Title
Bromodomain-containing Proteins in HIV Transcription &amp; Latency

Permalink
https://escholarship.org/uc/item/49q648jw

Author
Conrad, Ryan J.

Publication Date
2016

Peer reviewed|Thesis/dissertation
Bromodomain-containing Proteins in HIV Transcription & Latency

by

Ryan James Conrad

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences & Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
Dedication

This dissertation is dedicated to the women in science who inspired and advanced my interests in biochemistry & molecular biology – specifically, in chronological order – Grace Kim, Erica Crespi, Jodi Schwarz, Jennifer Kennell, Deanna Kroetz, Danica Galonic Fujimori, and Melanie Ott.
Acknowledgements

It really takes a village to raise a scientist, and I am grateful to the many people without whom this academic labor would not have been possible. I am foremost appreciative of Melanie Ott, my research advisor – a cunning scientist, a fearless optimist, and overall a talented, compassionate, and extremely affable human being. My thesis committee members Danica Galonic Fujimori, Hiten Madhani, and Eric Verdin all provided essential insight and reinforcement throughout my years as a graduate student. I acknowledge Deanna Kroetz and Alan Frankel for their guidance during my days as a rotation student, and Nadav Ahituv for serving on my qualifying exam committee. I am indebted to Sebastian Schröder, who took me under his wing in Melanie’s lab and taught me nearly everything I know as a bench molecular biologist. Tyler Faust also provided me invaluable practical lab experience, and was all along a great buddy with whom to discuss all things transcription. Aparna Chhibber and Sean Thomas were greatly helpful in positioning me to become somewhat competent as an autonomous bioinformatician. John Carroll is an inspirational and patient graphic designer who is responsible for most of the visual beauty bestowed to the science presented in this work. Veronica Fonseca, Debbie Acoba-Idlebi, Rebecca Brown, Nicole Flowers, and Julia Molla provided necessary administrative and personal support. The majority of experiments in this dissertation were performed listening to the music of Joanna Newsom.

This dissertation encompasses five and a half years of graduate education and research. During my time as a graduate student, Melanie generously presented me with unique opportunities to review relevant fields of research for peer-reviewed publications. Accordingly, four of the chapters herein (Chapters II-V) are adapted directly from previously published review articles. The citations for these publications in the chronological order of their appearance in this text are as follows:


I especially thank Mark Y. Jeng, Daniela Boehm, and Melanie Ott for their contributions as co-authors on these review publications. **Chapter VI** represents the formal narrative of my scientific progress as a doctoral student, and is work currently under peer review for publication. For this chapter, I am grateful to co-authors Parinaz Fozouni, Sean Thomas, Hendrik Sy, and Melanie Ott. **Chapter VII** presents preliminary data summarizing advancement on my first project in the Ott lab, and for this chapter I acknowledge Sebastian Schröder, Qiang Zhang, Martina Schnölzer, Ming-Ming Zhou, and Melanie Ott. I also thank all members of the Ott and Verdin laboratories for their scientific and personal guidance throughout my tenure as a graduate student at the Gladstone Institutes.

There are many scientists whose original contributions could not be directly cited here due to space limitations. This applies primarily to the review articles adapted for inclusion in this dissertation, where individual journals restricted citation numbers. In an effort to not excessively cite, I have limited citations in the first and last two chapters to only those works that have not been cited elsewhere within this text. I apologize to those researchers whose work could not be directly cited within this dissertation.

Lastly, I would like to acknowledge and express gratitude toward members of my personal support system – mom and dad for making me and giving me every chance of success and happiness, davey for his iridescent love and for being a great man, adrian for being the best person ever to go through graduate school & life with, lily, jj, court, eva, lee, jason, hannah, lauren, and john for being my sf family, alan, dennis, jaime, dave, alyssa, erica, janice, and nana for being my real family, and weetz, kev, mallory, madison, teo, and Zachie for being life-long friends.
Bromodomain-containing Proteins in HIV Transcription & Latency

Ryan James Conrad

Thesis Committee:
Melanie Ott (Chair)
Danica Galonic Fujimori
Hiten Madhani
Eric Verdin

Abstract

HIV-1 latency is a phenomenon of transcriptional silencing wherein components of the host epigenetic machinery impose reversible restrictions to viral chromatin. Bromodomain-containing factors are emergent epigenetic effector proteins that recognize acetyl-lysine residues, and have been implicated in the regulation of HIV-1 transcription & latency. This dissertation presents a review of HIV-1 transcription and viral epigenetics, with a particular focus on the roles of protein acetylation and bromodomain-containing proteins in these processes. Original scientific work describing a novel corepressive function of the bromodomain and extraterminal domain (BET) subfamily member bromodomain-containing protein 4 (BRD4) is reported. Specifically, we establish that the understudied short isoform of BRD4 interacts with SWI/SNF chromatin remodeling machines to maintain transcriptional repression of latent HIV-1 via nucleosome positioning. Through genetic and pharmacological loss of function, chromatin biology, and immunoprecipitation experiments, we detail the physical and functional interaction between BRD4 and BRG1, a catalytic SWI/SNF subunit. Although primarily a gene-centered study surrounding the latent HIV-1 promoter, this dissertation also includes high throughput sequencing data establishing genomic co-binding of BRD4 and BRG1 at both coding and non-coding regions that is disrupted under pharmacological inhibition of BET bromodomains. These data i.) uncover SWI/SNF remodelers as critical downstream targets of BET inhibitors in the reversal of HIV-1 latency, which is a promising therapeutic strategy to achieve eradication in infected individuals, ii.) support the hypothesis that BRD4 executes isoform-specific functions, iii.) underscore the essential role of chromatin in HIV-1 latency maintenance and iv.) provide a global view of genomic regulation by BRD4 and SWI/SNF. Lastly, this dissertation includes preliminary data demonstrating that the long isoform of BRD4 is itself subject to reversible acetylation. We have mapped a cluster of acetylation-competent lysine residues within the long BRD4 C-terminus, generated modification-specific antibodies, and begun to characterize the effects of this BRD4 modification on its transactivation capacity via the P-TEFb kinase.
Collectively, the work presented herein advances our knowledge of both isoforms of the bromodomain-containing protein BRD4, and further develops our understanding of the role of chromatin in HIV-1 transcription & latency.
Table of Contents

Chapter I: Introduction                                                                                                            1
References .............................................................................................................................................. 17

Chapter II: Tat Expression and Function                                                                                             19
References .............................................................................................................................................. 33

Chapter III: Bromodomain Proteins in HIV Infection                                                                             34
References .............................................................................................................................................. 47

Chapter IV: Therapeutics Targeting Protein Acetylation Perturb Latency of Human Viruses                                       53
References .............................................................................................................................................. 72

Chapter V: Activating Latent HIV by Inhibiting Bromodomain Proteins                                                              81
References .............................................................................................................................................. 95

Chapter VI: The Short Isoform of BRD4 Promotes HIV-1 Latency by Engaging Repressive SWI/SNF Chromatin Remodeling Complexes     101
References ............................................................................................................................................ 121

Chapter VII: BRD4 is Subject to Reversible Acetylation                                                                          139
References ............................................................................................................................................ 155

Chapter VIII: Conclusions & Outlook                                                                                               156
References ............................................................................................................................................ 161
List of Tables

Table 2-1 – Tat posttranslational modifications................................................................. 27
Table 2-2 – Extra-transcriptional effects of intracellular and extracellular forms of Tat................................................................. 30
Table 3-1 – Selected reported bromodomain inhibitors tested in models of HIV latency 45
Table 4-1 – Key facts of virus families in discussion............................................................ 56
Table 6-S1 – Mapping statistics for high throughput sequencing experiments................. 137
Table 6-S2 – PCR primers used......................................................................................... 138
Table 7-1 – PCR primers used......................................................................................... 154
**List of Figures**

Figure 2-1 – Tat expression is fueled by a positive feedback loop .............................................. 21

Figure 2-2 – BLOSUM sequence alignment of consensus Tat sequences from HIV-1 ................................................................. 23

Figure 3-1 – The human bromodomain family ...................................................................... 36

Figure 3-2 – Role of bromodomain proteins in HIV infection ......................................................... 39

Figure 4-1 – Drugs targeting catalysis and recognition of protein acetylation ............................. 58

Figure 4-2 – Effects of acetylation-targeted therapeutics on latency of HIV, EBV, and HPV ........................................................................ 62

Figure 5-1 – Acetylation writers, erasers, and readers implicated in HIV latency and transcription ........................................................................ 84

Figure 5-2 – P-TEFb complexes relevant to HIV transcription ....................................................... 88

Figure 5-3 – Potential mechanisms of BET inhibitor action at the HIV LTR ................................. 90

Figure 6-1 – Short BRD4 promotes HIV latency ........................................................................ 105

Figure 6-2 – Chromatin disruption at the HIV promoter upon JQ1 treatment .............................. 106

Figure 6-3 – The BAF chromatin remodeling complex is required for JQ1-mediated latency reversal and nuc-1 remodeling .................................................................................. 109

Figure 6-4 – Short BRD4 interacts with BRG1 via bromo- and ET domains ............................... 112

Figure 6-5 – Short BRD4 recruits BRG1 to the latent HIV provirus .............................................. 114

Figure 6-6 – BRD4 and BRG1 exhibit genome-wide co-binding that is disrupted upon BET inhibition ............................................................................................. 115

Figure 6-7 – BRD4:BRG1 co-binding across genomic features and endogenous LTR sequences .................................................................................................................. 117

Figure 6-S1 – Targeted screen of chromatin remodeling ATPases reveals BRG1 as necessary for JQ1-mediated reversal of HIV latency ........................................................................ 133

Figure 6-S2 – BRD4S immunoprecipitates BRG1 more efficiently than BRD4L ....................... 134

Figure 6-S3 – JQ1 minimally induces Pol II recruitment at the HIV promoter .......................... 135

Figure 6-S4 – BET inhibition and BRD4 knockdown do not alter BRG1 protein levels ... 136

Figure 7-1 – The BRD4 C-terminus is acetylated in cells ............................................................. 143

Figure 7-2 – A conserved cluster of C-terminal lysine residues in BRD4 is subject to acetylation .................................................................................................................................. 144

Figure 7-3 – Several histone acetyltransferases are sufficient to induce BRD4 hyperacetylation ................................................................................................................................. 145

Figure 7-4 – BRD4 is hyperacetylated in the presence of overexpressed p300 and class III HDAC inhibitors .................................................................................................................. 146

Figure 7-5 – Cellular BRD4 is acetylated at K1195/1197 ............................................................. 147
Figure 7-6 – Modification-specific BRD4 antibodies ................................................................. 148
Figure 7-7 – Acetyl-BRD4 antibodies specifically recognize acetyl-lysine cluster within BRD4 ................................................................................................................................. 149
Figure 7-8 – Acetylation-deficient BRD4 displays decreased transactivation capacity to viral and cellular core promoters, enhancers, and super-enhancers ........................................ 150
Figure 7-9 – Acetylation-deficient BRD4 displays reduced binding to cyclin T1 of P-TEFb ........................................................................................................................................ 151
Figure 7-10 – Proposed model for posttranslational regulation of BRD4 by acetylation 152
Chapter I

Introduction

Viruses, HIV, shock & kill

Viruses were once described as “evidence for the existence of a twilight zone between living and non-living matter inhabited by shadowy creatures,” (Laurence, 1936). This portrayal depicts viruses as spectral entities caught somewhere between life and death, perhaps malicious in intent yet also possessing a certain awareness unknowable to the living. The metaphor is not entirely foolish. Viruses are truly on the fringe – non-autonomously replicative infectious vehicles that deftly exploit only the most fundamental biological features of their hosts. It is because of this ability that the study of viruses has been transformative in our understanding of biology. Historical examples are abundant, including the study of bacteriophage as instrumental in the assignment of DNA as genetic material (Hershey and Chase, 1952) and the discovery of oncogenes through studies of the Rous Sarcoma Virus (Levinson et al., 1978). The study of viruses can be viewed as an important catalyst for the dawn of molecular biology.

Apart from being enigmatic and essential tools for biologists, viruses simultaneously represent destructive agents capable of causing disease across all kingdoms of life. In healthy humans, viruses variably cause benign (i.e., herpesviruses), moderate (i.e., influenza), to severe (i.e., filoviruses) disease. While the 20th century witnessed the development of the vaccine and also several effective antivirals, this period also beheld the emergence of novel and deadly viruses, notably the human immunodeficiency virus, type 1 (HIV-1). In 1981 California, members of the gay community fell ill with what appeared to be an undiagnosed defect in cell-mediated immunity, symptomatized by opportunistic infections and later termed Acquired Immunodeficiency Syndrome (AIDS). Approximately two years later, Barré-Sinousi, Gallo, and others described that what was once termed “gay cancer,” was in fact viral in origin. Thus, the field of HIV-1 research was born.

Roughly 35 years after the initial HIV-1 outbreak, the epic struggle to combat this virus has arguably been heralded a success. Small molecule therapeutics targeting viral enzymes (reverse transcriptase, protease, and integrase) are effective in combination at suppressing viremia in infected individuals. These drugs have transformed HIV-1 infection from an acute, fatal illness into a chronic yet clinically manageable disease. Under antiretroviral therapy, HIV-infected individuals rarely develop AIDS and have near normal life expectancies. However, a glaring issue with the current state of treatment for HIV-1 infection remains; antivirals do not eradicate the virus, are not curative, and thus require life-long adherence. The cost of anti-HIV
medication during the lifespan of an infected individual approaches $400,000 (CDC, 2015). Equally as alarming, access to these medications in developing countries, where the majority of the 36.7 million people currently infected with HIV reside, remains limited. Thus, efforts to cure HIV or, at minimum, to improve the existing status of treatment persist. My thesis research has largely focused on the molecular characterization of a novel class of small molecules that disrupts HIV-1 latency (described below), which at this time is a predominant approach in HIV-1 eradication research. In this section of Chapter I, I provide a brief summary of the HIV-1 life cycle, present the concept of viral latency, and introduce a therapeutic avenue for HIV-1 eradication efforts.

HIV-1 is a retrovirus, a family of diploid single stranded RNA viruses utilizing a DNA intermediate that integrates into host chromatin to execute their replication cycle (Coffin, 1997). The HIV-1 virion is spherical in form, and is encapsulated by a host-derived lipid membrane and the viral matrix protein. Transmembrane proteins include virally-encoded and host modified glycoproteins (gp120 and gp40) that enable fusion to target cells. Viral capsid proteins form a rod-like structure that encloses the RNA genomes bound to nucleocapsid, viral enzymes, and certain host factors including heat shock proteins and lysyl-tRNA synthetase. HIV-1 displays primary tropism for CD4+ T cells, either circulating or tissue-resident. HIV-1 also infects macrophages and dendritic cells, among other cell types, which may serve as important sites of HIV-1 replication upon virally induced CD4+ T cell depletion and in immune-privileged anatomical sanctuaries. Attachment of viral particles to the target cell is achieved via binding of gp120 to CD4 and either the CXCR4 or CCR5 co-receptor on the host cell plasma membrane. A series of coordinated structural changes lead to the fusion of the viral envelope and the plasma membrane and subsequent deposition of the viral capsid structure into the cytoplasm. Capsid undergoes uncoating to release viral enzymes and RNA, which continue the process of reverse transcription.

Reverse transcription is a hallmark of the retroviral cycle and describes the conversion of RNA to DNA, initially inconsistent with the central dogma of molecular biology. Once thought to be restricted to viruses, reverse transcription is now known to occur in uninfected human cells, notably via telomerase and endogenous retroviruses. Reverse transcription of the HIV-1 genome is catalyzed enzymatically via reverse transcriptase, an RNA-dependent DNA polymerase that is a proteolytic fragment of the viral pol gene product. This step of the viral life cycle was the first to be therapeutically targeted by nucleoside analogues such as azidothymidine (AZT). The reaction catalyzed by reverse transcriptase generates a linear double stranded cDNA genome that is recognized by the viral integrase, a distinct pol cleavage
product. Integrase catalyzes 3’-end processing of the viral cDNA to generate sticky ends, upon which it also catalyzes a strand transfer reaction that inserts the viral DNA into the human genome. Integrase is also involved in nuclear import and targeting of the viral DNA to transcriptionally dynamic areas of host chromatin, mediated via importins and LEDGF/p75, respectively. The site of HIV-1 integration is non-random, and the virus is mainly directed toward intronic, AT-rich regions at the major groove of nucleosomal DNA.

Integration of viral DNA into host chromatin is an irreversible step in the HIV-1 replication cycle, upon which viral DNA becomes indistinguishably coupled to the fate of its host cell. A variety of factors contributes to the variable events occurring post-integration. Typically, infection by HIV-1, and many other viruses, results in one of two outcomes – a productive or latent infection. Under a productive infection, the virus completes its replication cycle, and progeny virions are assembled and released from the infected cell. This is the most common route upon HIV-1 infection of activated, permissive CD4+ T cells. During a productive infection, the long terminal repeat (LTR) at the 5’-end of the HIV-1 genome acts as an inducible promoter within host chromatin to successfully drive processive transcription of integrated viral DNA. HIV-1 RNA is exported to the cytoplasm where structural (Gag, Pol, Env), essential regulatory (Rev, Tat), and accessory (Vpr, Vif, Nef, and Vpu) proteins are translated and processed. Progeny virions are assembled in the cytoplasm and bud from the infected cell. Recent work has established that productive HIV-1 infection of CD4+ T cells provokes an inflammatory type of cell death termed pyroptosis (Doitsh et al., 2010; Doitsh et al., 2014), which releases damage-associated molecular patterns and various inflammatory cytokines from the dying cell. This results in the recruitment of immune effector cells to the site of HIV-induced cell death. These bystander cells are then infected with newly assembled HIV-1 particles, and subsequent killing proceeds. Over time, HIV-mediated destruction of immune cells results in the loss of cell-based immunity, immunodeficiency, opportunistic infections, and ultimate mortality.

A small proportion of HIV-1 infection events leads to transcriptional silencing of the viral integrant (provirus) in host chromatin. This is termed latency, or a reversible state of nonproductive infection where viral genetic material is stably present within the host cell yet viral gene expression is restricted. Whereas a productive infection tends to be highly cytopathic, a latent infection allows the virus to persist within its host cell, and may very well represent a unique evolutionary strategy with ostensible fitness benefits. The phenomenon of latency can be simplistically attributed to a switch in viral transcription. Under latency, heterogeneous yet reversible mechanisms imposed by both the virus and its host cell enforce transcriptional silencing of the HIV-1 locus in the context of chromatin. With correct stimuli and conditions, viral
DNA can be derepressed to trigger productive replication and subsequent reinfection within the host.

Latent infection by HIV-1 is an extremely rare phenomenon, with a frequency of $1/10^6$ resting T cells in blood isolated from aviremic infected individuals, with this ratio slightly augmented in lymphoid tissue. Although a subset of peripheral resting memory CD4+ T cells is the best characterized compartment in which HIV latency occurs, macrophages, microglial cells, gut associated lymphoid tissues, hematopoietic progenitor cells, and other cell types are important, yet relatively uncharacterized reservoirs. Generally speaking, these cell types have long half-lives, low activation states, and undergo homeostatic proliferation, which all contribute to the persistence of latent virus. HIV-1 latency is established via heterogeneous mechanisms that include i.) the locus of integration, as HIV-1 integrates in nonrandom manner primarily into accessible, actively transcribed regions potentially with functional importance to the virus and/or the host cell type ii.) the orientation of integration, either in the same (transcriptional read through) or reverse (convergent transcription) orientation as the sense of the integration site transcript iii.) the absence of host factors required for HIV-1 transcription, often inextricably tied to the type and/or activation state of the host cell and iv.) a firm on-off switch encouraged by stochastic transcriptional changes and the strong positive feedback loop driven by the viral transactivator of transcription (Tat).

The current state of treatment for HIV-1 infection is not curative due to the durable persistence of replication-competent proviruses archived in the latent reservoir. This reservoir of latently infected cells does not decay significantly throughout the lifespan of an infected individual; models predict that it would take 73 years for the latent reservoir decline without intervention (Finzi et al., 1999). Several clinical studies have shown that intensification of antiretroviral therapy does not impact reservoir size (Dinoso et al., 2009). If eradication of HIV-1 from infected patients is to remain a major public health objective, alternative therapeutic strategies must be devised and tested.

Cure research efforts thus far in the early 21st century have largely been directed toward the exploration of an eradication strategy termed “shock and kill.” (Deeks, 2012) This approach relies on a latency-reversing agent (LRA), typically a small molecule, to stimulate HIV-1 transcription. The ideal LRA would activate viral transcription specifically and among all anatomical compartments harboring latent virus. Administration of an LRA would occur as an adjunctive treatment to antiretroviral therapy. Antiretroviral therapy should effectively suppress active reinfection by newly assembled virions within the individual. The ultimate goal of shock and kill is achieve what is termed a “sterilizing cure,” in which viral DNA is eradicated and
undetectable in patients after therapy. At minimum, shock and kills aims to achieve a “functional
cure,” wherein patients may still harbor viral DNA, but the latent reservoir has been depleted to
an extent as to allow patients to go off antiretroviral therapy for extended periods of time or
indefinitely.

Shock and kill has been tested clinically with somewhat disheartening results. Initial
clinical efforts focused on the cytokines IL-2/IFN-γ as the shock portion (Chun et al., 1999).
Cytokines are among the most potent inducers of latent HIV, as latent virus is exquisitely
sensitive to the activation state of its host cell. The use of cytokine therapy, however, is
extremely toxic and therefore is no longer considered a viable method to reverse latency in vivo.
Current efforts to stimulate HIV-1 production from latently infected cells include small molecules
that activate protein kinase C (PKC) signaling cascades. PKC agonism is not a specific HIV-1
inducer per se, but rather stimulates HIV expression via global T cell activation, not dissimilar to
cytokines. Leading hypotheses for the mechanisms of PKC agonist-mediated T cell stimulation
include influx of Ca²⁺ levels and increased phosphorylation of and consequent inactivation of
IkB, and inhibitor of NF-κB (Jiang and Dandekar, 2015). Original PKC agonists and classical
activators of HIV-1 transcription include phorbol esters, such as PMA. These compounds have
oncogenic properties and are thus unsuitable for in vivo administration. Other PKC agonists are
mostly plant-derived and include prostratin and ingenol. These small molecules exhibit
significant in vivo toxicity in non-human primates, with risks of cytokine storm and sepsis.
Analogs of PKC agonists are currently being optimized to curb toxicity in in vivo systems, and
one of these molecules, bryostatin-1, has completed phase I clinical trials for the treatment of
HIV latency. Patients in this study did not have elevated PKC activity nor any detectable
induction of HIV-1 transcription with the doses used (Gutierrez et al., 2016).

The second predominant LRA class is small molecules that target epigenetic
mechanisms in place to enforce transcriptional silencing of latent HIV-1. Inhibitors of the histone
decetylase (HDAC) family of enzymes have long been known to reactivate latent HIV-1 in cell
line models, and have begun clinical trails for eradication purposes. Pilot single-arm studies of
the FDA-approved HDAC inhibitor vorinostat have shown that this LRA effectively induces
unspliced, intracellular HIV-1 mRNA in patients. This finding has also been extended in the
clinic for the HDAC inhibitors panobinostat and romidepsin. In some cases, most notably with
romidepsin, these agents effectively induce plasma HIV-1 mRNA levels (Sogaard et al., 2015),
allusive of successful virion production. None of these HDAC inhibitors, however, reduce levels
of viral DNA, which serves as a proxy measurement for the number of latently infected CD4⁺ T
cells. These data suggest that HDAC inhibitors may only serve modest utility as an LRA in vivo, at least as monotherapies.

Initial clinical proof of concept data for shock and kill has been underwhelming, and it is therefore clear that next generation LRAs are necessary if this approach is to be successful in HIV-1 eradication efforts. Inhibitors of the bromodomain and extraterminal domain (BET) family of proteins are a newly described class of LRAs. Similar to HDAC inhibitors, BET inhibitors target chromatin-engaged transcriptional regulators belonging to the protein acetylation pathway. My thesis work has largely focused on understanding the molecular mechanisms of BET inhibitor action at the latent HIV-1 promoter. In this next section of Chapter I, I provide an overview of protein acetylation, the factors involved, and drugs designed to disrupt this pathway.

Protein acetylation

The HIV-1 provirus is subject to the same regulatory components of the nuclear machinery as human chromatin. The nucleus of a human cell is a highly dynamic environment in which genetic material is maintained, replicated, and processed; the layers of regulation orchestrating these processes are immeasurable. In addition to DNA cis-elements that are naturally limited in their regulatory capacity, the structure and modification status of chromatin provide rich avenues for fine-tuning of DNA-based transactions. Chemical modifications to chromatin, either DNA (i.e., methylation, hydroxymethylation) or histone (i.e., methylation, acetylation) constituents, alter its physiochemical properties and thereby function. Such modifications can disrupt local electrostatic interactions, but they also can generate novel binding interfaces for protein domains that specifically recognize a given mark. In this way, these epigenetic reader domains can effect functional outcomes associated with particular chemical modifications on chromatin. The predominant reader domain that recognizes protein acetylation marks is the bromodomain.

Protein acetylation describes the transfer of an acetyl group from acetyl-coenzyme A (CoA) to the primary amine in the ε-position of a lysine side chain within a protein. Advances in mass spectrometric technologies have uncovered that >2000 proteins in the human cell undergo reversible acetylation. Historically, much attention has been paid to acetylation in the nucleus, where this mark regulates nucleosome structure and interpretation. Histone acetylation is generally correlated with DNA accessibility and transcriptional activation. This can be at least partly attributed to antagonism of the electrostatic interactions between the lysine residue that is positively charged under physiological conditions and negatively charged DNA. For example, H3K56 acetylation occurs in the globular core of H3 at the DNA entry-exit site of the
nucleosome and has been shown to transiently loosen DNA:nucleosome contacts to allow for DNA breathing (Neumann et al., 2009). Alternatively, H3 and H4 tails contain several acetylation-competent lysine residues that have been studied extensively (i.e., H3K4/9/14/27 and H4K5/8/12/16) and, when acetylated, generally mark euchromatic, transcriptionally active loci. Given that these tails protrude from the DNA-engaged nucleosome core, acetylation of these residues likely promotes transcription by modulating local protein:protein interactions on chromatin. Notably, nuclear acetylation is not restricted to the histones, but also numerous transcription factors and chromatin-engaged proteins are subject to acetylation (i.e., NF-κB, Sp1, P-TEFb, RNA polymerase II, etc.). Acetylation of these factors can impact protein function through altering affinity for DNA and proteins, but can also affect stability and localization. Invading viral proteins (i.e., HIV-1 Tat) can also become acetylated under a cellular context, and may have very well coevolved to utilize this mode of regulation to fine-tune replicative capacity.

Protein acetylation is catalyzed enzymatically in the cell by histone/lysine acetylation transferases (HATs, also known as KATs). The ~25 human HATs encoded in the human proteome predominate in the nucleus where they modify histones in addition to other chromatin-associated factors, generally facilitating transcription. Opposing HAT activity are HDACs (also known as KDACs), of which there are four distinct classes based upon structural homology and catalytic mechanism. Class I, II, and IV HDACs are comprised by HDAC1-11, while class III HDACs are referred to as sirtuins of which there are seven (SIRT1-7) in humans. Class I HDACs (HDAC1,2,3,8) localize mainly to the nucleus, Class II (HDAC4-7,9,10) and Class IV (HDAC11) HDACs generally shuttle between the nucleus and cytoplasm, while different sirtuins localize to the cytoplasm (SIRT2), nucleus (SIRT1,6,7), and mitochondria (SIRT3-5). Nuclear HDACs generally negatively regulate transcription and are often found in large corepressive complexes (i.e., CoREST/REST, NuRD, N-CoR). Mitochondrial acetylation may not be of enzymatic origin due to the high pH and acetyl-CoA levels found in this cellular compartment. Non-enzymatic catalysis of acetylation is slow and may be important for cell types with long half-lives.

Small molecules interfering with the protein acetylation pathway have been characterized since their discovery in the late seventies. In fact, chemical inhibitors of deacetylation were known prior to the characterization of acetyltransferases and deacetylases. The short chain fatty acid butyrate was the first HDAC inhibitor described, previously known to modulate gene expression and induce differentiation of acute erythroid leukemia cells (Candido et al., 1978; Riggs et al., 1977; Vidali et al., 1978). In the early nineties, the fungal hydroxamate tricostatin A and tetrapeptide trapoxin were identified as HDAC inhibitors (Kijima et al., 1993),
with a potency orders a magnitude above that of butyrate. The fact that these compounds inhibited deacetylation, rather than promoting acetylation, was experimentally supported, yet the enzymes behind these activities remained a mystery. That is until 1996, when Taunton and Schreiber used a trapoxin affinity matrix to isolate its binding partners from cellular extracts. They identified a mammalian nuclear factor related to yeast Rpd3 (Taunton et al., 1996), which was later assigned as HDAC1.

An assortment of chemically diverse HDAC inhibitors is currently in existence, and these small molecules predominantly target class I, II, and IV HDACs non-selectively. Emergent inhibitors are active against a more restricted range of HDACs, yet a highly specific HDAC inhibitor has yet to be described. Most HDAC inhibitors chelate the divalent metal ion required for catalysis, although not all inhibitors exploit this mechanism (i.e., the inhibition mechanism of butyrate is still unknown, although it is non-competitive). Specific inhibitors of class III HDACs, or sirtuins, are more limited, although they do exist (i.e., EX-527 and sirtinol) and likely function by interfering with NAD+ cofactor binding.

A unified mechanism behind the biological activities of the HDAC inhibitors remains unavailable. Early studies showed that a relatively small percentage (2-10%) of the transcriptome is significantly altered, demonstrating that these drugs target expression of a relatively narrow range of genes rather than the majority of the genome. Surprisingly, most HDAC inhibitors induce global histone acetylation as detectable by Western blotting. Together these findings indicate that other regulatory pathways are in place to preserve gene expression patterns under global hyperacetylation. Mechanistic hypotheses for HDAC inhibitor action include the accumulation of DNA damage and subsequent cell cycle checkpoint failure, accumulation of reactive oxygen species via thioredoxin induction, and transcriptional activation of pro-apoptotic TRAIL and/or the cyclin-dependent kinase inhibitor p21. To date, three HDAC inhibitors are in the clinic, all with cutaneous T cell lymphoma as the indication. Vorinostat, also known as suberanilohydroxamic acid (SAHA), was the first approved HDAC inhibitor to enter the clinic. Toxicities are largely manageable, but do include dose-limiting levels of cardiotoxicity, thrombocytopenia, and gastrointestinal side effects.

Although not as abundant and well characterized as HDAC inhibitors, inhibitors of HATs have been described. Available HAT inhibitors mostly target p300/CBP, but PCAF inhibitors exist as well. Original inhibitors were bi-substrate acetyl-CoA mimics, but the Cole laboratory has since pioneered the discovery of smaller synthetic compounds, such as C646. Interestingly, several phytochemicals act as HAT inhibitors, such as curcumin, gercinol, and anacardiac acid. A very recent study demonstrated that salicylate is a p300/CBP inhibitor operating via competition
with acetyl-CoA for catalytic cleft binding (Shirakawa et al., 2016). The relevance of p300/CBP inhibition activity to the well-known anti-inflammatory properties of aspirin, of which salicylate is the active metabolite, is unknown and likely considerable. While preclinical data is advancing, to date no specific HAT inhibitors have reached the clinic.

Apart from enzymatic regulation, protein acetylation is intimately controlled by the bromodomain, the epigenetic reader that specifically binds acetyl-lysine residues. The bromodomain is approximately 110 amino acids in length, and there are 61 distinct bromodomains encoded by 46 proteins in the human proteome. Most bromodomain-containing proteins encode one bromodomain, with BET subfamily members containing two and Polybromo-1 containing 6 bromodomains as notable exceptions. All bromodomain-containing proteins are nuclear factors that bind chromatin to regulate its structure and interpretation. To date, they are mostly implicated as transcriptional coactivators (i.e., p300, BRD4), yet repressive functions have been ascribed to certain bromodomain-containing proteins (i.e., BAZ2A, ZYMND11).

The first reference to the bromodomain is traced to the characterization of the Drosophila gene brahma (brm), a regulator of homeotic genes now known as a core catalytic component of SWI/SNF chromatin remodelers (Tamkun et al., 1992). In the tradition of imaginative fly geneticists, the brm gene was named after the Hindu four-headed god who created the universe. The conserved structural motif discovered in the brm gene was thus somehow termed a bromodomain, and it is etymologically distinct from elemental bromine. Apart from its frequent occurrence in transcriptional regulators, the bromodomain was relatively uncharacterized from the time of its discovery in 1992 to the determination of its structure by Zhou and colleagues in 1999. NMR studies of the PCAF bromodomain revealed that this motif binds histone acetyl-lysine residues and described the structural details of this interaction (Dhalluin et al., 1999).

