Characterization and Elimination of the HIV Reservoir

by

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Dedication

To my professors and my family

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Abstract

The current HIV epidemic is highlighted by the remarkable success of combination anti-retroviral therapy (cART). Unfortunately, life-long and continuous cART is necessary for infected individuals due to persistent HIV infection and rebound viremia if cART is interrupted. The ability of HIV to establish a reservoir within long-lived cells is a major barrier to improving treatment and affording a cure.

Here, I first describe our work to characterize the HIV reservoir within hematopoietic stem and progenitor cells (HSPCs) and determine the contribution of infected HSPCs to residual viremia *in vivo*. We found that HSPCs bear HIV proviral genomes in almost half of donors tested (24 of 43). Furthermore, we found that HSPC-derived proviral genomes are represented within residual plasma viral sequences about 3-fold more than proviruses derived peripheral blood and bone marrow mononuclear cells. We also discovered that HSPC-derived proviruses that were genetically identical to plasma virus are very often associated with expanded clonal HIV proviruses found in peripheral blood and bone marrow mononuclear cells. Additionally, we identified a signature deletion within proviral sequences derived from HSPCs and mature PBMCs and $CD4^+$ T cells, providing evidence that HIV-infected HSPCs can propagate the HIV genome to progeny through differentiation. This study sheds light on the heterogeneity of the HIV reservoir and presents evidence in support of a novel pathway for the establishment and maintenance of persistent HIV *in vivo*.

Finally, I describe our work investigating strategies to potently reverse HIV latency and induce reservoir elimination using histone deacetylase inhibitors (HDIs). We determined that class 1-selective HDIs are superior to pan-HDIs in their ability to act additively with the PKC agonist bryostatin-1 to reverse latency, induce potent viral outgrowth, and promote elimination of latently-infected cells. We also found that pan-HDIs suppress potent viral reactivation; an effect that is correlated with their unique inhibitory effects on important pro-viral cellular factors, NF-κB and Hsp90.

This work will significantly inform future studies that will further characterize the heterogeneity of the *in vivo* HIV reservoir and develop clinically pragmatic and effective latency-reversing agents that may lead to improved HIV therapy aimed at reducing the burden of persistent viral infection and hopefully affording a cure.

Chapter 1

Introduction

Combination anti-retroviral therapy (cART) significantly reduces morbidity and mortality associated with HIV infection. Nevertheless, even long-term, continuous cART does not cure infected individuals. The existence of long-lived reservoirs of latently-infected cells is a major barrier to affording a cure. The goals of the work described in this dissertation are to gain a better understanding of the HIV reservoir and to develop strategies that may lead to reservoir elimination. In this chapter I will first introduce the current knowledge of HIV epidemiology, pathogenesis, treatment and biology. I will then discuss our current understanding of the HIV reservoir within infected individuals. Finally, I will review several pharmacologic agents that affect HIV latency and have been proposed to potentially reduce the viral reservoir *in vivo*. Therefore, in this chapter I present the background information necessary for the original discoveries by our group that I describe in chapters 2 and 3 of this dissertation.

The beginning of the HIV pandemic

The signs and symptoms of acquired immunodeficiency syndrome (AIDS) were first described in the United States of America in 1981 when an increasing number of men began to succumb to opportunistic infections and rare malignancies (Centers for Disease,

1981; Centers for Disease, 1982; Hymes et al., 1981; Masur et al., 1981). Two years later the causative infectious agent was identified as human immunodeficiency virus type-1 (HIV-1; HIV) (Barre-Sinoussi et al., 1983; Gallo et al., 1983; Popovic et al., 1984). Shortly thereafter diagnostic tests for HIV infection were developed and public health measures were implemented to limit the spread of this devastating disease. By 1986 over 30,000 cases of HIV/AIDS had been identified globally with about 60% of the documented cases occurring in the American population (UNAIDS, 2016). Six years after the first reports of patients with AIDS, the United States Food and Drug Administration (FDA) approved the first effective anti-retroviral drug, zidovudine (Fischl et al., 1987). It was; however, not until 1995 when the discovery of a mechanistically unique anti-retroviral drug was incorporated into a combination therapy with AZT that the era of combination anti-retroviral therapy (cART) began. The introduction of cART dramatically changed the outlook on the HIV pandemic as combination therapy was able to decrease the morbidity and mortality associated with HIV infection by over 60% (UNAIDS, 2016).

By the turn of the century, HIV/AIDS was the fourth most-common cause of death worldwide and the leading cause of death in Africa. Moreover, despite cART, cases of HIV infection continued to rise dramatically, especially in Africa and Asia (UNAIDS, 2016). In developed nations, where infected people had greater access to cART, medical professionals were encouraged by the remarkable success of cART in reducing viral replication in infected people and the entire community looked forward to what they believed would soon be a day where HIV infection could be cured with sufficient cART.

The HIV pandemic today

Since the introduction of cART, there has been a dramatic reduction in the rate of new HIV infection events and of the frequency of HIV/AIDS-related deaths (Palella et al., 1998). Nevertheless, in 2016 over 36 million people were living with HIV and 1 million died due to the infection. The greatest burden of the HIV pandemic today is undoubtedly upon those affected in Africa where access to cART is limited and the rates of infection are dramatically higher than in any other region (UNAIDS, 2016).

On the other hand, in developed nations, with continuous and compliant cART, HIV infection can be effectively controlled to levels where even the most sensitive clinical assays often do not detect any evidence of viral replication or disease progression. In these cases, life-long cART represents the only practical option for most HIV-infected people to avoid recrudescence of viral replication and progression to AIDS. However, there remain several significant challenges to life-long cART compliance including: the life-long financial burden of treatment compliance, the associated side-effects of therapy, and the persistent social stigma.

Clinical features of HIV infection

Viral Transmission

Infection with HIV often occurs through sexual intercourse, exposure to infected blood, or perinatal transmission (Tindall et al., 1988). The proportion of individuals who become HIV infected by one of these modes of transmission differs globally and is often related to specific risk factors such as: viral load of transmitting individuals, sexual behavior, male circumcision, and genetic background. The highest rates of HIV

transmission events per exposure occur in men who have sex with men (1 in 72 exposures) and intravenous drug users (1 in 150 exposures). Transmission of HIV through occupational exposure to HIV-contaminated body fluids is extremely low (1 in 10,000 exposures) (UNAIDS, 2016).

Natural history of untreated infection

The acute phase of HIV infection occurs over approximately the first 3 months post-exposure and is characterized by a period of rapid viral replication and a precipitous loss of $CD4^+$ T lymphocytes ($CD4^+$ T cells); the main target cell of HIV (**Figure 1.1a**). Approximately 50-70% of infected individuals will experience symptoms of acute HIV infection, including fever, lymphadenopathy, myalgia, arthralgia, and headache (Braun et al., 2015; Daar et al., 2001; Niu et al., 1993). Interestingly, some studies have described a worsened clinical prognosis in infected people who had prolonged symptoms (>14 days) during the acute phase (Daar et al., 2008; Kelley et al., 2007; Lavreys et al., 2006). Within several weeks, infected individuals seroconvert and begin producing clinically detectable levels of antibodies against HIV antigen. This response, along with a robust cell-mediated reaction against high viral titers reduces serum viremia to a viral set point and recovers $CD4^+$ T cells count nearly to a normal level (Musey et al., 1997; Pantaleo et al., 1997). Critically, the viral set point has been associated with prognosis of untreated disease (Lavreys et al., 2006; Mellors et al., 1995). Following the resolution of primary infection, a period of asymptomatic infection occurs where HIV and the host immune system engage in a dynamic competition. This period can last for months to years, during which low-level viral replication in $CD4^+$ T cells causes an insidious decrease in $CD4^+$ T cell counts at a

rate of about 30 to 60 cells/mm^3/year (Mellors et al., 1997; Munoz et al., 1997; Phair et al., 1992). Once below 200 CD4$^+$ T cells/mm^3, an infected person is considered to have AIDS, which is characterized by severe opportunistic infections and rare malignancies including: *P. jirovecii* pneumonia, candidiasis, disseminated histoplasmosis, toxoplasma encephalitis, and cryptococcal meningitis (Chaisson et al., 1987; Flanigan et al., 1992; Phair et al., 1990). When CD4$^+$ T cell counts drop below 50 cells/mm^3, generalized deficiency in cellular immunity is profound and infected individuals are ever more susceptible to additional opportunistic pathogens (Masur et al., 1989). Most individuals whose HIV infection progresses to AIDS, often die due to an AIDS-related illness within only 12-18 months (Phillips et al., 1992) (**Figure 1.1a**).

Combinational anti-retroviral therapy

The advent of cART dramatically changed outcomes of HIV infection. By combining antiretroviral agents that inhibit distinct events in the viral replication cycle, development of drug resistant virus is incredibly unlikely. Maintained on cART continuously, plasma viremia is often below the limit of detection of even the most sensitive clinical assays (< 50 HIV RNA genome copies/mL plasma) and patients are able to live relatively healthy lives (Egger et al., 2002; Hogg et al., 2001; Palella et al., 1998). Occasionally; however, intermittent viral "blips" (> 50 HIV RNA genome copies/mL plasma) can be detected in treated patients (Nettles and Kieffer, 2006; Nettles et al., 2005). Interruption of cART, even after years of continuous therapy, leads to a rapid rebound viremia (Chun et al., 1999; Davey et al., 1999; Joos et al., 2008). Thus, cART is an effective treatment for HIV-infection, but not curative (**Figure 1.1b**).

Elite controllers

A very small percentage of HIV-infected people exhibit spontaneous effective control of HIV infection for at least 12 months even in the absence of cART. Indeed, these patients are HIV-positive by standard clinical assays for antibodies against the virus but lack any sign of robust viral replication (undetectable plasma virus by standard clinical assays) and can remain so for decades (Dinoso et al., 2008; Klein and Miedema, 1995; Pereyra et al., 2009). These individuals, termed elite controllers, make up only about 1 in 300 people infected with HIV (Walker, 2007). Thus far, studies with the goal to understand the mechanisms of this phenomenon have pointed to at least two factors that are strongly correlated with spontaneous control, including: asymptomatic acute infection (Madec et al., 2005) and the certain HLA B alleles (Emu et al., 2008). Interestingly, elite controllers also bear $CD8^+$ cytotoxic T lymphocytes (CTLs) that are qualitatively more functional compared to CTLs from HIV-infected progressors (Betts et al., 2006; Migueles et al., 2002; Zimmerli et al., 2005).

The HIV replication cycle

HIV is a member of the Lentivirus genus within the Retrovirus family. Therefore, HIV virions are enveloped, spherical particles, about 100 nm in diameter that contain a conical protein capsid core, inside which are two copies of the single-stranded RNA genome (Gelderblom et al., 1987). The HIV genome is approximately 9 kilobases in size and encodes for 3 structural proteins (Gag, Pol, and Env), 2 regulatory proteins (Tat and

Rev), and 4 accessory proteins (Vif, Vpr, Vpu, and Nef) the functions of which will be discussed below (**Figure 1.2a**).

Binding and entry

HIV infection begins when subunit gp120 of the viral Env protein binds its cognate target cell receptor CD4 and co-receptor CCR5 or CXCR4 (Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; McDougal et al., 1986). This initiates a conformational change in the Env protein, which approximates the lipid bilayers of the virion and target cell leading to fusion and release of the inner protein capsid core of the virion into the host cell cytoplasm (Wilen et al., 2012) (**Figure 1.2b**).

Reverse transcription

Shortly after entry, the viral capsid disassembles and the viral RNA genome is reverse transcribed by HIV reverse transcriptase (RT) into a complementary double-stranded DNA molecule (**Figure 1.2b**) (Hu and Hughes, 2012). HIV reverse transcription begins at the 5' end of the genome where the 3' hydroxyl end of a producer-cell derived tRNA(Lys3) is bound to the primer binding site (pbs) of the HIV genome. Minus-strand cDNA [(-) cDNA] is synthesized to the end of the 5' RNA genome. As the (-) cDNA—RNA hybrid complex is formed, RNaseH activity of RT degrades the RNA template, leaving a short, single-stranded (-) cDNA molecule. Next the (-) cDNA anneals to the its complementary R region of the 3' end. (-) cDNA elongation continues over the length of the RNA genome. Again, as cDNA—RNA hybrids are formed, RNaseH degrades the RNA template leaving a single-stranded (-) cDNA molecule. Critically, the polypurine tract

(PPT) is spared RNaseH digestion and serves as the primer for positive strand cDNA [(+) cDNA] synthesis, which proceeds to the 5' end of the complementary (-) cDNA and extends to include complementary cDNA to the pbs sequence. Following RNaseH digestion of the tRNA primer the single-stranded, complementary pbs sequences of the (-) cDNA and (+) cDNA anneal and permit the completion of reverse transcription by elongation of (+) cDNA. The final product is a linear, double-stranded cDNA with long terminal repeats (LTRs) at either end of the genome (**Figure 1.3**).

HIV RT lacks any proof-reading activity and, as a result, is estimated to make about 2 errors per 10,000 bases pairs, or about 2 mutations per genome (Hu and Hughes, 2012). Furthermore, cDNA recombinants are frequently formed during (-) cDNA synthesis. These recombination events allow for successful completion of reverse transcription, but may also lead to significant deletions, insertions, and duplications of regions of the genome yielding defective proviral genomes.

Nuclear import and integration

A distinguishing feature of lentiviruses is their ability to usurp host cell machinery to actively transport their genome across intact nuclear envelopes, thereby allowing them to productively infect non-dividing cells. Following reverse transcription, the viral cDNA genome associates with cellular and viral proteins to form the nucleoprotein pre-integration complex (PIC), which passes through nuclear pores into the nucleus. Once in the nucleus, via the activities of HIV integrase and host DNA repair enzymes, the cDNA genome is integrated into the host genome, often in actively transcribed host genetic regions (Craigie and Bushman, 2012; Lewinski et al., 2005; Marini et al., 2015) (**Figure 1.2b**).

Provirus transcription

Once in the host genome, the integrated viral cDNA, or provirus, is dependent upon host cell machinery to transcribe and translate its genome. The 5' HIV LTR serves as a viral gene promoter, with several DNA sequences that can be bound by host and viral proteins necessary for robust viral gene transcription (**Figure 1.2b**). Many of these factors will be discussed in more detail below.

RNA processing and translation

Splicing and processing events regulate the abundance of viral RNA species and ensures efficient and complete expression of the viral genome (Purcell and Martin, 1993). The first RNA species to be transcribed and processed by host spliceosomes are multiply-spliced transcripts, which encode the viral accessory protein Nef as well as important regulatory proteins, Tat and Rev. Only when Tat and Rev accumulate to sufficient levels can they facilitate Tat-mediated transactivation of RNA polymerase II-dependent transcription and Rev-mediated nuclear export of unspliced HIV RNA species (Kim et al., 1989; Klotman et al., 1991). Further discussion on the importance of Tat of Rev in regulating viral gene expression and HIV latency are discussed later in this chapter. Incompletely or singly-spliced RNA transcripts are next processed and encode the structural protein Env, as well as three accessory proteins, Vif, Vpr, and Vpu. Finally, unspliced, or full-length RNA transcripts, encode the Gag-Pol polyprotein and serve as the genomic RNA to be incorporated into newly forming virions (Purcell and Martin, 1993) (**Figure 1.2b**).

Virion assembly, release, and maturation

HIV Gag is both necessary and sufficient to form virus-like particles. Gag is synthesized as a polyprotein, which accumulates on the cytoplasmic side of the cell membrane and multimerizes with other Gag polyproteins to form a budding structure (Freed, 1998). Gag is also essential for loading of specific cargo into the forming virus particle including Env, Vif, Vpr, the Gag-Pol precursor, and two copies of the viral RNA genome (Clever et al., 1995; Cosson, 1996; Muller et al., 2000; Murakami and Freed, 2000). The budding virion is eventually released by membrane fission (von Schwedler et al., 2003). Following release, viral protease processes the Gag and Gag-Pol polyproteins into constituent mature Gag proteins: matrix, capsid (p24), nucleocapsid, and p6; and fully processed viral enzymes, protease, reverse transcriptase, and integrase (Peng et al., 1989). Processing of the Gag precursor induces functionally necessary structural changes within the virion, including the formation of the distinctive inner conical capsid core (**Figure 1.2b**).

HIV accessory proteins

In addition to the structural proteins necessary for infectious virion production, HIV encodes four accessory proteins that mediate host immune system evasion and can contribute to pathogenicity of infection.

Vif binds and directs the degradation of the cytosine deaminase APOBEC3G within infected cells, thereby limiting APOBEC3G's encapsidation within newly produced

virions, and avoiding APOBEC3G-mediated hypermutation of proviral genomes that could render the virus non-infectious (Sheehy et al., 2002; Sheehy et al., 2003).

Vpr is another virion-incorporated protein which has several functions both during delivery by the infecting virus particle and during post-integration viral protein production. Interestingly, Vpr appears to only provide a significant fitness advantage to HIV during macrophage infection and not in T cells. Nevertheless, Vpr and its homologs are highly conserved accessory proteins in all primate lentiviruses and thus have a non-dispensable role *in vivo*. Some of the many functions of Vpr that have been described include: promotion of nuclear importation of the pre-integration complex, G2 cell cycle arrest, and induction of apoptosis (Romani and Cohen, 2012). More recently, some have proposed that Vpr plays a role subverting the host cell DNA damage response to promote efficient proviral integration. Moreover, investigation of the importance of Vpr in macrophage infection by our lab has pointed towards a potential macrophage-specific restriction factor that is counteracted by Vpr and promotes efficient virus release and spread in T cell co-cultures (Collins et al., 2015; Mashiba et al., 2014).

Vpu is a unique accessory protein among lentiviruses whose main function is mediation of BST-2/tetherin degradation. As a result, Vpu Bst2/tetherin is unable to restrict virion release from infected cell surfaces and limit pathogenicity of infection (Neil et al., 2008; Van Damme et al., 2008).

Finally, Nef is a pleiotropic accessory protein that greatly increases pathogenicity of HIV infection. Three cellular targets of Nef are: SERINC5, cell surface CD4, and major histocompatibility class I (MHC-I). Nef-mediated exclusion of SERINC5 from newly forming virions promotes efficient subsequent infections (Usami et al., 2015) (Rosa et al.,

2015). Downmodulation of cell surface CD4 enables HIV to avoid CD4-mediated retention of virions at the cell surface through interactions with virion Env protein (Lama et al., 1999; Ross et al., 1999). Finally, downmodulation of MHC-I prevents infected cells from CTL recognition and promotes infected cell survival (Collins et al., 1998).

Regulation of HIV gene expression

HIV proviral gene expression is exquisitely regulated by host factors as well as viral regulatory proteins. In a transcriptionally favorable cellular environment, HIV often establishes an active infection with robust viral gene expression and post-transcriptional replication events (Lewinski et al., 2006). Alternatively, in unfavorable cellular environments, proviral gene transcription can be significantly suppressed resulting in a latent infection and a transcriptionally dormant provirus within the host genome. Importantly, HIV latency can be reversed, leading to robust active viral replication (Brooks et al., 2001; Zack et al., 1990). The critical elements in the process of HIV gene expression are the viral promoter and *cis*-acting elements of the 5' LTR. Binding of necessary transcription factors to the core promoter is sufficient to induce HIV transcription; however, the degree of transcription is dramatically affected by several factors and can dramatically change the outcome of any single infection event (Nabel and Baltimore, 1987b; Rittner et al., 1995). Below I describe some of the important processes and factors that regulate HIV gene expression.

Epigenetic regulation

Epigenetic modifications of HIV 5' LTR-associated nucleosomes, Nuc-0 and Nuc-1, lead to dramatic changes in HIV transcription efficiency and regulation of HIV latency. Nucleosome and chromatin structure around the HIV promoter is remarkably consistent. Between Nuc-0 and Nuc-1 exists the nucleosome-free HIV core promoter, a scaffolding for the assembly of the RNA transcription machinery (Verdin et al., 1993). In an inactive state, the HIV core promoter is the only accessible region of the LTR to transcription factors due to compact or 'closed' chromatin structure (Demarchi et al., 1993). This state leads to inefficient transcription initiation and latency. Methylation and deacetylation of histone tails by histone methyltransferases and histone deacetylases (HDACs), respectively, sustains chromatin compaction and HIV latency (Blazkova et al., 2009; Kauder et al., 2009; Pearson et al., 2008) (**Figure 1.4a**). Interestingly, of the 4 classes of mammalian HDACs, only class 1 HDACs (HDAC1, HDAC2, and HDAC3) are recruited to and modulate the HIV LTR (Keedy et al., 2009). On the other hand, histone acetyltransferases (HATs) acetylate histone tails, promote euchromatic structure around the 5' LTR and provide greater access for transcriptions factors to proviral enhancer sites (Steger et al., 1998; Struhl, 1996; Struhl and Moqtaderi, 1998) (**Figure 1.4b and Table 1.1**).

Tat-mediated transcription transactivation

HIV transcription occurs in two phases. Early gene expression is characterized by relatively low levels of HIV gene products due to obstruction of RNA Pol II by negative elongation factor (NELF) and inefficient HIV mRNA elongation (Narita et al., 2003;

Yamaguchi et al., 1999; Zhang et al., 2007). Therefore, early, multiply-spliced HIV RNAs slowly accumulate and are translated, including those encoding for HIV Tat. Once produced, Tat liberates positive transcription elongation factor b (P-TEFb) from its inhibitory complex of HEXIM 1 and 7SK RNA and recruits P-TEFb to the transactivation-responsive element (TAR) in nascent HIV mRNA. There, P-TEFb, phosphorylates the C-terminal domain of stalled RNA Pol II which releases the NELF-dependent suppression and dramatically increases the efficiency of HIV mRNA elongation and late gene transcription (Kim et al., 2002; Parada and Roeder, 1996). Therefore, the Tat-TAR axis is composed of several proteins and whose relative abundance and activity can potently regulate HIV gene transcription and latency (**Figure 1.5 and Table 1.1**).

Cellular factors that regulate HIV transcription

Several cellular transcription factors bind to the HIV promoter and regulate HIV transcription. Below I discuss some of the most important factors necessary for transcription initiation and elongation. Nuclear factor kappa B (NF-κB) is a family of five transcription factor monomers. Three monomers contain transcription activation domains (p65, c-Rel, and RelB), while the other two monomers (p50 and p52) serve as transcription inhibitors. NF-κB transcription factors exert their effects as dimers made up of combinations of the five monomers (Gilmore, 2006). All dimers have the potential to occupy NF-κB binding motifs within the HIV 5' LTR and the relative abundance of monomers determines which dimers will bind to and influence the promoter. Typically, the two most abundant monomers are p65 and p50 (Baldwin, 1996; Siebenlist et al., 1994).

Therefore, p50/p50 homodimers and p50/p65 heterodimers are the two dimers most responsible for HIV transcription suppression and enhancement, respectively. Often, cellular physiology determines the relative abundance of the uninhibited monomers available to bind to and affect HIV transcription. As T cell activation is associated with induction of NF-κB activity, activated, dividing T cells often have a greater abundance of p65-containing dimers available to bind to the HIV promoter (Ghosh et al., 1998; Nabel and Baltimore, 1987a; Thanos and Maniatis, 1995). On the other hand, quiescent T cells usually have relatively low levels of active p50/p65 dimers due to IκB-mediated inhibition. This regulation of NF-κB dimer activation involves a complex network of upstream factors with strict negative feedback regulation and can be affected by many diverse cellular stimuli (Stancovski and Baltimore, 1997). Another important HIV transcription factor is c-Myc, which associates with SP1 proteins bound to the HIV 5' LTR and recruits inhibitory HDACs to the HIV LTR (Jiang et al., 2007) (**Figure 1.4, Figure 1.5, and Table 1.1**).

As described above, following transcription initiation, RNA Pol II stalls shortly after the transcription start site and production of long mRNA transcripts is incredibly inefficient. The role of P-TEFb in removing this block is critical for potent viral gene expression and active infection. The mechanism of P-TEFb transactivation is summarized above. In addition to inhibition by the hetero complex of HEXIM 1 and 7SK RNA, P-TEFb can be sequestered in the cytoplasm and unavailable to transactivate HIV gene expression, by the bromodomain protein Brd4 (Bisgrove et al., 2007; Urano et al., 2008). If in abundance, Brd4 competitively inhibits Tat binding and recruitment of P-TEFb to the nascent HIV mRNA and can promote HIV latency (Bisgrove et al., 2007; Ott et al., 2011; Schroder et al., 2012) (**Figure 1.5 and Table 1.1**).

Finally, heat shock protein 90 (Hsp90) is a ubiquitous cellular chaperone protein. Hsp90 activity is necessary for the activation of NF-κB dimers and mediates assembly of P-TEFb subunits (Pan et al., 2016; Zhang and Burrows, 2004). Hsp90 activity is also responsible for the proper folding of many other factors also involved in HIV gene expression, including STAT5 (Xu et al., 2003). Therefore, the activity of Hsp90 is a key regulator of HIV gene transcription (**Table 1.1**).

Rev-dependent HIV mRNA nuclear export

Incompletely spliced RNA transcripts are typically degraded within the nucleus. However, HIV has evolved a mechanism to avoid this fate for its incompletely spliced late viral gene transcripts and unspliced RNA genome. HIV Rev interacts with the Rev-response element (RRE), a highly-conserved RNA structure contained within incompletely spliced HIV transcripts (Daly et al., 1989; Heaphy et al., 1990; Malim et al., 1989; Sodroski et al., 1986). Once bound by Rev, HIV mRNA is directed through the nuclear pore by canonical nuclear export processes. Thus, Rev is responsible for the nuclear export of incompletely processed, late HIV transcripts, which then can be translated into viral protein. As with the Tat-TAR axis, the Rev-RRE axis is critical for efficient active HIV infection and mutations to either Rev or the RRE can dramatically alter the course of infection (**Table 1.1**).

Persistent HIV infection and the HIV reservoir

As discussed above, cART efficiently suppresses HIV replication *in vivo* but does not eliminate all forms of the virus as HIV replication can rebound following interruption of cART. Two hypotheses have been proposed to explain this phenomenon.

The first hypothesis to explain the persistence of HIV despite cART is that low-level replication occurs even during therapy. It is proposed that; indeed, cART is effective but may not affect or reach all forms of the virus in sufficient quantity. In other words, there may be anatomic regions of low penetrance of antiretroviral agents where viral replication can occur. Evidence in support of this proposal includes: an increase of unintegrated HIV DNA when cART was intensified with the integrase inhibitor raltegravir, suggesting that new integration events were being blocked that would have otherwise gone on to cause new infections (Buzon et al., 2010); and evidence in several patients for residual plasma virus evolution, which would only be possible due to ongoing replication (Shiu et al., 2009; Tobin et al., 2005). Furthermore, one group, through mathematical modeling, has proposed that it is within lymphoid tissue where concentrations of antiretroviral drugs are insufficient, that ongoing, low-level HIV replication may occur (Lorenzo-Redondo et al., 2016). However, several other groups have not been able to repeat these results (Besson et al., 2012; Dinoso et al., 2009; Gandhi et al., 2010; Hatano et al., 2011). Thus, it is possible that in some patients, some forms of HIV evade potent antiretroviral therapy and can replicate at very low levels; however, it cannot account for HIV persistence in every case.

The second hypothesis to explain the ability of HIV to persist despite cART, proposes that a population of long-lived cells bearing latent HIV provirus establishes a reservoir of transcriptionally silent HIV that can be reactivated to produce new virions even

decades after initial infection. Several studies have demonstrated that indeed HIV sequences from cART-treated, infected people do not evolve but persist (Joos et al., 2008; Kieffer et al., 2004; Tobin et al., 2005). Below I describe our current understanding of the HIV reservoir.

The resting $CD4^+$ T cell HIV reservoir

Activated $CD4^+$ T cells are the most susceptible and primary cellular target of HIV. The cellular state of these cells; however, does not support latent HIV infection. As a result, most infected $CD4^+$ T cells bear actively replicating HIV that can induce viral cytopathicity and produce antigen that is presented and detected by circulating anti-HIV CTLs (Ho et al., 1995; Wei et al., 1995). A very small proportion of activated and actively infected $CD4^+$ T cells survive viral cytopathicity and immune responses and transition to a resting state, characterized by relative physiologic quiescence (Chun et al., 1995). It also appears that in some cases, HIV is able to directly infect resting $CD4^+$ ($rCD4^+$) T cells or $CD4^+$ T cells in the process of transitioning to a resting state (Cameron et al., 2010; Chavez et al., 2015; Pace et al., 2012; Shan et al., 2017; Vatakis et al., 2009). In these cases, infection is incredibly inefficient and the likelihood of host cell death is reduced. It is within this small population of infected $rCD4^+$ T cells that HIV latency is supported.

The contribution of $rCD4^+$ T cells to rebound and residual viremia is currently a topic of great interest to the field. Because instances of treatment failure or treatment interruption and rebound viremia are rare, many groups look to the residual viremia that can be detected by ultra-sensitive laboratory assays to investigate the contribution of $rCD4^+$ T cells to these circulating viral species. Interestingly, multiple studies have demonstrated

that residual plasma virus sequences can only infrequently be attributed to any detected rCD4$^+$ T cell reservoir (Bailey et al., 2006; Brennan et al., 2009; Chun et al., 2000; Sahu et al., 2009; Tobin et al., 2005). It has also been discovered that the CD4$^+$ T cell HIV reservoir rapidly accumulates defective proviral genomes during establishment of the reservoir, which is believed to be a result of frequent deleterious and inactivating recombination events that occur during reverse transcription (Bruner et al., 2016; Cohn et al., 2015). Thus, other cellular sources of persistent functional HIV may exist and also contribute to residual and rebound viremia. Emerging evidence suggests that macrophages may also form a durable reservoir of HIV (Arainga et al., 2017; Honeycutt et al., 2017). Although infection is incredibly inefficient in these cells, macrophages can be long-lived and appear to be less susceptible to viral cytopathicity compared to infected CD4$^+$ T cells (Collman et al., 1989; Ho et al., 1986).

Hematopoietic stem and progenitor cells as an HIV reservoir

Following birth, all human blood cells are progeny of bone marrow-derived hematopoietic stem and progenitor cells (HSPCs). Hematopoiesis is a tightly regulated and ordered process that sustains appropriate blood cell production according to physiological needs for the life of an individual. Therefore, hematopoietic stem cells and even the early progenitor cells are long-lived (Busch et al., 2015; Sun et al., 2014). Hematopoietic stem cells (HSCs) are the most primitive and multipotent sub-population and have the greatest capacity for self-renewal. In instances where they differentiate, HSCs can produce common myeloid progenitors (CMPs), which give rise to the myeloid lineage of mature cells, or common lymphoid progenitors (CLPs), which differentiate into all lymphoid lineages of

mature cell (Doulatov et al., 2012). These HSCs, CMPs, and CLPs, can reliably be distinguished from other blood cell types based on their surface antigens CD133 and CD34. More committed progenitors derived from CMPs and CLPs lose surface expression of CD133 (Berenson et al., 1991; Leary et al., 1985; Yin et al., 1997) (**Figure 1.6**).

Understanding the susceptibility of HSPCs to HIV infection *in vivo* has been a question of great interest since the start of the pandemic. Multiple studies have demonstrated that HSPCs do, indeed, express the cognate HIV receptor CD4 as well as the necessary co-receptors CCR5 and CXC4 (Alexaki and Wigdahl, 2008). Early attempts to determine whether HIV could infect HSPC *in vivo*; however, were plagued by inefficient cell purification methods, which could not adequately rule-out contamination by other cell types, including $CD4^+$ T cells (Folks et al., 1988; Neal et al., 1995; Stanley et al., 1992; Zauli et al., 1992). More recently, our group sought to determine whether HIV could infect HSPCs using flow cytometric and PCR assays capable of single cell and single genome resolution. Indeed, work from our group determined that HSPCs are susceptible to HIV infection and could support both active and latent infection *in vitro*, and that HSPCs from about half of clinically suppressed, HIV-positive people tested contained detectable HIV DNA (Carter et al., 2010). Shortly thereafter, our group again demonstrated that in about half of HIV-positive donors tested, HSPC-derived HIV proviral DNA could be detected (McNamara et al., 2013). In order to assuage any concerns of T cell contamination confounding these results, we also described a statistical test used to demonstrate that T cell contamination is an extremely unlikely explanation for the detection of HIV proviral DNA in our HSPC samples (McNamara et al., 2013). Other groups that have also investigated the HSPC reservoir were unable to detect HIV DNA from HSPCs (Josefsson

et al., 2012; Neal et al., 1995; Shen et al., 1999; Weichold et al., 1998). Importantly, most of these studies recruited a relatively small cohort of donors from whom they isolated HSPCs. Moreover, one of these studies used flow cytometric sorting to isolate a highly purified population of HSPCs, but excluded $CD4^+$ cells (Josefsson et al., 2012). Most recently, we demonstrated that it is the population of the $CD4^+$ HSPCs that are most susceptible to HIV infection *in vitro* (Sebastian et al., 2017). Therefore, it is likely that these studies were both underpowered and experimentally flawed in order to confidently state that HSPCs are not a source of persistent HIV provirus *in vivo*. Therefore, it is imperative to also investigate whether HSPCs constitute a clinically-relevant, persistent functional HIV reservoir that contribute to residual and rebound viremia.

