5 cell line model of latency. Together, these results suggest that HDAC inhibitors specific for a limited number of class I HDACs may offer a targeted approach to the disruption of persistent HIV-1 infection.

INTRODUCTION

Persistent proviral human immunodeficiency virus type 1 (HIV-1) infection, primarily within a small population of long-lived resting CD4+ T cells, is a central obstacle to the clearance of established HIV-1 infection. This is due, in part, to the occasional silencing of proviral HIV-1 genomes in resting CD4+ T cells, shielding this reservoir from immunological or pharmaceutical attack. To overcome proviral latency, potent and clinically tolerable agents capable of inducing expression of latent HIV-1 must be identified. Such efforts will be aided by an understanding of mechanisms that regulate transcription from the HIV-1 5' long terminal repeat (LTR) promoter.

Once integrated, HIV-1 resides in the host chromatin environment where its genome is packaged around histone octamers (25). The N-terminal tails of histones can be chemically modified by multiple enzymes including histone acetyltransferases (HATs) and histone deacetylases (HDACs) (reviewed in reference (5)). Acetylation of the lysine residues in histone tails by HATs allows transcriptional machinery access to the DNA template and serves as a signal for recruitment of transcription factors and complexes that upregulate gene expression. Conversely, HDACs are a family of lysine deacetylases that act on multiple targets, including histone tails. Deacetylation of histones creates a chromatin environment unfavorable to transcription, in part by creating a platform for the recruitment of histone methyltransferases and other factors that repress transcription. There are 18 known human HDACs that are divided into 4 classes based on amino acid sequence and domain organization (reviewed in reference (5)). Class I HDACs (HDAC1, -2, -3, and -8); class II HDACs (HDAC4, -5, -6, -7, -9, and -10); and the class IV HDAC, HDAC11, are all sensitive to global HDAC inhibitors such as trichostatin A (TSA). Class III HDACs, also known as the

sirtuins, are structurally unrelated to the other classes and are not sensitive to traditional HDAC inhibitors.

Evidence suggests that mechanisms of acetylation and deacetylation regulate HIV-1 proviral expression (reviewed in reference (22)). HDACs are recruited to the initiator and enhancer regions of the HIV-1 promoter by several transcription factors and corepressor complexes. The recruitment of HDAC1 to the latent HIV-1 LTR was first shown to be mediated by YY1 and LSF (4), and later studies demonstrated that NF-κB p50 homodimers (26), AP-4 (10), CTIP2 (20), Sp1 and c-Myc (12), and CBF-1 (23) could all participate in HDAC1 recruitment. CTIP2 was also found to recruit HDAC2 to the Sp1 binding site of the LTR in microglial cells (20). Furthermore, HDAC3 has been shown to associate with the HIV-1 LTR (18). HDAC3 occupancy at the LTR is further supported by evidence demonstrating recruitment of the HDAC3-containing corepressor complexes, N-CoR and SMRT, by unliganded thyroid hormone receptor in a *Xenopus laevis* oocyte model system for chromatin assembly (9).

The existence of multiple mechanisms that recruit HDACs to the proviral promoter may be of high therapeutic significance. Nonselective HDAC inhibition with potent agents such as TSA can induce HIV-1 expression in cell line models of latency (15, 16, 24). Furthermore, HDAC inhibitors induce viral expression in resting CD4+ T cells obtained from aviremic, HIV-1-positive patients (1, 27). Inhibitors specific for the individual HDACs relevant to HIV-1 LTR regulation may provide a more selective targeting of the viral promoter and avoid toxicities that can accompany robust, global HDAC inhibition.

To define the specific HDACs that regulate HIV-1 transcription during latency, we characterized mRNA expression and protein expression and localization of HDAC1 to -11 in

the resting CD4+ T cells of HIV-1-positive patients. We evaluated HDAC occupancy at the HIV-1 LTR and the effect of targeted HDAC inhibition on HIV-1 transcriptional activation in cell line models of HIV-1 latency. We find that HDACs 2 and 3 play a prominent role in the regulation of HIV-1 expression. These enzymes may be important targets for selective antilatency therapies.

MATERIALS AND METHODS

Primary CD4+ T cells and cell lines. Resting CD4+ T cells were obtained from aviremic (<50 HIV-1 RNA copies/ml plasma), HIV-1-positive patients on stable antiretroviral therapy with CD4+ T-cell counts >300/μl via continuous-flow leukapheresis. Peripheral blood mononuclear cells were purified from leukapheresis products using a Ficoll gradient and were subjected to a negative-selection purification using an antibody cocktail of anti-CD8, anti-CD14, anti-CD16, anti-CD19, anti-CD56, anti-glycophorin A, anti-CD41, anti-CD25, and anti-HLA-DR as previously described (2) to obtain purified (>97%) resting CD4+ T cells defined as a $CD4^+$ $CD45^+$ $CD3^+$ $CD69^-$ $CD25^-$ $CD8^-$ $CD14^-$ $HLA\text{-}DR^-$ population by fluorescence-activated cell sorting analysis. J89GFP cells (16) were maintained in RPMI1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). HeLa P4/R5 cells (14) were cultured in phenol red-free Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.5 mg/ml Puromycin (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin.

RNA extraction and microarray analysis. RNA was extracted from purified resting CD4+ T cells of aviremic, HIV-1-positive patients and hybridized to a microarray of 23,500

60-mer oligonucleotides from Agilent Technologies (Palo Alto, CA) as previously described (12). Intensities of HDAC mRNA expression were normalized to an Agilent internal standard and converted to log ratio values using the Rosetta Resolver system (Rosetta Biosoftware, Seattle, WA).

Western blot analysis of protein expression and localization. For fractionation of nuclear and cytoplasmic proteins, purified resting CD4+ T or J89GFP cells were first lysed for 10 minutes on ice in a buffer containing 10 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1% NP40, 10 mM NaF and protease inhibitor cocktail (Sigma). Following centrifugation, cytoplasmic extracts were removed and nuclei were incubated in RIPA buffer with protease inhibitor cocktail (Sigma) and 10 mM NaF for 10 minutes on ice. Cellular debris were removed by centrifugation, and nuclear extracts were collected. Protein concentrations were determined using Bradford protein assays (Bio-Rad). To denature proteins, extracts were heated at 95˚C for 5 minutes in NuPAGE LDS sample buffer and NuPAGE sample reducing agent (Invitrogen). Protein extracts were separated on 4 to 12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were probed with the following antibodies: anti-HDAC1 (sc-7872x), anti-HDAC2 (sc-7899x), anti-HDAC3 (sc-11417x), anti-HDAC6 (sc-11420), anti-HDAC7 (sc-11421x), anti-HDAC8 (sc-11405), or anti-HDAC9 (sc-28732) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HDAC4 (40969), anti-HDAC5 (40970) (Active Motif, Carlsbad, CA); anti-HDAC10 (AP1110a), anti-HDAC11 (AP1111b) (Abgent, San Diego, CA); anti-lamin B1 (ab16048), anti-alpha tubulin (ab7291) (Abcam, Cambridge, MA); anti-glyceraldehyde-3-phosphate dehydrogenase (MAB374) (Millipore, Billerica, MA). Membranes were then washed and incubated with the corresponding

horseradish peroxidase-conjugated secondary antibodies from ECL-Amersham (GE Healthcare, Piscataway, NJ). Proteins were visualized using ECL or ECL Plus detection reagent and developed on Hyperfilm ECL (GE Healthcare). Whole-cell protein extracts from resting CD4+ T cells were collected by incubating cells in RIPA buffer as described above. Membranes were stripped by incubating in 62 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, and 2.7% β-mercaptoethanol for 25 minutes at 56˚C and reprobed with antibodies as needed.

ChIP assays. J89GFP cells were fixed with 1% formaldehyde for 10 min at room temperature and subjected to the chromatin immunoprecipitation (ChIP) procedure using a ChIP assay kit (Millipore) following the manufacturer's protocol. Each immunoprecipitation was performed with approximately 1 million cells and 5 to 10 µg of the following ChIP-validated antibodies: anti-HDAC1 (sc-7872x), anti-HDAC2 (sc-7899x), and anti-HDAC3 (sc-11417x) from Santa Cruz, anti-HDAC4 (40969) or anti-HDAC6 (40971) from Active Motif, or anti-HDAC7 (ab1441) from Novus Biologicals (Littleton, CO). Rabbit immunoglobulin (IgG) serum (5 to 24 µg; Sigma) was used to control for nonspecific immunoprecipitation of DNA. After formaldehyde de-cross-linking and proteinase K digestion (Roche, Nutley, NJ), DNA was purified using a QIAquick PCR purification kit (Qiagen, Germantown, MD) in accordance with the manufacturer's protocol.

Immunoprecipitated DNA was amplified using LTR -109F/LTR+82R (27) or LTR7F/LTR8R (12) primers to detect enrichment of the HIV-1 LTR and visualized by separating PCR products on an 8% acrylamide gel stained with ethidium bromide. Images were obtained using an InGenius L gel documentation system and GeneSnap software (Syngene, Frederick, MD). For quantitative PCR assays, ChIP DNA was amplified using

LTRrt8 (5'-TAGCCAGAGAGCTCCCAGGCTCAGA-3') and LTRrt9 (5'-AGCCCTCAGATGCTACATATAAGCA-3') primers and Power SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA). Values represent the enrichment over the IgG negative control using the threshold cycle ($2^{-\Delta\Delta ct}$) method.

siRNA transfection and β-galactosidase assays. Three distinct siRNAs were designed to each HDAC transcript based on predictors of on- and off-target activity (Sigma-Proligo). The sense sequences of siRNAs were as follows: HDAC1-1 CGCCAAGUGUGUGGAAUUU, HDAC1-2 CGAAUCCGCAUGACUCAUA, HDAC1-3 CUCAUAAUUUGCUGCUCAA; HDAC2-1 CAAAUACUAUGCUGU CAAU, HDAC2-2 CUCAUUAUCUGGUGAUAGA, HDAC2-3 CAGUGAUGAG UAUAUCAAA; HDAC3-1 CCAAGAGUCUUAAUGCCUU, HDAC3-2 GGCACC CAAUGAGUUCUAU, HDAC3-3 CAUUCAGGAUGGCAUACAA; HDAC8-1 CAUUCAGGAUGGCAUACAA, HDAC8-2 GACCGUGUCCCUGCACAAA, HDAC8-3 CAGUAUGGUGCAUUCUUUG. Individual siRNAs were reconstituted in nuclease-free water to achieve a 20 μM solution. The siRNAs targeting a single HDAC transcript were pooled at a 1:1:1 ratio prior to transfection. The siCONTROL Non-Silencing 1 (NS1) negative control was purchased from Dharmacon (Lafayette, CO) and reconstituted according to the manufacturer's protocol.

All siRNA transfection experiments were performed using HeLa P4/R5 cells. Briefly, cells were plated in 96-well, white tissue culture-treated plates (Corning, Corning, NY) in 80 μl of assay medium (phenol red-free Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 0.5 mg/ml Puromycin [Sigma], 100 U/ml penicillin and 100 μg/ml streptomycin [Invitrogen]) at $1x10^5$cells/ml and incubated overnight at 37° C and 5% CO_2. Prior to transfection, siRNA-Oligofectamine (Invitrogen) complexes

were established according to the manufacturer's protocol and then added to each well to achieve a final concentration of 50 nM siRNA and 0.5% Oligofectamine. After incubation at 37° C and 5% CO_2 for 20 h, medium was removed from the cells and replaced with assay medium containing either 1 μM TSA or 1% dimethyl sulfoxide (DMSO). Following 20 h incubation at 37° C and 5% CO_2, LTR-mediated β-galactosidase activity was measured using Gal-Screen (Applied Biosystems) according to the manufacturer's protocol.

Cell proliferation assays. Cell proliferation was evaluated following transfection with HDAC siRNA and incubation with TSA or DMSO using the alamarBlue assay (Trek Diagnostic Systems, Cleveland, OH) according to the manufacturer's protocol.

Statistical analysis. Normalized β-galactosidase activity and cell proliferation following siRNA-mediated HDAC knockdown were analyzed by the non-parametric Kruskal-Wallis test. To make individual comparisons against the mock control, the Mann-Whitney U test was applied post-hoc with Bonferroni's correction. Analyses were performed using SPSS (Version 16.0) software (Chicago, IL). A *P* value less than 0.05 was considered statistically significant.

RESULTS

HDAC1, -3, and -7 are highly expressed in resting CD4+ T cells. To determine which HDACs are expressed in resting CD4+ T cells, the primary reservoir for latent HIV-1 infection, we calculated the relative intensities of mRNA expression for HDAC1 to -11. Levels of HDAC mRNA expression were similar between patients. HDAC1, -3 and -7 had the highest mRNA expression levels (Fig. 2.1A). However, as these levels were derived

from microarray data, they provide information on only the relative intensities of HDAC expression in the resting CD4+ T cells of the patients and not absolute expression levels.

To evaluate HDAC protein expression, we obtained whole-cell extracts from the resting CD4+ T cells of three additional HIV-1-positive patients and performed Western blot analysis using antibodies specific for HDAC1 to -11. HDAC1 to -11 were detected in all three patients, and levels of HDAC protein expression were relatively constant between patients (Fig. 2.1B). As a positive control, we performed Western blotting on 2 μg of lysate from 293T cells transfected with an HDAC10 expression plasmid (lysate obtained by Abgent) to verify the ability of the HDAC10 antibody to function in Western blotting. Although HDAC10 was undetectable in 20 μg of whole-cell extracts as shown in Fig. 2.1B, HDAC10 was detected when Western blot analysis was performed on 40 μg of whole-cell extracts (data not shown). However, the low levels of HDAC10 expression in resting CD4+ T cells suggest that it is unlikely to contribute to HIV-1 latency.

HDAC5, -8, and -11 are predominantly cytoplasmic in resting CD4+ T cells. To determine the localization of HDACs within resting CD4+ T cells, we separated cellular lysates into nuclear and cytoplasmic fractions and evaluated HDAC protein expression by Western blot analysis. Most HDACs were expressed in the nucleus, and many were detectable in both the nuclear and the cytoplasmic fractions (Fig. 2.2). However, HDACs 5, 8, and 11 were primarily localized to the cytoplasm. These findings suggest that it is unlikely that HDAC5, -8, or -11 plays a direct role in regulating transcription driven by the LTR at the level of chromatin modification in the latent reservoir of resting CD4+ T cells, although it is possible that some amounts of these enzymes reside in the nucleus below the level of detection of the antibodies used in this assay. Furthermore, it does not exclude a possible

contribution to transcriptional regulation by these HDACs in a pathway upstream of the LTR through deacetylation of cellular factors in the cytoplasm. HDAC10 expression levels were too low to permit appropriate analysis of localization in resting CD4+ T cells.

Because latency is a phenomenon that occurs in resting CD4+ T cells and not activated CD4+ T cells, we examined whether there was a differential regulation of HDAC expression and/or localization in these two cell types. Most HDACs had similar expression levels and localization before and after mitogen stimulation with phytohemagglutinin A (PHA) (Fig. 2.2). However, following T-cell activation, HDAC7 expression dramatically increased and became almost exclusively sequestered in the cytoplasm. Additionally, although HDAC11 remained cytoplasmic following T-cell activation, there was a noticeable decrease in HDAC11 protein expression. The differential regulation in the expression and localization of HDAC7 and HDAC11 in resting compared to activated CD4+ T cells suggests that they may be serving different functions in the two cell populations. However, siRNA-mediated knockdown of HDAC7 or HDAC11 in the HeLa P4/R5 cell line model of HIV-1 latency did not induce LTR activation (data not shown). Thus, although HDAC7 and HDAC11 are differentially regulated in resting versus activated CD4+ T cells, we found no evidence that they contribute to silencing of the proviral HIV-1 LTR.

HDAC1, -2, and -3 are resident at the HIV-1 LTR. Of HDAC1 to -11, there is evidence that HDAC1, -2, and -3 are recruited to the LTR in model cell lines of HIV-1 latency (4, 10, 12, 18, 20, 23, 26). Other HDACs could play an important role in HIV-1 regulation and could be critical for effective therapeutic targeting of latent infection. The frequency of latent HIV-1 in resting CD4+ T cells has been estimated at less than 1 in 10^7 cells (3). Because proviral latency is an extremely rare event, it is impossible to perform

ChIP assays on resting CD4+ T cells obtained from patients, due to the small amount of target DNA. Thus, to address experimentally the question of which HDACs occupy the HIV-1 LTR, we utilized J89GFP cells. J89GFP cells are a Jurkat cell line that contain a stably integrated, full-length HIV-1 provirus (strain 89.6) with a green fluorescent protein (GFP) reporter incorporated into the viral genome (16). The viral genome in J89GFP cells is transcriptionally silent. However, upon appropriate stimulation, such as with the NF-κB inducer tumor necrosis factor alpha or the HDAC inhibitor TSA, viral transcription is activated and viral expression can be measured by GFP production (16). Critically, this cell line reproducibly returns to quiescence when stimuli are withdrawn.

First we determined the protein expression and cellular localization of HDAC1 to -11 in J89GFP cells by Western blot analysis of 30 μg of nuclear and cytoplasmic protein fractions. Due to the abundance of cell line extract, we were able to use twice as much protein in these Western blot assays as in those performed with scarce material extracted from available aviremic, HIV-1-infected patients' cells. We were able to detect all 11 HDACs in J89GFP cells (Fig. 2.3A). The nuclear and cytoplasmic distribution of HDACs in J89GFP cells mirrors that of resting CD4+ T cells with the exception that HDAC1 and HDAC8 are detected in both the nucleus and cytoplasm of J89GFP cells. Although these slight differences in HDAC expression between the J89GFP cells and resting CD4+T may be relevant to HIV-1 latency, importantly, all HDACs that are expressed in the nuclei of resting CD4+ T cells are also present in the nuclei of J89GFP cells. Thus, J89GFP cells are a reasonable model cell line to evaluate HDAC recruitment to the integrated HIV-1 LTR by ChIP.

To assess the occupancy of the various HDACs at the HIV-1 LTR, we first performed ChIP assays using antibodies directed against the class I HDACs that were detected in the nuclear protein extracts of resting CD4+ T cells: HDAC1, -2, and -3. We observed HDAC1, -2, and -3 at the HIV-1 LTR (Fig. 2.3B) of J89GFP cells. These results agree with previous findings in diverse cell systems that HDAC1, -2, and -3 are recruited to the HIV-1 LTR (4, 10, 12, 18, 20, 23, 26). However, to our knowledge this is the first report of HDAC2 occupying the LTR in a T-lymphocytic cell line. Although the amount of HIV-1 LTR DNA immunoprecipitated with an HDAC3 antibody was smaller than the amount immunoprecipitated with HDAC1 or HDAC2 antibodies, it was a consistent finding observed over multiple experiments. Such results do not necessarily indicate that there are fewer HDAC3 molecules associated with the HIV-1 LTR, as they may simply reflect the ability of the particular antibody to perform in ChIP assays, the distance of the target enzyme from the DNA, or the availability of corresponding epitopes following formaldehyde fixation.

Next we examined the HIV-1 LTR occupancy levels of the predominant, nuclear class II HDACs in resting CD4+ T cells: HDAC4, -6, and -7. We did not detect significant levels of HIV-1 LTR DNA enrichment over the IgG negative control following ChIP with ChIP-validated antibodies targeting HDAC4, -6, or -7 (Fig. 2.3C). In these experiments, HDAC2 was used as a positive control to verify success of the ChIP assay. However, as we are unaware of a positive-control region of DNA in J89GFP cells to verify the ability of these antibodies to work in ChIP assays in our hands, we cannot completely exclude the possibility that some levels of these enzymes may associate with the LTR. Taken together, these findings suggest that the class I HDACs HDAC1, -2, and -3 are the predominant HDACs to occupy the HIV-1 LTR in the J89GFP model of latency.

HDAC2 and HDAC3 regulate LTR-driven gene expression. To determine the impact of individual HDACs on the regulation of HIV-1 transcription, we treated the HIV-1 latency cell line model, HeLa P4/R5 cells (14), with siRNAs targeting the class I HDACs, HDAC1, -2, -3 and -8. HeLa P4/R5 cells are a variant of HeLa Magi cells that express both CXCR4 and CCR5. They contain an integrated *lacZ* gene under the control of the HIV-1 minimal LTR and were selected for low background β-galactosidase expression. As these cells contain only an integrated LTR promoter driving a LacZ reporter, they permit identification of factors that exert strong regulation over transcription from the LTR in the absence of Tat.

Silencing of HDAC2 mRNA led to a statistically significant increase in LacZ production as measured by β-galactosidase assays (Fig. 2.4A). Isolated knockdown of HDAC1, -3 and -8 did not result in a significant increase in LTR-driven LacZ expression. Using the alamarBlue assay, we evaluated cell proliferation following siRNA-mediated knockdown of HDAC1, -2, -3, and -8 and observed no differences in cell viability when compared to the mock control (Fig. 2.4C). Thus, the lack of LTR induction seen with siRNA-mediated silencing of HDAC1, -3, and -8 is not due to a decrease in the viability or proliferation of cells following HDAC knockdown.