The bromodomain structure is well characterized, with >400 high-resolution X-ray crystal structures currently available and near complete coverage throughout the family. The bromodomain is composed of four left-handed α-helices (αZ, αA, αB, and αC) connected by two loops (ZA and BC loops). This structure forms a hydrophobic cavity that serves as the acetyl-lysine recognition site. A hydrogen bond mediated by a conserved bromodomain asparagine residue and the acetyl-lysine carbonyl is the ligand recognition mechanism. Tyrosine residues lining the bromodomain cleft also play a significant role in ligand positioning and hydrogen bond formation. While the helical regions are moderately conserved among different bromodomains, the loop regions are highly variable in both length and sequence composition. This loop
variation is thought to impart structural distinctions in the hydrophobic acetyl-lysine binding core that guide the ligand specificity observed among members of the bromodomain family. Certain bromodomains display cooperativity in binding multiply acetylated peptides, while others are regulated by adjacent posttranslational modifications. Generally speaking, the bromodomain:acetyl-lysine interaction is low affinity in vitro, with $K_d$ values typically in the low micromolar range. However, these domains are rarely found alone in a given protein. It is likely that neighboring protein domains (i.e., helicase, SAND, distinct bromodomain) modulate affinities in an in vivo context.

The bromodomain is conserved from yeast to humans, and is encoded by an increasing number of factors as higher eukaryotes expanded. In mammals, bromodomains can be divided into several distinct subfamilies based mostly on structural homology. Perhaps the most recognized subfamily of bromodomain-containing proteins is the BET subfamily. Members of the BET subfamily encode not one, but two N-terminal bromodomains and a secondary conserved domain, the extraterminal domain. The two bromodomains exhibit limited sequence homology (~40%), and may bind distinct sets of acetyl-lysine residues. Emergent evidence suggests that the first BET bromodomain (BD1) binds more avidly to acetyl-lysine residues in histones, while the second BET bromodomain (BD2) engages non-histone ligands. Regardless, these tandem bromodomains generate a unique protein architecture that distinguishes the BET subfamily. The extraterminal domain, also unique to BET members, is a protein:protein interaction interface that binds mainly to chromatin modifiers. Notably, viral gene products bind to the ET domain, including papillomaviral E2 and KSHV LANA, underscoring the potential functional significance of this domain. While the significance of the space between the ET and bromodomains is still being unraveled, it is known to be phosphorylated and to bind mononucleosomes.

The BET family comprises three ubiquitously expressed members (BRD2, BRD3, and BRD4) as well as one testis-specific member (BRDT). BRD4 is the best characterized of the family members, and encodes at least two isoforms, a short and a long isoform. Notably, the long isoform of BRD4, as well as of BRD3, encodes a third functional domain that interacts with P-TEFb, or the P-TEFb-interacting domain (PID). As discussed below, P-TEFb is a powerful activator of transcript elongation at many human genes as well as at the HIV-1 promoter. Long BRD4 is a positive regulatory component of P-TEFb, and is mutually exclusive from inactive 7SK snRNP-bound P-TEFb molecules. Although mechanistic details currently remain limited, BRD4 is thought to actively dissociate the 7SK snRNP to liberate catalytically active P-TEFb via its PID. Accordingly, long BRD4 is a critical coactivator of many genes via P-TEFb activation and recruitment of active P-TEFb to key loci potentially through acetyl-histone:bromodomain
interactions. The functional significance of the short isoform of BRD4 is relatively unknown, although this is the subject of Chapter VI.

Small molecule inhibition of the bromodomain is the most recent development in efforts to pharmacologically target the protein acetylation network. Rather than affecting enzymatic catalysis, these drugs target protein:protein interactions by disrupting binding between the bromodomain and its acetyl-lysine residue-containing ligand. The first drug discovery efforts aimed at bromodomains were in the HIV-1 field, after the assignment of acetylated Tat as a critical ligand for the PCAF bromodomain in HIV-1 transactivation. The use of NMR screening lead to the discovery of two compounds with relatively low potency in inhibiting the Tat:PCAF interface in vitro. Several years later, Mitsubishi Pharmaceuticals published a patent describing thienodiazepines as BET bromodomain binders. The Bradner group heeded this patent and synthesized thienodiazepine derivatives to discover a lead molecule, JQ1, that had therapeutic activity against a rare squamous epithelial cancer called the NUT midline carcinoma. This form of cancer is cytogenetically defined by a translocation of the BRD4 gene that results in an in-frame fusion with the nuclear protein in testis (NUT), a tissue specific acetyltransferase (French, 2014). This translocation disrupts BRD4 function by fusing the short isoform of BRD4 (and rarely BRD2) to NUT, and also by abrogating long BRD4 function. Both aspects are likely oncogenic, yet the BRD4-NUT fusion has been functionally characterized to promote unrestrained and aberrant histone modifications. Outside of the NUT midline carcinoma context, BET proteins can promote progression of distinct cancers by driving expression of critical oncogenes, including MYC. Accordingly, the development of BET inhibitors is of intense interest in the oncology field.

Current BET inhibitors target both BET bromodomains with comparable affinities, yet no BET inhibitor is available that can distinguish among subfamily members. Therefore, biological phenotypes associated with BET inhibitors are likely a result of combined inhibition of BRD2, BRD3, and BRD4. The use of BET inhibitors has undoubtedly helped to elucidate the pleiotropic functions of these factors. While BET inhibitors are rapidly advancing as viable therapeutics, inhibitors of non-BET bromodomains are also being pursued. At this time, they are mostly chemical probes with affinity for the bromodomains of acetyltransferases (i.e., p300/CBP) and of chromatin remodeling components (i.e., BRD7, BRG1). It is clear that druggability varies considerably among individual bromodomains.

Since JQ1 was first reported as a preclinical chemotherapeutic, its activity against an impressive number of cancers has been described. Owing to this, JQ1 and derivatives (i.e., OTX015) are already the subjects of >20 ongoing clinical trails, even though they were only first
described five years ago. Several trials have completed Phase I or at least have reported tolerability and partial clinical outcomes of early trails (Berthon et al., 2016; Odore et al., 2016). Thus far, BET inhibitors appear well tolerated with dose-limiting side effects as diarrhea, fatigue, and reversible thrombocytopenia. Trough plasma concentrations following an oral dose reach between 500nM-3µM, which is well within the concentration range of many preclinical studies demonstrating favorable therapeutic outcomes. These clinical trials are mostly to treat a diverse array of cancers, although BET inhibitors are also being clinically evaluated for use against cardiovascular disease and diabetes. Preclinical data indicate that BET inhibitors may also be useful for the treatment of inflammatory diseases, male fertility, and HIV-1 latency.

In the next section of Chapter I, I provide a review of basic mechanisms regulating HIV-1 transcription and latency, with particular attention paid to the role of factor acetylation in these processes.

**HIV-1 transcription & latency**

Productive HIV-1 transcription is orchestrated by the virally-encoded Tat protein. The study of Tat serves as a beautiful example of a viral protein that has illuminated our understanding of molecular biology. HIV-1 Tat is an essential regulatory protein that functions as a powerful transcriptional activator. Early studies of HIV-1 transcription established that the HIV-1 LTR readily initiates RNA synthesis in host chromatin, yet does not proceed past 30-100nt of synthesis. Tat was sufficient to relieve this stalling of transcripts, leading to its assignment as an “anti-termination” factor. Several years later, components of the positive transcription elongation factor b (P-TEFb) were identified as critical Tat cofactors. Decades of elegant molecular work have identified that i.) Tat is an RNA binding protein with exquisite specificity for the transactivation response element (TAR), an RNA structure at the leading 5’-end of short HIV-1 transcripts and ii.) P-TEFb is a kinase complex that catalyzes phosphorylation of Pol II and other transcription components to promote the elongation phase of the transcription cycle. Although P-TEFb was discovered through studies of HIV-1 Tat, its relevance has since been extended to encompass a central role in the mammalian transcription cycle. Namely, activity of P-TEFb relieves what is known as “proximal promoter pausing.”

Proximal promoter pausing describes the stalling of Pol II holoenzymes immediately downstream (<nt +100) of the transcription start site (TSS). Pausing is enforced owing to significant molecular obstacles imposed downstream of the TSS. Negative transcription elongation factors, notably NELF and DSIF, sterically hinder Pol II passage past the proximal pause site. Action of P-TEFb and associated factors, such as the super elongation complex
(SEC), simultaneously allows for disassembly of chromatin and negative elongation factors in addition to enhancement of Pol II catalytic activity. P-TEFb is a heterodimer composed of a Cyclin T subunit and the CDK9 kinase subunit. CDK9 permits elongation via catalysis of phosphorylation events on the Pol II CTD (at $S_2$ and other heptad serines), but also critically on NELF and DSIF to relieve their inhibitory effects. CDK9 activity is dynamically regulated by a posttranslational means, including incorporation into an inhibitory small nuclear ribonucleoprotein (the 7SK snRNP) and covalent modifications. The SEC, which comprises active P-TEFb, stimulates elongation mainly through ELL2, a noncatalytic subunit that prevents Pol II backtracking and keeps the nascent RNA in line with the Pol II catalytic cleft.

Although studies of HIV-1 were critical in uncovering molecular details of pausing, it was also originally described by the Lis laboratory in studies of heat shock protein gene transcription in *Drosophila melanogaster* (Lis and Wu, 1993). Since this time, and with important technical advances including ChIP-seq, proximal promoter pausing is estimated to occur at a large fraction of human genes, particularly those that encode important cell type-specific regulators of fate and plasticity (i.e., *MYC* and *FOS*). Having initiation-component RNA polymerase II holoenzymes preassembled at promoters is thought to confer a strategy enabling rapid induction of gene expression in response to a given signal. It is also likely intimately linked to regulating chromatin structure and co-transcriptional splicing events. While the mechanisms governing pausing are still being unraveled, it is clear this is predominant method of transcriptional regulation in higher eukaryotes and their viruses.

The first phase of HIV-1 transcription is characterized by proximal promoter pausing, or the production of initiated short RNA species that generally are not transcribed past nt +59 (the TAR element). These short transcripts are inadequate to support productive viral replication, and occur in the context of latency. It is thought that stochastic fluctuations in molecular events during this phase of HIV-1 transcription allow for the rare production of a fully transcribed viral message. This mRNA is successfully exported and translated to produce viral proteins, notably Tat. Tat then feeds-back to HIV chromatin via TAR to enhance transcript elongation and usher the second phase of HIV-1 transcription, which is characterized by fully processive transcription and consequent productive infection. In this way, escape from HIV-1 latency can be viewed as a relief of proximal promoter pausing.

Chromatin is a natural barrier to an elongating polymerase, and thus a variety of epigenetic factors play a role in both the maintenance and reversal of HIV-1 latency. A discussed previously, HDACs surveil HIV-1 nucleosomes under latency to enforce a transcriptionally restricted chromatin environment. Indeed, HIV-1 chromatin becomes hyper-acetylated and
transcriptionally permissive under HDAC inhibition. Repressive histone methylation marks (i.e., H3K27me3) and associated factors (i.e., Suv39H1, EZH2, and HP1) have been reported to occupy the latent HIV-1 promoter. Chromatin remodeling complexes have importantly been associated with discrete nucleosome positioning to maintain HIV-1 latency, most notably the SWI/SNF BAF complex (discussed in later chapters). In contrast, active HIV-1 transcription occurs in the context of the Tat-dependent recruitment of several acetyltransferases (i.e., p300, PCAF, GCN5) and chromatin remodelers (BRM-containing SWI/SNF complexes). The structure and modification status of chromatin is thus a key determinant of HIV-1 latency. This is further evidenced by the fact that epigenetic-based drugs that target these chromatin transactions are currently lead LRA candidates. Therefore, the molecular details of the chromatin events occurring during the reversal of HIV-1 latency are of critical importance.

Targeting the recognition of protein acetylation, particularly by the BET subfamily, on viral chromatin holds promise for HIV-1 eradication strategies. In the next section of Chapter I, I provide a brief overview of BET inhibition as a means to reawaken HIV-1 from transcriptional latency and introduce the major topic of this dissertation.

**BET inhibitors as HIV latency reversing agents**

BET inhibitors are newly described LRAs, and several lines of preclinical evidence indicate that these small molecules may serve as *bona fide* pharmacological tools for the treatment of HIV-1 latency. Firstly, the BET inhibitors JQ1, I-BET151, MS417, and OTX015 all reactivate latent HIV-1 across different cell line models, including U1, ACH2, JΔK, and J-Lat cells. BET inhibitors also induce HIV-1 expression in certain, but not all, primary models of viral latency. As an *ex vivo* monotherapy in cells isolated from infected aviremic patients, JQ1 displays minimal latency reversing activity. However, in combination with low dose PKC agonists, JQ1 induces HIV-1 transcription to a similar extent as T cell activation achieved via PMA/Ionomycin. Along with romidepsin, BET inhibition scored as the most potent LRA in combination with low dose bryostatin-1 in these *ex vivo* analyses. Furthermore, it has been shown that I-BET-151, in combination with broadly neutralizing antibodies (bNAbs), other LRAs, and antiretroviral therapy, can significantly delay viral rebound in a humanized mouse model of latency. These and emerging studies lend credence to the use of BET inhibitors as a novel class of LRAs in the fight against HIV-1.

The molecular mechanisms underlying the activation of HIV-1 transcription in response to BET inhibition have remained elusive. This is mostly due to the fact that currently ascribed functions of BET proteins in HIV-1 transcription are enigmatic. The long isoform of BRD4 has
been shown to independently transactivate HIV-1 via its PID, supporting the widely accepted role of BET proteins as transcriptional coactivators. The finding that BET inhibition induces HIV-1 transcription, however, is unanticipated and seemingly contradicts this finding. One prevailing hypothesis for BET inhibitor action is that these compounds achieve P-TEFb activation via 7SK snRNP dissociation, which has been shown to occur in certain cell line models. Conversion of inactivated P-TEFb reservoirs to freed complexes harboring catalytically active CDK9 would theoretically allow for Tat to more effectively capture active P-TEFb molecules for delivery to the HIV-1 provirus. However, three findings cast doubt on this as the primary mechanism of BET inhibitor action. The first is that Tat can autonomously eject P-TEFb from the 7SK snRNP, and this model implies that BRD4 is more efficient at P-TEFb release than Tat, which has not been shown. Secondly, D’Orso et al demonstrated that it is necessary for the 7SK snRNP to be recruited to the HIV-1 promoter and then locally dismantled for robust stimulation of transcription. Lastly, the relevance of the 7SK snRNP in primary T cells is still a matter of debate, and it is speculated that these complexes may not be present in significant amounts in primary cells. Another hypothesis for BET inhibitor action at the latent HIV-1 promoter is the relief of competition between BRD4 and Tat, which both bind a finite pool of active P-TEFb molecules. Removal of BET proteins from chromatin may give Tat a competitive advantage in binding active P-TEFb molecules. The finding that BET inhibitors reverse latency in models devoid of Tat discredit this hypothesis as the primary mechanism of latency reversal. BET proteins regulate transcription of a vast array of genes, and it is possible that inhibition of these factors causes indirect transcriptional effects that then feed back to activate the HIV-1 promoter. However, experiments with the protein biosynthesis inhibitor cycloheximide demonstrate that indirect effects account for a minority of the mechanism for JQ1 (data not shown). Collectively, the mechanism of action through which BET inhibition reverses HIV latency is largely undefined.

**Thesis Objective & Hypothesis**

The objective of my dissertation research was an in-depth molecular characterization of BET inhibitor activity in the reversal of HIV-1 latency. As described above, a well-defined mechanism through which the newly described LRAs function to activate HIV-1 transcription remains undetermined. This information, however, is critical to further our understanding of the biology controlling HIV-1 latency.

We hypothesized that BRD4, the prototypical BET protein, enforces HIV-1 latency by maintaining repressive positioning of the +1 nucleosome (nuc-1) at the latent HIV-1 promoter. We investigated this hypothesis through a variety of techniques including enzymatic digestion of
chromatin, shRNA-mediated knockdowns, and chromatin immunoprecipitation. The scientific investigation of this hypothesis is formally presented as Chapter VI.
References


Chapter II

Tat Expression and Function

Ryan J. Conrad*, Mark Y. Jeng*, Melanie Ott#
Gladstone Institute of Virology and Immunology
University of California, San Francisco
1650 Owens Street, San Francisco, CA 94158
*equal contributions
#Correspondence: mott@gladstone.ucsf.edu

Definition

HIV-1 hijacks the cellular transcription machinery by expressing the virally encoded transactivator of transcription (Tat) protein. Tat is a 14–16-kDa nuclear/nucleolar protein that serves as an essential adaptor for cellular transcription elongation factors and other transcriptional regulators. This RNA-binding protein recognizes a stem-loop structure called transactivation response element (TAR) in the 5’ extremity of initiating viral transcripts. The structural plasticity of Tat enables versatile interactions with host proteins, as well as host and viral RNAs, and forms the basis for Tat’s critical role in HIV transcription and pathogenesis. No current drug treatment targets Tat, but recent progress in deciphering the structure and modification status of Tat provides novel impetus for drug development that may help tackle HIV infection and latency at the transcriptional level.

(1) Tat regulates its own expression
When HIV integrates into the human genome, the proviral cDNA undergoes chromatinization and becomes subject to cellular RNA polymerase II–mediated transcription, like a human gene. However, basal HIV transcription is not consistently efficient, as the human RNA polymerase II (pol II) complex pauses shortly after the transcriptional start site and, in the absence of additional elongation signals, yields only so-called short transcripts that are insufficient to support viral replication (Kao et al., 1987). While human genes depend on activation or differentiation signals to recruit transcription elongation factors, HIV encodes its own specialized viral factor called Tat to overcome this block preventing viral transcript elongation. Tat binds avidly to a cellular protein complex, the positive transcription elongation factor b (P-TEFb)—a factor first discovered to play a fundamental role in HIV transcription, but now known to regulate a large number of human genes. P-TEFb, composed of cyclin T proteins (cyclin T1, T2A and T2b with only cyclin T1 supporting Tat transactivation) and the cyclin-dependent kinase 9 (CDK9), promotes transcriptional elongation not only by enhancing the catalytic rate of the
polymerase, but also by dissociating negative elongation factors that are physically blocking processive transcription. In this way, Tat exploits this host elongation factor by recruiting it to the TAR element for robust stimulation of viral transcription and the completion of a productive viral replication cycle.

The two phases of HIV-1 transcription have distinct characteristics. The early, Tat-independent phase promotes low-level expression of viral gene products. It is driven by interactions of host transcription factors and cis-acting elements located within enhancer and promoter regions in 5' long-terminal repeat (LTR) sequences of the proviral DNA. In the context of activated CD4+ T cells, the preferred targets of HIV infection, cellular transcription factors, such as NF-κB, bind to tandem NF-κB recognition sites within the HIV promoter and induce expression of full-length HIV transcripts through Tat-independent recruitment of P-TEFb. However, as this recruitment is transient and less robust than with Tat, this first phase is characterized by a proportionally higher production of short or prematurely terminated transcripts that do not support HIV replication (Figure 2-1).

The second Tat-dependent phase promotes high-level viral protein expression. It is driven by adequate levels of the Tat protein generated from full-length viral transcripts produced in the first phase. These transcripts undergo multiple splicing events before being exported to the cytoplasm to produce early viral gene products, such as the viral Nef and Rev proteins. Importantly, these transcripts also promote production of Tat, which jumpstarts full-length HIV transcript production and fuels persistent expression of Tat in a positive autoregulatory feedback loop (Figure 2-1). As a result, the majority of full-length HIV RNA chain assembly, followed by successful viral replication, occurs in the presence of adequate Tat expression and activity. While Tat’s role in transcription elongation is well established and the focus of intense studies in the HIV and transcription fields, additional studies point to potential functions of Tat in initiating HIV transcription by recruiting histone-modifying and chromatin-remodeling complexes as well as the stimulation of transcription initiation in the absence of TATA-box-binding protein-associated factors (TAFs).

Most models attribute the early expression of Tat to full-length transcript production from integrated proviral DNA as described above, but alternative or complementary models exist. 1) Tat may be expressed before proviral integration from nonintegrated viral DNA, a common feature of HIV and other retroviruses. Tat transcripts have been detected as early as 1 hour post-infection in the absence of integration; these may increase the initial pool of Tat proteins fueling early viral transcription. 2) Small amounts of Tat may be incorporated into viral particles and may jumpstart early viral transcription. Support for this model comes from observed defects
Figure 2-1 – Tat expression is fueled by a positive feedback loop.
of Tat-deficient viruses in pre-transcription steps of the viral lifecycle, such as reverse transcription. Moreover, Tat peptides have been detected by mass spectrometry in highly purified preparations of HIV-1 virions produced by macrophages. However, most studies fail to detect Tat in viral particles. 3) Tat may translocate from neighboring infected cells and activate HIV transcription. Tat contains a protein domain that allows cell membrane penetration, and release of biologically active forms of Tat by infected cells has been reported. While the release and uptake of extracellular Tat has been observed in vitro and may contribute to transcriptional and pathogenic effects of Tat, especially in sanctuaries such as the brain, the quantity and in vivo biological activity of extracellular Tat remain unclear.

(2) Tat is a small versatile factor with protein and RNA-binding properties
Tat is a small nuclear/nucleolar protein of 72–101 amino acids (aa) that displays a net positive charge, low hydrophobicity, and low sequence complexity. In the early phases of transcription, a full-length protein (101 aa) is encoded by two tat exons and expressed from highly spliced HIV transcripts, whereas later in infection, an additional one-exon form (72 aa) is produced from unspliced transcripts because of a stop codon in the intron after the first tat exon. An 86-aa form of Tat is a variant found in the laboratory-adapted strain HXB2 and in subtype D and H Tats (Figure 2-2).

Traditionally, Tat is divided into six conserved regions, based on functional contributions and protein or RNA interactions (Figure 2-2). The acidic and proline-rich N-terminus (aa 1–21) is required for LTR transactivation and binds the human transcriptional coactivator and acetyltransferase CREB-binding protein (CBP). The adjacent cysteine-rich region (aa 22–37) features seven clustered cysteine residues, all of which except Cys31 are essential for Tat’s transcriptional activity, in a sequence reminiscent to zinc finger motifs common to many human transcription factors. The Tat core (aa 38–48) displays high sequence conservation among subtypes (Figure 2-2) and, with the N-terminus and cysteine-rich region, forms the so-called activation domain (AD, aa 1–48). The Tat AD provides the primary interaction surface for P-TEFb and is essential for the powerful transactivation of the HIV LTR by Tat. In a recent crystallographic structure of Tat/P-TEFb, the Tat AD extensively interacted with P-TEFb with Tat being almost completely buried into a cleft between the first and second cyclin boxes of cyclin T1 (Tahirov et al, 2010). Cyclin T1 is in contact with 88% of the covered Tat surface area; Tat formed more hydrogen bonds with P-TEFb than it did with itself. In addition, the N-terminus of Tat contacted the so-called T loop in CDK9, a part of CDK9 critically regulating its enzymatic activity. These findings support a widely held view that Tat is intrinsically structurally disordered and requires host cofactors for proper folding.
Figure 2-2 – BLOSUM sequence alignment of consensus Tat sequences from HIV-1 group M subtypes A-H, as obtained from http://www.hiv.lanl.gov/. The Tat sequence from the lab-adapted HXB2 isolate is also shown (Uniprot ID: P04608).
Next to the Tat AD lies the arginine-rich motif (ARM) (aa 49–57), which has general RNA-binding properties and in cooperation with cyclin T1 makes specific contact with TAR RNA. TAR spans positions +1 through +59 of the 5' ends of nascent viral transcripts, and is thus transcribed irrespective of Tat. TAR spontaneously adopts a stem-loop structure and a three-nucleotide pyrimidine-rich bulge, a unique and invariable secondary structure. Both of these features provide recognition surfaces for the Tat ARM, and cyclin T1 binds TAR only in complex with Tat. Tat:cyclin T1 binding to TAR is highly cooperative, suggesting that TAR functions to select Tat molecules in complex with P-TEFb. No crystal structure of the HIV Tat ARM in complex with TAR and P-TEFb has been solved so far, but a similar structure of the equine anemia infectious virus (EIAV) Tat protein shows that the retroviral ARM is helical and binds the major groove of the TAR stem-loop (Anand et al., 2008). The N- and C-terminal regions flanking the ARM interact with cyclin T1 to expose the helical ARM for TAR recognition, providing structural basis for cyclin T1 and Tat cooperativity in TAR binding. Importantly, the ARM also serves as a nuclear/nucleolar localization signal and harbors many of the known posttranslational modifications of Tat. Basic residues within the ARM are also essential for the ability of Tat to translocate across intact cell membranes.

The glutamine-rich region of Tat (aa 58–72) is implicated in altering cell fate by inducing T-cell apoptosis, without any specific role in transcription attributed to this region. Similarly, the Tat C-terminus (aa 72–101) has also been implicated in altering T-cell fate and activation. This region is entirely encoded by the second tat exon, and is thus only present in the full-length form of Tat. Historically, due to extensive studies of the truncated, 86-aa laboratory-strain mutant of Tat, it is likely that the biological activity of the full-length Tat C-terminus is not fully appreciated. The C-terminus in most strains contains a so-called RGD motif, which can bind surface integrins and, thus, in addition to the ARM, may play a role in the uptake of extracellular Tat.

(3) How does Tat deliver active P-TEFb to nascent viral transcripts?

The tripartite interaction among Tat, P-TEFb, and TAR RNA is at the core of productive HIV transcription (Wei et al., 1998). It is within this trimolecular complex that Tat enacts its leading function—enabling robust elongation of viral transcripts. Owing to the fact that P-TEFb controls transcriptional elongation of many human genes, CDK9 catalytic activity within the cell is tightly regulated. P-TEFb occurs in HeLa cells in roughly equal parts as a small, active complex and as a large, inactive complex, in which CDK9 and cyclin T proteins are sequestered by the small nuclear (sn) RNA 7SK and several accessory proteins termed the 7SK snRNP, which include the CDK9 inhibitors Hexim 1 or Hexim2, the La-related protein LARP7, and the
methylphosphate-capping enzyme MEPCE. CDK9 within this complex is inactive, but considered poised for rapid activity after the dissociation of the inhibitory factors. While this mechanism of P-TEFb regulation is well established in tumor cell lines, much less is known about the regulation of P-TEFb activity in non-transformed cells. In resting CD4+ T cells, cyclin T1 levels are low due to posttranscriptional regulation via microRNAs (Chiang et al., 2012).

It is mechanistically unclear how Tat delivers active P-TEFb to the site of TAR production. While active P-TEFb associates with the bromodomain-containing protein 4 (BRD4) to activate transcription elongation of cellular genes, BRD4 serves as a cellular competitor of Tat for P-TEFb binding. Tat might extract P-TEFb from its complex with BRD4 to deliver the cyclin T1/CDK9 core complex to the HIV promoter. However, structural similarities between the 7SK snRNP:P-TEFb and the Tat:TAR:P-TEFb complex provide alternative hypotheses. Indeed, a fraction of cellular Tat is found in complex with 7SK snRNA and several snRNP components except Hexim proteins (Sobhian et al., 2010), and the Tat ARM shares remarkable sequence similarity with the RNA-binding domain of Hexim. These findings support a model where Tat dismantles the 7SK snRNP through direct competition with Hexim1 for 7SK snRNA binding. A distinct model proposes that Tat competes with Hexim1 for cyclin T1 binding, as Tat and Hexim1 bind to the same region in cyclin T1 and Tat exerts higher affinity for this site in vitro.

Tat may employ one or more of these activation mechanisms with existing or newly forming P-TEFb complexes. Whether inactive P-TEFb complexes engage with chromatin and become locally activated during the transition to productive transcription remains open. Evidence exists that Tat targets the inactive P-TEFb relegated by the 7SK snRNP to the chromatinized provirus, suggesting that Tat liberates P-TEFb at the site of transcription. The synthesis of TAR has a critical role in the ejection of the 7SK snRNP once it is assembled at the viral promoter. In this model, the emergent TAR competitively displaces the 7SK snRNA and snRNP components, thus activating CDK9. The precise role of Tat in this process and the presence of TAR in short transcripts remain open questions. Tat function likely involves a combination of activation mechanisms to adapt HIV transcription to different cellular environments to ensure vigorous high-level HIV transcription.

(4) Tat binds cellular cofactors other than P-TEFb

While functional and structural data show that P-TEFb, in conjunction with TAR, is a crucial cofactor for Tat transactivation, several bioinformatic and experimental studies sought to determine the global catalogue of human factors that interacts with Tat. Approaches include algorithms designed to extract information from existing literature, large-scale yeast-two-hybrid
screens, siRNA screens, and tandem affinity purification coupled to mass spectrometry. These studies confirmed reported interaction partners and revealed novel Tat-interacting factors. The nearly 200 human Tat interaction partners include the ATP-dependent RNA helicase DDX5, the protein deacetylase SIRT1, the protein phosphatase 1G, and the E3 ubiquitin-protein ligase Praja2.

Proteomic approaches identified a novel transcription elongation complex with which Tat associates (He et al., 2010; Sobhian et al., 2010). This so-called super elongation complex (SEC) combines active P-TEFb with another elongation factor PAF1c and several proteins that are known as mixed lineage leukemia–fusion partners, including ELL2, AFF4, ENL, and AF9. ELL2 enhances transcriptional elongation by increasing the catalytic rate and preventing the pause by pol II. Several studies reproducibly showed that Tat interacts with the SEC to deliver the elongation complex to the site of viral transcription. In this way, Tat allows for the cooperative activities of P-TEFb, PAF1c and ELL2 to enhance HIV transcriptional elongation at multiple levels, including both the recruitment and activation of P-TEFb and the assembly and stability of the SEC.

Interestingly, Tat and P-TEFb both have functions beyond elongation and may regulate distinct phases of HIV transcription, such as transcript splicing. The HIV Tat-specific factor 1, necessary for efficient Tat-dependent transcription, was implicated in mRNA splicing through its interaction with components of the spliceosomal machinery. Similarly, a distinct Tat interaction partner, CA150, affects transcriptional elongation and splicing. P-TEFb itself localizes to nuclear speckles where splicing occurs and interacts with the splicing factor c-Ski-interacting protein, an interaction important for Tat’s transcriptional function. These findings and others suggest that Tat regulates HIV transcription at multiple levels.

(5) Posttranslational modifications fine-tune Tat function

Tat may accommodate interactions with different cofactors in a timely manner through its repertoire of posttranslational modifications. The list of these modifications—clustered mainly in the Tat ARM—is growing (Table 2-1). Posttranslational modifications add new protein:protein interfaces to Tat and allow for tunable, highly regulated interactions with host cell factors. Certain modifications provide recognition surfaces for specific protein domains, such as bromodomains, which recognize and bind acetyl-lysine residues. Indeed, acetylation of Tat at Lys50 enables interaction of the Tat ARM with the bromodomain of the acetyltransferase PCAF, thereby recruiting the acetyltransferase activity to the site of viral transcription (Mujtaba et al., 2002). In contrast, certain Tat modifications negatively regulate protein:protein and protein:RNA
<table>
<thead>
<tr>
<th>Modification</th>
<th>Tat Residue(s) Modified</th>
<th>Enzyme(s) Adding</th>
<th>Enzyme(s) Removing</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation</td>
<td>K28</td>
<td>PCAF</td>
<td>HDAC6</td>
<td>Enhances interaction among Tat, P-TEFb, and TAR</td>
</tr>
<tr>
<td>Acetylation</td>
<td>K50, K51</td>
<td>GCN5, p300</td>
<td>SIRT1</td>
<td>Dissociates TAR from Tat and P-TEFb Generates interaction interface for PCAF bromodomain</td>
</tr>
<tr>
<td>Methylation</td>
<td>R52, R53</td>
<td>PRMT6</td>
<td>Unknown</td>
<td>Inhibits formation of Tat:TAR:P-TEFb complex Increases Tat half-life</td>
</tr>
<tr>
<td>Methylation</td>
<td>K50, K51</td>
<td>SETDB1</td>
<td>Unknown</td>
<td>Knockdown of SETDB1 enhances Tat-mediated transcription</td>
</tr>
<tr>
<td>Methylation</td>
<td>K51</td>
<td>Set7/9</td>
<td>LSD1</td>
<td>Enhances interaction among Tat, P-TEFb, and TAR</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>S62, S68, T64</td>
<td>PKR</td>
<td>Unknown</td>
<td>Phosphorylation-deficient Tat triple-mutant reduces Tat-mediated transcription</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>S16, S46</td>
<td>Cdk2</td>
<td>Unknown</td>
<td>Phosphorylation-deficient Tat mutant reduces Tat-mediated transcription</td>
</tr>
<tr>
<td>Ubiquitination</td>
<td>K71</td>
<td>Hdm2</td>
<td>Unknown</td>
<td>Unclear; does not impact Tat half-life</td>
</tr>
</tbody>
</table>

Table 2-1 – Tat posttranslational modifications.
interactions. For example, methylation of Arg52 and Arg53 within the Tat ARM by the protein methyltransferase 6 (PRMT6) interferes with Tat binding to both TAR and P-TEFb.

The functional relevance of some known Tat modifications is unclear. For example, Tat mutants deficient in phosphorylation sites display reduced transactivation capacity, although the mechanism through which phosphorylation enhances transactivation is unknown. Similarly, while Tat is ubiquitinated, and this modification has little effect on the half-life of Tat, the molecular mechanism of how this modification enhances Tat transcriptional activity is not understood.