Pharmacological reversal of HIV latency and elimination of the HIV reservoir

Latent HIV infection within long-lived cellular reservoirs permits infected cells to survive by avoiding viral cytopathicity or immune-mediated clearance. One approach to promote the reduction of the viral reservoir is to reverse HIV latency, induce robust viral gene expression, and promote reservoir cell death (Deeks, 2012). Therefore, in addition to comprehensively characterizing the HIV reservoir, it has been the focus of the field to develop effective latency-reversing agents (LRAs) that promote the significant reduction of the viral reservoir *in vivo* and achieve durable viral remission without the need for antiretroviral therapy.

Assessing LRA effectiveness

Definitive evidence of LRA efficacy is viral remission following cART interruption. Some studies have performed an analytical treatment interruption (ATI); however, testing and developing LRAs *in vivo* using ATIs is impractical and unsafe. Alternatively, HIV latency cell line models provide a convenient and robust system in which to test the efficacy of LRAs. Their relative lack of physiologic significance; however, has become ever-more apparent as results in cell lines have not successfully translated into *in vivo* success (Delagreverie et al., 2016; Spina et al., 2013; Spivak and Planelles, 2016). Quantification of proviral load reduction *in vivo* within HIV reservoirs has been proposed as another method to evaluate LRA effectiveness. Unfortunately, as discussed above, most proviruses within infected individuals are defective. The relevant, functional proviruses that would be the target of LRA-based strategies make up only about 2-12% of HIV proviral genomes found within $rCD4^+$ T cells (Bruner et al., 2016; Hiener et al., 2017; Ho et al., 2013). Therefore, even a significant reduction in the functional reservoir would be difficult to discern by quantification of proviral load. The gold-standard that has emerged for the analysis of LRA effectiveness has become the *ex vivo* viral outgrowth assay (VOA), which tests the ability of proposed chemical compounds to induce viral outgrowth from $rCD4^+$ T cells from optimally-treated, HIV-infected people (Bullen et al., 2014; Laird et al., 2015).

Efficacy within $rCD4^+$ T cells has been the main focus of LRA studies. While the $CD4^+$ T cell reservoir constitutes the largest pool of persistent HIV, as I discussed above, additional, non-$CD4^+$ T cells likely also contribute to the persistent HIV reservoir, including HSPCs. Interestingly, our group has shown that the mechanism of HIV latency

within HSPCs is distinct from that within $CD4^+$ T cells and active P-TEFb is not limiting in *in vitro*, latently-infected HSPCs (McNamara et al., 2012). Moreover, unlike in latently-infected $CD4^+$ T cells, NF-κB activation by TNF-α was sufficient to induce latency reversal in HSPCs (McNamara et al., 2012). Whether additional differences in the establishment, maintenance, and reversal of HIV latency in HSPCs and $CD4^+$ T cell exist is unknown. Therefore, proposed LRAs must also be able to reactivate latent provirus within diverse cell types in order to significantly reduce all forms of persistent HIV.

T cell activation

Several classes of LRAs have been described. The earliest studies aimed at reversing HIV latency originated from the observation that HIV gene expression was correlated with T cell activation (Perelson et al., 1997; Zack et al., 1991). Therefore, two early clinical trials attempted to induce viral reactivation *in vivo* by co-treatment with anti-CD3 antibody and interleukin-2 (IL-2). Unfortunately, these treatments led to significant adverse events in study participants with no obvious change in the viral reservoir (Kulkosky et al., 2002; Prins et al., 1999). Therefore, general T cell activation is an unsafe and impractical method to induce viral reactivation. Affecting distinct pathways involved in T cell activation; however, may provide sufficient stimuli to reverse HIV latency while avoiding generalized T cell activation and toxicity. Protein kinase C (PKC) agonists have emerged as a potentially useful LRA that promote potent NF-κB activation (Jiang and Dandekar, 2015). Phorbol esters, including prostratin, were the first selective PKC agonists to observed to reverse HIV latency, followed by bryostatin-1 (DeChristopher et al., 2012; Kinter et al., 1990; Kulkosky et al., 2001; Mehla et al., 2010). Both prostratin and

bryostatin-1 have demonstrated moderate HIV latency reversal even in *ex vivo* model system; however, supratherapeutic levels are necessary to induce potent viral reactivation (Bullen et al., 2014; Kulkosky et al., 2004; Laird et al., 2015; Williams et al., 2004) (**Table 1.2**). Nevertheless, these proof-of-principle discoveries demonstrate that HIV latency-reversal can be achieved without complete T cell activation.

Targeting specific cellular pathways to reverse HIV latency

In addition to T cell activation, several screens of chemical libraries have generated evidence in support of LRAs that reverse HIV latency through novel mechanisms. First, the FDA-approved inhibitor of acetaldehyde dehydrogenase, disulfiram, was discovered to reactivate HIV latency in a highly-manipulated, *in vitro* primary $CD4^+$ T cell model of HIV latency (Xing et al., 2011). Further investigation discovered that disulfiram reversed HIV latency by depleting cellular phosphatase and tensing homolog (PTEN), thereby activating the Akt-NF-κB pathway (Doyon et al., 2013). In later studies; however, disulfiram was shown to not be effective *ex vivo* or *in vivo* (Elliott et al., 2015; Spivak et al., 2014). Second, the non-canonical NF-κB pathway is involved in very few cellular processes and is further characterized by a relatively slow onset and prolonged transcriptional effect compared to the canonical pathway (Sun, 2011). The ability of non-canonical NF-κB dimers to transactivate HIV gene transcription is a relatively understudied topic. Nevertheless, it was recently demonstrated that Smac mimetics that activate the non-canonical pathway were able to modestly reverse HIV latency from cell lines and patient-derived cells (Pache et al., 2015). Third, a previously underappreciated role for STAT5 in HIV latency reversal was discovered when benzotriazole derivatives demonstrated potent LRA activity (Bosque et

al., 2017). Fourth, hexamethylene bisacetamide (HMBA) and the BRD4 inhibitor JQ1 have been shown to reverse HIV latency *in vitro* by promoting P-TEFb activation (Antoni et al., 1994; Li et al., 2013; Vlach and Pitha, 1993). Again; however, when tested in more relevant *ex vivo* models, both compounds fail to induce viral outgrowth (Bullen et al., 2014; Darcis et al., 2015; Laird et al., 2015). Finally, one of the previously most promising category of novel LRAs were the histone deacetylase inhibitors (HDIs). These epigenetic modifying agents, as discussed above, can potently affect histone acetylation and promote HIV transcription (Archin et al., 2009; Rasmussen et al., 2013; Wei et al., 2014). Moreover, the HDIs tested as LRAs thus far are well-characterized and have been extensively tested *in vivo* for the treatment of several malignancies (Barbarotta and Hurley, 2015; Mann et al., 2007). Therefore, HDIs represent a clinically safe option for the reversal of HIV latency based on initial *in vitro* screens. Once again; however, the *in vitro* successes of the HDIs vorinostat, romidepsin, and panobinostat, have not translated into predictable or positive *in vivo* or even *ex vivo* results (Archin et al., 2012; Bullen et al., 2014; Elliott et al., 2014; Laird et al., 2015; Rasmussen et al., 2014; Sogaard et al., 2015). In fact, several studies have now shown that these HDIs are either immune-toxic or dramatically suppress CTL and NK cell immune function (Garrido et al., 2016; Jones et al., 2014; Pace et al., 2016). Thus far, the best-characterized HDIs have been those that affect more than 1 of the 4 classes of HDAC isoforms (pan-HDI). However, as discussed above, only class 1 HDAC isoforms have been shown to suppress HIV gene expression. Class 1-selective HDIs have demonstrated modest effects *in vitro,* but have not been thoroughly characterized beyond these initial screens (Bui et al., 2017; Savarino et al., 2009; Wightman et al., 2012). Whether an even more selective and targeted approach to HDI-mediated HIV latency-

reversal would be more efficient has largely been ignored in favor of ease of clinical translation (**Table 1.2**).

Combinations of mechanistically distinct LRAs to achieve maximum latency reversal

Development of a single effective LRA may not be a practical approach to efficient *in vivo* HIV latency reversal and reservoir reduction. Therefore, combinations of mechanistically distinct LRAs have been the focus of many recent studies. Moreover, pharmacologic synergy between LRAs may promote robust latency reversal and allow for lower doses of drugs to be administered, which may limit significant toxicity. In the several studies performed using patient-derived rCD4$^+$ T cells, investigators have focused on combinations of bryostatin-1 plus other mechanistically distinct LRAs (Bullen et al., 2014; Darcis et al., 2015; Laird et al., 2015; Martinez-Bonet et al., 2015). This is a rational approach given the availability of activating transcription factors that is necessary even for basal HIV gene transcription. Unfortunately, evidence of synergism between LRAs is lacking. Combinations of bryostatin-1 plus pan-HDIs or JQ1 have demonstrated additive effects on the amount of HIV transcription; however, no dramatic enhancement of viral outgrowth has been observed (Darcis et al., 2015; Laird et al., 2015). Fortunately, studies that assess LRA combinations are relatively recent and therefore still being developed. Moreover, because many of the initial LRAs identified are FDA-approved for treatment of other diseases, it has been possible to quickly translate modest *in vitro* efficacy into *in vivo* studies. Several clinical trials that test the effects of LRA combination on the *in* vivo HIV reservoir are ongoing or have just completed data collection (Delagreverie et al., 2016).

Summary

HIV replication is potently suppressed in infected people by cART. Unfortunately, HIV can persist in these individuals even after several years of continuous cART and may cause relapse of viremia and HIV disease. The presence of a persistent reservoir of long-lived, latently infected cells is a major barrier to affording a cure and improving HIV infection therapy. Therefore, it is necessary to better understand the HIV reservoir *in vivo* and to develop effective strategies that will reduce the persistent viral burden and lead to durable viral remission.

Resting $CD4^+$ T cells make up the largest and best-characterized reservoir of persistent HIV. However, not all residual and rebound plasma virus can be accounted for by the $rCD4^+$ T cell reservoir. Therefore, it is highly likely that other cell types contribute to the total HIV reservoir *in vivo*. Our group has presented evidence that HSPCs are an additional reservoir of HIV. We have demonstrated that HSPCs are susceptible to HIV infection both *in vitro* and *in vivo* (Carter et al., 2010; McNamara et al., 2013; Sebastian et al., 2017). We have also demonstrated that HSPCs can support HIV latency *in vitro* that is uniquely susceptible to latency reversal compared to HIV latency established in $rCD4^+$ T cells (McNamara et al., 2012). Finally, we have provided definitive evidence that infected HSPCs can maintain and propagate an integrated proviral genome to its progeny *in vivo* (Sebastian et al., 2017). The contribution of the HSPC-derived HIV reservoir to residual viremia; however, remains unknown. I address this point in Chapter 2.

The failure of an effective LRA regimen is a significant limitation of the field aimed at discovering a strategy to reduce the viral reservoir *in vivo*. HDIs are attractive LRA candidates, especially in combinations with other compounds that promote key

transcription factor activation. A focus on pan-HDIs; however, may be a major oversight in rational LRA selection and regimen design. Extensive evidence has been presented to confidently conclude that only class 1 HDAC isoforms contribute to HIV latency. Moreover, the negative effects of pan-HDIs on immune cell viability and function is a critical limitation for the use of these compounds in reservoir elimination strategies. Whether additional negative effects are elicited by pan-HDIs on other important physiological processes relevant to both potent viral reactivation and elimination of HIV reservoirs is unknown. Therefore, I propose that class 1-selective HDIs are superior to pan-HDIs with respect to potent viral reactivation and avoidance deleterious effects caused by inhibition of non-class 1 HDACs. I address these points in Chapter 3.

Together the work presented in this dissertation represents a significant contribution to the HIV cure field. We describe a novel and unique long-lived reservoir of HIV within HSPCs. Furthermore, our discovery of the benefit of class 1-selective HDIs compared to pan-HDIs is an important step towards a more rational and effective HIV latency-reversal regimen. By these two discoveries, I hope that this work will inspire future studies aimed at reducing the burden of persistent HIV and achieve a cure for this significant human pandemic.

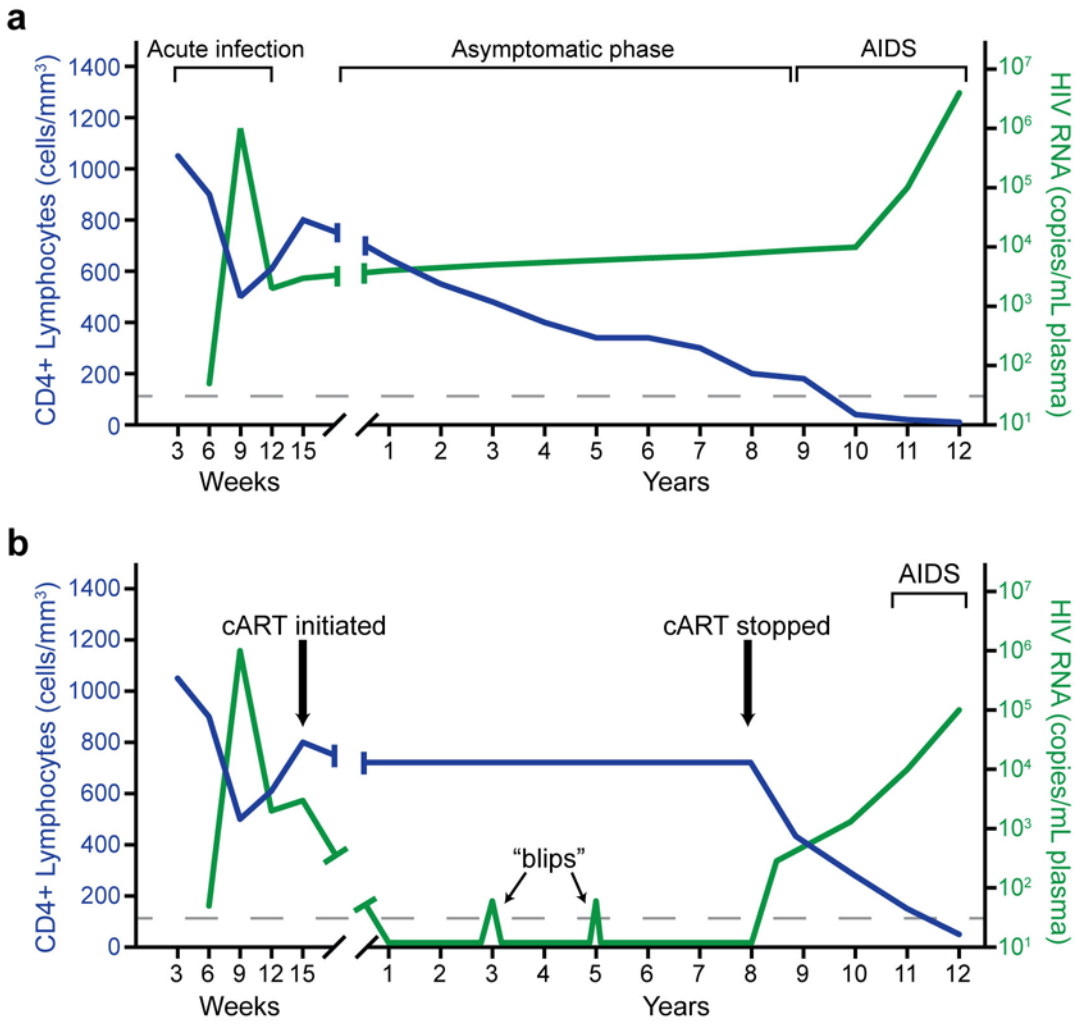

Figure 1.1. Clinical progression of untreated and treated HIV infection. Diagrammatic representation of the clinical course of HIV infection in terms of $CD4^+$ T cell count (left y-axis) and HIV plasma load (right y-axis). (**a**) Course of HIV infection in an untreated individual. (**b**) Course of infection in an individual treated with cART. Gray horizontal dashed line indicates the limit of clinical detection of plasma HIV RNA. Adapted from (Pantaleo et al., 1993).

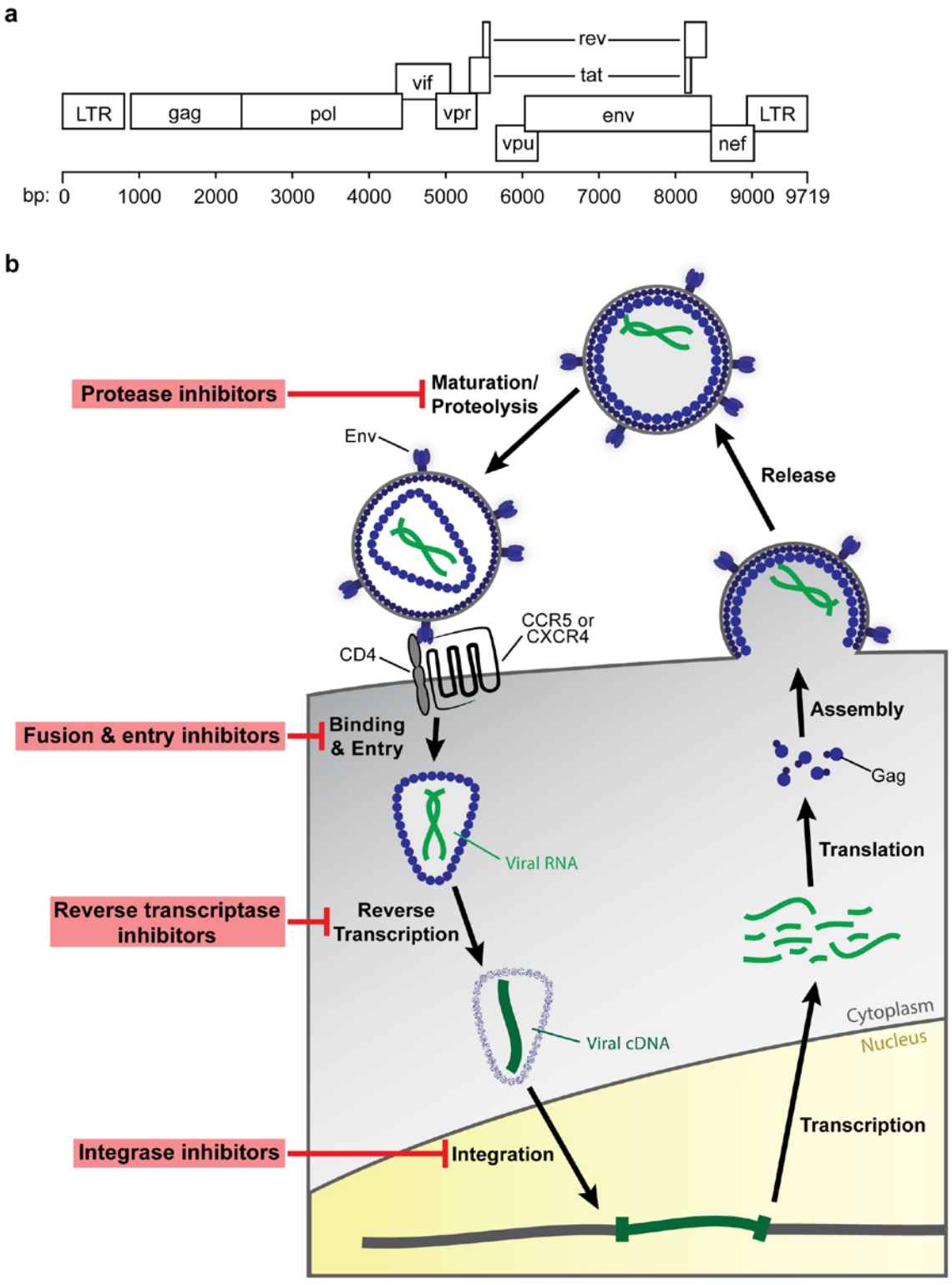

Figure 1.2. HIV genome and replication cycle. (**a**) Schematic of the HIV genome. (**b**) Diagram of the major steps in the HIV replication cycle. Classes of current antiretroviral drugs are shown in red boxed text on the left.

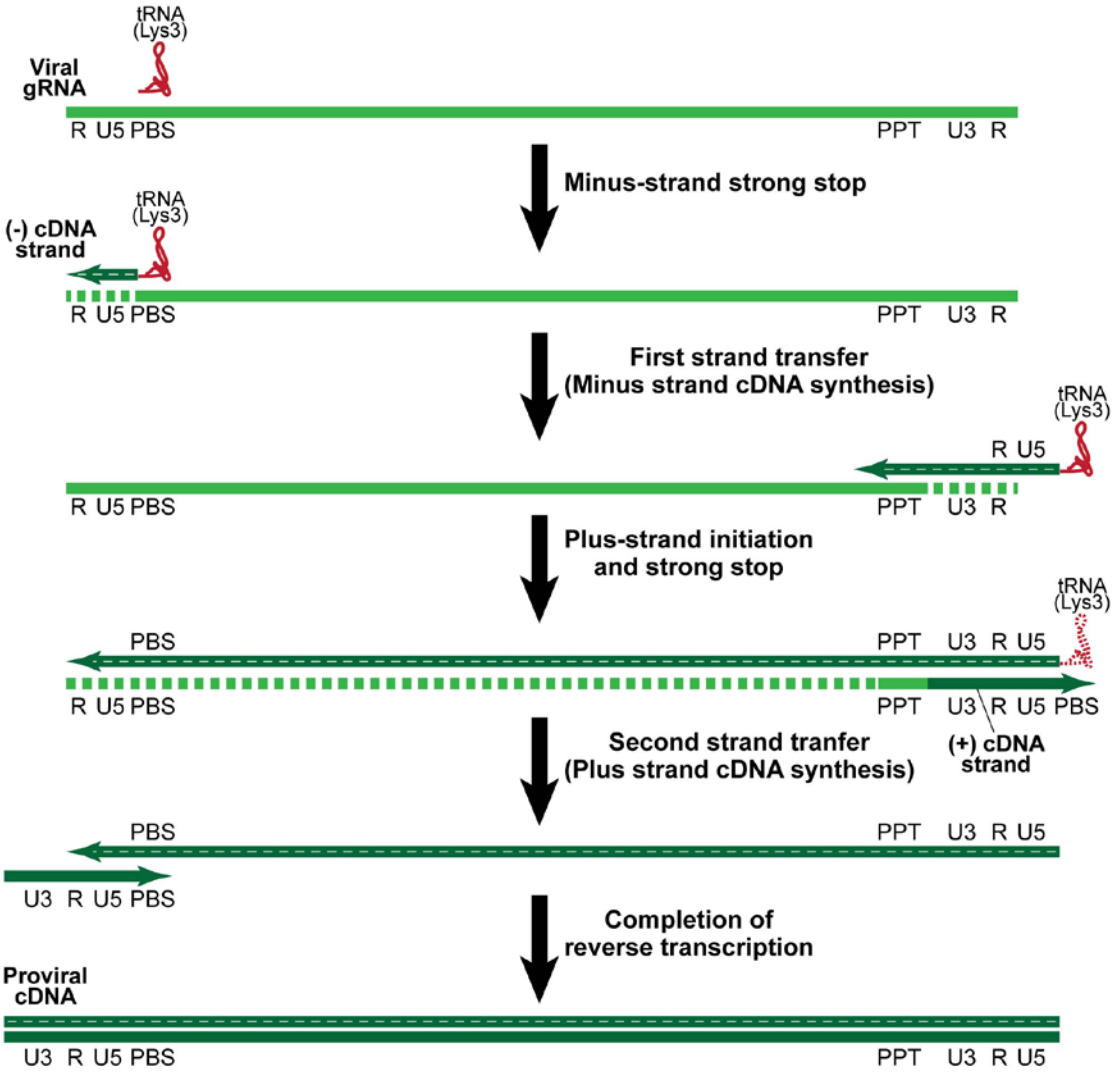

Figure 1.3. Summary of HIV reverse transcription. Schematic representation of the steps of HIV reverse transcription. Dotted lines indicate molecules digested by RNaseH. PBS, primer binding site; PPT, polypurine tract.

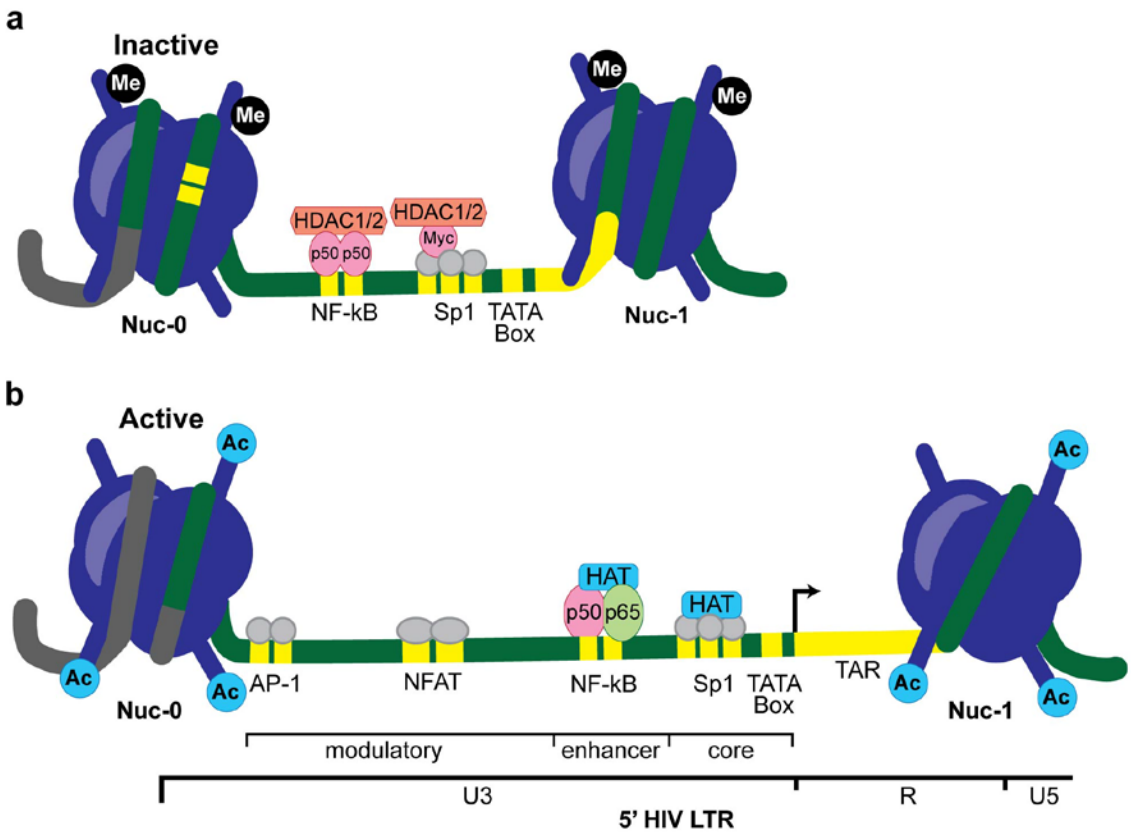

Figure 1.4. Epigenetic regulation of the HIV promoter. The HIV 5' LTR is associated with two key nucleosomes, Nuc-0 and Nuc-1. (a) In a closed chromatin structure, only the core promoter region of the 5' LTR remains accessible to transcription factors. Often HIV latency is concomitant with a cellular environment that does not provide activating transcription factors necessary to induce basal viral gene transcription. Instead, the inhibitory p50/p50 homodimer occupies NF-kB binding motifs of the promoter and recruits HDACs that promote and maintain heterochromatic structure. Histone methylation also promotes 'closed' chromatin structure. (b) Activation of HIV gene expression is enhanced when the chromatin structure is 'open'. Shown here, the activating p65/p50 heterodimer binds to the NF-kB binding site within the HIV promoter and recruits HATs that promote histone tail acetylation and euchromatic structure. This allows for additional transcription factors to bind to upstream enhancer and modulatory regions on the 5' LTR, affecting basal HIV gene transcription.

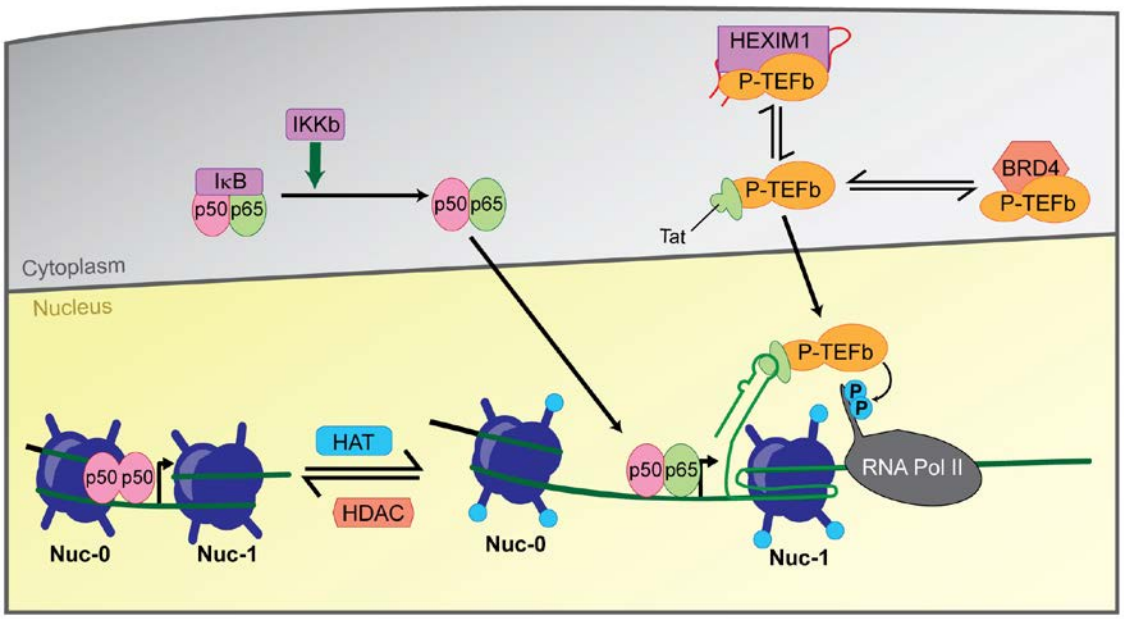

Figure 1.5. Summary of mechanisms that regulate HIV gene transcription. HIV gene expression is regulated by several host and viral factors, some of which are summarized in this cartoon representation of HIV gene expression regulation. Latent, or transcriptionally silent, HIV provirus (left) is sustained by the activity of HDACs and a lack of activating cellular transcription factors. Activation of the NF-κB pathway, leads to the IKKb-dependent release of the activating p50/p65 NF-κB heterodimer from its inhibitory IκB complex. Furthermore, activated and available P-TEFb is recruited to the HIV LTR by HIV Tat to transactivate HIV gene expression by eliminating RNA Pol II stalling soon after the transcription start site. The bromodomain protein, BRD4, is a competitive inhibitor of Tat as it too can bind and sequester P-TEFb away from the HVI LTR.

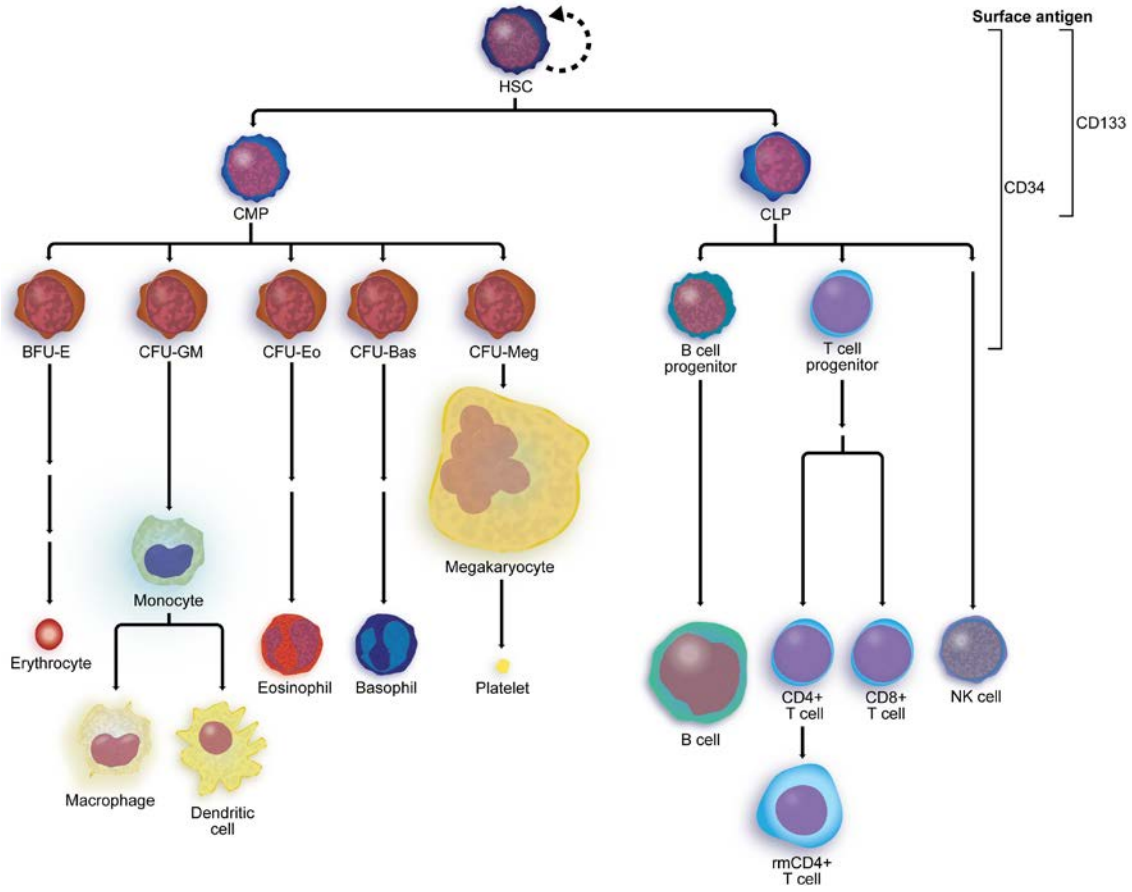

Figure 1.6. Schematic of hematopoiesis. Hematopoietic stem cells (HSCs) are the origin of all blood cells in the body. In addition to their multipotent potential, HSCs also have the ability to self-renew. HSCs can differentiate into a common myeloid progenitor (CMP) or common lymphoid progenitor (CLP), which still have multipotent potential, but are restricted to the myeloid and lymphoid lineages, respectively. CMPs and CLPs give rise to additional progenitor cells that are even more committed to specific cell types. Hematopoietic stem and progenitor cells (HSPCs) include all cells to this stage and can be distinguished by their cell surface markers CD133 and CD34. More immature HSPCs, including HSCs, express CD133 but not CD34. Mature blood cells do not express either CD133 or CD34.