When proteins like HDAC3 are expressed at high levels in cells, it can be difficult to achieve a full knockdown of expression using siRNA alone. We observed substantial reductions (93%) of HDAC mRNA expression following siRNA knockdown as determined by reverse transcriptase PCR (data not shown). However, to overcome the effect of residual HDAC protein that may persist despite HDAC mRNA knockdown, we combined chemical HDAC inhibition with siRNA-mediated knockdown. Based on the 50% to 90% effective

concentration for TSA induction of the LTR in HeLa P4/R5 cells, we treated P4/R5 cells with a submaximal concentration of TSA in conjunction with individual siRNA-mediated knockdowns of the class I HDACs. Combining TSA with individual siRNAs against HDAC1, -2, or -8 did not lead to a significant increase in LTR activation above that induced by TSA alone (Fig. 2.4B). Activation of the LTR by HDAC2 siRNA was not observed in this experiment because mock transfection combined with TSA treatment induced higher levels of β-galactosidase activity (~18-fold) than did isolated HDAC2 knockdown; thus, the backgrounds of the two experiments are different. Exposure to global HDAC enzymatic inhibition along with a targeted knockdown of HDAC3, however, led to a significant increase in LTR activation over that in the mock-transfected cells that were treated with TSA alone (Fig. 2.4B). There were no differences in cell proliferation following TSA treatment with targeted HDAC knockdowns when compared to the mock control (Fig. 2.4C). Taken together, these findings suggest that both HDAC2 and HDAC3 contribute to the restriction of HIV-1 LTR expression in HeLa P4/R5 cells.

DISCUSSION

In this study, we provide the first comprehensive assessment of the contribution of HDAC1 to -11 to HIV-1 latency in resting CD4+ T cells. Our results suggest that the class I HDACs HDAC1, 2, and 3 are recruited to the HIV-1 LTR and that HDAC2 and HDAC3 regulate HIV-1 transcriptional repression in cell line models of HIV-1 latency. HDAC1, -2, and -3 are expressed in the nuclei of resting CD4+ T cells (Figs. 2.1A, 2.1B, and 2.2), thus their contribution to HIV-1 transcriptional repression may extend to a clinically relevant viral reservoir.

Our data suggest that HDAC2 may be particularly important to the regulation of HIV-1 expression. Because ChIP is not feasible in resting cells CD4+ T cells from HIV-1-positive patients due to the rarity of target DNA, we performed ChIP assays in the J89GFP cell line model of HIV-1 latency. We observed HDAC2 associating with HIV-1 LTR DNA. Although HDAC2 was previously reported to be recruited to the LTR in microglial cells (20), to our knowledge this is the first report of HDAC2 being detected at the HIV-1 LTR in a T-lymphocytic cell line (Fig. 2.3B). Isolated knockdown of HDAC2 by siRNA resulted in a significant increase in LTR-driven gene expression (Fig. 2.4A) in HeLa P4/R5 cells. These results are similar to Marban et al. (20), who detected a modest 1.5-fold increase in LTR-driven expression of a luciferase reporter when HDAC2 was targeted by short hairpin RNA.

As previously reported (18), we found that HDAC3 was recruited to the HIV-1 LTR (Fig. 2.3B). Additionally, in combination with global HDAC enzymatic inhibition by TSA, siRNA-mediated knockdown of HDAC3 resulted in a synergistic increase in HIV-1 expression (Fig. 2.4B). We achieved a substantial knockdown of HDAC3 mRNA (93%; data not shown), but LTR upregulation was only seen when the HDAC3 knockdown was combined with submaximal global HDAC inhibition. Persistent activity of a large cellular pool of HDAC protein, despite mRNA inhibition, could explain this finding. Alternatively, as has been reported with other HDACs, HDAC3 may function as a transcriptional repressor via a function that does not depend on its deacetylase activity (7, 17, 28). In the presence of global HDAC enzymatic inhibition, HDAC 3 may continue to restrict LTR expression. This repression could be relieved by HDAC3 knockdown but would not be apparent without inhibition of other resident HDACs, e.g., HDAC1 and HDAC2.

It has been demonstrated by five independent research groups that HDAC1 is recruited to the LTR in cell line models of HIV-1 latency by an array of transcription factors (Fig. 2.3B) (4, 10, 12, 20, 23, 26). The existence of multiple mechanisms for the recruitment of this HDAC implies that it plays a role maintaining LTR repression. Furthermore, in a chemical library screen of small-molecule HDAC inhibitors at Merck Laboratories, a decreasing HDAC1 50% inhibitory concentration correlated with an increase of LTR activation in HeLa P4/R5 cells (8). However, targeted HDAC1 inhibition by siRNA did not substantially increase LTR expression (Fig. 2.4A). Thus, while HDAC1 may contribute to the maintenance of deacetylated histones at the latent LTR, other HDACs appear to compensate for the loss of HDAC1 when its expression is dampened.

Future efforts to evaluate the impact of targeted HDAC knockdown on latent viral outgrowth from the resting CD4+ T cells of HIV-1-positive patients are warranted to verify the importance of these findings to HIV-1 latency. Targeting a combination of selected HDACs (e.g., HDAC2 and HDAC3 or HDAC1, -2, and -3) may prove to be a more effective strategy for inducing HIV-1 expression in a broad population of cells *in vivo,* as HDAC expression is cell and tissue type specific (5). The discovery and development of new compounds with selective HDAC-inhibitory abilities are an area of intense research (reviewed in references (11 and 13)). HDAC inhibitors are currently in use in numerous clinical trials for cancer treatment (6, 21), and the selective HDAC inhibitor, vorinostat, has been approved for the treatment of subcutaneous T-cell lymphoma (19). We have recently shown that vorinostat can induce expression of HIV-1 from the resting CD4+ T cells of HIV-1-infected patients (1). Thus, selective HDAC inhibitors are viable candidates to explore as potential antilatency therapies.

Our results indicate that HDAC1, -2, and -3 are expressed in the nuclei of the latent reservoir of resting CD4+ T cells in HIV-1-positive patients. HDAC1, -2, and -3 occupy the HIV-1 LTR in the J89GFP model of HIV-1 latency, and siRNA-mediated knockdown of HDAC2 and HDAC3 can reactivate the integrated, quiescent LTR of HeLa P4/R5 cells. These observations suggest that the use of a class I-selective HDAC inhibitor, in particular one that acts on HDAC1, -2, and -3, may prove to be an attractive antilatency strategy with fewer of the toxicities that can accompany global HDAC inhibition.

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AUTHORSHIP CONTRIBUTIONS

K.S.K designed and performed all protein and chromatin studies. N.M.A. isolated and maintained resting CD4+ T cells and harvested mRNA from resting CD4+ T cells for microarrays. A.E. and D.J.H. were responsible for microarray analysis and A.T.G., A.E., and D.J.H. were responsible for siRNA and cell proliferation assays performed at Merck

Research Laboratories. K.S.K. and D.M.M. the analyzed data and wrote the paper with input from all authors.

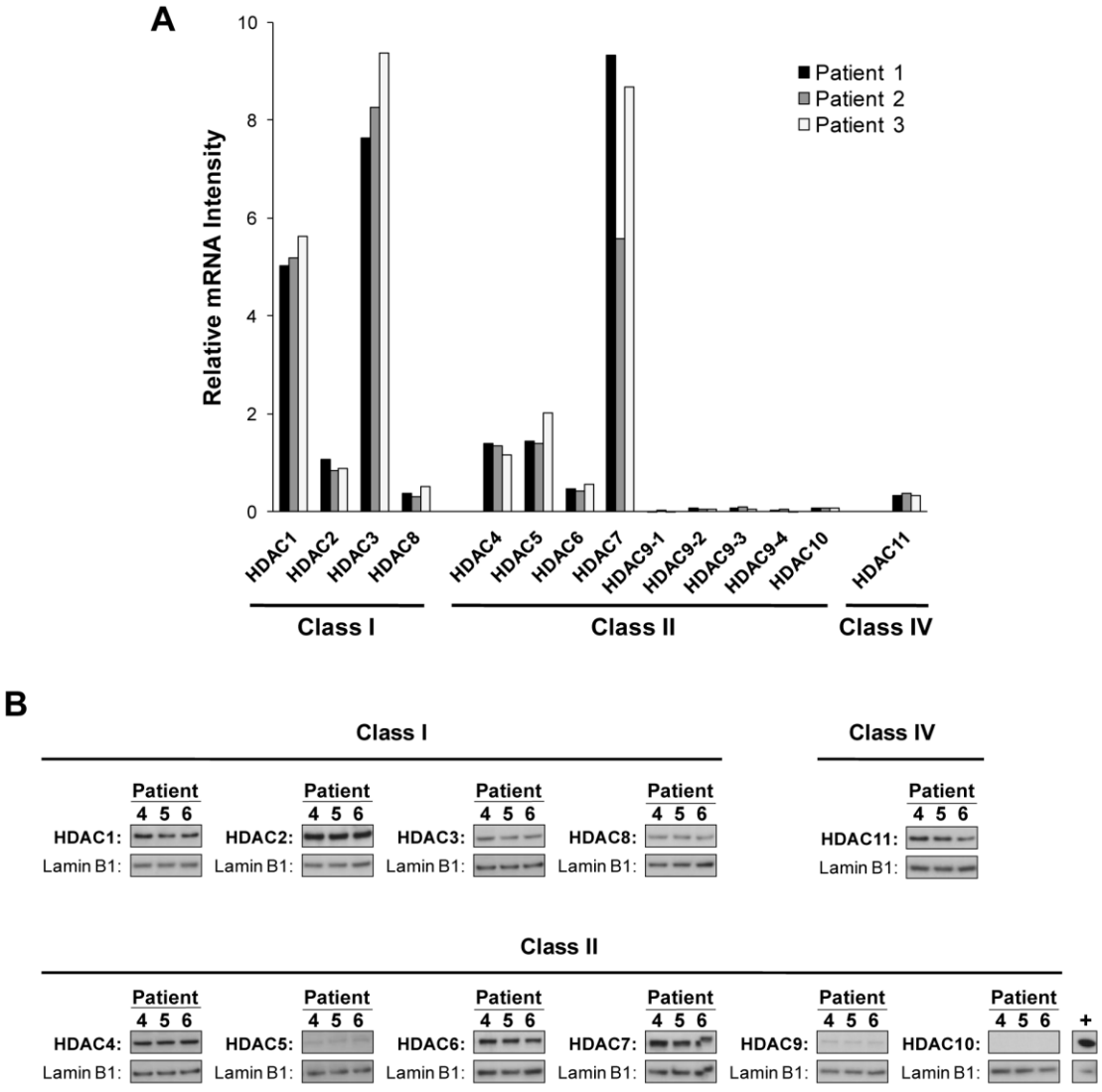

Figure 2.1 HDAC mRNA expression in resting CD4+ T cells from aviremic, HIV-1-positive patients. (A) HDAC1, -3, and -7 are the most highly expressed HDAC mRNAs in resting CD4+ T cells. Microarray analysis of mRNA expression in the resting CD4+ T cells from three HIV-1-positive patients provided relative intensities of HDAC mRNA expression. (B) HDAC1 to -11 are detectable in resting CD4+ T cells. Whole-cell extracts were obtained from the resting CD4+ T cells of HIV-1-positive patients, and 20 µg of protein was subjected to Western blot analysis with antibodies specific for HDAC1 to -11. An antibody against the nuclear envelope marker lamin B1 was used as a loading control. As a positive control (+) for the anti-HDAC10 antibody, 2 µg of whole-cell extracts from 293T cells transfected with an HDAC10 expression plasmid was subjected to Western blotting. Although HDAC10 was not detected in patient extracts in the experiment shown, it was detected when 40 µg of extracts was assayed (data not shown).

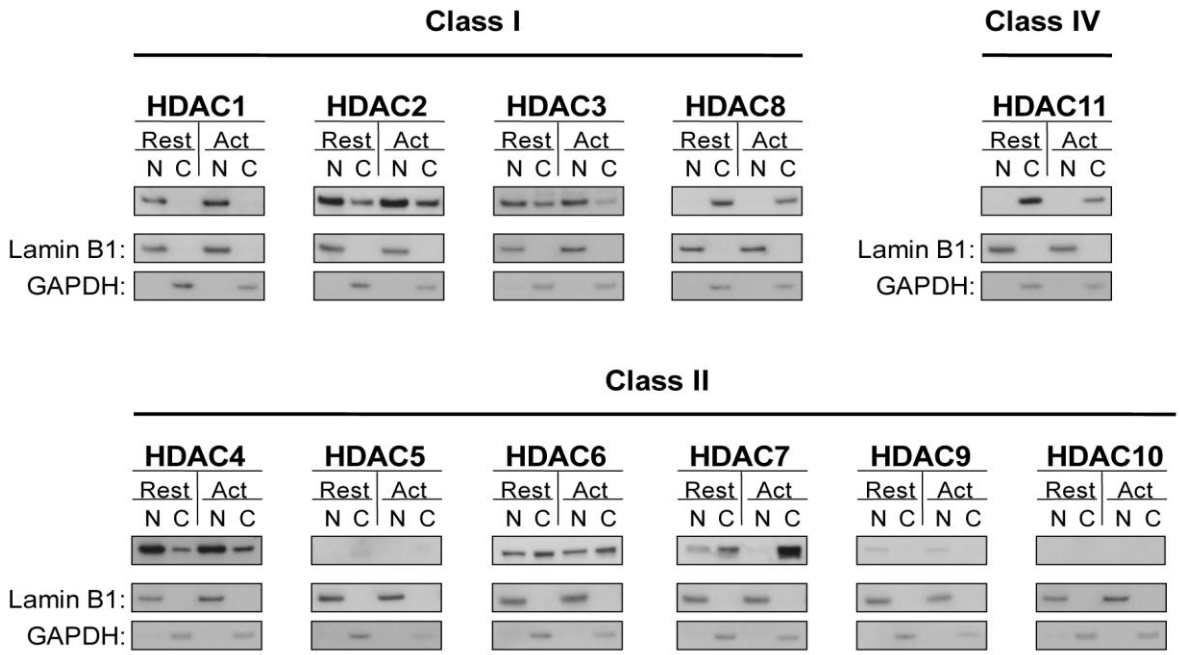

Figure 2.2 HDACs 5, 8, and 11 are excluded from the nuclei of resting CD4+ T cells. Resting CD4+ T cells from an aviremic, HIV-1-positive patient (patient 5 from Fig. 2.1A) were maintained in media (Rest) or activated by incubating with 1 µg/ml of the mitogen, phytohemagglutinin (PHA), overnight (Act) before harvesting cellular lysates. Proteins were separated into nuclear (N) and cytoplasmic (C) fractions and 15 µg of extracts were probed with antibodies targeting HDACs 1-11 in Western blot analysis. Antibodies against Lamin B1 and GAPDH were used to assess loading of nuclear and cytoplasmic lysates. HDACs 5, 8, and 11 were primarily localized to the cytoplasm in CD4+ T cells. Following T cell activation with PHA, HDAC7 expression increased and became sequestered in the cytoplasm, while HDAC11 expression decreased.

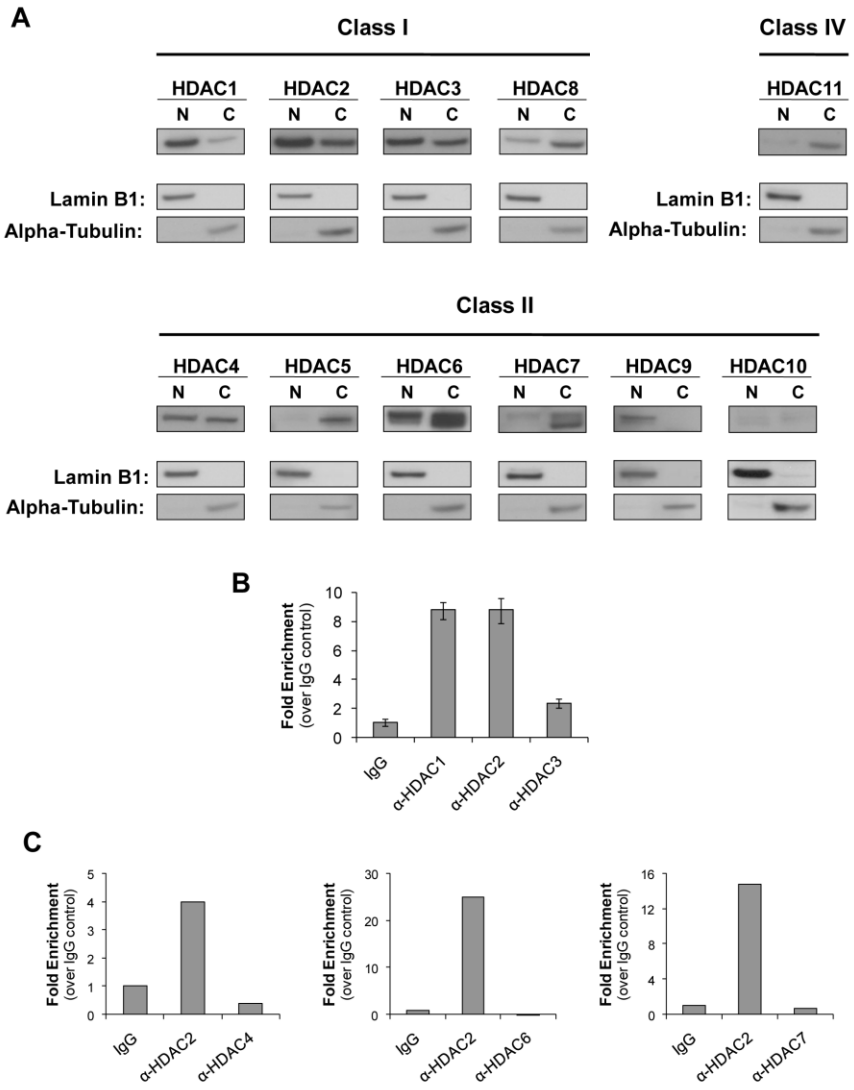

Figure 2.3 HDAC1, -2, and -3 are recruited to the HIV-1 LTR in the J89GFP cell line model of latency. (A) HDAC localization in J89GFP cells is similar to that in resting CD4+ T cells. Nuclear (N) and cytoplasmic (C) protein extracts (30 µg each) from J89GFP cells were probed with antibodies against HDAC1 to -11 in Western blot analysis. Antibodies targeting lamin B1 and alpha-tubulin were used as loading controls for nuclear and cytoplasmic extracts, respectively. (B) HDAC1, -2, and -3 associate with the HIV-1 LTR. Antibodies targeting the nuclear class I HDACs HDAC1, -2, and -3 were used in ChIP assays in J89GFP cells. Rabbit IgG serum was used to assay nonspecific immunoprecipitation of LTR DNA. (C) The nuclear class II HDACs HDAC4, -6 and -7 do not associate with the HIV-1 LTR. ChIP assays were performed in J89GFP cells. HDAC2 was used as a positive control, and rabbit IgG serum was used as a negative control. Values in panels B and C represent the enrichment of LTR DNA over the IgG negative control as determined by quantitative PCR. Experiments were performed on at least three occasions. Data are expressed as the means ± standard errors of the means.