(6) Is Tat involved in HIV-1 latency?
A key role in understanding Tat’s involvement in the establishment or maintenance of latency is rooted within the feed-forward mechanism regulating Tat expression (Figure 2-1). Viral latency is defined as a state of reversibly non-productive infection often associated with a transcriptionally silenced integrated provirus in subsets of resting memory CD4+ T cells. These latent reservoirs are found in every infected individual and are widely recognized as premier barriers to eradicating HIV from patients. The persistence of infection requires life-long treatment with anti-retroviral therapy and is associated with shortened lifespan and numerous HIV-associated comorbidities. While the role of Tat in the establishment, maintenance, and reversal of HIV-1 latency remains under investigation, reports point to levels of Tat expression and activity as central contributors to latency.

In latent cells, HIV LTR activity is expected to be silenced—resulting in a lack of Tat production. Key transcription factors, such as NF-κB and NFAT, are sequestered in the cytoplasm in resting central memory T cells, a critical reservoir for latent HIV infection in patients, and are therefore unavailable within the nucleus to activate viral transcription. In addition, levels of cyclin T1 are low in resting CD4+ T cells, preventing low levels of Tat from effectively transactivating HIV transcription.

Evidence that lack of functional Tat contributes to latency comes from in vitro experiments in which increased intracellular expression of Tat is sufficient to inhibit the establishment of latency and even reactivate most of the latent cell populations tested. Moreover, viruses that were recovered from non-productively infected CD4+ T cells in patients are enriched for Tat variants with attenuated transactivation activity, suggesting that impaired Tat activity contributes to the establishment and/or maintenance of latency. Interestingly, small stochastic fluctuations in Tat gene expression are sufficient to drastically affect the Tat feedback loop and determine whether viral latency or productive infection ensues (Weinberger et al., 2005). Thus, similar to actively infected cells, Tat activity in latent cells autoregulates its own production, or in this case, the
lack of its production; this lack of production contributes critically to the robust silencing of HIV transcription in latency. The precise mechanisms of how Tat function is inactivated in latently infected cells and full-length transcript production is kept low are currently the subject of intense research.

(7) **Tat is an important effector of HIV pathogenesis**

In addition to its role in regulating HIV-1 transcription and latency, numerous intracellular and extracellular activities have been attributed to Tat. They include modulating host immune responses, mediating survival and apoptotic processes, and stimulating cellular growth/proliferation, ultimately ensuring viral persistence and effective viral spread (Table 2-2). Some of these effects are linked to Tat interaction partners, such as the (NAD$^+$)-dependent deacetylase SIRT1. Tat binds avidly to SIRT1 and inhibits SIRT1’s catalytic activity, thus preventing the timely deacetylation of downstream SIRT1 targets, such as NF-κB. As acetylated NF-κB is transcriptionally more active, this effect of Tat causes increased NF-κB transcriptional activity and elevated expression of immune activating cytokines that contribute to a state of generalized immune activation observed in HIV infection. Increased immune activation is a hallmark of HIV infection directly linked to the progression to AIDS and the unrestricted spread of HIV infection.

Tat also associates with host gene promoters *in vivo*. A genome-wide chromatin immunoprecipitation approach identified ~450 cellular promoters that were occupied by Tat, including the promoters of the phosphatase PTEN and two subunits of the phosphatase PP2A (Kim et al., 2010). This study links Tat with the activation of phosphoinositide 3-kinase-mediated apoptotic pathways in HIV-infected CD4+ T cells, a possible explanation of how Tat contributes to the depletion of CD4+ helper T cells in patients. Other research has demonstrated the ability of Tat to bind and inhibit the function of the host protein Dicer, an RNase III-like enzyme that processes microRNA (miRNA) and short interfering RNA (siRNA). Because RNA silencing may also serve as a defense mechanism against viral infection, Tat suppression of Dicer’s ribonuclease-directed processing activity may allow HIV-1 to more effectively evade cell-based immunity.

The ability of Tat to translocate to neighboring cells theoretically amplifies its pathogenic effect and may be specifically relevant in distinct sanctuaries, such as the central nervous system (CNS). Before crossing the plasma membrane and accessing the extracellular space, Tat is recruited and inserted into the membrane via its basic ARM in a phosphatidylinositol (4,5) biphosphate-dependent manner. Outside the cell, Tat functions as a promiscuous ligand that
Table 2. Extra-transcriptional effects of intracellular and extracellular forms of Tat

<table>
<thead>
<tr>
<th>Functions</th>
<th>Cell-type Affected</th>
<th>Mechanisms</th>
<th>Contributions to Pathogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune Activator</td>
<td>T-Cells</td>
<td>Induces expression of pro-inflammatory cytokines: IL-1, IL-6, TNF-α</td>
<td>Recruits and activates immune cells for propagation of infection</td>
</tr>
<tr>
<td></td>
<td>Dendritic Cells</td>
<td>Increase CCR5, CXCR4, and CD40 expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Binds chemokine receptors: CXCR4, CCR2, and CCR3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microglia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune Suppressor</td>
<td>T-Cells</td>
<td>Reduces expression of MHC Class I molecules, IL-2, and NO</td>
<td>Promotes evasion of immune surveillance</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Induces expression of IL-4 and IL-10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Degrades RON receptor tyrosine kinase</td>
<td></td>
</tr>
<tr>
<td>Pro-apoptotic</td>
<td>T-Cells</td>
<td>Perturbs microtubule dynamics and promotes mitochondria-dependent apoptosis (i.e. Bim)</td>
<td>Increases susceptibility to infections by weakening immune system</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Increases TRAIL, caspase-3, and caspase-8 activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Modulates Fas/FasL system</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associates with PTEN and PP2A promoters</td>
<td></td>
</tr>
<tr>
<td>Anti-apoptotic</td>
<td>T-Cells</td>
<td>Increases Bcl-2 expression</td>
<td>Allows for viral replication</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Reduces expression of p53</td>
<td>Oncogenic</td>
</tr>
<tr>
<td>Neurotoxic</td>
<td>Neurons</td>
<td>Alters neuronal calcium flux</td>
<td>Promotes development of dementia</td>
</tr>
<tr>
<td></td>
<td>Microglia</td>
<td>Activates excitatory amino acid receptors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Astrocytes</td>
<td>Effects inflammatory and oxidative processes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endothelial</td>
<td>Interferes with Nerve Growth Factor (NGF)</td>
<td></td>
</tr>
<tr>
<td>Growth Factor</td>
<td>Kaposi’s Sarcoma</td>
<td>Stimulates growth of Kaposi’s sarcoma cells</td>
<td>Oncogenic</td>
</tr>
<tr>
<td></td>
<td>Endothelial</td>
<td>Activates VEGFR-1, VEGFR-2, and integrins</td>
<td>Angiogenic</td>
</tr>
<tr>
<td>Gene Regulator</td>
<td>T-Cells</td>
<td>Activates NF-κB activity through ROS production, degradation of IκB-α, interaction with p50-p65 heterodimers, and suppression of SIRT1 deacetylase activity</td>
<td>Promotes HIV-1 transcription</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td>Regulates mRNA capping and splicing</td>
<td>Enhances pleiotropic cellular effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Binds and inhibits Dicer function to suppress production of host siRNA</td>
<td>Subverts cellular defense</td>
</tr>
</tbody>
</table>

Table 2-2 – Extra-transcriptional effects of intracellular and extracellular forms of Tat.
binds a number of chemokine and integrin surface receptors on a variety of cell types. The majority of these proposed Tat “receptors” (e.g., lipoprotein receptor–related protein, CXC chemokine receptor type 4, and heparin sulphate proteoglycans) are endocytic receptors that may internalize Tat upon binding. In addition, the Tat ARM functions as a protein transduction domain (PTD), a stretch of basic amino acids also found in antennapedia, a *Drosophila* homeodomain transcription factor, and VP22, a herpes simplex virus 1 structural protein, that may allow for spontaneous translocation of Tat across plasma membranes through direct electrostatic interactions with negatively charged phospholipids or through the formation of inverted micelles.

(8) **Therapeutic strategies surrounding Tat**

Because Tat plays essential roles in HIV replication and pathogenesis as well as in protein transduction, it is not surprising that many therapeutic approaches have focused on Tat. These include therapeutic attempts to disrupt Tat transcriptional activity and binding to TAR RNA, a Tat–based vaccine, and the use of Tat peptides as vehicles for cellular delivery of various macromolecules into cells.

A main focus of Tat–based therapy research is small molecules that target the crucial interaction between Tat and TAR RNA, with the promise to halt HIV replication at an early stage. One example is 7-chloro-5-(2-pyrryl)-3H-1,4-benzodiazepine-2(H)-one, which inhibits Tat–dependent transcription in a manner relying on TAR. This compound inhibits replication of HIV-1 and HIV-2. Despite the demonstrated promise for this molecule *in vitro*, a limited therapeutic window and observed toxicity of this drug were issues that preempted its further development. Other examples of compounds that inhibit the Tat:TAR interface include quinoline derivatives, diphenylfuran derivatives, beta-carboline derivatives, and aminoglycoside-arginine conjugates.

Tat is also considered a promising candidate target for an anti-AIDS vaccine. Tat is the most conserved HIV protein, thus limiting the risk for the development of resistance. In both primates and humans, cellular and humoral immune responses against Tat are rapid and positively correlated with slower progression to AIDS. Results from recent cross-sectional and longitudinal studies have demonstrated that a Tat vaccine based on the biologically active Tat protein alone is safe and may reduce HIV-1 replication and disease progression.

Since the discovery that the Tat ARM serves as a PTD, research into the use of short peptides mimicking Tat in the cellular uptake of various molecular cargo has dramatically expanded. A key property of these cell-penetrating peptides is that they are cationic and
typically enriched in positively charged residues, such as lysines or arginines, as found in the Tat ARM. Peptide fragments encoding tyrosine 47 through arginine 57 of the Tat ARM have been used to transport DNA, proteins, and other materials through the cell membrane and into the cell nucleus. Prominent examples of Tat fusion peptides include a Tat-Bcl-xL fusion to inhibit apoptosis in ischemia, a Tat-p53 fusion to increase the tumor suppressive activity of p53 in tumor cells, and gp91dsTat, which blocks the assembly of the NAD(P)H oxidase complex to alleviate the increase in systolic blood pressure caused by angiotensin II injections. Although the use of Tat peptides holds great promise for gene transfer and drug delivery, current use is limited by a lack of tissue specificity and an incomplete understanding of uptake modes.

**Conclusion**

Research into Tat biology has been successful in providing an increasing molecular understanding of HIV transcription and latency. Studies of Tat have fueled important biological discoveries, such as the central role of P-TEFb in transcriptional regulation and the design and usage of Tat-adapted PTDs for the transport of molecular cargo into cells. The “pathogenetic” functions of Tat (i.e., in modulating immune activation and cell survival of infected and neighboring cells) have gained molecular shape in part by identifying novel Tat interaction partners. The urgency in better understanding and eliminating viral latency has refocused the field on possible therapeutic interventions targeting HIV transcription. Future pursuits will inevitably need to acknowledge the prominent role of Tat as a central regulator of HIV transcription and address the powerful autoregulatory feedback loop through which Tat regulates its own expression.
References


Chapter III
Bromodomain Proteins in HIV Infection

Daniela Boehm 1,2#, Ryan J Conrad 1,2# and Melanie Ott 1,2*

1 Gladstone Institute of Virology and Immunology, San Francisco, CA 94158, USA;
2 Department of Medicine, University of California, San Francisco, CA, 94158, USA;
E-Mails: daniela.boehm@gladstone.ucsf.edu (D.B.); Ryan.Conrad@ucsf.edu (R.J.C.);
mott@gladstone.ucsf.edu (M.O.)
# Authors contributed equally.
* Author to whom correspondence should be addressed; E-Mail: mott@gladstone.ucsf.edu
  (M.O.);
  Tel.: +1-415-734-4807; Fax: +1-415-355-0855.

Abstract: Bromodomains are conserved protein modules of ~110 amino acids that bind acetylated lysine residues in histone and non-histone proteins. Bromodomains are present in many chromatin-associated transcriptional regulators and have been linked to diverse aspects of the HIV life cycle, including transcription and integration. Here, we review the role of bromodomain-containing proteins in HIV infection. We begin with a focus on acetylated viral factors, followed by a discussion of early structural studies defining the interaction of the acetylated HIV Tat transactivator with the KAT2B/PCAF bromodomain as a drug target. We end with an overview of promising new studies of bromodomain inhibitory compounds for the treatment of HIV latency.

Keywords: Bromodomains; HIV; PCAF; PBAF; p300; CBP; TRIM28; BRD2, BRD4

1. The bromodomain protein family
Reversible modifications of nucleosome components are increasingly acknowledged for their regulatory potential (Holloway and Oakford, 2007). One such modification is acetylation, which involves the transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine side chains on histone and non-histone proteins (rev. in (You et al., 2012)). This modification is catalyzed by lysine acetyltransferases (also known as KATs, or HATs) (You et al., 2012). Their action is reversed by lysine deacetylases (KDACS, also known as HDACs) or sirtuins (SIRTs) (You et al., 2012). Histone acetylation is a well-studied modification that antagonizes the nucleosomal DNA-protein interaction, promoting chromatin accessibility and transcriptional activation (Graff and Tsai, 2013). However, aside from causing physiochemical
changes in the histone core, acetylation also generates novel and unique interaction interfaces for the assembly of macromolecular complexes important for a variety of cellular processes.

The bromodomain is a conserved protein module of ~110 amino acids that recognizes and binds ε-N-acetylated lysine residues in histone and non-histone proteins (Wu and Chiang, 2007). Recognition of acetyl-lysine residues by bromodomain-containing proteins is at least partly responsible for the functional consequences linked to protein acetylation (Wu and Chiang, 2007). The first reference to a bromodomain can be traced to the Drosophila gene brahma (brm) (Tamkun et al., 1992), and the human bromodomain family to date includes 46 distinct proteins and 61 unique bromodomains (Figure 3-1A) (Filippakopoulos et al., 2012b). Select transcriptional regulators (i.e, BRD4, TAF1, TIF1), chromatin-modifying enzymes (i.e., p300, PCAF, MLL), and nucleosome remodelers (i.e., SMARAC2, PB1, BAZ1B) contain bromodomains (Filippakopoulos and Knapp, 2012).

Structurally, bromodomains are comprised of four left-handed α-helices (αZ, αA, αB, and αC) connected by two loops (ZA and BC loops) (Figure 3-1B) (Dhalluin et al., 1999). This structure forms a deep hydrophobic cavity that serves as the acetyl-lysine recognition site (Filippakopoulos et al., 2012b). While the helical regions are moderately conserved among different bromodomains, the loop regions are highly variable in both length and sequence composition (Filippakopoulos et al., 2012b). This loop variability guides the substrate specificity observed among members of the bromodomain family. Co-crystal structures of bromodomains with various acetylated peptides demonstrate that the neutralized acetyl-lysine residue forms a hydrogen bond with an asparagine residue found in most bromodomains (Figure 3-1C). Some bromodomains exhibit higher affinity for multiply acetylated substrates, while the affinities of others are regulated by additional post-translational modifications of the substrate, such as phosphorylation (Filippakopoulos et al., 2012b; Moriniere et al., 2009).

2. HIV infection and reversible acetylation

Reversible acetylation of histone and non-histone proteins plays a key role in HIV transcription and is a lead target in preclinical and clinical efforts to reverse HIV latency (Archin et al., 2012; Contreras et al., 2009; Edelstein et al., 2009; Kelly et al., 2003; Kent et al., 2013). Upon integration into the human genome, the HIV proviral cDNA is organized into higher-order
Figure 3-1 – The human bromodomain family. (A) Phylogenetic tree of 57 human bromodomains. Those bromodomain-containing proteins that have been shown to interact with HIV proteins are denoted in red with the corresponding viral factor indicated alongside. Phylogenetic trees were generated using Seaview v4.4.1 with individual bromodomain sequences obtained from (23). (B) Structure of the first bromodomain of BRD4 (purple) in complex with a diacetylated histone peptide (blue). Histone acetyl-lysine residues are shown in yellow. Structural representations in Figure 3-1B and 3-1C were rendered using Chimera (UCSF) with PDB:3UVW. (C) Top view of interaction between first bromodomain of BRD4 and a diacetylated histone peptide. The hydrogen bond between the canonical bromodomain asparagine residue (N140 in BRD4) and the histone acetyl-lysine residue is shown in red with an estimated length of 2.94Å.
chromatin and becomes subject to regulation by host chromatin-modifying enzymes, including acetyltransferases and deacetylases (Verdin et al., 1993). Indeed, it has been shown that following stimulation with phorbol esters, distinct lysines in histone H3 (H3K9 and H3K14) become rapidly acetylated within a single nucleosome (nuc-1) located immediately downstream of the viral transcription start site (Lusic et al., 2003). However, the HIV provirus differs from cellular genes because it encodes a viral protein called transactivator of transcription (Tat). Tat is essential for HIV replication. It relieves a powerful block to the elongation of HIV transcripts by cooperatively binding to (1) an RNA stem-loop structure called TAR present at the 5’ end of all nascent viral transcripts and (2) the positive transcription elongation factor b (P-TEFb), which together with other elongation factors, including PAF1c, forms a “super-elongation complex” (Sobhian et al., 2010).

Additionally, Tat is known to recruit several acetyltransferases to the HIV LTR, thus enhancing HIV transcription in the context of chromatin. These include KAT3B/EP300/p300 (Marzio et al., 1998), KAT3A/CREBBP/CBP (Marzio et al., 1998), KAT2A/GCN5 (Lusic et al., 2003), and KAT2B/PCAF (Lusic et al., 2003). Conversely, several HDACs have been shown to bind to the HIV promoter located in the 5’ long terminal repeat (LTR) through interactions with cellular transcription factors, including YY1 (Coull et al., 2000), LSF (Coull et al., 2000), NF-κB (Williams et al., 2006), AP-4 (Imai and Okamoto, 2006), CBF-1 (Tyagi and Karn, 2007), c-Myc and Sp1 (Jiang et al., 2007). HDAC inhibitors, which are known to activate HIV from latency in cell culture models (Van Lint et al., 1996), are being clinically tested for their potential to reactivate HIV from transcriptional latency (Archin et al., 2012).

Tat itself is subject to reversible acetylation. Tat is acetylated by KAT2B/PCAF at lysine 28 within a characteristic cysteine-rich region required for its interaction with P-TEFb (Kiernan et al., 1999). Tat is also acetylated by KAT3B/EP300/p300 and the close KAT2B/PCAF homologue KAT2A/GCN5 at lysines 50 and 51 located in its basic RNA-binding domain (Col et al., 2001; Kiernan et al., 1999; Ott et al., 1999). Both acetylation events positively support Tat’s transcriptional activity (Bres et al., 2002; Kaehlcke et al., 2003) and are reversed by the deacetylase activities of HDAC6 and SIRT1 (Huo et al., 2011; Pagans et al., 2005). In addition to Tat, HIV integrase, a DNA-binding protein that catalyzes 3’ processing and strand transfer of the viral genome, is acetylated by KAT3B/EP300/p300 at lysines 264, 266 and 273 (Cereseto et al., 2005). These residues are also subject to acetylation by KAT2A/GCN5, in addition to lysine 258 (Terreni et al., 2010). Integrase acetylation increases the affinity of the enzyme for DNA and also enhances strand-transfer catalysis in vitro. Lastly, Vpr, a viral protein implicated in nuclear translocation of the HIV pre-integration complex, HIV-mediated G2/M arrest, and transcription of
viral and cellular promoters, interacts with KAT3B/EP300/p300 (Kino et al., 2002). Mutational analysis suggests that the Vpr-p300 interaction occurs independently of the p300 bromodomain (Kino et al., 2002), although it is unclear whether Vpr is acetylated.

3. Interactions between bromodomain-containing proteins and HIV proteins

3.1 Acetylation-dependent interactions

3.1.1 p300/CBP-associated factor (PCAF)

KAT2B/PCAF (p300/CBP-associated factor) is a histone acetyltransferase that contains a N-acetyltransferase domain and a C-terminal bromodomain. KAT2B/PCAF also participates in the reversible acetylation of various non-histone proteins, including p53, β-catenin, retinoblastoma protein (Rb), and several transcriptional regulators, such as the general transcription factors TFIIEβ and TFIIIF and the sequence-specific transcription factors E2F1, c-Myc, and MyoD (reviewed in (Nagy and Tora, 2007)(Wang et al., 2013a)). In HIV infection, KAT2B/PCAF acetylates Tat at lysine 28 (D’Orso and Frankel, 2009; Kiernan et al., 1999). Acetylation of Tat on lysine 28 facilitates recruitment of P-TEFb kinase, resulting in the efficient phosphorylation of heptaserines in the carboxyl terminus of cellular RNA polymerase II and thus promoting HIV transcript elongation (Bres et al., 2002; D’Orso and Frankel, 2009; Kiernan et al., 1999). KAT2B/PCAF is recruited to Tat through an acetylation-dependent mechanism (Figure 3-2). Acetylated K50 in Tat acts as a specific binding partner of the KAT2B/PCAF bromodomain, an interaction that was examined by NMR spectroscopy at the structural level and characteristically involves additional residues in Tat that interact with the KAT2B/PCAF bromodomain in an acetylated K50-dependent manner (Dorr et al., 2002; Mujtaba et al., 2002). KAT2B/PCAF binding to Tat positively supports HIV transcription either through enhanced lysine 28 acetylation in Tat, or enhanced local histone acetylation during HIV transcriptional elongation. Consequently, mutations in the KAT2B/PCAF bromodomain that suppress interactions with acetylated Tat or treatment with small molecules that specifically bind the KAT2B/PCAF bromodomain effectively suppress Tat transactivation, supporting the concept that this interaction could serve as a specific target for anti-HIV transcription therapeutics (Dorr et al., 2002; Zeng et al., 2005).

3.1.2 Switch/Sucrose Non-Fermentable (SWI/SNF)

Members of the SWI/SNF family form evolutionarily conserved chromatin-remodeling complexes that utilize the energy of ATP hydrolysis to induce nucleosome remodeling. Two important and distinct SWI/SNF complexes have been described in humans: BAF and PBAF. These complexes contain either Brg-1 (also known at SMARCA4) or BRM (also known as
Figure 3-2 – Role of bromodomain proteins in HIV infection. Bromodomain proteins implicated in HIV transcription, HIV integration, and cell cycle progression are schematized. Briefly, Tat acetylated at K50/51 interacts with BAF180 and Brg-1 within the PBAF complex to support viral transcription. Acetylated Tat also interacts with PCAF to induce local acetylation of histones and potentially other factors at the site of viral transcription. BRD4 is present at the HIV LTR, yet is hypothesized as an intracellular competitor of Tat, while the role of BRD2 in HIV transcription is unknown. Tat also interacts with TAF1 to repress select cellular promoters. Acetylated integrase displays enhanced enzymatic activity, yet generates an interaction interface for the TRIM28 bromodomain that in turn recruits the HDAC1 deacetylase, negatively impacting HIV integration. BRD4 and SP140 are both cell cycle regulators that interact with Vif, yet the bromodomain-dependence and functional significance of these interactions remain unclear.
SMARCA2) as the major catalytic subunit together with several accessory proteins. BAF is distinguished by the presence of the BAF250a/b subunit, while PBAF contains BAF180 (also known as Polybromo or PB1), BAF200, and BRD7 subunits. BAF has been shown to repress HIV transcription by positioning the nuc-1 nucleosome immediately downstream of the transcription start site in a manner that encumbers processive transcription (Rafati et al., 2011). Such repressive remodeling events may be crucial for the maintenance of HIV latency in the absence of Tat. Conversely, the PBAF complex is required for robust Tat-mediated transactivation of HIV expression (Mahmoudi, 2012; Van Duyne et al., 2011). Both shared constituents of BAF and PBAF, Brg-1 and BRM, interact with Tat. These interactions are regulated by acetylation of Tat, with K50 acetylation enhancing the Tat-Brg1 interaction (Mahmoudi et al., 2006) and inhibiting the Tat-BRM interaction (Treand et al., 2006). The interaction between acetylated Tat and Brg-1 was mapped to the Brg-1 bromodomain, while the Tat-BRM interaction was shown to be bromodomain-independent (Treand et al., 2006). Tat displays enhanced interaction with the PBAF-specific constituent BAF200 in a K50/K51-dependent manner (Easley et al., 2010). In addition, Tat acetylated at K50/K51 interacts with PBAF through the BAF180 subunit, an interaction that facilitates Tat-mediated transactivation (Figure 3-2) (Rafati et al., 2011). It is likely, though not experimentally confirmed, that acetylated Tat interacts with one or more of the six bromodomains present in BAF180. These findings evoke a model in which Tat acetylation at K50/K51 functions to switch the repressive BAF complex with the activating PBAF complex through specific interactions with several bromodomain-containing proteins in the PBAF complex, including Brg-1 and BAF180.

3.1.3 Bromodomain-containing protein 4 (BRD4)

BRD4 is a mitotic chromosome-associated protein that serves as an important regulator of post-mitotic transcription by recruiting various transcriptional regulators to acetylated chromatin. BRD4 is also required for maintaining a proper higher-order chromatin structure (Dey et al., 2003; Hubner et al., 2012; Wu and Chiang, 2007). BRD4 is a member of the bromodomain and extraterminal domain (BET) family of bromodomain proteins. Members of the BET family are distinguished by the presence of two functional domains – tandem bromodomains and a so-called extraterminal domain, the latter of which may serve to mediate protein-protein interactions (Wu and Chiang, 2007). BRD4 contains a third functional domain termed the P-TEFb-interacting domain (PID) (Bisgrove et al., 2007). The PID serves to recruit and activate the Tat cofactor P-TEFb, a heterodimer composed of cyclin T1 and CDK9 that when complexed with the HEXIM1 inhibitor is part of an inactive ribonucleoprotein complex found in HeLa cells and other tumor cell lines (Schroder et al., 2012). While BRD4 is an important factor recruiting
P-TEFb to the HIV promoter in the absence of Tat, Tat and the BRD4 PID compete for P-TEFb binding, making BRD4 a negative factor in Tat transactivation (Bisgrove et al., 2007; Yang et al., 2005). In addition, BRD4 negatively regulates HIV transcription by inducing an inhibitory phosphorylation event on CDK9 (Zhou et al., 2009).

The cyclin T1 subunit of P-TEFb is acetylated at four defined residues by KAT3B/p300 (Cho et al., 2009). Three of the four acetylated residues (K380, K386, K390) bind the second bromodomain of BRD4 defining a second acetylation-dependent P-TEFb interaction site in BRD4 besides the PID. Interestingly, while this acetylation-dependent interaction is required for basal HIV LTR activity and cellular gene expression, it is not necessary for Tat-mediated transactivation of HIV transcription, supporting the model that Tat- and BRD4-mediated activities at the HIV promoter are mutually exclusive (Bisgrove et al., 2007; Yang et al., 2005). Vollmuth et al. determined the crystal structures of the two bromodomains of BRD4 and showed that acetylated K390 weakly bound to the second bromodomain of BRD4 (Vollmuth et al., 2009). This binding is markedly enhanced when K380 and K386 are also acetylated (Schroder et al., 2012), supporting an emerging model that bromodomains of BET proteins have a preference for binding to multiply acetylated proteins.

3.1.4 Tripartite motif–containing protein 28 (TRIM28)

TRIM28 was originally identified as an interaction partner of members of the family of Krüppel-associated box (KRAB) domain–containing zinc finger transcription factors. It is also named KRAB-associated protein 1 (KAP1), KRAB-A-interacting protein 1 (KRIP1), and transcription intermediary factor (TIF) 1β. Its protein architecture includes an N-terminal tripartite motif (TRIM) that acts as a protein-protein and oligomerization interface, and contains a RBCC (Ring finger, two B-box zinc fingers, and a coiled coil) domain, a central heterochromatin protein 1 (HP1)-binding domain, a TIF1 signature sequence (TSS) domain, and a C-terminal plant homeodomain (PHD) and bromodomain. The TRIM28 bromodomain is a 100-amino acid stretch consisting of four α-helices that, similar to other bromodomain-containing proteins, has a conserved hydrophobic core and recognizes the backbone of histone tails (Zeng et al., 2008).

Structural analysis of the tandem PHD and bromodomain (PB) of TRIM28 between amino acids 624 and 812 revealed that both domains function as a cooperative unit to facilitate lysine sumoylation, which is required for TRIM28 co-repressor activity in gene silencing (Zeng et al., 2008). In a yeast two-hybrid screen, TRIM28 was identified as an interaction partner of acetylated HIV-1 integrase (Allouch et al., 2011). TRIM28 in vitro and in vivo preferentially binds integrase when acetylated at K264, K266 and K273, rather than the unmodified protein, implicating that this interaction is mediated by the TRIM28 bromodomain. Integrase K264, K266
and K273 are targeted for acetylation by KAT3B/p300, which is a prerequisite for the interaction of TRIM28 with integrase and leads to subsequent recruitment of HDAC1. As a consequence, TRIM28 inhibits HIV-1 integration through integrase deacetylation by HDAC1 (Figure 3-2) (Allouch and Cereseto, 2011).

3.2 Other interactions with bromodomain-containing proteins

3.2.1 Transcription initiation factor TFIIID, subunit 1 (TAF1)

TAF1, also referred to as TAF1_250, is the largest component of transcription factor TFIIID that is composed of TATA-binding protein (TBP) and a variety of TBP-associated factors. TAF1 contains N- and C-terminal serine/threonine kinase domains, but can also function as an acetyltransferase and an ubiquitin-activating/conjugating enzyme. It contains two tandem bromodomains, both located in the C-terminus of the protein. Structurally, the two bromodomains form two side-by-side, four-helix bundles, each with an acetyl lysine binding pocket at its center, recognized by diacetylated histone H4 peptides (Jacobson et al., 2000). When Weissman et al. identified TAF1 as a Tat interaction partner, the interaction was mapped to amino acids 80 to 83 in Tat and a site in TAF1 that overlaps the acetyltransferase domain, inhibiting TAF1 acetyltransferase activity (Weissman et al., 1998). TAF1 was not required for Tat transactivation of the HIV LTR; instead, the interaction with TAF1 was linked to Tat-mediated transcriptional repression, i.e. of the MHC class I promoter (Figure 3-2) (Weissman et al., 1998). These findings could be explained by a model in which the interaction with Tat inhibits TAF1 acetyltransferase activity, thereby recruiting an inactive Tat/TAF1 complex to actively transcribing gene promoters containing hyperacetylated histone H4.

3.2.2 Nuclear body protein SP140

SP140, also referred to as lymphoid-restricted homolog of Sp100 (LYSsp100) is a component of a subset of nuclear bodies in lymphoid cells. It is involved in the pathogenesis of acute promyelocytic leukemia and has been shown to be an autoantigen in primary biliary cirrhosis (Bloch et al., 1996; Dent et al., 1996; Granito et al., 2010). Its protein architecture includes a N-terminal homogeneous staining region (HSR) domain, a central SAND domain which mediates DNA binding, a PHD-type zinc finger, and a C-terminal bromodomain. In a yeast two-hybrid screen, SP140 was identified as an interaction partner of the HIV Vif protein (Madani et al., 2002). Vif enhances the infectivity of HIV virions released from so-called non-permissive cells by inducing the degradation of an antiviral restriction factor APOBEC3G (Sheehy et al., 2003). SP140 was found specifically in non-permissive cells, and HIV-1 infection induced its dispersal from nuclear bodies into cytosolic colocalization with Vif (Madani et al., 2002). SP140 interacts with the N-terminal and central regions of Vif (amino acids 1–112) in the
yeast two-hybrid screen, and the SP140 prey cDNAs that were isolated encoded the C-terminal region between amino acids 527 and 836, which includes the SAND domain, the PHD-type zinc finger, and the bromodomain (Madani et al., 2002). Further studies are needed to determine whether an acetyl-lysine-bromodomain interaction is involved in the interaction between Vif and SP140.

3.2.3 BRD2

BRD2, formerly named RING3 (really interesting new gene 3) or Fshrg1 (female sterile homeotic related gene 1), is a nuclear serine/threonine kinase possessing chromatin binding and transcription activity. Along with BRD4, BRD2 is a member of the BET family, but lacks a C-terminal PID domain.

However, BRD2 was found to coimmunoprecipitate with CDK9/Cyclin T1 or Cyclin T2 (Malovannaya et al., 2011). BRD2 also functions as a Tat-independent suppressor of HIV transcription in latent cells (Boehm et al., 2013a). In cell lines containing latent HIV, lentiviral shRNA–mediated depletion of BRD2 resulted in activation of the HIV LTR, and this effect was independent of Tat. The fact that BRD2 is known to bind co-repressor complexes including HDACs (Denis et al., 2006) supports a model whereby BRD2, by recruiting repressor complexes to the latent HIV LTR, directly suppresses HIV transcription. It remains to be seen whether BRD2, like BRD4, interacts with P-TEFb in an acetylation-dependent manner.

3.2.4 BRD4

In addition to its role in HIV transcription, BRD4 has been implicated in Vif-mediated cell-cycle progression. By mass spectrometry, Wang et al. identified BRD4 and CDK9 as Vif interactors required for Vif-mediated acceleration of cell-cycle transition from the G1-to-S phase (Wang et al., 2011). BRD4 also regulates the G2-to-M transition and stimulates cell-cycle progression from G1 to S through recruitment of P-TEFb to chromosomes and stimulation of G1 gene expression during late mitosis (Mochizuki et al., 2008; Yang et al., 2008). It is unknown whether the Vif-BRD4 interaction involves the BRD4 bromodomains.