Table 1.1. Processes and factors that regulate HIV gene transcription.

	Factor	Mechanism of regulation	Effect on HIV gene expression
Epigenetic regulation	Histone Acetyltransferase (HAT)	Chromatin remodeling; "open" chromatin structure	Enhance
	Histone deacetylase (HDAC)	Chromatin remodeling; "closed" chromatin structure	Suppress
	Lysine methyltransferase	Chromatin remodeling; "closed" chromatin structure	Suppress
Regulatory viral proteins	Tat	Recruitment of P-TEFb complex (see below)	Enhance
	Rev	Post-transcriptional; Necessary for nuclear export of incompletely spliced HIV RNA transcripts	Enhance
Regulatory cellular proteins	p65 homo-/ hetero-dimer (NF-κB)	Recruitment HATs and other factors that promote transcription	Enhance
	p50 homodimer (NF-κB)	Recruitment of HDACs	Suppress
	Negative elongation factor (NELF)	Sterically hinders RNA Pol II close to the promoter and leads to inefficient mRNA elongation	Suppress
	P-TEFb	Phosphorylates C-terminal domain of stalled RNA Pol II leading to transcription elongation	Enhance
	BRD4	Binds and sequesters P-TEFb away from the HIV LTR	Suppress
	c-Myc	Recruits HDACs to Sp-1 promoter sites	Suppress
	Hsp90	Necessary for the assembly or activation of P-TEFb and NF-κB	Enhance

Table 1.2. Summary of several classes of latency-reversing agents.

Mechanistic Classes		LRA	Summary of effects
NF-κB activator	**PKC agonist**	Prostratin	- Modest induction of *ex vivo* viral outgrowth - Induces potent, global T cell activation; not tested *in vivo*
		Bryostatin-1	- Modest *ex vivo* viral outgrowth induction - Too toxic to test *in vivo*
	PTEN inhibition	Disulfiram	- Modest increase in HIV transcription *in vivo*, but no outgrowth or reduction of reservoir size - No global T cell activation detected
Non-canonical NF-κB activator		Smac mimetics	- Reactivates HIV in cell lines - Modest induction of *ex vivo* viral outgrowth - No T cell activation detected
STAT5 activator		Benzotriazole derivatives	- Reactivates HIV in primary cell model and induces *ex vivo* viral outgrowth only with co-treatment of IL-2 - No T cell activation detected
P-TEFb activator	**PI3K/Akt pathway activation**	HMBA	- Not tested *in vivo*
	BRD4 inhibition	JQ-1	- Modest induction of *ex vivo* viral outgrowth
Histone deacetylase inhibitor (HDI)	**Pan-HDI**	Vorinostat	- Induction of HIV gene transcription, but not *ex vivo* viral outgrowth
		Romidepsin	- Induction of HIV transcription, but little (romidepsin) to no induction (vorinostat and panobinostat) of viral outgrowth, and no reduction of HIV reservoir *in vivo*
		Panobinostat	
	Class 1-selective HDI	Entinostat	- No *ex vivo* or *in vivo* studies performed

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Chapter 2

Hematopoietic stem and progenitor cells are a unique HIV reservoir that contribute to persistent viremia in suppressed patients[1]

Abstract

Long-lived reservoirs of HIV are a major barrier to a cure. Thus, it is important to develop a better understanding of the cell types that contribute to virus production in HIV-infected people on anti-retroviral therapy. $CD4^+$ hematopoietic stem and progenitor cells (HSPCs) have the capacity for life-long survival, self-renewal, and are susceptible to HIV infection *in vitro* and *in vivo*. Whether HSPCs harbor infectious virus or contribute to plasma viremia is unknown. Here, we investigate this question in 24 optimally-treated, HIV-infected people. Single-genome PCR products from highly purified bone marrow-

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derived HSPCs and residual plasma virus were directly sequenced to assess the extent to which HSPCs harbored proviral genomes that matched plasma virus. For comparison, we also obtained proviral genome sequences from peripheral blood and bone marrow mononuclear cells. Remarkably, residual plasma virus sequences often matched clusters of HSPC-associated identical proviruses (CHIPs). One CHIP that matched multiple copies of clonal plasma virus had a deletion in the primer binding site. Loss of this element is predicted to allow viral transcription and virion production, but the packaged genome would not be competent for reverse transcription. Thus, this CHIP was likely generated by cellular proliferation of an infected progenitor rather than by coincidental infection of multiple cells by genetically similar viruses. We also found that HSPCs make an important contribution to plasma virus; a higher proportion of CHIPs matched residual plasma virus than proviral genomes from bone marrow and peripheral blood mononuclear cells that did not form CHIPs. Furthermore, an analysis of near full-length genomes isolated from HSPCs provided evidence that HSPCs can harbor functional HIV proviral genomes with open reading frames that often match plasma virus. These results support the conclusion that HIV-infected HSPCs form a distinct and functionally significant reservoir of persistent HIV in infected people.

Introduction

Combination anti-retroviral therapy (cART) efficiently blocks HIV spread in vivo. However, the virus is able to persist within infected individuals and rebound viremia frequently occurs if cART is interrupted (Davey et al., 1999). Failure of cART is thought to result from persistent long-lived cells that harbor integrated HIV genomes that are

unaffected by anti-retroviral therapy. Resting memory $CD4^+$ T cells are a well-characterized HIV reservoir (Chun et al., 1997; Finzi et al., 1999; Finzi et al., 1997). In addition, emerging data support the possibility that non-$CD4^+$ T cells may also form a persistent HIV reservoir (Arainga et al., 2017; Avalos et al., 2016; Honeycutt et al., 2017; Zhu et al., 2002). In particular, hematopoietic stem and progenitor cells (HSPCs) are a long-lived cell type that has been shown to be infected in vivo and capable of propagating integrated provirus to $CD4^+$ and $CD4^-$ progeny (Carter et al., 2010; Sebastian et al., 2017). Moreover, HSPCs are capable of producing HIV virions upon latency-reversal *in vitro* (Zaikos et al., 2018). Therefore, it is possible that HSPCs could contribute to persistent and rebound viremia directly or by serving as a source of infected daughter cells that can be activated to produce virus.

Residual plasma virus (PV) can be detected in treated people by ultra-sensitive techniques even when virus is undetectable by standard clinical tests. Sequence analysis of residual PV and rebounding PV in HIV-infected people indicates that virions likely come from the activation of latent provirus that had been archived since before the initiation of therapy rather than from low-level replication and spread of cART-resistant virus (Eisele and Siliciano, 2012; Kearney et al., 2014).

The cellular source of residual virus remains poorly understood. A number of studies have shown that proviral DNA from $CD4^+$ T cells and other peripheral blood mononuclear cells (PBMCs) frequently does not match residual PV (Bailey et al., 2006; Brennan et al., 2009; Buzon et al., 2014; Chun et al., 2000; Sahu et al., 2009). One small study of two donors found that virus induced *ex vivo* from a subset of activated T cells matched PV, leading the authors to conclude that it is possible that residual PV may

originate from a minor population of circulating $CD4^+$ T cells (Anderson et al., 2011). Whether HSPCs contribute to PV is unknown.

Genetic characterization of provirus from $CD4^+$ T cell has demonstrated that many are defective and that defective proviruses rapidly accumulate (Bruner et al., 2016; Hiener et al., 2017; Imamichi et al., 2016). Analyses of near full-length proviral genomes indicate that 2-12% of $CD4^+$ T cell-derived HIV proviral genomes have full open reading frames (Bruner et al., 2016; Hiener et al., 2017; Ho et al., 2013). Whether provirus from HPSCs is defective has not previously been examined.

A determination that proviral genomes from HSPCs contain open reading frames that match plasma virus would provide evidence that HSPC-associated genomes are indeed functional. Here, we examine provirus and plasma virus from HSPCs and other cell types from a cohort of 24 donors, providing evidence in support of this conclusion.

Results

Single genome amplification (SGA) of proviral DNA from HSPCs and other cellular subsets

Figure 2.1 depicts a summary of the donors who were recruited and included in this study. Of the 53 recruited donors, ten were excluded for insufficient sample size or suboptimal HSPC sort purity. We excluded HSPCs samples that were <80% positive for hematopoietic cell markers ($CD133^+$ or $CD34^+$) or >1% positive for $CD3^+$ cells. In most cases, samples were well within our inclusion criteria with mean values of 90 - 94% $CD133^+$ or $CD34^+$ purity and mean $CD3^+$ T cell contamination rates of 0.2 - 0.3% (**Table**

2.1). Of the 43 donors included in the study (**Table 2.2**), 10 contributed samples more than once over periods spanning 4 months to nearly 5 years.

To determine whether HSPCs serve as a source of PV, we amplified individual HIV proviral genome sequences from highly purified HSPCs, HSPC-depleted bone marrow mononuclear cells (BMMCs), and PBMCs. For this analysis, we used a highly sensitive multiplex PCR protocol that was performed at limiting dilution to amplify individual genomes (Sebastian et al., 2017). Based on the sort purity and the frequency of HIV provirus in $CD3^+$ samples, we performed a statistical analysis to determine the likelihood that provirus was amplified from a contaminating cell type rather than an HSPC (McNamara et al., 2013; Sebastian et al., 2017). Only samples that yielded HIV proviral DNA that was statistically not likely due to T cell contamination were included in our final analyses.

Additionally, all viral sequences were compared phylogenetically to every previously acquired sequence and lab strain to rule out cross contamination. All included donor sequences clustered appropriately except for outliers which contained frameshifts.

We determined that 25 of the 43 included donors had detectable provirus in HSPC DNA. Sort purity and $CD3^+$ T cell contamination levels were very similar between the donors who had detectable HIV provirus in HSPC DNA and those that did not (**Table 2.1**).

Isolation and characterization of residual plasma virus

For each donor, we also harvested plasma from which we isolated PV. Purified viral RNA was converted to cDNA and HIV sequences were amplified using the multiplex PCR described above. We successfully detected PV from 24 out of 25 donors who had

provirus isolated from HSPC DNA (**Figure 2.1**), including donor 503501 who initiated cART in acute phase.

To identify potential cellular sources of PV, we compared all PV sequences to all proviral DNA sequences. The total number of sequences from each source is summarized in **Table 2.3**. As expected, some of the PV matched provirus from PBMC and BMMC, a large percentage of which are T cells. However, phylogenetic analysis revealed that proviral sequences isolated from highly purified HSPCs exactly matched amplified residual PV in 8 of 24 donors (**Figure 2.2Figure** 2.9). Higher mean CD4 counts at the time of sampling and number of PV amplicons recovered per donor were the only clinical and experimental parameters which significantly differed between these 8 donors and the 16 without matching HSPC and PV sequences (**Table 2.4 and Table 2.5**).

Sequence identity across entire amplicons is suggestive that PV was derived from the matching proviral genome. More precisely, the likelihood that sequences identical over the C2-V3 *env* region represent identity over the entire HIV genome can be estimated at 83% (Laskey et al., 2016). In some cases, the multiplex SGA PCR simultaneously generated an 828 bp fragment from the 5' LTR into the *gag* open reading frame as well as 429 bp of *env* C2-V3 allowing us to link two amplicons to the same genome (**Figure 2.2, Figure 2.4, Figure 2.5, Figure 2.6, and Figure 2.8**). When matching was achieved at both locations, the likelihood that the genomes were clonal is even higher. Thus, our findings of sequence identity across multiple locations within the HIV proviral genome, strongly support the conclusion that matching PV sequences were derived from the matching proviral genome.

Evidence that residual plasma virus is derived from clusters of HSPC-associated identical proviral genomes (CHIPs)

It was relatively common in our cohort for PV to exactly match proviral sequences from HSPCs; across the 24 donors analyzed, when unique sequences were compared, a higher proportion of proviral genomes from HSPCs matched PV (9% of *gag* and 13% of *env* amplicons) than genomes derived from PBMC or BMMC (3% *gag* and 5% *env*; $p<0.05$; **Figure 2.10a**). All of the HSPC-associated proviral genome sequences that matched PV also matched identical clusters of proviral genomes from PBMCs or BMMCs (**Figure 2.10b**). When these matching sequences include at least four proviral amplicons from non-HSPC cells, we refer to them as clusters of HSPC-associated identical proviruses (CHIPs).

The impact of CHIPs is apparent when comparing **Figure 2.10a** (unique sequences only) to **Figure 2.10c** (all sequences). Because CHIPs commonly match PV, when all identical copies of provirus from BMMCs and PBMCs were included in the analysis, significant differences observed in **Figure 2.10a** were lost. This result is consistent with a model in which latent proviral genomes in HSPCs expand and differentiate to produce CHIPs. Remarkably, approximately 40-50% of CHIPS could be matched to PV (**Figure 2.10b**).

Evidence that virions matching CHIPs are often predominant in the plasma

Five donors, (434423, 436000, 449000, 454304, and 458311) had large groups of identical PV sequences that qualify as predominant plasma clones (PPCs) (Bailey et al., 2006). For two donors, these sequences matched CHIPs (**Figure 2.11**). In the remaining

three donors, no cellular source of PPCs were identified. Thus, CHIPs account for PPCs in at least a subset of donors.

A signature deletion found in both a CHIP and a PPC supports HSPCs as an original source of viremia

While exact matches over *gag* and *env* C2-V3 regions provide strong evidence that genomes are clonal, it remains theoretically possible that sequence differences are present elsewhere in the genome. This is an important consideration as infection by genetically similar viruses could mimic clonality that arises by cellular proliferation and expansion of genetically identical viruses. We were able to exclude coincidental infection as an explanation in one donor (**Figure 2.2**). In this donor, proviral genomes from multiple cellular sources, including HSPCs, contained a signature deletion. This deletion, which likely occurred at the time of reverse transcription, eliminated the tRNA(Lys3) primer binding site (*pbs*), the dimerization initiation site and the first two packaging stem loops. Due to the loss of the *pbs*, virus generated from this proviral genome cannot initiate reverse transcription and is thus noninfectious (**Figure 2.2a**). However, the defects harbored by the provirus are not expected to block transcription or packaging of the RNA genome as SL3 is sufficient for RNA genome packaging (Abbink and Berkhout, 2008). Consistent with this, we identified 23 clonal copies of PV harboring the deletion (**Figure 2.2**). The spread of this noninfectious provirus amongst multiple cells most likely occurred by cellular proliferation originating from an infected progenitor cell. Each of the infected daughter cells and/or the originally infected progenitor would be competent to produce noninfectious virions containing the defective viral genome.

HSPCs harbor intact, near full-length HIV genomes

In addition to the multiplex SGA, we sought to amplify near full-length HIV genomes from HSPCs of 28 donors. For this assay, we used first-round PCR primers within the viral LTRs and screened these products for near-full length genomes using primers that amplify a ~400 bp *env* fragment in a second-round reaction. Positive first round reactions were amplified with internal primers and sequenced to determine whether or not each individual near full-length genome was functional. In all, we identified 22 near full-length genomes from 10 donors (**Figure 2.12**). Twenty amplicons from 9 donors were statistically highly unlikely to have been due to T cell contamination of our HSPC samples. We obtained PCR products sufficient to assess the integrity of 18 HIV genomes and fully sequenced 9 (**Figure 2.13a**). Five of 18 genomes, or 28%, had open reading frames for all 9 HIV genes (**Figure 2.13b**). The near full-length genomes contained intact *cis* elements (based on 3'LTR sequencing). One proviral genome from donor 409000 was missing the tRNA(Lys3) pbs (**Figure 2.13a**).

Strikingly, of the 5 intact genomes, 3 (60%) exactly matched PV sequences as well as proviral genomes from PBMC and BMMC. Donor 409 produced 2 intact genomes, one of which had identity to a PV *env* amplicon (**Figure 2.13a and Figure 2.6**). From donor 421, we found an HSPC-derived near full-length sequence that was also identical to a PV *env* amplicon (**Figure 2.13a and Figure 2.7**). Lastly, both *env* and *gag* regions from the near full-length genome isolated from donor 454 HSPC were identical to PV from this donor (**Figure 2.13a and Figure 2.4**). These intact genomes account for 10-14% of unique plasma viruses found in these donors. Moreover, sequence identify to proviral genomes

from PBMCs and BMMCs provides evidence that these genomes can clonally expand *in vivo* (**Figure 2.13**).

Evidence that virions matching HSPC proviral genomes are persistently present in plasma

Three of the 8 donors with identical PV and HSPC-derived viral sequences provided tissue samples on more than one occasion (435412406, 454304, 434423). For each of these donors, we were able to demonstrate persistent matching between PV and HSPC proviral genomes over time.

Donor 435412406 contributed blood and bone marrow three times over 10 months. and we were able to detect unique proviral genomes in HSPCs from all three donations. Of note, a CHIP from the first donation matched sequences found in PV from the third donation. Conversely, a CHIP from the third donation was identical to PV from the first donation **(Figure 2.3 and Table 2.4**).

Donor 454304 had been suppressed for 3.3 years at the time of the first donation and provided tissue again almost 4 years later. Described as donor 304000 in an earlier study, HSPCs from this donor's first donation had detectable HIV provirus by a *gag* qPCR assay (McNamara et al., 2013). Using our multiplex SGA, we isolated PV from both donations in which the *env* amplicon was identical to a near full-length genome from an HSPC isolated from the second donation (**Figure 2.2, Figure 2.6, and Table 2.4**). A *gag* amplicon derived from plasma from the second donation also exactly matched this near full-length genome.

Donor 434423 provided blood and bone marrow twice over 4 months. A CHIP from the second donation, 434, matched large clonal clusters of PV for both *gag* and *env*

amplicons (**Figure 2.5 and Table 2.4**). Among the reactions that were performed at limiting dilution, 2 PV PCRs produced both *gag* and *env* that were identical to HSPC-derived *gag* and *env* amplicons (**Figure 2.5a**). As discussed above, the presence of identical *gag* together with *env* sequences in a single genome amplification reaction for both HSPC and PV strongly supports identity across the entire genome. These examples provide strong support for the conclusion that HSPC-associated genomes are functional and contribute to persistent residual viremia.

Discussion

Here, we confirm a prior studying providing evidence that HSPCs spread HIV proviruses by cellular proliferation (Sebastian et al., 2017) and provide new evidence that these clonally expanded proviruses contribute to PV. Proviral genome sequences from HSPCs were often identical to sequences isolated from PBMCs and BMMCs. These clusters of HSPC-associated identical proviruses (CHIPs) often matched PV and in two donors, predominant plasma viral clones exactly matched CHIP sequences. Thus, HSPCs and their daughter cells containing clonal genomes are capable of producing clonal virus that is the predominant sequence isolated from plasma.

We also provide evidence that HSPC-associated HIV genomes are functional. A relatively high percentage of proviral genomes isolated from HSPCs contained complete open reading frames for all 9 viral genes (28%). This compares to 2-12% for CD4+ T cells (Bruner et al., 2016; Hiener et al., 2017; Ho et al., 2013). Remarkably, HSPC-derived near full-length genomes often matched plasma virus (60%). This "*in vivo* outgrowth assay" provides strong evidence that HSPCs can harbor infectious HIV.

The relative paucity of HSPC-derived HIV sequences compared to those of PBMCs and BMMCs may have led to under-sampling of the genomes harbored within HSPCs. Given this difference, it is even more remarkable that HSPC-associated genomes matched PV more frequently than unique genomes from PBMCs or BMMCS. Based on the frequency with which *gag* and *env* PBMC-derived amplicons matched PV sequences (3% and 5%, respectively), we would have expected that only 2 (*gag*) and 3 (*env*) HSPC-derived proviral amplicons matched PV sequences. Instead, we found 5 (*gag*) and 8 (*env*) HSPC-derived amplicons matched PV. Based on the observed 3 (*gag*) and 5 (*env*) additional matching HSPC-derived amplicons and the expected proportion of matching sequences (3% and 5%), the probability that under-sampling overestimated that frequency of HSPC genomes matching PV is extremely low ($3x10^{-5}$ and $6x10^{-6}$, for *gag* and *env* amplicons respectively).

Determining whether genomes with identical sequences across an amplified segment are truly clonal can be challenging. It is theoretically possible that identical sub-genomic proviral sequences harbored by HSPCs and other cell types were derived from genetically similar viruses rather than truly clonal ones. However, this is an unlikely explanation for our results for a number of reasons: (1) identity was observed across the entire variable C2-V3 *env* amplicon, which is associated with a clonal prediction score of 83% (Laskey et al., 2016); (2) we were often able to exactly match both *gag* and *env* amplicons from the same first round SGA PCR reaction, increasing our confidence in clonality; (3) one CHIP that matched a PPC contained a deletion of the primer binding site (an 18-base pair sequence complimentary to tRNA(Lys3) that is used to prime reverse transcription). This defect permits viral gene expression and outgrowth; however, reverse

transcription in subsequent infection events cannot be primed and initiated. The most likely explanation for the presence of this deleted provirus in multiple cell sources is that an HSPC was infected by a virus that acquired this mutation during reverse transcription. The proviral genome then spread by cellular proliferation and differentiation.

Loss of the tRNA binding sites sequence by a genome that is producing large amounts of virus raises the possibility that this genetic element could regulate viral gene expression. Interestingly, a number of studies have shown that transcriptional repressors utilize tRNA binding sites in other retroviruses to restrict viral transcription. Specifically, this element is required for transcriptional silencing of murine leukemia viruses (MLVs) that have integrated into genomes of embryonic stem cells. The repression is largely mediated by *trans*-acting factors that recognize the primer binding (Wolf et al., 2008a; Wolf and Goff, 2008; Wolf and Goff, 2009; Wolf et al., 2008b). Whether a related trans-acting factor might similarly regulate HIV gene expression via the HIV tRNA binding site in cells with stem cell-like characteristics is not yet known.

Clonally-expanded, HIV-infected $CD4^+$ T cells have been described (Bruner et al., 2016; Hiener et al., 2017; Lee et al., 2017; Maldarelli et al., 2014; Simonetti et al., 2016; Sun et al., 2015; Wagner et al., 2014). In prior publications, this expansion has been explained by insertion of the provirus into genes that regulate cell growth and/or by antigenic stimulation of the T cell receptor (Cohn et al., 2015; Maldarelli et al., 2014; Simonetti et al., 2016). Here, we provide evidence that in some cases, expansion of clonal HIV genomes is driven by proliferation and differentiation of an infected hematopoietic progenitor cell.

In summary, our work provides evidence that HSPCs are a functionally significant reservoir of persistent HIV. Proviral genomes from HSPCs are associated with expanded clonal HIV proviral genomes found in peripheral blood and bone marrow that contribute to PV. These studies shed light on how the latent reservoir is maintained *in vivo* and suggest novel therapeutic options. Selective targeting of progenitor cells or therapeutics that limit cellular proliferation may enhance current regimens by reducing the number of clonally expanded HIV proviral genomes.

Methods

Ethics Statement and study subjects

HIV-infected individuals were recruited through the University of Michigan HIV-AIDS Treatment Program and the Henry Ford Health System. Written informed consent was obtained according to a protocol approved by the University of Michigan Institutional Review Board and Henry Ford Institutional Review Board (U-M IRB number HUM00004959 and HFH IRB number 7403). Donors were >18 years old, with normal white blood cell counts and plasma viral loads were <48 copies/ml for at least 6 months on antiretroviral therapy. 100 ml of peripheral blood and 20 ml of bone marrow were obtained from each donor. All collected samples were coded and anonymized.

Cell isolation and fractionation

HSPCs were isolated from bone marrow mononuclear cells, following Ficoll-Hypaque (GE Healthcare) density gradient centrifugation as described previously (Sebastian 2017). CD133+ HSPCs were positively selected, then remaining CD34+ HSPCs

were isolated from the first column flow through. Sort purity was ascertained by staining for CD133, CD34 and CD3 and flow cytometric analysis.

Flow cytometry and antibodies

Antibodies to the following human proteins were used for flow cytometry: CD133 (phycoerythrin [PE] conjugated; Miltenyi Biotec), CD34 (conjugated with fluorescein isothiocyanate [FITC]; BD Bioscience), CD3 (conjugated with allophycocyanine [APC]; eBioscience or APC-H7-conjugated; BD Bioscience). Nonviable cells were identified and excluded from sorts and analyses by staining with 7-aminoactinomycin D (7-AAD) or 4,6-Diamidino-2-phenylindole [DAPI]. Samples were analyzed using a BD FacsCanto cytometer.

Cellular DNA isolation

Cellular DNA was prepared using a MagNA Pure Compact System (Roche) according to the manufacturer's protocol.

Peripheral blood plasma HIV RNA isolation and cDNA synthesis

Peripheral blood plasma virus was pelleted from the post Ficoll plasma fraction by ultracentrifugation (25,000 x g, 2 hours, 4^{o}C). Virions were solubilized with TRIzol Reagent (Invitrogen) and stored at -80^{o}C. Two micrograms of control human Raji cell line RNA was spiked into donor Trizol samples prior to organic extraction. The aqueous phase of the Trizol suspensions was purified according to the manufacturer's protocol or with RNeasy Micro columns (Qiagen). RNA samples were treated with DNAse I during column

purification (Qiagen RNAse-free DNAse set) or after elution or resuspension with water (Amp Grade DNAse I, Invitrogen.) cDNA synthesis with oligo dT priming was performed using Superscript III First Strand cDNA Synthesis kit (Invitrogen) or qScript Flex cDNA Kit (Quanta Biosciences). cDNA yield was determined by real-time qRT-PCR for beta-actin (*ACTB*, TaqMan Gene Expression assay ID Hs99999903_m1), with TaqMan Gene Expression Mastermix (Applied Biosystems) on an Applied Biosystems 7300 thermocycler, with the following cycling conditions: 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds.

Single genome PCR assay

Cellular genomic DNA or cDNA prepared from peripheral plasma were used in a two-step, limiting dilution single-genome PCR. PCR was validated for single-copy sensitivity using ACH-2 cell DNA. Cut off for limiting dilution was 30% positive reactions. PBMC DNA from each donor was used to select a standard primer set that optimized HIV *gag* and *env C2-V3* detection. When necessary, donor specific primers were designed for compatibility with standard cycling conditions described previously (Sebastian 2017). For near full-length (NFL) genomes, first round reactions contained primers U5-577 and a Dengue-tagged version of LTR-pA-R (Sebastian 2017). Second round reactions were performed to generate a hemi-nested second round product of approximately 9 kilobase pairs (kbp) and three overlapping sub-genomic regions spanning 5' *LTR/gag* (~ 700 bp), 5' end of gag to 3' end of *env* (5.3 kbp), and Vif through the poly adenylation site (3.7 kbp). Reactions were amplified using a BioRad C1000 thermocycler. Amplicons were purified from agarose gel bands with the QIAQuick Gel Extraction kit (Qiagen) using

Machery Nagel Nucleospin columns, then sequenced directly in both directions by the Sanger method at the University of Michigan DNA Sequencing Core.

While the initial PCRs from samples with higher HIV copy numbers were often not at limiting dilution, the frequent prevalence of identical sequences resulted in unambiguous consensus sequences and we included these sequences in the phylogenetic trees. For quantitative analyses in which we concluded that *gag* and *env* amplicons were derived from the same genome, only sequences from reactions performed at limiting dilution were included.

Sequence data analyses

Forward and reverse sequence reads were assembled into consensus sequences for each amplicon with SeqMan Pro. Coding regions of consensus sequences were compared to all previously isolated experimental sequences and HIV-1 molecular clones used in our lab to rule out carryover and cross-contamination. Additionally, condensed consensus Maximum Likelihood phylogenetic trees of all *gag* and *env* C2-V3 amplicons were estimated with MEGA7 (Kumar et al., 2016) for appropriate clustering. Individual donor phylogenetic trees were inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter values as noted)). Trees were drawn to scale, with branch lengths

measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated.

Statistical analyses

Fisher's exact test to determine the probability of T cell contamination accounting HIV sequences found within HSPC samples was calculated as described previously (McNamara et al., 2013). Comparison of proportions of sequence populations was performed using online calculators for two-tailed Z-test comparison of 1 or 2 sample proportions.

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Table 2.1. Characteristics of donors with HIV provirus in HSPCs.

HSPC proviral DNA:	Positive	Below limit of detection
Number of donors	25	18
Number treated since acute infection	1	1
Male	22	16
Female	3	2
Black	4	4
White	20	14
Non-Hispanic	23	18
CD4 count (mean)	807	710
CD4 count (range)	215 - 2060	308 - 1183
Viral load	<20 - <50	<20 - <50
Duration of suppression, years (mean)	4.3	3.8
Range of suppression, years	0.5 - 13.8	0.6 - 9.2
Mean Sort 1 purity (CD133%, CD3%)	94, 0.3	93, 0.3
Mean Sort 2 purity (CD34%, CD3%)	91, 0.2	90, 0.2
Mean number of cells screened (per donor)	1,020,689	683,906
Range of cells screened	60,750 - 3,092,500	83,070 - 1,552,000

Table 2.2. Donor characteristics. Red, italic text indicates samples that did not meet criteria for purity. First 3 digits is donation number; subsequent groups of 3 digits are ID of previous donation(s) from the same individual, if any.

Donor ID	Gender/ Race	Viral load (co/mL)	Viral suppression before donation (years)	CD4 (cells/mm^3)	CD133$^+$ (%)	CD3$^+$ in CD133 sample (%)	CD34$^+$ (%)	CD3$^+$ in CD34 sample (%)
409000	M/W-NH	<48	1.1	853	99%	0.1%	98%	0.1%
410000	M/W-NH	<48	4.1	856	94%	0.2%	91%	0.4%
411000	M/W-NH	<48	4.2	588	95%	*1.4%*	94%	0.4%
414000	M/W-NH	<48	1.6	749	95%	0.2%	85%	0.3%
415000	M/W-H	<48	0.5	862	96%	0.1%	*72%*	0.0%
416000	M/W-NH	<48	1.1	722	97%	0.4%	93%	0.0%
417000	M/W-NH	<48	2.3	481	99%	0.0%	96%	0.0%
418000	M/W-NH	<48	8.8	671	96%	0.2%	80%	0.1%
420000	F/W-NH	<48	1.4	1315	99%	0.0%	92%	*1.0%*
421000	M/W-NH	<48	4.2	775	99%	0.2%	94%	0.1%
426000	M/W-NH	<40	7.1	1034	97%	0.3%	92%	0.2%
427000	M/W-NH	<48	5.3	1150	96%	0.3%	88%	0.7%
428408	M/W-NH	<48	9.2-9.6	303-444	91-95%	0.5-0.6%	86-91%	0.2-0.6%
429000	M/W-NH	<48	5.6	745	94%	0.5%	90%	0.4%
430000	M/W-NH	<40	6.4	308	98%	0.0%	94%	0.0%
431000	M/W-NH	<40	3.5	215	93%	0.2%	89%	0.2%
432000	M/W-NH	<40	5.2	565	98%	0.1%	99%	0.0%
433407	F/B-NH	<48	0.6-1.4	452-581	86-94%	*0.1-2.9%*	83-90%	0.2-0.5%
434423	M/Other	<48	0.9-1.2	1577-1693	92-94%	0.1-0.2%	84-89%	0.1%
435412406	M/W-NH	<48	4.7-5.5	1292-2060	95-99%	0.2-0.4%	83-99%	0.1-0.7%
436000	F/W-NH	<40	4.7	723	93%	0.9%	85%	0.3%
437000	F/W-NH	<40	1.7	418	94%	0.2%	92%	0.0%
438000	M/W-NH	<20	8.4	698	95%	0.1%	*78%*	0.4%
439000	M/B-NH	<20	0.8	540	96%	0.3%	93%	0.2%
440000	M/W-NH	<50	1.4	730	97%	0.4%	95%	0.2%
441000	M/B-NH	<20	2.6	618	90%	0.5%	86%	0.2%
442000	M/B-NH	<20	9.2	1112	94%	0.3%	95%	0.1%
443000	M/B-NH	<50	1.5	896	*71%*	*1.1%*	92%	0.4%
445000	M/B-NH	<20	1.1	495	87%	0.8%	89%	0.1%
446000	M/B-NH	<50	1.7	503	92%	0.5%	90%	0.2%
447000	M/W-NH	<50	0.6	544	98%	0.5%	94%	0.1%
449000	M/W-H	<20	0.6	1392	90%	0.2%	95%	0.0%
450000	M/W-NH	<40	4.3	1183	90%	0.6%	*77%*	0.2%
452000	M/W-NH	<40	3.1	618	86%	0.4%	*70%*	0.5%
453000	M/W-NH	<40	2.0	704	96%	0.8%	83%	0.2%
454304	M/W-NH	<48	3.3-7.1	533-594	92-98%	0.4%	93%	0.2%
455312101	F/W-NH	<48	2.2-6.0	812	87-99%	0.2%	83%	0.2%
456000	M/W-NH	<40	0.5	655	93%	0.8%	90%	0.3%
457413402	M/B-NH	<48	0.2-3.6	321-390	*69-94%*	0-0.2%	*70-91%*	0-0.2%
458311	M/W-NH	<48	8.2-13.0	564	85-99%	0.3%	91%	0.0%
459419	M/W-NH	<48	2.4-6.6	624	83-96%	0.0%	*78-91%*	0.1%
502000	M/W-NH	<40	0.7	603	84%	0.6%	*74%*	0.6%
503501	M/W-NH	<48	9.8-13.8	861	90-98%	0.1-0.6%	85-88%	0.1-0.4%

Table 2.3. Number of sequences obtained from each source of HIV. Abbreviations: PBMC, unfractionated peripheral blood mononuclear cells; BMMC, HSPC-depleted bone marrow mononuclear cell; HSPC, hematopoietic stem and progenitor cell; PV, plasma virus.