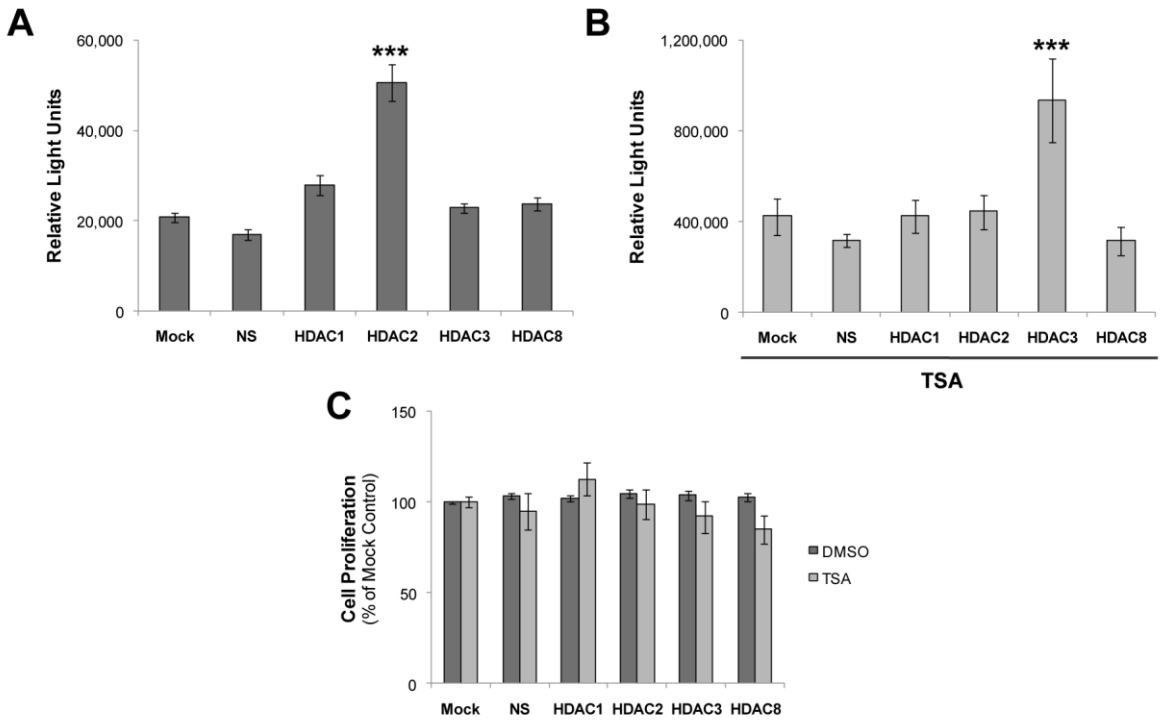

Figure 2.4 HDAC2 and HDAC3 negatively regulate the HIV-1 LTR. (A) siRNA-mediated knockdown of HDAC2 induced a significant increase in LTR-driven lacZ expression when compared to the mock control (n = 64; *** $P < 0.001$). P4/R5 cells were transfected with siRNAs targeting the class I HDACs HDAC1, -2, -3, and -8. Twenty hours post-transfection, cells were incubated with 1% DMSO (vehicle control) for an additional 20 h. As a control, cells were transfected with nonspecific (NS) siRNA. Values are displayed in relative light units and were derived from Gal-Screen assays of cellular lysates. (B) siRNA knockdown of HDAC3 in conjunction with exposure to a submaximal concentration of the HDAC inhibitor TSA upregulated LTR-driven lacZ expression compared to cells that were mock-transfected and exposed to TSA (n = 32, *** $P < 0.001$). Cells were treated as for panel A except that 1 μM TSA was added in place of DMSO. (C) There were no differences in cell proliferation following HDAC knockdown compared to the mock controls (n = 12, $P = 0.522$ for DMSO experiment; n = 12, $P = 0.307$ for TSA experiment). alamarBlue assays were used to assess cell viability in panels A and B. Data in panels A to C are the combined results of at least three experiments and are expressed as the means ± standard errors of the means.

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CHAPTER 3

HISTONE DEACETYLASES 1, 2, AND 3 CONTRIBUTE TO VARIEGATED REPRESSION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 PROVIRAL INTEGRANTS IN RESTING CD4+ T CELLS

Keedy, K. S., Archin, N. M., and D. M. Margolis

ABSTRACT

Selective histone deacetylase (HDAC) inhibitors have emerged as a potential antilatency therapy for persistent human immunodeficiency virus type 1 (HIV-1) infection. We utilized a small interfering (si)RNA-mediated knockdown strategy for delineating the minimal HDAC-inhibitory requirement for optimal induction of latent HIV-1 expression. Isolated knockdown of either HDAC2 or HDAC3 induced HIV-1 expression in the J89 latency cell line model. However, although selective enzymatic inhibition of HDAC1, -2, and -3 with a small molecule HDAC inhibitor was a potent inducer of HIV-1 expression in J89 cells, concurrent knockdown of HDAC1, -2, and -3 did not lead to HIV-1 expression. These results suggest that there is a fundamental difference between depletion of HDAC expression by siRNA and enzymatic inhibition with an HDAC inhibitor. When we examined the effects of isolated HDAC1, -2, or -3 knockdown on the *ex vivo* recovery of virus from the resting CD4+ T cells of aviremic patients, we observed outgrowth of selected proviral integrants. Thus, HDAC1, -2, and -3 contribute to variegated repression of proviruses in resting CD4+ T cells.

INTRODUCTION

Persistence of chronic human immunodeficiency virus type 1 (HIV-1) infection, despite highly effective antiretroviral therapy (ART), poses a formidable obstacle to eradication of HIV-1. The HIV-1 latent reservoir is established early during acute infection and persists in long-lived resting CD4+ T cells throughout an infected individual's life (9, 13, 14). With millions of people newly infected with HIV-1 each year, the health and economic costs of life-long antiretroviral regimens are a heavy burden and call for new approaches to eradicate HIV-1 (44). Design and testing of therapeutic eradication strategies requires a better understanding of the factors that establish and maintain HIV-1 latency.

HIV-1 enters and replicates productively in activated CD4+ T cells. Following reverse transcription, the HIV-1 genome translocates to the nucleus and integrates into cellular DNA. Like host DNA, HIV-1 is packaged around nucleosomes consisting of histone octamer complexes. Transcription of proviral DNA is regulated in part by nucleosome structure and histone modifications (45). The HIV-1 long terminal repeat (LTR) promoter associates with multiple nucleosomes that are positioned at discrete sequences in the proviral genome (46). The enhancer and promoter regions of the LTR are flanked by two nucleosomes: nuc-0 positioned at the 5'-end of the U3 region of the LTR and nuc-1 located just downstream of the transcription start site. Tight association of proviral DNA with these nucleosomes serves as an obstacle to HIV-1 transcription. However, in activated CD4+ T cells, transcriptional activators are available in abundance and localized in the nucleus, permitting low-level induction of elongated transcripts from the LTR (50).

The HIV-1 transactivator of transcription, *tat,* is one of the first viral genes transcribed. Once levels reach a certain threshold, Tat bolsters HIV-1 expression by

recruiting transcriptional activators, such as positive transcription elongation factor b (pTEF-b) and histone acetyltransferases (HATs), to the LTR (16, 36, 54). HATs, in turn, acetylate lysine residues on the histone tails of nuc-0 and nuc-1. This loosens the associations between proviral DNA and histones, facilitating RNA polymerase II loading and processivity (34). Additionally, the acetylated residues can serve as docking sites for transcriptional activators that possess bromodomains, protein motifs that recognize and bind to acetylated residues on histone tails (21, 22). Thus, infection of activated CD4+ T cells results in HIV-1 replication.

Resting CD4+ T cells are thought to be resistant to productive HIV-1 infection due to the quiescent phenotype of these cells, which encompasses low nuclear levels of the cellular transcription factors required for viral expression (15, 17, 38, 51). Although evidence exists that HIV-1 can occasionally overcome these barriers to directly infect resting CD4+ T cells, the resting cell latent reservoir is primarily thought to be generated when an activated CD4+ T cell is infected by HIV-1 as it transitions to the long-lived, resting memory CD4+ T cell state (32, 42). If HIV-1 genome integration has occurred, then a latent infection can ensue. Replication-competent virus can be recovered from latently-infected CD4+ T cells following mitogen stimulation or exposure to agents such as histone deacetylase (HDAC) inhibitors or protein kinase agonists (8, 53).

During latency, several restrictive factors associate with the HIV-1 LTR and block efficient transcription initiation and mRNA elongation. Among these factors are HDACs, a family of enzymes that regulate transcription of numerous cellular and viral genes by removing acetyl groups from lysine residues on both histones and non-histone proteins (10, 35). Deacetylation of histone tails leads to compaction of DNA and removal of important docking signals for activating transcription factors. The result is an overall repressive

transcriptional environment. HDACs are divided into four classes based upon amino acid sequence and domain organization (18). The class I HDACs include HDAC1, -2, -3, and -8. HDAC4, -5, -6, -7, -9, and -10 make up the class II HDACs, and HDAC11 is the sole member of class IV. Class III HDACs include sirtuins 1-7, which are nicotinamide adenine dinucleotide-dependent deacetylases structurally unrelated to class I, II, and IV HDACs. Class III HDACs are not sensitive to the type of HDAC inhibitors that induce HIV-1 expression.

Nonselective and class I-selective HDAC inhibitors are potent inducers of HIV-1 expression in both cell line models and *ex vivo* outgrowth assays using resting CD4+ T cells from HIV-1-infected individuals (3, 12, 40, 41, 53). The class I HDACs HDAC1, -2, and -3 are recruited to the LTR in cell line models of HIV-1 latency (25, 29, 35, 43, 49). These class I HDACs are highly expressed in the nuclei of resting CD4+ T cells and inhibitors selective for HDAC1, -2, and -3 are strong inducers of latent HIV-1 expression in resting CD4+ T cells (4, 29). In contrast, inhibitors selective for the class II HDACs do not induce expression of HIV-1 (4). Furthermore, we have not observed association of HDAC4, -6, or -7 with the HIV-1 LTR (29).

Although we are unaware of highly selective inhibitors for HDAC1, -2, or -3 in isolation, an inhibitor selective for HDAC1 and HDAC2—but not HDAC3—does not activate latent HIV-1 (4). This suggests that HDAC3 enzymatic inhibition is crucial for inducing HIV-1 expression, but it is unclear if simultaneous inhibition of HDAC1 and HDAC2 is also needed. Determining the minimum HDAC inhibition required to induce latent HIV-1 expression may focus efforts to identify and develop selective HDAC inhibitors

for antilatency therapies that would have fewer off-target effects when compared to pan-HDAC inhibition.

In an effort to better understand the role of individual class I HDACs in regulation of HIV-1 transcription, we chose to explore the impact of isolated and combination siRNA-mediated knockdown of HDAC1, -2, and -3 on HIV-1 expression in a T cell line model of latency. We extended these studies to resting CD4+ T cells isolated from HIV-1-infected patients. To our knowledge, this is the first study to examine the effects of HDAC knockdown on *ex vivo* latent HIV-1 outgrowth from resting CD4+ T cells.

MATERIALS AND METHODS

Isolation of resting CD4+ T cells and latency cell line models. To obtain a population of purified resting CD4+ T cells, aviremic (<50 HIV-1 RNA copies/ml plasma) HIV-1-positive patients on stable antiretroviral therapy (CD4 counts > 300/μl) were subjected to continuous-flow leukapheresis. Following Ficoll-purification, resting CD4+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) by a previously described (2) negative-selection purification procedure. The purity of the resting CD4+ T cell population, defined as $CD4^+CD45^+CD3^+CD69^-CD25^-CD8^-CD14^-$ HLA-DR^-, was verified by flow cytometry analysis. Resting CD4+ T cells were cultured in IMDM (Invitrogen; Carlsbad, California, USA) suplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 20 U/ml interleukin-2 (IL-2). Prior to use in outgrowth assays, resting CD4+ T cells were maintained in reverse-transcriptase and integrase inhibitors for two days. The J89 latency model cell line was

cultured in RPMI 1640 with 10% FBS, 100 U/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). All cell cultures were maintained at 37˚C under 5% CO_2.

Flow cytometry analysis. Resting CD4+ T cells were washed with cold phosphate-buffered saline (PBS) containing 2% FBS and incubated with antibodies for the indicated markers on ice for 30 minutes. Cells were then washed twice with cold PBS containing 2% FBS and resuspended in PBS containing 3.2% paraformaldehyde. Flow cytometry was performed using a FACScan or a FACSCalibur (Becton Dickinson, San Jose, California, USA) and analyzed with Cell Quest software (Macintosh; Sunnyvale, California, USA) or FlowJo software (Tree Star, Inc.; Ashland, Oregon, USA). Analysis of markers was performed on live cells, as determined by forward and side-scatter profiles.

Nucleofection of siRNA. J89 cells were nucleofected using Nucleofection Kit V (Lonza; Basel, Switzerland) following the manufacturer's protocol. Briefly, 1 or 2 million cells were centrifuged at 90 x g for 10 minutes. Medium was aspirated and cells were resuspended in nucleofection solution V along with siRNA duplexes. Sequences for the sense strand of siRNA duplexes were as follows: HDAC1 GUUAGGUUGCUUCAAUCUA, HDAC2 CCUUGAAUUACUAAAGUAU, and HDAC3 GCAUUGAUGACCAGAGUUA. For HDAC7 knockdowns, a pool of siRNA duplexes was used: HDAC7-1 CCACUUUGCCCAGUCCUUA, HDAC7-2 CUACCAUGUUUCUGCCAAA, and CCUAUGAAUCUCUAAGGCU. A nonspecific (NS) siRNA duplex that targets a luciferase sequence not expressed in the cell lines was used as a control (CGUACGCGGAAUACUUCGAUU). Cells were nucleofected using Lonza Nucleofector program X-001. Following nucleofection, cells were incubated at room temperature for 10 min and then placed into cell culture medium at 37˚C under 5% CO_2. For experiments with

the selective HDAC inhibitors MRK10 and MRK13 (Merck Research Laboratories) (4), drugs were added 24 h post-nucleofection and incubated for an additional 24 h before harvesting the supernatant for HIV-1 p24 ELISA. An equivalent volume of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri) vehicle was added to untreated cells as a control.

Purified resting CD4+ T cells were nucleofected with designated siRNA duplexes using Lonza's Human T Cell Nucleofection Kit following the manufacturer's protocol. Briefly, cells were centrifuged at 200 x g for 10 minutes. Medium was aspirated and cells were resuspended in the accompanying nucleofection solution. Resting CD4+ T cells were nucleofected at a density of 10 million cells per cuvette using program U-014 and placed into cell culture. Following an incubation period of 4 to 6 h at 37˚C under 5% CO_2, the cells were counted and placed into limiting dilution cultures for outgrowth assays.

RNA extraction and quantitative RT-PCR. RNA was extracted from cells using a QIAgen RNeasy Mini Kit (Valencia, California, USA) following the manufacturer's protocol. DNA was removed from RNA extracts by DNAse digestion (Promega; Madison, Wisconsin, USA) and cDNA was synthesized using the SuperScript III First-Strand Synthesis for RT-PCR kit from Invitrogen. Quantitative PCR was performed on cDNA with an Applied Biosystems 7500 Fast Thermocycler (Carlsbad, California, USA) using QuantiTect Multiplex PCR Mastermix (QIAgen) and the following primer pairs and 5' FAM-labeled probes: HDAC1 5' TGAGGACGAAGACGACCCT (forward), 5' CTCACAGGCAATTCGTTTGTC (reverse), and 5' CAAGCGCATCTCGATCTGCTCCTC (probe) (39); HDAC2 5' CTTTCCTGGCACAGGAGACTT (forward), 5' CTCATTGGAAAATTGACAGCATAGT (reverse), and 5'

AGGGATATTGGTGCTGGAAAAGGCAA (probe); and HDAC3 5' GGTGGTTATACTGTCCGAAATGTT (forward), 5' GCTCCTCACTAATGGCCTCTTC (reverse), and 5' AGCAGCGATGTCTCATATGTCCAGCA (probe). An HDAC7 mRNA FAM-labeled primer-probe mix was obtained from Applied Biosystems (Hs00248789_m1). A glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer pair and 5' HEX-labeled probe were included with each reaction for normalization: 5' GCACCACCAACTGCTTAGCACC (forward), 5' TCTTCTGGGTGGCAGTGATG (reverse), and 5' TCGTGGGAAGGACTCATGACCACAGTCC (probe) (47). Relative mRNA expression was calculated using the $2^{-\Delta\Delta ct}$ method.

Western blot analysis. Whole-cell extracts were obtained by lysing cells in RIPA buffer supplemented with 10 mM NaF and Protease Inhibitor Cocktail (Sigma-Aldrich). Protein concentrations were determined by Bradford protein assays using a BioRad Bradford reagent (Hercules, California) following the manufacturer's protocol. Protein extracts were analyzed using standard Western blot methods as previously described (29).

Cell proliferation assays. Cellular proliferation of J89 cells and resting CD4+ T cells was assayed 48 h post-nucleofection using an alamarBlue reagent (Invitrogen) following the manufacturer's protocol. Cellular proliferation was calculated as percent of proliferation compared to the NS siRNA control condition using the following formula: (((117, 216 x absorbance of test condition at 570 nM) - (80, 586 x absorbance of test condition at 600 nM))/((117, 216 x absorbance of NS siRNA control condition at 570 nM) – (80, 586 x absorbance of NS siRNA control condition at 600 nM))) x 100.

Limiting dilution cultures of resting CD4+ T cells. Limiting dilution outgrowth assays of resting CD4+ T cells from HIV-1-positive patients have been described previously

(2). In summary, to determine the patients' infected units per billion resting CD4+ T cells (IUPB), cells were maximally stimulated with 1 µg/ml phytohemagglutinin (PHA)-L (Remel, Lenexa, Kansas, USA) and a 5-fold excess of allogeneic irradiated PBMCs from a seronegative donor for 24 h, then washed and placed in limiting dilution culture. A negative control of unstimulated cells was also placed into limiting dilution culture. Stimulated CD8-depleted PBMCs from a CCR5-sufficient, seronegative donor were added to cultures to permit amplification of expressed virus. All cultures were maintained in IMDM with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 units of IL-2. Cultures were split and fresh medium was added as previously described (1) to ensure optimal growth conditions.

Enzyme-linked immunosorbant assay. Expression of the HIV-1 p24 gag protein was assayed by performing ELISA on supernatants from cell cultures 48 h post-nucleofection of siRNA in J89 cells and at day 15 and 19 of the limiting dilution outgrowth assay for resting CD4+ T cells using an HIV-1 p24 Antigen Capture Assay kit (Advanced BioScience Laboratories, Inc.; Kensington, Maryland, USA) following the manufacturer's protocol. For outgrowth assays with resting CD4+ T cells, wells that were positive for p24 expression on both days were considered positive for viral outgrowth. The recovered IUPB for each condition was calculated using a maximum likelihood method (37). Data points that fell below the limit of detection of the assay were excluded from final analyses.

Statistical analysis. A t test was used to compare the mean p24 expression in J89 cells following nucleofection with NS siRNA and HDAC1, -2, or -3 siRNAs. The differences in HIV-1 outgrowth between NS siRNA and HDAC siRNA conditions in resting CD4+ T

cells isolated from patients were analyzed using a paired t test on log-transformed IUPB values.

RESULTS

HDAC2 and HDAC3 negatively regulate HIV-1 expression in J89 cells. We first evaluated isozyme-specific HDAC knockdowns in the J89 cell line model of HIV-1 latency. J89 cells contain a single, full-length, transcriptionally silent HIV-1 genome (strain 89.6) integrated into cellular DNA. The J89 cell line was clonally selected for a low level of basal HIV-1 expression. However, viral expression is inducible by exposure to appropriate stimuli, such as tumor necrosis factor alpha (TNF-α) or HDAC inhibitors (31). J89 cells were nucleofected with a NS siRNA control sequence or with siRNAs targeting HDAC1, -2, or -3. HDAC mRNA levels following knockdown with HDACs were compared to the expression levels from the NS siRNA control condition 24 h post-nucleofection. HDAC1, -2, and -3 mRNA levels were reduced by 49%, 88%, and 73%, respectively (Fig. 3.1A). The knockdowns were specific to the HDAC targeted, and peak protein knockdown was observed by Western blot at 48 h post-nucleofection (Fig. 3.1B). We did not observe a change in cellular proliferation following HDAC1, -2, or -3 depletion when compared to the NS control at 48 h post-nucleofection as measured by alamarBlue assays (Fig. 3.1C).

We next assessed the impact of individual HDAC knockdown on HIV-1 expression in J89 cells by HIV-1 p24 ELISA on culture supernatants at 48 h post-nucleofection, the time-point where peak HDAC knockdown was observed. Depletion of HDAC2 or HDAC3 in J89 cells led to a modest, but statistically significant increase in HIV-1 p24 expression when compared to the NS control (Fig. 3.1D). HDAC1 knockdown did not induce HIV-1

expression in J89 cells. These results are consistent with our previous studies in the HeLa P4/R5 cell line model of latency, where HDAC2 and HDAC3 were found to regulate HIV-1 expression (29).

Concurrent knockdown of HDAC2 and HDAC3 abolishes the repressive effects of HDACs on HIV-1 expression in J89 cells. We previously reported that inhibitors selective for HDAC1, -2, and -3 are potent inducers of HIV-1 expression (4). We wanted to determine if a more selective induction of HIV-1 could be accomplished by inhibiting only two of these three HDACs. Thus, we evaluated the effects of combined siRNA-mediated HDAC knockdown on latent HIV-1 expression in J89 cells. Target HDAC mRNA expression levels following combination knockdowns were similar to those obtained following individual knockdowns at 24 h post-nucleofection (compare Figs. 3.1A and 3.2A). As seen with the previous experiments, peak protein knockdown was observed 48 h post-nucleofection (Fig. 3.2B). Cell viability was not affected at 48 h following combination HDAC knockdowns when compared to the NS siRNA control (Fig. 3.2C). However, although isolated knockdown of HDAC2 or HDAC3 resulted in a modest induction of HIV-1 expression (Fig. 3.1D), combined knockdown of HDAC2 and HDAC3 did not induce HIV-1 p24 expression (Figs. 3.2D). We also did not observe an induction in HIV-1 expression when HDAC1 and HDAC2 or HDAC1 and HDAC3 were inhibited together.