4. Bromodomain inhibitors and HIV infection

The characteristic architecture of the bromodomain-acetyl-lysine interface represents a potential target for the development of small-molecule inhibitors. In initial attempts to identify bromodomain inhibitors, NMR-based screens of commercial compound libraries were used to identify compounds that inhibit Tat transactivation at the Tat-PCAF interface (Zeng et al., 2005). Two lead compounds were discovered using this approach, both with relatively low IC50 values for the Tat-PCAF interaction in vitro. Recently, several high-affinity binding molecules for
bromodomains of the BET family were described (Dawson et al., 2011; Filippakopoulos et al., 2012a; Filippakopoulos et al., 2010; Nicodeme et al., 2010; Zhang et al., 2012). JQ1, a thienodiazepine derived from a BRD4 ligand developed by Mitsubishi Pharmaceuticals (Miyoshi et al., 2008, International Patent PCT/JP2008/073864 (US2010/0286127 A1)), was shown to bind the first bromodomain of BRD4 with high affinity and target the second bromodomain of BRD4, and those of BRD2, BRD3, and BRDT (Filippakopoulos et al., 2010). MS417, a BET inhibitor derived from JQ1, specifically targets the interaction between BRD4 and the acetylated p65/RelA subunit of the transcription factor NF-κB and has potent anti-inflammatory effects in a mouse model of HIV-associated kidney disease (Zhang et al., 2012). I-BET, a synthetic “histone mimic” identified using an ApoA1 reporter system, also functions as a potent anti-inflammatory agent that suppresses expression of pro-inflammatory genes in activated macrophages and confers protection against lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis (Nicodeme et al., 2010). These and other compounds are excellent tools to study BET protein function, but have also shown impressive preclinical promise for the treatment of the NUT midline carcinoma (JQ1, (Filippakopoulos et al., 2010)), specific types of leukemia (JQ1 (Zuber et al., 2011), I-BET151 (Dawson et al., 2011)) inflammation (I-BET (Nicodeme et al., 2010), MS417 (Zhang et al., 2012)), and viral infections, including HIV.

Current antiretroviral therapy only suppresses viral replication, requiring life-long adherence to continuously limit viral loads, but does not eradicate virus from most infected people. Therefore, novel treatments that eliminate persistent viral reservoirs and thereby cure patients are needed. One approach is to reactivate proviral genomes in latently infected cells in order to “purge” viral reservoirs, either through the immune system or through the cytopathic effects associated with viral reactivation. HDAC inhibitors, including valproic acid and vorinostat (SAHA), show great promise as anti-latency therapeutics and have already begun to be tested in clinical trials (Archin et al., 2012; Kent et al., 2013; Routy et al., 2012a; Routy et al., 2012b). Recently, a flood of publications demonstrated that BET bromodomain inhibitors reactivate HIV from latency in cell lines and primary T-cell models (summarized in Table 1). Notably, cells treated with drugs like JQ1 show little synergy with vorinostat, indicating that both drugs target similar pathways in HIV reactivation. However, it is unclear whether any of the current ex vivo models faithfully recapitulates the in vivo situation of latently infected cells; therefore, further studies are needed to evaluate the clinical potential of BET inhibitors in primary T cells.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Model of HIV latency</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JQ1</td>
<td>Ach2</td>
<td>reactivation</td>
<td>Banerjee et al.</td>
</tr>
<tr>
<td>JQ1</td>
<td>U1</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>J-Lat 10.6</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>Acutely infected primary CD4+ cells</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>JΔK</td>
<td>reactivation</td>
<td>Bartholomeeusen et al.</td>
</tr>
<tr>
<td>JQ1</td>
<td>J-Lat A2</td>
<td>reactivation</td>
<td>Li et al.</td>
</tr>
<tr>
<td>JQ1</td>
<td>Jurkat 1G5</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>HeLa NH1 and NH2</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>HeLa-T4</td>
<td>reactivation</td>
<td>Zhu et al.</td>
</tr>
<tr>
<td>JQ1</td>
<td>Primary CD4+ T cells</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>Primary CD4+ T cells</td>
<td>inhibition</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>J-Lat 6.3</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1 + Prostratin or PMA</td>
<td>J-Lat 8.4</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1 + Prostratin or PMA</td>
<td>J-Lat 9.2</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1 + Prostratin or PMA</td>
<td>J-Lat 15.4</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>J-Lat A2</td>
<td>reactivation</td>
<td>Boehm et al.</td>
</tr>
<tr>
<td>JQ1</td>
<td>J-Lat A72</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>infected primary Bc12-transduced CD4+ T cells</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>JQ1</td>
<td>infected primary nonpolarized T helper cells</td>
<td>no reactivation</td>
<td></td>
</tr>
<tr>
<td>I-BET</td>
<td>infected primary Bc12-transduced CD4+ T cells</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>I-BET</td>
<td>infected primary nonpolarized T helper cells</td>
<td>no reactivation</td>
<td></td>
</tr>
<tr>
<td>I-Bet151</td>
<td>J-Lat A2</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>I-Bet151</td>
<td>J-Lat A72</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>I-Bet151</td>
<td>infected primary Bc12-transduced CD4+ T cells</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>I-Bet151</td>
<td>infected primary nonpolarized T helper cells</td>
<td>no reactivation</td>
<td></td>
</tr>
<tr>
<td>MS417</td>
<td>J-Lat A2</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>MS417</td>
<td>J-Lat A72</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>MS417</td>
<td>infected primary Bc12-transduced CD4+ T cells</td>
<td>reactivation</td>
<td></td>
</tr>
<tr>
<td>MS417</td>
<td>infected primary nonpolarized T helper cells</td>
<td>no reactivation</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-1 – Selected reported bromodomain inhibitors tested in models of HIV latency.
5. Concluding remarks

The acetyl-lysine-bromodomain interface, first therapeutically explored in HIV infection, represents an important regulatory axis that controls many aspects of HIV infection, including viral integration, Tat transactivation, HIV latency, cell-cycle progression of infected host cells, and virally induced inflammation. Furthermore, it is likely that new molecular functions for bromodomain-containing proteins in HIV infection await discovery. We expect that the use of existing and the development of novel bromodomain inhibitors will facilitate both the study and the treatment of HIV infection.

Acknowledgments

We thank John Carroll for assistance with graphics and Gary Howard for editorial assistance. We gratefully acknowledge support from the NIH (R01 AI083139 and CARE Collaboratory (U19AI096113) to M.O.). R.J.C. was supported in part by NIH Training Grant T32 GM007175.

Conflict of Interest

The authors declare no conflict of interest.
References


crossover study of valproic acid and antiretroviral therapy to reduce the HIV reservoir. HIV Clin Trials 13, 301-307.


Chapter IV

Therapeutics Targeting Protein Acetylation Perturb Latency of Human Viruses

Ryan J. Conrad¹,²,³ and Melanie Ott¹,²,³,*
¹Gladstone Institute of Virology and Immunology, San Francisco, CA 94158, USA;
²Graduate Program in Pharmaceutical Sciences and Pharmacogenomics, University of California, San Francisco, CA, 94158;
³Department of Medicine, University of California, San Francisco, CA 94158, USA

*Correspondence to:
Melanie Ott, MD, PhD
Gladstone Institutes
University of California, San Francisco
1600 Owens Street
San Francisco, CA 94158
Tel: 415-734-4807
mott@gladstone.ucsf.edu

Abstract
Persistent viral infections are widespread and represent significant public health burdens. Some viruses endure in a latent state by co-opting the host epigenetic machinery to manipulate viral gene expression. Small molecules targeting epigenetic pathways are now in the clinic for certain cancers and are considered as potential treatment strategies to reverse latency in HIV-infected individuals. In this review, we discuss how drugs interfering with one epigenetic pathway, protein acetylation, perturb latency of three families of pathogenic human viruses—retroviruses, herpesviruses, and papillomaviruses.

Keywords:
Acetylation – A posttranslational modification in which the acetyl group from acetyl-CoA is transferred enzymatically to the amino group in the ε-position of a lysine side chain.

Lysine acetyltransferases (KATs, also known as HATs) – Enzymes that catalyze the transfer of the acetyl group from acetyl-CoA to lysine residues to form ε-N-acetyl-lysine.

Lysine deacetylases (KDACs, also known as HDACs) – Enzymes that catalyze removal of acetyl groups from acetyl-lysine residues. These enzymes can be ion dependent (class I, II, IV KDACs) or NAD⁺-dependent (class III KDACs, or sirtuins).

Bromodomain – A nuclear protein domain of ~110 amino acids that binds and recognizes acetyl-lysine residues.
Latency – A reversible state of non-productive viral infection in which viral genetic material is present, but little to no viral proteins are produced.

Retrovirus – A family of RNA viruses that reverse transcribe and integrate their genomes into host chromatin (i.e., HIV).

Herpesvirus – A family of large DNA viruses that are maintained as multicopy, extrachromosomal episomes (i.e., HSV, EBV, and KSHV).

Papillomaviruses – A family of DNA viruses that persist in infected cells as either a multicopy episome or as an integrant under certain conditions (i.e., HPV).

Viral epigenetics and the tussle between repression and activation in the nucleus

Certain viruses replicate in the cell nucleus and have evolved elaborate strategies with which to utilize the nuclear environment for their own genetic fitness. Generally, a viral infection results in one of two outcomes—i.) a productive infection, in which the virus completes its replication cycle and progeny virions are assembled, or ii.) a latent infection, in which viral genetic material is present, but little to no viral proteins are produced. The decision between these two fates is multifactorial, and is influenced by the differentiation state of the host cell, availability of select host/viral factors, and viral gene circuitry. While a productive infection often evokes a fierce immune response, a latent infection allows the virus to chronically persist for long durations without alarming host immune cells.

Upon transiting to the nucleus, viral genomes often are assembled into nucleosomes and chromatinized (Deshmane and Fraser, 1989; Favre et al., 1977; Knipe et al., 2013; Shaw et al., 1979; Verdin, 1991). The nucleosome is the organizational unit of eukaryotic chromatin in which ~146bp of DNA are tightly wrapped around an octameric core of 4 distinct acidic histone proteins (H2A, H2B, H3, and H4). The nucleosome represents a ~6 fold reduction in the physical space required to store DNA, and inherently poses a significant barrier to DNA-derived processes such as transcription (Li et al., 2007). Importantly, viral latency is a reversible phenomenon of transcriptional silencing achieved in part by appropriating host epigenetic processes. Such epigenetic processes include posttranslational modifications to histones, exchange of histone variants, and active assembly, disassembly, and remodeling of chromatin by molecular machines. This regulation of chromatin structure and DNA accessibility contributes to the decision between latent and productive states of viral infection. Viral DNA is often condensed into nucleosomes during latency, while during productive infection, viral chromatin is labile and bound by factors that maintain a permissive chromatin state.

Manipulation of viral latency, particularly via epigenetic-based approaches, is an emergent therapeutic avenue to combat latent viral infections in humans. Current antiviral
treatments are largely limited to drugs that specifically target viral enzymes only present during a productive infection. Latent virus is refractory to these treatment strategies, yet in many cases is sufficient to reseed productive infection within the host once treatment is stopped. Latency thus represents an attractive target for viral eradication in chronically infected patients. There are at least three approaches to tackle viral latency — i.) permanent suppression, in which latent virus is irreversibly inactivated, for example by a drug or an in vivo gene-editing method, ii.) forced reactivation (“shock and kill”) (Deeks, 2012), in which latent virus is specifically reactivated by one drug in the presence of antivirals that target replicative virus, theoretically allowing reduction or clearance of latently infected cells by the immune system, and iii.) immune modulation, in which an infected patient’s immune system is specifically primed to clear latently infected cells.

At this time, epigenetic drugs targeting protein acetylation, a posttranslational modification best studied with regard to histones, are the most clinically advanced (Arrowsmith et al., 2012). Here, we focus on protein acetylation and how this pathway may be therapeutically exploited for the treatment of three distinct families of human pathogenic viruses—retroviruses, herpesviruses, and papillomaviruses (Table 4-1). While these viruses share the capacity to co-opt the host nucleus and establish latent infections, they confront the nuclear environment in unique ways. Retroviruses such as HIV are RNA viruses that undergo reverse transcription and integrate resultant proviral DNA into host chromatin. Herpesviruses are large DNA viruses that shuttle their complex genomes to the nucleus where they associate with host chromatin as a circular episome, or “mini-chromosome.” Similar to herpesviruses, papillomaviruses are also maintained as episomes although may integrate into host chromatin under certain conditions. Small molecules targeting catalysis and recognition of protein acetylation are being explored for their effects on latency of these viruses and have distinct effects on viral transcription. Here, we review acetylation-targeted therapeutics and available preclinical and clinical data investigating the application of these drugs in latent viral infections.

Drugs targeting the protein acetylation network

Protein acetylation is one of the best-studied epigenetic mechanisms of transcriptional control (Verdin and Ott, 2015). While first discovered to modify histones (Phillips, 1963), acetylation is now known to regulate many epigenetic factors, including important transcriptional regulators such as NF-κB (Chen et al., 2001), P-TEFb (Cho et al., 2009), and RNA polymerase II itself (Schroder et al., 2013). Acetylation impacts protein function by altering stability, localization, and affinity for other proteins and nucleic acids. This is achieved via at least three
<table>
<thead>
<tr>
<th>Family</th>
<th>Retroviridae</th>
<th>Herpesviridae</th>
<th>Papillomaviridae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Members</td>
<td>HIV</td>
<td>HSV, EBV, KSHV</td>
<td>HPV</td>
</tr>
<tr>
<td>Global prevalence</td>
<td>0.8%</td>
<td>90% (HSV), 95% (EBV), 10-50% (KSHV)</td>
<td>12%</td>
</tr>
<tr>
<td>Genome size (kb)</td>
<td>9.8</td>
<td>150-210</td>
<td>8</td>
</tr>
<tr>
<td># gene products</td>
<td>9</td>
<td>70-90</td>
<td>8</td>
</tr>
<tr>
<td>Genome type</td>
<td>Integrated</td>
<td>Episomal</td>
<td>Episomal, integrated</td>
</tr>
<tr>
<td>Latency cell types</td>
<td>Resting CD4+ T cell, macrophages, microglia, GALT, HSC</td>
<td>Neurons (HSV), B lymphocytes (EBV &amp; KSHV)</td>
<td>Basal epithelial cells</td>
</tr>
<tr>
<td>Location</td>
<td>Active human genes</td>
<td>PML bodies</td>
<td>DNA repair foci</td>
</tr>
</tbody>
</table>

Table 4-1 – Key facts of virus families in discussion
mechanisms—i.) neutralization of the positively charged unmodified lysine residue, ii.)
generation of novel protein:protein interfaces, and iii.) suppression of other posttranslational
modifications that can occur on lysine residues (i.e., methylation, ubiquitination, etc.). These
mechanisms are not mutually exclusive, and likely work in concert to enact functional outcomes
associated with acetylation. For example, transcriptional activation correlating with histone
acetylation may be explained by both electrostatic antagonism of histone:DNA contacts and
site-specific recognition of histone acetyl-lysine residues by a bromodomain-containing effector
protein, as discussed below.

Protein acetylation is catalyzed enzymatically by lysine/histone acetyltransferases
(KATs, also known at HATs) (Berndsen and Denu, 2008; Lee and Workman, 2007). There are
currently ~25 known human KATs, and these enzymes are mostly known as transcriptional
activators. They fall in different classes based on structural and functional homology: GNAT
(i.e., GCN5, PCAF), MYST (i.e., TIP60, MOF, MOZ), p300 (p300, CBP), transcription factor-
related (i.e., TAF1/TBP), and nuclear receptor-related (i.e., CLOCK). KATs contain a conserved
glutamate residue that serves as a base to increase the nucleophilicity of the lysine ε-amino
group allowing addition to the acetyl-Coenzyme A (CoA) carbonyl, upon which an unstable
tetrahedral intermediate forms. Collapse of the tetrahedral intermediate releases reaction
products CoA and acetyl-lysine. MYST family KATs employ a slightly different reaction
mechanism using transfer of acetyl-CoA to a cysteine residue in the active site as an
intermediate step of catalysis.

KATs are only now emerging as important and feasible drug targets (Dekker and
Haisma, 2009), with p300/CBP at the forefront. Lys-CoA was the first synthetic inhibitor for the
p300/CBP KAT (Lau et al., 2000) operating via a unique mechanism in which the drugs
interferes with a long catalytic loop (L1) of the KAT domain that normally positions the substrate
and acetyl-CoA in a basic pocket to favor spontaneous acetylation (Figure 4-1A) (Liu et al.,
2008). C646 is a cell-permeable pyrazolone-furan p300/CBP inhibitor that is currently being
investigated mostly for cancers and inflammation (Bowers et al., 2010). Other KAT inhibitors
(KATi) include isothiazolones as PCAF inhibitors (Stimson et al., 2005), α-methylene-γ-
butyrolactones as GCN5 inhibitors (Biel et al., 2004), and 6-Alkylsalicylates as TIP60 inhibitors
(Ghizzoni et al., 2012). Remarkably, several natural products including curcumin
(Balasubramanyam et al., 2004b) (turmeric), garcinol (Balasubramanyam et al., 2004a)
(Garcinia indica fruit rind), and anacardic acid (Balasubramanyam et al., 2003) (cashew nuts)
inhibit KATs with considerable potency. Curcumin targets p300/CBP specifically via a yet
unknown mechanism while garcinol and anacardic acid are broader KAT inhibitors that may
Figure 4-1 – Drugs targeting catalysis and recognition of protein acetylation – A. Structure of p300 KAT domain (dark grey) in complex with bidentate Lys-CoA inhibitor (purple) with hydrogen bonds (red) formed between the L1 catalytic loop and Lys-CoA shown (left, PDB: 3BIY (Liu et al., 2008)). Chemical structures of select KATi (right). B. Structure of KDAC2 KDAC domain (dark grey) in complex with vorinostat (purple) with divalent zinc chelation shown (left, PDB: 4LXZ (Lauffer et al., 2013)). Chemical structures of select KDACi, with active moieties shadowed in red (right). C. Structure of first BRD4 bromodomain (dark grey) in complex with JQ1 (purple) with the hydrogen bond (red) formed between the bromodomain asparagine and JQ1 shown (left, PDB: 3MXF (Filippakopoulos et al., 2010)). Chemical structures of select BETi with active moieties shadowed in red (right).
function by mimicking acetyl-CoA. Recent evidence shows that salicylic acid, a well-known anti-inflammatory compound, is a potent inhibitor of the p300/CBP KAT and can delay onset and clinical development of Alzheimer’s disease in mice by inhibiting acetylation of the pathogenic tau protein (Min et al., 2015). Notably, KAT activators have been described, such as CTB (N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide) (Balasubramanyam et al., 2003), which alters the p300 structure to increase enzymatic activity through an unknown mechanism.

The enzymes opposing KAT activity are lysine deacetylases (KDACs, also known as HDACs) and are well-studied drug targets (Falkenberg and Johnstone, 2014). KDACs are comprised of four distinct classes based upon structural homology where class I, II, and IV KDACs utilize a divalent zinc ion coordinated in the active site to catalyze deacetylation upon nucleophilic attack of water on the acetyl-lysine residue. Class III KDACs or sirtuins operate via a distinct mechanism involving nicotinamide adenine dinucleotide (NAD⁺) in lieu of an active site metal. Class I KDACs localize mainly to the nucleus, where they largely repress cellular transcription, while Class II and IV KDACs generally shuttle between the nucleus and the cytoplasm. Sirtuins are found in the nucleus, cytoplasm and in mitochondria.

KDAC inhibitors (KDACi) primarily target class I and II KDACs, and some (i.e., vorinostat, romidepsin, panobinostat) are clinically approved for the treatment of certain types of cancer, such as T cell lymphomas and multiple myeloma. To date, there are over 500 ongoing/recruiting clinical trials involving KDACi for different indications. Although chemically diverse, these compounds share a basic structure of a variable cap that aids in recognition of the KDAC surface, an aliphatic spacer, and the active moiety functional group. KDACi mostly chelate the divalent zinc cation in the deacetylase active site to achieve reversible inhibition (Figure 4-1B). KDACi fall roughly into four structural classes—i.) short-chain aliphatic acids, such as valproic acid and sodium butyrate ii.) hydroxamates, such as trichostatin A (TSA) and vorinostat iii.) benzamides, such as entinostat and iv.) cyclic tetrapeptides and depsipeptides, such as trapoxin A and romidepsin. Class III KDACs, particularly SIRT1, are attractive drug targets given their connection with life- and health-span extension (Wood et al., 2004). EX-527 is a specific SIRT1 inhibitor and functions by engaging the enzyme during an NAD⁺-dependent intermediate step in catalysis (Gertz et al., 2013). In contrast, the natural phenol resveratrol as well as several small-molecule drugs activate SIRT1 activity through allosteric mechanisms (Hubbard et al., 2013).

Protein acetylation is controlled non-enzymatically via the bromodomain, an important “reader” module of ~110 amino acids that binds acetyl-lysine residues (Dhalluin et al., 1999). 61 distinct bromodomains are found encoded in 46 proteins within the human proteome, with 1-6
bromodomains found in a single protein. All are nuclear factors and comprise modifying enzymes, nucleosome remodelers, and transcription factors. The bromodomain:acetyl-lysine interaction has relatively low affinity in vitro (Filippakopoulos et al., 2012), yet the local chromatin environment and the complex in which the bromodomain-containing protein assembles may enhance bromodomain binding in vivo. The structure of the bromodomain:acetyl-lysine interaction is well characterized and serves as a valuable tool for drug development. The bromodomain adopts a distinctive structure comprised of four anti-parallel alpha-helices that generate a hydrophobic cleft in which the acetyl-lysine is bound. A key hydrogen bond is made between the acetyl-lysine and a conserved asparagine that is found in nearly all bromodomains. This asparagine is a critical target for inhibitors of the bromodomain:acetyl-lysine interaction (Figure 4-1C), most notably the benzodiazepine derivatives JQ1 (Filippakopoulos et al., 2010) and I-BET (Nicodeme et al., 2010), both inhibitors of the double bromodomain and extraterminal domain (BET) subfamily of proteins. Because JQ1 is active against certain cancers based on its ability to suppress expression of the oncogenic transcription factor c-Myc (Delmore et al., 2011), several BET inhibitors (BETi) are currently in early clinical trials.

**Human viruses, latency, and acetylation-targeted therapeutics**

*Retroviruses (Human immunodeficiency virus (HIV))*

Retroviruses like HIV encode small, diploid, single-stranded, positive-sense RNA genomes that are reverse transcribed and integrated into host chromatin. HIV infects primarily CD4+ T cells and macrophages to establish a life-long infection that, if untreated, leads to the development of acquired immunodeficiency syndrome (AIDS). It is estimated that 35 million people are living with HIV/AIDS, and the majority of infections are in developing countries. With the introduction of effective antiretroviral therapy, infected individuals rarely develop AIDS and have nearly normal life spans. However, treatment is not curative and requires life-long adherence due to a small reservoir of latently infected cells in which viral transcription is durably suppressed after integration into host chromatin (Siliciano and Greene, 2011). HIV latency occurs mainly in resting memory T cells, but also in macrophages, microglial cells, gut-associated lymphoid tissues, and hematopoietic progenitor cells. Establishment of HIV latency likely occurs early in infection (Chavez et al., 2015) and is influenced by the integration site (Jordan et al., 2001), availability of certain host factors (Tyagi et al., 2010), and stochastic transcriptional changes coupled to the strong positive feedback loop driven by the viral transactivator of transcription (Tat) (Razooky et al., 2015).
HIV transcription is orchestrated by a single promoter located within the viral 5′ long terminal repeat (LTR) that drives the expression of a multiply spliced mRNA that fuels viral replication (Ott et al., 2011). The HIV promoter behaves similarly to that of a signal-inducible human gene in that it is highly responsive to extracellular stimuli and readily initiates RNA synthesis in host chromatin, yet does not proceed past 30–100 nt of synthesis. This phenomenon is known as proximal promoter pausing of the host RNA polymerase II complex and also occurs at a large fraction of human genes, particularly those that encode important cell type-specific regulators of fate and plasticity (Adelman and Lis, 2012). Negative transcription elongation factors, such as NELF and DSIF, sterically hinder polymerase passage past the proximal pause site (Figure 4-2A). Productive HIV transcription relies on the recruitment of the positive transcription elongation factor b (P-TEFb) and associated “super elongation” factors by HIV Tat to TAR, an RNA element present in the 5’ extremity of initiating viral transcripts. Thus, HIV transcription represents a positive feedback loop in which minimal processive transcription can result in synthesis of Tat that feeds back to fuel robust transcription elongation.

The latent integrated HIV provirus is readily chromatinized to adopt several well-defined nucleosomes independently of the integration site, including a nucleosome positioned immediately downstream of the HIV transcription start site (nuc-1) (Verdin et al., 1993). Factor acetylation has long been recognized as a critical regulator of the HIV locus, as early studies showed that KDACi reactivated viral transcription in latently infected T cell lines by disrupting nuc-1 positioning (Van Lint et al., 1996). These studies have been extended to different KDACi spanning all structural classes in different models of latency, including Jurkat latency (J-Lat) cell lines and primary cells (Shirakawa et al., 2013). These findings indicate that during HIV latency, nuc-1 undergoes constitutive deacetylation by KDACs to maintain transcriptional silencing. Indeed, class I KDACs are recruited to the HIV promoter (i.e., via c-Myc, Sp1 (Jiang et al., 2007), and YY1 (He and Margolis, 2002)) and TSA treatment induces nuc-1 histone H4 hyperacetylation (He and Margolis, 2002; Sheridan et al., 1997). Importantly, Tat cooperates with KATs (i.e., p300/CBP, PCAF (Benkirane et al., 1998)) to further mediate local acetylation of the HIV locus. The importance of KATs in Tat-mediated transcription is underscored by the potent transcriptional inhibition mediated by KATi curcumin (Barthelemy et al., 1998). In addition to histones, acetylation of trans-acting factors regulates HIV transcription, with targets including Tat (Ott et al., 1999), P-TEFb (Cho et al., 2009), and subunits of NF-kB (Chen et al., 2001). Latency reversal with KDACi is robust in cell line models and has been tested clinically (Archin et al., 2012), but appears most promising in patient cells when combined with T cell activators such as protein kinase C (PKC) agonists (Laird et al., 2015). Although many targets of
Figure 4-2 – Effects of acetylation-targeted therapeutics on latency of HIV (A), EBV (B), and HPV (C). A. Latent HIV is disrupted by KDACi via nuc-1 hyperacetylation and acetylation of trans-acting factors such as Tat and P-TEFb. BETi reverses latency through relief of potentially repressive BET complexes that inhibit transcription via as yet undefined mechanisms. Conversely, HIV transcription is inhibited by KATi via hypoacetylation of nuc-1 and trans-acting factors. B. Latent EBV is reversed by KDACi via hyperacetylation of EBV chromatin, particularly the Zta locus. KATi inhibit EBV transcription by reducing acetylation marks on EBV chromatin. C. BETi induces amplification of HPV chromatin by removing BRD4:E2 tethered HPV episomes from human chromatin.
Acetylation act at the HIV promoter directly, indirect effects of KDACi on cellular genes involved in regulation of HIV transcription remain to be rigorously explored. Importantly, it is estimated that KDACi treatment manipulates expression of 2-10% of total genes (VanLint et al., 1996), and what the effect of these host transcriptomic changes are on viral latency reactivation is underappreciated.

The functional outcomes of acetyl-lysine recognition by bromodomain-containing proteins are complex at the HIV promoter. Acetylated Tat was originally shown to generate an interaction surface for the PCAF bromodomain (Dorr et al., 2002), and initial attempts to target this interaction therapeutically to inhibit Tat transactivation yielded two compounds (Zeng et al., 2005). In contrast, BETi reactivate HIV from latency across different experimental systems, including primary cells (Banerjee et al., 2012; Boehm et al., 2013a; Li et al., 2013; Zhu et al., 2012). The BET protein BRD4 and Tat are competitive for limiting levels of P-TEFb (Bisgrove et al., 2007), which in part explains how displacement of BET proteins from chromatin favors Tat-mediated transactivation (Li et al., 2013). BETi nonetheless reverse latency in models devoid of Tat (Boehm et al., 2013a), suggesting that other mechanisms contribute to the activity of these compounds in HIV latency reversal. Plausible hypotheses include increasing levels of active P-TEFb required for HIV transcription, BET proteins exerting direct repressive functions at the HIV promoter, and indirect transcriptional effects (Boehm et al., 2013b). Similar to KDACi, BETi synergize with low dose PKC agonists in primary cells (Laird et al., 2015). Importantly, KDACi and BETi do not synergize in HIV latency reactivation (Zhu et al., 2012), suggesting that these compounds work through similar pathways.

**Herpesviruses (Herpes simplex virus (HSV), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV))**

Herpesviruses are extremely pervasive viruses that maintain their large double-stranded DNA genomes as circular episomes topologically associated with host chromatin. Under a productive infection, herpesvirus DNA is scarcely associated with nucleosomes and is transcribed in a highly coordinated and sequential manner; groups of genes are expressed at immediate early (IE), early (E), and late (L) stages of infection. IE genes are transcribed and translated in the absence of de novo viral protein synthesis and maintain a chromatin environment permissive to transcription by destroying nuclear compartments important for subsequent virion formation (i.e., PML nuclear bodies), reducing nucleosome occupancy, and/or altering the activity of histone-modifying enzymes. E genes function to replicate viral DNA, while L genes are expressed only after DNA replication has begun. Productive or lytic infection is brief
as infected cells are mainly destroyed due to cytopathic effects of the virus. The herpesvirus family has thus evolved intricate mechanisms to persist in a latent form in certain cell types.

Herpesviral latency is complex, and characterized by the variable gain of a restricted gene expression program that allows for production of proteins and noncoding RNAs to enforce latency and alter the host. Strikingly, herpesviruses regulate their chromatin in a locus-specific manner with select regions open and active during latency. We focus here on an α-herpesvirus, HSV, and two γ-herpesviruses, EBV and KSHV. HSV establishes latency in the nervous system, allowing long-term persistence and access to epithelial tissues where productive infection occurs (Roizman and Whitley, 2013). The γ-herpesviruses are tumor viruses whose latency programs are causally associated with certain cancers (Wen and Damania, 2010; Young and Rickinson, 2004).

HSV

While HSV productively infects oral (HSV-1) or genital (HSV-1, -2) epithelial cells causing cold sores or genital lesions, it is also neuroinvasive and establishes latency in ganglionic neurons innervating the site of primary exposure. HSV-1 is quite common both in developed and developing countries, with >80% of the population being infected by the age of 50. HSV-2 is less common, with ~30% of the population being infected by age 50. There are several models how HSV latency is established in neurons (Roizman and Whitley, 2013): i.) VP16, a viral tegument protein required for IE expression, is lost during retrograde transport to the neuronal nucleus ii.) host factors required for VP16-mediated IE expression (i.e., Oct-1 and HCF1) are missing or limited in neurons and iii.) VP16 is not properly posttranslationally modified in neurons. Importantly, latent HSV retains expression of latency-associated transcripts (LATs) thought to cause RNA-mediated heterochromatinization of the HSV genome, antisense inhibition of IE transcription, and inhibition of neuronal apoptosis. Stress to the host (i.e., fever, UV light, physical/emotional stress, nerve damage) can reactivate latent HSV in neurons, leading to viral particle assembly, anterograde transport to the epidermis where productive, symptomatic infection and transmission can occur.

The latent HSV genome that persists in neurons is largely heterochromatinized and displays a regular pattern of host-derived nucleosomes bearing modifications indicative of repressed transcription (i.e., H3K9me3 and H3K27me3) (Cliffe et al., 2013; Wang et al., 2005). Accordingly, acetylation levels on the latent HSV genome are low, although the neuronal-specific LAT promoter bears hyperacetylated histones (Kubat et al., 2004). Acetylation of HSV-associated histones at lytic genes is a hallmark of productive infection(Kent et al., 2004).
Curcumin is a potent inhibitor of lytic HSV (Kutluay et al., 2008), but also blocks latency reactivation in ganglionic cultures stimulated with nerve growth factor (Du et al., 2013). The effects of curcumin on HSV latency are independent of p300/CBP inhibition (Kutluay et al., 2008), and genetic knockdown of KATs does not decrease characteristic histone acetylation marks (i.e., H3K9ac and H3K14ac) at IE promoters during induction from latency (Kutluay et al., 2009). This suggests non-histone targets or indirect effects explain the effects of KAT inhibition on HSV expression. Interestingly, activation of the p300/CBP KAT activity by CTB promotes latency reactivation by inducing IE expression and inhibiting LAT expression (Du et al., 2013), supporting the model that KATs are positive regulators of HSV IE gene expression.

KDACi, including short-chain aliphatic acids and hydroxymates, consistently reactivate HSV from latency both in cell-based (Arthur et al., 2001) and animal models of latency (Neumann et al., 2007). KDAC-mediated surveillance of viral chromatin maintains latency through KDAC1/KDAC2-containing CoREST/REST repressive complexes associated with HSV chromatin (Gu and Roizman, 2007). KDACs are thought to inhibit CoREST/REST complexes at IE genes, which is supported by the potency of entinostat, a benzamide KDAC inhibitor that shows specificity for KDAC1 at low doses, in murine trigeminal ganglia (Du et al., 2013). Interestingly, the viral IE gene product ICP0 serves as an autonomous KDAC inhibitor by disrupting the interaction between CoREST and KDAC1/KDAC2 (Gu and Roizman, 2007). Overall, these findings support a model wherein a dynamic equilibrium of acetylation at HSV chromatin locally governs the decision between lytic and latent infection (Knipe and Cliffe, 2008). KDACs induce the formation of progeny virions from neurons, but it remains to be tested whether forced reactivation in the presence of antiviral agents can lead to clearance of viral reservoirs.

