Regulation of Cellular and HIV-1 Gene Expression by Positive Transcription Elongation Factor B: a Dissertation

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REGULATION OF CELLULAR AND HIV-1 GENE EXPRESSION BY POSITIVE TRANSCRIPTION ELONGATION FACTOR B

A Dissertation Presented
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Program in Biochemistry and Molecular Pharmacology
October 26, 2010
DEDICATION

This thesis is dedicated to my parents, Patrick and Sheila O’Brien.
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CONTRIBUTIONS

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Synthesis of flavopiridol and flavopiridol analog compounds
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ABSTRACT

RNA polymerase II-mediated transcription of HIV-1 genes depends on positive transcription elongation factor b (P-TEFb), the complex of cyclin T1 and CDK9. Recent evidence suggests that regulation of transcription by P-TEFb involves chromatin binding and modifying factors. To determine how P-TEFb may connect chromatin remodeling to transcription, we investigated the relationship between P-TEFb and histone H1. We show that P-TEFb interacts with H1 and that H1 phosphorylation in cell culture correlates with P-TEFb activity. Importantly, P-TEFb also directs H1 phosphorylation during Tat transactivation and wild type HIV-1 infection. Our results also show that P-TEFb phosphorylates histone H1.1 at a specific C-terminal site. Expression of a mutant H1.1 that cannot be phosphorylated by P-TEFb disrupts Tat transactivation as well as transcription of the c-fos and hsp70 genes in HeLa cells. P-TEFb phosphorylation of H1 also plays a role in the expression of muscle differentiation marker genes in the skeletal myoblast cell line C2C12. Additionally, ChIP experiments demonstrate that H1 dissociates from the HIV-1 LTR in MAGI cells, stress-activated genes in HeLa cells, and muscle differentiation marker genes in C2C12 cells under active P-TEFb conditions. Our results overall suggest a new role for P-TEFb in both cellular and HIV-1 transcription through chromatin.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CTD</td>
<td>Carboxy terminal domain</td>
</tr>
<tr>
<td>DRB</td>
<td>5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole</td>
</tr>
<tr>
<td>DSIF</td>
<td>DRB sensitivity inducing factor</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HEXIM</td>
<td>Hexamethylene bis-acetamide inducible protein</td>
</tr>
<tr>
<td>HMBA</td>
<td>Hexamethylene bis-acetamide</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type-1</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MAGI</td>
<td>Multinucleate activation of galactosidase indicator cells</td>
</tr>
<tr>
<td>NELF</td>
<td>Negative elongation factor</td>
</tr>
<tr>
<td>P-TEFb</td>
<td>Positive transcription elongation factor b</td>
</tr>
<tr>
<td>Pol II</td>
<td>RNA Polymerase II</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>TAR</td>
<td>Trans-activation responsive</td>
</tr>
<tr>
<td>Tat</td>
<td>Trans-activator of transcription</td>
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CHAPTER I: LITERATURE REVIEW
Introduction

Gene expression is a highly regulated and multistep process. According to the central dogma of biology, information from DNA is transferred to RNA transcripts that are then translated into protein [1]. While recent studies have revealed the functional importance of RNA transcripts that do not encode proteins, the work in this thesis centers on the regulation of protein-coding gene expression. In mammals, the RNA polymerase (RNA Pol) family catalyzes the transcription of DNA to RNAs. RNA pol I is responsible for transcribing ribosomal RNA (rRNA), while RNA pol II transcribes messenger or protein-coding RNAs (mRNAs) and microRNAs (miRNAs), and RNA pol III transcribes transfer RNAs (tRNAs), and other small RNAs [2]. The three RNA pol isoforms are large holoenzymes comprised of many protein components [2, 3]. All three are also regulated at multiple steps including 1. preinitiation, when the polymerase and its associated factors assemble at a promoter; 2. initiation, when the promoter is melted and transcription begins; 3. elongation, the formation of full transcripts; and 4. termination of the transcript [4].

Transcription of mRNAs by RNA Polymerase II

Transcription of DNA into mRNAs involves the coordination of multiple processes. The recruitment of factors for transcription initiation, elongation, and termination, as well as mRNA processing and splicing factors is tightly controlled and timed. General transcription factors (GTFs) are recruited first to the
promoter by specific transcription factors to form a preinitiation complex (PIC) [5]. TFIID, a complex comprised of the TATA binding protein (TBP) and TATA-associated factors (TAF) bind to the TATA box if the particular gene contains one [6, 7]. Then, TFII B binds to TBP and recruits RNA Pol II together with TFII F [8]. Finally, to activate transcription, TFII E is recruited to melt DNA and and TFII H unwinds DNA using helicase activity [2, 9].

Pol II phosphorylation is crucial in activating transcription. Pol II is phosphorylated on the heptapeptide repeat YSPTSPS that comprises the C-terminal domain (CTD) of its largest subunit, Rpb1 [10]. During initiation of transcription, Pol II is phosphorylated by CDK7/Cyclin H, a component of TFII H, on Ser5 of this heptapeptide repeat. With this phosphorylation, Pol II is activated and begins to transcribe [11]. At many genes that are activated rapidly in response to stress or other stimuli, Pol II pauses after transcribing 50 to 100 nucleotides. To reactivate Pol II, elongation factors such as positive transcription elongation factor b (P-TEFb), CDK12, and CDK13 are needed. P-TEFb binds and phosphorylates Ser2 of the Pol II CTD as well as negative factors such as the negative elongation factor (NELF) and 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) sensitivity inducing factor (DSIF) [12-15]. While P-TEFb was originally thought to be the sole Ser2 kinase, recent work has shown that CDK12 and CDK13 phosphorylate the CTD to promote elongation [15]. These phosphorylation events result in an increase in accessibility of some factors for Pol II binding and a decrease in affinity of other factors [16]. As Ser2-
phosphorylated Pol II transcribes, RNA processing factors are recruited, including capping enzyme and splicing and polyadenylation factors [17, 18]. These phosphorylation events thereby relieve the inhibition of negative elongation factors and stimulate Pol II activity.

**Discovery of P-TEFb**

P-TEFb is the complex of cyclin-dependent kinase 9 (CDK9) and Cyclin T1. CDK9 was discovered in a search for novel kinases homologous to the cell division control (CDC2) family. CDK9 was originally called PITALRE because it contained a PSTAIRE sequence similar to other cyclin-dependent kinases, and it shared approximately 40% sequence identity to CDC2 and CDK2 [19]. PITALRE is also ubiquitously expressed, as determined by Northern blot analysis of a number of tissues from mice, and localizes to the nucleus [20]. Like other PSTAIRE proteins, it is a serine/threonine kinase, and it phosphorylates retinoblastoma protein (Rb) and myelin basic protein (MBP) *in vitro*. Unlike other CDKs, PITALRE’s activity remained the same throughout the cell cycle [19].

The function of CDK9 was not immediately clear, however, and its role in Pol II elongation was determined later. In early *in vitro* transcription experiments, it was noted that RNA Pol II pauses while transcribing certain genes. To identify the factors involved in relieving this restriction, *in vitro* transcription was carried out with glycerol gradient fractions of *Drosophila* Kc cells [21]. Fractions that contained elongation activity were run on SDS-PAGE, silver stained, and
individual proteins were identified. The P-TEFb complex was first purified from Drosophila by the Price lab, who later cloned human Cyclin T1 and T2 by expressed sequence tag (EST) homology search [22, 23]. Cyclin T1 was also identified as an HIV-1 Tat interacting factor [24]. In in vitro transcription reactions, P-TEFb was found to increase run-off formation of full transcripts dose-dependently [13].

In vitro experiments showed that the CTD of Pol II is important for transcription elongation. Digesting the CTD with chymotrypsin was found to inhibit run-off transcription, and while P-TEFb relieved pausing by full length Pol II, it had no effect on ΔCTD Pol II [13]. In vitro kinase assays confirmed that P-TEFb did indeed phosphorylate the tail of RNA Pol II. Mutation of the catalytic aspartic acid of CDK9, D167, to asparagine was found to disrupt CTD phosphorylation [25]. Studies of HIV-1 transcription in vitro showed that active P-TEFb, in addition to Tat and TAR RNA, was necessary for elongation [26, 27]. Moreover, overexpression of PITALRE in HeLa or Jurkat cells enhanced Tat transactivation [28]. Compounds that inhibited Tat-activated transcription also inhibited P-TEFb kinase activity in vitro [26].

**Structure of P-TEFb**

The components of P-TEFb are similar to other CDK/Cyclin complexes. The major CDK9 isoform, CDK9-42, is a 42 kDa protein comprised of 372 amino acids [29]. Residues 19-315 make up the Ser/Thr kinase domain, with amino
acids 25-48 forming the ATP binding site, and Asp 167 as the catalytic aspartic acid residue [19]. The CDK9-55 isoform, which is expressed in specific cell types such as muscle, is transcribed from a site upstream of the CDK9-42 start site, and has an additional 120 amino acids of unknown function [30]. At 81 kDa and 726 amino acids, Cyclin T1 has a cyclin domain at amino acids 43-142, which it uses to bind CDK9 [31]. Cyclin T1 also binds other factors using its coiled coil domain at residues 393-417 [28, 32]. The other P-TEFb-associated Cyclins, Cyclin T2a and T2b, which are 74 and 81kDa respectively, have similar structure with Cyclin domains spanning amino acids 42-242 [23]. Cyclins T2a and T2b arise from alternative splicing events, and their expression patterns differ based on cell type [23].

The crystal structure of CDK9 complexed with Cyclin T1 was solved only recently [33]. Previous assumptions about P-TEFb structure had been made by modeling CDK9 and Cyclin T1 on the crystal structure of CDK2 and Cyclin A [34]. This approach had been problematic in that the CDKs share 40% amino acid sequence homology and the cyclins are non-homologous. The current structure of P-TEFb is quite different from that of CDK2/Cyclin A. Cyclin T1 is bound to CDK9 in a position that is rotated compared to Cyclin A binding to CDK2 [33]. This difference is thought to exist because P-TEFb binds to the large Pol II holoenzyme and a number of other proteins, while CDK2 and Cyclin A act independently. The difference in these structures also sheds light onto how CDK9 is activated. For example, CDK9 autophosphorylates, so these serine and
threonine residues are exposed while their counterparts on CDK2 and other cell cycle CDKs, which do not autophosphorylate, are buried [33, 35].

The structure of P-TEFb bound to the HIV-1 transcription activating protein Tat has also been solved [36]. HIV-1 Tat binds P-TEFb and recruits it to bind the TAR RNA stem loop, formed from nascent HIV-1 mRNA transcripts [26, 37]. The ability of Tat to influence P-TEFb activity is apparent in the complex’s structure: the Tat-Cyclin T1 and CDK9 interface is twice as large as the surface area taken up by most protein-protein interactions [36]. Tat was also found to bind the T loop, or activating loop, of CDK9, and this interaction may account for an increase in kinase activity in the presence of Tat [36].

**Phosphorylation of RNA Pol II Ser2 is necessary for transcription elongation**

The CTD of RNA Pol II consists of 52 repeats of the heptapeptide YSPTSPS [10]. This heptapeptide can be phosphorylated at Ser2 and Ser5, and each of these phosphorylation events recruits machinery that works cotranscriptionally [37, 38]. Ser5 is phosphorylated by the CDK7/Cyclin H complex during transcription initiation, and thereby activates Pol II [11]. Ser2 is phosphorylated after Pol II pauses, and this event reactivates the polymerase to promote elongation [21, 38]. The differential phosphorylation by these kinases was identified by the use of antibodies specific to pSer5 and pSer2 as well as truncated CTD mutants [13]. In *S. cerevisiae*, when Ser5 is phosphorylated, the
Set1 methyltransferase is recruited to trimethylate Histone H3 at Lys4 and the 5’ capping enzyme is recruited to stabilize nascent RNAs [17]. When Ser2 is phosphorylated, the Set2 methyltransferase is recruited to trimethylate H3K36, and the 3’ end-processing factor Pcf11 is recruited along with mRNA splicing machinery [17]. In mammals, Ser2 phosphorylation also results in the recruitment of mRNA processing factors [17, 39]. The Pol II CTD is thus thought to act as a scaffold upon which all these complexes form and its phosphorylation is necessary for mRNA processing.

**Ser2 of Pol II is phosphorylated by P-TEFb, CDK12, and CDK13**

While P-TEFb was originally thought to be the main Ser2 kinase, recent work has identified two other kinases that exhibit CTD kinase activity. In *S. cerevisiae*, both CTD kinases Ctk1 and Bur1 were previously thought to be CDK9 homologs [40], but kinase domain swap of CDK9 with Bur1 and Ctk1 in yeast identified CDK9 as the Bur1 homolog [15]. In *Drosophila*, depletion of CDK12 resulted in a dramatic reduction in Ser2 phosphorylation, and depletion of CDK12 and CDK12 in human cells showed marked differences in Ser2 phosphorylation patterns [15]. CDK12 ChIP also overlapped more closely with Ser2 ChIP along heat shock genes than CDK9 [15]. Together, these results suggested that CDK12 and CDK13 may function primarily in phosphorylating Pol II to promote elongation, while P-TEFb’s role may be more focused on phosphorylation of negative transcription elongation factors and other substrates to activate elongation.
P-TEFb alleviates inhibition of Pol II by negative elongation factors

In order for elongation to proceed at genes with poised Pol II, the repressive activity of negative transcription elongation factors needs to be overcome. In addition to the necessity for Pol II phosphorylation, NELF and DSIF provide another block to Pol II transcription. NELF and DSIF were both identified as the factors responsible for conferring Pol II sensitivity to the transcription inhibitor DRB in vitro [41, 42]. The subunits of DSIF (Spt4 and Spt5) and NELF (NELF A-E) were identified by fractionation of nuclear extracts and testing for DRB sensitivity in in vitro transcription experiments [43]. Spt5 was also phosphorylated during elongation in stepwise transcription experiments [44].