Selective enzymatic inhibition of HDAC1, -2, and -3 induces latent HIV-1 expression, but combined knockdown of HDAC1, -2, and -3 has no effect. Because we have previously observed induction of HIV-1 expression in J89 cells with inhibitors that selectively target HDAC1, -2, and -3, it is possible that optimal induction of HIV-1 may require simultaneous inhibition of all three HDACs (4). Thus, we decided to test the effects

of combined knockdown of HDAC1, -2, and -3 on HIV-1 expression. HDAC mRNA levels were reduced at 24 h post-nucleofection with HDAC siRNAs when compared to nucleofection with an equivalent concentration of NS siRNA (Fig. 3.3A). Additionally, protein levels were reduced following nucleofection with siRNAs targeting HDAC1, -2, and -3 when compared to the NS control at 48 h (Fig. 3.3B). Concurrent knockdown of HDAC1, -2, and -3 led to increased cellular proliferation at 48 h post-nucleofection when compared to the NS control (Fig. 3.3C). However, we did not observe HIV-1 p24 expression following combined HDAC1, -2, and -3 knockdown at 48 h (Fig. 3.3D). Induction of HIV-1 expression was also not observed at 24 or 72 h post-nucleofection (data not shown).

As we have previously observed a potent induction of HIV-1 in J89 cells treated with class I-selective HDAC inhibitors (4), we evaluated whether the nucleofection procedure itself was somehow inhibiting HIV-1 expression in these cells. 24 h post-nucleofection with either NS control siRNA or combined HDAC1, -2, and -3 siRNAs, cells were exposed to 200 nM of the HDAC inhibitors MRK10 or MRK13 for an additional 24 h before supernatant was harvested for ELISA. At this concentration, MRK10 selectively inhibits the class II HDAC, HDAC6, and MRK13 targets HDAC1, -2, and -3. As a control, untreated cells were exposed to an equivalent volume of the DMSO vehicle. We have previously reported that MRK13 is a potent inducer of HIV-1 expression in J89 cells, whereas MRK10 has no effect (4). We observed a strong induction of HIV-1 p24 expression following treatment with MRK13, but not with MRK10 or DMSO in both the NS control siRNA and combined HDAC siRNAs conditions (Fig. 3.3E). Thus, the nucleofection procedure itself does not have an inhibitory effect on HIV-1 expression. Furthermore, these results suggest that simultaneous

knockdown of HDAC1, -2, and -3 expression does not have the same effect on HIV-1 transcription as simultaneous enzymatic inhibition of HDAC1, -2, and -3.

Nucleofection of resting CD4+ T cells induces a selective and transient increase in CD69 expression. One method to test of the effects of HDAC knockdown on latent HIV-1 is to assess the impact of siRNA-mediated HDAC knockdown on *ex vivo* recovery of latent virus from the resting CD4+ T cells of HIV-1-positive patients. Because the combination HDAC knockdowns described above were not as effective at inducing HIV-1 in J89 cells as isolated HDAC knockdowns, we decided to focus on knocking down the class I HDAC1, -2, and -3 in isolation. To obtain a population of purified resting CD4+ T cells, we acquired leukapheresis products from stably suppressed HIV-1-positive patients. Following isolation using a Ficoll gradient, PBMCs were subjected to a previously described negative-selection purification protocol (2). The purity of the resting CD4+ T cells, defined as the $CD4^+CD45^+CD3^+CD69^-CD25^-CD8^-CD14^-HLA\text{-}DR^-$ population, was regularly between 97-99% as determined by flow cytometry analysis (Fig. 3.4A).

To deliver target siRNAs to the resting CD4+ T cells, we utilized a Lonza nucleofector following the manufacturer's protocol for unstimulated human T cells. Following nucleofection, we observed similar rates of cell proliferation between nucleofected and un-nucleofected resting CD4+ T cells at 24 h as measured by alamarBlue assays (Fig. 3.4B). Thus, the nucleofection procedure does not significantly impact T cell viability or induce proliferation. In contrast, when resting CD4+ T cells were activated by mitogen exposure (5 µg/ml PHA), we observed a 2-fold increase in cell proliferation.

We also assessed the activation status of the cells following nucleofection with siRNAs by staining with anti-CD69 and anti-CD25 antibodies and evaluating by flow

cytometry. With both the NS siRNA control (shown) and HDAC siRNAs (not shown), the nucleofection procedure resulted in a selective upregulation of CD69 mean fluorescence intensity (MFI) 24 h post-nucleofection (Fig. 3.4C). We did not observe any changes in CD69 expression when cells were exposed to the nucleofection solution alone (data not shown), thus the increase in CD69 expression is specific to the nucleofection procedure. CD69 expression decreased at 48 h and 72 h, hence the increase was transient. The activation marker CD25 did not increase at 24, 48, or 72 h post-nucleofection. As comparative controls, un-nucleofected resting CD4+ T cells and un-nucleofected CD4+ T cells treated with 5 µg/ml PHA were examined for activation markers at each timepoint. As expected, treatment with PHA activated the resting CD4+ T cells leading to prolonged upregulation of CD69 and CD25. Thus, while nucleofection transiently increased CD69 expression, the nucleofected cells maintained an overall resting phenotype.

Isolated knockdown of HDAC1, -2, or -3 induces outgrowth of some latent HIV-1 proviruses from the resting CD4+ T cells of patients. We observed a selective knockdown of each target mRNA when we nucleofected resting CD4+ T cells with siRNAs specific for HDAC1, -2, or -3 (Fig. 3.5A). Because resting CD4+ T cells may be differentially affected by decreases in HDAC expression when compared to J89 cells, a proliferating cell line, we again examined the effects of targeted HDAC knockdown on cell viability 48 h post-nucleofection. We did not observe any differences in cell viability following siRNA-mediated knockdown of HDAC1, -2, or -3 when compared to nucleofection with the NS control siRNA using an alamarBlue assay (Fig. 3.5B).

After nucleofection with specific siRNAs, surviving cells were counted and placed into a limiting dilution culture assay as described in Materials and Methods. The recovered

IUPB for each condition was determined using a maximum likelihood method of analysis (37). To establish the size of the resting cell latent reservoir for each patient, resting CD4+ T cells were maximally stimulated with PHA, IL-2, and irradiated, allogeneic PBMCs before analysis in limiting dilution culture. Table 3.1 contains the IUPB of each patient as determined by maximal stimulation of the patient's resting CD4+ T cells.

HDAC1 knockdown induced latent HIV-1 outgrowth above the NS siRNA control in four out of four experiments (Fig. 3.5C). The difference between these two conditions was statistically significant ($P < 0.01$). Patients in panels C-E of Figure 3.5 are arranged along the x-axis in order of increasing IUPB as determined by maximal mitogen stimulation (Table 3.1). We obtained resting CD4+ T cells from patient 6 on two separate occasions and observed similar rates of latent HIV-1 outgrowth following HDAC1 knockdown both times.

Following nucleofection with HDAC2 siRNA, we observed outgrowth of latent virus from resting CD4+ T cells isolated from five out of nin patients (Fig. 3.5D). With cells from patients 1 and 8, whom had similar rates of resting cell infection (Table 3.1), the fold-increase in outgrowth was large, 4.6- and 3.6-fold, respectively. Furthermore, in patient 1 the outgrowth of latent virus following HDAC2 knockdown was, in fact, slightly higher than the outgrowth induced by maximal mitogen stimulation.

HDAC3 knockdown induced latent HIV-1 outgrowth over the NS siRNA control in cells from four out of eight patients (Fig. 3.5E). Latent virus was activated by HDAC3 knockdown three-fold over the NS siRNA control in cells isolated from patients 2 and 9, both of whom had relatively low rates of resting cell infection (Table 3.1). With the exception of cells isolated from three patients, the IUPB calculated following knockdown of either HDAC1, -2, or -3 was not as high as that measured in the maximal mitogen stimulation

condition. Thus, knockdown of HDAC1, -2, or -3 only induces outgrowth from a subset of proviruses within an individual patient's latent HIV-1 reservoir.

Because some of the fold-increases in HIV-1 outgrowth over the NS siRNA control observed following HDAC knockdown were relatively modest, we decided to evaluate HIV-1 outgrowth following knockdown with a class II HDAC as a negative control. We have previously observed that inhibitors that target class II HDACs do not induce HIV-1 expression. The class II HDAC HDAC7 is highly expressed in resting CD4+ T cells (29). When we evaluated HIV-1 outgrowth following HDAC7 knockdown in resting CD4+ T cells isolated from patients, we did not observe an induction of latent HIV-1 outgrowth over the NS siRNA control in three out of three experiments (Fig. 3.5F). Thus, even modest increases in HIV-1 outgrowth measured following knockdown with HDAC1, -2, or -3 may be significant.

DISCUSSION

HDAC inhibitors selective for the class I HDACs HDAC1, -2, and -3 are potent inducers of HIV-1 transcription (4, 41). In an effort to narrow down the minimal HDAC-inhibitory requirement for optimal induction of latent HIV-1, we employed an siRNA-mediated strategy to deplete both individual and various combinations of the class I HDACs HDAC1, -2, and -3 in the J89 T cell line latency model. We found that isolated knockdown of either HDAC2 or HDAC3 expression led to a modest, but statistically significant induction of latent HIV-1 expression in J89 cells (Fig. 3.1D). These findings are consistent with our previous studies on the effects of class I HDAC knockdown in the HeLa P4/R5 cell line (29).

Surprisingly, when HDAC2 and HDAC3 siRNAs were combined we did not observe an induction of HIV-1 expression (Fig. 3.2D). This cannot be attributed to a significant decrease in the efficiency of HDAC knockdown as target mRNA expression levels are comparable in experiments with single siRNA or multiple siRNAs (Figs. 3.1A and 3.2A). Another study reported similar results when analyzing expression of the cyclin D1 by quantitative RT-PCR following knockdown of either HDAC1, HDAC2, or HDAC1 and HDAC2 in combination. In that study, isolated knockdown of HDAC1 reduced cyclin D1 mRNA levels by 75% and knockdown of HDAC2 led to a 50% reduction in cyclin D1. However, when HDAC1 and HDAC2 were knocked down concurrently, no reduction in cyclin D1 expression of was observed (11). Thus, concurrent depletion of two HDACs can abolish the repressive effects of either HDAC.

We and others have previously reported induction of latent HIV-1 expression with HDAC inhibitors that selectively target the class I HDACs HDAC1, -2, and -3 (4, 41). However, simultaneous depletion of HDAC1, -2, and -3 by siRNA in J89 cells did not induce HIV-1 expression (Fig. 3.3D). This was not due to an inhibitory effect of the nucleofection procedure itself on HIV-1 expression. When nucleofected J89 cells were incubated with the class I selective HDAC inhibitor MRK13, we observed a dramatic increase in HIV-1 p24 expression (Fig. 3.3E). Concurrent knockdown of HDAC1, -2, and -3, however, did result in a significant increase in cellular proliferation. This is again in contrast to selective enzymatic inhibition of HDAC1, -2, and -3 by MRK13, which does not alter proliferation of J89 cells (4). Thus, depletion of HDAC protein levels and enzymatic HDAC inhibition can impact different cellular processes. This could conceivably be due to disruptions in protein-protein

interactions following HDAC depletion that are not affected by binding of small molecule HDAC inhibitors.

It worth noting that the rate of knockdown achieved by any HDAC siRNA in this study ranged from 50 to 90% (Figs. 3.1A, 3.2A, 3.3A, and 3.5A), thus there was not a complete elimination of any of the HDACs targeted. Because HDAC1, -2, and -3 are all known to associate with the HIV-1 LTR (29), it is possible that some combination of these HDACs may remain at any one viral promoter following knockdown by siRNA. Furthermore, nucleofection with siRNAs results in a slow and transient depletion of protein expression. This slow reduction in HDAC levels may permit time for other repressive factors to compensate. In contrast, chemical HDAC enzymatic inhibition occurs rapidly and persists until the drug is cleared and the active enzyme is reconstituted.

One of the goals of this study was to determine the minimal HDAC inhibition required to induce latent HIV-1 expression. Using selective HDAC inhibitors, we had previously shown that selectively targeting HDAC1 and HDAC2 was not sufficient to induce latent HIV-1 expression (4). In contrast, HDAC inhibitors selective for HDAC1, -2, and -3 can reactivate latent HIV-1 (Fig. 3.3E). However, the results discussed in this paper suggest that there is a difference in the effects achieved between HDAC depletion by siRNA and HDAC enzymatic inhibition by pharmacological agents. Thus, it is still unclear whether or not a more selective HDAC inhibitor (i.e., one that targets HDAC1 and HDAC3 or HDAC2 and HDAC3, or even HDAC3 alone) would induce expression of latent HIV-1. Such an evaluation will require the identification or development of even more selective class I HDAC inhibitors.

Although the impact of isolated knockdown of HDAC2 and HDAC3 on HIV-1 expression in J89 cells was modest, studies of HIV-1 latency in proliferating cell line models cannot definitively address the impact that HDAC depletion would have on latent HIV-1 proviruses in the resting CD4+ T cells of HIV-1 infected patients. Therefore, we decided to expand the clinical relevance our studies by performing *ex vivo* latent HIV-1 outgrowth assays with resting CD4+ T cells isolated from aviremic patients following HDAC knockdown. As resting CD4+ T cells readily express latent HIV-1 upon T cell activation, we began our studies with an evaluation of the effects of the nucleofection procedure itself on the resting CD4+ T cell phenotype. We first assessed cellular proliferation, a hallmark of T cell activation, and did not observe a difference between nucleofected or un-nucleofected resting CD4+ T cells (Fig. 3.4B).

We next examined expression of T cell activation markers following nucleofection. We did not observe changes in expression levels of the IL-2 receptor, CD25, at any timepoint following post-nucleofection (Fig. 3.4C). However, we did measure a transient increase in CD69 MFI at 24 h post-nucleofection that steadily decreased by 48 and 72 h. In contrast, resting CD4+ T cells activated with PHA displayed a substantial and persistent increase in both CD69 and CD25 expression levels at each timepoint.

To our knowledge, this is the first report of a transient increase in CD69 expression following nucleofection of resting CD4+ T cells with a Lonza nucleofector. Other groups have reported that nucleofection does not alter CD69 expression in resting CD4+ T cells. However, these groups examined CD69 expression at 72 h (23, 52) and 96 h (7) post-nucleofection. We observed a progressive decrease in CD69 expression by 48 and 72 h. The

significance of a selected early upregulation of CD69 on the resting cell, despite the transient nature of this expression, is unknown and warrants further attention (Fig. 3.4C).

When we examined HDAC1, -2, and -3 mRNA expression levels in resting CD4+ T cells following HDAC knockdown, target mRNA were selectively depleted at 24 h post-nucleofection (Fig. 3.5A). As with J89 cells (Fig. 3.1C), resting cell viability was not affected by isolated HDAC knockdown (Fig. 3.5B). However, unlike J89 cells, we consistently recovered latent HIV-1 from patient resting CD4+ T cells following HDAC1 knockdown (four out of four experiments, Fig. 3.5C). The fold-induction of viral outgrowth over the NS siRNA control condition was modest (2-fold or less in each experiment), but statistically significant ($P < 0.01$). Furthermore, the fact that HDAC1 has been shown to be recruited to the HIV-1 LTR in a diverse range of cell systems by numerous mechanisms suggests that it serves some function in regulating HIV-1 latency (10, 24, 25, 29, 35, 43, 49). Importantly, when we examined virus outgrowth following knockdown of the class II HDAC, HDAC7, we did not measure an increase in HIV-1 reactivation when compared to the NS siRNA control in three out of three experiments. Thus, the increase in HIV-1 outgrowth we have observed following HDAC1 knockdown is specific and unlikely to be attributed to chance.

When we nucleofected resting CD4+ T cells with HDAC2 or HDAC3 siRNAs, we observed increased outgrowth of latent HIV-1 above the NS siRNA control in over half of the experiments (Figs. 3.5D and E). Increased HIV-1 outgrowth was detected with cells from five out of nine patients following HDAC2 knockdown and four out of eight patients following HDAC3 knockdown. Some of these fold-increases in latent HIV-1 outgrowth were quite large, as high as 4.6-fold following HDAC2 knockdown and 3-fold following HDAC3 knockdown.

Among resting cell pools where an increase in latent HIV-1 outgrowth was detected following HDAC knockdown, the recovered IUPB from HDAC knockdown was lower than that achieved with maximal mitogen stimulation (Table 3.1) in all cases except with cells from three patients (IUPB values for HDAC knockdown not shown). In the majority of experiments, only a selected population of latent proviruses within an individual patient's resting pool responded to HDAC knockdown. The reasons for variegated virus outgrowth following HDAC knockdown are not known, but multiple possibilities exist.

One potential explanation is that sequence variation in the LTR between proviral genomes could account for different responses to HDAC knockdown. The majority of cells used in these studies were obtained from patients who initiated therapy during acute infection with HIV-1, i.e., within three months of the estimated date of infection. Only three, patients 3, 7, and 9, started on therapy during the chronic phase of infection. Individuals treated during the acute phase of HIV-1 infection are less likely to have significant viral diversity within their infected resting cell reservoir. However, multi-virus transmission has been reported and viral evolution during early infection does occur (30). Thus, viral sequence diversity is one possible explanation for a differential response among proviral integrants.

An alternative possibility is that the variegated outgrowth of proviruses following siRNA-mediated depletion of HDACs reflects integration site-dependent controls on viral latency. Latent HIV-1 proviral genomes in resting CD4+ T cells are primarily integrated into the introns actively transcribed genes (19) and transcriptional interference from host genes can influence HIV-1 gene expression (20, 33). Studies have shown that viral clones integrated at different sites in the host genome are differentially activated by stimulation with the NF-κB activator TNF-α or the HDAC inhibitor TSA (26, 27). Thus, recovery of latent

HIV-1 outgrowth following HDAC knockdown could be affected by the site of proviral integration into host DNA.

Variations in epigenetic modifications at the proviral LTR could also account for different responses to HDAC knockdown. Some proviruses isolated from resting CD4+ T cells have been found to be methylated on cytosine residues at CpG dinucleotide motifs in the HIV-1 LTR (5). These groups of CpG dinucleotides are referred to as CpG islands. The HIV-1 LTR contains two CpG islands, the second of which is located between nuc-1 and nuc-2. Heavy DNA methylation at this specific CpG island has been associated with recruitment of HDAC2 to this region of the LTR by methyl-CpG binding domain protein 2 (28). There are wide variations in the amount of and location of methylated cytosines between proviral integrants. Even within a clonal population of cells, proviruses with different methylation patterns respond differently to stimuli (5, 28). Thus, the differential response seen between proviral integrants following HDAC knockdown could be due to variations in the epigenetic environment at the HIV-1 LTR.

Divergent proviral response to HDAC depletion could be due to stochastic fluctuations of factors within the infected cells at the time of HDAC knockdown that converge to induce latent virus outgrowth. Certain factors that positively regulate HIV-1 transcription, including NFAT, NF-kB p50-p65, and PTEF-b, are expressed at very low levels in the nuclei of resting CD4+ T cells (15, 17, 38, 51). When factor pools are low and initiation of transcription is inefficient, as is the case with HIV-1 transcription in resting CD4+ T cells, stochastic fluctuations can influence gene expression resulting in phenotypic bifurcation (6). In other words, small differences in factor pools between cells influences whether a specific stimuli, such as HDAC knockdown, is effective at turning on a silent gene.

Phenotypic bifurcation has been demonstrated in the case of HIV-1 latency in clonal cell line populations, where small fluctuations in the availability of Tat during the initial stages of transcription determine whether cells within a clonal population express HIV-1 (48).

Finally, in some of the experiments, the nucleofection procedure itself induced outgrowth of HIV-1. This was determined by comparing the IUPB recovered following nucleofection with NS siRNA to the IUPB recovered from of a negative control of undisturbed resting cells (data not shown). A higher IUPB was observed in the NS siRNA condition than in the negative control condition in one-third of the experiments performed. In these instances, induction of virus outgrowth by nucleofection alone increased experimental background levels and may have made it more difficult to observe an effect from HDAC knockdown.

Selective HDAC inhibitors that target a limited number of the class I HDACs have potential as antilatency therapies with fewer host-toxicities and off-target effects than pan-HDAC inhibitors. We previously demonstrated that inhibitors that selectively target HDAC1, -2, and -3—but not HDAC1 and HDAC2 alone—are potent inducers of latent HIV-1 (4). In this study, we evaluated whether a more selective induction of HIV-1 could be achieved using an siRNA-mediated strategy of HDAC knockdown. However, our results suggest that depletion of HDAC levels within a cell by siRNA does not have the same impact on latent HIV-1 expression as enzymatic inhibition of HDACs by pharmacological agents. Thus, it remains to be determined whether more selective inhibitors that simultaneously target HDAC2 and HDAC3, HDAC1 and HDAC3, or possibly even HDAC3 alone are effective inducers of latent HIV-1.

In contrast to HDAC knockdown in J89 cells, where a modest increase in HIV-1 expression was only observed following knockdown of HDAC2 or HDAC3, in resting CD4+ T cells isolated from patients, latent HIV-1 was recovered by knockdown of HDAC1, -2, or -3. However, HIV-1 outgrowth was only from a selected number of latent proviruses within the infected resting cell reservoir. Taken together, these findings suggest that HDAC1, -2, and -3 contribute to variegated repression of HIV-1 proviral integrants in resting CD4+ T cells.