**EBV**

EBV causes mononucleosis and in its latent form is associated with different types of cancer, such as Burkitt’s and Hodgkins lymphomas (Young and Rickinson, 2004). EBV productively infects endothelial cells in the oropharynx during oral contact, yet the virus eventually reaches the B cell compartment where it can establish a latent infection that persists lifelong. EBV is widespread, with >90% of US adults having detectable antibodies indicative of current or previous EBV infection. EBV latency is complex and consists of four types (0-III) that may reflect distinct differentiation states of infected host cells. EBV latency types are ranked in order of increasing gene expression: latency type 0 (resting B cells) displays no detectable gene expression, latency type I (Burkitt’s lymphoma, proliferating B cells) expresses EBV nuclear
antigen 1 (EBNA1) and viral microRNAs, latency type II (Hodgkin’s lymphoma, carcinomas) expresses EBNA1, microRNAs, and latency membrane proteins (LMPs), and latency type III (immortalized lymphoblastoid cell lines) expresses six EBNA proteins and three LMPs. Molecular mechanisms of type III latency are best studied, likely due to the tractable experimental system of immortalized lymphoblastoid cell lines. During primary infection, EBNA2 accumulates and, through interaction with host transcription factor PU.1, activates the Cp promoter that in turn is responsible for activation of type III latency. Type III latency (also known as “Cp on latency”) leads to the production of a ~120kb polycistrionic RNA that is differentially spliced to encode for EBV latency proteins that drive immortalization of B cells in vitro. It is important to note that latency type III can relax to more restrictive patterns of gene expression of type II and I, although this phenomenon is not well understood. Lytic induction requires activation of the Zta promoter that drives expression of the IE gene product Zta (also known as BZLF1), a transcription factor required for expression of lytic genes (Figure 4-2B).

The EBV episome is well studied with respect to epigenetic regulatory mechanisms, including DNA methylation, histone posttranslational modifications, chromatin assembly/disassembly, and long-range chromosomal interactions with itself and the human genome (Lieberman, 2013; Tempera and Lieberman, 2010). Histone acetylation marks, including those at histone H3K9 and H3K27, are present at the Cp region under type III latency, at the Zta promoter during lytic induction, and more generally at LMP and microRNA promoters in lymphoblastoid cell lines (Alazard et al., 2003; Deng et al., 2003). This indicates that histone acetylation is involved in the regulation of EBV latency type decision and lytic induction in a locus-specific manner. The KAT inhibitor curcumin negatively regulates EBV reactivation and EBV-mediated B cell immortalization, underscoring the functional importance of cellular KATs in activating lytic infection. Conversely, structurally diverse KDACi, including valporic acid, vorinostat, and panobinostat, induce lytic EBV gene expression in different models (Feng and Kenney, 2006; Ghosh et al., 2012; Mentzer et al., 1998). Certain cell lines carrying EBV, however, are refractory to induction of lytic infection by KDACi, even though global histone hyperacetylation occurs in these models (Countryman et al., 2008). In responsive cell lines, it is assumed that hyperacetylation of the Zta promoter and consequent transcriptional activation causes lytic gene expression. In resistant cell lines, the model is that indirect, inhibitory transcriptional effects of the drugs dominate. When combined with drugs targeting replicative virus, such as ganciclovir (Perrine et al., 2007), KDACi become toxic to B cells undergoing lytic EBV infection. This is a first indication that forced reactivation may reduce latent EBV reservoirs and block cancer development.
Various bromodomain:acetyl-lysine interactions are functionally important for the EBV life cycle. Firstly, the bromodomain of the CBP KAT is necessary for Zta-facilitated histone acetylation and lytic induction (Zerby et al., 1999). Secondly, the BET protein BRD4 interacts with EBNA1 and is critical for its full transactivation capacity (Lin et al., 2008). Accordingly, the BET inhibitor JQ1 reduces Cp transcription via loss of P-TEFb (Palermo et al., 2011) and reduces activity of virally induced “super-enhancers,” or areas of active transcription with high densities of the transcriptional coactivator complex mediator and BRD4 that uniquely tie together the fate of the EBV genome and the cell state of its lymphoblastoid host (Zhou et al., 2015).

KSHV

KSHV is causally linked to several malignancies in immunocompromised individuals, including Kaposi’s sarcoma and primary effusion lymphoma (PEL) (Wen and Damania, 2010). It is estimated that 10% of the population in North America is infected with KSHV, and this number is considerably higher in developing countries. KSHV displays tropism for lymphocytes, fibroblasts, epithelial, and endothelial cells. Similar to other herpesviruses, KSHV latency is characterized by a restricted pattern of gene expression, although several proteins (i.e., LANA, vFLIP, and vCyclin) and noncoding RNAs are expressed and function in latency maintenance, immune modulation, and cell transformation. Most infected cells in KSHV-associated tumors display a latent phenotype, suggesting that latency is required for oncogenesis, although lytic KSHV is linked to cancer development as well (Mesri et al., 2010). The switch between lytic and latent states is governed by the viral IE gene product RTA (also known as ORF50), which activates lytic gene expression through interactions with several host transcription factors (i.e., AP1, C/EBPα) and has E3 ubiquitin ligase activity.

Epigenetic regulation of KSHV latency is mechanistically similar to EBV, in that locus-specific chromatin modifications contribute to the latent phenotype. During latency, histone hypoacetylation is observed at the RTA promoter (Lu et al., 2003), while nucleosomes encompassing the LANA binding sites required for episome replication are hyperacetylated (Toth et al., 2010). Very little work has been done on the effects of KATi on KSHV latency, although these enzymes are functionally important in the KSHV life cycle, and several viral factors directly associate with KATs (i.e., RTA with p300/CBP (Gwack et al., 2001)). Different KDACi, including valproic acid, TSA, and sodium butyrate, reverse KSHV latency (Lu et al., 2003; Yu et al., 1999), suggesting that class I and II KDACs constitutively act to maintain KSHV chromatin in a latent state. Modulation of class III KDAC activity also disturbs KSHV latency.
Nicotinamide and sirtinol reactivate KSHV latency, accompanied by increases in histone acetylation at the RTA promoter (Li et al., 2014). Interestingly, SIRT1 interacts with RTA and binds its promoter. The SIRT1 activator, resveratrol, inhibits KSHV reactivation, concordant with a model of continuous surveillance of KSHV chromatin by class III KDACs. Importantly, KSHV LANA is acetylated in infected cells (Lu et al., 2006) and may play an unappreciated role in the response of latent KSHV to KDACi.

KSHV LANA directly interacts with the BET ET domain (You et al., 2006), and this interaction may be functional in altering host cell cycling and in tethering the replicative viral episome to chromatin during mitosis. The role of BET proteins in KSHV transcription remains relatively unexplored although it is known that JQ1 does not stimulate KSHV lytic induction (Tolani et al., 2014). However, JQ1 effectively kills PEL cells by targeting c-Myc (Tolani et al., 2014), and may therefore be valuable in treating KSHV-associated cancers.

_Papillomaviruses (Human papillomavirus (HPV))_

HPVs encompass a group of >200 different DNA viruses that infect squamous epithelial cells to produce a variety of lesions, including genital/anal warts and certain cancers (Doorbar, 2005). HPV is considered the most common sexually transmitted disease, with ~79 million people infected in the United States. The viral genome is ~8kb in size and is maintained normally as an episome, although integration of the HPV genome does occur and is a major risk factor for carcinogenesis. The HPV life cycle is intimately coupled to the differentiation state of its host cell in the epithelial striatum; latent virus persists at a low copy number in mitotically slowed cells in the basal layer, while productive infection is initiated as the latently infected cell asymmetrically divides and migrates to the upper epithelium. Different HPV genotypes are classified as high-risk or low-risk according to their capacity to malignantly transform cells. Oncogenesis can arise from high-risk HPV infection (i.e., HPV16, -18) mainly due to increased expression of viral E6 and E7 oncoproteins, which deregulate p53 and Rb, respectively, to induce cell transformation. The HPV E2 protein, a sequence-specific regulatory factor important for maintenance of the replicative episome and viral transcription, tightly limits expression of E6 and E7. Loss of E2 function, notably through an integration event disrupting the E2 gene, leads to E6 and E7 expression and virally induced carcinogenesis.

Epigenetic alterations to viral chromatin during high-risk HPV infection and cervical carcinogenesis have been studied, with methylation of viral DNA being the best characterized (Szalmas and Konya, 2009; You, 2010). Nucleosomes readily form over HPV DNA at the early and late promoter regions both _in vitro_ and in cell lines harboring extrachromosomal
(del Mar Pena and Laimins, 2001) and integrated HPV genomes (Rosl et al., 1989; Stunkel and Bernard, 1999). Strikingly, the nucleosomes of the early and late promoters bear acetylated histones in undifferentiated cells, suggesting that HPV chromatin is in an open state throughout its replication cycle (Wooldridge and Laimins, 2008). These histones become further acetylated during differentiation (Wooldridge and Laimins, 2008). Interestingly, curcumin inhibits E6 and E7 expression in HeLa cells, suggesting that histone hyperacetylation may be important for active transcription of these viral factors (Prusty and Das, 2005). However, HPV proteins also manipulate acetylation of nonhistone factors. E6 inhibits p300-mediated acetylation of the tumor suppressor p53 (Patel et al., 1999), and E7 upregulates SIRT1 to induce constitutive deacetylation of p53 (Allison et al., 2009), both outcomes implicated in p53 deregulation and oncogenesis. KDACi such as valproic acid and sodium butyrate may restore p53 function by inducing its hyperacetylation and consequently inhibiting its degradation. Cervical cancer cells are sensitive to KDACi, likely through upregulation of the p21 tumor suppressor. Although in various cell lines and model systems KDACi induce expression and copy number of HPV, in primary tumors E6 and E7 expression was unchanged during valproic acid treatment (de la Cruz-Hernandez et al., 2007). Collectively, these findings indicate that KDACi do not target HPV chromatin directly, but rather disrupt the ability of the virus to control acetylation pathways in the infected cell.

Several groups have shown that BRD4 physically interacts with E2, an interaction functionally implicated in both episome maintenance and its transcriptional regulation (McPhillips et al., 2006; Wu et al., 2006; You et al., 2004). BRD4 tethers E2 to mitotic chromatin ensuring proper segregation and replication of the viral genome. The interaction between E2 and BRD4 is mutually exclusive with the active BRD4:P-TEFb complex (Yan et al., 2010), as both E2 and P-TEFb engage the extreme C-terminus of BRD4. Disruption of active P-TEFb by E2 may account for E2-mediated viral oncogene repression, although other host factors are likely involved (Schweiger et al., 2007). Interaction with BRD4 is also required for full transactivation capacity of E2, likely through E2-independent P-TEFb recruitment. JQ1 dissociates BRD4:E2 from chromatin, disrupts viral episome maintenance, and causes a transient increase in viral genome amplification (Figure 4-2C) (Helfer et al., 2013; Sakakibara et al., 2013; Wang et al., 2013). In addition, JQ1 inhibits HPV transcription presumably via P-TEFb (Helfer et al., 2014). These findings suggest that JQ1 may have therapeutic effects against HPV by i.) transiently stimulating HPV genome amplification and possibly immune detection and ii.) generally suppressing HPV transcription. Further studies are needed to validate these hypotheses.
Conclusions, outlook, and perspective

It is clear that latency programs of distinct viruses utilize protein acetylation as a means to regulate chromatin structure, transcription, and viral fate. Acetylation-targeted therapeutics perturb these latency programs in different ways with several commonalities among the discussed viruses—i.) all encode viral-specific transactivators that reverse latency (Tat, VP16, Zta, RTA, E2) via a mechanism that includes recruitment of KATs, therefore KATi generally enforce latency and inhibit lytic induction, ii.) latent viral chromatin appears to undergo constitutive deacetylation via KDACs, and therefore KDACi generally reactivate viruses from latency, and iii.) BET proteins play distinct roles in viral latency and can impact both viral transcription (i.e., HIV, EBV, and HPV) and replication (HPV).

Given the prevalence of chronic latent infections, particularly by herpesviruses, the effects of epigenetic-based therapies on these viruses should be considered in the clinic, either with therapeutic intention or with awareness of potential adverse drug reactions. Ideally, the potency of these agents to regulate viral transcription programs might be therapeutically harnessed to durably suppress (KATi) or reactivate (KDACi) latent virus. While a “shock and kill” approach has advanced to clinical stages in chronic HIV infection with KDACi (Archin et al., 2012), the concept of durable viral suppression is currently only emerging (Mousseau et al., 2015) and further studies are required to validate KATi in this therapeutic strategy. Development of BETi to treat various cancers is rapidly advancing, but their therapeutic use in viral latency or detailed knowledge about side effects on latent viruses is still in very early stages.

Notably, many epigenetic pathways apart from protein acetylation are currently being explored as therapeutic targets mainly in cancer treatment. These include inhibitors of DNA methyltransferases approved for clinical use and modestly active in reactivating latency of HIV and HSV, with more consistent data for EBV. Protein methyltransferase inhibitors, although not currently in the clinic, may also prove useful in the reactivation of latent viruses, given strong links between several methyltransferases (i.e., EZH2) and latency of the discussed viruses. Conversely, demethylase inhibitors, such as inhibitors of the lysine-specific demethylase (LSD1) and Jumonji proteins, may support durable viral suppression strategies.

Collectively, these epigenetic-based therapies—alone or in combination—represent an exciting new class of drugs that should be fully explored to reduce, clear, or permanently enforce latency of human viruses in infected patients.
Acknowledgements

We thank V. Fonseca for administrative support and D. Nolan for assistance with graphics. We gratefully acknowledge support from the NIH (R01 AI083139 and U19 AI096113 to M.O.). R.J.C. was supported in part by NIH Training Grant T32 GM007175. We apologize to authors whose original work could not be cited due to citation limits.
References


Chapter V

Activating Latent HIV by Inhibiting Bromodomain Proteins

Ryan J. Conrad¹,², Daniela Boehm¹,², and Melanie Ott¹,²,*

¹ Gladstone Institute of Virology and Immunology, San Francisco, CA 94158, USA; ² Department of Medicine, University of California, San Francisco, CA 94158, USA

E-Mails: Ryan.Conrad@ucsf.edu; daniela.boehm@gladstone.ucsf.edu
*Author to whom correspondence should be addressed; E-Mail: mott@gladstone.ucsf.edu; Tel.: +1-415-734-4807; Fax: +1-415-355-0855.

Abstract: Pharmacologically inhibiting bromodomain:acetyl-lysine recognition has recently emerged as a strategy to activate latent HIV reservoirs for the purpose of eradication. Bromodomains are present in many chromatin-associated factors and have been functionally linked to the HIV replication cycle, particularly at the transcription phase. Here, we provide a review of HIV latency and transcription, of the emergent role of bromodomain-containing proteins in HIV biology, and of studies with chemical inhibitors of bromodomains to activate HIV from latency.

Introduction

Thirty years after it was established that human immunodeficiency virus type 1 (HIV-1) causes AIDS (Gallo et al., 1984; Popovic et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984), HIV infection remains incurable. Currently, more than 30 different anti-HIV drugs are approved for clinical use. However, treatment with these drugs does not eradicate HIV from the host and cannot fully restore the health of an infected individual. HIV-1 patients treated with antiretroviral therapy (ART) have shorter lifespans and are more susceptible to non-AIDS-related conditions, such as inflammation, immunosenescence, cardiovascular disease, liver and kidney dysfunction, and cancer (d'Arminio et al., 2004; Deeks, 2011).

HIV infection persists in ART-treated patients due to physiological reservoirs that harbor transcriptionally silenced—or “latent”—provirus (Finzi et al., 1999). This latent reservoir remains “hidden” from ART, which only targets viruses actively engaged in replication (Sedaghat et al., 2007). When ART is interrupted or discontinued, plasma viral load rebounds rapidly. A current strategy to eradicate the virus is the “shock and kill” approach, which involves stimulating latent proviruses by activating HIV transcription without causing systemic inflammatory responses in
the host (“shock”), followed by clearance of these latently infected cells in the presence of ART (“kill”). While this approach is promising, many difficulties remain.

As part of current efforts to identify a broad-based cure for HIV infection, research has focused on combining antiretroviral treatments with latency-purging strategies. This results in virus-producing cells that either die from direct cytopathic effects of the virus or are cleared by host mechanisms and may accelerate the depletion of latent reservoirs and lead to a cure (Geeraert et al., 2008). Experimental approaches to reactivate latent HIV include nuclear factor kappa B (NF-κB)-inducing agents, histone deacetylase (HDAC) inhibitors, methyltransferase inhibitors, immune modulators, and pro-apoptotic and cell-differentiating molecules. Recently, a number of studies have described the therapeutic potential of pharmacologically inhibiting members of the bromodomain and extraterminal (BET) family of human bromodomain proteins (Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010). Small-molecule BET inhibitors, such as JQ1 (Delmore et al., 2011; Filippakopoulos et al., 2010), I-BET (Nicodeme et al., 2010), I-Bet151 (Dawson et al., 2011), and MS417 (Zhang et al., 2012) successfully activate HIV transcription and reverse viral latency in clonal cell lines and certain primary T-cell models of latency. In this chapter, the latest developments in activating transcription of latent HIV by targeting bromodomains are presented.

**HIV Latency**

**Establishment and Maintenance of HIV Latency**

Successful completion of the HIV lifecycle depends strongly on the activation state of CD4+ T cells. In resting T cells, HIV replication is blocked at multiple levels, including reverse transcription (Meyerhans et al., 1994; Zack et al., 1990) and nuclear import (Bukrinsky et al., 1992). Latent HIV infection occurs in resting CD4+ T cells in the form of preintegration latency, whereby nonintegrated HIV-1 cDNA will either degrade or integrate into the host genome upon activation. Conversely, postintegration latency occurs when integrated proviruses are transcriptionally silenced. Postintegration latency produces a more stable latent pool than preintegration latency. A popular model of how postintegration latency is established is that antigen-specific CD4+ naive or memory T cells become preferentially infected during activation, evade cell death, and revert to a resting “memory” state, thereby silencing HIV transcription. Upon antigenic restimulation or exposure to cytokines, silencing is reverted, and HIV transcription reactivated. This model can be successfully modeled ex vivo in several primary T-cell models of latency (Bosque and Planelles, 2009; Yang et al., 2009). Further support comes from the finding that a substantial part of the latent reservoir resides in central and transitional
CD4⁺ memory T cells in patients (Chomont et al., 2009). In addition, after stimulation with chemokine receptor ligands (CCL19, CXCL9 and CXCL10, CCL20) or exposure to high doses of concentrated viral stock, resting CD4⁺ T cells establish latent infection ex vivo indicating that when en route to lymphoid tissues and guided by chemokine receptors, naive or memory T cells can establish latent infection directly without being activated first (O'Doherty et al., 2000; Saleh et al., 2007). To date, other potential reservoirs include monocytes, macrophages, astrocytes, and microglial cells as well as epithelial cells in tissues (Siliciano and Greene, 2011).

Postintegration latency is established when viral DNA integrates into the host’s chromatin, and complex molecular mechanisms epigenetically silence active viral transcription (Mbonye and Karn, 2014). Although HIV integrates preferentially into actively transcribed genes of infected cells (Han et al., 2004; Schroder et al., 2002), the provirus is transcriptionally silenced by heterogeneous corepressor complexes that may contain histone acetyltransferases and deacetylases (Figure 5-1), histone and DNA methyltransferases, heterochromatin proteins, and other epigenetic regulators (Blazkova et al. 2009, Coull et al. 2000, He et al. 2002, Kauder et al. 2009, Van Lint et al. 1996, Verdin et al. 1993 and Williams et al. 2006). The nucleosomal organization of the proviral DNA occurs independently from the proviral integration site (Verdin et al., 1993), but the proximity to eu- or heterochromatic environments within the genomic location may dictate which modifying enzymes or chromatin remodeling complexes are recruited (Jordan et al., 2003). Other factors that contribute to the latent proviral state include the unique cellular milieu within resting T cells, which limits the availability of critical HIV cofactors such as the positive transcription elongation factor b (P-TEFb) and NF-κB (Budhiraja et al., 2013; Duverger et al., 2009; Tyagi et al., 2010), transcriptional interference with the host transcription machinery that is engaged on adjacent cellular genes (Lenasi et al., 2008), and integration into critical genes controlling clonal expansion (Maldarelli et al., 2014). Notably, the virally encoded transactivator of transcription (Tat) autoregulates its own production and recruits the P-TEFb cofactor and many epigenetic regulators to the elongating RNA polymerase II complex associated with the HIV provirus (Cujec et al. 1997, Herrmann et al. 1995, Jones et al. 1994, Kao et al. 1987, Selby et al. 1990 and Tyagi et al. 2010). Consequently, Tat may not reach critical levels in resting T cells, and the lack of robust Tat expression or function may be a key factor in establishing and maintaining HIV latency.

Epigenetic silencing of a provirus can be reversed by drugs that target chromatin-associated factors, including HDAC inhibitors (Hakre et al., 2012). In addition, productive HIV transcription is most strongly activated by signals delivered through the T-cell receptor (TCR–
Figure 5-1 – Acetylation writers, erasers, and readers implicated in HIV latency and transcription. (A) Latent proviruses are characterized by the presence of histone deacetylases (HDACs) 1, 2 and 3, the SWI/SNF chromatin-remodeling complex BAF, and BET proteins. Recognition of acetylated histones, and perhaps non-histone proteins, by BET family members may play a role in their repressive effects on HIV transcription. (B) Active HIV transcription is distinguished by the presence of the p300, PCAF, and GCN5 acetyltransferases, the SWI/SNF chromatin-remodeling complex PBAF, and BRD4. Recognition of acetylated histone and nonhistone proteins (P-TEFb/cyclin T1, NF-κB/p65, and Tat) by bromodomain proteins (BRD4, PCAF) functions in the transcriptional activation at the LTR.
CD3 complex and CD28 co-receptor) that increase levels of critical cofactors, such as NF-κB and P-TEFb, and boost Tat expression and function. Drugs that interrupt the molecular pathways associated with epigenetic silencing, mimic T-cell activation, or activate Tat function are all promising candidates to help clear the latent reservoir. Some of these drugs, including HDAC inhibitors, are now being clinically tested for their potential to reactivate HIV in patients (Archin et al., 2012).

**Basic Mechanisms of HIV Transcription**

Transcription of the HIV genome is a pivotal step in the postintegration phase of the viral lifecycle. Under basal conditions, transcription of the HIV provirus is remarkably inefficient, such that transcription produces mostly incomplete transcripts that cannot fuel viral replication. This phenomenon occurs because the cellular RNA polymerase II complex, which is responsible for HIV transcription, pauses shortly downstream of initiation (called “promoter-proximal pausing”). While this was long regarded a unique feature of the HIV provirus, this pausing is now known as a common way to regulate inducible cellular genes that require fast or synchronous activation (Adelman and Lis, 2012; Hargreaves et al., 2009). HIV has evolved a unique mechanism to overcome the transcriptional elongation block; the virally encoded transactivator Tat relieves the pausing by recruiting and activating P-TEFb.

**Nucleosomal structure of the HIV long terminal repeat (LTR).** Upon integration into chromatin, the HIV proviral DNA becomes organized into higher-order nucleosomal structures that do not depend on the genomic integration site (Verdin et al., 1993). The first (+1) nucleosome (nuc-1) located immediately downstream of the transcription start site (TSS) was long thought to prevent efficient elongation of HIV transcripts because when the virus is activated from latency, nuc-1 becomes significantly remodeled (Figure 5-1). Recent genome-wide studies showed that, indeed, the +1 nucleosome is a barrier to the elongating RNA polymerase II complex for essentially all genes, including those undergoing regulated pausing (Weber et al., 2014). At the latent HIV provirus, the +1 nucleosome is actively positioned in an energetically unfavorably position by the SWI/SNF remodeling complex containing BAF250 (Rafati et al., 2011). When the HIV provirus becomes active, the PBAF complex is recruited and nuc-1 is repositioned to its predicted location (Mahmoudi et al., 2006). Several histone modifications have been described at nuc-1, including acetylation of H3K9 and H3K14 (Lusic et al., 2003), and these modifications are thought to impact viral transcription.

**Tat and P-TEFb.** After cellular activation, cellular transcription factors, such as NF-κB, bind to cis-acting elements in the viral promoter. These factors transiently increase the elongation
capacity of RNA polymerase II and trigger production of the viral Tat protein. Tat is a small, intrinsically disordered RNA-binding protein whose folding and function largely depend on binding of host factors, most notably P-TEFb (Tahirov et al. 2010). Tat and the associated P-TEFb complex bind to an RNA structure called TAR that spans the positions +1 to +59 of all initiating HIV transcripts. P-TEFb is a heterodimeric complex composed of a regulatory cyclin T subunit protein (cyclin T1, T2A, or T2b, only cyclin T1 supports Tat transactivation) and the cyclin-dependent kinase 9 (CDK9). Phosphorylation events catalyzed by CDK9 markedly stimulate transcriptional elongation, specifically the phosphorylation of negative elongation factors, negative elongation factor (NELF) and 5,6-dichloro-beta-D-ribofuranosyl-benzimidazole (DRB) sensitivity-inducing factor (DSIF), which, when hypophosphorylated, sterically hinder the polymerase from passing beyond the proximal pause site at the viral genome (Guo and Price, 2013; Zhou et al., 2012). Tat and P-TEFb are part of a larger “super elongation” complex (SEC) that strongly stimulates HIV transcription and is composed of ELL1/ELL2, AFF1/AFF4, ENL, and AF9 (He et al., 2010; Sobhian et al., 2010). Of note, the ELL2 protein represents an independent elongation factor that advances transcriptional elongation by keeping the 3’-hydroxyl group of the nascent transcript in line with the polymerase and by preventing polymerase backtracking (Shilatifard et al., 1997; Shilatifard et al., 1996). AFF4 acts as a scaffolding protein that brings P-TEFb and ELL2 close together, whereby they cooperatively stimulate elongation competence of the same polymerase molecule (Chou et al., 2013). Tat stabilizes the normally short-lived ELL2 protein to encourage formation of the SEC and stimulate robust HIV transcription (He et al., 2010).

Cellular P-TEFb activity, at least in tumor cells, is regulated by a unique posttranslational mechanism that partitions active and inactive P-TEFb into roughly equal parts (Peterlin and Price, 2006; Yik et al., 2003). Catalytically active P-TEFb is composed of the P-TEFb heterodimer and associates with the bromodomain-containing protein 4 (BRD4), which acts as an adaptor protein delivering active P-TEFb to transcriptionally active genomic loci by interacting with acetylated histones (Hargreaves et al., 2009; Jang et al., 2005; Yang et al., 2005). Inactive P-TEFb resides in a larger complex composed of P-TEFb, La-related protein 7 (LARP7), 7SK snRNA methylphosphate capping enzyme (MEPCE), the small non-coding RNA 7SK, and the CDK9 inhibitor Hexim1. In this large complex, termed the 7SK snRNP, Hexim1 suppresses CDK9 catalytic activity. Cellular stress and interaction with BRD4 or Tat trigger the liberation of P-TEFb from the inactive 7SK snRNP and transiently augments the transcription of stress-response genes or HIV. Importantly, Tat and BRD4 compete for P-TEFb binding, and
BRD4 inhibits Tat-mediated transactivation of viral transcription (Bisgrove et al., 2007) while coactivating basal HIV transcription in the absence of Tat (Jang et al. 2005; Yang et al. 2005).

**Bromodomain-Containing Proteins in HIV Infection**

Although protein acetylation has long been functionally implicated in HIV replication and is now clinically targeted to activate HIV transcription from latency, the role of bromodomain proteins in HIV infection is only just now being appreciated (Boehm et al., 2013b). In the human proteome, 46 bromodomain proteins encompass 57 unique bromodomains that are almost exclusively encoded by chromatin-associated factors. BRD4 belongs to the family of bromodomain and extraterminal domain (BET) proteins that is distinguished by two tandem N-terminal bromodomains and a conserved extraterminal domain that coordinates protein:protein interactions (Florence and Faller, 2001; Rahman et al., 2011). Other BET proteins include the widely distributed BRD2 and BRD3 proteins, and the testis-specific BRDT protein.

Bromodomains are *bona fide* recognition domains for acetylated lysines within histone and nonhistone proteins alike. The bromodomain is approximately 110 amino acids in length and adopts a unique, left-handed four-α-helix bundle. This structure generates a large hydrophobic pocket in which the acetyl-lysine residue is recognized (Dhalluin et al., 1999; Filippakopoulos et al., 2012). The first structural characterization of a bromodomain and its recognition of an acetyl-lysine residue occurred with the bromodomain of the PCAF acetyltransferase (Dhalluin et al., 1999). The viral Tat protein, when acetylated at lysine 50, is recognized and bound by the PCAF bromodomain to promote transcriptional activation of the HIV LTR (Dorr et al., 2002; Mujtaba et al., 2002) (Figure 5-1).

In addition to the Tat:PCAF interaction, the second direct evidence for bromodomain involvement in HIV transcriptional regulation arrived with the finding that the cyclin T1 component of P-TEFb is acetylated, and tri-acetylated cyclin T1 specifically binds the second bromodomain of BRD4 (Cho et al., 2009; Schroder et al., 2012; Vollmuth et al., 2009). This is in addition to the C-terminal P-TEFb-interacting domain (PID) of BRD4 that binds to both the cyclin and CDK9 components of P-TEFb (Bisgrove et al., 2007) (Figure 5-2). This contrasts with Tat-mediated transactivation, in which Tat competes with the PID of BRD4 for P-TEFb binding. A model emerges under which BRD4 is required for initial HIV transcription in the absence of Tat (Jang et al., 2005; Ott et al., 2011; Yang et al., 2005) However, upon Tat production, BRD4 is functionally replaced by the viral factor that delivers active P-TEFb to the LTR (Bisgrove et al., 2007; Yang et al., 2005).
Figure 5-2 – P-TEFb complexes relevant to HIV transcription. Tat competes with the PID of BRD4 for P-TEFb binding to recruit P-TEFb and drive processive HIV transcription. Besides the PID, a secondary site of BRD4/P-TEFb interaction is the recognition of the second bromodomain of BRD4 by triacetylated cyclin T1.
This model has received considerable attention since the exciting discovery and characterization of small molecules that selectively bind BET bromodomains to competitively inhibit recognition of acetyl-lysine residues (Filippakopoulos et al., 2010; Nicodeme et al., 2010). The first such compounds, JQ1 and I-BET, are structurally distinct; yet, both harbor a triazole ring that forms a hydrogen bond with a canonical asparagine residue within the bromodomain that otherwise forms a hydrogen bond with the coordinated acetyl-lysine residue. JQ1 is a benzodiazepine-derived molecule with highest affinity for the first bromodomain of BRD4, yet it has considerable affinity for bromodomains of all other BET family members. This small molecule was first explored therapeutically in the NUT midline carcinoma, a rare epithelial cancer genetically defined by oncogenic BRD4 fusions, but has since been applied in preclinical models of many different cancers (Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010; Lockwood et al., 2012; Mertz et al., 2011; Puissant et al., 2013; Zuber et al., 2011). In contrast, I-BET was developed by GlaxoSmithKline as a pharmaceutical histone mimic with slightly more degenerate affinity among BET family members. I-BET functions as a strong immunosuppressant that abrogates expression of several key proinflammatory cytokines in bone-marrow-derived macrophages challenged with lipopolysaccarides (Nicodeme et al., 2010). Thus, the development and novel preclinical application of I-BET have widened the use of bromodomain inhibitors from cancer to inflammation. Interestingly, MS417, a compound structurally similar to JQ1, blocks the interaction between acetylated NF-κB and BRD4 and suppresses HIV-induced renal damage in a model of HIV-induced kidney disease (Zhang et al., 2012).

Because of the central role of the BRD4/P-TEFb complex in HIV transcription, several independent groups tested the effect of BET inhibitors on HIV transcription (Banerjee et al., 2012; Bartholomeeusen et al., 2012; Boehm et al., 2013a; Li et al., 2013; Zhu et al., 2012). All found that BET inhibitors activate HIV transcription in latently infected tumor cell lines, thus adding to the list of epigenetic drug candidates for the “shock and kill” strategy. However, the precise molecular mechanisms of how these drugs activate HIV transcription remain unknown. Mechanisms specific for HIV or potentially shared with cellular genes that respond to BET inhibitors with transcriptional upregulation are being considered. Current models of BET inhibitor function in HIV transcription are described below and schematized in Figure 5-3.