When DSIF and NELF are added to Pol II in vitro transcription assays, transcription is paused at 50-100nt. This pausing occurs when both factors are added together, but not when only one factor is added [41]. When P-TEFb is added to these reactions, the inhibition caused by DSIF and NELF is relieved, allowing for formation of full transcripts. In vitro kinase assays also showed that the Spt5 protein of the DSIF complex was phosphorylated by P-TEFb in its C-terminal region [41, 42]. Adding ATP along with P-TEFb to in vitro transcription assays with NELF and DSIF also alleviates their inhibition, reinforcing the importance of DSIF phosphorylation during transcription [41]. P-TEFb phosphorylation of DSIF is also enhanced at the HIV-1 LTR in the presence of the Tat protein [45]. Once P-TEFb phosphorylates Pol II and DSIF, NELF dissociates from the Pol II complex, while phosphorylation of DSIF transforms it
into a positive transcription elongation factor [46-49]. How DSIF activates transcription at this step is still unknown. Notably, NELF and DSIF only act at promoters that are sensitive to Pol II pausing, and knockdown of NELF in cells stimulates transcription at genes that normally have an elongation checkpoint [48, 49].

**The Human Immunodeficiency Virus**

The human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS). The virus is comprised of 15 proteins and a single-stranded RNA genome of ~9 kb [50]. First observed in clinics in 1981, HIV infection is now considered pandemic, with an estimated 33 million people living with HIV worldwide in 2007 [51]. Due to the high error rate of its reverse transcriptase enzyme, HIV-1 has a high rate of mutation, which has proven difficult for therapy [52, 53]. The widest range of genetically different strains occurs in the areas of the world most impacted by HIV, namely Sub-Saharan Africa and Southeast Asia [54]. This constant evolution of the virus poses a challenge to developing inhibitors, and strains that are resistant to highly active antiretroviral therapy (HAART) have recently emerged [55]. While many currently available drugs target reverse transcriptase and HIV protease, one new approach is to target cellular factors that are used by the virus. One example of a cellular target is P-TEFb, which is necessary for the transcription of HIV mRNAs.
HIV-1 Life Cycle

In order to understand how P-TEFb functions as an accessory to HIV infection, we first need to know its place within the life cycle of the virus. First, a virion binds to CD4 receptor or the CXCR4 or CCR5 coreceptors [56]. The virion then fuses with the cell membrane, releasing its contents into the cell. Once in the cell, the viral RNA genome is reverse transcribed into double stranded DNA, which is then integrated into the host cell DNA by the HIV integrase protein. The location of integration is important because it can determine the extent of viral latency: if the provirus is integrated into a highly transcribed region, it may be transcribed and replicate more readily than if it is integrated into a transcriptionally silent region of chromatin [57]. The provirus is then transcribed into protein-coding mRNAs and copies of the full length RNA genome [50, 52]. At first, only basal transcription occurs, resulting in abortive transcripts, but occasionally RNA Pol II will transcribe full mRNAs. Once the Tat protein is transcribed and translated, it creates a feedback loop, activating transcription of more viral RNAs by recruiting P-TEFb to hyperphosphorylate Pol II and promote elongation [26, 27, 58]. The mRNAs are then transported into the cytoplasm, where they are translated into protein. When the viral proteins are expressed, they assemble along with viral RNA and immature virions then bud from the plasma membrane [50, 56, 59]. The HIV-1 protease protein then cleaves viral polypeptides in the virion, allowing for the formation of mature viral particles [50, 52, 60]. These mature virions can go on to infect other cells.
Interaction of P-TEFb with HIV-1 Tat-TAR complex

While P-TEFb is recruited to many Pol II-transcribed genes, it is also necessary for the transcription of HIV-1 genes. Like many other Pol II transcripts, Pol II initiates transcription and then pauses shortly thereafter [26]. To reactivate transcription, HIV usurps the activity of P-TEFb for its own benefit through the action of TAR RNA and the Tat protein. The HIV-1 LTR precedes the viral genome and acts as a promoter, but only short transcripts are observed at a basal level in the absence of Tat. In order to productively transcribe these HIV-1 genes, trans-activation by the HIV-1 regulatory protein Tat has to occur by binding Cyclin T1 and recruiting P-TEFb to Pol II [26, 27, 61].

TAR RNA is a stem loop structure comprised of the first 59 nt of HIV-1 transcripts, and it serves as a scaffold for binding the HIV-1 Tat protein [50, 58]. Tat binds to TAR via its highly basic C-terminal region, while it interacts with Cyclin T1 via a Zn$^{2+}$-mediated bridge at the cysteine-rich domain at its N-terminus. Cyclin T1 binds to the Tat/TAR complex via its coiled-coil domain [24, 62]. P-TEFb is therefore recruited to the HIV-1 LTR, allowing both Cyclin T1 and Tat proteins to contact the TAR stem loop. This recruitment of P-TEFb to Tat and TAR brings it within close proximity to the Pol II CTD, which it phosphorylates to promote elongation [27]. *In vitro* transcription reactions have identified that both Tat and P-TEFb are necessary for transcription of TAR-encoding genes. Depletion of Tat, CDK9, or Cyclin T1 results in an inability of Pol II to transcribe HIV templates [26, 63, 64].
The interaction with HIV-1 Tat not only serves to recruit P-TEFb to the HIV LTR, but it also enhances P-TEFb activity. In *in vitro* kinase assays, adding Tat to P-TEFb results in increased Pol II CTD phosphorylation [27]. While Ser2 phosphorylation was improved, P-TEFb phosphorylated Ser5 as well in the presence of Tat, as shown by Western blot with phosphorylation site-specific antibodies [65]. Microarray studies have shown that a number of genes are activated when cells are infected with HIV, and many of these are activated when Tat is expressed [66, 67]. Therefore, Tat may also recruit P-TEFb to cellular genes to promote HIV infection.

**Activation of P-TEFb by Brd4**

While P-TEFb can be recruited to specific genes by binding transcription factors such as NFκB or Sp1, it is also recruited by a more general chromatin-binding factor called Brd4. Brd4 is a bromodomain-containing, BET family protein that binds acetylated lysine residues on histone H4 [68]. Brd4 is also a component of the Mediator complex, which binds to transcription factors at the promoter and recruits Pol II. P-TEFb and Brd4 co-elute together in the small P-TEFb complex in glycerol gradient centrifugation [69]. As the large P-TEFb complex dissociates when cells are stressed with ActD or UV irradiation, P-TEFb association with Brd4 increases [70]. Expression of P-TEFb regulated oncogenes such as *c-myc* or *c-jun* is enhanced in the presence of Brd4, as is basal HIV-1 transcription [68, 70]. P-TEFb may interact with Brd4 as it binds Tat, because Brd4 does not enhance Tat-mediated transcription of HIV genes [70]. Overexpression of Brd4
also increases global Pol II Ser2 phosphorylation, so Brd4 may therefore recruit P-TEFb to many inducible cellular genes [71, 72].

**P-TEFb inhibitory complex: 7SK and HEXIM1/2**

P-TEFb exists in two distinct cellular complexes. P-TEFb can be recruited to Pol II by Brd4 and specific transcription factors, but it also exists in an inactive complex with the snRNA 7SK and the HEXIM1 and HEXIM2 proteins [37, 42]. These two complexes were characterized after fractionation of HeLa cells showed that P-TEFb eluted in a lower molecular weight complex and a higher molecular weight complex [73]. *In vitro* kinase assay using these lower and higher molecular weight fractions showed that only the smaller complex was able to phosphorylate RNA pol II [73]. Additionally, treatment of cell lysates with RNase A shifted all P-TEFb into the lower molecular weight complex. However, 7SK alone is not sufficient to inhibit P-TEFb. The Bensaude and Zhou labs characterized the interaction of P-TEFb with the HEXIM1 protein in the large complex [74-77]. Adding both 7SK and HEXIM1 to P-TEFb significantly reduced its ability to phosphorylate CTD *in vitro* [75, 77].

The large P-TEFb complex forms by using 7SK as a scaffold. On one 7SK molecule, two Cyclin T1 proteins and two HEXIM1 or HEXIM2 proteins can bind the complex [78]. By truncating 7SK and performing gel shift experiments or expressing each hairpin in cells, researchers have determined that the stem loop formed by bases 24-87 interacts with HEXIM1. Specifically, U30 was found to
bind directly to HEXIM1 in crosslinking experiments [79]. Since P-TEFb interacts with the 7SK complex by binding HEXIM1, these binding experiments were not possible with P-TEFb and 7SK alone. Other proteins that bind 7SK such as LARP7 utilize its other three stem loops [80-82].

P-TEFb and HEXIM binding to the 7SK complex was determined by two-hybrid experiments. Cyclin T1 binds to HEXIM1 via a portion of its cyclin box domain, aa 188-290, while HEXIM1 was found to bind Cyclin T1 with aa181-359, an Asp and Glu-rich NLS section [76]. Importantly, swapping this region of HEXIM1 for the NLS of Tat restores HEXIM1 function, supporting the theory that Tat and TAR RNA displaces HEXIM1 and 7SK during HIV infection [83]. Once bound to Cyclin T1, HEXIM1 binds the 7SK 5’ stem loop using its N-terminal basic domain [77, 84].

About half of the total P-TEFb in the nucleus is free and active, and the other half is bound to the 7SK/HEXIM complex. The equilibrium of these complexes is quite fluid, as cellular stresses are known to cause a release of P-TEFb from the large complex. Treatment of cells with the transcription inhibitors Actinomycin D (ActD) or DRB, UV irradiation, or hexamethylene bisacetamide (HMBA) causes the release of P-TEFb from the large complex [76, 77, 84]. These treatments result in an increase in P-TEFb-regulated transcription, such as from HIV-LTR reporter gene constructs [76, 77]. After extended HMBA treatment, however, P-TEFb returns to the large complex and transcription is inhibited [85]. The way this equilibrium is maintained remains unclear, but protein levels appear to shift
with unaltered expression. Depletion of Cyclin T1 or Brd4 results in a decrease in HEXIM1 expression, and HEXIM1 depletion results in the preexisting free HEXIM1 binding CDK9 [85].

Another example of P-TEFb activation from 7SK/HEXIM occurs during HIV-1 infection. An increase in P-TEFb activity during infection was noted before the 7SK/HEXIM complex was identified. Additionally, HIV-1 Tat protein expression also draws P-TEFb from the large complex [80, 86]. One hypothesis for this shift is that the interaction of Tat and TAR RNA with P-TEFb mimics the 7SK/HEXIM1 interaction, and therefore a direct displacement occurs.

Another HEXIM family member, HEXIM2, was identified as part of the large P-TEFb complex [87, 88]. HEXIM1 and 2 are expressed in different tissues, and HEXIM2 has a higher affinity for CyclinT2 than CyclinT1 [88, 89]. Despite these differences, HEXIM2 binds the 7SK complex and P-TEFb similarly, and P-TEFb bound to HEXIM2 and 7SK is inactive [87, 88].

In addition to the 7SK and the HEXIM proteins, the large P-TEFb complex contains other proteins that do not bind P-TEFb directly. Larp7 is one such protein that functions in stabilizing the 7SK complex by binding HEXIM1 [82]. Depletion of Larp7 results in an increase in Tat transactivation and Hsp70 transcription [82]. BCDIN3 is another protein that binds the large complex via 7SK, and was identified as the 7SK capping methyltransferase [90]. BCDIN3 depletion by siRNA also destabilizes the complex by increasing the likelihood of 7SK degradation [80].
Inhibition of P-TEFb by small molecules and depletion by siRNA

In addition to being regulated by the 7SK/HEXIM complex in the nucleus, P-TEFb can be inhibited by small molecules or siRNA depletion. The compound flavopiridol is a CDK inhibitor with high specificity for CDK9: its IC$_{50}$ for CDK9 is 9 nM, while its IC$_{50}$ for CDK2 and other CDKs is greater than 200 nM [91]. Flavopiridol therefore inhibits transcription much more effectively than DRB, whose IC$_{50}$ is 2.7 μM [92]. Because it can also target other CDKs, flavopiridol activity on tumor growth has been studied, and it is currently undergoing clinical trials as a chemotherapy drug [93, 94]. However, its specificity for CDK9 makes flavopiridol a great tool for studying P-TEFb activity in cell culture. Further, structure-activity relationship (SAR) studies of flavopiridol have generated a derivative that is nearly three times more specific for CDK9 than other CDKs. Both flavopiridol and this derivative can inhibit HIV-1 replication in the H9 T cell line and Pol II CTD phosphorylation in vitro in the low nanomolar range with low toxicity [91]. Flavopiridol has also been shown to inhibit mRNA polyadenylation and cleavage, which are activated downstream of P-TEFb [95]. While flavopiridol effectively inhibits P-TEFb activity, it has been shown that treatment of cells with this compound results in a release of P-TEFb from the 7SK/HEXIM complex, presumably to compensate for a reduction in activity [92]. How this release is triggered remains unclear.