Sub-genomic region	Source of DNA	Amplicons (No.) Total	Unique*
gag	PBMC	622	496
	BMMC	266	240
	HSPC	58	58
	PV	201	61
env	PBMC	684	508
	BMMC	285	225
	HSPC	62	62
	PV	329	107

Table 2.4. Characteristics of donors with HSPC provirus identical to plasma virus.

HSPC proviral DNA identical to plasma virus:	Yes	No	
Number of donors	8	16	
Number treated since acute infection	0	1	
Male	7	16	
Female	1	2	
Black	0	4	
White	7	14	
Non-Hispanic	7	17	
CD4 count (mean)	1128	620	p = 0.0005
CD4 count (range)	533 - 2060	215 - 1315	
Year of diagnosis (median)	2007	2002	
Number of donors diagnosed prior to 1997 (%)	2 (25%)	5 (31%)	
Duration of suppression, years (mean)	3.3	5.0	
Range of suppression, years	0.6 - 7.1	0.5 - 13.8	
Mean Sort 1 purity (CD133%, CD3%)	95, 0.3	94, 0.3	
Mean Sort 2 purity (CD34%, CD3%)	91, 0.2	90, 0.2	
Mean number of cells screened (per donor)	1,452,147	827,503	
Range of cells screened	167,375 - 3,092,500	60,750 - 1,715,000	
Mean number of HSPC amplicons per donor	6	4	
Range of HSPC amplicons per donor	3 - 10	1 - 19	
Mean number of plasma virus amplicons per donor	42	12	p = 0.04
Range of plasma virus amplicons per donor	5 - 112	1 - 92	

Table 2.5. Characteristics of donors with HSPC provirus identical to plasma virus. Red, italic text indicates samples that did not meet criteria for purity. First 3 digits is donation number; subsequent groups of 3 digits are ID of previous donation(s) from the same individual, if any.

Donors with HSPC provirus identical to plasma virus								
Donor ID	**Gender/ Race**	**Viral load (copies/mL)**	**Viral suppression before donation (years)**	**CD4 (cells/mm^3)**	**CD133$^+$ (%)**	**CD3$^+$ in CD133 sample (%)**	**CD34$^+$ (%)**	**CD3$^+$ in CD34 sample (%)**
409000	M/W-NH	<48	1.1	853	99%	0.1%	98%	0.1%
414000	M/W-NH	<48	1.6	749	95%	0.2%	85%	0.3%
421000	M/W-NH	<48	4.2	775	99%	0.2%	94%	0.1%
434423	M/Other	<48	0.9-1.2	1577-1693	92-94%	0.1-0.2%	84-89%	0.1%
435412406	M/W-NH	<48	4.7-5.5	1292-2060	95-99%	0.2-0.4%	83-99%	0.1-0.7%
436000	F/W-NH	<40	4.7	723	93%	0.9%	85%	0.3%
449000	M/W-H	<20	0.6	1392	90%	0.2%	95%	0.0%
454304	M/W-NH	<48	3.3-7.1	533-594	92-98%	0.4-2.8%	93%	0.2%
		Average:	**3.3**	**1128**	**95%**	**0.3%**	**91%**	**0.2%**

Donors without HSPC provirus identical to plasma virus								
Donor ID	**Gender/ Race**	**Viral load (copies/mL)**	**Viral suppression before donation (years)**	**CD4 (cells/mm^3)**	**CD133$^+$ (%)**	**CD3$^+$ in CD133 sample (%)**	**CD34$^+$ (%)**	**CD3$^+$ in CD34 sample (%)**
411000	M/W-NH	<48	4.2	588	95%	*1.4%*	94%	0.4%
415000	M/W-H	<48	0.5	862	96%	0.1%	*72%*	0.0%
420000	F/W-NH	<48	1.4	1315	99%	0.0%	92%	*1.0%*
426000	M/W-NH	<40	7.1	1034	97%	0.3%	92%	0.2%
428408	M/W-NH	<48	9.2-9.6	303-444	91-95%	0.5-0.6%	86-91%	0.2-0.6%
431000	M/W-NH	<40	3.5	215	93%	0.2%	89%	0.2%
432000	M/W-NH	<40	5.2	565	98%	0.1%	99%	0.0%
437000	F/W-NH	<40	1.7	418	94%	0.2%	92%	0.0%
439000	M/B-NH	<20	0.8	540	96%	0.3%	93%	0.2%
446000	M/B-NH	<50	1.7	503	92%	0.5%	90%	0.2%
453000	M/W-NH	<40	2.0	704	96%	0.8%	83%	0.2%
456000	M/W-NH	<40	0.5	655	93%	0.8%	90%	0.3%
457413402	M/B-NH	<48	0.2-3.6	321-390	*69-94%*	0-0.2%	*70-91%*	0-0.2%
458311	M/W-NH	<48	8.2-13.0	564	85-99%	0.3%	91%	0.0%
459419	M/W-NH	<48	2.4-6.6	624	83-96%	0.0%	*78-91%*	0.1%
503501	M/W-NH	<48	9.8-13.8	861	90-98%	0.1-0.6%	85-88%	0.1-0.4%
		Average:	**5.0**	**620**	**94%**	**0.3%**	**90%**	**0.2%**

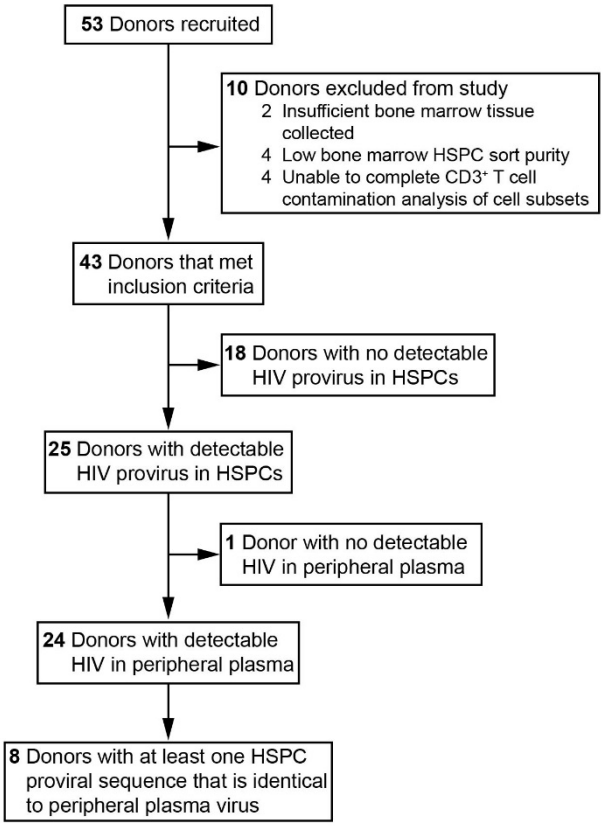

Figure 2.1. Donor inclusion and summary of study cohort sub-groups.

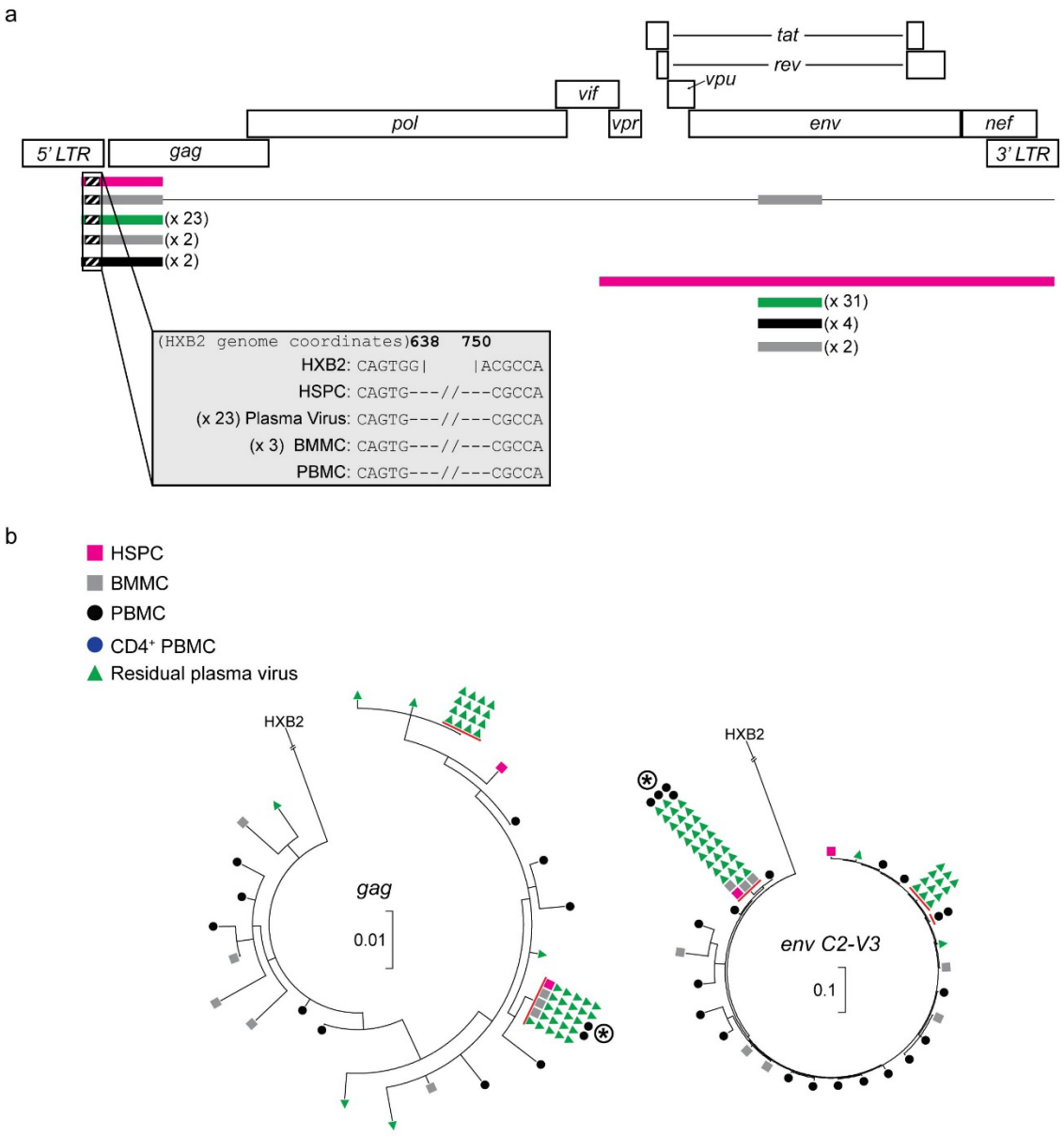

Figure 2.2. Phylogenetic analysis of HIV sequences from donor 436000. (**a**) HIV genome map and identical viral sequences from indicated tissue source. Solid bars indicate fully-sequenced, genetically intact amplicons. Black and white hatched bars indicate the location of a signature deletion. The thin line connecting gag and *env* amplicons indicates sequences that originated from the same first-round reaction performed at limiting dilution. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from the indicated tissue sources. Identical sequence groups are designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled asterisk. Scale indicates nucleotide substitutions per site.

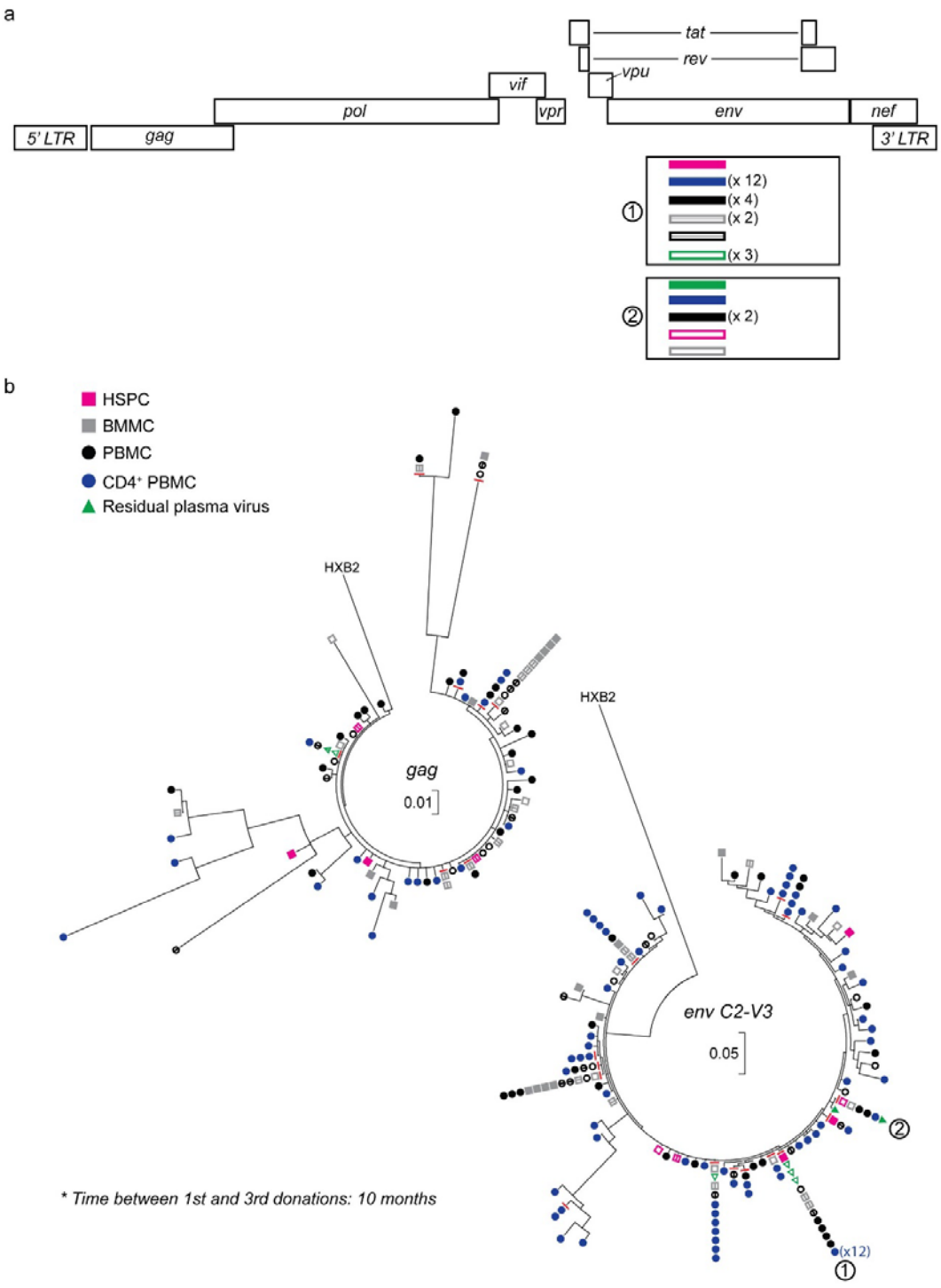

Figure 2.3. Phylogenetic analysis of HIV sequences from donor 435412406. (**a**) HIV genome map and identical viral sequences from the indicated tissue source. Boxes indicate fully-sequenced, genetically intact amplicons. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from indicated tissue sources. Identical sequence groups are designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled numbers. Scale indicates nucleotide substitutions per site. Sequences from the

first, second, and third donations are represented by symbols that are empty, struck-through, and filled, respectively.

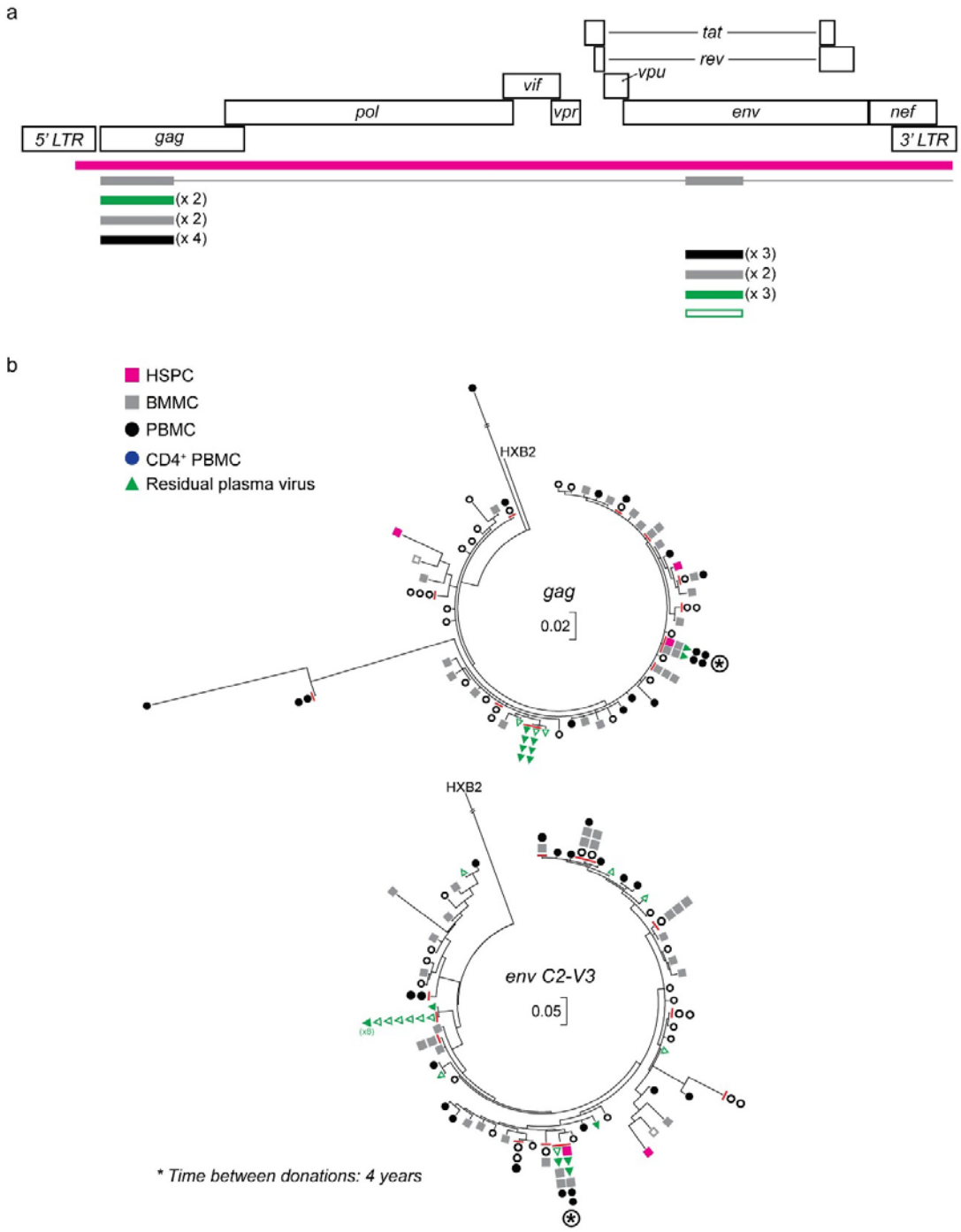

Figure 2.4. Phylogenetic analysis of HIV sequences from donor 454304. (**a**) HIV genome map and identical viral sequences from indicated tissue source. Solid bars indicate fully-sequenced, genetically intact amplicons. Thin line connecting gag and *env* amplicons indicate sequences that originated from the same first-round reaction performed at limiting dilution. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from the indicated tissue sources. Identical sequence groups are designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled asterisk. Scale

indicates nucleotide substitutions per site. Sequences from the first and second donations are represented by symbols that are empty and filled, respectively.

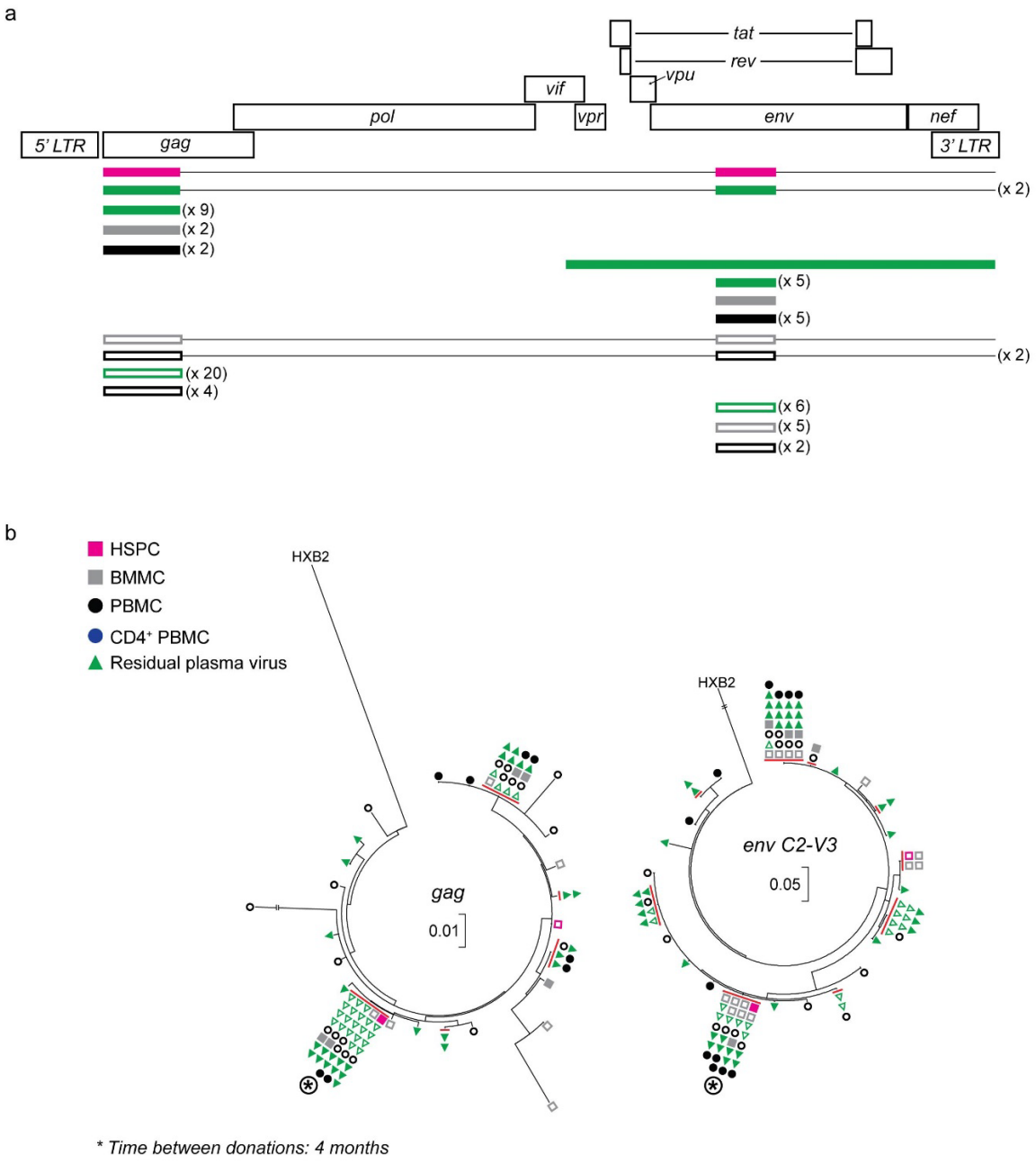

Figure 2.5. Phylogenetic analysis of HIV sequences from donor 434423. (**a**) HIV genome map and identical viral sequences from indicated tissue source. Solid bars indicate fully-sequenced, genetically intact amplicons. Thin lines connecting gag and *env* amplicons indicate sequences that originated from the same first-round reaction performed at limiting dilution. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from indicated tissue sources. Identical sequence groups are designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled asterisk. Scale indicates nucleotide substitutions per site. Sequences from the first and second donations are represented by symbols that are empty and filled, respectively.

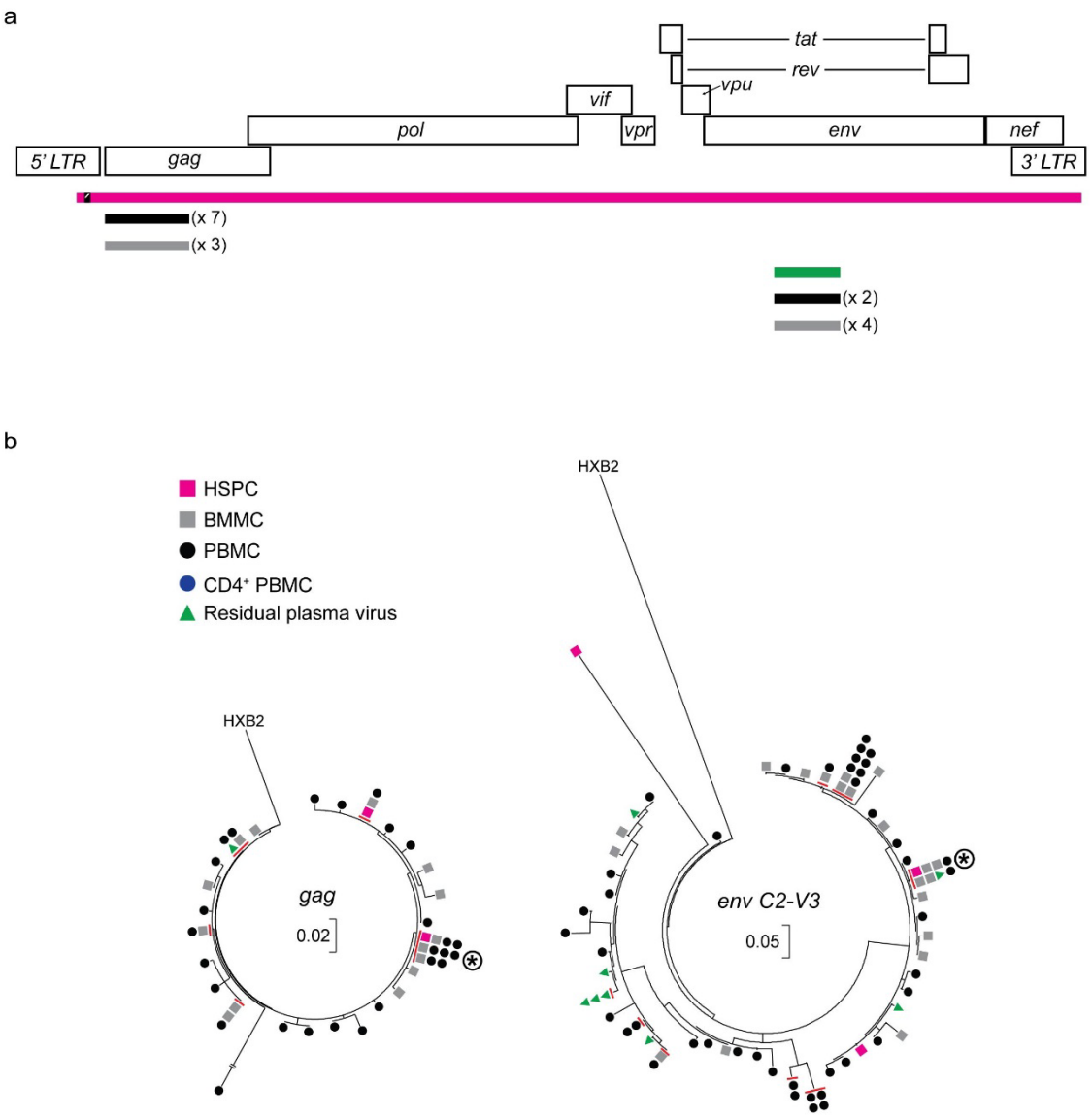

Figure 2.6. Phylogenetic analysis of HIV sequences from donor 409000. (**a**) HIV genome map and identical viral sequences from indicated tissue source. Solid bars indicate fully-sequenced, genetically intact amplicons. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from indicated tissue sources. Identical sequence groups are designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled asterisk. Scale indicates nucleotide substitutions per site.

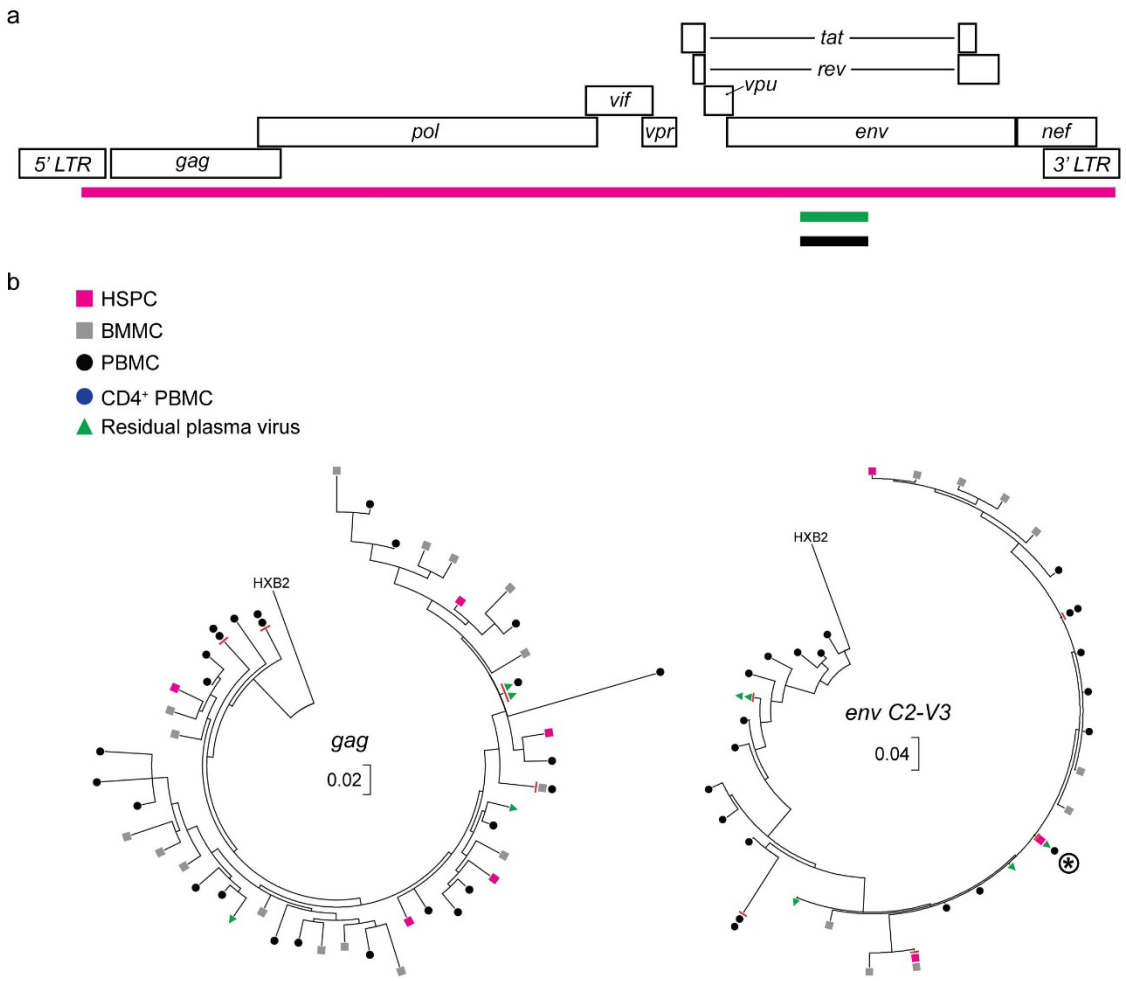

Figure 2.7. Phylogenetic analysis of HIV sequences from donor 421000. (**a**) HIV genome map and identical viral sequences from indicated tissue source. Solid bars indicate fully-sequenced, genetically intact amplicons. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from indicated tissue sources. Identical sequence groups are designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled asterisk. Scale indicates nucleotide substitutions per site.