**BET Inhibitors Boost Tat Activity**

Because BRD4 and Tat compete for P-TEFb binding, an obvious and relevant hypothesis is that BET inhibitors dissociate the BRD4/P-TEFb complex from the LTR, thereby
Figure 5-3 – Potential mechanisms of BET inhibitor action at the HIV LTR. The BET inhibitor JQ1 binds the bromodomain (PDB: 2OSS from (Filippakopoulos et al., 2012) and rendered using Chimera (UCSF)) and competitively inhibits its recognition of acetyl-lysine residues. BET inhibitors may function by relieving the competition between Tat and BRD4, stoichiometrically increasing free active P-TEFb, relieving repressive BET complexes, or via indirect transcriptional effects.
increasing the recruitment of the Tat/P-TEFb complex to the site of viral transcription. Supporting this model, robust reactivation from latency is observed in a Jurkat-derived latency cell line (J-Lat) called A2 (Jordan et al., 2003), containing an HIV “minivirus” where the promoter activity within the HIV 5′-LTR drives expression of Tat and GFP (Tat-IRES-GFP) (Boehm et al., 2013a; Li et al., 2013; Zhu et al., 2012). Furthermore, Li et al. demonstrated that in A2 cells, BRD4 at the HIV LTR blocks the Tat/SEC formation, a function alleviated by adding JQ1 (Li et al., 2013). JQ1 dissociates BRD4 from the HIV promoter so that Tat can recruit the SEC and stimulate HIV transcriptional elongation. Similar results were reported by Bartholomeeusen et al., showing that AFF4 and ELL2 occupancy increase at the LTR and in viral coding regions upon JQ1 treatment. Zhu et al. found that the activatory function of JQ1 relies on an intact TAR element, which is normally co-occupied by Tat and the cyclin T1 subunit of P-TEFb (Li et al., 2013; Wei et al., 1998). In addition, Banjeree et al examined the effects of JQ1 on cellular gene expression and showed potent upregulation of chromatin-modifying genes (Banerjee et al., 2012), including the deacetylases SIRT1 and HDAC6, previously shown to deacetylate Tat (Huo et al., 2011; Pagans et al., 2005).

**BET Inhibitor Treatment Releases P-TEFb from the Inactive Complex**

An interesting aspect of the HIV transcriptional activation caused by BET inhibitors is the posttranslational regulation of P-TEFb activity. Bartholomeeusen et al. showed that JQ1 treatment transiently released active P-TEFb from the inactive 7SK snRNP, increasing the levels of free P-TEFb and the BRD4/P-TEFb complex (Bartholomeeusen et al., 2012). This is concordant with stoichiometric increases of both CDK9 and other SEC components at the HIV promoter LTR. The increase is transient because JQ1 also strongly increases transcription of the Hexim1 gene, thus promoting the reassembly of the 7SK snRNP after JQ1 treatment. Under this model, BET inhibition activates P-TEFb from the 7SK snRNP, allowing Tat to capture it. The Tat/P-TEFb complex or AFF4/SEC components are then recruited to the HIV LTR. Importantly, knockdown of the ELL2 elongation complex reduced this activation, underscoring the importance of the SEC to HIV transcription and the function of the BET inhibitor. Interestingly, BRD4 itself liberates P-TEFb from the inactive 7SK snRNP via its PID (Bisgrove et al., 2007; Krueger et al., 2010; Schroder et al., 2012). The release of BRD4 from chromatin upon JQ1 treatment may therefore shift the equilibrium between inactive and active P-TEFb. Alternatively, Tat may recruit P-TEFb from chromatin-bound 7SK snRNP, thus implicating the 7SK snRNP directly in HIV transcription (D’Orso and Frankel, 2010).
**BRD4 and BRD2 Act as Repressors at the HIV LTR**

Despite the robust response in A2 cells that supports the model of Tat-dependent activity of BET inhibitors in HIV transcription, activatory effects on the HIV promoter were also reported in the absence of Tat (Boehm et al., 2013a; Zhu et al., 2012). Boehm et al. showed that, in A72 cells, a J-Lat cell line, in which GFP expression is driven by the HIV LTR in the absence of Tat (Jordan et al., 2003), JQ1 and other BET inhibitors I-BET, I-BET151, and MS417 activated HIV transcription with similar efficiencies as in A2 cells (Boehm et al., 2013a). Zhu et al. also reported that weak activatory effects of JQ1 were seen with the HIV promoter alone (Zhu et al., 2012). Boehm et al. further showed that knockdown of BRD2 in A72 cells spontaneously activated HIV transcription to similar levels as treatment with JQ1 and that the response to JQ1 was attenuated in BRD2 knockdown cells. Activation levels were higher in BRD2 than in BRD4 knockdown cells, implicating BET family members other than BRD4 in reactivating HIV and as targets of JQ1. Although JQ1 binds with highest affinity to the first bromodomain of BRD4, it binds efficiently to all bromodomains of all BET proteins, and it binds with low efficiency to several other bromodomain proteins (Filippakopoulos et al., 2010). It is unclear how BRD2 interacts with P-TEFb, as P-TEFb remains necessary for the BET inhibitor effect in A72 cells, since treatment with the P-TEFb inhibitor DRB suppressed JQ1-induced reactivation of HIV transcription in these cells (Boehm et al., 2013a).

**BET Inhibitor Effects on the HIV Promoter are Indirect**

Because BET inhibitors induce widespread changes in the transcriptional profiles of treated cells, indirect effects might contribute to the latency-purging effects of these drugs. To test this hypothesis, Banjeree et al. performed microarray studies in JQ1- and control-treated cells to examine global transcriptional changes induced by these compounds. Interestingly, a variety of genes functionally implicated in HIV transcription showed considerable changes in their expression upon JQ1 treatment. These included various P-TEFb and SEC constituents, such as cyclin T1, Hexim1, AFF4, AFF1, and AF9, which were induced upon JQ1 treatment. Expression of other relevant epigenetic regulators, such as PCAF, MLL3, and several lysine demethylases was also upregulated. In contrast, NELF complex members NELF-A and NELF-C, relevant for the pausing of RNA polymerase II (Zhang et al., 2007), were downregulated upon JQ1 treatment, in addition to a variety of T-cell activation genes, such as CD3, CD28, and the CXCR4 chemokine receptor. The functional involvement of these changes in HIV reactivation remains to be determined.
Challenges to BET Inhibitor Treatment in Primary T cells

Although reactivation of latent HIV-1 by BET inhibitors was robustly demonstrated in several latently infected tumor cell lines, including the U1 monocytic cell line (Zhu et al., 2012), in several Jurkat-derived cell lines such as JΔK (lacking functional NF-κB sites within the HIV promoter), A2 and A72 (Banerjee et al., 2012; Bartholomeeusen et al., 2012; Boehm et al., 2013a; Li et al., 2013; Zhu et al., 2012), the responses to BET inhibitors in primary T-cell models of latency were less robust. Boehm et al. demonstrated about 10% of maximal activation of latently infected cells in a primary T-cell model of HIV latency transduced with the Bcl-2 survival factor (Yang et al., 2009), while in another system modeled after the latent reservoir in central and transitional memory T cells (Bosque and Planelles, 2009), JQ1 or other BET inhibitors did not show any effect. Banjeree et al. tested JQ1 in resting CD4^+ T-cell ex vivo cultures prepared from HIV-1-infected patients under antiretroviral therapy. They detected HIV-1 recovery in only one out of three samples (Banerjee et al., 2012). Zhu et al reported robust reactivation by JQ1 in combination with several known HIV-1 inducers (phorbol esters, TNFα, prostratin) in ex vivo cultures of CD8^+-depleted peripheral blood mononuclear cells isolated from 19 ART-treated patients with undetectable viral loads (Zhu et al., 2012). Importantly, in this study, JQ1 alone did not induce HIV-1 recovery (Zhu et al., 2012). One explanation for these results might be the low cellular P-TEFb and low nuclear NF-κB levels found in resting T cells that are boosted upon T-cell activation (Budhiraja et al., 2013; Chiang et al., 2012). Further studies are clearly needed to better understand and increase the therapeutic potential of BET inhibitors in primary resting T cells.

Conclusion
The development of BET inhibitors has highlighted the functional importance of the bromodomain:acetyl-lysine regulatory axis in HIV transcription and latency. While the molecular mechanisms through which these compounds activate HIV expression are still not fully understood, these compounds are emerging as promising new tools for both the molecular study and the clinical treatment of HIV latency. As drug combinations are likely the most promising approach to effectively purge the latent reservoir, BET inhibitors may join other epigenetic modulators and T cell activators in the development of a curative treatment for HIV patients.
Acknowledgments
We thank John Carroll and Teresa Roberts for assistance with graphics, Gary Howard and Celeste Brennecka for editorial and Veronica Fonseca for administrative assistance. We gratefully acknowledge support from the NIH (R01 AI083139 and CARE Collaboratory (U19AI096113) to M.O.) and the California HIV/AIDS Research Program (F13-GI-316 to D.B.). R.J.C. was supported in part by NIH Training Grant T32 GM007175.

Conflict of Interest
The authors declare no conflict of interest.
References


cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224, 500-503.


Chapter VI

The Short Isoform of BRD4 Promotes HIV-1 Latency by Engaging Repressive SWI/SNF Chromatin Remodeling Complexes

Ryan J. Conrad¹,²,³, Parinaz Fozouni¹,³, Sean Thomas¹, Hendrik Sy¹, and Melanie Ott¹,²,³*

¹Gladstone Institutes, University of California, San Francisco
²Graduate Program in Pharmaceutical Sciences & Pharmacogenomics, University of California, San Francisco
³Department of Medicine, University of California, San Francisco

* To whom correspondence should be directed:

Melanie Ott, MD, PhD
Gladstone Institutes
University of California, San Francisco
1650 Owens Street
San Francisco, CA, 94158
Tel: 415-734-4807
Email: mott@gladstone.ucsf.edu
www.ottlab.uscf.edu

Summary

BET proteins commonly activate cellular gene expression, yet inhibiting their recruitment paradoxically reactivates latent HIV-1 transcription. Here we identify the short isoform of BET family member BRD4 (BRD4S) as a novel corepressor of HIV-1 transcription. We find BRD4S enriched in chromatin fractions of latently infected T cells and more rapidly displaced from chromatin upon BET inhibition than the long isoform. BET inhibition induces marked nucleosome remodeling at the latent HIV promoter, which is dependent on the activity of BAF, a SWI/SNF chromatin-remodeling complex with previously described repressive functions in HIV-1 transcription. BRD4S binds BRG1, a catalytic subunit of BAF, via its bromo- and ET domains and is required for BRG1 recruitment to the latent HIV promoter. Using ChIP-seq combined with ATAC-seq data, we find that the latent HIV-1 promoter phenotypically resembles endogenous LTR sequences, pointing to a broader role of BRD4S-BRG1 complexes in genomic silencing of invasive retroelements beyond HIV-1.

Keywords: Bromodomain, BET protein, BRD4, JQ1, HIV, LTR, latency, chromatin, SWI/SNF, BRG1, BAF250a
Introduction

BRD4 is the prototypical member of the bromodomain and extraterminal domain (BET) family of proteins. BET proteins are distinguished by tandem N-terminal bromodomains that bind acetyl-lysine residues (Dhalluin et al., 1999; Filippakopoulos et al., 2012) and an extraterminal domain that mediates protein:protein interactions (Rahman et al., 2011). BRD4 interacts with multiple transcriptional regulators including Mediator (Jiang et al., 1998), P-TEFb (Bisgrove et al., 2007; Jang et al., 2005; Yang et al., 2005), and NF-kB (Huang et al., 2009), but also possesses intrinsic histone chaperone (Kanno et al., 2014) as well as kinase (Devaiah et al., 2012) and acetyltransferase (Devaiah et al., 2016) activities. Predominantly, BRD4 encodes two isoforms via alternative exon usage – a long isoform (isoform A or BRD4L, aa 1-1362) containing the P-TEFb-interacting domain (PID) at its extreme C terminus (Bisgrove et al., 2007) and a short isoform (isoform C or BRD4S, aa 1-722) that resembles other BET family members. A third isoform (isoform B, aa 1-794) restrains the propagation of DNA damage signaling along chromatin, yet has only been reported in osteosarcoma cells (Floyd et al., 2013).

While BRD4L is largely known to bind and activate P-TEFb complexes to positively support transcript elongation, the function of BRD4S remains relatively uncharacterized. Several lines of evidence suggest, however, that BRD4L and BRD4S have discrete functions in regulating transcription and chromatin structure. The two isoforms differentially regulate transcription of the neuronal genes Arc and GluA1 (Korb et al., 2015), and have opposing roles in the progression of metastatic breast cancer via divergent regulation of the TPX2 network (Hu et al., 2012). Overexpression and artificial tethering of N-terminal regions of BRD4 result in significant changes in chromatin structure (Floyd et al., 2013; Wang et al., 2012; Zhao et al., 2011). Furthermore, the two isoforms localize to distinct nuclear compartments and have unique histone-binding profiles, with BRD4S found in the nuclear periphery and possessing a broader and more avid acetyl-histone binding capacity than BRD4L (Alsarraj et al., 2013). These data point to a distinctive role for the short isoform of BRD4, yet mechanistic insight into this function is currently limited.

Latent infection by HIV-1 yields a transcriptionally inactive but replication-competent provirus integrated into host chromatin (Siliciano and Greene, 2011). It is widely recognized that the host epigenetic machinery participates in transcriptional repression during HIV-1 latency by imposing reversible restrictions on the chromatinized provirus, which can be overcome or enforced by epigenetic drugs (Gallo, 2016). Notably, such chromatin restrictions include the positioning of a nucleosome, termed nuc-1, immediately downstream of the HIV-1 transcription start site (TSS) that is disrupted during transcriptional reactivation in response to TNFα or
histone deacetylase inhibition (Rafati et al., 2011; Van Lint et al., 1996; Verdin, 1991; Verdin et al., 1993). Positioning of nuc-1 during latent infection occurs over thermodynamically unfavorable cis-elements of the provirus and is actively mediated by the SWI/SNF remodeling complex BAF, characterized by the presence of the ATPases BRM or BRG1 and the ARID-domain containing BAF250a subunit (Rafati et al., 2011; Van Lint et al., 1996; Verdin, 1991; Verdin et al., 1993).

The described roles of BRD4 in HIV-1 transcription are currently contradictory (Boehm et al., 2013b). Initially, BRD4 was shown to play a positive role in HIV transcription by critically supporting basal HIV-1 transcription through recruitment of P-TEFb (Jang et al., 2005; Yang et al., 2005), a necessary cofactor for processive HIV-1 transcription (Wei et al., 1998; Zhu et al., 1997). However, BRD4 also competes with the virally-encoded transactivator of transcription Tat for limiting reservoirs of P-TEFb, and as such negatively impacts HIV-1 transcription via P-TEFb sequestration (Bisgrove et al., 2007). These studies have exclusively focused on the long isoform of BRD4. BET inhibitors, such as JQ1, reactivate HIV-1 from latency across a variety of experimental models (Banerjee et al., 2012; Bartholomueusen et al., 2012; Boehm et al., 2013a; Halper-Stromberg et al., 2014; Laird et al., 2015; Li et al., 2013; Zhu et al., 2012), further supporting a repressive role for BET proteins in HIV-1 latency (Boehm et al., 2013b). Importantly, BET inhibitors reverse latency in a T cell model in which HIV-1 transcription functions in the absence of Tat (Boehm et al., 2013a), indicating that BET proteins perform yet unknown repressive functions at the latent HIV promoter apart from interfering with the Tat:P-TEFb axis.

Here we characterized the repressive functions of BRD4 in HIV transcription and identified its short isoform as a novel negative transcriptional regulator in latently infected T cells. We show that BRD4S is highly chromatin-bound and recruits a BRG1-containing BAF chromatin-remodeling complex to maintain a repressive nucleosome structure at the latent HIV promoter. This process is not only relevant for HIV-1 but also is in place at endogenous LTR-containing elements, pointing to a more general silencing mechanism to restrict expression of retroviral promoter sequences.

Results

Short BRD4 enforces HIV latency

Our initial results demonstrated that knockdown of BRD4 had no substantial effect on reactivation of HIV-1 from latency (Boehm et al., 2013a). However, the shRNAs used at the time exclusively targeted the 3’ UTR unique to long BRD4 mRNAs, not affecting expression of the
short isoform (Figure 6-1A, shRNA#1). We therefore tested shRNAs directed against 5' sequences present in mRNAs encoding both BRD4S and BRD4L (Figure 6-1A, shRNA#2). Transduction of J-Lat A2 cells, Jurkat T cells harboring a latent HIV GFP reporter virus encoding Tat (Jordan et al., 2003), with shRNA#2 resulted in efficient knockdown of both BRD4 isoforms and robust latency reversal, with 30-fold induction of GFP+ cells (Figure 6-1A). Importantly, cells treated with shRNA#2 failed to respond to JQ1 (Figure 6-1B), indicating that BRD4S is a relevant target of the BET inhibitor in this system.

To further characterize BRD4S function, we processed T cell nuclei into nucleoplasmic and chromatin fractions to interrogate the localization of BRD4 isoforms. We found BRD4S highly enriched in the chromatin fraction of latently infected T cells, while BRD4L was present in both fractions (Figure 6-1C). Treatment with JQ1 induced rapid depletion of BRD4S levels in chromatin fractions (<1 h) with levels of BRD4L decreasing more slowly and less markedly over time (Figure 6-1C). The same difference was observed when increasing concentrations of JQ1 were tested; chromatin-bound BRD4S responded to ten fold lower JQ1 concentrations than BRD4L (0.18µM vs. 1.8µM, Figure 6-1D). Nucleoplasmic and chromatin-associated levels of Cyclin T1, a core component of P-TEFb, were not altered by JQ1 treatment over time (Figure 6-1C). Notably, nucleoplasmic levels of BRD4S and BRD4L did not consistently increase when chromatin levels decreased under JQ1 treatment, suggesting that chromatin-bound forms were either exported to the cytoplasm or degraded. These results establish that BRD4S is abundant in chromatin fractions of T cells and is highly responsive to JQ1 treatment.

**JQ1 disrupts HIV chromatin architecture independently of transcription**

The +1 nucleosome of HIV (nuc-1) is a key restraint to processive HIV transcription during viral latency (Van Lint et al., 1996; Verdin, 1991; Verdin et al., 1993). To test whether JQ1 treatment is linked to nuc-1 remodeling, we performed enzymatic digestion of J-Lat chromatin first by AflII, a restriction enzyme with a cognate recognition site located within sequences occupied by nuc-1 (Van Lint et al., 1996). Limited cutting is observed when nuc-1 is properly positioned at the latent HIV promoter, yet upon remodeling this site becomes progressively more accessible, leading to increased cutting and a decrease in successful PCR product amplification spanning this site (Figure 6-2A). Digestion of naked DNA was used as control, and data were expressed as fold increase in AflII cutting over untreated cells. We found that treatment with JQ1 doubled AflII accessibility in J-Lat A2 cells, indicating significant nuc-1 remodeling, which occurred to similar levels in cells treated with TNFα, a well-known latency-reversing cytokine (Figure 6-2B, left panel). Importantly, comparable remodeling was observed
Figure 6-1 – Short BRD4 promotes HIV latency. A – J-Lat A2 cells were transduced with lentiviral shRNAs (#1 and #2) targeting N- and C-terminal sequences of BRD4 as indicated or scramble non-targeting shRNAs as controls. Flow cytometry of GFP indicating HIV transcriptional activity (mean of three independent experiments analyzed in triplicate ± SEM) and western blotting of BRD4 to assess knockdown. *ns depicts non-specific band that was resistant to shRNA treatment. B – Flow cytometry of J-Lat A2 cells transduced with shRNAs described in (A) treated with JQ1 (625 nM for 18h). Means of three independent experiments analyzed in triplicate ± SEM is shown. C – Western blotting of nuclear and chromatin fractions isolated from J-Lat A72 cells treated with JQ1 (625 nM) with indicated antibodies. Representative experiment of three replicates is shown. D – Quantification of chromatin fraction band intensities expressed relative to untreated controls from J-Lat A72 cells, treated with increasing concentrations of JQ1. Average of two biological replicates is shown.
Figure 6-2 – Chromatin disruption at the HIV promoter upon JQ1 treatment. A – Schematic of AflII assay to measure nuc-1 accessibility. Nuclei were isolated from cells, digested \textit{in vivo} with AflII, DNA was purified and subjected to qPCR using primers flanking the AflII site. Data is normalized to both uncut DNA and naked DNA cut \textit{in vitro}. B – AflII accessibility of J-Lat A2 cells treated with TNF\(\alpha\) (10 ng/mL) or JQ1 (625 nM) with or without flavopiridol (5\(\mu\)M) for 18h. Average of three independent experiments performed in triplicate (\(\pm\) SEM) is shown. C – Quantitative RT-PCR of HIV RNA levels of same experiments as in (B). Average of three independent experiments performed in triplicate (\(\pm\) SEM) is shown. D – Schematic of DNase I digestion coupled to LM-PCR for measuring nuc-1 positioning. Nuclei were digested with DNase I (20 U/mL), DNA was purified, linkers were ligated to first-strand synthesized DNA, and PCR was performed using a linker primer and a primer specific for the 5'-LTR. Naked gDNA cut \textit{in vitro} with 1 U/mL DNase I was used to control for DNase I specificity and aberrant PCR amplification. PCR products were labeled with a 6-FAM-modified primer and product size, corresponding to DNase I cutting, was detected using capillary electrophoresis. E, F – Raw amplicon intensity as measured by fragment analysis of LM-PCR signals from J-Lat A72 cells treated with DMSO (E) or 625 nM JQ1 (F) for 18h before DNase I digestion. Purple signal represents naked gDNA. One representative experiment is shown. G – LOWESS regression curves of LM-PCR signal for DMSO and JQ1 treatments as in (E, F). gDNA signal was subtracted from DNA cut \textit{in vivo}. Data are representative of two biological replicates. H – ATAC-seq of viral chromatin in response to JQ1 treatment. Chromatin from J-Lat A72 cells treated with either DMSO or 625 nM JQ1 for 18h was transposed and resultant libraries were paired-end sequenced. ATAC density (above) and inferred nucleosome density (below) for the integrated provirus are shown. One representative experiment is shown.
when HIV-1 mRNA production was blocked with the transcriptional cyclin-dependent kinase inhibitor flavopiridol (Figure 6-2B, C, right panels). This excludes the possibility that nuc-1 remodeling by JQ1 occurred as a mere consequence of transcriptional activation and polymerase passage.

Next, we performed DNase I digestion coupled to ligation-mediated PCR (LM-PCR) to gain positional accessibility information of nuc-1 over broader ranges of sequence (Carey et al., 2009b). We altered the original protocol, which relies on radiolabeled PCR probes (Verdin et al., 1993), and introduced a 6-carboxyfluorescein (6-FAM)-labeled probe, enabling sensitive detection of PCR amplicon sizes using fragment analysis by capillary electrophoresis (Figure 6-2D). Using a nested 6-FAM labeled primer anchored at nt +281 relative to the HIV TSS (Verdin et al., 1993), we captured PCR fragments corresponding to the 3'-edge of nuc-1 (Figure 6-2E, F). Upon smoothing of LM-PCR amplicon curves via LOWESS non-parametric regression and normalization to naked DNA, we observed in untreated cells a region of protection immediately downstream of the TSS corresponding to nuc-1 (Figure 6-2G, DMSO curve). This protected area was lost and DNase I accessibility was increased upon JQ1 treatment (Figure 6-2F, G), supporting the model that JQ1 increases chromatin accessibility downstream of the HIV TSS by altering nuc-1 positioning.

We also examined the status of HIV chromatin using the assay for transposase-accessible chromatin coupled to high throughput sequencing (ATAC-seq) (Buenrostro et al., 2013), a new restriction enzyme-independent method to interrogate chromatin structure. Chromatin from control or JQ1-treated J-Lat cells was transposed, and resultant libraries were sequenced to obtain long (100 bp) paired-end reads. We chose paired-end sequencing to enable proper assignment of homologous 5'- and 3'-LTR regions. Using this method, we observed a high degree of transposase accessibility centered just downstream of the HIV TSS that sharply decreased over sequences occupied by nuc-1 (Figure 6-2H, top panel). The accessible area broadened and transposition density decreased under JQ1 treatment, indicative of nuc-1 remodeling (Figure 6-2H, top panel). This was confirmed upon inference of nucleosome densities from ATAC-seq data, which under basal conditions positioned nuc-1 at a similar position downstream of the TSS as Verdin and colleagues (Verdin et al., 1993). Upon JQ1 treatment, nuc-1 shifted further downstream away from the TSS with overall less density, indicative of repositioning and remodeling during transcriptional activation (Figure 6-2H, bottom panel). In addition, we observed considerable depletion of the upstream nucleosome, nuc-0, under JQ1 treatment. Interestingly, nuc-1 remodeling was not mirrored in the 3'LTR, where the corresponding nucleosome shifted into the same direction as nuc-1, possibly disfavoring
antisense transcription from the 3’LTR. Collectively, these data uncover detailed chromatin remodeling processes at the latent integrated HIV provirus in response to BET inhibitor treatment.

**JQ1-mediated latency reversal and nuc-1 remodeling are dependent on the BAF complex**

Given that JQ1 treatment substantially modifies chromatin structure downstream of the HIV TSS (Figure 6-2), we hypothesized that a chromatin-remodeling complex underlies these effects. The SWI/SNF family of chromatin remodeling machines has previously been linked to the regulation of HIV transcription and latency via nucleosome positioning (Easley et al., 2010b; Mahmoudi et al., 2006; Rafati et al., 2011; Treand et al., 2006; Van Duyne et al., 2011). We first performed knockdown studies of the two catalytic subunits of SWI/SNF remodelers, BRM and BRG1, in J-Lat A72 cells (Jordan et al., 2003). This cell model of HIV latency lacks Tat and is thus useful in studying early, Tat-independent events preceding latency reversal. We observed that while BRM was dispensable for JQ1 to induce HIV transcription, its close homologue BRG1 was required to robustly reverse latency (Figure 6-3A). Notably, BRG1 was the most robust hit among eight different chromatin remodeling ATPases spanning all four families (SWI/SNF, CHD, ISWI, and INO80, Figure 6-S1).

SWI/SNF remodeling complexes are partitioned into at least two mutually exclusive complexes—BAF and PBAF, discriminated by BAF250a and BAF180 subunits, respectively. Previous research has established that PBAF cooperates with Tat to enhance HIV transcription (Easley et al., 2010a; Mahmoudi et al., 2006; Treand et al., 2006), whereas BAF plays an opposing role in the maintenance of HIV latency via enforcement of repressive nuc-1 positioning (Rafati et al., 2011). Knockdown of the BAF-specific BAF250a protein, but not of the PBAF component BAF180, decreased JQ1 activity even at high doses of JQ1 (Figure 6-3B, C). In addition, expression of stable components found in both BAF and PBAF complexes, BAF155 and BAF170, were also necessary for JQ1-mediated latency reversal (data not shown). These data support the model that BET inhibitors target repressive BRG1-containing BAF complexes at the latent HIV promoter.

We tested the effects of BAF knockdown on the nucleosome structure of HIV using DNase I digestion followed by LM-PCR. Both knockdown of BRG1 and BAF250a resulted in marked increases in accessibility of DNA sequences normally occupied by nuc-1 as shown by LOWESS regression (Figure 6-3D), consistent with their designated roles in actively positioning nuc-1 during latency. Similar results were obtained in multiple independent experiments, which were averaged by quantifying the area under the LM-PCR amplicon curve (Figure 6-3E).
Figure 6-3 – The BAF chromatin remodeling complex is required for JQ1-mediated latency reversal and nuc-1 remodeling. A – Flow cytometry and western blotting of J-Lat A72 cells transduced with two lentiviral shRNAs targeting BRG1 or BRM to measure transcriptional reactivation. Selected cells were treated with 625 nM JQ1 for 18h. Average of three independent experiments (± SEM) is shown for flow cytometry. B, C – Flow cytometry and western blotting of J-Lat A72 cells transduced with two lentiviral shRNAs targeting BAF250a, specific for the BAF complex (B) or two lentiviral shRNAs targeting BAF180, specific for the PBAF complex (C). Selected cells were treated with indicated titration of JQ1 for 18h. Representative data of at least three independent experiments (± SD) is shown for flow cytometry. D – LOWESS regression curves of normalized LM-PCR signals obtained from J-Lat A72 cells transduced with lentiviral shRNAs targeting BRG1 or BAF250a. Average of three independent experiments is shown. E – Area under the curve (AUC) for LM-PCR signal across.
three independent replicates (± SEM). F–H – Representative normalized LM-PCR signals from J-Lat A72 cells transduced with scramble control shRNAs (F) or shRNAs targeting BRG1 (G) or BAF250a (H) treated with either DMSO or 625 nM JQ1 for 18h. Average of two independent experiments is shown. I — Fold change in AUC values for LM-PCR signals across three replicates (± SEM).
Importantly, no further increase in nuc-1 accessibility was observed upon JQ1 treatment in BRG1 and BAF250a knockdown cells, while cells transduced with control shRNAs showed a robust increase as expected (**Figures 6-3F–I**). Collectively, these data establish a novel connection between JQ1 action and repressive BAF chromatin-remodeling activities.

**Short BRD4 binds SWI/SNF complexes via bromo- and ET domains**

We next hypothesized that BRD4S physically engages SWI/SNF complexes. To test this, we first mined published affinity purification/mass spectrometry data and found two studies examining interaction partners of BRD4S (Crowe et al., 2016; Rahman et al., 2011). BRG1 was detected in both data sets with a mean z-score of 1 (**Figure 6-4A**), which was in a similar range as NSD3 (z=1.2), a validated interaction partner of BRD4S (Rahman et al., 2011; Shen et al., 2015). Interestingly, the second ATPase specific for SWI/SNF complexes, BRM, was not detected in either data set. Additional SWI/SNF family members, BAF155 and BAF170, were also weakly detected with mean z-scores of 0.07 and 0.002, respectively. As expected, bait protein BRD4S scored highly in both experiments (**Figure 6-4A**). These data implicate BRG1-containing SWI/SNF complexes as physical interaction partners of BRD4S.

We confirmed this finding in co-immunoprecipitation experiments using antibodies directed against the N-terminus of BRD4. Immunoprecipitations in combined nuclear and chromatin extracts isolated from J-Lat A72 cells effectively recovered both BRD4 isoforms as well as endogenous BRG1, but not BRM, proteins (**Figure 6-4B**). Remarkably, BRG1 and BRM share >70% sequence homology yet incorporate into distinct SWI/SNF complexes (Kadoch and Crabtree, 2015). Overexpression of a FLAG-tagged BRD4S protein confirmed that the N-terminus of BRD4 binds endogenous BRG1 efficiently (**Figure 6-4C**, WT lane), more efficiently than BRD4L (**Figure 6-S2**). To our surprise, deletion of individual bromodomains (ΔBD1 or ΔBD2) or the ET domain (ΔET) in BRD4S did not affect BRG1 binding (**Figure 6-4C**), indicating that none of these conserved domains is alone necessary for the BRD4:BRG1 interaction.

Individual FLAG-tagged proteins corresponding to the two bromodomains (BDS, 47-459 aa) or the ET domain-containing C-terminus of BRD4S (601-722 aa) were, however, sufficient to co-immunoprecipitate endogenous BRG1 (**Figure 6-4D**). The interdomain space (IDS, aa 458-608) located between the bromo- and ET domains did not interact with BRG1. All constructs expressing individual BRD4 domains also encoded SV40 nuclear localization signals to ensure proper subcellular localization. Both bromodomain and IDS fragments ran at a higher molecular size than predicted, consistent with the presence of posttranslational modifications in

111
Figure 6-4 – Short BRD4 interacts with BRG1 via bromo- and ET domains. A – Standard score plot of two N-terminal BRD4 IP/MS experiments (Rahman et al., 2011, Crowe et al., 2016) indicating BRD4 (bait), NSD3 (positive control, Rahman et al., 2011 and Shen et al., 2015), and SWI/SNF components BRG1, BAF155, and BAF170. B – Endogenous immunoprecipitation of N-terminal BRD4 from J-Lat A72 cells nuclear-chromatin extracts followed by western blotting of BRD4 and SWI/SNF catalytic subunits BRG1 and BRM. C – Immunoprecipitation of overexpressed FLAG-BRD4 domain deletions purified from 293T cells, followed by western blotting using FLAG and BRG1 antibodies. D – Immunoprecipitation of overexpressed FLAG-BRD4 domains purified from 293T cells followed by western blotting using FLAG and BRG1 antibodies. Representative blots of three independent experiments with similar results are shown, and immunoprecipitations represent ~0.4% input.
these regions including phosphorylation, as described for the IDS (Wu et al., 2013). Collectively, these results support a model wherein BRG1-containing SWI/SNF chromatin remodeling complexes physically interact with the short isoform of BRD4 via bromo- and ET-domains.

**Short BRD4 recruits BRG1 to the HIV promoter**

Next, we treated J-Lat A72 cells with JQ1 and performed chromatin immunoprecipitation (ChIP) experiments with antibodies directed against the N-terminus of BRD4 and against BRG1. Immunoprecipitated DNA was subjected to qPCR using two primers specific for the HIV 5’-LTR. We observed robust enrichment of BRD4- and BRG1-specific signals at the latent promoter, which decreased upon JQ1 treatment, consistent with the model that both BRD4 and SWI/SNF proteins are dissociated from latent HIV chromatin by JQ1 (Figure 6-5A, B). We observed no significant change in RNA polymerase II (Pol II) occupancy upon JQ1 treatment (Figure 6-S3) as previously described (Li et al., 2013), consistent with fact that Pol II is paused at the HIV-1 promoter, and paused polymerases show limited occupancy changes following induction of gene expression (Adelman and Lis, 2012).