The structure of flavopiridol bound to CDK9 has been determined. Contrary to an earlier report, the crystal structure of P-TEFb shows that flavopiridol binds
CDK9 in its ATP-binding pocket [33]. This finding makes sense in that flavopiridol is an ATP analog, and its binding to CDK9 prevents further ATP binding and catalysis. The position of Cyclin T1 when bound to CDK9 makes the kinase more apt to bind flavopiridol than the cell cycle CDKs [36].

Another way to inhibit P-TEFb function and the expression of HIV-1 and other genes is by RNA interference (RNAi). siRNA-mediated depletion of CDK9 or Cyclin T1 individually by sequence-specific siRNAs has been shown to inhibit Tat transactivation in the HeLa-MAGI cell line [63]. Further, depletion of CDK9 and Cyclin T1 also inhibits HIV replication in the H9 T cell line, as determined by RT assay. This study also showed the necessity of Cyclin T1 for CDK9 activity and stability in that CDK9 expression is decreased when Cyclin T1 is depleted [63]. Depletion of P-TEFb did not affect cell viability as long as 66 hr posttransfection, so Pol II transcription, while seriously affected at many genes, can continue at housekeeping genes [63, unpublished data].

**P-TEFb activation of transcription in immune cells**

Many Pol II-transcribed genes are controlled at the point of elongation. These polymerases sit poised to transcribe, and elongation is the rate-limiting step for which P-TEFb provides a necessary function [37, 42].

P-TEFb is recruited to the HIV-1 LTR by Tat and TAR to promote transcription elongation. During infection, however, a number of cellular genes are activated as well. A number of studies have examined changes in cellular expression when
cells are infected with HIV. The most drastic changes in gene expression occur during the acute phase of infection, when immune response genes and immune suppression-related genes are expressed simultaneously [96]. The transcription of some of these genes may be due to Tat, and therefore P-TEFb, activity. To identify exclusively Tat-responsive genes, Tat protein was expressed in dendritic cells, and microarray analysis was performed [67]. The results of this study showed that Tat expression upregulated immune response elements such as chemokines, the STAT family, and IFN-γ related genes, as well as genes regulating cell metabolism. Tat expression alone was also sufficient for activation of T cells and monocytes, implying that Tat and P-TEFb can prepare cells for HIV replication by changing their transcriptional program [67].

**P-TEFb activity during stress-activated cellular transcription**

While P-TEFb is recruited to many Pol II transcribed genes, the necessity of P-TEFb for transcription has been studied with only a handful of genes. Microarray experiments have shown that P-TEFb depletion results in reduced expression of genes involved in transcription regulation, cellular organization, nuclear transport, and metabolism, processing, or export of mRNAs [96, Rana lab unpublished data]. P-TEFb is also recruited to heat-shock loci on *Drosophila* polytene chromosomes [97]. ChIP experiments have additionally shown that when the MEK/ERK pathway is activated in neuroendocrine cells by thyrotropin-releasing hormone, P-TEFb is recruited into the nucleus and to the oncogenes *c-fos* and *c-jun* to stimulate elongation [98]. ChIP- high throughput sequencing (ChIP-Seq)
experiments with human cells also showed that P-TEFb is recruited to primary response genes induced by serum within one hour of stimulation [99]. During this stress response, P-TEFb is recruited to these genes by Brd4, and Brd4 depletion prevents P-TEFb recruitment to primary response genes [99].

**P-TEFb activity in development and differentiation**

The necessity of P-TEFb for general cell viability has been contested, and P-TEFb may be dispensable depending on tissue type or developmental stage of an organism. CDK9 and Cyclin T1 can both be depleted by siRNA in HeLa cells with no effect on cell viability or growth [63]. However, P-TEFb activity has proven necessary for differentiation and development. When CDK9 is knocked down in *Drosophila* embryos, the flies die during metamorphosis, and murine CDK9 knockouts die before the blastocyst stage [12, 100]. Additionally, the Pgc protein in *Drosophila* binds P-TEFb and inhibits P-TEFb-controlled gene expression in pole cells of the developing embryo [101]. The timing of P-TEFb activity is therefore thought to be important during embryogenesis, since many promoters in pluripotent cells are not constitutively active, but have RNA Pol II poised to transcribe.

While these results suggested a more general role for CDK9 in development, other studies have described a role for P-TEFb in the differentiation of specific cell types. For example, morpholino-directed depletion of CDK9 in zebrafish results in impaired erythropoesis [102]. In addition to issues associated with loss
of P-TEFb function, increased P-TEFb activity is problematic. Mice that have hypermorphic P-TEFb mutations were found to die of cardiac hypertrophy, since P-TEFb enhances the growth of cardiac muscle [103]. In cultured myocytes, hypertrophic signals such as calcineurin or mechanical stress cause a release of P-TEFb from 7SK and HEXIM1 [104]. This release of P-TEFb then activates transcription from promoters such as myogenin, a muscle-specific transcription factor, MCK (muscle creatine kinase), or myosin [105]. To stimulate these genes, P-TEFb is recruited to muscle-specific promoters by transcription factors such as MyoD. Changes in P-TEFb activity also have a clear impact on cell morphology: expression of dominant negative CDK9 in the C2C12 muscle cell line inhibits the formation of myotubes from the original myoblasts [105, 106]. Therefore, regulation of P-TEFb activity is critical for the timing and control of differentiation and development associated gene expression.

**P-TEFb activity and chromatin remodeling**

While a direct relationship between P-TEFb and chromatin remodeling machinery has not been determined, there are some clues regarding how P-TEFb activates transcription in a chromatin context. The phosphorylation of Pol II recruits mRNA processing proteins in both yeast and human cells. At the HIV-1 LTR, P-TEFb binds the SKIP splicing factor, which recruits c-Myc. C-Myc in turn interacts with TRAPP, which recruits histone acetyltransferases [107, 108]. HIV-1 Tat, in addition to recruiting P-TEFb to the HIV LTR, also binds histone acetyltransferases such as p300, Tip60, and pCAF [109-111]. CDK9 knockdown
in human cells causes a decrease in global histone H3K36 trimethylation, a mark associated with transcription elongation [112]. In *S. cerevisiae*, P-TEFb homolog Ctk1 is necessary for both Ser2 phosphorylation and recruitment of the H3K36 methyltransferase Set2, as well as the H2B deubiquitinase. Knockdown of Ctk1 results in broadened patterns of H3K4 trimethylation, a mark associated with transcription initiation [113, 114]. Knockout of Set2 does not result in any changes in H3K4 methylation, suggesting a role for Ctk1 in creating boundaries of these chromatin marks [115].

In addition to regulation of these methylation events, P-TEFb interacts with chromatin binding and modifying factors. In the absence of Tat or other specific transcription factors, P-TEFb is recruited to cellular genes by the bromodomain-containing protein Brd4 [69, 70]. Brd4 binds acetylated histone H3, which marks sites of active transcription. P-TEFb also interacts with acetyltransferases such as p300 and PCAF [116, 117]. Additionally, in order for P-TEFb to activate elongation through chromatin, the FACT complex is necessary for Pol II to transcribe efficiently through nucleosomes [118]. While these connections of P-TEFb to chromatin modifications have been made, a more direct relationship with chromatin during transcription has not been discovered.

**Genomic DNA is packaged into chromatin**

In order for the eukaryotic genome to fit into the nucleus of the cell, it needs to be carefully packaged. A human cell needs to accommodate $3.2 \times 10^9$ base pairs, or
nearly 2 meters, of DNA base pairs and it needs the help of chromatin proteins [2]. The organization of chromatin by nucleosomes and other chromatin binding and modifying proteins provides the cell with dynamic and static regions of DNA and helps prevent aberrant gene expression. The most basic unit of chromatin is the nucleosome, in which 146 bp of DNA is wrapped around an octamer of histone proteins, containing two molecules each of the core histones H2A, H2B, H3, and H4. Nucleosomes form the “beads on a string” shape [2]. The highly abundant chromatin protein histone H1 binds to the DNA in between nucleosomes and facilitates higher order packing of chromatin into the 30 nm fiber [119, 120]. These 30 nm fibers further compact to form heterochromatin, tightly packed chromatin that is transcriptionally inactive, and euchromatin, less tightly packed regions that can be readily transcribed.

In order to be transcribed, chromatin must be accessible to RNA polymerase and transcription factor assembly. Chromatin remodeling allows for dynamic unwrapping and re-wrapping of DNA triggered by signals to activate transcription. Binding of transcription factors to specific sequences in a promoter can recruit histone-modifying enzymes and ATP-dependent chromatin remodeling complexes, making chromatin along that gene accessible and clearing the way for RNA polymerase [2].
**Histone H1**

Histone H1 is known as the linker histone. It binds to nucleosomes and the stretches of DNA that link them and thus facilitates protection and compaction of chromatin. Structure studies of nucleosomes containing H1 have shown that H1 contacts the dyad, where DNA enters and leaves the nucleosome, and 10 nt on either side of the dyad with its globular domain [121]. Its highly positively charged C-terminal domain tail contacts the linker DNA between nucleosomes [119].

The different isoforms of histone H1 have arisen due to gene duplication events, with the number of isoforms increasing along with organism complexity. Humans have five somatic isoforms, H1.1-H1.5, along with the testes and oocyte-specific isoforms H1t and H1oo, and H1.0, considered a replacement for other isoforms [122]. These isoforms are highly homologous (>90% sequence identity) in their globular domains, but vary in their C-terminal tails, which allows for preferential binding to certain regions of chromatin. While H1.4 and H1.5, which have the longest C-terminal tails, bind heterochromatin, H1.1, H1.2, and H1.3 tend to bind more highly transcribed regions of chromatin [122, 123]. The isoforms of H1 also localize to different places in the nucleus. Immunofluorescence with isoform-specific antibodies has shown that histone H1.5 localizes to the nuclear periphery, while histone H1.3 and H1.4 both stain in nuclear speckles [123].

Unlike the core histones, the relationship between H1 and DNA is more fluid. H1 binds chromatin less stably and therefore in equilibrium between a free and
bound state [124, 125]. This equilibrium allows H1 to compete with other factors for DNA binding, such as general chromatin binding proteins like the high mobility group (HMG) family or specific transcription factors [119]. This dynamic nature allows for the incorporation of affinity-tagged or fluorescent protein-tagged H1 into chromatin when these constructs are expressed in cells. The kinetics of DNA binding by H1 have also been approximated by fluorescence recovery after photobleaching (FRAP) experiments. The isoforms of histone H1 each have different binding characteristics and residence times on DNA, reflecting their residence on euchromatin or heterochromatin [122, 125, 126].

**Histone H1 phosphorylation**

Posttranslational modifications of H1 can also affect chromatin binding, and phosphorylation is known to enhance H1 mobility [119]. Histone H1 phosphorylation during the cell cycle has been well studied; H1 is phosphorylated during the G1/S phase transition by CDK2 to promote its dissociation from DNA [127, 128]. This step is necessary for replication fork progression during DNA synthesis in S phase. As the cell prepares for mitosis in late G2 phase, H1 is phosphorylated again by Cdc2, and this phosphorylation triggers chromosome compaction and nuclear envelope breakdown [129].

The C-terminal tail of histone H1 is highly positively charged. Phosphorylation of H1 adds negative charge to this tail, but it is not enough to induce the repulsion of DNA. Rather than simply interrupting charge-charge interactions between H1
and DNA, phosphorylation may induce a conformational change in the tail, making its structure suboptimal for DNA binding [130, 131]. Fourier transform IR experiments have also shown that the DNA-bound C-terminus of H1 has mostly $\alpha$-helical structure, while the fully phosphorylated H1 has more $\beta$-structure [131]. Because of the lack of structure in the C-terminal domains of H1 isoforms, it seems as though the extent of phosphorylation, rather than the phosphorylation of specific sites, affects mobility [122].

**Histone H1 and gene expression**

Histone H1 was originally thought to act as a general repressor of transcription due to its role in chromatin compaction, and that its removal would lead to a global increase in gene expression. Early studies showed that the addition of histone H1 inhibited transcription *in vitro* and that H1-containing genes were transcribed less than genes without H1 [132, 133]. This repression was found to be reversible, as addition of specific transcription factors activated transcription in the presence of H1 [132]. *In vivo*, however, the relationship between histone H1 and transcription did not prove to be as clear. H1 knockouts in both yeast and *tetrahymena* did not have global increases in transcription, nor was nucleosome spacing radically changed [133-135]. The Skoultchi group found the general repressor theory to be false when they knocked out multiple H1 isoform genes from mice [136, 137]. Embryonic cells from triple H1 knockout mice expressed half the total histone H1 of a wild-type mouse, and the group found that this extreme knockout only results in 20 upregulated genes [136]. Similar results
were seen in H1 knockout *Drosophila*, where H1 depletion resulted in altered structure of polytene chromosomes and depletion of heterochromatic marks such as H3K9 methylation [138]. To prevent changes in gene expression, nucleosomes moved closer together, resulting in massive changes in nuclear architecture [120, 136].