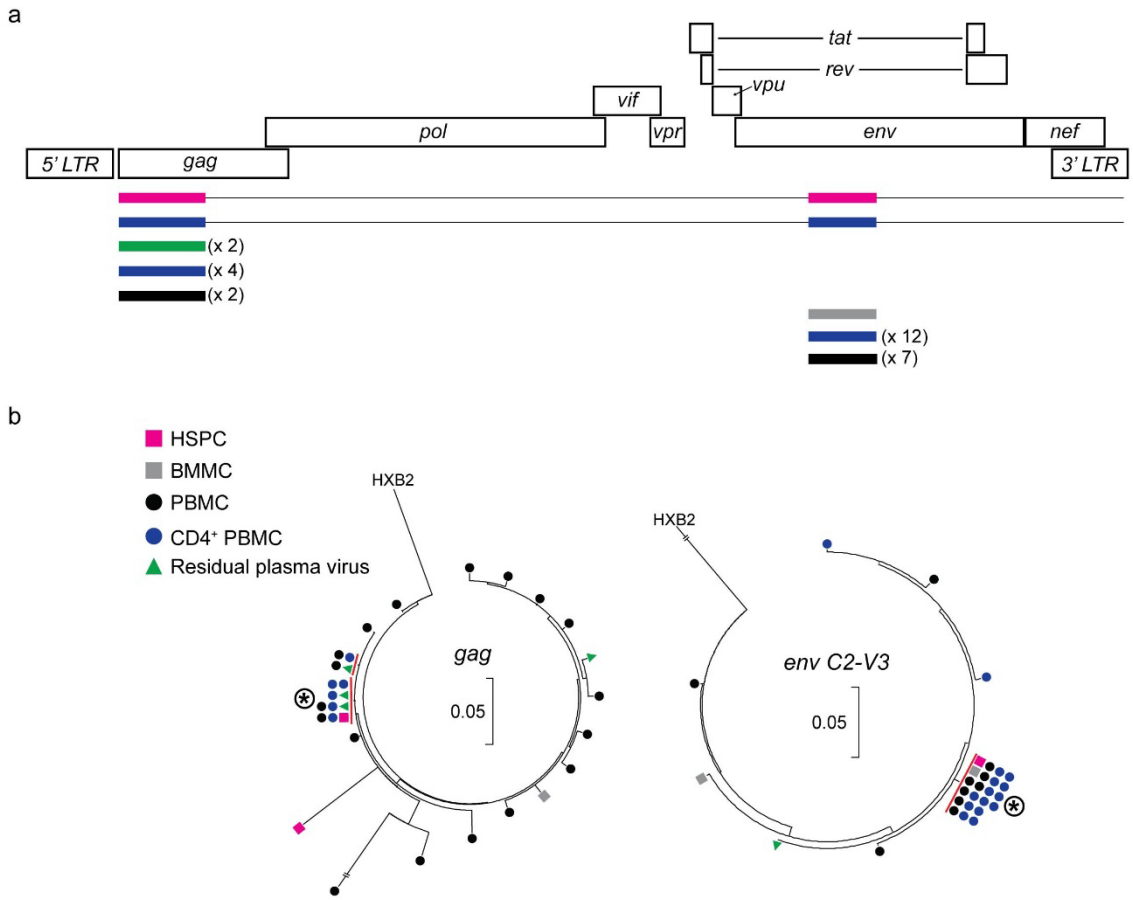

Figure 2.8. Phylogenetic analysis of HIV sequences from donor 414000. (**a**) HIV genome map and identical viral sequences from indicated tissue source. Solid bars indicate fully-sequenced, genetically intact amplicons. The thin line connecting gag and *env* amplicons indicates sequences that originated from the same first-round reaction performed at limiting dilution. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from indicated tissue sources. Identical sequence groups are designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled asterisk. Scale indicates nucleotide substitutions per site.

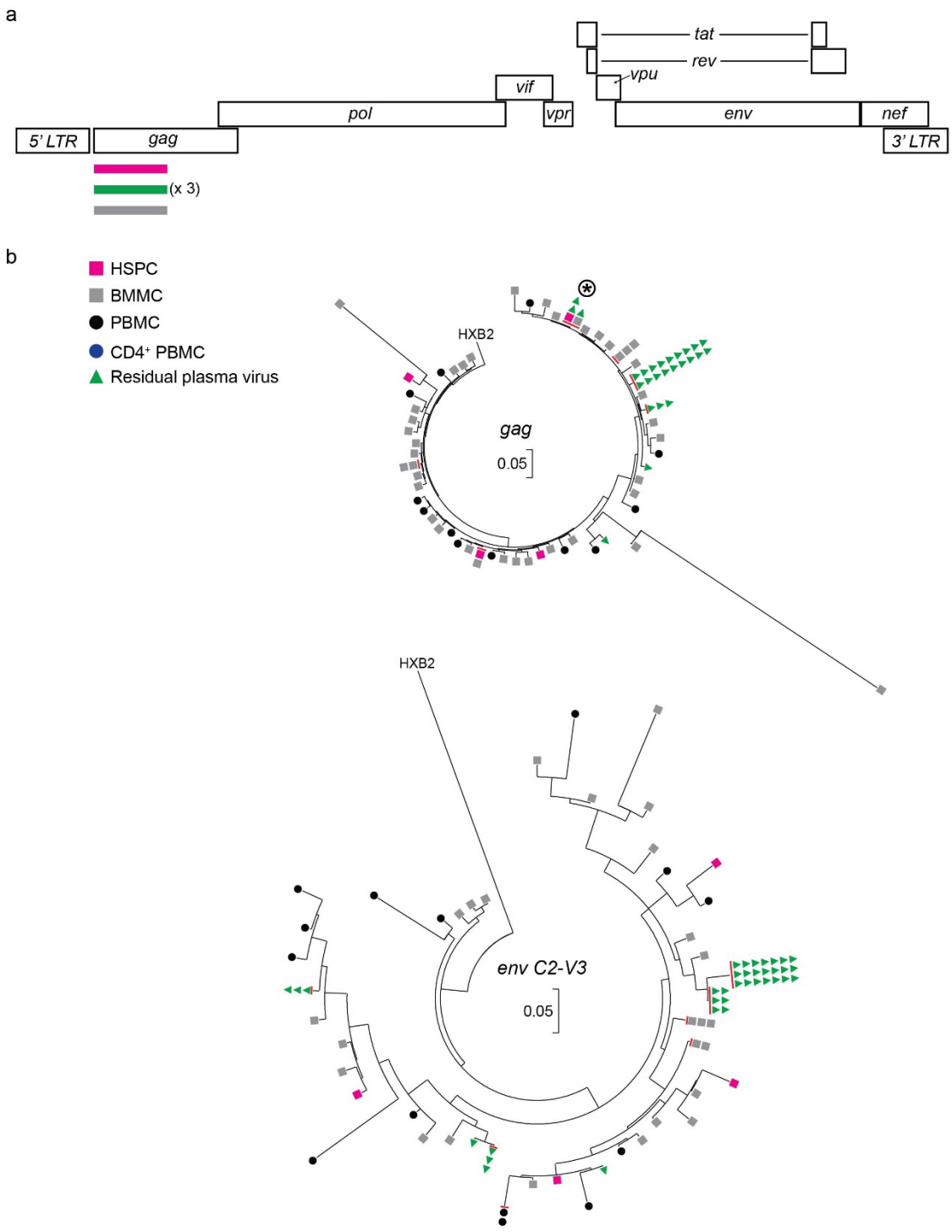

Figure 2.9. Phylogenetic analysis of HIV sequences from donor 449000. (**a**) HIV genome map and identical viral sequences from indicated tissue source. Solid bars indicate fully-sequenced, genetically intact amplicons. (**b**) Maximum likelihood phylogenetic trees of *gag* and *env* HIV sequences from indicated tissue sources. Identical sequence groups are

designated with a red bar. Identical sequence groups depicted in panel (a) are marked with the circled asterisk. Scale indicates nucleotide substitutions per site.

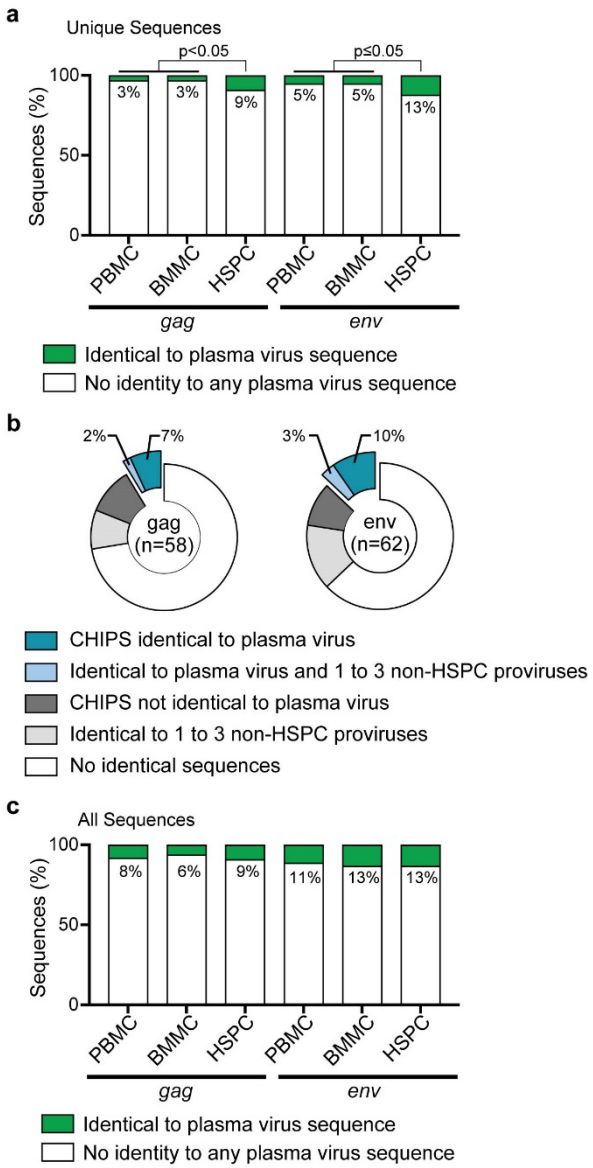

Figure 2.10. Residual plasma virus is often derived from groups of clusters of HSPC-associated identical proviral genomes (CHIPs). (**a**) Proportions of unique *gag* and *env* proviral sequences from HSPCs, PBMCs, and BMMCs with identity to plasma virus. (**b**) Proportions of HSPC-derived *gag* and *env* sequences with sequence identity to plasma virus or other proviruses. CHIPs represent groups of identical proviral sequences from at least 1 HSPC-derived amplicon and 4 or more non-HSPC cell sources. (**c**) Proportions of HSPC, PBMC, and BMMC proviral sequences that are identical to plasma virus when all sequences are analyzed, including genomes with evidence of clonal expansion. Analysis included sequences from donors from whom we detected HIV nucleic acid in HSPCs and peripheral blood plasma (n=24). The number of sequences analyzed is shown in Table 2.3. Statistically significant differences between sample proportions were calculated by two-tailed Z-tests of proportions for 2 samples.

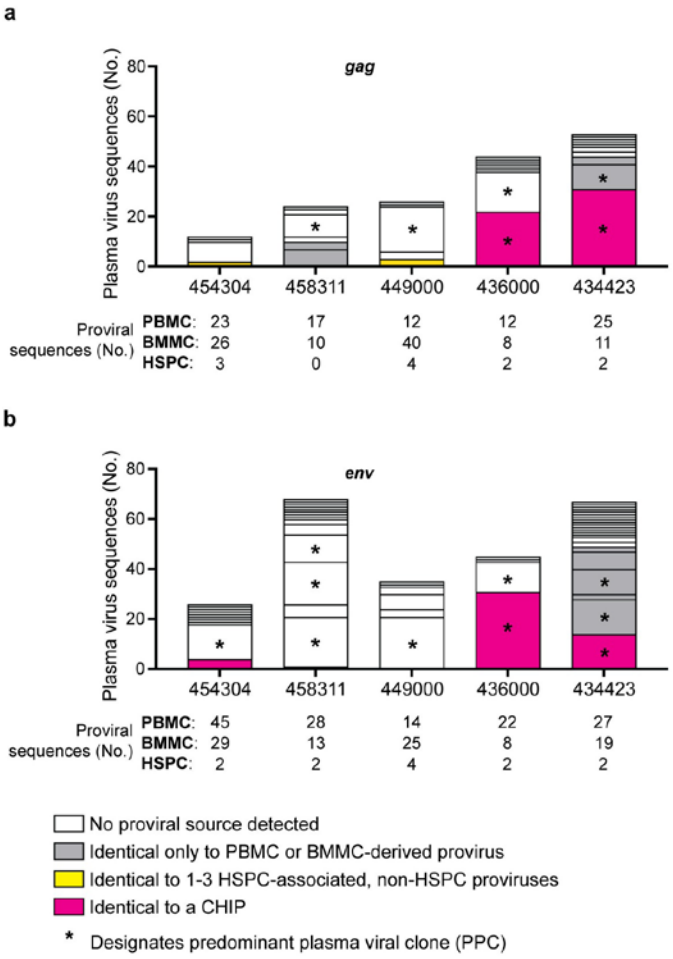

Figure 2.11. Predominant plasma clones that can be attributed to a cellular source often match CHIPs. Bar graphs showing number of (**a**) *gag* and (**b**) *env* plasma virus amplicons that matched amplicons from the indicated groups of cellular proviruses from the five donors from whom we identified PPCs. Groups of identical plasma virus sequences are indicated by horizontal bars within each stacked bar graph. Groups of PPCs are indicated with an asterisk. Number of proviral sequences analyzed is shown below x-axis donor labels.

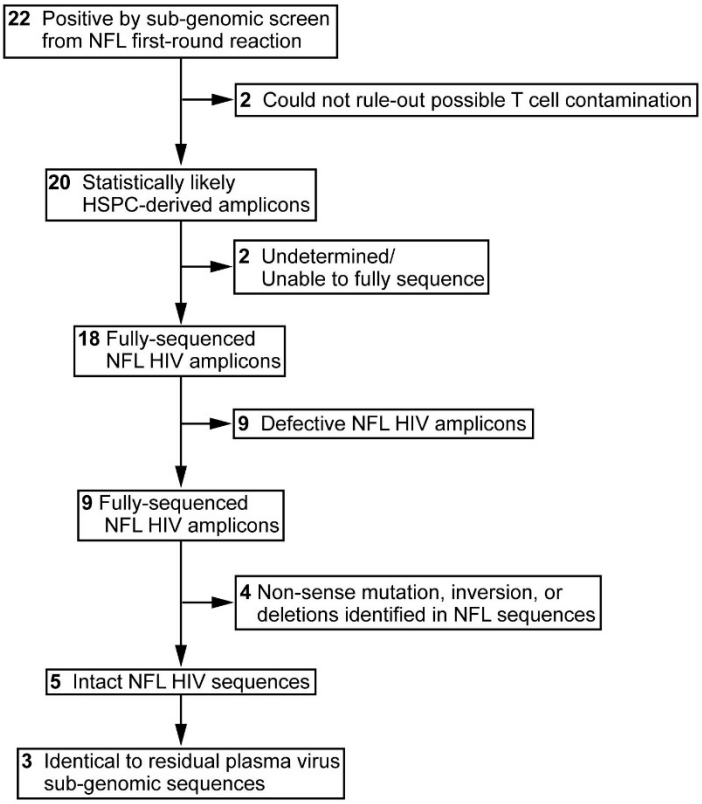

Figure 2.12. Summary of HSPC-derived near full-length (NFL) HIV genome assay results.

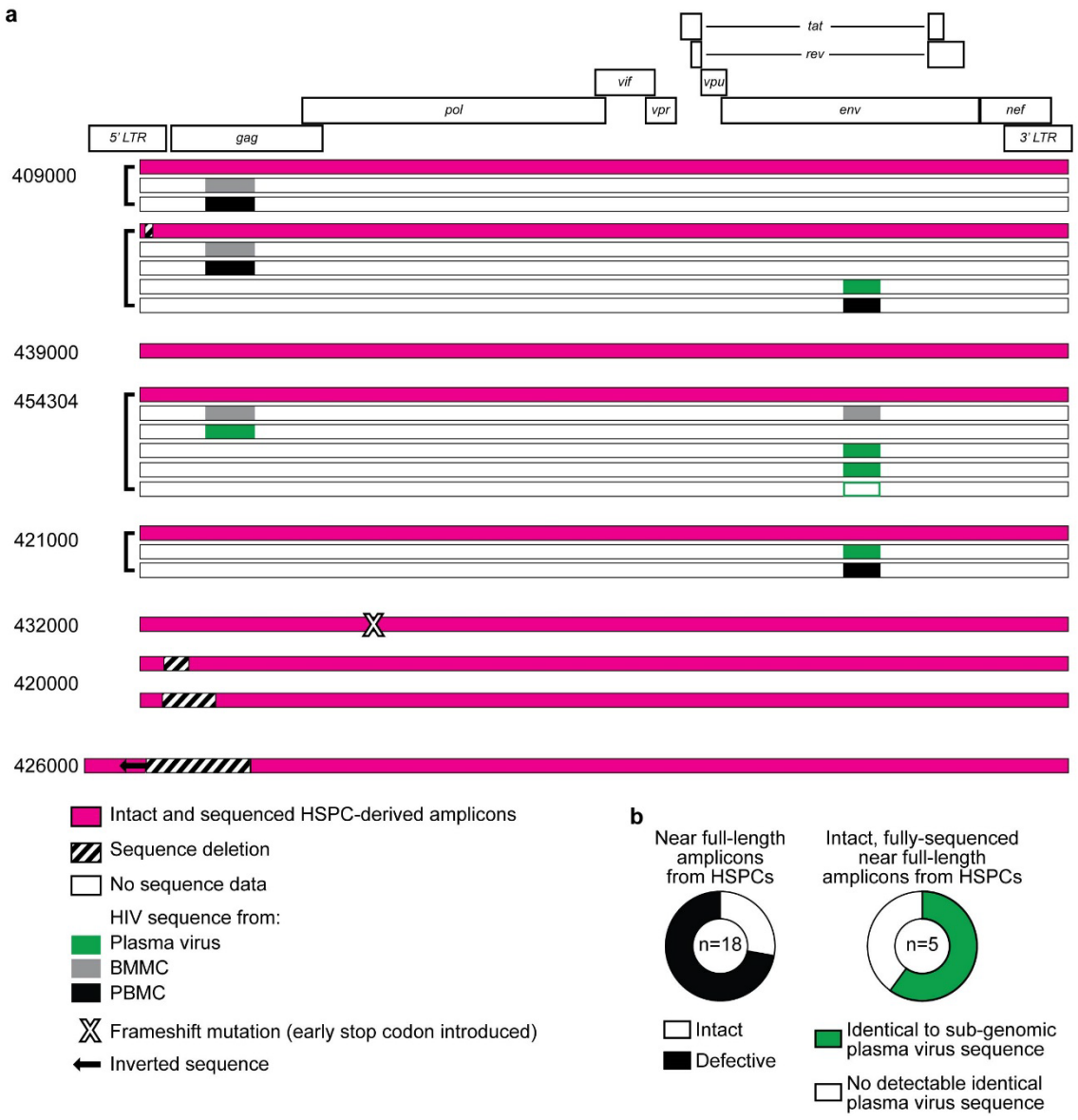

Figure 2.13. HSPCs contain intact, near full-length HIV genomes, which are represented in residual plasma virus at a high rate. (**a**) HIV genome map and near full-length proviral sequences from HSPCs and genetically identical sub-genomic amplicons from other tissue sources. All amplicons represented are from PCRs that were performed at limiting dilution. Donor IDs for groups of amplicons are indicated on the left. For donor 454304, amplicons from the first and second donations are represented by empty and filled bars, respectively. (**b**) Pie graphs showing the proportion of intact, fully-sequenced near full-length HIV genomes from HSPCs (left) and the proportion of these intact sequences that are genetically identical to sub-genomic amplicons from plasma virus (right).

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Chapter 3

Class 1-selective histone deacetylase inhibitors enhance HIV latency reversal while preserving the activity of HDAC isoforms necessary for maximal HIV gene expression[2]

Summary

Combinations of drugs that affect distinct mechanisms of HIV latency aim to induce robust latency reversal leading to cytopathicity and elimination of the persistent HIV reservoir. Thus far, attempts have focused on combinations of PKC agonists and pan-histone deacetylase inhibitors (HDIs) despite the knowledge that HIV gene expression is regulated by class 1 histone deacetylases. We hypothesized that class 1-selective HDIs would promote more robust HIV latency reversal in combination with a PKC agonist than pan-HDIs because they preserve the activity of pro-viral factors regulated by non-class 1 histone deacetylases. Here, we show that class 1-selective agents used alone or with the

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PKC agonist bryostatin-1 induced more HIV protein expression per infected cell. In addition, the combination of entinostat plus bryostatin-1 induced viral outgrowth, whereas bryostatin-1- combinations with pan-HDIs did not. When class 1-selective HDIs were used in combination with pan-HDIs, the amount of viral protein expression and virus outgrowth resembled that of pan-HDIs alone, suggesting that pan-HDIs inhibit robust gene expression induced by class 1-selective HDIs. Consistent with this, pan-HDI-containing combinations reduced the activity of NF-κB and Hsp90, two cellular factors necessary for potent HIV protein expression, but did not significantly reduce overall cell viability. An assessment of viral clearance from *in vitro* cultures indicated that maximal protein expression induced by class 1-selective HDI treatment was crucial for reservoir clearance. These findings elucidate limitations of current approaches and provide a path towards more effective strategies to eliminate the HIV reservoir.

Introduction

Latent HIV within long-lived cells constitutes a reservoir of persistent virus that cannot be eliminated by antiretroviral therapy (ART) and is a major barrier to a cure (Chun et al., 1997; Finzi et al., 1997). Pharmacologic reversal of HIV latency has been proposed as a means to induce viral gene expression, rendering infected cells susceptible to viral cytopathic effect and immunological clearance (Deeks, 2012). Thus far, work towards this goal has led to the identification of mechanistically distinct drugs that affect several regulatory factors of HIV gene expression including histone deacetylase inhibitors (HDIs) and protein kinase C (PKC) agonists, such as bryostatin-1 (Archin et al., 2009; Mehla et al., 2010; Wei et al., 2014). Despite promising *in vitro* results, attempts to reduce the viral

reservoir *in vivo* using these latency reversal agents (LRAs) have failed at clinically achievable levels.

High level viral gene expression is needed for viral proteins to accumulate to toxic levels and kill the infected cells (Shan et al., 2012). Both HDIs and PKC agonists have the capacity to activate viral gene expression in patient-derived resting $CD4^{pos}$ ($rCD4^{pos}$) T cells as measured by cell-associated HIV RNA, but only PKC agonists used at supra-therapeutic levels have the capacity to induce viral gene expression sufficient for viral outgrowth (Bullen et al., 2014). In attempts to lower the concentrations of individual agents to clinically achievable levels while still maximizing viral gene expression, efforts have focused on combinations of HDIs and PKC agonists, which demonstrate synergistic induction of cell-associated HIV mRNA expression in cell line and primary cell models of HIV latency. However HDI combinations tested thus far have failed to maximally enhance gene expression as assessed by viral outgrowth assays using patient $rCD4^{pos}$ T cells tested *ex vivo* (Bullen et al., 2014; Laird et al., 2015) .

There are three main classes of histone deacetylases (HDACs) and efforts to reverse HIV latency have largely focused on HDIs that act on a broad range of HDAC classes (pan-HDIs). Because broadly acting agents inhibit a wider range of HDACs, they are more likely to have deleterious effects that could limit maximal latency reversal. A more narrowly focused regimen that targets the subset of HDACs (class 1) involved in HIV latency (Keedy et al., 2009) may allow more efficient activation of HIV gene expression. Indeed, previous work has demonstrated that class 1-selective HDIs can reverse HIV latency in cell line models and are superior to pan-HDIs when used in combination with PKC agonists in *in vitro* primary cell HIV latency model systems (Bui et al., 2017; Savarino et al., 2009;

Wightman et al., 2012). These studies, however, are significantly limited by the use of latency models that do not fully recapitulate the effects of LRA treatment in rCD4^{pos} T cells from HIV-infected people and by focusing on the frequency of reactivated proviruses rather than the potency of the induced viral gene expression, viral outgrowth, and clearance of infected cells (Bullen et al., 2014). Moreover, the efficacy of selective agents in hematopoietic stem and progenitor cells (HSPC), which may constitute a small but important long-lived reservoir *in vivo* (Carter et al., 2010; Sebastian et al., 2017) has not been demonstrated.

Therefore, using an *in vitro* model system of HIV latency in HSPCs and rCD4^{pos} T cells from optimally treated HIV-infected donors, we compared the efficacy of pan-HDIs to class 1-selective HDIs on viral reactivation and outgrowth from latently infected cells. We show that class 1-selective HDIs are unique amongst HDIs tested thus far in that they enhance the ability of PKC agonists to induce viral outgrowth from latently infected HSPCs and from rCD4^{pos} T cells from HIV-infected people. Moreover, our results provide the first evidence that class 1-selective HDIs are superior at inducing viral outgrowth due to reduced off-target inhibition of cellular factors necessary for optimal HIV gene expression.

Results

A robust model of HIV latency in HSPCs allows measurement of reactivation frequency and viral protein production per reactivated cell

To compare the ability of HDIs to induce HIV latency reversal we used a latency model system in which purified umbilical cord blood HSPCs from healthy donors were infected with a replication-defective, NL4-3-based HIV molecular clone that expresses

green fluorescent protein fused to a short Env peptide (E-GFP; NL4-3-ΔGPE-GFP; **Figure 3.1a and Figure** 3.1**b**). Three days post-infection, actively infected (E-GFP^{pos}) HSPCs were removed using fluorescence-activated cell sorting **(Figure 3.1c**) and the remaining cells, which include latently infected cells, were immediately treated with each LRA regimen for 24 hours. An integration inhibitor (raltegravir) was also added to block new integration events, ensuring that we only scored reversal of post-integration latency. Because latency occurs relatively frequently in the HSPC model system, the relative amount of viral protein production in cells bearing a reactivated provirus (E-GFP^{pos}) could be accurately measured by flow cytometry via the E-GFP mean fluorescence intensity (MFI).

Consistent with our previous work (McNamara et al., 2012), when latently infected HSPCs were treated with TNF-α, we observed potent latency reversal compared to 0.1% DMSO solvent control (**Figure 3.1d**). The capacity to quantitatively assess the effect of LRAs on viral protein expression in an unmodified primary cell model of latency is a unique feature of our system that may allow the identification of combinations of drugs that will maximally reverse latency and have the greatest capacity to promote eradication

Based on our previous work (Painter et al., 2017), reactivation occurring in the solvent control is due to spontaneous differentiation of HSPCs and does not reflect latency reversal activity by the DMSO solvent. Compared to untreated cells, 0.1% DMSO did not significantly affect the frequency of E-GFP^{pos} cells (**Figure 3.1e**) or the E-GFP MFI (**Figure 3.1f**). In addition, the concentration of DMSO used in our assay was not sufficient to induce acetylation of histone or tubulin (**Figure 3.1i**).

HSPCs gradually lose some expression of the CD34 cell surface marker under culture conditions that support HSPC proliferation. Moreover, differentiation as assessed by CD34 expression is associated with reactivation from latency in the HSPC model system (Painter et al., 2017). However, the solvent DMSO does not affect differentiation of HSPCs as assessed by cell surface CD34 expression (**Figure 3.1g**) and all cells remain lineage negative (**Figure 3.1h**).

Class 1-selective HDIs induce more viral Env (E-GFP) production per infected cell than pan-HDIs

To assess the efficacy of HDIs on latency reversal in HSPCs, we performed a titration over a range of drug concentrations and assessed the frequency of GFP^{pos} cells (**Figure 3.2a**) and the amount of GFP expressed per cell (E-GFP MFI, **Figure 3.2b**). Optimal concentrations were those that maximized latency reversal while minimizing toxicity and were consistent with results from other studies (Savarino et al., 2009; Shan et al., 2014; Wei et al., 2014). Work from other groups has shown that selectivity of class 1-selective HDIs is maintained at concentrations well above those tested here (Beckers et al., 2007; Khan et al., 2008; Lauffer et al., 2013). Nevertheless, to confirm selectivity of HDIs at the concentrations chosen for our assays, we assessed their effects on histone H4 and tubulin acetylation. Histone H4 is deacetylated by class 1 HDACs, therefore both pan-HDIs and class 1-selective HDIs should promote histone H4 acetylation. In contrast, tubulin is deacetylated by the class 2 HDAC HDAC6 and should be affected by pan-HDIs but not class 1-selective HDIs. Indeed, we observed that at the concentrations chosen for our study,

the pan-HDI vorinostat, but not the class 1-selective HDI entinostat, inhibited deacetylation of tubulin, whereas both drugs inhibited deacetylation of histone H4 (**Figure 3.1i**).

A summary of results for HSPCs from at least four independent donors indicate that, on average, every HDI tested induced E-GFP expression in a smaller fraction of latently infected cells than the positive control, TNF-α (**Figure 3.2c**). Among HDIs, pan-HDIs (vorinostat, panobinostat, and romidepsin) induced E-GFP expression in a significantly greater fraction of latently infected cells than the class 1-selective HDIs (entinostat, tacedinaline and mocetinostat) at the concentrations tested (**Figure 3.2c**).

In contrast, all class 1-selective HDIs tested induced significantly more viral protein expression per cell than pan-HDIs based on E-GFP MFI (1.5-fold, **Figure 3.2d**). Moreover, this difference in efficacy was observed across a range of concentrations; increasing the concentration of pan-HDIs above that used in Figure 3.2d did not overcome this limitation (**Figure 3.2b**). In addition, these differences were not due to toxicity as cell viability was similar at HDI concentrations chosen for our assays (**Figure 3.2e**)

We also assayed cell surface MHC-I expression in cells bearing reactivated virus, which has been shown to be inversely proportional to Nef expression levels (Liu et al., 2001). We observed that treatment of latently infected HSPCs with entinostat induced an 8-fold Nef-dependent downmodulation of MHC-I in cells bearing reactivated virus compared to E-GFP^{neg} cells (**Figure 3.3a**). Compared to vorinostat, we observed significantly more downmodulation of cell surface MHC-I on cells treated with entinostat (1.3-fold; **Figure 3.3b**), which corresponds to the degree of induction of viral protein (E-GFP) production (**Figure 3.2d**). Together these data demonstrate that the class 1-selective HDI entinostat induces greater expression of both early and late HIV genes than pan-HDIs.

Pan-HDIs suppress HIV protein production induced by class 1-selective HDIs

The ideal LRA would maximally reverse latency in all infected cells to promote cytopathicity and clearance. Thus, we next asked whether combinations of pan- and class 1-selective HDIs would maximize both frequency of cells expressing E-GFP as well as the amount of E-GFP expressed per infected cell. Indeed, we observed that the addition of pan-HDIs to class 1-selective HDIs maintained the higher frequencies of E-GFP^{pos} cells observed with pan-HDIs alone (**Figure 3.2c**). However, these combinations failed to achieve the high E-GFP MFIs observed with class 1-selective HDI treatment alone (**Figure 3.2d**). The inhibitory effect of pan-HDIs could not be attributed to additive toxicity as there was no significant difference in the frequency of viable HSPCs treated with HDIs alone or in combination (**Figure 3.2e**). Based on these results, we hypothesized that the broad inhibitory effects of pan-HDIs had undesirable negative effects on HIV gene expression that did not occur with class 1-selective HDIs.

Class 1-selective HDIs plus a PKC agonist maximally reactivate viral gene expression and pan-HDIs are inhibitory when added to these combinations

Reversal of HIV latency has also been observed by activating protein kinase C (PKC) and NF-κB using bryostatin-1. Recent studies have shown that bryostatin-1 acts synergistically with pan-HDIs to increase HIV cell-associated RNA transcription, but not viral protein production or viral outgrowth in latently infected primary CD4^{pos} T lymphocytes (Laird et al., 2015; Martinez-Bonet et al., 2015; Perez et al., 2010). To determine whether the higher HIV protein expression observed with class 1-selective HDIs

could overcome limitations observed with pan-HDIs, we next investigated the effect of bryostatin-1 combinations. To accomplish this, we used an HIV molecular clone that expresses full length Gag and the human placental alkaline phosphatase (PLAP) gene within the HIV *env* open reading frame (HXB2-ΔE-PLAP; **Figure 3.4a and Figure** 3.4**b**). HSPCs latently infected with HXB2-ΔE-PLAP were isolated using magnetic bead sorting to deplete actively infected cells ($PLAP^{pos}$; **Figure 3.4c**). We then treated $PLAP^{neg}$ cells and scored for HIV latency reversal (**Figure 3.4d**).

Using this system, we again observed that all HDIs in combination with bryostatin-1 had similar frequencies of $PLAP^{pos}$ cells (**Figure 3.4e**). In addition, we confirmed that class 1-selective agents induced the highest levels of viral protein per infected cell when used alone, or in combination with bryostatin-1 (**Figure 3.4f**).