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AUTHORSHIP CONTRIBUTIONS

K.S.K contributed to the design, performance, and analysis of all experiments described in this chapter. N.M.A. assisted with design and analysis of outgrowth experiments

and led the studies for the isolation and culturing of resting CD4+ T cells for the outgrowth experiments. D.M.M. supervised the design and analysis of all experiments. The data presented in this chapter are currently in preparation for submission to a peer-reviewed journal.

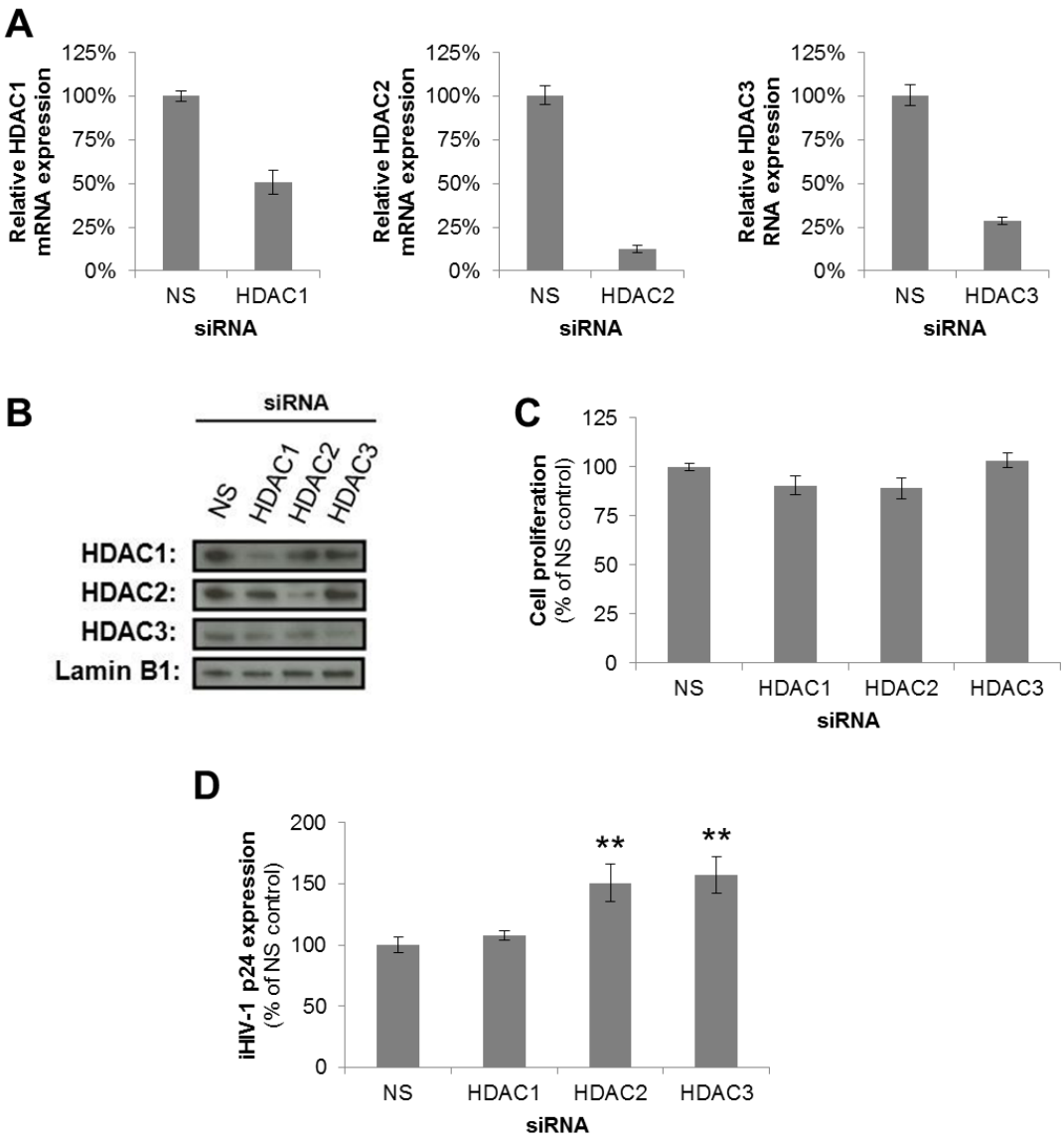

Figure 3.1 HDAC2 and HDAC3 negatively regulate HIV-1 expression in the J89 latency cell line model. A) Isozyme-specific siRNAs reduce target HDAC mRNA expression 24 h post-nucleofection. The relative knockdown of HDAC mRNA was normalized to the nonspecific (NS) siRNA control as determined by quantitative RT-PCR B) HDAC protein expression is reduced by nucleofection with corresponding HDAC siRNAs. Cell extracts were assessed for HDAC expression by Western blot analysis 48 h post-nucleofection. C) Depletion of HDAC1, -2, or -3 does not alter cell viability 48 h post-nucleofection as determined by alamarBlue assays. D) Targeted knockdown of HDAC2 or HDAC3 increases HIV-1 p24 expression when compared to nucleofection with NS control siRNA (**, $P < 0.01$). Expression of HIV-1 was determined by HIV-1 p24 ELISA 48 h post-nucleofection. Data represent the combined results of multiple experiments and are presented as the means ± standard errors of the mean.

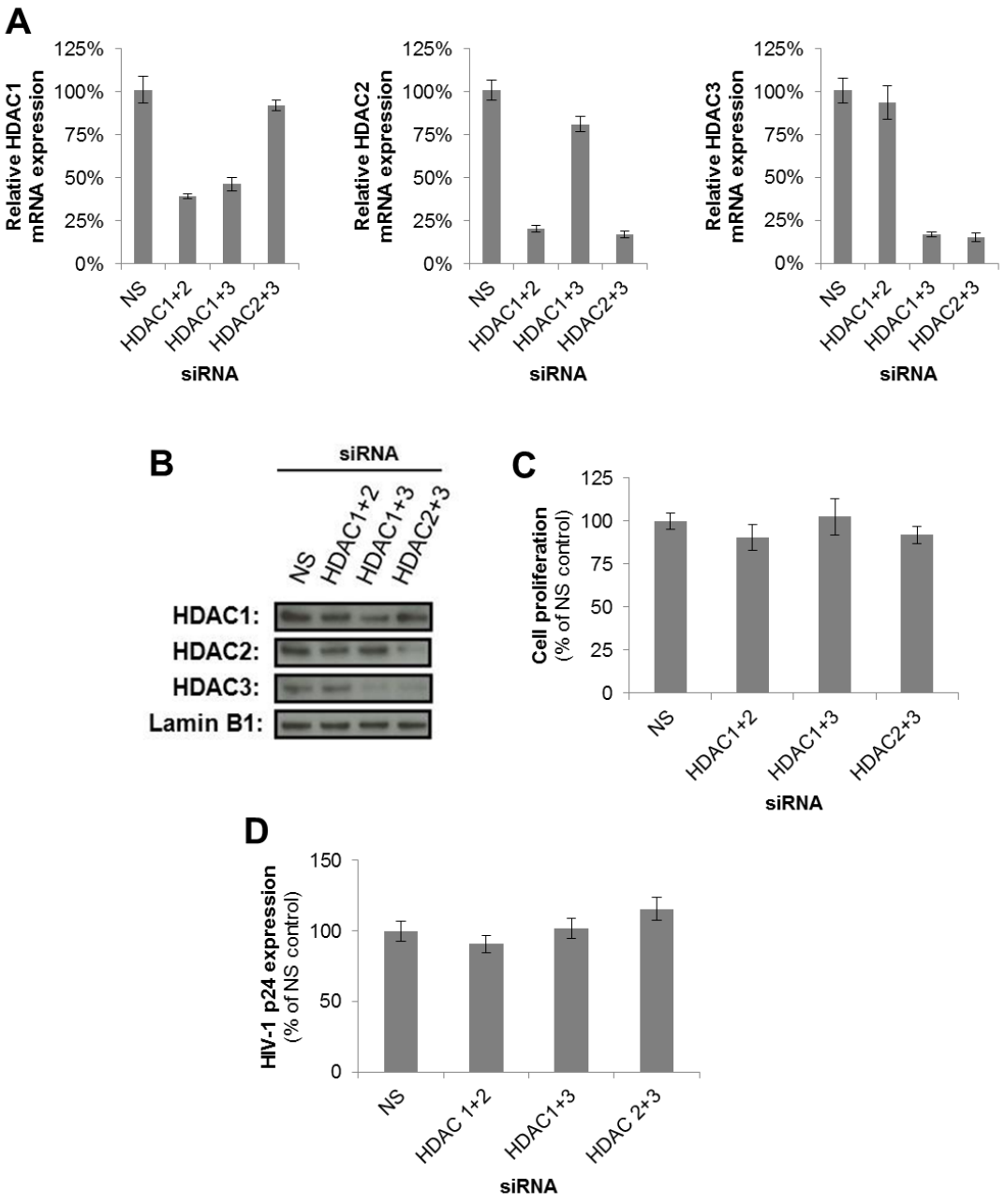

Figure 3.2 Combined knockdown of HDAC2 and HDAC3 does not induce latent HIV-1 expression in J89 cells. A) Nucleofection with multiple HDAC siRNAs downregulates target mRNA. J89 cells were nucleofected with siRNAs targeting two HDACs. HDAC mRNA expression levels were quantified by RT-PCR 24 h post-nucleofection. Values were normalized to HDAC mRNA from cells nucleofected with an equal concentration of NS siRNA. B) Combined HDAC knockdown reduces target protein levels in J89 cells 48 h post-nucleofection as determined by Western blot analysis. C) Concurrent knockdown of two HDACs does not affect cell viability. alamarBlue cell proliferation assays were performed on

cells 48 h post-nucleofection. D) Simultaneous depletion of two class I HDACs does not induce HIV-1 expression. HIV-1 expression was measured by HIV-1 p24 ELISA on culture supernatants and normalized to expression in the NS control siRNA condition. Data represent the combined results of multiple experiments and are presented as the means ± standard errors of the means.

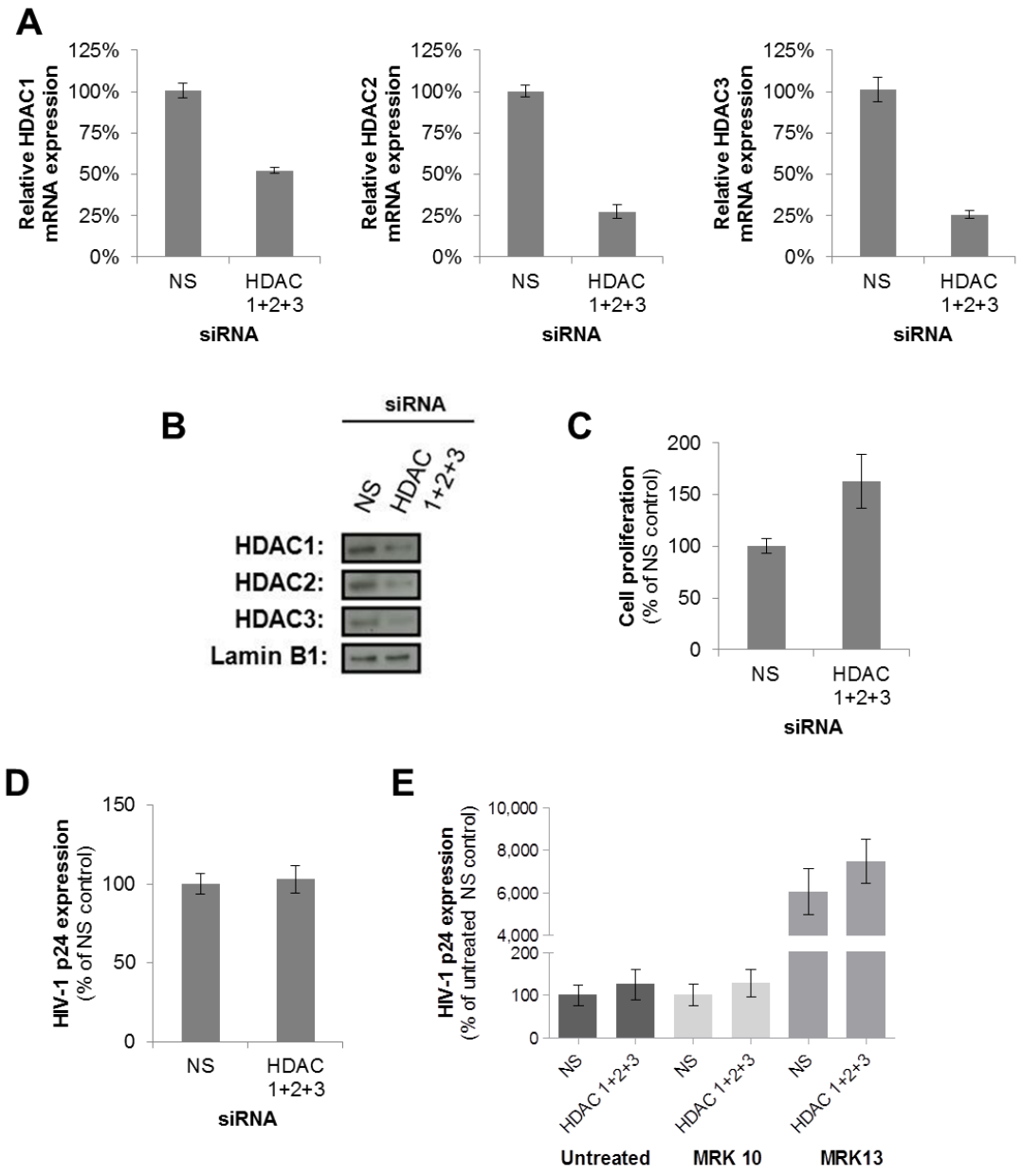

Figure 3.3 Enzymatic inhibition, but not cellular depletion of HDAC1, -2, and -3, stimulates latent HIV-1 expression in J89 cells. A) Concurrent knockdown of HDAC1, -2, and -3 depletes target mRNA. HDAC mRNA expression levels were quantified by RT-PCR and normalized to nucleofection with an equivalent concentration of NS siRNA. B) Western blot analysis of cellular extracts 48 h post-nucleofection shows significant knockdown of HDAC protein expression. C) Combined depletion of HDAC1, -2, and -3 leads to an increase in cellular proliferation. Cells were assessed for changes in proliferation compared to the NS siRNA control condition by an alamarBlue assay 48 h after nucleofection with HDAC siRNAs. D) Despite increased proliferation, simultaneous knockdown of HDAC1, -2, and -3 does not induce HIV-1 expression as evaluated by HIV-1 p24 ELISA. E) Enzymatic

inhibition of HDAC1, -2, and -3 leads to a potent induction of HIV-1 expression in cells nucleofected with either NS siRNA or siRNAs targeting HDAC1, -2, and -3 when compared to cells nucleofected with NS siRNA and treated with DMSO. Cells were nucleofected with a NS siRNA or siRNAs specific for HDAC1, -2, and -3. 24 h post-nucleofection, cells were treated with either MRK10, an HDAC6-selective inhibitor, MRK13, an HDAC1, -2, and -3 inhibitor, or with a corresponding volume of the DMSO vehicle control for an additional 24 h before culture supernatants were harvested for HIV-1 p24 ELISA. Data represent the combined results of multiple experiments and are presented as the means ± standard errors of the means.

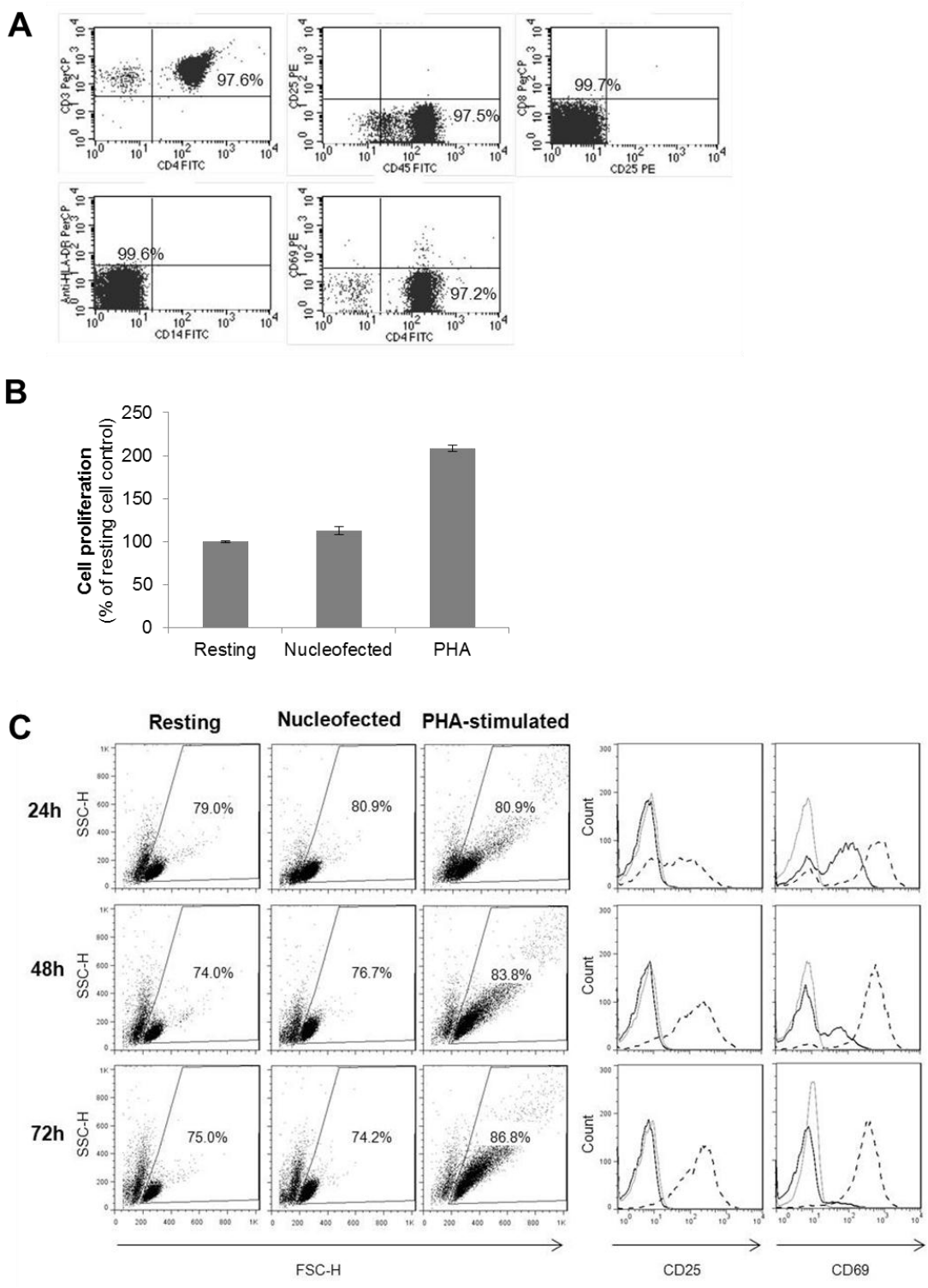

Figure 3.4 Nucleofection of resting CD4+ T cells induces a selective and transient increase in expression of the T-cell activation marker CD69. A) Resting CD4+ T cells obtained from HIV-1 positive patients by a negative selection procedure are regularly between 97%-99% pure as determined by flow cytometry. Data shown are from a single patient and are representative of the purity of resting cells used in this study. B)

Nucleofection of resting CD4+ T cells does not increase cell proliferation as compared to un-nucleofected resting CD4+ T cells. Purified resting CD4+ T cells were either left untreated (resting), nucleofected with NS siRNA (nucleofected), or stimulated with 5 µg/ml of the mitogen PHA and subjected to alamarBlue cell proliferation assays at 48 h. Data represent the combined results of multiple experiments and are presented as the means ± standard errors of the means. C) Nucleofected resting CD4+ T cells experience a selective and transient increase in CD69 expression 24 h post-nucleofection that progressively decreases at 48 and 72 h. Cells were either un-nucleofected (resting, light grey line), nucleofected with NS siRNA (nucleofected, black line), or activated with 5 µg/ml PHA (PHA-stimulated, dashed line) and collected for analysis by flow cytometry at the indicated times. Live cells were gated on forward (FSC-H) and side (SSC-H) scatter profiles. Mean fluorescence intensities of CD25 and CD69 expression at 24, 48, and 72 h are displayed. Data shown are from a single patient and representative of the population studied.