While this global approach did not identify many targets of H1 control of transcription, the role of histone H1 in transcription from the MMTV promoter is well characterized. When dexamethasone induces transcription from the MMTV promoter, H1 is phosphorylated and then dissociates from chromatin [139]. The kinase inhibitor roscovitine prevents this phosphorylation and therefore prevents dissociation from the MMTV promoter [140]. During a refractory period after activation, MMTV transcription is inhibited and H1 binds to the promoter once again [139, 140].

**Histone H1 DNA binding & mobility**

The mobility of histone H1 has been correlated with its binding to chromatin. While the histone proteins of the nucleosome bind DNA very stably, H1 is thought to be in equilibrium with chromatin: binding one site, releasing, and then binding another site. The residence time of H1 has been measured to be up to 3 min, whereas the core histone proteins can remain stable for hours [124]. This dynamic association is affected by a number of factors, such as posttranslational
modifications and competition with specific transcription factors or more general chromatin binding proteins for DNA binding [119, 124].

The rate of H1 movement in the nucleus can be measured by FRAP, where fluorescently tagged H1 protein is bleached in part of the nucleus and the recovery of fluorescence to that area is measured over time [125]. The higher the affinity of an H1 molecule for DNA, the longer the $T_{1/2}$, or recovery time, in FRAP.

FRAP has been used rather extensively to study the effect of H1 phosphorylation on DNA binding. Treatment of cells with the general kinase inhibitor staurosporine increases the residence time of H1 [141]. Deletion of the C-terminal tail of H1, where the phosphorylation sites are located, drastically decreases the recovery time in FRAP [141]. More specifically, H1 constructs that have phosphorylation sites mutated to lysine have increased recovery time, while phosphorylation mimic constructs that have threonine or serine sites mutated to glutamic or aspartic acid recover more quickly than wild type [141, 142]. Thus, phosphorylation is one way that histone h1 binding and function is regulated.

The binding of histone H1 to regions of DNA is also one way to determine the likelihood of expression of a particular gene. Global studies of H1 binding by ChIP-seq methods have shown that there is a loss of H1 at active transcription start sites [143]. The loss of H1 at intergenic areas may denote regulatory sites where specific factors may bind. Several studies have shown specific displacement of H1 when transcription is activated, e.g. at the MMTV promoter
as previously discussed, as well as the HTLV promoter [144, 145]. We also report the P-TEFb-specific removal of H1 from the c-fos and hsp70 genes during transcription [146, Chapter II], and from muscle-differentiation related genes in Chapter III.
Figure 1. Life cycle of HIV-1. 1. The HIV-1 virion binds to receptors on the cell surface. 2. The virion fuses with the cellular membrane, releasing its contents. 3. Viral RNA is reverse transcribed into double stranded DNA. 4. HIV-1 DNA is imported into the nucleus. 5. The provirus is integrated into cellular DNA. 6. Proviral DNA is transcribed into RNA. 7. Viral mRNAs are spliced and translated. 8. Viral proteins and RNA assemble along the plasma membrane for packaging. 9. Immature virions bud from the plasma membrane. 10. Immature viral proteins are cleaved to form mature virions. Reproduced from [147].
**Figure 2. Activation of transcription elongation by P-TEFb.** RNA polymerase II initiates transcription and transcribes for 50-100 nucleotides, then pauses due to the action of negative elongation factors. P-TEFb is recruited to the Pol II holoenzyme, where it phosphorylates Ser2 in the heptapeptide repeats of the Pol II CTD. P-TEFb also phosphorylates the DRB Sensitivity Inducing Factor, relieving its repression of Pol II and causing NELF to dissociate. Together, these actions reactivate transcription to promote the formation of full-length mRNAs.
Figure 3. Equilibrium of active and inactive P-TEFb complexes in the nucleus. P-TEFb exists in two complexes in the nucleus: one active complex, which can bind activating factors and phosphorylate Pol II, and one inactive complex bound to the snRNA 7SK and the HEXIM1 and HEXIM2 proteins. The equilibrium between these complexes can be shifted to provide more active P-TEFb under stress conditions. Treatment with Actinomycin D, UV irradiation, or HMBA cause the release of P-TEFb from the 7SK/HEXIM complex. HIV infection or Tat expression also causes a release of P-TEFb from the inactive complex, so more P-TEFb can be recruited to the HIV-1 LTR. Modified from [63].
CHAPTER II: P-TEFB PHOSPHORYLATES HISTONE H1 TO PROMOTE HIV-1 AND CELLULAR TRANSCRIPTION
**Introduction**

The transcription of viral and many eukaryotic genes is controlled at the point of mRNA elongation by positive transcription elongation factor b (P-TEFb), a complex of cyclin dependent kinase 9 (CDK9) and cyclin T1. This control has been shown in the HIV-1 genes encoding proviral peptides [37], oncogenes such as c-fos [148], and inducible genes such as hsp70 [39]. The transcription of these genes is initiated by RNA polymerase II (Pol II), but is inhibited shortly thereafter when Pol II pauses [37, 41, 149]. This pause was originally thought to be alleviated by P-TEFb, which increases the processivity of Pol II by phosphorylating its C-terminal domain [37, 38, 44]. P-TEFb hyperphosphorylates Ser 2 of the heptapeptide repeat YSPTSPS in the Pol II C-terminal domain, thus promoting the transition from abortive to productive mRNA elongation [13, 22, 150, 151].

The effect of P-TEFb on transcription of the HIV-1 genome has been well studied. During HIV transcription, Pol II processes the first 59 nucleotides of the nascent transcript, forming the trans-activation responsive region (TAR) RNA stem loop, and then pauses. TAR then recruits both P-TEFb and HIV-1 Tat protein to the long terminal repeat (LTR) promoter to hyperphosphorylate Pol II, allowing HIV transcription to proceed [58, 151-153]. Importantly, HIV mRNA transcription can be prevented by inhibiting P-TEFb, such as by siRNA-mediated
knockdown of CDK9 or treating infected cells with the potent CDK9 inhibitor, flavopiridol [63, 154, 155].

P-TEFb activity is also controlled at the protein level by shifting the equilibrium between its active and inactive complexes. The active complex consists of CDK9 and cyclin T1 alone, and the inactive complex is bound to 7SK small nuclear RNA (snRNA) and the proteins, hexamethylene bisacetamide-induced protein 1 (HEXIM1) and HEXIM2 [37, 74, 75, 77, 85]. Active P-TEFb may be favored by shifting the equilibrium between these two complexes, which can occur under cellular stresses such as UV irradiation, treatment with the transcription inhibitor actinomycin D, or expression of HIV-1 Tat [74, 77, 86, 156].

Active P-TEFb also binds multiple chromatin-binding and -modifying proteins. P-TEFb that has been freed from the 7SK/HEXIM1/2 complex binds to the bromodomain-containing protein, Brd4 [69, 70]. Brd4 recruits P-TEFb to promoters by binding both acetylated histone proteins and components of the Mediator complex [68-70], which is essential in binding transcription factors and recruiting Pol II to promoters [157, 158]. P-TEFb not only interacts with Brd4 at cellular promoters, but also binds specific transcription factors such as NFκB during cellular and HIV transcription [159, 160]. P-TEFb may also play a role in histone methylation during transcription; the budding yeast homolog of P-TEFb, Ctk1, also recruits the Set2 histone methyltransferase to Ser2-phosphorylated Pol II [161, 162]. This recruitment results in methylation of histone H3 at K4 and K36, which occur alongside elongating Pol II. These methylation events recruit
histone acetyltransferases, ATP-dependent chromatin remodeling machinery, and then deacetylases, signaling an opening and then closing of chromatin as Pol II transcribes [161, 162]. Thus, P-TEFb may play a role in coupling chromatin remodeling to transcription.

Another highly abundant chromatin-binding protein is histone H1, the linker histone that binds to nucleosomes and their linker DNA [120]. H1 facilitates the compaction of chromatin, thus maintaining chromatin patterns during differentiation and development [119, 137, 163]. In addition to its role in chromatin compaction, H1 exists in equilibrium between chromatin-bound and free states [119, 124]. Moreover, this equilibrium between free and bound H1 shifts to free H1 when it is phosphorylated [124, 126, 164, 165]. H1 is phosphorylated by CDK2 during the G1/S phase transition to promote its dissociation from DNA during DNA replication fork progression [127]. Besides this global phosphorylation event, histone H1 may be phosphorylated in the transcription of particular genes; for example H1 is phosphorylated at the MMTV promoter when transcription is activated and then dephosphorylated when transcription is inhibited [139, 140, 144, 165-167]. However, H1 phosphorylation during cellular transcription has not been examined.

To probe the relationship between P-TEFb and chromatin, we explored the relationship between P-TEFb and histone H1. We found that P-TEFb phosphorylated the C-terminal domain of histone H1 at S/TPXK sequences that are important in regulating chromatin binding [122, 127, 141]. Inhibiting or
promoting P-TEFb activity also decreased or enhanced H1 phosphorylation, respectively, in vivo. Importantly, H1 was also phosphorylated during HIV infection, but only in the presence of functional Tat. We hypothesized that phosphorylation of these S/TPXK sites by P-TEFb would likely destabilize interactions of the C-terminal tail with chromatin, thereby shifting the H1 equilibrium to promote free H1. Using fluorescence recovery after photobleaching (FRAP), we showed that inhibiting P-TEFb decreased H1 mobility, which may be correlated with stabilization of chromatin binding [168]. P-TEFb activity correlated with H1 phosphorylation as well as H1 dissociation from DNA in chromatin immunoprecipitation (ChIP) assay. Additionally, expression of a mutant histone H1.1 that is not phosphorylated by P-TEFb also inhibits Tat transactivation of the HIV-1 LTR in a reporter cell line. Because P-TEFb phosphorylation of H1 is necessary for both H1 mobility and HIV-1 transcription, we propose a new role for P-TEFb in transcription as a histone H1 kinase.

**Experimental Procedures**

**Expression and Purification of P-TEFb** Recombinant baculovirus was generated using BaculoGold™ DNA (BD Pharmingen), and the plasmid pBAC-HuCDK9-T1 was kindly provided by Dr. D. Price [22]. Sf9 cells were infected with recombinant baculovirus, incubated for 3 days, and harvested by centrifugation. The cell pellet was lysed in insect cell lysis buffer (BD Pharmingen) supplemented with insect cell protease inhibitor cocktail (BD Pharmingen). The cell lysate was centrifuged 20,000 g for 45 min. Ni-NTA beads
(Qiagen) were added to the supernatant and incubated at 4°C with constant mixing. The beads were centrifuged at 300 g and 4°C for 5 min and applied to a small screening column. The beads were washed and P-TEFb was isolated using the 6-His purification kit (BD Pharsing).

**Expression of H1.1-His variants** In order to assay CDK9 and CDK2 phosphorylation of histone H1.1, we subcloned WT, T152A, and S183A H1.1 from the pEGFP-C1 vector (gifts from M. Hendzel [125, 126, 141]) into the pQET-1 vector. The T152A/S183A H1.1 mutant was made using the Quickchange Mutagenesis kit (Stratagene). Expression of H1.1-His in BL-21 cells was induced at 25°C for 8 hr using 0.5 mM IPTG. Cells were lysed using 50 mM Tris-Cl pH 7.5, 300 mM NaCl, and 1% Triton X-100, sonicated, and centrifuged at 12,000 g for 18 min. Expression of His-tagged fusion proteins was then performed as described [75], and the HisPur kit (Pierce) was used for purification. Purified H1.1 was subsequently used as substrate for kinase assays.

**In vitro Kinase Assays** To measure the phosphorylation activity of P-TEFb in vitro, kinase assays were performed with purified P-TEFb and purified histone H1 (Upstate Biotechnologies, Lake Placid, NY) or His-H1.1 fusion proteins. For commercial histone H1 assays, P-TEFb (10 ng) was incubated for 0 to 120 min at 30°C with 1 μg histone H1 in P-TEFb kinase buffer (described above) containing a mix of 50 μCi γ32P-labeled ATP in 3.5 μM cold ATP. For His-H1.1 assays, 50 ng P-TEFb or CDK2/Cyclin A (Calbiochem) was incubated with 2 μg H1.1 for 30 min. Reactions were stopped using Laemlli buffer and run on 4-20%
gradient SDS-PAGE. Gels were exposed to phosphor-storage screens, which were scanned using a Fujifilm phosphorimager. Quantitation was performed using ImageGauge software (FujiFilm).