To test whether BRD4S is required for SWI/SNF occupancy at the latent promoter, we performed ChIP experiments in J-Lat A72 cells transduced with shRNAs targeting either BRD4L (shRNA#1) or both isoforms (shRNA#2). Treatment with both shRNAs decreased BRD4 occupancy levels at the integrated provirus (Figure 6-5C, shRNA#2 > shRNA#1) and total BRD4 protein levels (Figure 6-5E), as expected. However, only depletion of both isoforms, and not BRD4L alone, markedly reduced BRG1 occupancy at viral chromatin (Figure 6-5D). Importantly, JQ1 treatment and BRD4 knockdown did not significantly alter BRG1 protein levels (Figure 6-S4). These data support the model that BRD4S is necessary to recruit the repressive SWI/SNF chromatin-remodeling complex to the latent HIV promoter.

**Genome-wide co-occupancy of BRD4 and BRG1**

To extend our findings beyond the HIV promoter, we performed high-throughput sequencing of ChIP DNA recovered using N-terminal BRD4 (recognizing short and long isoforms) and BRG1 antibodies. These experiments revealed a quantitative relationship between BRD4 and BRG1 tag densities at all peaks identified (Pearson’s r=0.66; Figure 6-6A). Both BRD4 and BRG1 occupancies were diminished upon JQ1 treatment, with occupancies of BRD4 more significantly shifted than for BRG1 after drug treatment (Figure 6-6B, C). We subsampled BRD4 peaks to determine the percentage overlap with BRG1-bound peaks, and
Figure 6-5 – Short BRD4 recruits BRG1 to the latent HIV provirus. A-B – ChIP-qPCR analysis with BRD4 N (A) and BRG1 (B) antibodies at two positions downstream of the HIV TSS from J-Lat A72 cells treated with DMSO or 625 nM JQ1 for 18h. Nonspecific IgG values have been subtracted from displayed data. Average of three biological replicates (± SEM) is shown. C-D – ChIP-qPCR analysis with BRD4 N (C) or BRG1 (D) antibodies at nt +155 from J-Lat A72 cells transduced with scramble shRNA or shRNAs targeting either the N- or C-terminus of BRD4, as in Figure 6-1. Average of three biological replicates (± SEM) is shown. E – Western blotting of BRD4 to assess knockdown efficiency in J-Lat A72 cells.
Figure 6-6 – BRD4 and BRG1 exhibit genome-wide co-binding that is disrupted upon BET inhibition. A – Correlation of tag densities between N-terminal BRD4 and BRG1 across all peak regions. Red line is the line of best-fit. B, C – Tag density (DMSO) vs. fold change in ChIP signal between JQ1 (625 nM) and DMSO treatments for N-terminal BRD4 (B) and BRG1 (C). Red line represents the moving average. D – Percentage overlap of subsampled BRD4 peaks and BRG1 peaks ±625nM JQ1 shown for two replicates.
found that >80% of BRD4 peaks overlapped a BRG1 peak (Figure 6-6D). Upon JQ1 treatment, the percentage overlap between N-terminal BRD4 and BRG1 reduced to 20–40% (Figure 6-6D), demonstrating that JQ1 treatment significantly disrupts the overlap between BRD4 and BRG1. These results establish that BRD4 and BRG1 occupancies are correlated genome-wide, and that roughly half of BRD4/BRG1 co-binding is disrupted under BET inhibition.

We next performed unsupervised non-hierarchical clustering of all genomic regions on the basis of ChIP signal obtained using each antibody. We also included ChIP-seq data for Pol II and ATAC-seq data (Figure 6-2H) obtained under the same experimental conditions. This analysis revealed 9 distinct clusters, each partitioned by unique occupancy-accessibility profiles (Figure 6-7A). We annotated elements in each cluster for genomic feature, and calculated z-scores through permutation testing to quantify deviations from the average genomic distribution of each feature per cluster (Figure 6-7B).

Clusters self-segregated into two major classes based upon genomic feature (Figure 6-7B, dendrograms). Clusters 4–7 were enriched for coding sequences, containing promoter, exon, and transcription termination site sequences, while clusters 1-3, 8, and 9 were enriched for non-coding sequences containing intergenic, intronic, and class I (LINEs, SINEs, & LTRs) and class II (DNA) transposon sequences. Significant BRD4:BRG1 co-binding was observed at both coding (i.e., clusters 6 and 7) and non-coding elements (i.e., clusters 1 and 2), establishing that BRD4:BRG1 co-occupancy is not restricted to genes. Notably, clusters 1 and 2 harbor multiple endogenous LTR-containing sequences evolutionarily related to HIV-1.

To further interrogate the response of endogenous LTR sequences to BET inhibition, we performed average profiling of all focal LTR peaks defined in our combined ChIP-/ATAC-seq analyses and found striking similarities between these sequences and the latent HIV-1 LTR. Firstly, occupancies of BRD4 and BRG1 were diminished under JQ1 treatment (Figure 6-7C), as with the latent HIV promoter (Figure 6-5). Furthermore, polymerase occupancy was unchanged, if not modestly increased, similar to what we found at the HIV LTR (Figure 6-S4). Lastly, transposition density decreased over endogenous LTR sequences (Figure 6-7C), as we had observed with latent HIV-1 chromatin (Figure 6-2H). Collectively, these data identify repressive functions of BRD4:BRG1 complexes at ancestral LTR sequences, pointing to a broader role in transcriptional silencing of retroviral promoters.

**Discussion**

Here we report a novel functional and physical interaction between the short isoform of BRD4 and SWI/SNF complexes that is involved in maintaining HIV latency. Our data indicate
Figure 6-7 – BRD4:BRG1 co-binding across genomic features and endogenous LTR sequences. A– Heatmap corresponding to binned ChIP/ATAC signal across the genome with clusters displayed on the left. B – Heatmap of z-scores (scale range: -10 to +10) displaying enrichments of genomic features across all clusters. SVA – SINE/VNTR/Alu, SR – simple repeat, DNA_t – DNA transposons. C – Average profiles of ChIP/ATAC signal across LTR peaks (n=334). D – Schematic of model for JQ1 action at the latent HIV-1 promoter.
that BRD4S is a relevant target of the BET inhibitor JQ1 in reversing HIV latency, operating via relief of a BRG1-containing BAF complex and subsequent nucleosome remodeling to facilitate transcription. We propose that BRD4 exerts isoform-specific functions at the latent HIV promoter: while BRD4L can transactivate HIV-1 transcription via its known interaction with P-TEFb, BRD4S functions as a new corepressor in the context of viral latency by tethering a repressive SWI/SNF BAF chromatin-remodeling complex to the viral promoter (Figure 6-7D).

Cooperation between BET proteins and SWI/SNF members has been previously reported in the literature, both via physical (Denis et al., 2006; Shi et al., 2013) and functional (Liu et al., 2014) interactions. BRD2, another BET family member, was previously shown to interact with BRG1 and BAF155 (Denis et al., 2006). We extend these findings by showing that BRD4S also interacts with SWI/SNF proteins in T cells. Functionally, BRD4 was shown to recruit BRG1 to the Nanog locus in mouse embryonic stem cells to activate Nanog production (Liu et al., 2014). Vakoc and colleagues reported that BRD4 and BRG1 regulate a common set of genes but did not find a significant effect of JQ1 on BRG1 occupancy or an effect of BRG1 knockdown on BRD4 occupancy in this gene set (Shi et al., 2013).

These last results differ from our findings at the latent HIV promoter, where BRD4S is required to maintain BRG1 occupancy. This is likely due to unique aspects of retroviral versus cellular gene regulation, as we show that HIV LTR chromatin phenotypically resembles endogenous LTR elements and not coding sequences. Similar to our findings, Vakoc and colleagues find BRD4S to be sufficient to immunoprecipitate BRG1. However, only the ET domain was necessary for mediating this interaction, while in our study bromo- and ET domains independently engaged BRG1. Importantly, our cell lysates included chromatin extracts, and we speculate that interactions between the BRD4 bromodomains and BRG1 might require chromatin-engaged factors, thus offering a possible explanation for these differing findings.

An important question that remains unanswered is how BRD4S is recruited to latent HIV chromatin. An interesting but also puzzling candidate is histone H3K27, which when acetylated binds the first bromodomain of BRD4 (Filippakopoulos et al., 2012) and co-localizes genome-wide with BRD4 in human CD4+ T cells (Zhang et al., 2012). H3K27 is also trimethylated by Polycomb complexes, and H3K27, when unmethylated, enhanced the response of latent HIV to JQ1-mediated activation (Tripathy et al., 2015). Polycomb activities are classically antagonized by Trithorax chromatin modifiers, which include SWI/SNF chromatin remodelers as well as the Drosophila homolog of BRD4 and the only fly BET protein, fs(1)h (Digan et al., 1986). Genomic LTR sequences are known Polycomb targets (Ishak et al., 2016), and Polycomb is required to prevent endogenous murine leukemia virus element mobilization (Leeb et al., 2010). How
BRD4S and SWI/SNF complexes act in concert with other silencing mechanisms such as Polycomb to transcriptionally restrict parasitic retroviral elements is the focus of future studies.

Our current studies underscore the critical role of chromatin in regulating the transcriptional activity of the integrated HIV provirus and other LTR-containing sequences. Previous MNase digestion experiments showed nuc-1 encompassing nt -2 to +142 relative to the HIV TSS (Verdin et al., 1993), which was recapitulated in analogous experiments in vitro using pre-assembled nucleosome arrays (Pazin et al., 1996; Steger et al., 1998). Using DNase I coupled to LM-PCR, we find a protected site corresponding to nuc-1 to be positioned at nt -64 to +82 (Figure 6-2G, DMSO curve), but the ATAC-seq data position nuc-1 close to the original position at nt +12 to +160. Differences in nuc-1 positioning can be attributed to documented sequence specificities for both DNase I (Koohy et al., 2013) and the Tn5 transposase (Madrigal, 2015), although we cannot formally exclude other technical differences such as PCR bias or sequencing methods.

Our data highlight SWI/SNF complexes as critical downstream targets of BET inhibitor action in the reversal of HIV-1 latency, and this finding has clinical implications. BET inhibitors show variable results in reactivating latent virus in primary T cell models of HIV latency (Boehm et al., 2013a) and in patient cells (Laird et al., 2015). We speculate that heterogeneity of SWI/SNF complexes may explain these differential responses, as the composition of SWI/SNF complexes is highly variegated and modulated by cell identity, developmental contexts, and disease states (Kadoch and Crabtree, 2015). We further derive from our data the risk to transcriptionally reawaken endogenous retroviral sequences with BET inhibitor treatment, although multiple posttranscriptional restriction mechanisms likely remain in place to curb gene expression from these fossilized sequences (Yang and Kazazian, 2006). Future work is aimed at defining SWI/SNF complex compositions in primary human T cell subsets and the transcriptional activity of endogenous LTR sequences in response to BET inhibition.

Author contributions
R.J.C. performed the experiments with help from P.F., R.J.C. and S.T. analyzed the data, H.S. first raised the idea of BRD4:BRG1 interactions, M.O. supervised, guided, and funded the research, R.J.C. and M.O. wrote the paper, with input from P.F. and S.T.

Acknowledgements
We thank all members of the Ott laboratory for sharing reagents and advice throughout the preparation of this manuscript. We also thank Eric Verdin, Danica Galonic Fujimori, and
Hiten Madhani for guidance throughout the course of this work. We thank Veronica Fonseca and John Carroll for administrative and graphical support, respectively. We gratefully acknowledge support from the NIH (R01 AI083139 and U19 AI096113 to M.O.). R.J.C. was supported in part by NIH Training Grant T32 GM007175. This publication was made possible with help from the University of California, San Francisco-Gladstone Institute of Virology & Immunology Center for AIDS Research (CFAR), an NIH-funded program (P30 AI027763).
References


Experimental Procedures

Cell fractionation and western blotting

Exponentially growing J-Lat cells, obtained from Dr. Eric Verdin (The Buck Institute for Research on Aging, Novato, CA) were seeded at a density of 500,000 cells/mL and treated with indicated concentrations of JQ1 (Tocris) for a specified times. Cell fractionation was performed via the Dignam & Roeder method (Carey et al., 2009a), with minor modifications. For further details, please refer to Supplemental Experimental Procedures.

The following antibodies were used for Western blotting: BRD4 N (Abcam, ab128874), BRD4 C (Bethyl, A301-985A100), Sp1 (Santa Cruz, H-225, sc-14027), Cyclin T1 (Thermo, PA1-31486), BRG1 (Abcam, ab110641), BRM (Abcam, ab15597), BAF250a (Santa Cruz, PSG3, sc-32761), BAF180 (Millipore, ABE70), Tubulin (Sigma, SAB3501071), and FLAG (Sigma, F7425).

ShRNA-mediated knockdown experiments

Knockdown experiments were performed essentially as described (Boehm et al., 2013a). For shRNA TRC numbers, see Supplemental Experimental Procedures.

Flow cytometry

All flow cytometry experiments were performed using the IntelliCyt HTFC with samples in each experiment analyzed in at least triplicate. Cell viability was estimated using forward and side scatter parameters. Roughly 5-20,000 live cells were analyzed per condition, gated such that ~1% of control samples were assigned as GFP⁺.

RNA isolation, reverse transcription, and qPCR

RNA for qPCR experiments was isolated from approximately 10⁶ J-Lat cells via RNA STAT-60 (AMSBIO) and Zymo-Spin IIC columns (Zymo Research) including on-column DNase I digestion. Isolated RNA primed with random hexamers (Thermo) was reverse transcribed using the AMV reverse transcriptase (Promega) according to manufacturer’s instructions. cDNA was analyzed via the 7500 Fast Real-Time PCR system (Applied Biosystems).

Accessibility of nucleosomal DNA to restriction enzymes

Enzymatic digestion of chromatin was performed essentially as described (Verdin et al., 1993). AflII accessibility was defined as (Cₜ,digested-Cₜ,undigested)nuclei/(Cₜ,digested-Cₜ,undigested)naked DNA, with Cₜ as the real-time qPCR threshold cycle number. For ligation-mediated PCR, a standardized protocol (Carey et al., 2009b) was used with several adaptations, notably to
amend the technique to fragment analysis using the 3730xl DNA Analyzer (Applied Biosystems, UC Berkeley DNA Sequencing Facility). Please refer to Supplemental Experimental Procedures for details.

**Immunoprecipitations**

Detailed experimental procedures for protein and chromatin immunoprecipitations are provided in Supplemental Experimental Procedures.

**ChIP-seq**

1–2ng of ChIP DNA was processed using the Ovation Ultralow Library Systems V2 (NuGEN) before quality control of libraries using BioAnalyzer 2100 (Agilent) and KAPA quantification. ChIP samples were sequenced on the HiSeq 2500 (Illumina) at the UCSF Institute for Human Genetics under the PE100 protocol. For mapping statistics, please see Table 6-S1.

**ATAC-sequencing**

ATAC-seq was performed essentially as described (Buenrostro et al., 2013), with the exception of outer membrane lysis prior to transposition. This step was removed from the original protocol to reduce contamination from mitochondrial DNA. Libraries were assessed for quality control on the BioAnalyzer 2100 (Aglient) to ensure nucleosomal phasing and complexity. Sequencing was performed on the HiSeq 2500 (Illumina) at the UCSF Institute for Human Genetics under the PE100 protocol. For mapping statistics, please see Table 6-S1.

**Bioinformatic analysis**

All reads were trimmed for adapters and quality using the fastq-mcf tool. Reads were then aligned to the hg19 human genome assembly using bowtie2 (Langmead and Salzberg, 2012). Tags that did not map to the genome uniquely (mapq≥30) were not kept. Separately, all data was aligned to the A72 HIV integrant (sequenced via primer walking, available upon request) without removing non-uniquely mapping reads in order to allow alignment to homologous LTR regions. Peaking calling was performed via thresholding methods. For further details, please refer to Supplemental Experimental Procedures.
Supplemental Experimental Procedures  
*Cell fractionation and western blotting*

Cells were sedimented at 1000g for 5min at 4°C and washed with ice cold PBS. Pelleted cells were resuspended in 5 volumes of DR Buffer A (10mM HEPES-KOH, 10mM KCl, 1.5mM MgCl$_2$, 0.5mM DTT, supplemented with Halt) and incubated on ice for 10min. Cell suspensions were homogenized with 10 strokes of a Dounce tight pestle (Wheaton), and nuclei were pelleted at 10000rpm for 20min, 4°C. Supernatants were decanted, the resulting pellet was gently resuspended in DR Buffer C (20mM HEPES, 0.42M NaCl, 1.5mM MgCl$_2$, 0.2mM EDTA, 25% w/v glycerol, 0.5mM DTT, 0.5mM PMSF, 1X Halt) at ~3x10$^9$ cells/mL and rotated for 1h at 4°C. The suspension was centrifuged at 10000rpm for 30min at 4°C, and supernatants were collected as the nuclear fraction. The remaining insoluble pelleted was resuspended in digestion buffer (10mM Tris-HCl pH=7.6, 10mM MgCl$_2$, 50mM NaCl, 5mM CaCl$_2$, 1mM DTT, 100µg/mL BSA, 0.1mM PMSF, 1X Halt) and incubated with MNase (60U/µL, NEB) for 20 minutes at room temperature. Samples were briefly spun down and sonicated (Cole Parmer Ultrasonic Processor) with 40% amplitude, pulsing 10s on, 2s off, for a total of 10 pulses, two times spinning down briefly in between sonication steps. Samples were spun down at maximum speed for 5 minutes at 4°C, with the resulting supernatant collected as the chromatin fraction. Protein concentrations of nuclear and chromatin fractions were determined using a Bradford assay (BioRad), and normalized among samples per experiment before analysis via Western blotting using standard techniques. Band intensities were quantified using ImageJ.

*Plasmids*

The majority of BRD4 plasmids were generous gifts from Dr. Eric Verdin (Buck Institute). The BDS, IDS, and ET constructs (Figure 6-4D) were cloned into pFLAG-CMV-2 (Sigma) via standard restriction enzyme-dependent methods using primers listed in Table 6-S2. SV40 NLS sequences were inserted using a linker. Sequences of all plasmids are available upon request.

*ShRNA-mediated knockdown experiments*

The following shRNAs were obtained from Sigma: BRD4 #1 (BRD4L) – TRCN0000021424, BRD4 #2 (BRD4S + L) – TRCN0000021427, BRG1 #1 – TRCN0000231099, BRG1 #2 – TRCN0000380723, BRM #1 – TRCN0000330380, BRM #2 – TRCN0000330445, BAF250a #1 – TRCN0000059091, BAF250a #2 – TRCN0000358749, BAF180 #1 – TRCN000015994, BAF180 #2 – TRCN0000235890, CHD1 – TRCN0000359159, CHD3 –
TRCN0000382066, CHD4 – TRCN0000021359, SMARCA1 – TRCN0000303644, INO80 – TRCN0000365461, SRCAP – TRCN0000281131.

Accessibility of nucleosomal DNA to restriction enzymes

3-5x10^6 J-Lat cells (per condition) were spun down at 1000g for 5 minutes at 4°C and washed once with ice cold PBS. Cells were resuspended at 2.5x10^6 cells/mL in buffer A (10mM Tris-HCl pH=7.6, 10mM NaCl, 3mM MgCl₂, 0.3M sucrose, 0.3mM spermidine, 1X Halt) and incubated on ice for 5min. An equal volume of buffer A supplemented with 0.2% NP-40 (Surfact Amps, Thermo) was added and suspensions were incubated on ice for 5min. Nuclei were pelleted by centrifugation at 300g for 5min at 4°C, and resuspended at 1x10^8 nuclei/mL in either buffer B (for DNase digestion: 10mM Tris-HCl pH=7.6, 10mM MgCl₂, 1mM CaCl₂, 50mM NaCl, 1mM DTT, 100µg/mL BSA, 0.1mM PMSF, 1X Halt) or 1X CutSmart (for AflII digestion, NEB). Reactions were stopped with the addition of 2mM EDTA and 1% SDS, upon which proteinase K was added at a concentration of 400µg/mL and incubated at 55°C overnight. RNase was added at a concentration of 100µg/mL and incubated at 37°C for at least 1h. DNA was purified via QIAquick PCR purification kit (Qiagen).

To control AflII experiments, naked genomic DNA was incubated either with or without AflII and digested to completion for 20min at 37°C and heat inactivated at 65°C for 20mins. 10-80ng of DNA, normalized among samples, was subjected to qPCR using the 7500 Fast Real-Time PCR system (Applied Biosystems).

For DNase I-treated DNA, first strand synthesis was performed on 1µg of control or in vivo digested DNA, using Pfu II Ultra. Pre-annealed, unidirectional linkers were ligated to DNA overnight at room temperature using T4 DNA ligase (NEB). Ligated DNA was precipitated and amplified using a linker primer and a primer specific for the 5'-LTR. NEBNext High Fidelity 2X PCR MasterMix (NEB) and the following cycling conditions were used for amplification: 98°C 2min, 98°C 30s, 58°C 2min, 72°C 2min, steps 2-4 repeated 29x, 72°C 5 min, 4°C hold. A 6-FAM labeled probe was added and reactions were cycled for an additional three rounds of amplification: 98°C 2min, 98°C 1min, 62°C 2min, 72°C 10min, steps 2-4 3x, 4°C hold. Amplified, labeled DNA was precipitated and resuspended in 30µL HiDi Formamide. 1-5µL of DNA was mixed with 0.35µL GeneScan 1200 LIZ and analyzed on the 3730xl DNA Analyzer. Amplicon peaks were called using PeakScanner, and data was imported to GraphPad Prism for visualization and analysis by LOWESS regression.
**Immunoprecipitations**

Immunoprecipitation was performed on nuclear/chromatin extracts from either J-Lat A72 (for endogenous complexes) or 293T cells (for domain mapping). Roughly $1.8 \times 10^7$ 293T cells were transfected with 20µg of the indicated FLAG-BRD4 construct using PolyJet (Signagen) and harvested 48-72h post transfection. For endogenous IPs from J-Lat cells, roughly $2 \times 10^7$ cells were used per extraction.

Nuclear/chromatin extracts were prepared exactly as described for cell fractionation (see above), except upon resuspension in DR Buffer C, extracts were directly sonicated and cleared. 1-2mg of extract was diluted in FLAG-IP buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% Triton-X-100, and 1X Halt) and ~2-4µg of indicated antibody was added (BRD4 N (Abcam, ab128874), FLAG (Sigma, M2, F1804)). Dynabeads Protein A/G (Thermo, 50µL/IP) were washed in FLAG-IP buffer, added to samples, and rotated overnight at 4°C. IPs were washed 3x with FLAG-IP buffer before elution in 2X Laemmli buffer and processing via Western blotting.

**Chromatin immunoprecipitation**

Exponentially growing J-Lat A72 cells (~$3 \times 10^7$ cells/IP) were fixed with 1% formaldehyde (Sigma-Aldrich) for 20 minutes at room temperature. Cross-linking was quenched with 125mM glycine for 5 minutes, after which cells were sedimented at 1000rpm for 5 minutes at 4°C and washed 3x with ice-cold PBS. Outer membranes were lysed with Lysis Solution #1 (5mM PIPES pH 8.0, 85mM KCl, 0.5% Surfact Amps NP-40, 1X Halt) for 15min on ice and nuclei were spun down at 1000rpm. Supernatants were discarded, and the resulting pellet was washed once in MNase digestion buffer (10mM Tris-HCl pH=7.6, 10mM MgCl$_2$, 50mM NaCl, 5mM CaCl$_2$, 1mM DTT, 100µg/mL BSA, 1X Halt) and resuspended in the same buffer supplemented with 60U/µL MNase (NEB). Digestion reactions were rocked at room temperature for 20 minutes, stopped with 2mM EDTA, and centrifuged at 10000rpm for 5 minutes. Supernatants were discarded and the pellet was washed in Lysis Solution #2 (0.1% SDS, 10mM EDTA, 50mM Tris-HCl pH=8.0, 1X Halt). Samples were sonicated (Cole Parmer Ultrasonic Processer) with 40% amplitude, pulsing 10s on, 2s off, for a total of 10 pulses, two times spinning down briefly in between sonication steps. Samples were spun down for 5 minutes at 4°C, with the resulting supernatant collected as the chromatin fraction.

A small fraction (1/30) of isolated chromatin was used for quantification via reverse cross-linking with ~600mM NaCl, boiling, RNase-treatment, and DNA purification via QIAQuick PCR purification kit (Qiagen). Purified DNA was quantified and used to estimate chromatin concentration. DNA was also analyzed via agarose gel electrophoresis to ensure proper
shearing of DNA to 200-1000bp. Chromatin concentrations were normalized among samples and pre-cleared with Protein A/G Dynabeads (Thermo, 20µL/IP). Approximately 30-100µg of chromatin was used for each IP, diluted in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, 167mM NaCl, 1X Halt). Antibodies were added (BRD4 N (Abcam, ab128874): 3µg, BRD4 C (Bethyl, A301-985A100): 4µg, BRG1 (Abcam, ab110641): 0.3µg, Pol II (Santa Cruz, N-20, sc-899): 4µg), along with the equivalent of 50µL of Protein A/G Dynabeads washed in ChIP dilution buffer. Immunoprecipitations were carried out overnight at 4°C and washed as follows: 3x in low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 150mM NaCl), 1x in high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH=8.0, 500mM NaCl), 1x in LiCl buffer (0.25M LiCl, 1% NP-40, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris-HCl pH=8.0), and 1x in TE buffer. DNA was eluted from the beads 2x with elusion buffer (50mM NaHCO₃, 1%(w/v) SDS) at 65°C with shaking. NaCl was added to a final concentration of ~600mM and samples were reverse cross-linked overnight at 65°C. 10µg RNase A (Thermo) was added and samples were incubated for 20 minutes at 37°C, before purification of ChIP DNA on QIAquick columns. For qPCR experiments, ChIP DNA was analyzed using the 7500 Fast Real-Time PCR system (Applied Biosystems).

Bioinformatic analysis

To calculate tag density measurements for ATAC-seq and ChIP-seq data, reads were first processed in an experiment-specific manner, with the center point of each ChIP-seq fragment location being taken as the most likely position of factor binding, and ATAC-seq reads being shifted by 4bp from the edge of the fragment to more precisely indicate the location of transposition. To later estimate nucleosome occupancy, ATAC-seq fragments longer than the span of a nucleosome were kept and the center point of each fragment retained as the most likely position of a nucleosome for that fragment. After sample-specific processing of tags, tag densities were calculated. The genome was divided into 20bp bins and the number of tags that mapped to within 75bp of each 20bp bin was used as the tag count (tc) for each bin. Tag density was calculated using those counts as follows:

\[
\text{tagDensity} = \text{tc} \times \#\text{binsInGenome} / \#\text{tagsTotal}
\]

and for ChIP data, input normalization was calculated for each bin as:

\[
\text{normalizedDensity} = \text{tagDensity(chip)} - \text{tagdensity(input)}
\]
Peaks were called for ChIP-seq and ATAC-seq by thresholding and merging bins with tag densities greater than 60. Replicate concordant peaks were identified as peaks that were observed in multiple replicates.

A region of the same size as each peak was taken from 10kb upstream of each peak to serve as a background region. The median peak score across all backgrounds regions for each sample was used to normalize the tag density values to correct for varying signal-to-noise ratios between experiments. All subsequent peak-based analyses were performed using the maximum normalized density score at each peak for each sample. Clustering of peaks was performed by first performing k-means clustering with 100 clusters, and then using hopach to collapse similar clusters. Peak overlap analysis was performed by sampling without replacement 1000 of the replicate concordant peaks and then observing the distribution of percent overlap with other factors. Genome feature enrichment was calculated by a permutation analysis in which the feature associations were shuffled 1000 times and the fraction of each peak cluster observed to map to each feature was observed. The mean and standard deviation these random associations served to model the significance of the enrichment of each peak cluster with each genomic feature.
Figure 6-S1 – Targeted screen of chromatin remodeling ATPases reveals BRG1 as necessary for JQ1-mediated reversal of HIV latency – Flow cytometry measuring HIV reactivation in the presence 625nM JQ1 for 18h in J-Lat A72 cells transduced with shRNAs targeting indicated factors (top). Knockdown was assessed via Western blotting for BRG1 (Figure 6-3), BRM (Figure 6-3), and for SMARCA1 (Abcam, ab37003, bottom left), and via qPCR for the remaining ATPases (bottom right). Screen flow cytometry data represents the average of three independent experiments ±SEM, while knockdown assessment is representative of three experiments.
Figure 6-S2 – BRD4S immunoprecipitates BRG1 more efficiently than BRD4L – Western blotting of immunoprecipitated FLAG-tagged BRD4 proteins and endogenous BRG1 from transfected 293T nuclear/chromatin extracts.
Figure 6-S3 – JQ1 minimally induces Pol II recruitment at the HIV promoter — Pol II ChIP-qPCR data from J-Lat A72 cells treated with DMSO or 625nM for 18h. X-axis numbers represent nt distance relative to the HIV-1 TSS. Data are the mean of three independent experiments normalized to non-specific IgG values ±SEM.
Figure 6-S4 – BET inhibition and BRD4 knockdown do not alter BRG1 protein levels – A — Western blotting of J-Lat A72 nuclear/chromatin extracts from cells treated with DMSO or 625nM for 18h. Bottom histogram represents blot quantifications from four independent experiments ±SEM. B — Western blotting of J-Lat A72 nuclear/chromatin extracts from cells transduced with BRD4 shRNAs (see Figure 6-5C-E) Bottom histogram represents blot quantifications from two biological and two technical replicates ± SEM.
<table>
<thead>
<tr>
<th>Sample_name</th>
<th>Num_total_tags</th>
<th>Num_mapped_tags</th>
<th>Final_mapped_tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO_BRD4.1</td>
<td>39787057</td>
<td>38402467</td>
<td>29858129</td>
</tr>
<tr>
<td>DMSO_BRD4.2</td>
<td>35118886</td>
<td>34290080</td>
<td>28113934.5</td>
</tr>
<tr>
<td>DMSO_BRD4.3</td>
<td>47073281</td>
<td>43519248</td>
<td>31577568.5</td>
</tr>
<tr>
<td>DMSO_BRG1.1</td>
<td>37494392</td>
<td>35522186</td>
<td>26321023.5</td>
</tr>
<tr>
<td>DMSO_BRG1.2</td>
<td>35160019</td>
<td>32726945</td>
<td>23176423.5</td>
</tr>
<tr>
<td>DMSO_ATAC.1</td>
<td>70258268</td>
<td>67469014</td>
<td>37504792</td>
</tr>
<tr>
<td>DMSO_input.1</td>
<td>30240996</td>
<td>25798593</td>
<td>14608789</td>
</tr>
<tr>
<td>DMSO_input.2</td>
<td>35835457</td>
<td>29793598</td>
<td>12724158</td>
</tr>
<tr>
<td>DMSO_input.3</td>
<td>32765315</td>
<td>26815133</td>
<td>22095675</td>
</tr>
<tr>
<td>DMSO_polII.1</td>
<td>52937018</td>
<td>43868906</td>
<td>47836714</td>
</tr>
<tr>
<td>DMSO_polII.2</td>
<td>39917419</td>
<td>38652036</td>
<td>30106794</td>
</tr>
<tr>
<td>JQ1_BRD4.1</td>
<td>41040011</td>
<td>39262978</td>
<td>30221687.5</td>
</tr>
<tr>
<td>JQ1_BRD4.2</td>
<td>30768417</td>
<td>25107028</td>
<td>27082278</td>
</tr>
<tr>
<td>JQ1_BRD4.3</td>
<td>51920881</td>
<td>41604201</td>
<td>47781928</td>
</tr>
<tr>
<td>JQ1_BRG1.1</td>
<td>45587840</td>
<td>44010500</td>
<td>33391015.5</td>
</tr>
<tr>
<td>JQ1_BRG1.2</td>
<td>46728098</td>
<td>45461766</td>
<td>35484389</td>
</tr>
<tr>
<td>JQ1_ATAC.1</td>
<td>100675510</td>
<td>96900178</td>
<td>88931468</td>
</tr>
<tr>
<td>JQ1_input.1</td>
<td>32474538</td>
<td>27561140</td>
<td>18436492</td>
</tr>
<tr>
<td>JQ1_input.2</td>
<td>39716940</td>
<td>33755427</td>
<td>22250918</td>
</tr>
<tr>
<td>JQ1_input.3</td>
<td>47759352</td>
<td>45997031</td>
<td>33876990</td>
</tr>
<tr>
<td>JQ1_polII.1</td>
<td>49831921</td>
<td>48331980</td>
<td>36514763.5</td>
</tr>
</tbody>
</table>

Table 6-S1 – Mapping statistics for high throughput sequencing experiments.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Application</th>
<th>Forward (5' to 3')</th>
<th>Reverse (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AflII_HIV</td>
<td>AflII accessibility</td>
<td>TGGGAGCTCTCTCT GTGTAATCT</td>
<td>CTGGTTTCCTTTT CGATTTC</td>
</tr>
<tr>
<td>LM-PCR_A</td>
<td>LM-PCR 1st strand</td>
<td>-</td>
<td>ATTTTTGGGCTAC TCACCGTC</td>
</tr>
<tr>
<td></td>
<td>synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM-PCR_linkers</td>
<td>LM-PCR ligation</td>
<td>GCGGTGACCAGGG AGATCTGAGTC</td>
<td>GAATTCAGATCG</td>
</tr>
<tr>
<td>LM-PCR_B</td>
<td>LM-PCR amplification</td>
<td>GCGGTGACCAGGG AGATCTGAGTC</td>
<td>ATTTTTGGGCTAC TCACCGTC</td>
</tr>
<tr>
<td>LM-PCR_C</td>
<td>LM-PCR labeling</td>
<td>-</td>
<td>TACTCACCAGCTG CCGCCCCCTGCGG TCTTG</td>
</tr>
<tr>
<td>HIV_ChIP_155</td>
<td>ChIP-qPCR</td>
<td>AGTGTGCTGCCCAG TCTGTGGT</td>
<td>TTCGCTTTTACAGGT CCGGCTGGT</td>
</tr>
<tr>
<td>HIV_ChIP_489</td>
<td>ChIP-qPCR</td>
<td>GCAAGCAGGGAG CTAGAAGCG</td>
<td>GGATGGTGGTGA TAGCTGCCAGG</td>
</tr>
<tr>
<td>HIV_GFP</td>
<td>RT-qPCR</td>
<td>ATGGTGAGCAAG GGGAGAGGAG</td>
<td>GTGGTGTCAGATT AACTTCAG</td>
</tr>
<tr>
<td>RPL13A</td>
<td>RT-qPCR</td>
<td>GCCCTACGACAA GAAAAAGCG</td>
<td>TACTTCCAGCCAA CCTGCAGA</td>
</tr>
<tr>
<td>CHD1</td>
<td>RT-qPCR</td>
<td>AAACATGATGG AGAATCAGACCC</td>
<td>AGAATCGAACC AGATCCAGAG</td>
</tr>
<tr>
<td>CHD3</td>
<td>RT-qPCR</td>
<td>CCGTCAGCATGG GGTGTAAG</td>
<td>TCTTGGTTCCTTC GGGTTTTC</td>
</tr>
<tr>
<td>CHD4</td>
<td>RT-qPCR</td>
<td>GGAGCTCAATTC ATCTGGTCTCG</td>
<td>GTGAGGGTTGGA TAATCCCTCCT</td>
</tr>
<tr>
<td>INO80</td>
<td>RT-qPCR</td>
<td>TGGAGCACAGCT GAGCTATCT</td>
<td>CTGGGGCAATAA TGGATTACGT</td>
</tr>
<tr>
<td>SRCAP</td>
<td>RT-qPCR</td>
<td>AAATGGCCGCTAT GCTTATCTGG</td>
<td>CTGGTCGTAAGAG GAGATTC</td>
</tr>
<tr>
<td>BDS</td>
<td>Cloning</td>
<td>CGACAAAGGCTTCC CCCAGAGACCTC</td>
<td>GACGGATCCCTA CTCAGGCTCGTC</td>
</tr>
<tr>
<td>IDS</td>
<td>Cloning</td>
<td>CGACAAAGGCTTATG CCGGAGCAGCTCCG</td>
<td>GACGGATCCCTA CTTGCACCTTTGTCGTC</td>
</tr>
<tr>
<td>ET</td>
<td>Cloning</td>
<td>CGACAAAGGCTTTCC GGAGGAGAGGAGCAAG</td>
<td>GACGGATCCCTA TTGAGGTTTTCCTTTTCT</td>
</tr>
<tr>
<td>SV40_NLS_linker</td>
<td>Cloning</td>
<td>GACAGGCTTCCAA AGAAGAAGAGCGA AGGTGCTAGCTTG</td>
<td>ATCAAGCTTGACC TCCGGTTTCTCTTTGGAAGCTTGTC</td>
</tr>
</tbody>
</table>

Table 6-S2 – PCR primers used
Chapter VII

BRD4 is Subject to Reversible Acetylation

Gene expression cascades critical to cellular function converge on chromatin, where histone acetylation has emerged as an important modification controlling the access of the transcriptional machinery to DNA. Apart from effecting direct physiochemical changes in the nucleosome core, histone acetylation simultaneously generates binding platforms for the bromodomain, a specialized protein module that recognizes and binds acetyl-lysine residues. Many transcriptional regulators, nucleosome remodelers, and chromatin-modifying enzymes encode bromodomains, and it is postulated that these domains are in part responsible for executing the functional consequences linked to protein acetylation. Among the 46 human bromodomain proteins, the bromodomain and extraterminal domain (BET) subfamily has been classified based on a unique domain organization of twin bromodomains and an extraterminal domain, the latter of which mediates protein:protein interactions. A well characterized BET protein is the bromodomain-containing protein 4 (BRD4), a ~200 kDa transcriptional regulator whose significance and therapeutic tractability have recently been underscored in preclinical successes using BET inhibitors for the treatment of select cancers, inflammatory diseases, and viral infections.