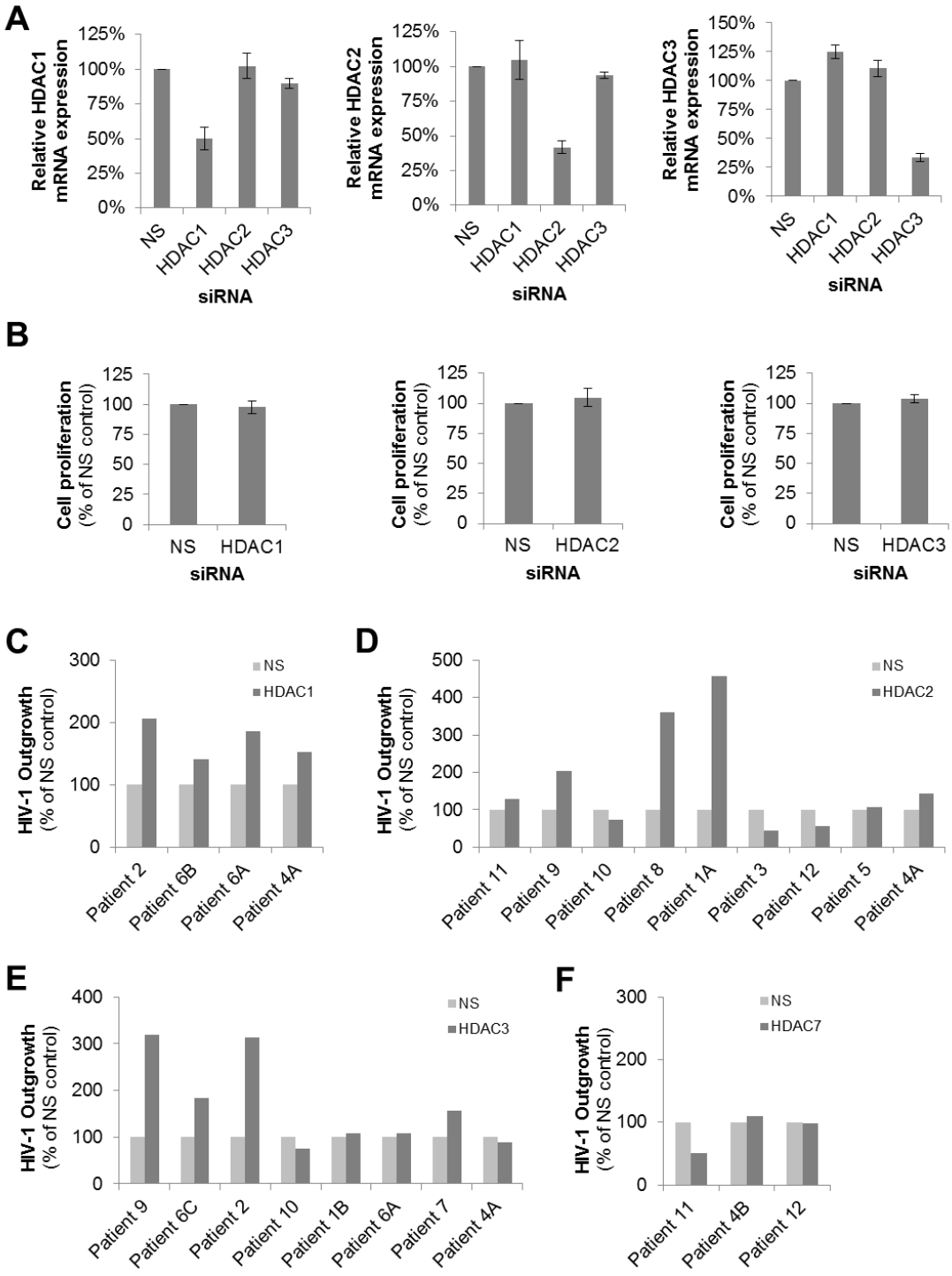

Figure 3.5 Knockdown of HDAC1, -2, or -3 induces selective latent virus outgrowth from the infected resting CD4+ T cells of patients. A) Nucleofection with siRNA results in targeted knockdown of HDAC mRNA in resting CD4+ T cells 24 h post-nucleofection. Cells were nucleofected with a single HDAC siRNA or an equivalent concentration of NS siRNA and HDAC mRNA levels were quantified by RT-PCR and normalized to the NS siRNA control condition. B) Nucleofection of resting CD4+ T cells with siRNAs targeting HDAC1,

-2, or -3 does not alter cell proliferation compared to the NS siRNA control as determined by alamarBlue assays at 48 h. C-F) Depletion of HDAC1, -2, or -3 by siRNA induces outgrowth of select HIV-1 proviruses from the infected cells of patients. In contrast, knockdown of HDAC7 does not induce outgrowth. Data are represented as fold-increase in recovered infected units per billion resting CD4+ T cells over nucleofection with the NS control siRNA. Patients are displayed on the x-axis in order of increasing rates of resting cell infection as determined by maximal mitogen stimulation.

Table 3.1 Recovery of HIV-1 from resting CD4+ T cells isolated from 11 aviremic, ART-treated individuals by maximal mitogen stimulation.

Infected units per billion resting CD4+ T cells following maximal mitogen stimulation[a]		
Patient	**IUPB**	**Patient Type**[b]
1$_{\text{visit A}}$	983	Acute
1$_{\text{visit B}}$	750	Acute
2	242	Acute
3	1,419	Chronic
4 $_{\text{visit A}}$	10,294	Acute
4 $_{\text{visit B}}$	1,105	Acute
5	7,559	Acute
6 $_{\text{visit A}}$	754	Acute
6 $_{\text{visit B}}$	571	Acute
6 $_{\text{visit C}}$	177	Acute
7	2,200	Chronic
8	923	Acute
9 $_{\text{visit A}}$	174	Chronic
9 $_{\text{visit B}}$	49	Chronic
10	649	Acute
11	5,124	Acute

[a] PHA 1 µg/ml, IL-2 20 U/ml, 5-fold excess irradiated allogeneic PBMCs

[b] Acute patients initiated therapy within three months of the estimated date of infection. Chronic patients initiated therapy more than three months after the estimated date of infection.

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CHAPTER 4

DISCUSSION

INTRODUCTION

A significant contributor to the persistence of human immunodeficiency virus type 1 (HIV-1), in spite of potent antiretroviral therapies (ART), is the occasional silent integration of the HIV-1 genome into the DNA of long-lived, resting CD4+ T cells, a phenomenon referred to as viral persistence or latency. Transcriptionally silent proviruses evade immune detection and are impervious to current ART. Latent infection of resting CD4+ T cells permits propagation of the virus upon immune homeostatic proliferation or activation of infected cells.

The exact mechanisms regarding how HIV-1 is able to maintain a state of proviral latency and the pathways that induce its reactivation are not fully understood. However, they are most assuredly multi-factorial and include a lack of activating transcription factors in the nuclei of resting cells (11, 12, 32, 44), transcriptional interference of integrated viruses by active host genes (15, 28), inefficient nuclear export of viral RNA (26), cellular microRNAs that prevent translation of HIV-1 proteins (17), and repressive epigenetic regulation of proviral DNA (5, 9, 22, 31, 34).

Once integrated, HIV-1 resides in the chromatin environment of the host genome (35, 40). In eukaryotic cells, DNA is packaged around histone octamers in repeating units of nucleosomes. Post-translational covalent modifications of the amino-terminal tails of histones can influence whether or not a gene has the potential for being active. Histone acetyltransferases (HATs) acetylate lysine residues on the histone tails surrounding nucleosomes, providing docking signals for activating transcription complexes with matching bromodomains and creating an open chromatin environment conducive to transcription (16). Conversely, histone deacetylases (HDACs) deacetylate histones tails, thereby blocking the

recruitment of activating factors and producing a closed, transcriptionally repressive environment. HATs and HDACs regulate HIV-1proviral transcription, and inhibitors of HDACs can reactivate latent HIV-1 (39, 41, 46).

In order for current or future ART to be successful in eliminating HIV-1 from infected individuals, simultaneous strategies to purge the latent reservoir must be employed. Although pan-HDAC inhibitors can induce latent virus expression *ex vivo*, more potent and selective strategies will likely be required to fully eradicate HIV-1 in an infected individual (2). The classical family of HDACs is comprised of 11 isoforms, divided into three classes based on amino-acid sequence and domain organization (13). Class I HDACs include HDAC1, -2, -3 and -8, class II HDACs include HDAC4, -5, -6, -7, -9, and -10, and class IV is comprised solely of HDAC11. Identification of the specific HDACs relevant to HIV-1 latency would pave the way for developing more selective viral derepression strategies. Furthermore, defining the mechanisms by which these specific HDACs induce latent HIV-1 may suggest alternative means of targeting latent HIV-1 *in vivo*.

FINDINGS AND IMPLICATIONS

In this dissertation, I hypothesized that specific HDACs are recruited to the HIV-1 long terminal repeat (LTR) promoter during latency to maintain transcriptional repression. To test this hypothesis, I utilized a combination of biochemical, genetic, and pharmacological studies. In Chapter 2, I characterized expression of HDAC1 to -11 in resting CD4+ T cells, the primary reservoir of latent HIV-1. Relative HDAC mRNA expression levels were evaluated in purified resting CD4+ T cells from HIV-1+ patients by comparing intensities of HDAC mRNA expression from microarray data obtained from three patients. HDAC1, -3,

and -7 had the highest levels of mRNA expression in this cell population, whereas HDAC9 and HDAC10 had relatively low levels of expression. Because latency is a phenomenon of resting CD4+ T cells and not activated CD4+ T cells, I investigated whether there was a difference in the protein expression or cellular localization of HDACs in these two cell types by Western blot analysis of nuclear and cytoplasmic protein extracts. Although most HDACs had similar levels of expression and localization in resting and activated CD4+ T cells, HDAC7 was enriched in the cytoplasm following T cell activation. The predominant nuclear HDACs in resting CD4+ T cells included HDAC1, -2, -3, -4, -6 and -7.

I next sought to identify the specific HDACs that associate with the HIV-1 LTR during latency, focusing on the HDAC isoforms expressed in the nuclei of resting CD4+ T cells. Chromatin immunoprecipitation (ChIP) assays permit the identification of proteins that interact with specific DNA sequences in cells. ChIP assays cannot be performed on HIV-1 proviral DNA in resting CD4+ T cells harvested from patients due to the rarity of target DNA (estimated at 1 replication-competent integrant per 10^6-10^7 resting CD4+ T cells in aviremic patients on ART). Thus, to analyze proteins that associate with the HIV-1 LTR during latency, latency cell line models must be employed. I first evaluated the validity of using the J89 T cell line, which contains a single, silently-integrated HIV-1-genome, as a model for HDAC recruitment to the HIV-1 LTR during latency (25). In order to relevantly reflect the potential recruitment of HDACs to the LTR in resting cells, the model cell line should at a minimum show the same expression of nuclear HDACs as resting CD4+ T cells. Thus, I evaluated HDAC localization in J89 cells by Western blot. HDAC1, -2, -3, -4, -6, and -7 were the predominant HDACs detected in the nuclei of resting CD4+ T cells. All of these HDACs were also present in the nuclei of J89 cells. Next, I evaluated the association of these

nuclear HDACs with the LTR in J89 cells using ChIP analysis. Antibodies targeting the class I HDACs HDAC1, -2, and -3 immunoprecipitated LTR DNA. This was the first demonstration of HDAC2 associating with the HIV-1 LTR in T cells, which had previously been observed in a microglial HIV-1 cell line model of latency (31). I was unable to detect LTR DNA in ChIP eluates obtained using antibodies targeting the class II HDACs HDAC4, -6, or -7. Taken together, these data demonstrate that the class I HDACs HDAC1, -2, and -3 are highly expressed in the nuclei of resting CD4+ T cells, the primary latent reservoir of HIV-1, and that these HDACs are recruited to the HIV-1 LTR.

Based on these results, we chose to primarily focus our efforts on the class I HDACs. Initially, we silenced HDAC mRNA expression using small interfering (si)RNA in the HeLa P4/R5 cell line model of latency (experiments performed at Merck Research Laboratories). P4/R5 cells are a MAGI-derived cell line that contain an integrated HIV-1-LTR driving the reporter gene *lacZ* (24). We chose this cell line because it has a high transfection efficiency of siRNA. Additionally, as these cells only possess the HIV-1 promoter, we would be able to test specific LTR-driven regulation of transcription. We transfected cells with siRNAs targeting the class I HDACs: HDAC1, -2, -3, and -8. Silencing of HDAC2 resulted in a significant increase in LTR-driven LacZ expression (~ 2-fold over mock transfection). Next, we combined individual class I HDAC knockdowns with exposure to a submaximal concentration of the pan-HDAC inhibitor trichostatin A (TSA). In these experiments, HDAC3 depletion led to a 2.5-fold increase in β-galactosidase activity over cells treated with TSA alone. Thus, while HDAC2 independently regulates HIV-1 transcription, in the context of pan-HDAC inhibition, HDAC3 is the most significant contributor to HIV-1 repression in HeLa P4/R5 cells.

The results presented in Chapter 2, point to class I HDACs as regulators of HIV-1 latency and suggest that use of class I-selective HDAC inhibitors may be a potential antilatency therapeutic strategy. In the course these studies, we published a report on the effects of various novel and selective HDAC inhibitors synthesized by our collaborators at Merck Research Laboratories on latent HIV-1 expression (see Appendix for the report by Archin et al.) (3). The findings from these studies support the conclusions derived in Chapter 2. We found that inhibitors that primarily target the class I HDACs HDAC1, -2, and -3 were potent inducers of latent HIV-1 in both the J89 cell line model of latency and in resting CD4+ T cells obtained from HIV-1-positive patients. In contrast, inhibitors selective for class II HDACs did not stimulate latent HIV-1 expression. Interestingly, an inhibitor selective for HDAC1 and HDAC2 alone did not activate latent HIV-1. This implies that targeting HDAC3 is required for inducing HIV-1 expression with HDAC inhibitors. However, as we are unaware of inhibitors that target HDAC3 in isolation, or HDAC1 and HDAC3 or HDAC2 and HDAC3 in tandem, we were unable to delineate the minimal HDAC inhibitory requirement for optimal induction of latent HIV-1 expression.

In an effort to narrow down the specific combination of HDAC inhibition required to most effectively induce HIV-1 expression, in Chapter 3 I employed a series of individual and combination knockdown experiments in more clinically relevant systems. I first tested the significance of our observations with siRNA-mediated HDAC knockdown in P4/R5 cells to HIV-1 latency by repeating these experiments in J89 cells. I found that both HDAC2 and HDAC3 independently regulate HIV-1 latency in this model system. The level of induction following depletion of either HDAC2 or HDAC3 was relatively modest (~ 1.5-fold over the nonspecific (NS) siRNA control), but statistically significant.

Next, I examined the effects of combined HDAC depletion on HIV-1 expression. When two class I HDACs were knocked down in tandem, HDAC1 and HDAC2, HDAC1 and HDAC3, or HDAC2 and HDAC3, I did not observe an induction of latent HIV-1 expression. This was not due to decreases in cell viability or decreased efficiency of HDAC mRNA knockdown. These results were surprising as at a minimum I had anticipated to see induction of HIV-1 expression following concurrent HDAC2 and HDAC3 knockdown comparable to levels induced by isolated HDAC2 and HDAC3 knockdown.

However, our studies with selective HDAC inhibitors have suggested that the minimal HDAC inhibitory requirement for optimal HIV-1 expression may be HDAC1, -2, and -3 (3). I therefore decided to test the effects of simultaneous depletion of HDAC1, -2, and -3 on HIV-1 expression. In contrast to our results with selective HDAC inhibitors, combined knockdown of HDAC1, -2, and -3 did not induce latent HIV-1 expression. This was not due to a decreased viability of these cells following knockdown. In fact, depletion of HDAC1, -2, and -3 pools led to an increase in cellular proliferation when compared to the NS control. Lack of induction of HIV-1 expression was likewise not attributable to an inhibitory effect of the nucleofection procedure on HIV-1 transcription as nucleofected cells were readily induced by MRK13, an HDAC inhibitor selective for HDAC1, -2, and -3. These findings suggest that there is a fundamental difference between depletion of HDAC expression and inhibition of HDAC enzymatic activity with small molecule inhibitors. The difference between these two modes of HDAC inhibition, could be due to the fact that knockdown of HDAC expression with siRNAs is a slow process and does not completely deplete cellular HDAC pools. In these studies, HDAC mRNA knockdown ranged from 50-90% and peak protein knockdown was not observed until 48-hours post-nucleofection. Thus,

at any one promoter some combination of residual HDAC1, -2, or -3 could continue to repress the HIV-1 LTR. In contrast, HDAC inhibition with small molecule inhibitors is a rapid and comprehensive block to HDAC enzymatic activity. Alternatively, depletion of HDAC expression could disrupt protein-protein interactions that are unaffected by small molecule inhibitors, leading to alterations in other cellular pathways that regulate HIV-1 expression. HDACs have been reported to have additional functions independent of their deacetylase activity (14, 27, 29, 48, 49) .

Based on the seemingly fundamental difference between the effects of knockdown of HDAC expression and enzymatic HDAC inhibition on HIV-1 latency, our findings do not allow us to narrow down the minimal HDAC inhibitory requirement for optimal HIV-1 expression beyond HDAC1, -2, and -3. Continued efforts to identify and develop more selective inhibitors are warranted to determine whether a more targeted induction of HIV-1 expression by selective HDAC inhibitors is a feasible antilatency strategy.

I next sought to determine the impact of individual class I HDAC knockdown on *ex vivo* latent HIV-1 proviral expression in resting CD4+ T cells from HIV-1-positive patients. Although I did not observe a large effect on HIV-1 expression following individual knockdown of HDAC2 or HDAC3, there are fundamental differences between proliferating cell line models of latency and quiescent, resting CD4+ T cells that may lead to different results. Indeed, although the HDAC inhibitor VPA is only a modest inducer of HIV-1 expression in the J89 cell line model, in latent virus outgrowth assays using patient resting CD4+ T cells, VPA is as effective at inducing HIV-1 expression as maximal mitogen stimulation with phytohemagglutinin A and allogeneic PBMCs (46).

When we measured *ex vivo* virus outgrowth from patient resting CD4+ T cells following HDAC1, -2, or -3 knockdown, we observed reactivation of a selected number of proviral integrants. In contrast to our cell line experiments with HDAC siRNAs, knockdown of HDAC1 led to latent HIV-1 outgrowth in four out of four experiments. However, the fold induction of HIV-1 expression with HDAC1 knockdown over the NS siRNA control was modest, ranging from 1.4- to 2-fold. When HDAC2 or HDAC3 was knocked down by siRNA, we observed relatively large fold-increases in virus recovery from cells isolated from a subset of patients. HDAC2 knockdown induced outgrowth in five out of nine experiments, with a range from 1.4- to 4.6-fold over the NS siRNA control condition. Knockdown of HDAC3 reactivated latent HIV-1 in four out of eight experiments, with a range of 1.6-fold to 3.2-fold. In contrast to the class I HDAC knockdowns, knockdown of HDAC7, a class II HDAC, did not induce latent HIV-1 outgrowth in three out of three experiments.

With the exception of a few experiments, the infected units recovered per billion (IUPB) resting CD4+ T cells following HDAC knockdown were not as high as the IUPB following maximal mitogen stimulation. Thus, not all of the replication-competent proviral integrants were induced within a particular patient's resting CD4+ T cell pool. We have observed similar results with some HDAC inhibitors, where the IUPB following exposure to an HDAC inhibitor is sometimes not as high as that measured following maximal mitogen stimulation (1, 3). Variations in proviral outgrowth following HDAC knockdown compared to HDAC enzymatic inhibition could be due to differences in proviral genomic sequences, cell-to-cell fluctuations in the availability of transcriptional regulatory factors (6, 42), DNA methylation patterns at CpG islands in the proviral promoters (5, 22), or integration site-dependent controls on proviral expression (15, 20, 21, 28).

In conclusion, the studies described in Chapters 2 and 3 suggest that the class I HDACs HDAC1, -2, and -3 are recruited to the HIV-1 LTR to repress HIV-1 transcription and that class I-selective HDAC inhibitors are potential antilatency therapeutics that may have lower toxicities and fewer off-target effects than pan-HDAC inhibitors. The resting CD4+ T cell latent reservoir within an infected individual is not a homogeneous population. The mechanisms that maintain HIV-1 transcriptional repression may vary from cell to cell. Additionally, differences in levels of transcriptional regulatory factors in an infected cell at a given point in time and variations in the epigenetic environment at the site of proviral integration may affect the inducibility of a particular latent provirus by HDAC inhibitors. More potent HDAC inhibitors could possibly overcome these restrictions. Alternatively, as multiple mechanisms lead to HIV-1 latency, a combined approach that targets HDACs and other blocks to HIV-1 expression in resting CD4+ T cells may be more effective at inducing a diverse population of proviral integrants *in vivo.*

REMAINING QUESTIONS AND FUTURE DIRECTIONS

The experiments described in this dissertation increased our understanding of the regulation of HIV-1 latency by HDACs. However, they also led to many questions that remain to be answered. Future studies aimed at resolving the following questions could guide identification and development of more effective eradication strategies.