**Cell Culture** HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin/streptomycin (Invitrogen). Cell growth was slowed by incubation in serum-starvation medium, DMEM supplemented with 0.5% FBS and 20 mM HEPES pH 7.9, for at least 16 hr before lysis. H9 cells were maintained in RPMI medium 1640 (Invitrogen) containing 25 mM HEPES pH 7.9 and L-glutamine with 10% FBS and 100 μg/mL penicillin and streptomycin.

**SiRNA Transfections** To knock down CDK9, cells were transfected with a custom-designed siRNA (Dharmacon) perfectly matched (5’ UGACGUCCAUGUUCGAGUA 3’) to CDK9 mRNA. CDK2 was targeted using a previously designed and characterized siRNA (5’ AGUUGUACCUCUCCGGAU 3’) [169]. siRNAs (100 nM) were transfected into 4x10^5 cells/well in 6-well plates using Lipofectamine reagent (Invitrogen) in Opti-mem (Invitrogen). Transfection mixes were replaced after 4-6 hr with serum-starvation media and nuclear extracts were prepared 48 hr later.

**Transient Plasmid Transfections** For inhibition of P-TEFb in cell culture, we used pCMV-WT and D167N CDK9-HA expression constructs, obtained from Dr. X. Grana [20]. Each construct was transfected into HeLa cells using
Lipofectamine (Invitrogen), transfection mixes were replaced with serum-starvation medium, and nuclear extracts were prepared 48 hr after transfection.

For Tat transactivation experiments in MAGI, pCMV-WT, T152A, S183A, S183E and T152A/S183A GFP-H1.1 or GFP alone were cotransfected with WT or C22G Tat-RFP plasmid into MAGI cells using Lipofectamine.

**Drug and UV treatment** To inhibit CDK9 *in vivo*, serum-starved HeLa cells were treated with 1-25 nM flavopiridol or vehicle (DMSO) at a final concentration of 0.1% DMSO and incubated overnight. For UV treatment, cells were serum-starved 24-48 hr and irradiated as described previously [77]. Cells were allowed to recover for 3 hr and nuclear extracts were prepared.

**Nuclear Lysates** Nuclear lysates were prepared from cells by washing culture dishes twice with cold PBS, scraping cells from dishes, and lysing with Buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.1% Triton X-100) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Lysates were spun 5 min at 3700 g and 4°C in a microfuge and nuclear pellets were resuspended in Buffer C (30 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.5 mM DTT), incubated for 30 min with mixing at 4°C, and were spun again at 3700 g for 10 min.

**Coimmunoprecipitation** To determine interactions among proteins, nuclei were extracted from treated cells, and target proteins were coimmunoprecipitated using a Cyclin T1 antibody (Santa Cruz). Proteins from nuclear extracts (250 µg)
were brought to a total volume of 500 µL with PBS. Samples were then incubated with protein A/G beads (Santa Cruz Biotechnology) for 30 min after incubation with 2 µg anti-goat polyclonal cyclin T1 antibody (Santa Cruz Biotechnologies). Protein A/G beads were washed 3 times with PBS and boiled in Laemmli buffer.

**HIV-1 infection** To prepare virions, 293T cells were transfected using Lipofectamine 2000 (Invitrogen) with the pNL-Luc-E·R strain. At 48 hr posttransfection, supernatants were collected, clarified by centrifugation at 500 g for 5 min, and filtered through 0.2 µm filters (Pall Corporation). Virus titer was determined using a p24 capsid ELISA kit (Zeptomatrix). For infection of H9 cells, 0.5µg pNL4-3-Luc virus or DMEM (mock infection) was incubated with 8x10^5 cells/well of a 12-well plate. Cell suspensions were centrifuged at low speed for 90 min at 37°C. Cells were washed once with PBS and transferred to H9 medium. Infected cells were incubated at 37°C for 16-24 hr and lysed with M-PER lysis buffer (Pierce Biotechnology). H9 cells infected with the rtTA viral strain were also treated with 5 µg/mL doxycycline (Sigma) to activate transcription.

**Western Blotting** Aliquots of total lysate protein (1-10 µg) were separated by 4-12% SDS-PAGE, transferred to PVDF membrane using a semidry transfer apparatus (BioRad), and blocked with 5% milk in TBS-T. Membranes were blotted with anti-rabbit cyclin T1, anti-rabbit CDK9, anti-mouse histone H1 (Santa Cruz Biotechnologies), anti-mouse IgM phospho-H1 (Upstate Biotechnologies),
anti-mouse RFP (BD Pharmingen) or anti-mouse p24 (AIDS Vaccine Program). Membranes were then washed and incubated with anti-rabbit or anti mouse IgG-HRP secondary antibody (GE Healthcare) or anti-mouse IgM-HRP secondary antibody (Santa Cruz Biotechnology). SuperSignal West Dura™ HRP Substrate (Pierce Biotechnology) was used for detection. Chemiluminescence images were taken using an Imagereader LAS-3000 LCD camera (FujiFilm).

**Fluorescence Recovery After Photobleaching (FRAP)** To measure the mobility of GFP-tagged histone H1, fluorescence recovery after photobleaching was used. In experiments where CDK9 was inhibited, 2x10^5 WT GFP-H1.1 stable cells were plated onto MatTek 35 mm cell culture dishes with glass bottoms in serum-starvation medium, and 4 hr later either 100 nM flavopiridol in DMSO or vehicle alone was added to the medium for overnight incubation. In experiments with HeLa cells stably expressing WT, T152A, and S183A GFP-H1.1, the stable cell lines described above were plated in serum-starvation medium and incubated at 37°C overnight. Before assessing FRAP of cells (see next step), DMEM serum-starvation medium was replaced with CO₂-independent medium supplemented with 0.5% FBS (Gibco).

FRAP was measured at ~37°C with a Leica TSC-SP2 AOBS confocal microscope equipped with a heated 63x 1.4 NA oil objective (Leica). Cells in glass bottom dishes were mounted on a heating chamber (QE-1, Warner Instruments). Maximal Argon laser power was used to bleach half a nucleus for 2 s, and 10% power of the 488 nm laser line was used to record images. For all
experiments, 5 images were prepared for pre-bleach and 60 images for post-bleach. The first 10 images of the post-bleach were set at a 2 s interval (fast recovery fraction), and the remaining 50 images were set at an 8 s interval (slow recovery fraction). Recovery curves were normalized as described [125]. Final recovery curves represent the mean of more than 20 cells analyzed for each condition. Each recovery curve was fitted with a 2 exponential rate equation, where T_{1/2} represents the time to achieve 50% fluorescence recovery in the bleach area. Differences in mean recoveries were tested for significance by Student’s t-test.

**Chromatin Immunoprecipitation** Chromatin immunoprecipitation (ChIP) was performed as by the Imbalzano lab [170] with the following modifications. HeLa or MAGI cells were crosslinked with 37% formaldehyde for 5 min at room temperature before lysis. Fifty micrograms of lysate were used per immunoprecipitation, and were precleared for at least 2 hr with protein G agarose/ BSA and salmon sperm DNA (Millipore). Immunoprecipitations were then performed 1hr to overnight with 3 μg anti-Histone H1, clone AE-4 (Millipore), anti-rabbit pH1 (Millipore), anti-Histone H3 (Abcam), anti-GAPDH (Novex), or no antibody (mock sample) and collected using protein G Dynabeads (Invitrogen). After washing and reversing the crosslinks, DNA was isolated by phenol/chloroform extraction. H1 binding to the HIV-1 LTR, β-galactosidase, c-fos, actin, and 18s was determined by qPCR using Absolute Blue qPCR reaction mix (Abgene). Fifty nanograms of purified DNA and a primer concentration of
100nM was used for each qPCR reaction. Quantitation was performed by a \( \Delta \Delta C_t \) method: \( \Delta \Delta C_t = C_{t \text{ sample}} - C_{t \text{ input}} - C_{t \text{ mock IP}} \), and H1 enrichment = \( 2^{-\Delta \Delta C_t} \).

The following primers were used for detection of ChIP products:

LTR -80: 5' GGG AGG TAC CAG GGA GGC GTG G 3'; LTR +3: 5' CAG AGG ATC CCA GTA CA 3'

\( \beta \)-gal +1613: 5' GAT GGG TAA CAG TCT TGG CGG TT 3'; \( \beta \)-gal +1718: 5' AAT CAG CGA CTG ATC CAC CCA GT 3'

Fos -72: 5' TTG AGC CCG TCA CGT TTA CAC TC 3'; Fos +176: 5' GTT GAA GCC CGC GAA CAT CAT CG 3'

Fos +2646: 5' AGC TGG TGC ATT ACA GAG AGG AG 3'; Fos +2879: 5' GCC TGG CTC AAC ATG CTA CTA AC 3'

Actin + 101 5' GTC GAC AAC GGC TCC GGC 3'; Actin + 335 5' GGT GTG GTG CCA GAT TTT CT 3'

18s rDNA +46 5' AGC CAT GCA TGT CTA AGT ACG 3'; 18S +146 5' CAA GTA GGA GAG GAG CGA GCG ACC A 3'

**Detection of mRNAs by qPCR** After treatment of WT, T152A, S183A, S183E or T152A/S183A GFP-H1.1-expressing HeLa cells with DMSO or Flavopiridol, RNA was collected by extraction with Trizol (Invitrogen). Superscript II reverse transcriptase (Invitrogen) was used to generate cDNA from these RNA samples. QPCR was then performed using Absolute Blue qPCR reaction mix (Agene)
using primers to recognize GAPDH, c-Fos, and hsp70 cDNAs. Quantitation was performed using a ΔΔC_t method, normalizing to GAPDH signal and WT DMSO-treated samples.

The following primers were used for cDNA detection:

GAPDH FWD: 5’ GAA GGT GAA GGT CGG AGT C 3’; REV: 5’ GAA GAT GGT GAT GGG ATT TC 3’

Fos FWD: 5' GTC TCC AGT GCC AAC TTC ATT 3'; REV: 5' CCT CCT GTC ATG GTC TTC ACA 3'

Hsp70b FWD: 5' CAT CGC CTA TGG GCT GGA C 3'; REV: 5' GTC AAT GGA GAG AAC CGA CAC 3'

β-actin FWD: 5' ACT CTT CCA GCC TCC TCC 3'; REV 5' ATC TCC TTC TGC ATC CTG TC 3'

18s rRNA FWD 5’ AGC CAT GCA TGT CTA AGT ACG 3’; REV 5’ CAA GTA GGA GAG GAG CGA GCG ACC A 3’

**Beta-Galactosidase Assays** HeLa-MAGI cells were transfected with WT or C22G Tat-RFP and WT, T152A, S183A, S183E or T152A/S183A GFP-H1.1 plasmids. Cells were then lysed 48 hr later using reporter lysis buffer (Promega). Lysates (10 μg) were then incubated overnight with All-in-One beta galactosidase substrate (Pierce). Beta-galactosidase activity was measured after incubation by reading absorbance at 405 nm.
Results

P-TEFb phosphorylates H1 in vitro and interacts with H1 in vivo.

To explore the relationship between P-TEFb and histone H1, we first investigated potential substrates of P-TEFb by performing kinase microarray experiments containing known protein phosphorylation sites. Four separate array experiments showed that P-TEFb phosphorylated H1 isoforms at $S/TPXK$ consensus phosphorylation sites (Appendix I). These C-terminal $S/TPXK$ sites, which are normally phosphorylated by CDK2 during the cell cycle, are thought to be important for both C-terminal domain folding and chromatin binding [141, 171]. Because of the role of histone H1 binding in chromatin compaction [119] and the necessity of chromatin remodeling for transcription, we examined whether P-TEFb phosphorylates $S/TPXK$ sites in full-length H1 protein. Using in vitro kinase assays, we found that P-TEFb did indeed phosphorylate full-length H1 (Fig. 1A). The $K_m$ for this phosphorylation was $5.8 \pm 0.9 \, \mu M$, slightly more than the calculated $K_m$ for CDK2 in vitro phosphorylation of H1, $3.4 \, \mu M$ [172].

Next, we examined whether P-TEFb also interacted with H1 in human cells. To that end, endogenous cyclin T1 was immunoprecipitated from nuclear extracts of HeLa cells. We found that endogenous histone H1 as well as CDK9, Brd4, and HEXIM1 coimmunoprecipitated with Cyclin T1 (Fig. 1B). Importantly, Cyclin T1 did not coimmunoprecipitate CDK2. We then tested the activity of immunoprecipitated P-TEFb on H1 in a kinase assay. P-TEFb
immunoprecipitated using a Cyclin T1 antibody did phosphorylate H1 in vitro whereas beads from a goat IgG mock IP sample did not (Fig. 1C).

**P-TEFb preferentially phosphorylates the S183 phosphorylation site of histone H1.1.**

Since the above experiments showed that P-TEFb interacted with histone H1 in cells and phosphorylated H1 in vitro, we asked whether P-TEFb phosphorylated specific sites on histone H1. To that end, we examined P-TEFb phosphorylation of the H1.1 isoform of histone H1, whose C-terminus contains only two S/TPXK phosphorylation sites: Thr152 and Ser183. Thus, P-TEFb activity at one site could be examined in H1.1 mutated at the other site.