We next asked whether the inhibitory effect we observed with pan-HDIs on class 1-selective HDI-mediated E-GFP expression from Figure 3.2 was also observed when added to combinations of bryostatin-1 plus class 1-selective HDIs in this modified latency model system. Indeed, when any of the pan-HDIs tested were added in combination with bryostatin-1 plus a class 1-selective HDI, PLAP MFI was significantly reduced in almost every case (**Figure 3.4f**). An effect that could not be attributed to additive toxicity (**Figure 3.4g**).

Entinostat plus bryostatin-1 maximally induces viral outgrowth and pan-HDIs are inhibitory when added to this combination

Complete HIV latency reversal is considered to be the induction of virus outgrowth from latently infected cells, which is needed for maximal cytopathicity. Therefore, we

tested the ability of LRA combinations to induce virus outgrowth from latently infected HSPCs. To accomplish this, we performed experiments as described in Figure 3.4 and assayed culture supernatants for HIV virion-associated RNA by RT-qPCR with primers specific for genomic HIV mRNA. Alone, HDIs induced only modest viral outgrowth, whereas bryostatin-1 induced more than any HDI ($p < 0.01$; **Figure 3.5**). When combined with bryostatin-1, entinostat significantly increased the potency of viral outgrowth compared to either LRA alone (**Figure 3.5**). When pan-HDIs were added to combinations of bryostatin-1 plus entinostat, we observed significant suppression of viral outgrowth, concordant with the suppressive effects on viral protein production described earlier (compare **Figure 3.5 and Figure 3.4f**). Importantly, latently infected HSPCs treated with triple-LRA combinations demonstrated neither dramatic differences in the frequencies of $PLAP^{pos}$ cells induced, nor significant cellular toxicity following treatment compared to relevant double combinations (**Figure 3.4e Figure 3.4f**). Therefore, entinostat plus bryostatin-1 induced the greatest viral protein production and outgrowth, which was suppressed by co-treatment with pan-HDIs.

Entinostat plus bryostatin-1 induces maximal viral outgrowth and gene expression from latently infected resting $CD4^{pos}$ T cells from optimally-treated HIV-infected individuals

While previous work has demonstrated that pan-HDIs enhance the ability of bryostatin-1 to stimulate cell-associated RNA from patient-derived $rCD4^{pos}$ T cells ex vivo, pan-HDIs did not significantly increase bryostatin-1-induced virus outgrowth (Laird et al., 2015). To determine whether class 1-selective HDIs could achieve this important goal, we treated $rCD4^{pos}$ T lymphocytes obtained from the donors listed in **Table 3.1** with

bryostatin-1 alone or in combination with entinostat and assayed culture supernatants at 24 and 48 hours for virion-associated genomic HIV mRNA. We also measured cell-associated HIV mRNA from cell pellets harvested at 48 hours. Remarkably, in three of four donors, we detected greater virus outgrowth with the combination of bryostatin-1 plus entinostat compared to bryostatin-1 alone (5 to 11-fold; **Figure 3.6a**). Concomitant with this increased viral outgrowth detected in the culture supernatant, we also detected more cell-associated HIV mRNA with bryostatin-1 plus entinostat treatment compared to treatment with bryostatin-1 alone (**Figure 3.6b**). Interestingly, in the one donor from whom we did not detect bryostatin-1-induced virus outgrowth (419000) we nevertheless observed induction of cell-associated HIV mRNA with bryostatin-1 treatment as well as enhanced expression with the combination of bryostatin-1 plus entinostat (**Figure 3.6b**).

In both donors (412406 and 445000) from whom sufficient cells were available, we observed a 2 to 5-fold reduction in viral outgrowth with the addition of vorinostat to entinostat plus bryostatin-1 (**Figure 3.6a**). This was correlated with similar reductions of cell-associated RNA expression (**Figure 3.6b**). Collectively, these data suggest that even in *ex vivo* models, class 1-selective HDIs enhance bryostatin-1-mediated viral outgrowth, whereas pan-HDIs have an inhibitory effect that limits maximum viral gene expression and virion outgrowth.

Entinostat plus bryostatin-1 leads to the greatest reduction of LRA-induced actively infected cells

The ultimate test of the effectiveness of an LRA is the ability to reduce the HIV reservoir. To compare the ability of LRAs to reduce the inducible latent viral reservoir *in*

vitro, we modified our HSPC viral outgrowth assay by washing and culturing LRA-treated cells an additional three days following treatment and quantified the frequency of $PLAP^{pos}$ cells that remained in culture 4 days after treatment (**Figure 3.7a**). We found that the combination of entinostat plus bryostatin-1 was the only combination that led to a significant reduction of infected ($PLAP^{pos}$) cells based on flow cytometry (**Figure 3.7b and Figure** 3.7**c**). Interestingly, we observed an inverse correlation when we compared the frequency of residual $PLAP^{pos}$ cells that remained in culture 4 days after the initial LRA regimen treatment to the potency of latency reversal (**Figure 3.7d**), suggesting that more potent latency reversal is more likely to induce clearance of affected cellular reservoirs. Finally, to compare the amount of residual inducible latent HIV 4 days after initial LRA treatment, we treated every condition with TNF-α and quantified viral outgrowth after 24 hours (**Figure 3.7a**). The only condition that lead to a significant reduction in the relative amount of induced viral outgrowth between the first and second treatments was bryostatin-1 plus entinostat (**Figure 3.7e**). Thus, the capacity of LRAs to maximally induce virus release correlates with likelihood of affected cellular reservoirs being eliminated from culture.

Vorinostat inhibits NF-κB and Hsp90

HDIs regulate the acetylation of many proteins, including non-histone substrates that can negatively affect HIV reactivation and replication (Yang and Seto, 2008). For example, NF-κB, which is required for HIV transcription, is known to be regulated by acetylation. Thus, differential effects of pan- and class 1-selective HDIs on NF-κB could, in part, explain the inhibitory effects of pan-HDIs (Buerki et al., 2008; Chen et al., 2002).

Interestingly, we found that vorinostat significantly inhibited bryostatin-1-induced NF-κB/p65 activation in PBMCs, whereas entinostat did not (**Figure 3.8a**). This modest inhibition of bryostatin-1-mediated NF-κB/p65 activation by vorinostat is concordant with the suppression of viral outgrowth observed for this combination, compared to bryostatin-1 treatment alone (**Figure 3.5 and Figure 3.6**).

Heat shock protein 90 (Hsp90) is a molecular chaperone that is required for the normal functioning of a number of client proteins and recent studies have demonstrated that Hsp90 activity is required for reversal of HIV latency (Anderson et al., 2014; Joshi et al., 2016; O'Keeffe et al., 2000; Pan et al., 2016; Roesch et al., 2012). Additional work has also shown that Hsp90 deacetylation by the class 2 HDAC, HDAC6, activates Hsp90 activity (Bali et al., 2005; Scroggins et al., 2007) and vorinostat, which inhibits HDAC6, causes acetylation of Hsp90 and inhibits its function (Li et al., 2011; Muhlenberg et al., 2009). In contrast, the class 1-selective HDI entinostat does not affect the acetylation of HDAC6 client proteins under the conditions of our assay (**Figure 3.1i**). Consistent with this, an acetylome analysis that compared protein acetylation by entinostat versus vorinostat directly demonstrated that a subset of lysine residues on Hsp90 are acetylated by vorinostat but not by entinostat treatment (Choudhary et al., 2009). To confirm that vorinostat inhibits Hsp90 activity in HIV-infected HSPCs and PBMCs, we assessed Hsp70 levels, which are induced upon inhibition of Hsp90 activity in a heat shock factor-1-dependent pathway (Guo et al., 2005; Isambert et al., 2015; Yong et al., 2016). As a positive control, we treated HSPCs and PBMCs with a specific Hsp90 inhibitor (17-AAG), which induced a dramatic increase in Hsp70, compared to the solvent control (**Figure 3.8b and Figure** 3.8**c**). In addition, we observed that vorinostat, but not entinostat, induced Hsp70

expression in both HSPCs and PBMCs when used in combination with bryostatin-1 (**Figure 3.8b and Figure** 3.8**c**). Treatment with vorinostat alone also induced Hsp70 expression in PBMCs, which was not observed with entinostat treatment (**Figure 3.8c**). Together these results provide evidence that vorinostat but not entinostat inhibits cellular factors necessary for robust viral gene expression and outgrowth.

Discussion

Multiple failed attempts to reduce the viral reservoir *in vivo* using pan-HDI-based LRA regimens emphasizes a need for a change in strategy. Shan and colleagues demonstrated that latency reversal regimens that induced greater viral gene expression led to more rapid death of infected cells by viral cytopathicity (Shan et al., 2012). Here, we describe effects of pan-HDIs that limit potent HIV latency reversal and provide evidence for a superior and effective strategy using class 1-selective HDIs.

We observed potent inhibition of bryostatin-1 plus entinostat-induced viral protein production and outgrowth by all three pan-HDIs tested. Importantly, these effects of pan-HDIs were not due to combined toxicity of the three drugs. In fact, mocetinostat plus bryostatin-1 treatment was the most toxic of the LRA combinations tested and addition of pan-HDIs modestly increased cell viability relative to the two-LRA combination. Interestingly, inhibition by pan-HDIs affected protein expression and viral outgrowth but not the frequency of reactivated cells. As clearance in our study correlated with the amount of protein expressed per cell, our results highlight potential limitations of previously published work that only compared the frequency of proviruses affected by HDI treatment. While it will ultimately be important to reverse latency in all latently infected cells, it is

critical that reversal is sufficient to promote reservoir elimination. Thus, assays measuring potency of viral latency reversal within each cell are important to inform successful LRA regimen development.

In addition, we show that pan-HDIs inhibited at least two cellular factors that are necessary for robust viral gene expression and outgrowth (NF-κB and Hsp90). To our knowledge this is the first study to identify these limitations of pan-HDIs on potent latency reversal and clearance of infected cells. Together, with previously described negative effects of pan-HDIs on cytotoxic T lymphocytes and NK cells (Garrido et al., 2016; Jones et al., 2014; Pace et al., 2016), these results suggest that LRA regimens using pan-HDIs are not likely to lead to robust reservoir elimination *in vivo*.

While we describe a significant limitation of pan-HDIs in reservoir elimination strategies, we also provide evidence for an alternative strategy employing class 1-selective HDIs in combination with bryostatin-1 that maximally induce viral protein production, viral outgrowth, and infected cell death. The demonstration that HDI combinations can promote viral clearance without added cytotoxic T lymphocytes (CTLs) is important because we also, for the first time to our knowledge, demonstrate Nef-dependent downmodulation of cell-surface MHC-I on cells bearing reactivated provirus. If we consider CTL-mediated killing of reservoirs as the major mechanism of reservoir elimination, a paradoxical barrier becomes evident that the most potent latency reversal that promotes robust antigen production and MHC-I loading will simultaneously induce the greatest Nef-dependent MHC-I downmodulation and limit antigen presentation and CTL-killing. Robust viral gene expression is necessary for efficient CTL-mediated killing of reservoirs, a degree of induced viral gene expression that is not achieved by vorinostat

treatment alone (Shan et al., 2012). Therefore, until a specific and effective method to inhibit Nef-dependent MHC-I downmodulation is developed, it will be important to focus on latency reversal strategies that induce robust cytotoxic viral protein production and CTL-independent killing of reservoir cells through cytopathicity, as we show is possible in this study.

With the increasing understanding of the diversity of persistent HIV reservoirs (Chomont et al., 2009; Leng et al., 2014), it is important that any proposed latency reversal regimen affects a diverse pool of reservoir cell subtypes, despite their physiologic differences. Our data demonstrate that the combination of a class 1-selective HDI and a PKC agonist is optimal in both rCD4^{pos} T lymphocytes and HSPCs, which, along with other cell types may constitute a small but important long-lived reservoir *in vivo* (Carter et al., 2010; Sebastian et al., 2017). This result is highly-encouraging especially since HIV latency biology in HSPCs is known to be distinct from that in rCD4^{pos} T cells (McNamara et al., 2012).

Some assumptions and limitations of this study warrant further discussion. First, the selectivity of the HDIs in this study used at the indicated concentrations is an important assumption, but one based on many studies that investigated these HDIs for their ability to inhibit HDAC isoforms at various concentrations. Indeed, entinostat does not significantly inhibit class 2 HDACs even when used at 3-10-fold higher concentrations than used here (Khan et al., 2008; Lauffer et al., 2013). Nevertheless, we confirmed this selectivity in HSPCs at the concentrations used in our assays by demonstrating that vorinostat, but not entinostat, induced tubulin acetylation, which is mediated by the class 2 HDAC, HDAC6.

In addition, our HIV latency model system in HSPCs demonstrates a background of spontaneous latency reversal in the absence of LRAs. We recently determined that this spontaneous latency reversal is associated with HSPC proliferation and differentiation; culture conditions that induce quiescence and prevented differentiation decrease viral gene expression (Painter et al., 2017). Moreover, undifferentiated cells expressing higher levels of the HSPC markers CD34 and CD133 are more likely to remain latent as compared to more lineage-restricted HSPCs with lower expression of HSPCs markers (Painter et al., 2017; Sebastian et al., 2017).

Finally, the clearance assay described in Figure 3.7 also has some limitations as an alternative interpretation is that previously activated ($PLAP^{pos}$) cells treated with entinostat plus bryostatin-1 preferentially revert to $PLAP^{neg}$ cells that are refractory to a second round of latency reversal stimulus. However, we believe this explanation is unlikely as it would require that entinostat plus bryostatin-1 behave in ways that have not previously been described to selectively suppress HIV gene transcription. Attempts to further investigate this possibility by quantifying proviral DNA were confounded by the presence of unintegrated HIV DNA present in all samples, including in the raltegravir-treated control. Nevertheless, the simplest and most logical conclusion based on the potency of latency reversal induced is that the combination of entinostat plus bryostatin-1 killed reactivated HIV-infected cells, clearing them from the culture more rapidly than the other treatment conditions because higher levels of toxic viral proteins were achieved.

In conclusion, while these studies identify a path towards the development of an effective latency reversal regimen, further studies are required to identify less toxic alternatives to bryostatin-1 such as recently identified bryologs that still need to be assessed

in clinical studies as well as other novel, more targeted approaches to latency reversal (Beans et al., 2013; Bosque et al., 2017). Ultimately, the identification of a more effective and more targeted latency reversal regimen is an important step towards the goal of eliminating the viral reservoir in HIV-infected people.

Materials and Methods

Ethics statement

HIV-infected individuals were recruited through the University of Michigan HIVAIDS Treatment Program and the Henry Ford Health System. Written informed consent was obtained according to a protocol approved by the University of Michigan Institutional Review Board and Henry Ford Institutional Review Board (U-M IRB number HUM00004959 and HFH IRB number 7403). Donors were >18 years old, with normal white blood cell counts and plasma viral loads were <48 copies/ml for at least 6 months on antiretroviral therapy. 100 ml of peripheral blood and 20 ml of bone marrow were obtained from each donor. All collected samples were coded. Whole umbilical cord blood from uninfected donors was obtained from the New York Blood Center. All collected samples were anonymized.

HSPC isolation and culture

HSPCs were isolated and cultured as previously described. Briefly, mononuclear cells were isolated from whole umbilical cord blood by Ficoll-Paque (GE Healthcare) density gradient centrifugation and were frozen in BSA (7.5% in PBS; Gibco) and DMSO (10%; Sigma-Aldrich) or used fresh. Mononuclear cells were adherence depleted for 1-2

hours at 37°C in Stemspan II medium (STEMCELL Technologies). $CD133^{pos}$ cells were isolated by magnetic separation using CD133 magnetic beads according to the manufacturer's protocol (Miltenyi Biotec), with the modification that 1.5 times as many magnetic beads were used to increase purity of sorted cells (≥ 90% $CD133^{pos}$). $CD133^{pos}$ cells were cultured at 37°C with 5% CO_2 in Stemspan II medium supplemented with CC110 cytokine cocktail (100 ng/ml stem cell factor, 100 ng/mL thrombopoietin, and 100 ng/mL Flt3 ligand; STEMCELL Technologies) and 100 ng/mL insulin-like growth factor binding protein 2 (R&D Systems).

Resting $CD4^{pos}$ ($rCD4^{pos}$) T lymphocyte isolation and culture

Peripheral blood mononuclear cells (PBMCs) of HIV-infected donors with <48 HIV copies/mL were purified by density gradient centrifugation from whole blood samples as described above and frozen in FBS with 10% DMSO. Frozen PBMCs were thawed and washed twice by drop-wise addition of RPMI medium (Gibco) plus DNase I (Invitrogen). Thawed PBMCs were enriched for $CD4^{pos}$ T lymphocytes by negative selection using a $CD4^{pos}$ T cell isolation kit (Miltenyi Biotec). $CD4^{pos}$ T lymphocytes were further enriched for resting $CD4^{pos}$ T lymphocytes by magnetic bead depletion of cells that expressed CD69, CD25, or HLA-DR (CD69 MicroBead Kit II, CD25 MicroBeads, and HLA-DR MicroBeads, Miltenyi Biotec). Purity of $rCD4^{pos}$ T lymphocytes was assessed by flow cytometry and was greater than 90%. Cells were immediately used in viral outgrowth assays in which $rCD4^{pos}$ T lymphocytes were cultured in T cell culture medium (RPMI supplemented with 10% FBS, 20 mM HEPES (Invitrogen), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine (PSG; Gibco), and 5.6 µg/mL plasmocin).

Virus constructs and infection of HSPCs

NL4-3-ΔGPEN-E-GFP (ΔGPEN) was generated by disrupting Nef expression from NL4-3-ΔGPE-E-GFP (ΔGPE) (McNamara et al., 2012) by Xho1 digestion followed by Klenow polymerase fill-in and re-ligation. Generation of HXB2-ΔE-PLAP has been previously described (Chen et al., 1996). Infectious supernatants were prepared by transfection of proviral plasmids into 293T cells (ATCC) using polyethylenimine. Proviral plasmids were co-transfected with plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G). For plasmids that lacked HIV structural genes (ΔGPE and ΔGPEN), a helper plasmid (pCMV-HIV) was also co-transfected to provide necessary structural proteins *in trans* and permit infectious particle formation. 48 to 72 hours after transfection, culture supernatants were harvested and filtered through a 0.45 μm syringe filter (GE Healthcare). 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), PSG, and 5.6 μg/mL plasmocin (Invivogen). HSPCs were infected by spin inoculation at 1,049 x g for 2 hours at room temperature. Mock infected controls were treated with 293T cell culture medium. Following spin infection, viral supernatant was removed and cells were cultured in STIF medium for 3 days.

Sorting infected HSPCs

HSPCs infected with ΔGPE or ΔGPEN viruses were sorted by fluorescence-activated cell sorting (FACS) using a FACSAria III (BD Biosciences) flow cytometer to remove E-GFP^{pos} (actively infected) cells and enrich for E-GFP^{neg} (latently and uninfected)

cells. Gating was determined based on mock infected control. HSPCs infected with HXB2-ΔE-PLAP were sorted using magnetic beads to isolate PLAP^{neg} cells. Briefly, infected HSPC cultures were incubated on ice in magnetic-activated cell sorting (MACS) buffer (PBS, 0.5% BSA, and 2mM EDTA) for 20 minutes with FcR blocking reagent (Miltenyi Biotec) and biotinylated antibody raised against PLAP (Serotec). Cells were washed with MACS buffer and then incubated at 4^{o}C for 15 minutes in MACS buffer with streptavidin-conjugated magnetic beads (Miltenyi Biotec). Cells were washed, placed on a magnetic column, and cells that did not bind to the column (PLAP^{neg}) were collected. Column-bound cells were also collected by elution with 5 mL of MACS buffer and used to check sort efficiencies.

Treatment of sorted E-GFP^{neg} HSPCs

Sorted, E-GFP^{neg} cells (50,000-80,000 cells) were incubated for 24 hours at 37^{o}C with 5% CO_2 in 200 µL of Stemspan II medium plus 8 µM raltegravir (Selleck Chemicals) with the following drugs and controls: 0.1%[v/v] DMSO (Sigma-Aldrich), 3 ng/mL tumor necrosis factor alpha (TNF-α; R&D Chemicals), 1 µM vorinostat (Cayman Chemical or Selleck Chemicals), 25 nM panobinostat (Selleck Chemicals), 25 nM romidepsin (Selleck Chemicals), 10 µM entinostat (Cayman Chemical or Selleck Chemicals), 10 µM tacedinaline (Selleck Chemicals), 10 µM mocetinostat (Selleck Chemicals). Unless otherwise indicated, concentrations of drugs remained the same in all experimental conditions, including for those testing drug combinations.

Treatment of sorted PLAP^{neg} HSPCs

Sorted, PLAP^{neg} cells (250,000-750,000 cells) were incubated for 24 hours at 37^{o}C with 5% CO_2 in 1 mL of Stemspan II medium plus 8 µM raltegravir and with the following drugs and controls: 0.1%[v/v] DMSO, 3 ng/mL TNF-α, 1 µM vorinostat, 25 nM panobinostat, 25 nM romidepsin, 10 µM entinostat, 10 µM tacedinaline, 10 µM mocetinostat, 5 nM bryostatin-1 (Sigma-Aldrich). Unless otherwise indicated, concentrations of drugs remained the same in all experimental conditions, including for those testing drug combinations.

For experiments presented in Figure 3.7, sorted, PLAP^{neg} cells (800,000-1,000,000 cells) were incubated at 37^{o}C with 5% CO_2 in 1 mL of Stemspan II medium plus 8 µM raltegravir and with the indicated drugs at the concentrations described above. After 24 hours, supernatants were harvested as described below, 10% of cells were collected and stained for flow cytometric analysis, and the rest of the cells were washed once in PBS before being plated in STIF medium (1 mL) and cultured without stimulus for 3 days. After 3 days in culture, 10% of cells were again collected from each sample and stained for flow cytometric analysis. The rest of the cells were re-plated in Stemspan 2 medium with raltegravir and TNF-α at the concentrations described above. After 24 hours, supernatants were harvested and cells were collected and stained for flow analysis.

Treatment of rCD4^{pos} T lymphocytes

Five million rCD4^{pos} T lymphocytes were incubated for 24 and 48 hours in 1 mL of T cell culture medium with the indicated drugs at the following concentrations: 50

ng/mL PMA (Sigma-Aldrich), 1 µM ionomycin (Sigma-Aldrich), 5 nM bryostatin-1, 10 µM entinostat, 1 µM vorinostat.

Measurement of MHC-I downmodulation on treated E-GFP^{neg} HSPCs

E-GFP^{neg} HSPCs from ΔGPE or ΔGPEN infected cultures were isolated by FACS, treated with indicated drugs for 24 hours, and stained for flow analysis. Fold downmodulation of cell surface MHC-I on infected cells was determined by dividing the median fluorescence intensity (MedFI) of the surface MHC-I stain for E-GFP^{neg} cells by that of the E-GFP^{pos} cells for each condition.

Measurement of supernatant HIV mRNA from treated PLAP^{neg} HSPC cultures

Culture supernatants were harvested at 24 hours after addition of drugs and cell debris was removed by centrifugation (700 x g; 5 minutes; 4^{o}C). Supernatant RNA was extracted from clarified supernatant with TRIzol LS reagent according to the manufacturer's protocol (Invitrogen). cDNA synthesis was performed using qScript cDNA Supermix (Quanta Biosciences). Real-time qPCR was performed using TaqMan Gene Expression Mastermix (Applied Biosystems) on an Applied Biosystems 7300 thermocycler, with the following cycling conditions: 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds. Primers and probes have been previously described (Bullen et al., 2014). Molecular standard curve was generated as previously described (Laird et al., 2015).

Measurement of supernatant HIV mRNA from treated rCD4^{pos} T lymphocyte cultures

Culture supernatants were harvested at 24 and 48 hours after addition of drugs and cell debris was removed by centrifugation. Supernatant RNA was extracted from clarified supernatant with Trizol LS reagent according to the manufacturer's protocol. cDNA was synthesized and real-time qPCR was performed as described above with the modification that TaqMan Fast Advanced Mastermix (Applied Biosystems) was used for real-time qPCR.

Measurement of cell-associated HIV mRNA from treated rCD4^{pos} T lymphocytes

Cell-associated HIV RNA transcripts were measured as previously described (Laird et al., 2015). Briefly, cell-associated RNA was extracted from treated rCD4^{pos} T lymphocytes cells with TRIzol reagent according to the manufacturer's protocol. Contaminating genomic DNA was removed by DNase I digest (Invitrogen). cDNA synthesis was performed using qScript cDNA Supermix. Real-time qPCR was performed using TaqMan Fast Advanced Mastermix on an Applied Biosystems 7300 thermocycler. Levels of RNA polymerase II (PolR2A) were measured for each sample as a control. Molecular standard curves for HIV mRNA were generated as described above.

NF-κB/p65 Binding ELISA

Whole cell lysates from treated PBMCs were obtained using a commercial kit according to the manufacturer's protocol (Active Motif). Total protein amounts of lysates were quantified by Bradford assay (Thermo Fisher) and an equal total protein amount was loaded for each sample in subsequent assays. The activated form of the NF-κB monomer

p65 was detected using a commercial kit according to the manufacturer's protocol (Active Motif).

Hsp70 immunoblot

1-3 million HSPCs or PBMCs were treated with the indicated drugs for 24 hours and lysed with Laemmli lysis buffer. Proteins from whole cell lysates were separated by gel electrophoresis, transferred to a membrane and detected using antibodies against Hsp70 (Enzo Life Science), Hsp90 (Abcam), and GAPDH (Sigma-Aldrich).

Acetylated histone H4 and tubulin immunoblot

500,000 HSPCs were treated with the indicated drugs for 9 hours and lysed with Laemmli lysis buffer. Proteins from whole cell lysates were separated by gel electrophoresis, transferred to a membrane and detected using antibodies against acetylated alpha-tubulin (Sigma-Aldrich), acetylated histone-H4(Lys12) (Millipore), and GAPDH (Sigma-Aldrich).

Flow cytometry antibodies and staining

Antibodies raised against the following antigens were used for flow cytometry: CD133 (phycoerythrin (PE) conjugated; Miltenyi Biotec), CD34 (fluorescein isothiocyanate[FITC] conjugated, allophycocyanin (APC) conjugated, and PE-Cy7 conjugated; all from Miltenyi Biotec), hematopoietic lineage markers (eBioscience), MHC-I (clone Bw4 and Bw6, PE-Cy7-conjugated; Miltenyi Biotec), PLAP (eFluor660-conjugated, eBioscience), and HIV Gag (clone KC57, FITC- or PE-conjugated; Beckman Coulter). Flow data were collected with a BD FACS Canto cytometer or a BD FACScan

cytometer with Cytek 6-color upgrade. For staining of surface proteins, cells were suspended in FACS buffer (PBS with 2% FBS, 1% human serum, 2mM HEPES, and 0.025% sodium azide) and antibodies, incubated on ice for 10 minutes (CD34, CD133) or 30 minutes (MHC-I and PLAP), washed, and then fixed in 2% paraformaldehyde in PBS. Intracellular HIV Gag was stained by permeabilizing fixed cells with 0.1% Triton X-100 in PBS for 5 minutes at room temperature, then incubating in FACS buffer with antibody against HIV Gag for 30 minutes at room temperature, and washing before analyzing.

Statistics

Statistical analyses were performed using Microsoft Excel software. The statistical tests used to calculate p-values are indicated in the corresponding figure legend.

Acknowledgements

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Table 3.1. HIV-infected donor characteristics.

Donor ID	Age	Sex	Race	Year of diagnosis	ART regimen	Time on ART (months)	Duration of viral suppression (months)
412406	45	M	W	2007	3TC, AZT, EFV	62	60
419000	53	M	W	1986	FTC, TDF, ATV/r	192	28
445000	59	M	B	2009	FTC, TDF, EFV	32	13
457413402	50	M	B	2011	FTC, TDF, EFV	59	55

M, male; F, female; W, white; B, black; 3TC, lamivudine; AZT, zidovudine; ATV/r, atazanivir boosted with ritonavir; EFV, efavirenz; FTC, emtricitabine; TDF, tenofovir

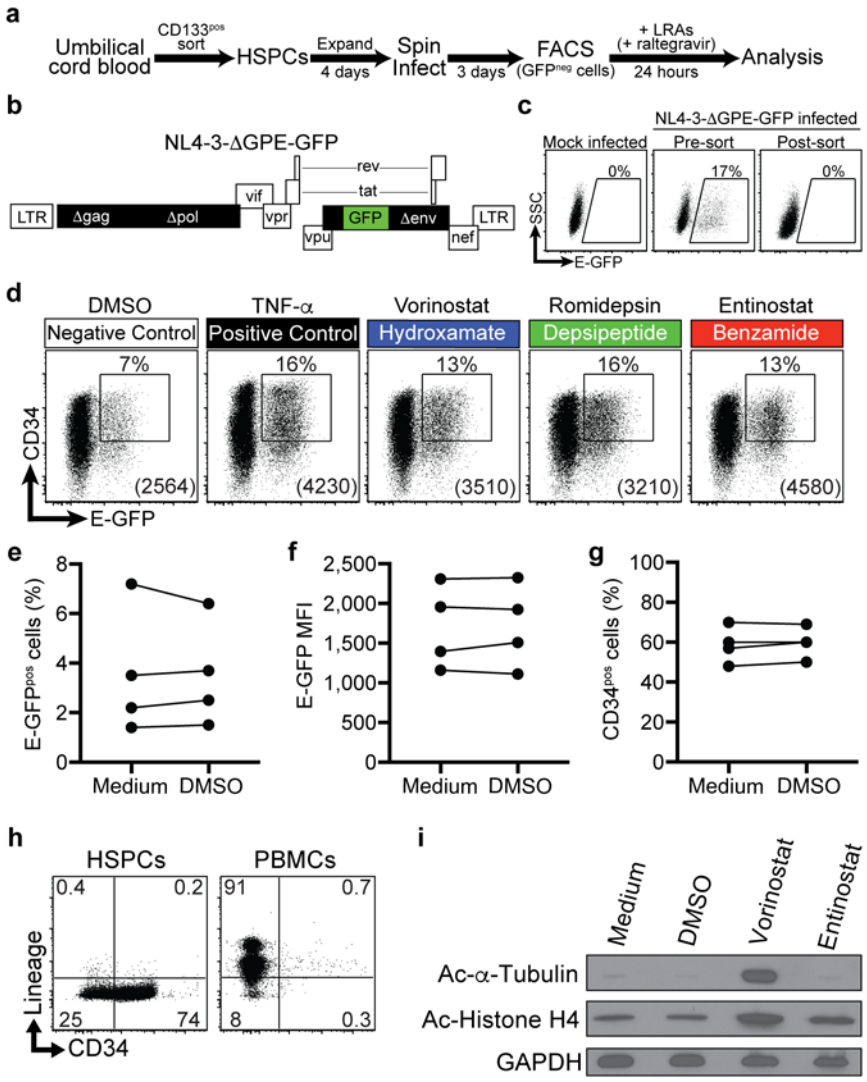

Figure 3.1. Model system of HIV latency in HSPCs. (**a**) Schematic illustration of HIV activation assay in HSPCs. (**b**) Schematic diagram of NL4-3-based HIV molecular clone used for activation studies. Filled black and green rectangles indicate genes that have been deleted from or added to the wild-type molecular clone, respectively. (**c**) Flow cytometric analysis of mock infected and sorted, infected HSPCs used for activation studies. Numbers above gates indicate the frequency of live E-GFP^{pos} cells. Live cells were gated based on FSC and SSC parameters. (**d**) Representative flow cytometric analysis of infected HSPCs treated for 24 hours with the indicated LRAs. Live cells were gated based on FSC, SSC, and 7-AAD. Numbers above gates indicate the frequency of CD34^{pos}, E-GFP^{pos} cells. Italicized numbers in brackets indicate E-GFP MFI of cells within the gate. Gates are based on mock infected cells and staining with an isotype control antibody. Summary graphs showing the frequency of (**e**) E-GFP^{pos} cells, (**f**) E-GFP MFI, and (**g**) frequency of CD34^{pos} HSPCs following 24 hour treatment with medium and 0.1% (v/v) DMSO. (**h**) Flow cytometric analysis of HSPCs and PBMCs for cell surface CD34 and lineage markers. (**i**)

Immunoblot analysis of whole cell lysates from HSPCs treated for 9 hours with indicated conditions demonstrating HDI selectivity.