Why are there multiple mechanisms of HDAC recruitment to the HIV-1 LTR?

Multiple mechanisms have been reported to recruit HDACs to the HIV-1 LTR during latency. Five different factors have been demonstrated to recruit HDAC1 to the LTR

including Yin Yang 1 (YY1) and late SV40 factor (LSF) (9), NF-κB (43), activator protein-4 (AP-4) (18), chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (CTIP2) (31), c-Myc and specificity protein 1 (Sp1) (19), and C-promoter binding factor-1 (CBF-1) (37). CTIP2 and methyl-CpG binding domain protein 2 regulate recruitment of HDAC2 (22, 31). HDAC3 also associates with the LTR and this interaction has been proposed to be mediated by transcription factor II-I (TFII-I) (23, 30).

What remains to be determined is whether all of these factors recruit these HDACs simultaneously or at different times. Are HDAC1, -2, and -3 present at the LTR concurrently? Furthermore, are these multiple reports of HDAC recruitment illustrative of a redundant mechanism for LTR repression, or do multiple transcription factors cooperatively recruit HDAC-containing complexes to the LTR? Questions such as these can begin to be addressed by sequential chromatin immunoprecipitation (ChIP) assays, which permit the detection multiple proteins at specific sequences of DNA at the same point in time.

Furthermore, although knockdown of any one of the above transcription factors leads to reduced levels of the corresponding HDAC at the LTR, it has not been investigated whether the other transcription factors and HDACs remain associated with the LTR. Examining the effects of siRNA-mediated knockdown of the above transcription factors on the association of HDAC1, -2, and -3 and other transcription factors with the LTR may reveal which of these factors are dominant to HDAC recruitment. Determining the dominant mechanisms that mediate HDAC recruitment to the LTR may suggest alternative strategies to target HIV-1 expression.

Is the effect of HDAC inhibition on the LTR mediated in part by non-histone substrates?

The most widely studied mechanism of HDAC action is the deacetlation of histones. However, phylogenetic analyses suggest that HDACs actually predate histone evolution (13). Furthermore, HDAC-related proteins are present in bacteria, which do not express histones. Thus, despite their name, HDACs are fundamentally lysine deactylases and can catalyze the removal of acetyl groups from non-histone proteins as well. Multiple transcription factors are regulated by reversible acetylation including transcription factors such as YY1, c-myc, and NF-κB (7, 8, 33, 45). For example, HDAC3-mediated deacetylation of the activating NF-κB p65 subunit results in export to the cytoplasm. Thus, HDAC3 regulates the duration of NF-κB p65 mediated transcription.

Because exposure to HDAC inhibitors leads to acetylation of histones 3 and 4 at the HIV-1 LTR, we have primarily considered the targets of HDAC1, -2, and -3 to be histone tails (1, 3). However, the idea that HDACs could also be regulating transcription factors at the LTR via deacetylation is an intriguing possibility that deserves further consideration. The fact that known HDAC substrates colocalize with HDACs at the LTR warrants particular attention. Examination of histone and transcription factor acetylation at the LTR by ChIP assays following HDAC knockdown could reveal additional pathways for HDAC-mediated repression of HIV-1 expression.

Why does knockdown of class I HDACs not have the same effect on HIV-1 expression as enzymatic inhibition with small molecule inhibitors?

Our findings in Chapter 3 suggest that there is a fundamental difference between the effects of depletion of HDAC pools by siRNA and inhibition of HDAC catalytic activity with

small molecule inhibitors on HIV-1 expression. Non-enzymatic functions of HDACs have been reported (14, 27, 29, 48, 49). It is possible that disruption of key protein-protein interactions following HDAC knockdown could alter cellular pathways that lead to activation of the LTR. Alternatively, because siRNA knockdown leads to a slow and incomplete reduction in cellular HDAC levels, it is possible that some combination of the remaining pool of HDACs continue to associate with proviral promoters to repress LTR expression. The fact that multiple pathways exist to recruit HDACs to the LTR suggests that the latent LTR has a high affinity for HDACs. This possibility could be evaluated by ChIP assays of HDAC1, -2, and -3 at the HIV-1 LTR following both isolated and combination siRNA-mediated HDAC knockdowns. Such experiments could provide a mechanistic explanation for the discrepancy in our studies comparing HDAC knockdown and HDAC inhibition.

Will class I-selective HDAC inhibitors deplete the HIV-1 latent reservoir in vivo?

The ultimate goal of the studies described in this dissertation is to identify and evaluate strategies that could be used to therapeutically target latent HIV-1 in patients. Our findings suggest that class I-selective HDAC inhibitors have potential as antilatency therapeutics. However, whether use of these inhibitors will translate into a targeted depletion of resting cell infection *in vivo* remains to be determined. Small animal models of HIV-1 infection have been established, including bone marrow-liver-thymus mice (10, 36) and humanized (hu-) $Rag2^{-/-}\gamma_c^{-/-}$ mice (47). Thus, *in vivo* pre-clinical evaluation of class I HDAC inhibitors as an effective antilatency therapy is possible. Furthermore, as some selective HDAC inhibitors are currently in use in the clinic and in clinical trials, evaluation of temporally-contained selective HDAC inhibition on depletion of resting cell infection may be

possible in human subjects (4). Persistent HIV-1 infection affects millions of people world-wide, with thousands of new infections occurring each day (38). Innovative translational studies that target persistent HIV-1 expression are urgently needed.

CONCLUDING REMARKS

The ability of the HIV-1 provirus to stably integrate into host DNA and adopt a state of latency is one of the biggest contributors to viral persistence. Unfortunately, there are no therapies at present that specifically target the latent reservoir. HDACs have emerged as potential targets for antilatency therapeutic strategies. This dissertation addressed the role of HDACs in regulation of HIV-1 latency. I showed that the class I HDACs HDAC1, -2, and -3 associate with the latent LTR and contribute to variegated repression of integrated proviruses in the resting CD4+ T cell reservoir. These findings point to class I-selective HDAC inhibitors as potential therapies for eradication of HIV-1 that deserve further pre-clinical evaluation.

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APPENDIX

EXPRESSION OF LATENT HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 IS INDUCED BY NOVEL AND SELECTIVE HISTONE DEACETYLASE INHIBITORS

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ABSTRACT

Objectives: A family of histone deacetylases (HDACs) mediates chromatin remodeling and repression of gene expression. Deacetylation of histones within the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) by HDAC1 plays a key role in the maintenance of latency, while acetylation of histones about the LTR is linked to proviral expression and escape of HIV-1 from latency. Global HDAC inhibition may adversely affect host gene expression, leading to cellular toxicities. Potent inhibitors selective for HDACs that maintain LTR repression could be ideal anti-latency therapeutics.

Methods: We investigated the ability of selective HDAC inhibitors to de-repress the HIV LTR in both a cell line model of latency and in resting CD4+ T cells isolated from patients who were aviremic on antiretroviral therapy (ART).

Results: We found that inhibition of class I HDACs increased acetylation of histones at the LTR, but that LTR chromatin was unaffected by class II HDAC inhibitors. In a latently infected cell line, inhibitors selective for class I HDACs were more efficient activators of the LTR than inhibitors that target class II HDACs. Class I HDAC inhibitors were strikingly efficient inducers of virus outgrowth from resting CD4+ T cells of aviremic patients, whereas HIV-1 was rarely recovered from patient's cells exposed to class II HDAC inhibitors.

Conclusions: Further development of selective HDAC inhibitors as part of a clinical strategy to target persistent HIV-1 infection is warranted.

INTRODUCTION

Latent infection of resting CD4+ T cells is established early during HIV-1 infection, making eradication of HIV-1 unachievable with current ART (1-3) . Following integration of viral DNA into the cellular genome, the HIV-1 LTR promoter can revert to transcriptional silence in the absence of stimulation (4-6). One of the mechanisms through which HIV-1 latency is maintained is by the action of HDACs at the HIV-1 LTR (reviewed in (7)).

HDACs are lysine deacetylases that modify histones and induce transcriptional repression, but can also exert influences on cellular activities that are independent of transcriptional repression (reviewed in (8)). HDACs are generally divided into 3 classes. The class I HDACs comprise HDAC 1, 2, 3 and 8; while class II HDACs include 4, 5, 7 and 9 (subclass IIa) and 6 and 10 (subclass IIb). The catalytic domain of HDAC11 shares homology with both class I and II and this enzyme is sometimes classified as a class IV HDAC. The class III HDACs, the sirtuins, differ from the other classes in that they require NAD to function and are not affected by HDAC inhibitors active against class I, II and IV.

HDACs repress transcription mainly through their ability to covalently modify the lysine tail of core histones of nucleosomes through deacetylation. Deacetylation of lysine residues on histone tails decreases the access of transcription factors to the DNA, and recruits other histone modifying complexes that result in further transcriptional repression.

In tissue culture models of latent HIV-1 infection, HDAC1 is recruited to the LTR by multiple DNA-binding complexes. HDAC recruitment was first reported by the transcription factor LSF in concert with YY1 (9). Later studies suggested that AP-4(10) , heterodimers of the activation domain-deficient NF-κB p50 subunit (11), C-myc through interaction with Sp1 (12), and CBF-1 by binding near the NF-κB /NFAT enhancer element (13) could also recruit

HDAC1. CTIP2 was reported to recruit HDAC1 or HDAC2 to the Sp1 binding site of the LTR (14) . Finally, it has been suggested that HDAC3 associates with the LTR (15) .

Disruption of HDAC1 recruitment to the LTR by specific DNA-binding molecules, or inhibition of HDAC activity by global HDAC inhibitors (HDACi), leads to LTR activation and the escape of HIV-1 from latency in both cell line models and primary cells obtained from patients (9, 16, 17). Furthermore, the HDAC inhibitors valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA; vorinostat) induce viral outgrowth from resting CD4+ T cells of aviremic HIV-1-infected individuals on ART (18, 19). HDAC 2 and 3 can also occupy a site at the HIV LTR, and may play a role in the repression of LTR expression (14, 15). These observations have led to the investigation of HDAC inhibition as a putative therapeutic strategy to induce HIV-1 from latency.

As global HDAC inhibition may have adverse effects on host cells, we studied the ability of selective HDAC inhibitors to de-repress the HIV-1 LTR in both a cell line model of latency and in CD4+ resting T cells isolated from aviremic patients on ART. We found that inhibitors that target class I HDACs 1, 2, 3 alone or in tandem with the class II HDAC 6 were efficient inducers of HIV-1 expression, yielding the outgrowth of replication-competent HIV-1 from the resting CD4+ T cells of patients. However, inhibition of HDAC 6 alone, or of other class II HDACs resulted in marginal LTR activation in cell line systems, and did not result in significant recovery of virus from patient's cells. Of note, a selective inhibitor targeting HDAC 1 and 2 was not very effective at inducing LTR activation in cell lines and virus outgrowth from resting CD4+ T cells of HIV-1-infected individuals. These finding suggest the design of inhibitors selective for a limited array of HDACs as therapies to target persistent HIV-1 infection.

MATERIALS AND METHODS

Cell culture. J89 cells, a latently infected Jurkat cell line encoding the enhanced green florescence protein (EGFP) as a marker for Tat-driven HIV-1 LTR expression (gift of D.N. Levy) (20), were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma, St Louis, MS), 100 U/ml penicillin and 100ug/ml of streptomycin (Invitrogen, Carlsbad, CA) at 37°C under 5% CO_2. Hela P4/R5 cells, a variant of HeLa Magi cells expressing both CXCR4 and CCR5 selected for low background beta-galactosidase expression via the HIV-1 minimal LTR (generous gift of N. Landau) (21) , were cultured in Phenol Red-free DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 0.5 mg/ml Puromycin (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen).

High throughput screening of LTR-activating compounds. Hela P4/R5 cells were seeded at 2000/well in 1536-well plates and incubated for 24 hrs. Test compounds were added and cells were incubated an additional 24 hrs and beta-galactosidase activity was measured by the use of the Tropix Gal-Screen Assay Kit (Applied Biosystem, Foster City, CA) and read with the Perkin Elmer Envision 2101 Multilabel Luminometer.

Selective HDAC inhibitors. The non-selective inhibitor valproic acid was obtained from Bedford Laboratories (Bedford, OH). Other inhibitors were synthesized at Merck Research Laboratories. MRK 1 is a selective inhibitor of the class I HDACs 1, 2, 3 and the class II HDAC 6 (22). MRK 4, apicidin, and MRK 13 are selective against HDACs 1, 2 and 3 (22, 23); MRK 10 is a selective inhibitor of HDAC6 (24). MRK 11 and MRK 14 selectivity inhibit the class II HDACs 4, 5, 6, and 7 and the class I HDAC 8 (25, 26). However, siRNA knockdown of HDAC8 does not induce HIV-1 LTR expression (A. Espeseth, unpublished observations). MRK12 is a selective inhibitor of HDACs 1 and 2 (22).

Flow cytometry. J89 cells were washed with PBS and incubated overnight with the indicated concentration of HDACi. Cells were washed and resuspended in PBS containing 2% paraformaldehyde. GFP expression was measured by FACScan (Becton Dickinson, San Jose, CA) and analyzed using Cell Quest software (Macintosh, Sunnyvale, CA). Live cells were gated and two-parameter analysis used to differentiate GFP-associated fluorescence from background fluorescence. A total of 10,000 gated events were collected and data represent the percent of GFP-expressing cells in total gated events.

Cytotoxicity assays. J89 cells were washed with PBS and incubated for 24 hrs in various concentrations of inhibitors. To measure proliferation and viability in the presence of drugs, cells were subjected to an MTT assay using a cell proliferation kit following the manufacturer's instructions (Roche Applied Sciences, Indianapolis, IN). The percentage of cells proliferating was calculated from cells cultured in drug-free medium.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (19) with the following modifications: J89 cells were cultured with HDACi at indicated concentration for 4 hrs. Formaldehyde crossed-linked cells were lysed for 20 minutes on ice using SDS Lysis buffer (Millipore, Billerica, MA) and sonicated to fragment chromatin to 500-1000 base pairs. 1×10^6 sonicated cells were used to set up each immunoprecipitation reaction using 5 μg of anti-acetyl histone 3 (Ac-H3, Millipore) or rabbit pre-immune immunoglobulin G (Sigma). PCR on immunoprecipitated or input DNA was performed as described previously (19) . The percent of input HIV-1 LTR DNA was determined by comparing the cycle threshold values of each reaction to a standard curve generated from input DNA. The fold enrichment of acetylated histone proteins at the

HIV-1 LTR region was calculated as a ratio of specific signal over untreated background signal.

Limiting dilution cultures of latently infected CD4+ T cells from HIV-infected donors. Lymphocytes were obtained by continuous-flow leukopheresis. Isolation of resting CD4+ T cells, recovery, and quantification of replication-competent virus were performed as previously described (27). For phytohemagglutinin A (PHA) conditions, 93.6 million resting CD4+ T cells were plated in replicate dilutions of 2.5 million (36 cultures), 0.5 million (6 cultures) and 0.1 million (6 cultures) cells per well and stimulated with 1 µg/ ml PHA-L (Remel, Lenexa, KS), a 5-fold excess of allogeneic irradiated PBMCs from a seronegative donor, and 20 U/ml interleukin 2 (IL-2) for 24 hours. For MRK HDACi, 33.5 to 48.6 million resting CD4+ T cells were plated in replicate dilutions of 2.5 million (12-18 cultures), 0.5 million (6 cultures) and 0.1 million (6 cultures) cells per well and stimulated with the indicated concentration of drugs for 24 hours.

After maximum mitogen stimulation or drug exposure, cells were washed and cultured as previously described (27) . Culture supernatants were collected on day 15 and 19 and assayed for virus production by p24 antigen capture ELISA (Zeptometrix, Buffalo, NY). Cultures were scored as positive if p24 was detected at day 15 and was increased in concentration at day 19. A maximum likelihood method was used to calculate the infectious units per million (IUPM) of resting CD4+ T-cells after exposure to PHA or HDACi. If all cultures were negative, the IUPM was estimated to have a value lower than if 1 culture of 2.5 million cells had been positive.

RESULTS

HDAC1 potency corresponds with activation of the LTR. To screen for novel HDACis that induce expression of the HIV-1 promoter, candidate inhibitors from the Merck Research Laboratories chemical library were tested for the ability to upregulate LTR expression in the P4/R5 LTR-reporter cells (21). As shown Fig. A.1A, by Spearman rank correlation there was a significant ($p<0.0001$) association between inhibitory potency against HDAC1 as measured in an in-vitro enzymatic assay, and increasing efficiency in LTR induction.

Class I but not class II inhibitors induce chromatin changes at the HIV-1 LTR leading to LTR expression. Class I HDACs have been shown to maintain histones within the nucleosome-bound provirus in a hypoacetylated state, facilitating LTR repression (9-15). Conversely, acetylation of histones by histone acetyltransferases (HATs) leads to neutralization of the net charge on lysine residues, increasing the access of transcription complexes to chromatin and recruiting other chromatin remodeling complexes, thus abrogating HDAC-mediated repression. However, the contribution of class II HDACs to this equilibrium is unexplored. We therefore compared the ability of class I and class II selective HDAC inhibitors to induce acetylation of nucleosome 1 (nuc-1) of the HIV-1 LTR by performing chromatin immunoprecipitation in J89 cells. Precipitated DNA was quantified using a set of primers spanning the nuc-1 region of the HIV-1 promoter. Cells were assayed after only 4 hours of treatment to minimize the impact of indirect, secondary effects expected to follow histone deacetylation. The percent of input for each immunoprecipitation was calculated and the relative fold occupancy of acetylated histones reported. We found that class I but not class II inhibitors induce acetylation of histones at the HIV-1 LTR (Fig. A.1B).

Class I selective HDAC inhibitors are better than class II at inducing LTR expression. To determine the ability of selective HDAC inhibitors to induce HIV-1 expression, selected candidate compounds were tested in the J89 Jurkat T cell model of HIV-1 latency (20) and LTR-driven enhanced green fluorescence protein (EGFP) reporter gene expression measured.

Class I selective HDAC inhibitors, MRK1, MRK4 and apicidin all induced LTR activation (Fig. A.1C). As expected, the weak, non-selective HDAC inhibitor VPA also effectively induced LTR expression, albeit at millimolar concentrations. However, little LTR expression was induced by the class II HDAC inhibitors MRK10, MRK11, and MRK14. This was not due to toxicity of VPA or MRK 10, 11, and 14, as concentrations used to induce LTR expression did not perturb cell proliferation (Fig. A.1D and ref. 18). These compounds were also evaluated in the HIV-1 latency model cell lines ACH-2, J1.1 and Hela P4/R5. A similar observation was made where only the class I and non-selective inhibitors robustly induced LTR expression (data not shown).

Class I selective HDAC inhibitors induce viral outgrowth more efficiently than class II HDAC inhibitors. We next examined the ability of the various HDACi to induce viral outgrowth from the resting CD4+ Tcells of HIV-1-infected volunteers. Resting CD4+ T-cells were isolated by negative selection from a total of 14 aviremic HIV-1-infected patients on ART (stable HIV-1 plasma RNA <50 copies/ml). Resting cells were obtained at multiple time points from six of the patients. The frequency of viral recovery after HDACi exposure in multiple limiting-dilution culture assays was compared to that after maximum mitogen activation with PHA, allogeneic PBMCs, and IL-2. As observed in J89 cells, the class I HDAC inhibitor MRK1 allowed more frequent recovery of replication-competent

HIV-1 from patient's resting CD4+ T cells than the class II selective inhibitors, MRK10 and MRK11 (Fig. A.2). Of note, in seven of eight patients studied, there was less than a two-fold difference in the frequency of recovery of HIV-1 in cells exposed to MRK1 compared to PHA, a difference that is within the variance of our resting cell outgrowth assay.

The non-selective inhibitor VPA was also effective at inducing virus from resting CD4+ T cells of aviremic HIV+ patients (Fig. A.2), as previously demonstrated (18, 19). The surprising activity of VPA in primary cells as compared to more potent and specific HDAC inhibitors is not well understood, but could be the result of effects of VPA on other cellular enzymes, such as glycogen synthase kinase-3β.

However, while the class I inhibitors apicidin and MRK4, were effective at inducing LTR expression in J89 cells (Fig. A.1C), induction of virus from CD4+ resting T cells by these inhibitors was suboptimal (Fig. A.3). As both MRK4 and apicidin appear to be slightly anti-proliferative in J89 T cells (Fig. A.1D), we cannot rule out the possibility that such an effect limits the recovery of virus in resting CD4+ T cell outgrowth assays.

As global HDAC inhibition may have effects on the host cell that lead to toxicities and adverse clinical outcomes, potent but selective inhibition of HDACs required to maintain LTR repression is desirable for potential therapeutics designed to disrupt HIV-1 latency. MRK4 and apicidin are both selective against HDAC1-3, induce LTR expression (Fig. A.1C), but may weakly induce viral outgrowth in patients' cells due to effects on the host cell (Fig. A.3).