To examine which H1.1 sites P-TEFb and CDK2 preferentially phosphorylate, we purified WT, T152A, S183A, and T152A/S183A mutant His-tagged H1.1 protein and performed in vitro kinase assays. Purified histone H1.1 was incubated with either P-TEFb or CDK2/Cyclin A and a mix of unlabeled and $\gamma^{32}$P-labeled ATP, samples were run on SDS-PAGE, and phosphorylation was determined by autoradiography. P-TEFb did indeed phosphorylate T152A H1.1 but phosphorylated S183A less, while CDK2 phosphorylated S183A more than T152A H1.1 (Fig.1D, Supplemental Fig. 1). The T152A/S183A double mutant exhibited background phosphorylation for both CDK9 and CDK2. Thus both CDK9 and CDK2 exhibited specificity for different H1.1 sites in vitro.
Histone H1 Phosphorylation in Serum-starved Cells Depends on P-TEFb Activity.

Since P-TEFb interacted in vitro with histone H1, we investigated the role of P-TEFb in H1 phosphorylation in vivo. Because histone H1 is predominantly phosphorylated by CDK2 during the G1/S and G2/M phase transitions of the cell cycle [127, 171], we studied CDK9 activity during serum starvation, when CDK2 is likely to be inactive [173]. HeLa cells were therefore serum-starved to slow their growth when examining the effect of P-TEFb activity on H1 phosphorylation. P-TEFb and CDK2 activity is effectively inhibited by siRNA-mediated knockdown [63, 169]. To block the expression of CDK9 and CDK2, custom siRNAs were used to target CDK9 or CDK2 mRNA, and we examined H1 phosphorylation in cells grown in media supplemented with 10% or 0.5% FBS (Fig. 2A).

In cells grown in media containing 10% serum, H1 phosphorylation was reduced in CDK2 knockdown samples but not in mock or CDK9 knockdown cells. However, in serum-starved cells, phosphorylation of H1 was lower in cells transfected with siRNA perfectly matched to CDK9 mRNA than in CDK2 knockdown or mock-transfected cells. Notably, serum-starved cells transfected with CDK2 siRNA had H1 phosphorylation levels comparable to mock-transfected cells. To determine if other CDKs were affected by these siRNAs, we examined the expression of CDK7, a kinase that participates in transcription initiation, whose protein levels remained unchanged. Thus CDK9 knockdown
decreased phosphorylation of histone H1 independently of CDK2 in serum-starved cells.

To further study the role of P-TEFb activity in H1 phosphorylation, we inhibited CDK9 with flavopiridol, a highly specific cyclin-dependent kinase (CDK) inhibitor with IC50 values of 9 nM for CDK9 and greater than 100 nM for CDK2 and other tested CDKs [154]. Therefore, by using low concentrations of flavopiridol, we inhibited CDK9 without affecting other CDKs. Treating serum-starved HeLa cells with 5-10nM flavopiridol decreased H1 phosphorylation in a dose-dependent manner (Fig. 2B).

Similarly, we examined H1 phosphorylation after increasing P-TEFb activity *in vivo*. UV irradiation of cells, performed as previously described [73], releases P-TEFb from the 7SK complex, thus increasing active P-TEFb [75]. UV-irradiated cells showed higher levels of H1 phosphorylation than untreated cells (Fig. 2C).

The role of P-TEFb in H1 phosphorylation was also evaluated by overexpressing wild-type (WT) and dominant-negative HA-tagged CDK9. Dominant-negative CDK9, which can still bind cyclin T1, contains a D167N mutation at its catalytic aspartic acid that negates its kinase activity [25]. Overexpressing this inactive CDK9 mutant decreased H1 phosphorylation, while WT CDK9 overexpression was comparable to mock-transfected cells (Fig. 2D).
**P-TEFb phosphorylates host-cell histone H1 during HIV-1 infection.**

Given our results connecting P-TEFb activity with histone H1 phosphorylation *in vivo*, we examined this interaction in HIV-1-infected cells. P-TEFb is activated in infected cells by HIV-1 Tat, which displaces HEXIM1 from cyclin T1, thus shifting the equilibrium to active P-TEFb [86, 156]. To determine whether Tat-mediated activation of P-TEFb induces histone H1 phosphorylation, we first examined H1 phosphorylation in serum-starved HeLa cells in the presence of RFP-tagged wild-type Tat or C22G Tat, which does not bind cyclin T1 [62]. In cells expressing WT Tat-RFP, but not C22G Tat-RFP, H1 phosphorylation increased (Fig. 3A).

Because expression of Tat on its own activated P-TEFb-mediated H1 phosphorylation, we then evaluated H1 phosphorylation in H9 T cells infected with the pNL4-3 HIV-1 strain. We found much higher levels of phosphorylated H1 in HIV-1-infected cells than in mock-infected cells (Fig. 3B).

Although H1 phosphorylation could be explained by Tat enhancement of P-TEFb activity and activation of HIV-1 transcription, we wanted to ensure that this increase was not due a cellular stress response or viral integration events, as with histone H2AX phosphorylation [174]. To address this issue, we used a doxycycline-inducible rtTA strain of HIV-1, which encodes a Y26A mutant Tat that cannot interact with cyclin T1, and an inactive TAR RNA with stem-loop mutations that prevent Tat and cyclin T1 binding [175]. H9 cells were infected with either WT pNL4-3 or rtTA HIV-1, and H1 phosphorylation was evaluated.
Again, H1 phosphorylation increased in cells infected with WT HIV-1, but not in rtTA-infected or mock-infected cells (Fig. 3C).

**Blocking P-TEFb phosphorylation of histone H1 decreases its mobility in serum starved cells**

The above results show the circumstances under which H1 may be phosphorylated by P-TEFb, but not the functional significance of this phosphorylation event. Because protein phosphorylation has been suggested to modulate histone H1 mobility [126, 141, 171], we examined the effect of P-TEFb-mediated phosphorylation of H1 on its mobility in G0 cells stably expressing GFP-histone H1.1. To that end, P-TEFb activity was blocked by inhibiting CDK9 in serum-starved GFP-H1.1 HeLa cells and measuring GFP-H1.1 mobility. Cells expressing GFP-H1.1 were treated with 100 nM flavopiridol or with vehicle (dimethyl sulfoxide, DMSO), and fluorescence recovery after photobleaching (FRAP) was used the next day to measure H1 mobility. In flavopiridol-treated cells, GFP-H1.1 fluorescence recovered much more slowly (T_{1/2} = 40.6 s) than in DMSO-treated control cells (T_{1/2} = 31.4 s) (Fig. 4A). Therefore, inhibiting CDK9 not only reduced phosphorylation of H1 histone, but also its mobility in G0 cells.

Since our *in vitro* kinase assays and coimmunoprecipitation experiments (Fig. 1) showed that P-TEFb phosphorylated and cyclin T1 interacted with wild-type and T152A GFP-H1.1 but not S183A GFP-H1.1, we examined the role of these phosphorylation sites in modulating H1 mobility in resting (G0) cells. In cycling
cells, when CDK2 is active as the major H1 kinase [171], the phosphorylation-site mutant T152K GFP-H1.1 was less mobile than WT or S183K GFP-H1.1, suggesting that CDK2 phosphorylated H1.1 at T152 [141]. Therefore, we examined H1.1 mobility in G0 (serum-starved) cells when CDK2 is inactive and CDK9 is active. In G0-synchronized HeLa cells stably expressing wild-type and the two phosphorylation-site mutant GFP-H1.1 constructs, H1.1 mobility was measured by FRAP. Among the three H1.1 variants, S183A GFP-H1.1 had the slowest recovery (Fig. 4B), indicating that in G0 cells the S183 phosphorylation site is important for H1.1 mobility. This result also suggests that loss of the S183 phosphorylation site increased the stability of H1.1 binding to chromatin. The other two H1.1 variants, however, were able to move more dynamically, implying that S183 is the phosphorylation site recognized by P-TEFb in vivo.

**WT Tat expression in MAGI Cells Causes A Loss of H1 from DNA, and Flavopiridol treatment of HeLa Cells results in increased H1 binding to the c-fos and Hsp70 genes.**

Since P-TEFb activity correlated with H1 phosphorylation, we wanted to discern whether phosphorylation of histone H1 by P-TEFb released H1 from chromatin. In addition, fluorescence recovery after photobleaching (FRAP) experiments using DMSO or Flavopiridol-treated serum-starved cells had shown that P-TEFb inhibition slows GFP-H1.1 recovery and movement in the cell (Fig. 4A). To that end, we performed chromatin immunoprecipitation (ChIP) of H1 using MAGI cells
transfected with WT or C22G Tat. Binding of H1 to the HIV-1 LTR and β-galactosidase gene was quantitated by qPCR of ChIP DNA fragments. In cells transfected with WT Tat, we observed less amplification of LTR and β-gal fragments relative to cells transfected with C22G Tat, which do not express β-galactosidase (Fig 5A). However, there was no change in H1 enrichment at the β-actin or 18s rDNA genes (Fig. 5D). We also did not see a difference in histone H3 binding to the LTR and β-gal gene when we performed ChIP with an antibody to histone H3 (Fig. 5F). ChIP with an antibody directed to phosphorylated H1, which does not bind DNA with high affinity, did not show significant binding. Therefore, in MAGI cells expressing WT Tat, P-TEFb is recruited to the LTR, and less histone H1 remains bound to DNA.

In addition to H1 binding at the HIV-1 LTR, we wanted to assess H1 binding at cellular genes that require P-TEFb for transcription. By performing H1 ChIP on cells treated overnight with DMSO or Flavopiridol, we could then directly determine H1 binding during active and inactive transcription. For these experiments, we chose to examine c-fos and hsp70, immediate early genes that are sensitive to P-TEFb activity [148]. We therefore immunoprecipitated histone H1 from HeLa treated with DMSO or 25 nM Flavopiridol, and examined binding to the c-fos and hsp70 transcription start sites and coding regions. When P-TEFb is inhibited using Flavopiridol, there is more H1 bound at both the c-fos start region and downstream coding region compared to DMSO treated samples (Fig. 5B). Likewise, more H1 is bound to the hsp70 coding region when P-TEFb
is inhibited. Due to the low levels of H1 bound to the hsp70 start site we were not able to detect a significant difference in the presence of Flavopiridol. Notably, H1 enrichment does not decrease at the β-actin or 18s genes when P-TEFb was inhibited (Fig. 5D). While there was a change in H1 enrichment at the c-fos and hsp70 genes, we saw no difference in histone H3 binding (Fig. 5F). Again, pH1 ChIP did not show significant enrichment in either gene.

**Phosphorylation of histone H1.1 S183 is necessary for both transcription from the HIV-1 LTR and transcription of the c-Fos and Hsp70 genes.**

*In vitro* kinase indicated that S183 is important for P-TEFb phosphorylation. FRAP studies using GFP-Histone H1.1 taught us that P-TEFb activity in serum-starved cells can control the rate of exchange of histone H1 on chromatin, and mutation of the S183 site to alanine decreased its recovery time (Fig. 4B). Taking this data together with our ChIP data, which showed a release of H1 from sites of P-TEFb-dependent transcription, we hypothesized that S183A H1.1 expression would have a negative effect on transcription. To examine whether H1 phosphorylation plays a role in HIV transcription, we expressed WT, T152A, S183A, or S183E GFP-H1.1 and WT or C22G Tat-RFP in MAGI cells. To quantify HIV transcription, we measured β-galactosidase activity 48 hr posttransfection. While expression of WT and T152A H1.1 had no effect on Tat transactivation, expression of the S183A mutant caused a decrease in β-
galactosidase expression (Fig. 6A). The S183E mutant, which mimics phosphorylation of S183 with negative charge, also had no negative effect on β-galactosidase expression. In the presence of C22G Tat and S183E H1.1, there was little β-galactosidase expression, presumably because P-TEFb could not be recruited to Pol II by the Tat/TAR complex.

Because expression of the S183A H1.1 mutant had a negative effect on HIV-1 transcription, we wanted to assess the effect this mutant H1 isoform had on endogenous transcription controlled by P-TEFb. To that end, we isolated RNA from cells transfected with GFP tagged WT, T152A, S183A, or T152A/S183A H1.1, and performed qPCR with primer sets to detect GAPDH and c-Fos, or Hsp70, two genes whose transcription is dependent upon P-TEFb [97, 148]. After normalizing the data to GAPDH signal, we found that both c-Fos and Hsp70 transcription is lower in cells expressing S183A H1.1 relative to WT, T152A, or S183E cells (Fig 6B-C). The double mutant T152A/S183A did not demonstrate a greater reduction in c-Fos and Hsp70 transcription than the S183A mutant. Moreover, when these cells were treated with 25 nM flavopiridol, the levels of c-Fos and Hsp70 transcripts in the S183A and T152A/S183A H1.1 cells did not change, while GFP-WT, T152A, and S183E H1.1 cells were sensitive to flavopiridol. Elimination of the S183 phosphorylation site of Histone H1.1 therefore diminishes P-TEFb-regulated transcription of c-Fos and Hsp70 mRNAs.
Discussion

Our results show that the activity of P-TEFb correlates with in vivo phosphorylation of histone H1 and its dissociation from DNA. P-TEFb mediated H1 phosphorylation in a Tat-specific manner in both HeLa cells transfected with Tat and in HIV-1 infected H9 cells. Furthermore, P-TEFb phosphorylated the H1.1 isoform at a specific C-terminal phosphorylation site to enhance histone mobility and transcription from the HIV-1 LTR and the c-fos and hsp70 genes. As determined by chromatin immunoprecipitation (ChIP), active transcription from the HIV-1 LTR in MAGI cells and the c-fos and hsp70 genes in HeLa cells resulted in a release of H1 from their respective transcriptional start sites and downstream coding regions. Taken together, our results imply histone H1 is a novel substrate for P-TEFb, suggesting a new role for this elongation factor in RNA Pol II transcription.