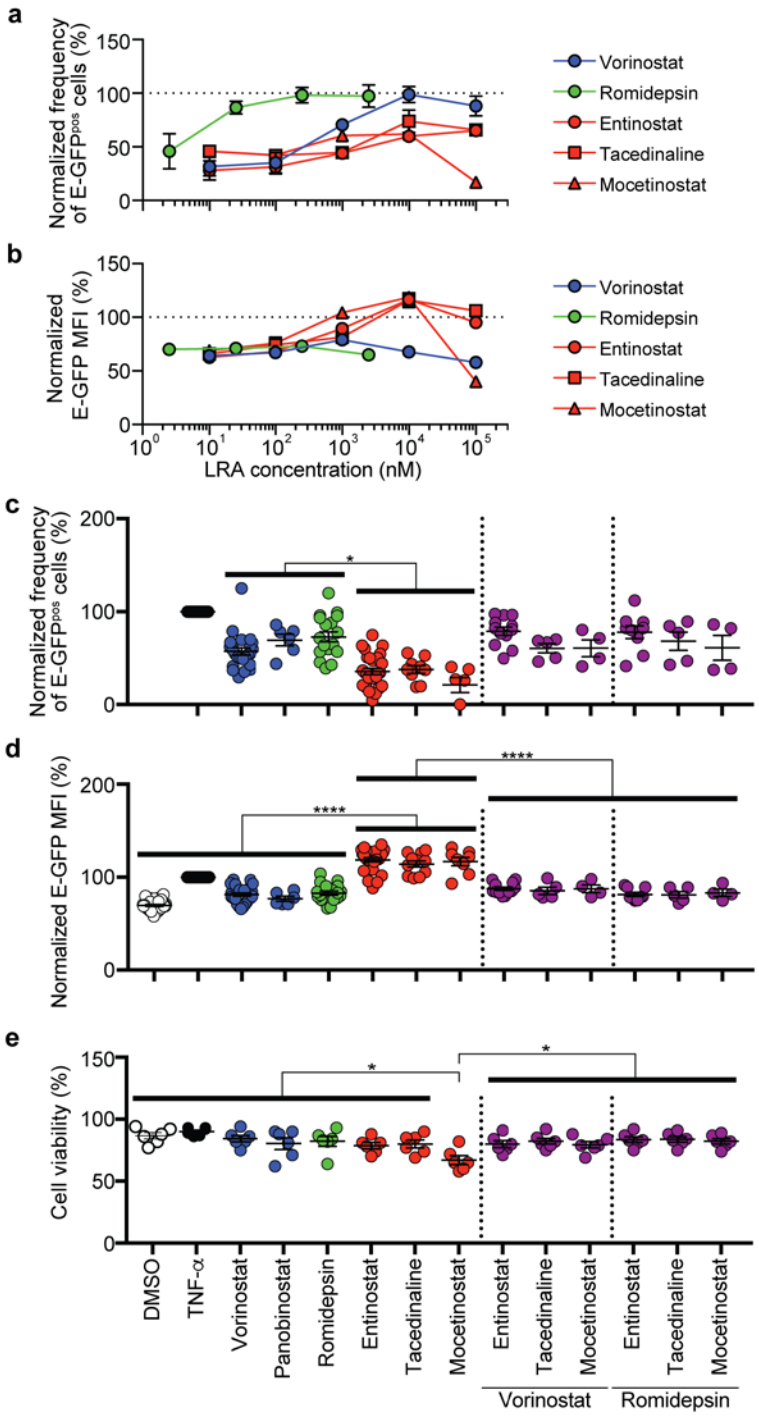

Figure 3.2. HDI combinations reveal an inhibitory effect of pan-HDIs that limits HIV protein production in HSPCs. Summary graphs of (**a**) frequency of E-GFPpos, CD34pos HSPCs and (**b**) E-GFP MFI in CD34pos cells bearing activated HIV following 24 hour treatment over the range of HDI concentrations indicated (mean ± s.e.m., n = 3). (**c**) Summary graph of the frequency of E-GFPpos, CD34pos cells following treatment with the indicated LRAs at the concentrations described in Materials and Methods and used in all subsequent assays. The frequency of spontaneous reactivation observed in DMSO solvent conditions was subtracted from each experiment to reflect the actual frequency of

reactivated provirus. Each symbol represents data from an independent experiment (mean ± s.e.m., $n \geq 4$). (**d**) Summary graph of E-GFP MFI in E-GFPpos, CD34pos cells (mean ± s.e.m., $n \geq 4$). (**e**) Summary graph of frequency of viable HSPCs following 24 hour treatment with HDIs alone and in combinations at the concentrations used for HIV latency-reversal studies. Cell viability was calculated using percentage of cells within live cell gating strategy based on FSC, SSC, and 7-AAD. Data are presented as a percentage of the effect of TNF-α. P values were calculated by two-tailed, unpaired Student's t-test. *$p < 0.05$, ****$p < 0.0001$. Solid bars indicate statistically significant differences between groups of conditions.

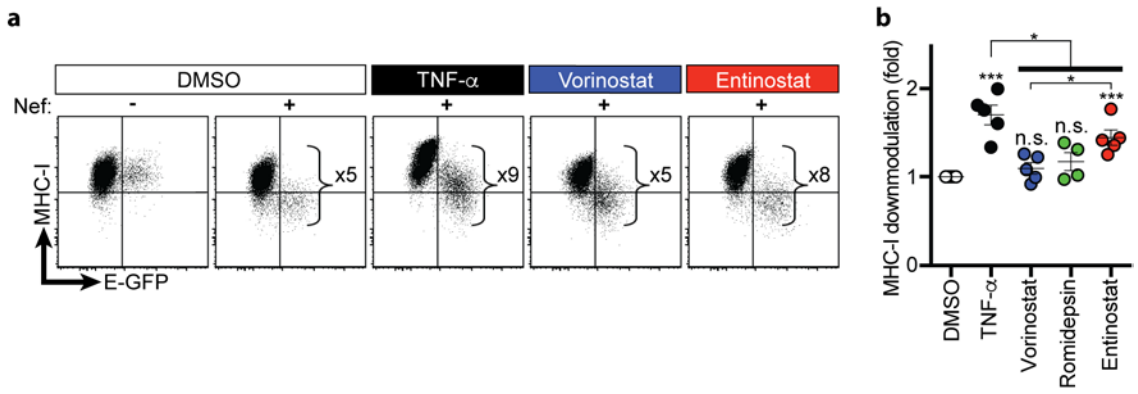

Figure 3.3. Entinostat induces more Nef-dependent downmodulation of MHC-I than pan-HDIs. (**a**) Representative flow cytometric analysis of MHC-I downmodulation on latently infected HSPCs treated with the indicated LRAs. The magnitude of MHC-I downmodulation in HSPCs with induced viral gene expression was calculated by dividing the MHC-I median fluorescence intensity (MedFI) of the E-GFP^{neg} population by that of the E-GFP^{pos} population for each sample (E-GFP$^{neg}{}_{\text{MHC-I MedFI}}$ / E-GFP$^{pos}{}_{\text{MHC-I MedFI}}$). (**b**) Summary graph of fold MHC-I downmodulation. Data are presented relative to DMSO (mean ± s.e.m., $n \geq 4$). Class 1-selective HDIs are shown in red and pan-HDIs are shown in green and blue. P values were calculated by two-tailed, unpaired Student's t-test. *$p < 0.05$, ***$p < 0.001$. Asterisks above each condition indicates statistically significant difference compared to DMSO. Solid bar indicates statistically significant differences between groups of conditions.

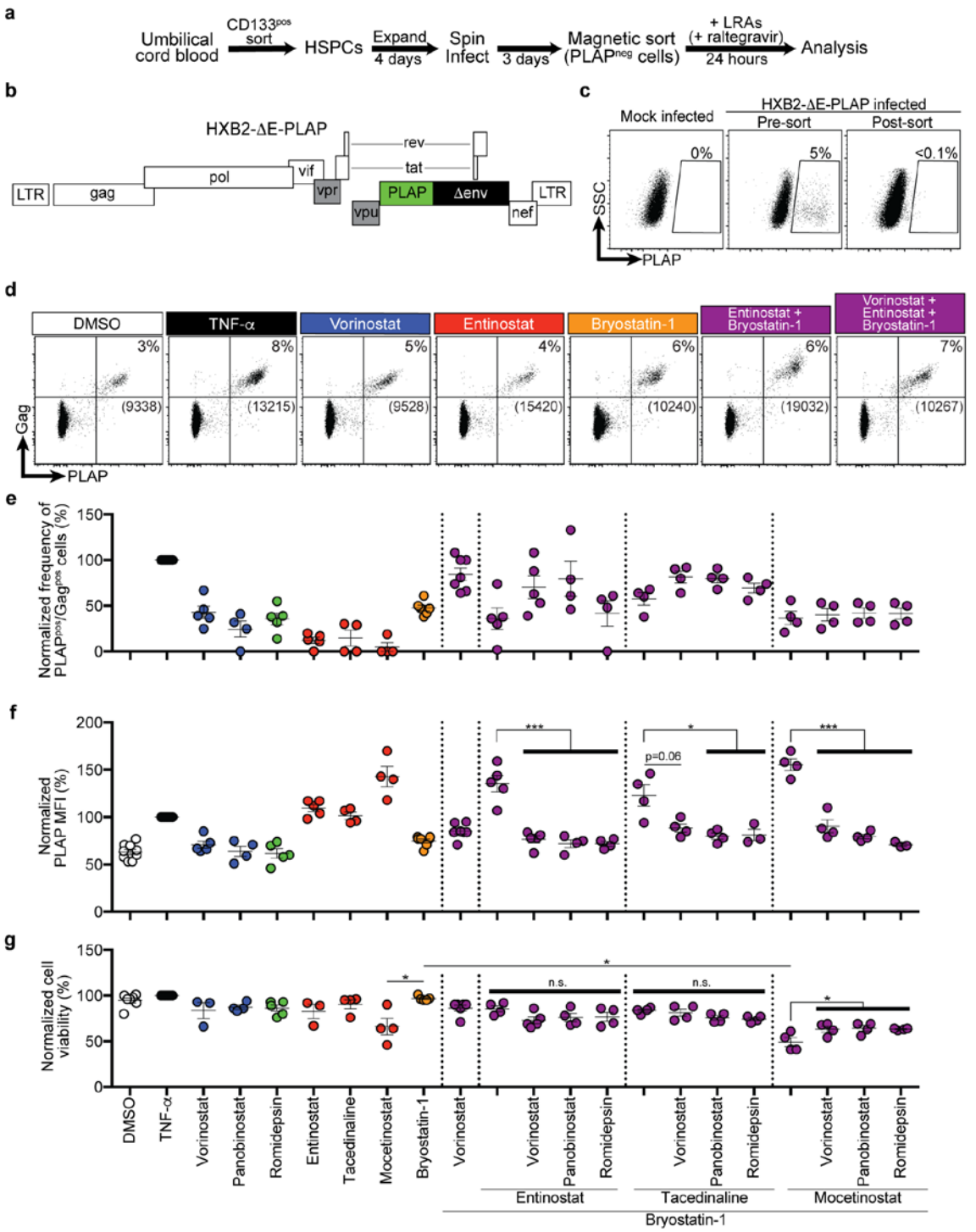

Figure 3.4. Class 1-selective HDIs plus bryostatin-1 maximally induce latency-reversal and viral protein production, whereas pan-HDIs are inhibitory when added to these combinations. (**a**) Schematic illustration of modified HIV latency-reversal model system in HSPCs. (**b**) Schematic diagram of HXB2-based HIV molecular clone. Filled black and green rectangles indicate genes that have been deleted from or added to the wild-type molecular clone, respectively. Filled gray rectangles indicate genes that are dysfunctional in the wild-type HXB2 molecular clone. (**c**) Flow cytometric analysis of

mock infected and sorted, infected HSPCs. Numbers above gates indicate the frequency of live $PLAP^{pos}$ cells. Live cells were gated based on FSC and SSC parameters. (**d**) Representative flow cytometric analysis of infected HSPCs treated for 24 hours with the indicated LRAs. Live cells were gated based on FSC and SSC. Numbers above gates indicate the frequency of $PLAP^{pos}$, Gag^{pos} cells. Italicized numbers in brackets indicate PLAP MFI of cells within the double-positive gate. Gates are based on mock infected cells and staining with an isotype control antibody. Summary graphs of (**e**) frequency of $PLAP^{pos}$, Gag^{pos} cells; (**f**) PLAP MFI of $PLAP^{pos}$, Gag^{pos} cells; and (**g**) relative cell viability following treatment with the indicated LRAs. The frequency of spontaneous reactivation observed in DMSO solvent conditions was subtracted from each experiment to reflect the actual frequency of reactivated provirus. Data are presented as a percentage of the effect of TNF-α (mean ± s.e.m., $n \geq 3$). P values were calculated by two-tailed, unpaired Student's t-test. *$p < 0.05$, ***$p < 0.001$.

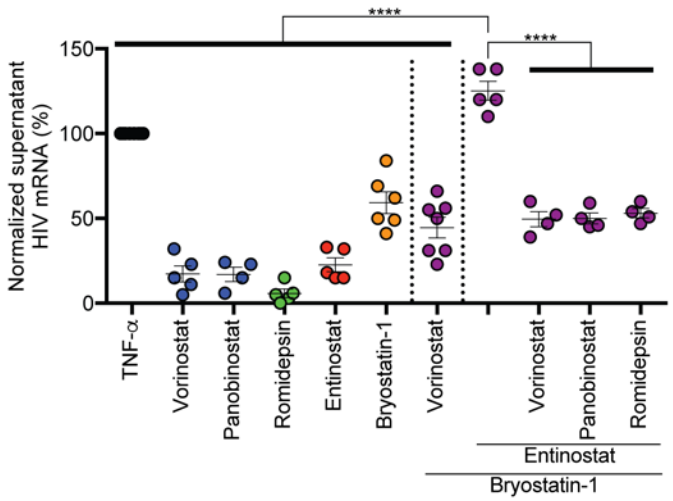

Figure 3.5. Entinostat plus bryostatin-1 induces maximum viral outgrowth from latently infected cells, whereas pan-HDIs are inhibitory when added in combination. Summary graph of genomic HIV mRNA copies/mL measured in supernatants from cultures of latently infected HSPCs treated as described in Figure 3.4. Data are presented as a percentage of the effect of TNF-α (mean ± s.e.m., $n \geq 4$). P values were calculated by two-tailed, unpaired Student's t-test. ****$p < 0.0001$.

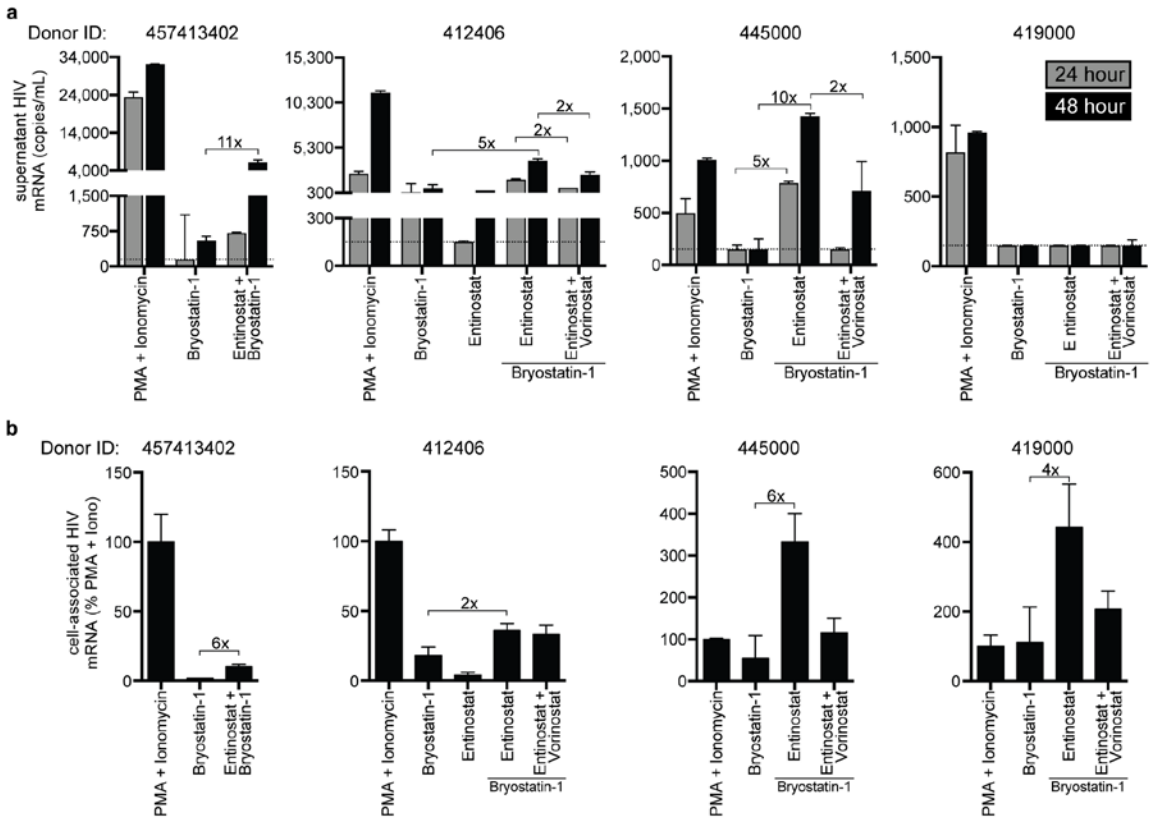

Figure 3.6. Entinostat plus bryostatin-1 induces maximum viral outgrowth and gene expression in latently infected rCD4pos T cells from HIV-infected individuals. (**a**) Graphical analysis of HIV released by patient rCD4^{pos} T cells following treatment with indicated LRAs (mean ± s.d. of experimental duplicates). Limit of quantification is indicated by dotted gray horizontal line in panel. (**b**) Induced cell-associated HIV mRNA in rCD4^{pos} T cells treated for 48 hours with indicated LRAs (mean ± s.d. of experimental duplicates).

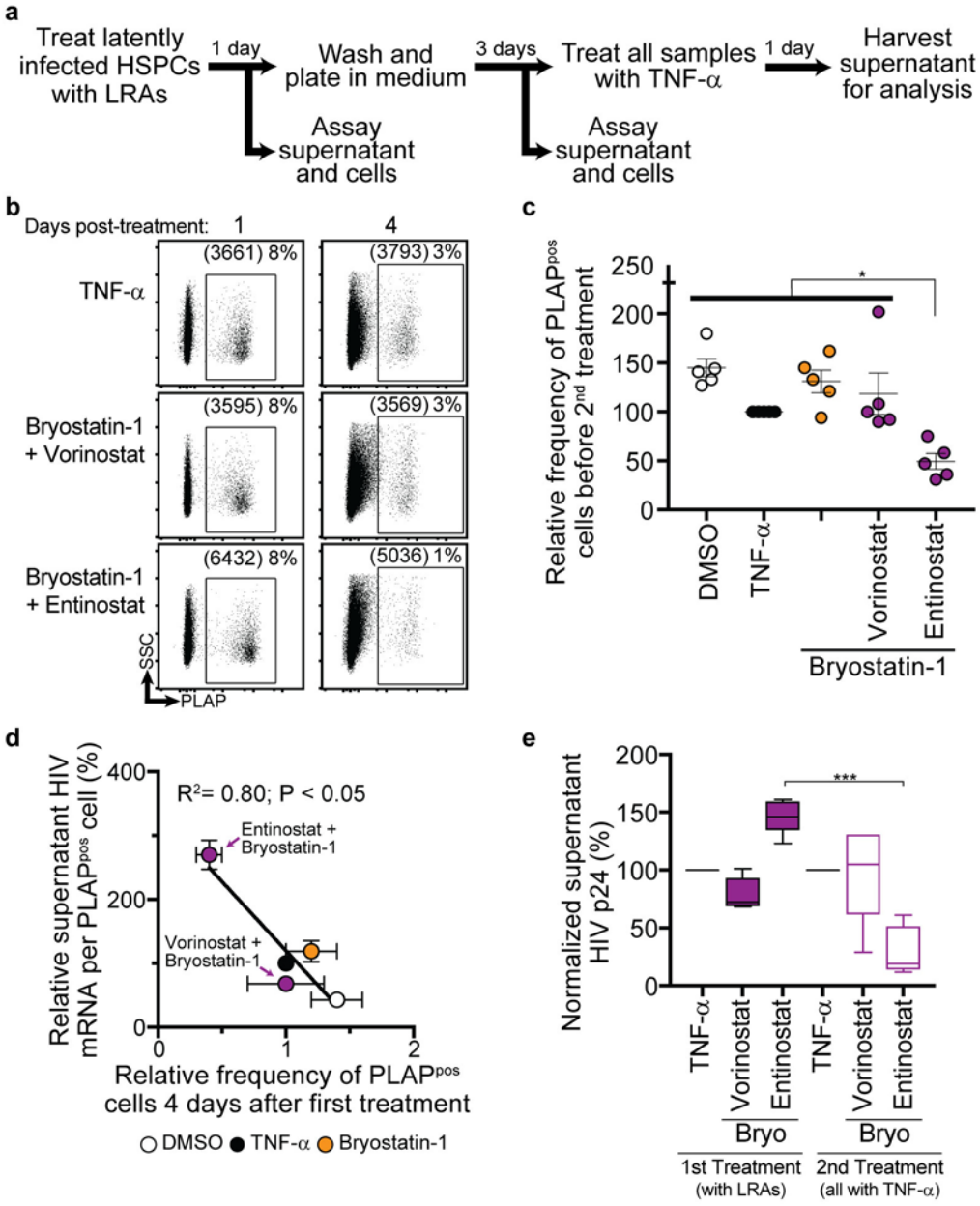

Figure 3.7. Potency of latency-reversal is correlated with survival of affected cells bearing reactivated HIV. (**a**) Schematic of *in vitro* assay to test the survival of latently-infected HSPCs following treatment with LRAs (**b**) Representative flow cytometric analysis of HSPCs 1 and 4 days after treatment with LRAs. (**c**) Summary graph showing the relative frequency of residual PLAP^{pos} cells in culture four days post-LRA treatment. (**d**) Summary graph showing inverse correlation between frequency of residual infected cells on 4 days after LRA treatment and potency of induced viral outgrowth by the indicated LRAs. Data were fit to a least-squares nonlinear regression. (**e**) Box and whisker plot (5-95 percentiles) of virus released into culture supernatant by HSPCs treated with TNF-α four days after initial LRA treatment. To facilitate comparison across multiple

experiments, data were normalized to TNF-α, n=5). P values were calculated by two-tailed, unpaired Student's t-test. *$p < 0.05$, ***$p < 0.001$.

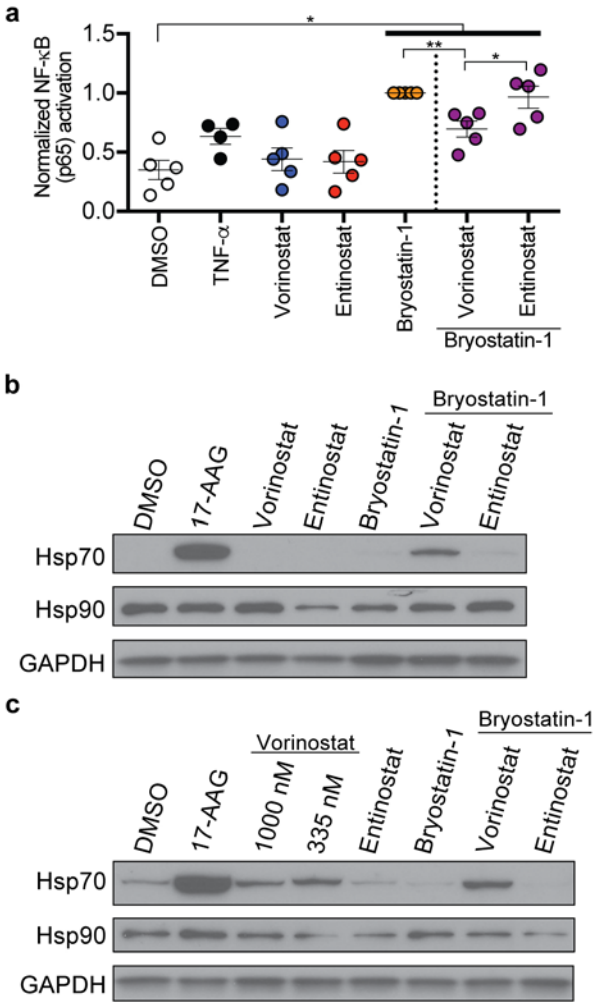

Figure 3.8. Vorinostat inhibits cellular factors important for robust HIV gene expression. (**a**) Summary graph showing abundance of activated NF-κB/p65 in PBMCs treated for 24 hours with indicated LRAs. Data were normalized to the effect bryostatin-1 (mean ± s.e.m., n ≥ 4). Immunoblot analysis of whole cell lysates from HSPCs (**b**) and PBMCs (**c**) treated for 24 hours with indicated LRAs. P values were calculated by two-tailed, unpaired Student's t-test. *p < 0.05, **p < 0.01.

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Chapter 4

Discussion

Durable remission of HIV disease without the need for antiretroviral therapy is an unattained objective for HIV cure research. The establishment of a persistent, latently-infected HIV reservoir of long-lived cells remains the greatest barrier to achieving this goal. Therefore, it is imperative to better understand and characterize the HIV reservoir and develop strategies to eliminate sources of persistent virus.

Work, thus far, on understanding the HIV reservoir has focused on the contribution of rCD4$^+$ T cells; however, evidence suggests that non-CD4$^+$ T cell reservoirs also exist. Previously, our group demonstrated that HSPCs are persistently infected in optimally-treated, HIV-infected people (Carter et al., 2011; McNamara et al., 2013; Sebastian et al., 2017). In chapter 2, I described our recent evidence demonstrating that HIV-infected HSPCs contribute to residual viremia in HIV-infected individuals. Furthermore, we provide evidence that large clusters of HSPC-associated identical [HIV] proviruses (CHIPs) from PBMCs, CD4$^+$ T cells, and BMMCs, may arise due to differentiation of an originally infected HSPCs. We also demonstrate that the HSPC reservoir of HIV provirus is uniquely functional and CHIPs are often genetically identical to predominant plasma virus sequences.

Potent HIV latency reversal aims to promote the reduction of the viral reservoir. Thus far, no proposed LRA has achieved this goal *in vivo*. In chapter 3, I described our

recently published work that demonstrates that class 1-selective HDIs can act additively with the PKC agonist bryostatin-1 to potently induce HIV reactivation in both latently-infected HSPC and rCD4$^+$ T cells from optimally-treated individuals. Furthermore, we provide evidence that while pan-HDIs can reactivate latent HIV, they suppress robust viral gene expression, in part, due to their inhibitory effects on the pro-viral cellular factors NF-κB and Hsp90. Finally, we demonstrate that the potent LRA combination of bryostatin-1 plus the class 1-selective HDI entinostat also promotes a dramatic elimination of latently-infected cells *in vitro*, whereas less potent, pan-HDI-containing combinations do not.

Together, these findings provide a more comprehensive understanding of HIV reservoir targets and strategies with which to eliminate persistent viral reservoirs. In this chapter, I will outline and discuss the significance of our findings within the current body of literature and describe future directions that can address the exciting new questions that are raised by our discoveries.

The HIV reservoir

A relatively high proportion of HSPC-derived HIV proviruses are functional

In chapter 2, I described our work to characterize the HIV reservoir within HSPCs from 43 treated, HIV-infected individuals who had clinically undetectable viremia for at least 6 months. From 13 of these donors, we were able to detect and fully analyze 18 HSPC-derived near full-length HIV proviral genomes. A surprisingly large proportion of these near full-length genomes were determined to contain intact open reading frames for all 9 viral genes and are likely replication-competent proviruses (5 of 18; 28%). Compared to estimates of the proportion of functional proviruses within rCD4$^+$ T cells (2-12%), it

appears that the HSPC-derived HIV reservoir may bear a greater proportion of replication competent and physiologically relevant proviruses (Bruner et al., 2016; Hiener et al., 2017; Ho et al., 2013). Importantly, no study that has investigated near full-length HIV proviral genomes, including ours, is methodologically directly comparable. Ho et al. initially asked whether HIV proviruses that could not be reactivated *ex vivo* contained functional genomes that would be capable of virus outgrowth. Therefore, the $CD4^+$ T cells from which their near full-length proviral genomes were derived had been extensively manipulated, including treatment with T cell activating stimuli and prolonged *in vitro* culture. Moreover, Ho et al. only fully analyzed near full-length sequences that had been positive by their initial screen for a *gag* sub-genomic fragment. This may have led to an overestimation of the frequency of intact genomes due to the omission of amplicons that were *gag*-negative and likely non-functional by their initial screen. Of the three studies conducted using patient-derived $CD4^+$ T cells, the estimated size of the functional HIV reservoir within $CD4^+$ T cells proposed by Ho et al. is the greatest and most discordant (12%). The other two studies that also estimated the size of the functional $CD4^+$ T cell reservoir used more unbiased methods to analyze their samples and report proportions that are much more concordant (2% and 5%) (Bruner et al., 2016; Hiener et al., 2017). Because we initially screened all near full-length first round PCR products with primers that amplify a sub-genomic fragment of HIV *env*, we may also be overestimating the proportion of intact HSPC-derived HIV proviruses; however, our samples were neither cultured nor exposed to any stimuli beyond those experienced during magnetic sorting, unlike cells analyzed by Ho and colleagues.

A study to compare the frequencies of intact near full-length genomes in multiple cell types from a large cohort of optimally-treated individuals has been proposed by our group. By performing all assays in exactly the same manner for samples of different viral sources, this work will permit the most direct comparison of the frequencies of intact near full-length proviruses from HSPCs, PBMCs, and $CD4^+$ T cells.

HIV-infected HSPCs contribute to residual plasma viremia

We also found that HSPC-derived provirus exactly matched plasma virus sequences in one third of the donors from whom we isolated both sources of virus. We discovered that HSPC-derived proviral sequences were about 3-fold more represented within plasma virus sequences compared to PBMC- or BMMC-derived proviral sequences. Previous studies examining the $CD4^+$ T cell proviral reservoir have consistently reported that these proviral sequences are underrepresented within residual plasma virus sequences (Bailey et al., 2006; Brennan et al., 2009; Chun et al., 2000). Moreover, others also demonstrated that $CD4^+$ T cell-derived provirus and plasma virus sequences were, in fact, genetically compartmentalized (Sahu et al., 2009; Sahu et al., 2010).

Whether HSPCs can contribute directly to residual and rebound viremia by viral outgrowth is an intriguing question. *In vitro* stimulation of myeloid differentiation of patient-derived HSPCs has been shown to induce intracellular HIV Gag expression and lead to rapid cell death (Carter et al., 2010). Therefore, differentiating HIV-infected HSPCs may reactivate latent provirus, produce new infectious virions, and lead to cell death (**Figure 4.1**). Using stimuli that do not promote HSPC differentiation, we have failed to detect inducible *ex vivo* viral outgrowth directly from highly purified, patient-derived

HSPCs. Based on our data in rCD4$^+$ T cells from optimally-treated people and in *in vitro* latently-infected HSPCs, it is unlikely that our stimuli were insufficient to induce latency reversal in HSPCs. Instead, it is more likely that our HSPC *ex vivo* viral outgrowth assays used far too few cells to have any reasonable level of confidence that an intact and inducible provirus was present in our pool of treated cells. Based on the rate of detected HIV infection of HSPCs *in vivo*, the frequency of predicted intact/functional HSPC-derived proviral genomes, and the frequency of rCD4$^+$ T cell-derived proviruses that are inducible by a single activating treatment (Ho et al., 2013), we estimate that over 60 million patient-derived HSPCs would need to be treated to have a 95% chance of detecting a single inducible, replication-competent provirus. Our current study design is unable to provide such a large number of HSPCs even if pooled from several donors. That is why future directions include modification of our study design to harvest larger volumes of bone marrow tissue and investigation of SIV *ex vivo* viral outgrowth from HSPCs obtained during necropsy of SIV-infected, cART-treated rhesus macaques.

Additionally, given the lack of evidence for a clonal HSPC-derived proviral reservoir, it is unlikely that we could have identified identical HSPC-derived and plasma virus sequences since induction of viral outgrowth from an infected HSPCs likely also lead to the death and elimination of that cell and proviral sequence from the HSPC reservoir. On the other hand, extensive evidence for the clonal expansion of the rCD4$^+$ T cell reservoir has been described (Chomont et al., 2009; Cohn et al., 2015; Maldarelli et al., 2014; Simonetti et al., 2016; Vandergeeten et al., 2012). In two of these studies, the authors present evidence that homeostatic proliferation of rCD4$^+$ T cells is at least one mechanism by which this reservoir may be expanded (Chomont et al., 2009; Vandergeeten et al., 2012).

Interestingly, *in vitro* induction of homeostatic proliferation in a subset of latently-infected rCD4$^+$ T cells has failed to induce HIV reactivation (Bosque et al., 2011; Wang et al., 2018). Therefore, it is possible that homeostatic proliferation and self-renewal of HSPCs may not reactivate HIV gene expression as potently as myeloid differentiation and allow for the expansion of a clonal HSPC reservoir of persistent HIV. Future studies looking at significantly more patient-derived HSPCs will be more powered to determine whether the HSPC-derived proviral reservoir is indeed unique as we have described or does establish a pool of clonally-expanded HIV-infected HSPCs.

Evidence in support of an HSPC origin for part of the HIV reservoir

One explanation for the identification of identical sub-genomic proviral sequences within HSPCs and other cellular compartments is coincidental infection of these different cells with genetically similar virus. We identified a 112 bp deletion of a portion of the HIV 5' LTR found within proviral sequences from a CHIP and PPC sequences that is predicted to allow proviral viral gene expression and outgrowth, but render newly produced virus non-infectious due to the deletion of the *pbs*, which is necessary for reverse transcription initiation in subsequent infection events. Therefore, it is most likely that this clonal viral reservoir within CHIPs originates from an initial infection event of an HSPC that then differentiated to give rise to a pool of mature peripheral blood cells. Further proliferation of these mature cellular reservoirs of HIV may further expand this CHIP (**Figure 4.1**).