To study the effect of HDACi selective for HDACs 1-3 that are without apparent host cell toxicity, we studied two additional compounds with selectivity against HDAC 1 and 2 (MRK12) and HDAC 1, 2, and 3 (MRK13). Although activation of LTR expression in the

J89 cell line by MRK13 (which inhibits HDAC 1-3) was comparable to that of MRK 1 (which inhibits HDAC1-3 and 6; Fig. A.4A), in six of the seven patients studied, recovery of HIV from cells exposed to MRK13 was over three-fold less frequent than from cells exposed to PHA (Fig. A.4B and A.4C). Surprisingly, the HDAC 1 and 2 inhibitor, MRK12, performed poorly in both cell line and primary resting CD4+ T-cells assays (Fig. A.4A and A.4C).

DISCUSSION

Selective HDAC inhibitors induce expression of the HIV-1 promoter and allow recovery of replication-competent HIV-1 from the resting CD4+ T cells of ART-treated, aviremic patients. Inhibition of class I but not class II HDACs resulted in an increase of acetylated histones at the nucleosome-bound LTR. We found that inhibitors that target the class I HDACs 1, 2 and 3 were more efficient activators of the HIV-1 LTR in a cell line model of HIV-1 latency than inhibitors that target the class II HDACs. Class II HDAC inhibitors also performed poorly at inducing virus outgrowth from resting CD4+ T cells isolated from aviremic HIV+ patients.

MRK12, an inhibitor selective against HDAC 1 and 2 failed to activate the LTR in a cell line model of latency, and also poorly induced virus outgrowth from resting CD4+ T cells. This finding is surprising given prior studies illustrating HDAC1, and to a lesser extent HDAC 2, activity at the HIV-1 LTR. However, our studies are the first to utilize selective inhibitors. HDAC 1 and 2 associate with the Sin3, NuRD or CoREST corepressor complexes to repress transcription (reviewed in (29)). It seems likely that HDACs 1, 2, and 3 cooperate as part of one or more multi-protein complexes to mediate HIV-1 LTR repression.

HDAC3 is found in complex with the nuclear hormone corepressors NCoR/SMRT. Whereas HDAC1 and 2 are reported to be global transcription repressors, HDAC3 is reported to be a more specific repressor with activity against genes involved in nuclear receptor signaling (reviewed in (29)). HDAC3 is reported to occupy a site at the HIV-1 promoter and may play a role in suppressing transcription (15).

We investigated the ability of four inhibitors (MRK1, MRK4, apicidin and MRK13) targeting HDACs 1, 2 and 3 to induce virus outgrowth from resting CD4+Tcells. Although all four compounds induced LTR transcription in J89 cells, only MRK1 robustly induced virus outgrowth from resting CD4+ T cells. In addition to its selectivity for HDACs 1, 2, and 3, this inhibitor also targets HDAC6. However, it should be noted that HDAC6 inhibition alone has little effect on HIV-1 LTR expression, as demonstrated (Fig. A.1C and A.2) by an inhibitor selective for HDAC6 (MRK 10). Of note, inhibition of HDAC6 may only be relevant in the study of patient's cells, as inhibition of HDAC 1, 2, and 3 is as effective in inducing LTR expression as inhibition of HDAC 1, 2, 3 and 6 in J89 cells. Interestingly, one study reported a predominantly cytoplasmic localization of HDAC6 in transformed, cancerous cells and a mostly nuclear localization in normal cells (30). However, as HDAC6 does not appear to act directly at the HIV-1 LTR (28), we speculate that the ability of MRK 1 to inhibit HDAC6 contributes to the outgrowth of virus from primary cells at another step in the viral lifecycle, or via other effects on the infected cell.

The mechanism by which HDAC6 might contribute to the suppression of HIV-1 expression requires further study. HDAC6 is a predominantly cytoplasmic enzyme, but can shuttle to the nucleus and is reported to mediate promoter repression in certain systems (30). For example, NF-κB p50 and p65 cooperate with HDAC6 to repress transcription of the H+-

K+-ATPase gene (31). Runt-related transcription factor 2 mediates repression of the p21 promoter via its interaction with HDAC6 (32). In yet another example of HDAC6 mediated repression, the enzyme binds to a domain of the HAT p300 leading to repression of its transcriptional activities. HDAC inhibition or siRNA knockdown of HDAC6 ablates this p300-mediated repression (33). Regardless of the role HDAC6 may be playing in LTR repression, defining the mechanisms involved may provide additional targets for anti-latency therapies.

Despite potent antiretroviral therapy, chronic HIV-1 infection remains a formidable problem that requires novel approaches. These findings suggest that selective HDAC inhibitors may contribute to therapeutic efforts to clear persistent HIV-1 infection.

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AUTHORSHIP CONTRIBUTIONS

N.M.A. and D.M.M. designed research; N.M.A. was responsible for setting up cell culture-based assays, chromatin studies and data analysis. K.S.K. provided help with experimental set-ups and data analysis; A.E., H.D. and D.J.H. were responsible for generating selective HDAC inhibitors and for high-throughput screening of compounds. N.M.A. and D.M.M. wrote the paper with input from all authors.

Table A.1 Selectivity of Novel HDAC Inhibitors.

HDACi	Selectivity
MRK1	HDAC 1, 2, 3, 6
VPA	Non-selective
MRK4	HDAC 1, 2, 3
Apicidin	HDAC 1, 2, 3
MRK10	HDAC 6
MRK11	HDAC 4, 5, 6, 7, 8
MRK12	HDAC 1, 2
MRK13	HDAC 1, 2, 3
MRK14	HDAC 4, 5, 6, 7, 8

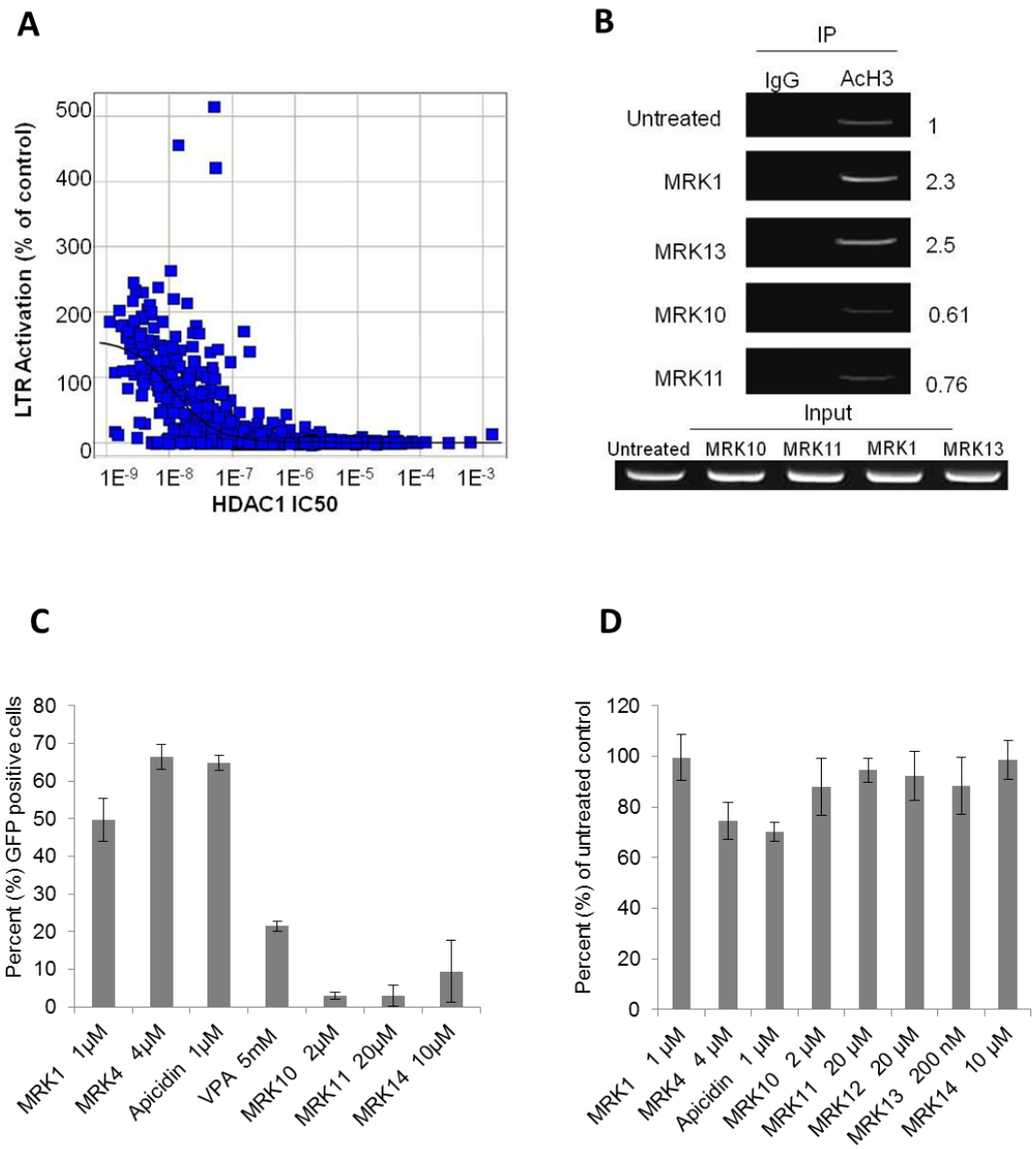

Figure A.1 Class I and non-selective HDAC inhibition induces LTR expression more effectively than class II HDAC inhibitors. (A) HIV-1 LTR expression is increased by HDAC inhibitors with increasing inhibitory potential for HDAC1**.** HeLa P4/R5 cells were seeded and incubated for 24 hours, followed by addition of HDAC inhibitors. β-galactosidase activity was measured after 24-hour incubation with inhibitors. LTR activation is reported as the percent β -galactosidase activity in treated cells over untreated control. (B) Class I but not class II HDAC inhibitors increase acetylation of nuc-1 at the HIV-1 LTR**.** J89 cells were treated with media or HDAC inhibitors: Class I-selective MRK1 or MRK13 at 500 nM and 300nM, respectively, and class II-selective MRK10 or MRK 11 at 200nM and 500nM, respectively. Cells were treated for four hours and assayed by chromatin immunoprecipitation (ChIP) with control rabbit IgG or anti-acetylated H3. DNA products of ChIP were quantitated in triplicate by real-time PCR. Assays are representative of 3

independent experiments, and real-time quantitation of the fold change relative to untreated control is shown. Only class I inhibitors demonstrate significant increases in histone H3 acetylation. (C) Class I and non-selective HDAC inhibition induces HIV-1 expression. J89 cells were incubated overnight with the indicated concentrations of the HDACi. LTR-driven green fluorescent protein (GFP) production was measured by flow cytometry as described in methods. The data presented are the mean ± SEM of 3 independent experiments. (D) Toxicity of HDAC inhibitors on J89 cells at concentrations used to measure promoter activation. J89 cells were cultured in the absence or presence of the indicated inhibitors for 24hrs at the concentrations displayed. MTT assays were performed in triplicate. The percentage of proliferating cells was calculated compared to cells cultured in standard media.

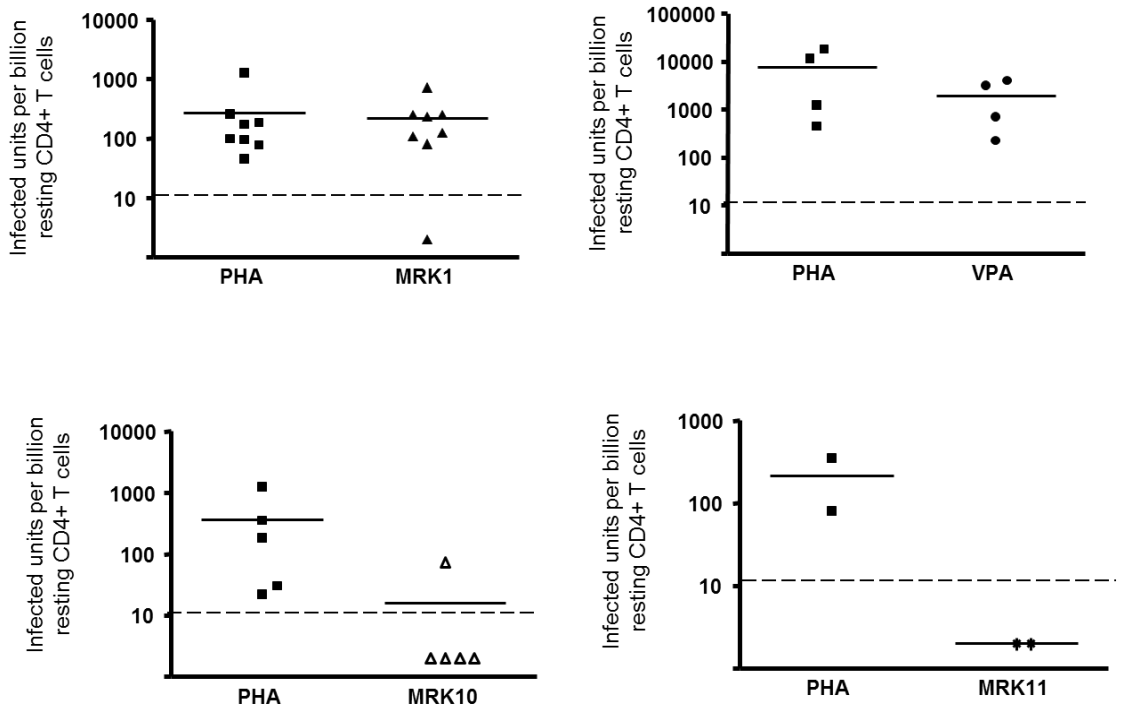

Figure A.2 Recovery of replication-competent HIV-1 from the resting CD4+ T cells of aviremic patients treated with HDAC inhibitors: MRK 1 (Class I HDACs 1, 2, and 3 and Class II HDAC 6), VPA (non-selective), or MRK 10 or 11 (Class II HDACs). Patient cells were subjected to maximum mitogen stimulation or exposed to 2 μM MRK1, 40 μM VPA, 2 μM MRK10 and 10 μM MRK11 for 24 hours. Cells were washed and co-cultured with CD8-depleted PBMC as detailed in methods. Frequency of virus outgrowth from cells treated with HDACi was compared to the frequency of outgrowth from maximally stimulated cells (PHA). Each icon represents independent studies of patient cell samples; patient samples were simultaneously tested with mitogen and HDACi on the same day. Dashed lines indicate the limit of detection of the assay.

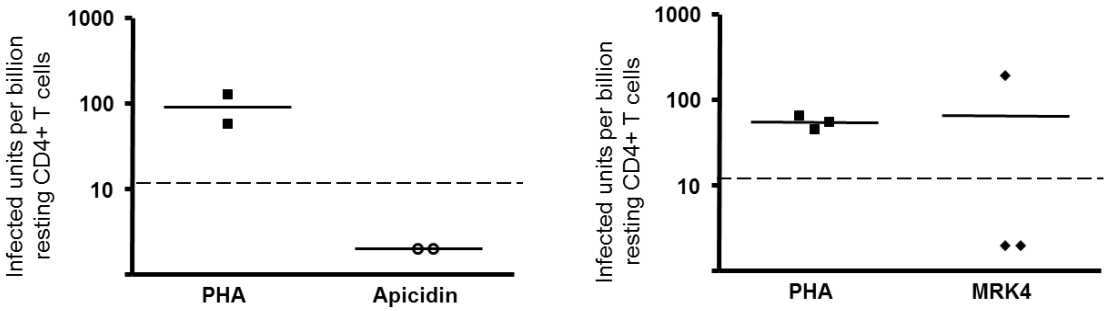

Figure A.3 Recovery of replication-competent HIV-1 from resting CD4+ T cells of aviremic patients treated with apicidin and MRK4, inhibitors selective only for the Class I HDACs 1, 2, and 3. Patient cells were subjected to maximum mitogen stimulation or exposed to 1 μM apicidin or 4 μM MRK4 for 24 hours. Cells were washed and co-cultured with CD8-depleted PBMC as described in methods. Frequency of virus outgrowth from cells treated with HDACi was compared to outgrowth after maximal mitogen activation by PHA. Each icon represents independent studies of patient cell samples; patient samples were simultaneously tested with mitogen and HDACi on the same day. Dashed lines indicate the limit of detection of the assay.

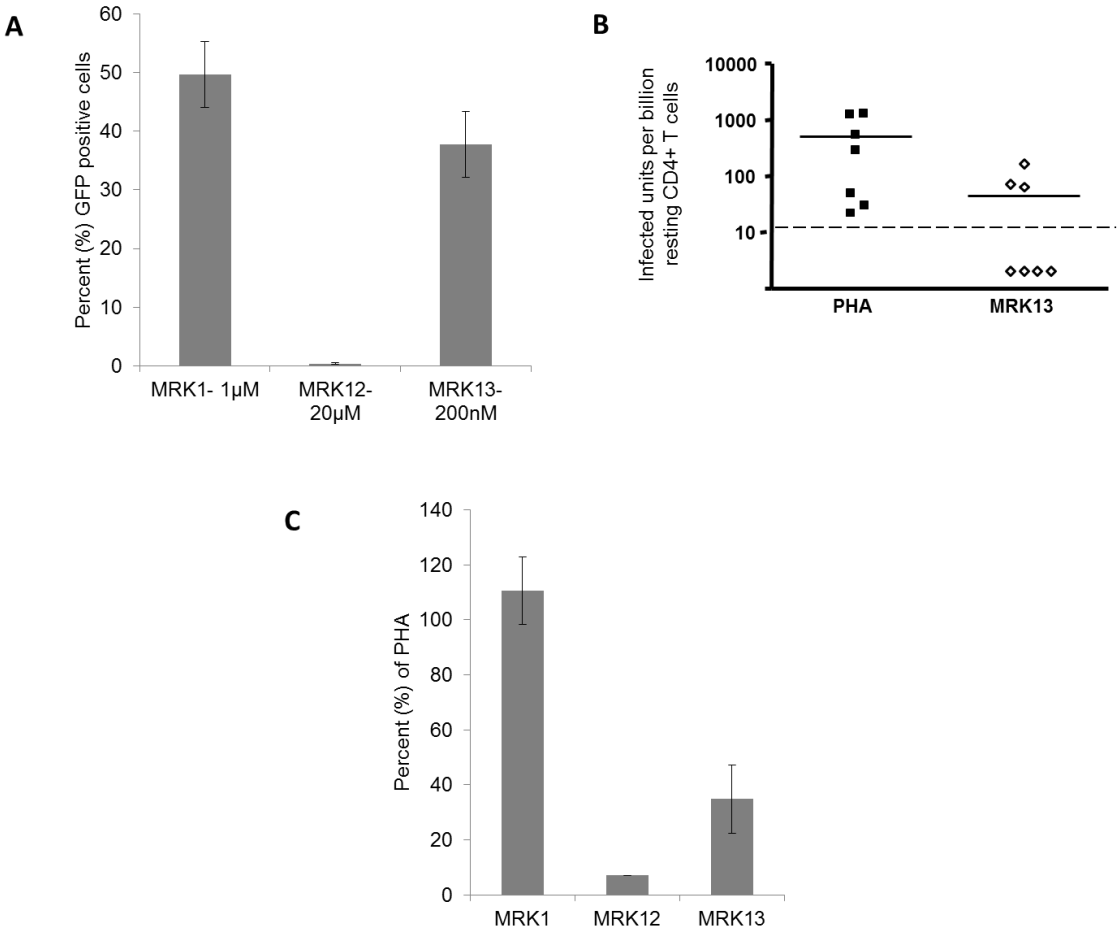

Figure A.4 HIV-1 expression and outgrowth is induced by inhibitors targeting HDACs 1, 2, and 3, but viral outgrowth is markedly improved by the tandem inhibition of HDAC 1-3 and the class II HDAC6. (A) J89 cells were incubated overnight with 1 μM MRK1 (selective for HDAC1, 2, 3 and 6), 2-20 μM MRK12 (HDAC1 and 2) or 200 nM MRK13 (HDAC1, 2, 3). LTR-driven GFP production was measured by flow cytometry as described in methods. The data presented are the mean ± SEM of 3 independent experiments. (B) Resting CD4+ T cells from patients were subjected to maximum mitogen stimulation or exposed to 200-300 nM MRK13 for 24 hours. Cells were washed and co-cultured with CD8-depleted PBMC as described in methods. Frequency of virus outgrowth from cells treated with MRK13 was compared to outgrowth after maximal mitogen activation. Each icon represents independent studies of patient cell samples; patient samples were simultaneously tested with mitogen and HDACi on the same day. Dashed lines indicate the limit of detection of the assay. (C) Frequency of virus outgrowth from resting CD4+ T cells of aviremic HIV+ patients exposed to 2 μM MRK1, 2-20 μM MRK12 and 300 nM MRK13 are shown as a percent of outgrowth obtained from corresponding maximal mitogen activation by PHA. MRK 1, n=8; MRK12, n=2; MRK13, n=7.

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