Although shown to have histone chaperone, kinase, and acetyltransferase activities, BRD4 is thought to globally control transcript elongation predominantly via the positive transcription elongation factor b (P-TEFb). BRD4 selectively associates with the active form of P-TEFb, a heterodimeric transcription elongation factor composed of cyclin T1 and the cyclin-dependent kinase 9 (CDK9). Kinase activity of CDK9 renders RNA polymerase II (Pol II) elongation competent primarily through the relief of negative elongation factors, but also increases Pol II enzyme processivity. Active P-TEFb is required for the majority Pol II-mediated transcription, supporting the hypothesis that elongation is a major rate-limiting phase of the mammalian transcription cycle.

While the body of knowledge on BRD4 is expanding, the field currently lacks an understanding of the modes of BRD4 regulation. Posttranslational modifications have been previously shown to regulate BRD4 function. N-terminal phosphorylation events enhance BRD4 chromatin binding and its interaction with transcription factors, notably p53 and the human papillomavirus transcription factor E2 (Wu et al., 2013; Wu et al., 2016). In this way, BRD4 phosphorylation is hypothesized to confer specificity in chromatin targeting of this factor. Large-scale proteomics studies have reported acetyl-lysine residues within BRD4 (Choudhary et al.,
2009), and BRD4 has been shown to auto-acetylate \textit{in vitro} (Devaiah et al., 2016). Confirmation, mapping, and functional characterization of BRD4 acetylation have not been reported.

Our efforts to address this have identified that the BRD4 long isoform is subject to acetylation (Figure 7-1). We overexpressed various FLAG-tagged BRD4 truncation mutants in 293T cells, pulsed cells with sorbitol (a potent inducer of acetylation, Sebastian Schröder, unpublished observations), and immunoprecipitated BRD4 proteins. We monitored BRD4 acetylation using pan-acetyl-lysine antibodies. These experiments revealed a BRD4 region between aa 882-1260 as containing acetylation-competent lysine residues. Importantly, we did not detect acetylation of the short BRD4 isoform (aa 1-722). We also observed that deletion of the extreme C-terminus (Δ1209-1362) enhanced BRD4 acetylation, even in the absence of sorbitol. Importantly, this region contains the P-TEFb interaction domain (PID) that is critical for BRD4 to promote transcript elongation.

We interrogated the enzymes responsible for catalyzing acetylation and deacetylation of BRD4. To this end, we overexpressed full length BRD4 in the presence of several acetyltransferases and monitored BRD4 acetylation via immunoprecipitation and blotting with pan-acetyl-lysine antibodies. We found that all acetyltransferases tested (p300, CBP, GCN5, PCAF, and TIP60) were sufficient to induce BRD4 hyperacetylation, with p300, CBP, and PCAF showing the most activity in this assay (Figure 7-2). We also tested pharmacological inhibitors of sirtuins, or class III histone deacetylase (HDAC) enzymes. Both nicotinamide, a pan-sirtuin inhibitor, and EX527, a specific inhibitor of the SIRT1 enzyme, induced BRD4 hyperacetylation under p300 co-expression (Figure 7-3). Notably, EX527 more strongly promoted BRD4 hyperacetylation, suggesting the specific involvement of SIRT1 in BRD4 deacetylation. From these experiments, we conclude that cellular BRD4 is acetylated likely by p300/CBP and PCAF, and deacetylated by SIRT1.

We next performed mass spectrometry to map acetyl-lysine residues within BRD4. These studies revealed a cluster of acetyl-lysine residues in the BRD4 C-terminus, at residues K1192, K1195, and K1197 (Figure 7-4). We observed that K1192 was acetylated only when K1195 was also acetylated (data not shown). Importantly, this cluster is conserved across BRD4 proteins from evolutionarily distinct vertebrates and also in BRDT, the testis-specific homolog of BRD4. These proteins are distinguished from other BET family members in that they encode PIDs, which are also conserved among species.

To confirm mapped acetyl-lysine residues discovered in mass spectrometry experiments, we sequentially mutated the BRD4 acetyl-lysine cluster to arginine. The K1192R mutant had no effect on BRD4 acetylation, yet mutation of K1195 completely abolished BRD4
acetylation (Figure 7-5). Mutation of all three residues (3KR) also exhibited no detectable BRD4 acetylation. These data confirm what was observed using mass spectrometry, and show that K1192 is dispensable for BRD4 acetylation.

Modification specific antibodies provide useful tools to examine the function of a given posttranslational modification, and we thus raised antibodies against acetyl-BRD4. Rabbits were immunized with peptides corresponding to the unmodified BRD4 acetylation-competent cluster (TPVAPKDLKIKNMGS), K1195Ac (TPVAPKKDLK[Ac]KINMGS), or a mixture of peptides containing K1192Ac and K1195Ac (TPVAPKK[Ac]DLK[Ac]KINMGS). Given that K1195 is the primary site of BRD4 acetylation (Figure 7-5), we selected this peptide for immunization. Furthermore, because this acetyl-lysine is observed with K1192ac, we also used a mixture of K1192 and K1195Ac peptides with the intention of reducing any possible epitope exclusion when both lysine residues are modified in proximity. Upon affinity-based isolation from rabbit sera, purified antibodies recognized spotted peptides via dot blot (Figure 7-6). Antibodies from rabbits immunized with unmodified peptides (R40, R41) recognized all spotted peptides, with the most affinity for the unmodified peptide. Antibodies targeting exclusively K1195Ac (R44, R45) also recognize all spotted peptides, except with considerably more affinity for modified peptides. Antibodies targeting both K1192Ac and K1195Ac (R42, R43), however, were specific for acetyl-BRD4. When tested against lysates containing overexpressed wild type or acetylation-deficient 3KR BRD4 in the presence or absence of p300, we observed that the signal of these antibodies (R42, R43) is sensitive to both p300 overexpression and mutation of the acetyl-lysine cluster, whereas signal using antibodies targeting unmodified BRD4 were not significantly altered (Figure 7-7). These data show that the purified antibodies are modification-specific.

The long isoform of BRD4 functions as a powerful transactivator of transcription via its PID. To test the hypothesis that BRD4 acetylation impacts its function as a transcriptional coactivator, we overexpressed wild type or acetylation-deficient 3KR BRD4 in the presence of various transcriptional reporter constructs. We included core promoters of the HIV-1 LTR, IκBα, E-Selectin, and c-Myc. We also included enhancer constructs bearing the c-Myc minimal promoter and also enhancer sequences from the TOP1 enhancer, and the IGLL5 and MED26 super enhancers. We find that acetylation-deficient BRD4 lacks the capacity to fully transactivate all of these reporters (Figure 7-8). These data indicate that BRD4 acetylation is a requirement for its function as a transcriptional coactivator at both core promoters and enhancers.

Given that BRD4 acetylation sites are conserved among PID-containing BET proteins, we hypothesized that BRD4 acetylation might affect its interaction with P-TEFb. To test this, we
performed co-immunoprecipitation experiments with overexpressed cyclin T1 and either wild type or 3KR BRD4. Upon immunoprecipitation of cyclin T1, we observed a robust decrease in the recovery of 3KR BRD4 as compared to wild type (Figure 7-9). These data indicate that BRD4 acetylation is required for its interaction with P-TEFb.

The data presented thus far begin the functional characterization of BRD4 acetylation. We propose a model wherein BRD4 acetylation promotes its interaction with P-TEFb, thereby enhancing its activity as a transcriptional coactivator (Figure 7-10).
Figure 7-1 – The BRD4 C-terminus is acetylated in cells – Western blot of input (left) and FLAG immunoprecipitations (right) from 293T cells transfected with pEV204 (empty vector) or indicated FLAG-BRD4 construct and treated with or without sorbitol for 2h to induce acetylation. BRD4 acetylation status was probed in FLAG immunoprecipitations using a pan-acetyl-lysine antibody (right, two exposures shown).
Figure 7-2 – A conserved cluster of C-terminal lysine residues in BRD4 is subject to acetylation – Domain architecture of BRD4 including first bromdomain (BD1), second bromodomain (BD2), extraterminal domain (ET), and PID. Acetylated lysine residues revealed by mass spectrometry (K1192, 1195, and 1197) are indicated. Alignments of BRD4/BRDT sequences from other vertebrates are shown.
Figure 7-3 – Several histone acetyltransferases are sufficient to induce BRD4 hyperacetylation – Western blot of input and FLAG immunoprecipitations from 293T cells transfected with full-length BRD4 and the indicated acetyltransferase construct.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Immunoprecipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEV</td>
<td>++-++-++-</td>
</tr>
<tr>
<td>FLAG-BRD4</td>
<td>++++++</td>
</tr>
<tr>
<td>p300</td>
<td>-+-+-+</td>
</tr>
<tr>
<td>HA-CBP</td>
<td>-+-</td>
</tr>
<tr>
<td>HA-GCN5</td>
<td>-+-+</td>
</tr>
<tr>
<td>HA-PCAF</td>
<td>-+-++</td>
</tr>
<tr>
<td>TIP60</td>
<td>-+-+</td>
</tr>
</tbody>
</table>

**Western Blot Images:**
- **α-FLAG**
- **α-HA**
- **α-tubulin**
- **α-pan acetyllysine**

:FLAG IP
Figure 7-4 – BRD4 is hyperacetylated in the presence of overexpressed p300 and class III HDAC inhibitors – Western blot of FLAG immunoprecipitations from 293T cells transfected with full length BRD4 constructs, either in the presence or absence of a construct expressing the p300 acetyltransferase. Co-transfected cells were treated with indicated HDAC inhibitors for 4h, lysed, and BRD4 acetylation status was measured using Western blotting with pan-acetyl-lysine antibodies.
Figure 7-5 – Cellular BRD4 is acetylated at K1195/1197 – Western blot of FLAG immunoprecipitations of 293T cells transfected with indicated BRD4 mutants. AcK = pan-acetyllysine.
**Figure 7-6 – Modification-specific BRD4 antibodies** – Dot blot using indicated peptides and purified antibodies from rabbits immunized with unmodified (R40,41), K1195Ac (R44, 45), or K1192Ac/K1195Ac (R42, 43) BRD4 peptides.
Figure 7-7 – Acetyl-BRD4 antibodies specifically recognize acetyl-lysine cluster within BRD4 – Western blot from 293T lysates containing overexpressed p300 and either wild type or 3KR BRD4, using purified antibodies targeting unmodified (R40,41) or K1192Ac/K1195Ac (R42, 43).
Figure 7-8 – Acetylation-deficient BRD4 displays decreased transactivation capacity to viral and cellular core promoters, enhancers, and super-enhancers – Luciferase activity of indicated reporter plasmids co-transfected into HeLa cells with BRD4 constructs (either WT or acetylation-deficient 3KR).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>HA-Cyclin T1</td>
<td>--</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FLAG BRD4 WT</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>FLAG BRD4 3KR</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
</tr>
</tbody>
</table>

**Figure 7-9** – Acetylation-deficient BRD4 displays reduced binding to cyclin T1 of P-TEFb. Western blot of coimmunoprecipitations performed in 293T cells after transient cotransfection of tagged BRD4 and cyclin T1 constructs. Immunoprecipitations represent ~0.2% input.
Figure 7-10 – Proposed model for posttranslational regulation of BRD4 by acetylation –
BRD4 is acetylated by p300, and redundantly by other acetyltransferases, a modification that is required for robust P-TEFb binding and transcriptional activation of target promoters/enhancers.
Materials & Methods

Plasmids and antibodies

All BRD4 constructs were generous gifts from Dr. Eric Verdin (Buck Institute), with the exception of BRD4 acetylation deficient mutants which were generated using site-directed mutagenesis with primers listed in Table 7-1. Acetyltransferase, cyclin T1 and the HIV-1 LTR, IkBa, and E-Selectin reporter constructs were also gifts from Dr. Verdin. The c-Myc minimal promoter in addition to the enhancer reporters were cloned into pGL3-Basic (Promega) in house using the primers listed in Table 7-1 and standard techniques.

The following antibodies were used for Western blotting: FLAG (Sigma, F7425), pan acetyl-lysine (Cell Signaling, #9441), actin (Sigma, A2228), tubulin (Sigma, T9026), and HA (Sigma, H3663).

Modification specific BRD4 antibodies were generated using the following peptides: unmodified: TPVAPKKDLKIKNMGSIC, K1195Ac: TPVAPKKDLK[Ac]IKNMGSIC, and K1192Ac/K1195Ac: TPVAPKK[Ac]DLK[Ac]IKNMGSIC. These peptides were supplied by Dr. Hans-Richard Rackwitz, Peptide Specialty Laboratories GmbH, Heidelberg, Germany. Rabbits were immunized with indicated peptides under the 118-day protocol (Covance). Antibodies were purified from sera on affinity columns loaded with the same immunogen (Affi-Gel Hz Immunoaffinity Kit, BioRad) as described previously (Pagans et al., 2011).

Cell lysis and immunoprecipitations

Cells were lysed in p300 buffer (7.6 mM NaH₂PO₄, 12.4 mM Na₂HPO₄, 250 mM NaCl, 30 mM NaPPi, 5 mM EDTA, 10 mM NaF, and 0.1% NP-40 [pH 7.0], and protease inhibitor cocktail [Sigma]). Protein concentrations were measured using a Bradford Assay (BioRad).

For FLAG immunoprecipitations, anti-FLAG M2 Affinity Gel (Sigma, A2220) was used. For HA immunoprecipitations, anti-HA agarose was used (Thermo, #26181). 1-2mg of extract was diluted in FLAG-IP buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1% Triton-X-100, and 1X Halt) and immunoprecipitations were carried out with indicated resin overnight at 4°C.

Transient transfections and reporter assays

All transfected were performed using PolyJet (Signagen) under the manufacturer’s suggested protocol. Cells from all transfection experiments were lysed approximately 48h post-transfection. For luciferase assays, cells were lysed in passive lysis buffer (Promega) and luciferase activity was measured using the Luciferase Assay system (Promega) and Analytical Luminescence Laboratory Monolight 2010.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1192R</td>
<td>CCAGTTGCGCCCAAAAAAG</td>
<td>CTTGATTTTCAGGTCCCT</td>
</tr>
<tr>
<td></td>
<td>GGACCTGAAAATCAAG</td>
<td>TTTGGGCAGCAACTGG</td>
</tr>
<tr>
<td>K1195R</td>
<td>CCAAAAAAGGACCTGAGA</td>
<td>GGGACCTGAAAATCAAG</td>
</tr>
<tr>
<td></td>
<td>ATCAAGAACATGGG</td>
<td>GGGACCTGAAAATCAAG</td>
</tr>
<tr>
<td>K1197R</td>
<td>GGACCTGAAAATCAGGA</td>
<td>CGGACTTGGTACCC</td>
</tr>
<tr>
<td></td>
<td>ACATGGGGCTCC</td>
<td>CGGACTTGGTACCC</td>
</tr>
<tr>
<td>K1195/97R-F</td>
<td>CCAAAAAAGGACCTGAGA</td>
<td>GTGTTTTTCTGGGAGCTCTT</td>
</tr>
<tr>
<td></td>
<td>ATCAGGAACATGGG</td>
<td>TACCCGAGGA</td>
</tr>
<tr>
<td>c-Myc_promoter</td>
<td>CCCGGAGCTCTCTGCTCAG</td>
<td>CCCGGAGCTCTCTGCTCAG</td>
</tr>
<tr>
<td></td>
<td>GGAGGAGCAGCAG</td>
<td>GGTTTTTCCTGGACTCTT</td>
</tr>
<tr>
<td>TOP1_enhancer</td>
<td>ACGCGGATCCGCTGTTG</td>
<td>TACGGCGTCGACGCAGGA</td>
</tr>
<tr>
<td></td>
<td>TTTAAGGCTGGGATG</td>
<td>GCCACAGAGCAAACAGT</td>
</tr>
<tr>
<td></td>
<td>GCTTCAATGAGC</td>
<td>TAAGTCC</td>
</tr>
<tr>
<td>IGLL5_super_enhancer</td>
<td>ACGCGGATCCGCTGTC</td>
<td>TACGGCGTCGACGCAGGA</td>
</tr>
<tr>
<td></td>
<td>CACTGAGCATCCAAATGT</td>
<td>GTCCTCACACCTGTCAA</td>
</tr>
<tr>
<td></td>
<td>GCCA</td>
<td>CCTTCT</td>
</tr>
<tr>
<td>MED26_super_enhancer</td>
<td>ACGCGGATCCGCTGCC</td>
<td>TACGGCGTCGACGCAGGA</td>
</tr>
<tr>
<td></td>
<td>CAGATCCTTCTCTCCCTCA</td>
<td>TACGGCGTCGACGCAGGA</td>
</tr>
</tbody>
</table>

Table 7-1 – PCR primers used
References


Chapter VIII

Conclusions & Outlook

In this dissertation, I have i.) provided a review of literature in the field of HIV-1 chromatin biology with a particular focus on the emerging roles of BET proteins in viral transcription and latency ii.) described a novel molecular mechanism through which BET inhibition reverses HIV-1 latency that involves a functional interaction between a specific BET factor and SWI/SNF chromatin remodeling machines and iii.) summarized initial work characterizing acetylation of BRD4 as a novel regulatory mechanism of protein function. Below in conclusion I recapitulate what has been learned about the biology of each BRD4 isoform, and provide an outlook on HIV-1 eradication efforts.

Short BRD4 cooperates with SWI/SNF chromatin remodelers to enforce HIV-1 latency

The vast majority of studies investigating BRD4 function has exclusively focused on the long isoform. Accordingly, this isoform has been classified as the canonical BRD4 isoform. Several studies, including experimental work presented in Chapter VI of this thesis, indicate that the short isoform of BRD4 has important and relevant functions in the regulation of transcription and chromatin. These two isoforms differentially regulate transcription, have unique effects on chromatin structure, localize to distinct areas within the nucleus, and have different acetyl-histone binding capacities. Importantly, their distinct relevance to the etiologies of the NUT midline carcinoma and breast cancer metastasis is well documented. These findings point to a functional distinction between BRD4 short and long isoforms.

Our studies have revealed the short isoform as a requirement for the enforcement of transcriptional silencing during HIV-1 latency. Only with genetic ablation of both isoforms do we observe robust latency reversal and abrogation of JQ1 function, indicative that JQ1 targets the short isoform. Furthermore, the short isoform is highly anchored on chromatin, and is more sensitive than long BRD4 to JQ1 treatment in terms of both time and dose. BET inhibition disrupts HIV-1 chromatin independently of transcription, increasing accessibility downstream of the transcription start site and within the transcription unit. A targeted screen of chromatin remodeling ATPases revealed the catalytic SWI/SNF component BRG1 as a critical requirement for JQ1-induced latency reversal, and we show that the activity of the BAF SWI/SNF remodeling complex is required for JQ1 to remodel HIV-1 chromatin. Short BRD4 is necessary for BRG1 occupancy on HIV-1 chromatin. Co-immunoprecipitation experiments show that this functional interaction is mediated by physical contact between BRG1 and short BRD4 via bromo- and ET-
domains. Collectively, these studies assign a novel role of short BRD4 as a chromatin regulator via SWI/SNF with transcriptionally repressive outcomes in HIV-1 latency.

The use of ChIP-seq allowed us to extend this newly described BET:BAF interaction across the human genome. Evident from this data was the fact that BRD4 and BRG1 co-localize genome-wide, and that JQ1 impairs this overlap. In fact, the majority of BRD4 peaks across the genome are co-bound by BRG1, and this is diminished by about half under JQ1 treatment. Although correlative, these findings demonstrate that cooperation between BRG1 and BRD4 may occur at a considerable number of loci across the human genome. We find that BRD4 and BRG1 co-occupy both coding and non-coding regions, which self-partitioned based on ChIP signal. While many, particularly coding, regions lose Pol II upon JQ1 treatment, we find that LTR sequences present in the human genome do not have significantly diminished Pol II and even have marginally elevated polymerase levels upon JQ1 treatment. The phenotypic similarity between these LTR regions and latent HIV-1 chromatin implies that the BET:BAF functional interaction may be a more general surveillance pathway in existence to silence endogenous retro-elements. As JQ1 disrupts HIV-1 latency, our data indicate that this drug may also disrupt endogenous LTR sequences via targeting of BET:BAF complexes. Future studies are required to validate these newly generated hypotheses based on our data.

The finding that short BRD4 cooperates with SWI/SNF raises new questions about the biology of these factors. SWI/SNF complexes are megadalton molecular machines with 10+ subunits, and our data indicate that BRD4 may be a member of these complexes under certain contexts. SWI/SNF composition is known to be cell type specific, and our data implicate short BRD4 as a member of BAF complexes in CD4+ T cells. The specific subunit composition of the BRD4-containing SWI/SNF complexes will be the subject of future work. Given the role of BRD4 in actively disrupting different P-TEFb complexes, we speculate that BRD4 may belong to specific SWI/SNF complexes and may even play a role in their redistribution. If and how the activity of BRD4 as a histone chaperone plays a role in BET:BAF-dependent chromatin remodeling is also an interesting question.

The contextual requirements to recruit short BRD4 to latent HIV-1 chromatin remain unknown. Although not a specific DNA-binding protein per se, BRD4 does bind sequence-specific transcription factors, and it is conceivable that a cis-element within the virus is responsible for tethering of short BRD4. More tempting is the possibility that a specific acetyl-lysine residue, or combination thereof, on histone or non-histone proteins at the latent HIV-1 locus is responsible for engaging BRD4 bromodomains. We are now interested in the histone H3K27 mark as a potential site of BRD4 recruitment. This residue is both acetylation and
methylation-component, and has been functionally associated with the JQ1 response at the latent HIV-1 promoter. This histone residue is trimethylated by repressive Polycomb complexes to induce silencing. Polycomb activity is antagonized by Trithorax proteins that mediate acetylation of this residue and chromatin remodeling. Importantly, SWI/SNF and possibly BRD4 are members of the Trithorax group. Future work will attempt to uncover the relevance of H3K27 in recruitment of BRD4 and SWI/SNF complexes to latent viral chromatin.

Collectively, these studies assign a novel, chromatin-based function of short BRD4 and underscore the working hypothesis that BRD4 executes isoform-specific functions in the regulation of transcription and chromatin structure.

**Long BRD4 is regulated by reversible acetylation**

Initial work presented in Chapter VII of this dissertation describes a cluster of acetylation-competent lysine residues within the long isoform of BRD4. These studies show that BRD4 acetylation is required for robust transactivation capacity at core promoters and enhancers, potentially via promoting its interaction with P-TEFb. We have generated novel, specific antibodies that adequately discriminate between unmodified and modified BRD4 forms. We envision that these antibodies will serve as valuable tools in our future in-depth characterization of BRD4 acetylation.

Our immediate future work will focus on the functional characterization of BRD4 acetylation under the hypothesis that this modification regulates its interaction with and/or activation of P-TEFb. Our preliminary immunoprecipitation experiments implicate BRD4 acetylation as necessary for P-TEFb binding, and we aim to confirm this using our newly generated modification-specific antibodies. Given that BRD4 actively dissociates P-TEFb from the 7SK snRNP, we do not exclude the hypothesis that BRD4 acetylation may modulate its capacity to disassemble 7SK snRNP complexes. To test this, we will perform *in vitro* P-TEFb release assays using immunoprecipitated unmodified or modified BRD4 complexes. Also, overexpression of BRD4 C-terminal regions, either wild type or acetylation deficient, followed by glycerol gradient sedimentation analysis will permit us to determine the precise effects of BRD4 acetylation on redistributing P-TEFb complexes.

In addition to the effects of BRD4 acetylation on P-TEFb binding and activation, we aim to explore the hypothesis that this modification promotes interactions with bromodomains. We consider the possibility that BRD4 acetylation may induce bromodomain recognition in *cis*, whereby acetylated residues in BRD4 are recognized by its own bromodomains. Precedent for the *cis* recognition model originates from an established mechanism in which the yeast
bromodomain protein, RSC4, is acetylated and this acetylation autoregulates protein function through an intramolecular interaction between acetylated residues and a RSC4 bromodomain (VanDemark et al., 2007). Furthermore, human ubiquitin binding proteins are autoregulated by ubiquitination (Hoeller et al., 2006). Preliminary NMR chemical shift perturbation assays did not show binding of BRD4 bromodomains to acetylated BRD4 peptides in vitro (data not shown), although these results need to be confirmed in cellular contexts. We also consider the hypothesis that that BRD4 acetylation may generate an interface for bromodomain recognition in trans. Recombinant bromodomain libraries with near complete coverage across the family are available, and we plan to collaboratively interrogate which bromodomains exhibit affinity for acetyl-BRD4 peptides using this experimental approach.

We anticipate that characterization of BRD4 acetylation may advance our mechanistic understanding of the protein and its inhibition. These studies may unveil acetylation of BRD4 as an important prognostic marker of protein function, applicable to its critical and broad roles in cells under normal and diseased states.

**Outlook on HIV-1 eradication**

BET inhibitors are among the most exciting next-generation LRAs for the purposes of HIV-1 eradication. While emergent data indicate that an LRA monotherapy is unlikely to impact reservoir size in vivo, promising preclinical data show that certain LRA combinations have desirable effects in targeting HIV-1 latency. We propose a model wherein BET inhibitors directly target HIV-1 chromatin, relieving repressive remodeling machines to critically enable transcriptional activation. PKC agonists, the other predominant class of LRAs, instead operate via NF-κB induction. Combinations of these agents synergize in latency reversal; it is conceivable how concerted relief of a transcriptionally non-permissive chromatin structure coupled to induction of transcription factors could result in robust transcriptional activation.

While shock and kill is an active and leading effort in HIV-1 eradication research, emerging clinical data supporting its efficacy are increasingly demoralizing. Available LRAs are either toxic (i.e., PKC agonists) or ineffective at reducing the latent reservoir size (i.e., vorinostat) as measured by proviral DNA loads. There is also no evidence that cytotoxic T cells are effective at killing reactivated cells, which is a major hypothesis as to how latently infected cells would be depleted. Cytotoxic T cells often become exhausted in the context of HIV-1 infection, their function can be affected by LRAs, and they may not penetrate all anatomical compartments of HIV-1 latency. Furthermore, viral escape mutations in cytotoxic T cell epitopes that abrogate killing are documented (Deng et al., 2015). It is unclear if cytopathic effects of
productive HIV-1 infection are alone sufficient to diminish the latent reservoir under a shock and kill strategy.

In addition to the discovery and characterization of novel LRAs, it is evident that several hurdles exist by the way of shock and kill. Not only are the molecular mechanisms of viral latency thought to be highly complex and heterogeneous, but LRAs exhibit different activities in different latency models. HIV-1 latency is an extremely rare phenomenon in vivo, and thus all available models have considerable caveats and limitations that affect drug screening. Additionally, drug response at the latent HIV-1 promoter may be more stochastic than deterministic, possibly reflecting this heterogeneity. Non-responsive proviruses may still be replication-component and thus sufficient to contribute to viral rebound. At this point, a sterilizing cure appears unlikely with current therapies.

Nonetheless, there are exciting new strategies on the horizon in the fight to eliminate HIV-1 infection. Notably, with the advent of the CRISPR/Cas9 system, several preclinical proof of concept studies have shown that LTR sequences can be targeted and excised by Cas9 (Ebina et al., 2013; Liao et al., 2015). Although considerable time is required for this gene-editing method to reach the clinic, there is clear potential for this technology to achieve a sterilizing HIV-1 cure. Broadly neutralizing antibodies derived from HIV-infected individuals that can bind to virions or infected cells and recruit cytolytic machinery are being characterized with intense interest (Halper-Stromberg and Nussenzweig, 2016). Priming of CD8+ T cells with Gag peptides makes them more effective at killing (Smith et al., 2016), which could be used to bolster killing strategies. Permanent suppression of latent viruses is now being considered as a functional cure strategy (Mousseau et al., 2015). Cortistatin-derived Tat inhibitors, which also inhibit the Mediator transcription complex (Pelish et al., 2015), effectively suppress HIV-1 transcription in various models and may be useful to “block and lock” latent virus.

The work presented in this dissertation underscores the fundamental role of chromatin in HIV-1 latency. Further in-depth characterizations of latent HIV-1 chromatin may reveal more unique, reversible epigenetic features imposed during latency that can be targeted by pharmacological agents. Combined with antiretroviral therapy and adjunctive strategies to disrupt viral latency, such efforts may push the concept of an HIV-1 cure further into our reality. Until then, we still have much to learn from and to be fearful about by way of the ghost of latent HIV-1.
References


Publishing Agreement
It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:
I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

[Signature]
Author Signature

12/21/2016
Date