The observation of identical CHIP and PPC sequences with a signature deletion spanning the *pbs* raises two intriguing possibilities to explain this discovery. First, the propagation of the CHIP by several possible mechanisms including HSPC self-renewal,

homeostatic T cell proliferation, and antigenic-mediated T cell proliferation would yield increasingly more proviral clones. Each of these expanded clones has the potential to stochastically provide the necessary stimuli for HIV latency reversal and lead to viral outgrowth. Therefore, the likelihood of a CHIP contributing to a PPC is directly correlated to the population of the CHIP (**Figure 4.1**).

Second, deletion of the HIV *pbs* in these CHIPs may promote greater basal viral gene transcription and outgrowth. A number of studies have demonstrated *trans*-acting factor-mediated, *pbs*-dependent repression of basal gene transcription of related retroviruses murine leukemia viruses (MLVs) in murine embryonic stem cells (Wolf et al., 2008a; Wolf and Goff, 2008; Wolf and Goff, 2009; Wolf et al., 2008b). Furthermore, disruption of the 18 bp *pbs* sequence even by a single nucleotide mutation near the 3' end of the motif has been shown to promote MLVs gene expression (Barklis et al., 1986). Our group has already begun designing experiments to test the hypothesis that the HIV pbs mediates viral gene repression through an, as yet unidentified, trans-factor, which limits robust viral outgrowth *in vivo*. We plan to perform *in vitro* experiments to compare the effects of *pbs* mutations on HIV gene expression in transfected HSPCs and $CD4^+$ T cells with and without stem cell-like characteristics from healthy donors (Buzon et al., 2014; Cieri et al., 2013; Gattinoni et al., 2011). In addition, sequencing 5' HIV LTRs of donor proviruses from various cell types has already begun. By accumulating these sequences of the non-coding region of HIV proviruses, we can investigate whether there is a correlation between 5' LTR mutations, especially within the *pbs*, and the size of the pool of genetically identical plasma virus.

Potent latency reversal as a means to reduce the HIV reservoir

Class 1-selective HDIs induce potent HIV latency reversal when combined with the PKC agonist bryostatin-1

In chapter 3, I described our work investigating the relative abilities of pan-HDIs and class 1-selective HDIs to potently reverse HIV latency and promote elimination of infected cells. We found that, in our model of HIV latency, class 1-selective HDIs were superior to pan-HDIs in their ability to induce potent viral gene expression. We also showed that class 1-selective HDIs, but not pan-HDIs acted additively with bryostatin-1 to induce the most robust viral outgrowth from our latently infected cells and from patient-derived rCD4$^+$ T cells. Furthermore, we provide evidence that inhibition of important pro-viral cellular factors is unique feature of pan-HDI treatment.

Our model of HIV latency within HSPCs is unique from those established in primary CD4$^+$ T cells in that we reliably obtain a large population of latently-infected primary cells that have not been manipulated beyond magnetic cell sorting with which to perform our experiments (Bosque and Planelles, 2009; Saleh et al., 2007; Swiggard et al., 2005; Tyagi et al., 2010; Yang et al., 2009). Nevertheless, our results for the effects of HDIs alone on HIV latency reversal are consistent with previous reports, which demonstrate consistent reactivation of latent HIV from various cell line and primary CD4$^+$ T cell models of HIV latency (Archin et al., 2009a; Archin et al., 2009b; Savarino et al., 2009; Wei et al., 2014; Wightman et al., 2013; Ylisastigui et al., 2004). Most of these studies; however, measured HIV latency reversal by the frequency of infected cell that bore reactivated virus rather than the quality or potency of the reactivation that was induced. Therefore, the class 1-selective HDI entinostat was only shown to reactivate HIV latency

in a relatively smaller frequency of latently-infected cells compared to pan-HDIs and the remarkable effect of entinostat on potent viral protein production went unappreciated and unreported (Savarino et al., 2009; Wightman et al., 2013).

While we report that reactivation of HIV latency in HSPCs by some proposed LRAs is consistent with effects observed in $CD4^+$ T cell models, it is possible that other candidate LRAs may only be effective in certain infected cell types. We have previously shown that P-TEFb levels are sufficiently high to allow viral gene expression in HSPCs; however, HIV latency with these cells is maintained by very low levels of NF-κB. Therefore, when the P-TEFb activating agent, HMBA, which has shown activity in $CD4^+$ T cell models of HIV latency, was used to treat latently-infected HSPC we observed no reactivation of HIV latency (McNamara et al., 2012). We have also discovered that disulfiram does not affect HIV latency in HSPCs, despite purported effects on latently-infected $CD4^+$ T cells (Doyon et al., 2013; Mohammadi et al., 2014; Xing et al., 2011). Collectively, our results support the use of latency model systems in multiple, relevant cell types to comprehensively test the efficacy of candidate LRAs.

One major limitation of our study and many others is the use of supratherapeutic levels of candidate LRAs. Current evidence for the use of bryostatin-1 to reverse HIV latency has been demonstrated using concentrations almost 100-fold higher than the average maximum serum level of bryostatin-1 observed in patients treated with a safe dose. Moreover, treatment of HIV-infected individuals with this safe dose of bryostatin-1 demonstrated no effect on HIV gene expression or the viral reservoir in vivo (Gutierrez et al., 2016). We show that at 10 μM, class 1-selective HDIs can potently reverse HIV latency; a concentration that is about 25-fold higher than the maximum achievable serum levels

with currently defined safe dosage (Batlevi et al., 2016; Gore et al., 2008; Siu et al., 2008). Therefore, while these proof-of-principle studies are important in delineating potentially effective mechanisms to achieve potent latency reversal, it is important to perform additional studies to understand the practical limitation of these findings and try to develop safer, effective LRAs. Fortunately, there is a clear path to develop clinically safe and potentially effective LRAs based on the knowledge from studies using compounds at supratherapeutic levels. The efficacy of bryostatin-1 as a single LRA has lead at least one group to chemically engineer and create bryostatin-1 analogs ('bryologs'), which have demonstrated significantly greater potency for both PKC agonism and HIV latency reversal in cell and mouse models of HIV latency (DeChristopher et al., 2012; Marsden et al., 2017).

Our data provide the foundation for similar class 1-selective HDI derivative development and discovery. The likelihood that such experiments and studies will occur receive the greatest support from those interested in the use of class 1-selective HDIs for the treatment of various cancers. As a relatively novel category of HDI, class 1-selective HDIs are underrepresented within the current pharmacopeia of anticancer treatment options (Li and Seto, 2016). However, their relative efficacy to suppress and kill specific types of cancerous cells and tumors has inspired the ongoing development and discovery of novel agents (Delcuve et al., 2013). Recently, largazoles and largazole derivatives were identified as a family of microbial-derived class 1-selective HDAC inhibitors that have potent anti-cancer activity and potently reactivate HIV in multiple cell line models of HIV latency at concentrations within the nanomolar range (Bui et al., 2017; Liu et al., 2010). However, the authors of this latter study failed to test the ability of largazole derivatives to induce viral outgrowth from patient samples. For this reason and for another that will be

discussed below, this study is limited in its relevance to the search for effective LRAs, but provides promising proof-of-principle that novel class 1-selective HDIs can be discovered and consistently induce potent HIV reactivation at least in *in vitro* models of latency.

We also observed disparate effects of class 1-selective HDIs on intracellular HIV protein production and viral outgrowth in our model of HIV latency in HSPCs. While entinostat induced the most robust viral protein production of any single LRA tested, alone entinostat demonstrated relatively weak induction viral outgrowth from cells treated in exactly the same manner. One explanation is that despite the potency of viral protein production induced by entinostat, a critical threshold of cellular viral protein abundance necessary for efficient viral outgrowth was not achieved. However, treatment with the PKC agonist bryostatin-1 alone was sufficient to induce significant viral outgrowth despite inducing relatively lower levels of viral protein. This observation may be related to the relative effects of entinostat and bryostatin-1 on NF-κB activation, which is a limiting factor for robust viral gene expression in HSPC (McNamara et al., 2012). Therefore, entinostat and other HDIs may, in fact, require additional stimuli that affect distinct pathways that promote efficient virion production and release. This mutual relationship between distinct categories of LRAs has also recently been demonstrated for HIV latency reversal using benzotriazole derivatives and IL-2 (Bosque et al., 2017). Thus, a rational approach to potent latency reversal using combinations of mechanistically unique LRAs may provide the most effective pathway to clinical success.

Elimination of latently-infected cells is correlated with potency of latency reversal and can be achieved by the combination of a class 1-selective HDI plus bryostatin-1

Individual LRAs have been shown to be ineffective at inducing robust *ex vivo* viral outgrowth and reservoir reduction in vivo (Archin et al., 2014; Bullen et al., 2014; Elliott et al., 2014; Sogaard et al., 2015). Therefore, the use of combinations of mechanistically distinct drugs aims to achieve synergism and permit safe and effective latency reversal. In chapter 3, we demonstrate that combinations of mechanistically distinct LRAs achieved the greatest effect on viral protein production, outgrowth, and reduction of infected cells in our HSPC model of HIV latency. Because of the previously demonstrated failure of vorinostat and other HDACs to induce synergistic latency reversal in combinations with bryostatin-1 (Laird et al., 2015), a recent study focused on combinations of bryostatin-1 and P-TEFb activating agents. Interestingly, the authors report that P-TEFb activating LRAs (HMBA and JQ1) significantly enhanced bryostatin-1-mediated HIV latency reversal from patient-derived PBMCs and CD4+ T cells. Using an *in vitro* model system of HIV latency, the authors also report that the ability of the combinations of bryostatin-1 plus JQ1 or HMBA was, predictably dependent on NF-κB and P-TEFb activation (Darcis et al., 2015). These results, especially the need for activated NF-κB and P-TEFb is precisely the kind of logical approach that future studies of LRA combinations should adopt.

Given the data we present in chapter 3, an intriguing next step would be to test triple-drug combinations for their ability to synergistically induce HIV latency reversal. As Darcis et al. demonstrated, both compounds included in their most effective combinations positively contributed to synergistic HIV latency reversal by activating the pathway they are known to affect. Therefore, I hypothesize that activation of NF-κB by bryostatin-1,

activation of P-TEFb by JQ1 or HMBA, and modification of LTR-associated chromatin structure by class 1-selective HDIs may lead to even greater latency reversal than what either Darcis et al or we have observed (**Figure 4.2**). Issues of combined drug toxicity would be the greatest concern with triple-drug therapy; however, we demonstrated that treatment of latently-infected HSPCs with three compounds did not significantly affect cell viability. Moreover, many of the current cART regimens are composed of three-drug combinations.

A major assumption of the current approach to reduce the HIV reservoir by latency reversal is that some of the infected cells will be cleared due to a robust anti-HIV immunological response by CTLs. However, as I discussed in chapter 1, the HIV accessory protein Nef limits MHC-I-dependent presentation of viral antigen on the cell surface, thereby evading CTL detection and elimination (Collins et al., 1998). There is no logical reason to assume that cellular reservoirs that bear reactivated, replication-competent HIV would behave any differently than the actively infected cells used to discover this paradigm. Indeed, we now have demonstrated, for the first time to our knowledge, that there is a direct correlation between the potency of viral reactivation and Nef-dependent downmodulation of MHC-I. Therefore, the most potent LRAs and LRA combo are also the most likely to most potently limit robust CTL responses against the infected cell. Interestingly, replication-defective proviruses that could be induced to at least express viral antigen, did render their reservoirs susceptible to CTL-mediated killing (Pollack et al., 2017). Interestingly, none of the defective proviruses that this group characterized were able to express Nef based on the lack of CD4 downmodulation on primary $CD4^+$ T cells transfected with patient-derived proviral plasmids (Pollack et al., 2017). Huang et al, also

demonstrated that patient-derived rCD4$^+$ T cells harboring replication-competent provirus exhibit an inherent resistance to elimination by CTLs even when stimulated with potent T cell activating stimulus (Huang et al., 2018). Consistent with previous data describing an HIV genome mutation hotspot in *nef* (Bruner et al., 2016), Huang et al. report a remarkably high frequency of hypermutated *nef* sequences within genomes that contained intact *gag* open reading frames, leading them to propose that these defective proviruses are preferentially eliminated, while intact proviruses may promote Nef-dependent reservoir immunoevasion. Thus, development of small molecules that inhibit Nef activity is an exciting and important field of research. To date, very few candidates have been described. Moreover, their true effectiveness in promoting reservoir elimination has not been tested (Emert-Sedlak et al., 2016; Mujib et al., 2017). Our group has also been searching for small molecules that antagonize HIV Nef and has recently identified 4 highly-purified, natural product extracts that have demonstrated potent inhibition of Nef-mediated downmodulation of MHC-I. It will be interesting when these highly-purified natural product extracts are fully-characterized and used in assays to assess their ability to promote immune elimination of infected cells.

Without an effective Nef inhibitor, immune elimination of the HIV reservoir is limited by the activity of HIV Nef. An alternative process that may reduce the viral reservoir is viral cytopathicity. Because this process is viral protein-dependent, the most potent viral cytopathicity would likely occur in the context of the most potent viral latency reversal. Indeed, we demonstrated that there is a direct correlation between potency of induced latency reversal and elimination of infected HSPC in our *in vitro* model. These data are consistent with a previous study that observed no viral cytopathicity of infected

CD4+ T cells treated with vorinostat (Shan et al., 2012). On the other hand, treatment of these cells with T cell activating stimuli induced relatively more potent viral latency reversal, leading to cytopathicity and a dramatic reduction of infected cells from culture (Shan et al., 2012). This study raised the alarming proposal that candidate LRAs that do not induce global T cell activation may not be potent enough to cause viral cytopathicity and reduce the viral reservoir. However, our results and those of at least one other group have demonstrated *in vitro* elimination of infected primary cells using distinct combinations of LRAs that are unlikely to induce global T cell activation (Bosque et al., 2017). Therefore, it may, indeed be possible to achieve reservoir reduction and avoid toxic global T cell activation. However, whether these *in vitro* effects on infected cells will translate into in vivo efficacy is one of the most important, as yet, unanswered questions raised by our results.

Pan-HDIs inhibit cellular pro-viral factors and suppress potent HIV latency reversal

Finally, we made the discovery that pan-HDIs suppress potent viral latency reversal, which is correlated with their inhibitory effects on important pro-viral cellular factors (NF-κB and Hsp90). Previous studies using pan-HDIs, even in combination with bryostatin-1 were likely unable to observe this effect since the potency of latency reversal induced by bryostatin-1 is relatively lower than that achieved with class 1-selective HDIs plus bryostatin-1. Therefore, the dynamic range over which the inhibitory effect of pan-HDIs could be observed was dramatically smaller (Darcis et al., 2015; Laird et al., 2015). Indeed, even in our HSPC model system of HIV latency, we observed a very small suppressive effect of vorinostat on bryostatin-1-induced viral outgrowth.

HDACs mediate de-acetylation of many non-histone substrates. Our work has bridged a gap in our knowledge on how pan-HDIs affect HIV gene expression by promoting Hsp90 acetylation (Anderson et al., 2014; Bali et al., 2005; Joshi et al., 2016; Li et al., 2011; Muhlenberg et al., 2009; O'Keeffe et al., 2000; Pan et al., 2016; Roesch et al., 2012; Scroggins et al., 2007). In addition to cellular factors affected by HDIs, thorough investigation of the "acetylome" of viral proteins within actively infected cells may lead to a more efficient search for highly-effective HDI LRAs. Based on our current knowledge of viral proteins whose functions are significantly affected by acetylation, the regulatory protein Tat is the most attractive candidate with which to initiate these studies. Tat activity is exquisitely regulated by the acetylation status of two key lysine residues (Dormeyer et al., 2003; Dorr et al., 2002; Kaehlcke et al., 2003; Mujtaba et al., 2002; Ott et al., 2004; Ott et al., 1999). Both the class II HDAC (HDAC6) and the class III HDAC (Sirt1) play roles in deacetylating Tat at specific times during Tat-mediated transcription transactivation (Huo et al., 2011; Pagans et al., 2005). Therefore, understanding the effects of HDIs on this viral protein that is critical for robust viral gene expression would be of great interest and important to designing novel and even more effective candidate LRAs.

Beyond their effects on HIV latency and gene expression, HDIs may have pleiotropic effects on treated cells and tissues, which may confound effective reservoir reduction. Indeed, several studies have now reported that pan-HDIs, especially vorinostat, has multiple deleterious effects with respect to $CD8^+$ T cell and CTL viability, mobility, and immune function; and to NK cell function (Garrido et al., 2016; Jones et al., 2014; Pace et al., 2016). Moreover, another pan-HDI panobinostat was recently shown to transiently increase the proportion of regulatory T cells (T_{regs}) *in vivo* and suppress *ex vivo*

inflammatory responses by whole blood induced by LPS. These results provide further evidence that pan-HDIs may limit potent HIV latency reversal and reservoir elimination by suppressing activation of signal pathways necessary for potent viral gene expression (Cary et al., 2016; Williams et al., 2007) and increasing the proportion of immune-suppressive T_{regs} *in vivo* (Tang and Bluestone, 2008). Therefore, it is critical to elucidate the specific effects of class 1-selective HDIs on immune cell function in order to better understand their practical potential as effective LRAs that may lead to HIV reservoir reduction.

Finally, throughout this entire section I've described data concerning very specific experimental outcomes; however, given our apparent lack of substantial knowledge on the diverse effects of different categories of LRAs on primary immune cells, more collaborative and unbiased studies are called for. The ability of unbiased and "-omic" studies to significantly contribute to the knowledge of a topic is best exemplified by some of the work performed and ongoing on the topic of elite controllers of HIV (Fellay et al., 2009; Fellay et al., 2007; International et al., 2010; Pelak et al., 2010; Zhang et al., 2018; Zhang et al., 2017). One study has compared the "acetylomes" within various cancer cell lines treated with entinostat or vorinostat and the results of this work informed our investigation of the effects of HDIs on Hsp90 activity (Choudhary et al., 2009). Similar unbiased analyses within primary cells treated with different HDIs or other candidate LRAs may shed light on processes affected by these treatments that may affect latency reversal and reservoir reduction.

Conclusions

In this dissertation, I have presented work from our group that has investigated the contribution of HSPCs to the HIV reservoir and residual viremia. We found that, compared to PBMC-derived HIV proviruses, proviruses detected in HSPCs are uniquely represented more frequently within sequences of residual plasma virus and are associated with clusters of HSPC-associated identical proviruses (CHIPs). Furthermore, we identified a signature deletion of the HIV primer binding site within a CHIP and PPC sequences that provides the strongest evidence, thus far, in support of HSPCs as an origin for part of the functional and clinically-significant HIV reservoir. As a result of these discoveries, I have modelled the possible fates of HIV-infected HSPCs *in vivo*, including the contribution and expansion of the HIV reservoir (**Figure 4.1**).

In this dissertation, I have also presented our work investigating of the efficacy of categories of HDIs to reverse HIV latency and promote elimination of latently-infected cells. We found that, compared to broadly-acting pan-HDIs, class 1-selective HDIs reversed HIV latency more potently and were able to act additively with the PKC agonist bryostatin-1 to achieve greater HIV reactivation both in latently-infected HSPCs and in patient-derived rCD4$^+$ T cells. Furthermore, we demonstrate that potent HIV latency reversal induced by the combination of a class 1-selective HDI plus bryostatin-1 lead to the greatest reduction of HIV-infected HSPCs in our *in vitro* model of HIV latency reversal and reservoir elimination. We also confirmed our hypothesis that the relative impotency of pan-HDIs in HIV latency reversal is due, in part, to their inhibitory effects on cellular factors necessary for robust viral gene expression (**Figure 4.2**). These findings suggest a possible explanation for the consistent failure of pan-HDIs to induce HIV latency reversal

either ex vivo or *in vivo*, as well as provide a more rational, targeted, and effective alternative for potent latency reversal using class 1-selective HDIs.

Together, the findings described in this dissertation provide novel information that will inform future research on the HIV reservoir and strategies to eliminate it from infected people. By this, we may be able to significantly improve therapy for HIV infection and lead us closer to a cure.

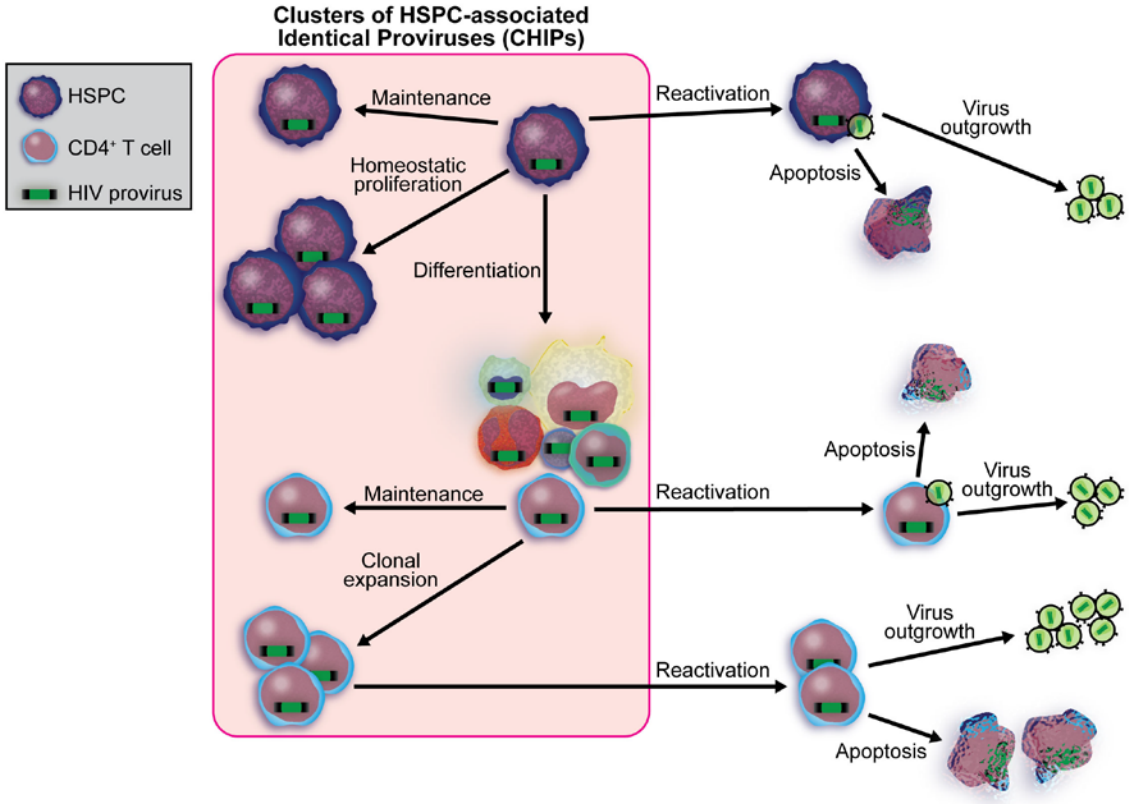

Figure 4.1. Proposed model of the contribution of clusters of HSPC-associated identical proviruses (CHIPs) to the HIV reservoir. HSPCs can be infected *in vivo* and can bear persistent HIV provirus for several years. Possible fates for an infected HSPC are summarized here. (1) As a long-lived cell, an HSPC that bears a latent provirus can avoid elimination due to active HIV infection and can be maintained in its bone marrow niche. (2) Alternatively, homeostatic proliferation may expand the HSPC-derived proviral reservoir. However, there is little evidence for clonal HSPC-derived proviral sequences. (3) Latently-infected HSPCs may also be reactivated and directly contribute to peripheral plasma virus. *In vitro*, HSPCs are capable of robust viral outgrowth. Demonstrating *ex vivo* viral outgrowth from infected HSPCs from optimally-treated people; however, is, currently, impractical due to the large number of HSPCs required to properly perform the assay. (4) Finally, infected HSPCs can differentiate into mature daughter cells including $CD4^+$ T cells. The possible fates of these $CD4^+$ T cells is shown here and discussed in (Sebastian and Collins, 2014). Propagation of the HSPC-derived HIV provirus to 4 or more mature daughter cells represents, as we have defined in our work, a cluster of HSPC-associated identical proviruses (CHIPs). As described in Chapter 2, in cases where a predominant plasma virus clone (PPC) can be attributed to a cellular proviral source, it is very often a CHIP that is identified as the probable source of PPCs.

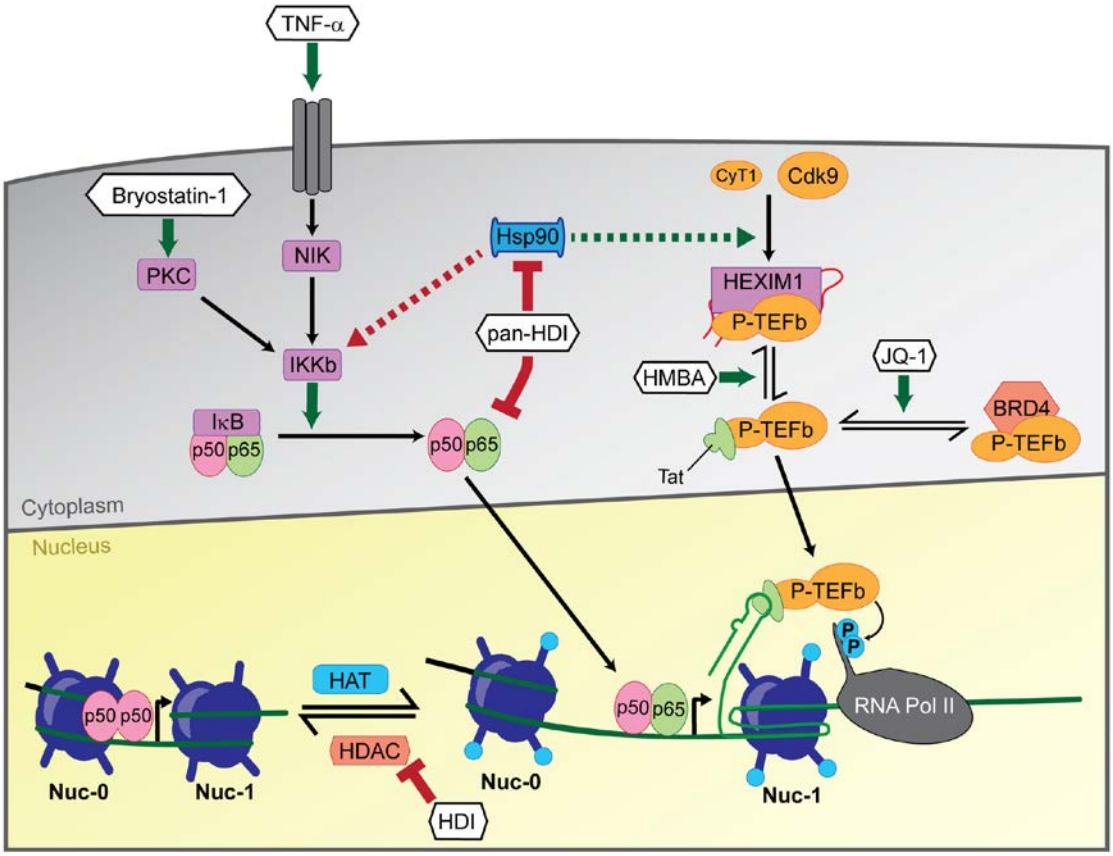

Figure 4.2. Summary of mechanisms of HIV latency reversal and effects of pan-HDIs on Hsp90 and NF-κB. As discussed in Figure 1.5, HIV gene transcription is regulated by several host and viral factors. Reactivation of HIV gene expression has been demonstrated using compounds (white polygons) that perturb these known mechanisms of HIV gene regulation. Activation of the NF-κB pathway can be achieved by the PKC agonist bryostatin-1 or by TNF-α. P-TEFb can become activated and made available for Tat-mediated transactivation by induction of its release from its inhibitory complex in the cytoplasm by HMBA or from the competitive antagonist BRD4. Both pan-HDIs and class 1-selective HDIs affect class 1 HDAC isoforms to promote histone acetylation and euchromatic structure at the HIV promoter. As a result of their relatively broad range, pan-HDIs also affect non-histone targets that are regulated by non-class 1 HDAC isoforms. In Chapter 3, I present evidence that the pan-HDI vorinostat modestly inhibits NF-κB activation and Hsp90 activity, which are both necessary for potent HIV gene expression.

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Appendix

Treatment of latently-infected HSPCs with entinostat plus bryostatin-1 leads to a reduction of the proviral load

In Chapter 3, I investigated the ability of candidate LRAs and LRA combinations to induce robust viral outgrowth and lead to the death and reduction of HIV-infected HSPCs. We demonstrated that the combination of entinostat plus bryostatin-1 induced the greatest reduction of the frequency of residual infected HSPCs and was the only LRA regimen that induced a significant reduction of the residual, TNF-α inducible reservoir (**Figure 3.7**). However, it is possible that these results could be explained by differences in rates of reversion to latency and sensitivity to TNF-α stimulus. Therefore, we next sought to quantify the number of integrated HIV proviruses within HSPCs that had been treated as described in Figure 3.7 using a quantitative PCR assay for Alu-HIV LTR.

Alu elements are the most numerous repetitive sequences that represent about 10% of the human genome. Alu sequences are random distributed throughout the genome about 5,000 bp apart and in random orientation (Lander et al., 2001). Therefore, several Alu PCR assays have been developed to amplify and quantify integrated HIV proviral genomes (Eriksson et al., 2013; Graf and O'Doherty, 2013; Liszewski et al., 2009; Vandergeeten et al., 2014). Amplification is performed using primers specific for the Alu element and the HIV LTR, so that proviruses integrated near Alu elements may be amplified and detected in downstream applications, including qPCR.

To determine whether infected HSPCs from experiments described in Figure 3.7 were being cleared from cultures faster with treatment that most potently induced HIV latency reversal, we performed qPCR assays on Alu-HIV amplicons that were generated as previously described (Vandergeeten et al., 2014). Consistent with our results in Figure 3.7, we observed the greatest reduction in the number of Alu-HIV amplicons detected in cultures of latently-infected HSPCs that were treated with entinostat plus bryostatin-1 (**Figure 0.1**). This result provides additional evidence in support of our conclusion that the combination of entinostat plus bryostatin-1 leads to the death and elimination of infected HSPCs from our *in vitro* model system of HIV latency. It is possible that these results may be explained by differential rates of proliferation of HSPC cell subsets within each treatment condition. This could be addressed in future experiments using a lipophilic, cell-tracking dye such as PKH26, whose fluorescence intensity decreases 2-fold with each symmetric cellular division. Moreover, as Alu elements exist throughout the human genome in both orientations, it will be necessary that future repeats of this experiment also include controls where HIV (Alu only) or Alu (HIV only) primers are left out to appreciate the contribution of first round Alu-Alu amplicons and background, unintegrated HIV DNA.

Methods

Quantification of integrated HIV DNA

DNA from treated HSPCs was isolated using the QIAGEN DNeasy Blood and Tissue Kit according to the manufacturer's protocol (QIAGEN).

Integrated HIV genomes were amplified and quantified as previously described (Vandergeeten et al., 2014). Briefly, integrated HIV DNA was first amplified using two

Alu-specific primers with 5’ Lambda T sequence tags and one HIV LTR-specific primer. The PCR cycle conditions were: 8 min at 95°C, and 12 cycles of amplification (95°C for 1 min, 55°C for 1 min, 72°C for 10 min), followed by 15 min at 72°C. The second round of PCR were performed using the TaqMan Gene Expression Mastermix (Applied Biosystems), primers specific for the Lambda T DNA sequence tag and the HIV U5 LTR, and a probe specific for the R region of the HIV LTR. All reactions were performed on an Applied Biosystems 7300 thermocycler, with the following cycling conditions: 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds

An assay standard curve was generated using DNA from ACH-2 cells which harbor a single copy of integrated HIV provirus (Clouse et al., 1989). Relative cell numbers were quantified using TaqMan Gene Expression Mastermix with the primers and probes specific for the human beta-globin (Forward: 5’-CCCTTGGACCCAGAGGTTCT-3’; Reverse: 5’-CGAGCACTTTCTTGCCATGA-3’; Probe:5’-VIC-ATCTGTCCACTC-CTGATGCTGTTATGGGC-QSY-3’) gene and the same PCR cycle conditions as described above.

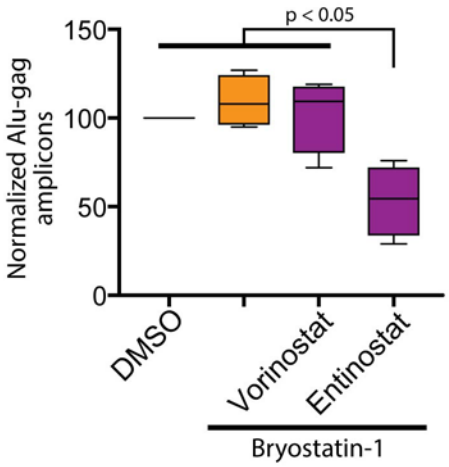

Figure 5.1. Treatment of latently-infected HSPCs with entinostat plus bryostatin-1 leads to a significant decrease in the proviral burden. Box and whisker plot (5-95 percentiles) of Alu-HIV amplicons detected by qPCR from DNA samples isolated treated HSPCs as described in Figure 3.7. Data were normalized to DMSO, n=4). P values were calculated by two-tailed, unpaired Student's t-test.

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