In this study, we have identified histone H1 as a potential substrate of P-TEFb both in vitro and in cell culture. Inhibiting P-TEFb activity by specific knockdown with siRNA, chemical inhibition by flavopiridol, or overexpression of dominant-negative CDK9 all decreased H1 phosphorylation. Conversely, releasing P-TEFb from its inactive complex with 7SK and HEXIM1/2, e.g. by UV irradiation or Tat expression, increased levels of phosphorylated H1. These experiments were performed in serum-starved cells to slow their growth, ensuring that CDK9 was active while CDK2 was inactive [173]. In sum, these data imply that P-TEFb phosphorylates H1 in the cell.
In addition to the dependence of histone H1 phosphorylation on P-TEFb activity, our data also indicate that H1 is phosphorylated in a Tat-dependent manner during HIV-1 infection. Because Tat is necessary to increase H1 phosphorylation during HIV-1 infection, this phosphorylation may be important in activating HIV-1 transcription. Moreover, H1 phosphorylation did not increase in cells infected with a Tat-mutant viral strain, ruling out the possibility that H1 was phosphorylated simply as part of a cellular stress response induced by the virus during integration. Since this mutant virus transcribes host DNA independently of Tat and P-TEFb, H1 phosphorylation during HIV-1 infection may be specific to Tat- and P-TEFb-mediated transcription. Because Tat activates the transcription of cellular genes in addition to viral genes [61, 66, 67], Tat and P-TEFb may also have synergistic effects on H1 phosphorylation at both cellular and HIV genes.

It is important to note that while we characterize H1 phosphorylation as a function of P-TEFb activity, there is a possibility that this effect is indirect. However, the methods used here inhibit or activate P-TEFb independently of most CDKs. Additionally, we cannot completely rule out the activity of other kinases, such as the newly characterized CDK12, on histone H1 and Pol II under these circumstances.

Having shown that P-TEFb phosphorylates histone H1 in vitro, we asked whether a specific site on histone H1 was phosphorylated. Although P-TEFb could phosphorylate multiple H1 isoforms, we used the H1.1 isoform because its two phosphorylation sites can be studied independently. Using histone H1.1
phosphorylation-site mutants, we found that P-TEFb preferentially phosphorylates WT and T152A H1.1 \textit{in vitro}. Additionally, S183A H1.1 expression in MAGI cells prevented full transactivation of the HIV-1 LTR, while WT, T152A, and the phosphorylation mimic S183E did not affect \(\beta\)-galactosidase expression. Importantly, the S183E mutant was sensitive to C22G Tat expression because the P-TEFb complex is not recruited to the LTR to phosphorylate Pol II. HeLa cells expressing GFP-S183A H1.1 also expressed lower levels of c-Fos and Hsp70 mRNA and were insensitive to flavopiridol treatment. The S183E phosphorylation mimic, however, did not reduce c-Fos and Hsp70 transcription. S183E expressing cells were also sensitive to Flavopiridol treatment because the prior step of Pol II phosphorylation is inhibited, thereby preventing elongation.

Taken together, we surmise that P-TEFb preferentially phosphorylates S183 \textit{in vivo}. Additionally, S183A H1.1 acts as a dominant negative mutant in P-TEFb-regulated transcription. This specificity of P-TEFb for S183 contrasts with previous results, suggesting that in actively dividing cells T152 is preferentially phosphorylated by CDKs [141]. We also found that CDK2 preferentially phosphorylates WT and S183A H1.1 \textit{in vitro}. Each H1.1 site could therefore be phosphorylated under different cellular conditions and during different processes, i.e. cellular replication or transcription. Whereas H1 is phosphorylated by CDK2 on a global scale at all genes [127], we observed changes in H1 enrichment at P-TEFb-specific genes but not \(\beta\)-actin or 18S, which are constitutively expressed.
Our results also show the potential function of P-TEFb phosphorylation of H1, namely, to increase H1 dissociation from actively transcribed DNA. The movement of histone H1 is inversely proportional to the stability of H1-chromatin binding [125, 126]. Likewise, GFP-H1.1 recovered more slowly in serum-starved cells treated with Flavopiridol than in DMSO treated control cells (Fig. 4A). Since decreased mobility of H1 has been correlated with its increased binding to DNA [125, 126], P-TEFb inhibition likely stabilized H1 binding to chromatin. To test this hypothesis, we performed histone H1 ChIP assays. In WT Tat-transfected MAGI cells, we observed a loss of H1 at both the LTR transcription start site and a downstream coding region of the β-galactosidase gene compared to C22G Tat-transfected cells. Likewise, when Flavopiridol was used to inhibit P-TEFb in HeLa cells, more H1 was bound to the transcription start site and downstream coding region of the c-Fos gene than when cells were treated with DMSO alone. The differences in H1 ChIP were more pronounced than our FRAP results, most likely because the ChIP was conducted in growing cells, which have more transcription activated overall than serum-starved cells. The loss of H1 at these sites of active P-TEFb-mediated transcription does support the mechanism suggested by the H1 FRAP data, namely, that P-TEFb phosphorylation of H1 leads to its dissociation from DNA.

Upon seeing the effect of WT Tat and P-TEFb together on H1 phosphorylation, and knowing the effect of P-TEFb activity on H1 mobility and DNA binding, we wanted to ascertain the importance of H1 phosphorylation for P-TEFb-mediated
transcription. We found that S183A H1.1 expression suppresses WT Tat transactivation, c-Fos and Hsp70 transcription compared to cells expressing WT, T152A, or the phosphorylation mimic S183E H1.1. From these data, we can deduce that P-TEFb phosphorylation of histone H1 might be a necessary step in the transcription of HIV genes as well as cellular genes that need active P-TEFb in order to be expressed.

The control of gene expression via H1 phosphorylation has been noted, and regulation of the mouse mammary tumor virus (MMTV) promoter is the most well-studied example [139]. Here we show the effect of P-TEFb-mediated phosphorylation of H1 on gene expression.

Additionally, P-TEFb is known to interact with proteins that bind and modify chromatin, and we report a specific and functionally relevant interaction between P-TEFb and histone H1. P-TEFb is recruited to cellular promoters by the bromodomain-containing protein Brd4[69, 70], and phosphorylation of the Pol II C-terminal domain by the S. cerevisiae homolog of CDK9 recruits the Set2 histone methyltransferase [162, 176]. This recruitment results in H3K36 methylation, marking chromatin at sites of Pol II elongation. Taking our data into account, H1 phosphorylation could potentially aid in chromatin remodeling by allowing chromatin modifying machinery to access nucleosomes. This increased accessibility would allow Pol II to transcribe through chromatin more efficiently.

Based on our results, we propose a model in which P-TEFb phosphorylates histone H1 during Pol II transcription. When activated from the 7SK/HEXIM
complex, P-TEFb would be recruited to phosphorylate Pol II complexes thereby promoting elongation. P-TEFb would also phosphorylate H1 concomitantly with Pol II transcription, increasing the processivity of the polymerase.

In summary, here we identify histone H1 as a substrate of P-TEFb and suggest a novel role for P-TEFb during elongation. Because P-TEFb phosphorylation of H1 could destabilize the interaction of H1 with chromatin, transcribed genes may be more readily remodeled. Our results describe a new mechanism of P-TEFb function in regulating the expression of both cellular and HIV-1 genes.
Figure 1. P-TEFb phosphorylates histone H1 in vitro and interacts with H1 in vivo. A. P-TEFb phosphorylates H1 in vitro. The in vitro kinase activity of purified P-TEFb was assayed with varying concentrations of histone H1. Bands on gel correspond to phosphorylated H1. B. P-TEFb interacts with H1 in vivo. Cyclin T1 was immunoprecipitated from nuclear extracts of HeLa cells, and interaction with CDK9, Brd4, HEXIM1, CDK2, and histone H1 was assessed by Western blot. C. Immunoprecipitated P-TEFb phosphorylates H1 in vitro. P-TEFb was immunoprecipitated using a Cyclin T1 antibody, and mock IP was performed using anti-goat IgG antibody. IPs were then used in a kinase assay with purified H1. A. CDK2 preferentially phosphorylates T152 of H1.1 in vitro, while P-TEFb preferentially phosphorylates S183 of H1.1 in vitro. Recombinant P-TEFb and CDK2/Cyclin A were incubated in a kinase assay with WT, T152A, S183A, and T152A/S183A His-H1.1.
**Figure 2.** Histone H1 phosphorylation in serum-starved cells depends on the activity of P-TEFb. H1 phosphorylation was evaluated in nuclear extracts from cycling or serum-starved HeLa cells by Western blot for cyclin T1, CDK9, phosphorylated H1 (pH1), and total histone H1. **A. CDK9 silencing decreases H1 phosphorylation in serum-starved cells independently of CDK2.** Cycling and serum-starved HeLa cells were transfected with siRNAs directed against CDK2 or CDK9 mRNA or were mock transfected, and nuclear extracts were prepared 48 hr later. **B. CDK9 inhibition blocks H1 phosphorylation.** Serum-starved HeLa cells were incubated overnight with 0-10 nM flavopiridol, a potent CDK9 inhibitor, or vehicle (DMSO), and nuclear extracts were prepared 48 hr later. **C. UV irradiation increases H1 phosphorylation.** HeLa cells were serum starved overnight and then UV treated the following day. Nuclear extracts were prepared 3 hr after irradiation. **D. Expression of enzymatically inactive CDK9 inhibits H1 phosphorylation.** HeLa cells were transfected with WT or D167N CDK9-HA, and nuclear extracts were prepared 48 hr later.
Figure 3. Histone H1 is phosphorylated during HIV infection in a Tat-dependent manner. A. H1 phosphorylation is elevated in HeLa cells expressing Tat. HeLa cells were transfected with WT and C22G Tat-RFP expression constructs and serum-starved for 48 hr. Nuclear extracts were prepared and evaluated by Western blot analysis for cyclin T1, CDK9, phosphorylated H1 (pH1), total histone H1, and RFP. B. H1 is phosphorylated in T lymphocytes infected with HIV-1. H9 cells were infected with pNL4-3 HIV-1, and lysates were prepared 24 hr later. p24 capsid protein was used as a marker for HIV. C. H1 is not phosphorylated in T cells infected with Tat-mutant HIV-1. H9 T cells were infected with WT and Tat rtTA viral constructs, and lysates were prepared 48 hr later.
A

![Graph A](image)

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**Figure 4.** Blocking P-TEFb phosphorylation diminishes GFP-histone H1.1 mobility in serum-starved cells.  

**A. Inhibiting CDK9 decreases GFP-H1.1 mobility.** Serum-starved GFP-H1.1 HeLa cells were treated overnight with flavopiridol (CDK9 inhibitor) or DMSO (control), and GFP-H1.1 mobility was measured by FRAP. Upper panel shows fluorescence recovery curves. Lower panel shows average half-recovery times (T$_{1/2}$).  

**B. In serum-starved cells expressing the H1 phosphorylation-site mutant, S183A, GFP-H1.1 mobility is decreased.** HeLa cells stably expressing WT GFP-H1.1 or its phosphorylation-site mutants, T152A (specific for CDK2 phosphorylation) and S183A (specific for CDK9 phosphorylation), were serum starved overnight, and GFP-H1.1 mobility was measured by FRAP. Upper panel shows fluorescence recovery curves. Lower panel shows average half-recovery times (T$_{1/2}$).
Figure 5. Active transcription of HIV-1, c-fos, and hsp70 genes causes loss of H1 from DNA. A. P-TEFb-activated transcription from the HIV-1 LTR results in Histone H1 dissociation from the HIV-1 LTR and β-galactosidase coding region. MAGI cells were transfected with WT or C22G Tat-RFP and chromatin immunoprecipitation of Histone H1, GAPDH, or pH1 was performed 24 hr later. To detect regions of DNA bound by H1, qPCR was performed using primers to detect the transcriptional start site of the LTR and the coding region of the beta galactosidase gene. Data is reported as fold enrichment above mock IP sample, normalized to input DNA. GAPDH IP was used as a negative control.

B-C. Inhibition of P-TEFb by Flavopiridol causes an accumulation of Histone H1 at c-fos and hsp70 coding region. HeLa cells were treated overnight with 25 nM Flavopiridol, and chromatin immunoprecipitation of Histone H1 or GAPDH was performed the following day. qPCR was performed using DNA isolated from the immunoprecipitations. D. Tat expression in MAGI does not cause a release of H1 from the β-actin and 18S genes. H1 and GAPDH ChIP and qPCR were performed as described above. E. Inhibition of P-TEFb by Flavopiridol does not cause an enrichment of H1 from the β-actin and 18S genes. H1 and GAPDH ChIP was performed after HeLa cells were treated overnight with Flavopiridol. F. Histone H3 binding at the HIV-1 LTR start site and β-galactosidase coding region does not change during transcription from the LTR in MAGI cells. MAGI cells were transfected with either WT or C22G Tat-RFP and chromatin IP of histone H3 was performed 24 hr later. G.
Histone H3 binding to the *c-fos* or *hsp70* start site and coding region does not change when P-TEFb is inhibited. HeLa cells were treated overnight with DMSO or 25 nM Flavopiridol and histone H3 chromatin IP was performed the following day.
Figure 6. S183A GFP-histone H1 expression inhibits P-TEFb activation of HIV-1, c-fos, and Hsp70 mRNA transcription. A. S183A GFP-H1.1 expression in MAGI cells reduces Tat-activated HIV transcription. MAGI cells were transfected with WT or C22G Tat-RFP and WT, T152A, S183A, T152A/S183A, or S183E GFP-H1.1. Cell lysates were taken and β-galactosidase activity was measured 48 hr posttransfection. B and C. S183A GFP-H1.1 expression in HeLa cells results in reduced c-Fos (B) and Hsp70 (C) mRNA expression and insensitivity to Flavopiridol. WT, T152A, S183A, S183E, or T152A/S183A GFP-H1.1 HeLa cells were treated with DMSO or 25 nM flavopiridol overnight, and mRNAs were isolated the following day. qPCR was performed to detect c-Fos and Hsp70 mRNA normalized to GAPDH message.
CHAPTER III: P-TEFB PROMOTES SKELETAL MUSCLE DIFFERENTIATION BY ACTIVATING TRANSCRIPTION OF EARLY AND LATE GENES
Introduction

The transcription elongation factor P-TEFb (positive transcription elongation factor b) participates in the activation of expression of many genes transcribed by RNA polymerase II (Pol II). Comprised of cyclin-dependent kinase CDK9 and Cyclin T1, P-TEFb phosphorylates the heptapeptide repeat of the largest subunit of RNA Pol II, increasing its processivity during elongation [12, 14, 42]. To enhance elongation, P-TEFb also phosphorylates other substrates such as the negative elongation factors DSIF and NELF. Our previous work has also shown that P-TEFb phosphorylates histone H1 to activate transcription. When phosphorylated by P-TEFb, H1 dissociates from DNA, allowing chromatin remodeling factors to access chromatin and Pol II to progress. We examined H1 phosphorylation in the context of HIV-1 and cellular-stress related transcription, but we wanted to know whether H1 phosphorylation is a requisite for transcription of other P-TEFb-regulated systems. As a model system for differentiation regulated by P-TEFb, we induced skeletal muscle differentiation in the myoblast cell line C2C12 and examined the activity of P-TEFb.

P-TEFb is indispensable for the transcription of HIV-1 genes as well as a number of stress-related genes such as c-fos and the heat shock genes [26, 97, 100, 151]. P-TEFb activity is also necessary for the differentiation of some tissues, particularly skeletal and cardiac muscle [105, 106]. Muscle differentiation is activated by four transcription factors of the myogenic regulatory factor (MRF)
family: MyoD, MyF5, Mrf4, and myogenin [177, 178]. In a process thought to be conserved from flies to mammals, the expression of these factors in the somites of developing embryos is activated together by the Wnt signaling pathway from the neurotubule, by Sonic Hedgehog activation from the notocord, and by other signaling molecules from the ectoderm [178]. While the MRFs are expressed at slightly different times in the mouse embryo, they are thought to work in either a redundant or linear manner. When MyoD is knocked out in mice, Myf5 expression is induced fourfold more, and skeletal muscle develops normally [177, 179]. When MyF5 is depleted by RNAi, skeletal muscle also develops normally, although the mutation is lethal due to ribcage deformation [180]. In cell culture, MyoD−/− cells cannot form myotubes but they divide normally, unlike differentiating cells [181]. Of the MRFs, MyoD and Myf5 are required for cells to start differentiation, while myogenin and Mrf4 are thought to act downstream as muscle-specific transcription factors [179, 181].

In skeletal muscle, P-TEFb is recruited to differentiation-specific promoters by MyoD and MEF2, a transcription factor responsible for the expression of structural genes [106, 180, 182]. MyoD regulates more than 300 genes in skeletal muscle, and it binds E-box sites (CANNTG) within these promoters [105]. MyoD also binds the histone acetyltransferases p300 and pCAF, and their activity in turn recruits the SWI/SNF complex to remodel chromatin [181]. In experiments using the myoblast cell line C2C12, MyoD was found to coimmunoprecipitate CDK9 and Cyclin T2, and ChIP experiments showed that P-
TEFb was recruited to the muscle marker genes myogenin and muscle creatine kinase (MCK) when C2C12 cells were stimulated with differentiation media [106]. Other studies have shown the interaction of P-TEFb and the transcription factor MEF2, which recruits P-TEFb to activate RNA Pol II in a similar fashion [182]. While both Cyclin T1 and Cyclin T2 are expressed in C2C12 myoblasts, only Cyclin T2 is recruited to myogenic genes by MyoD [180, 183].

P-TEFb activity is critical to myotube formation in C2C12 cells. Overexpression of wild type CDK9 promotes differentiation of C2C12 cells, while expression of dominant negative CDK9 slows the differentiation of C2C12 from myoblasts to myotubes [106]. Additionally, in vivo data shows that knockout of the murine HEXIM1 homolog Clp-1 causes cardiac hypertrophy [103, 104]. A hypermorphic mutation of murine CDK9 results in the same phenotype [103]. In sum, regulation of P-TEFb activity is necessary for proper muscle gene expression and function.

Previously, we characterized the phosphorylation of histone H1 by P-TEFb. We found that P-TEFb phosphorylation of histone H1 is necessary for the transcription of HIV-1 genes and the cellular immediate early genes c-fos and hsp70 [168]. In addition, P-TEFb activated-transcription caused a release of histone H1 from these genes, while inhibition of P-TEFb resulted in the maintenance of H1 levels. Therefore, we hypothesize that P-TEFb could modulate myogenic gene expression in C2C12 cells. In particular, we chose to examine the early muscle marker gene and transcription factor myogenin, and
the later-expressing markers muscle creatine kinase (MCK) and myosin heavy chain (MyHC), both essential to muscle contractile function. Using H1 phosphorylation site mutants, qPCR, H1 ChIP, and MyHC immunofluorescence, we show that P-TEFb phosphorylation of H1 is an important step in myogenic differentiation.

**Experimental Procedures**

**Cell Culture** C2C12 cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and were maintained at less than 50% confluency. To differentiate, cells were grown to greater than 50% confluency, and media was changed to DMEM containing 2% horse serum (Invitrogen).

**Transfections and Drug Treatment** C2C12 cells were transfected with GFP-H1.1 constructs using Lipofectamine 2000 (Invitrogen). Transfections were plated overnight, and differentiation was induced the following day. Flavopiridol (10-25 nM) was added to cells growing in DMEM supplemented with FBS, incubated overnight, and differentiation was induced with DMEM supplemented with horse serum the following day. Flavopiridol was added to the media again during induction, which was carried out for 24 hr before RNA or protein isolation.

**qPCR** After induction, RNA samples from C2C12 cells were prepared using Trizol (Invitrogen) and chloroform extraction followed by precipitation with isopropanol. RNA samples were reverse transcribed using a Superscript kit (Invitrogen) and EF1-α, myogenin, MCK, and MyHC transcripts were quantitated
using AbsoluteBlue Sybr Green Mix (Thermo) and a Chromo 4 quantitative PCR (BioRad). Transcripts were quantified using a ΔΔC(t) method, normalizing to EF1-α.

The following primers were used to detect muscle-specific mRNAs:

EF1-α Fwd: 5’ CAA CAT CGT CGT AAT CGG ACA 3’; Rev: 5’ GTC TAA GAC CCA GGC GTA CTT 3’

Myogenin Fwd: 5’ GAG ACA TCC CCC TAT TTC TAC CA 3’; Rev: 5’ GCT CAG TCC GCT CAT AGC C 3’

MCK Fwd: 5’ CTG ACC CCT GAC CTC TAC AAT 3’; Rev: 5’ CAT GGC GGT CCT GGA TGA T 3’

MyHC Fwd: 5’ CTC CAG GCT TAA GAG GAA 3’; Rev 5’ CCT GCT CCT AAT CTC AGC ATC C 3’

**Cell lysates and Western blot** For protein samples, total cell extracts were taken after induction as described. Total cell extracts (2.5-5μg) were run on 4-12% gradient gels, and blotted using anti-Cyclin T1, anti-CDK9, anti-histone H1, anti-myogenin, anti-MCK, anti-MyHC (all from Santa Cruz), and anti-phospho-H1 (Millipore).

**ChIP** ChIP using anti-histone H1 (Millipore) and anti-histone H3 (Abcam) was performed mainly as described in Chapter II. Uninduced samples were plated on 15 cm dishes (Fisher), while induced samples were plated on 10 cm dishes, and
incubated in DMEM with 2% horse serum for 24 hr. For each immunoprecipitation, 50 µg of protein was used in a 500 µL total volume.

Absolute Blue Sybr Green PCR mix (Thermo) and 50 ng of each ChIP DNA sample were used for quantitation of binding at EF1-α, myogenin, MCK, and MyHC coding regions.

The following primers were used to detect H1 binding at muscle-specific genes:

EF1-α Fwd: 5’ TAG ATT CCG GCA AGT CCA CCA CAA 3’; Rev: 5’ TAT ACT TGC CTC AGC AGC CTC CTT 3’

Myogenin Fwd: 5’ GGC CAA GCA TTT GCA GTG GAT CTT 3’; Rev: 5’ GGT CAG ACG GCA GCT TTA CAA ACA 3’

MCK Fwd: 5’ CAA GGG CAA GTA CTA CCC TAT GAA 3’; Rev: 5’ GGC TTG TCA AAC AGG AAG TGG TCA 3’

MyHC Fwd: 5’ AGG CAT GCA CAC AGT ACA CAC AGT 3’; Rev: 5’ ATG ACT GCT GAA CTC ACA GAC CCT 3’

**Immunofluorescence.** Indirect immunofluorescence was performed as described [184]. Staining was performed using anti-MyHC (Santa Cruz) on C2C12 cells incubated with differentiation media for 24-48 hours. Differentiation was quantitated by counting MyHC-positive cells relative to DAPI-stained number cells (total cells) in a field of vision at 100X amplification on an Axioscope (Zeiss).
**Results**

**Inhibition of CDK9 activity in C2C12 cells prevents muscle marker gene expression**

To evaluate the effect of P-TEFb inhibition on the differentiation of muscle cells, we treated C2C12 cells with flavopiridol, a specific inhibitor of CDK9 and depleted CDK9 using specific siRNAs. At 25 nM, flavopiridol can inhibit CDK9 but not the cell-cycle CDKs. We therefore plated C2C12 in growth media and treated cells with 25 nM flavopiridol or transfected C2C12 with CDK9 siRNA. To induce differentiation, the media on these cells was changed to DMEM containing 2% horse serum. We allowed differentiation to proceed for 24h, and then prepared RNA extracts for qPCR. To evaluate a range of muscle-specific mRNAs, we performed qPCR for myogenin, a transcription factor that expresses shortly after induction of differentiation and activates expression of many muscle-specific genes, and muscle creatine kinase (MCK) and myosin heavy chain (MyHC), two proteins necessary for muscle function. When cells were pretreated with flavopiridol or CDK9 siRNA, the expression of these genes was not induced (Fig. 1A, Fig. 1B). The effects of flavopiridol and CDK9 depletion were similar to the siRNA-mediated depletion of myogenin. This treatment was repeated and cell lysates were prepared to examine protein levels. Western blots also showed that treatment with flavopiridol inhibits the expression of myogenin, MCK, and MyHC protein (Fig. 1C). Importantly, histone H1
phosphorylation is increased in cells placed in differentiation media, and is inhibited in flavopiridol-treated samples (Fig.1C).

**Blocking H1 phosphorylation in C2C12 cells prevents muscle marker gene expression**

Previously, we identified the likely P-TEFb-specific phosphorylation site on histone H1.1. *In vitro*, the phosphorylation of the S183A mutant by CDK9 was diminished, while the T152A mutant was phosphorylated by CDK9 but not CDK2 [168]. In addition, expression of S183A GFP-H1.1 in HeLa cells inhibited expression of c-Fos and Hsp70 in HeLa cells, and HIV-1 transcription in HeLa-MAGI cells, which contain an integrated HIV-LTR β-galactosidase reporter gene. We therefore wanted to test whether inhibiting H1 phosphorylation with these mutants would have a negative effect on differentiation of C2C12 cells. WT, T152A, S183A, T152A S183A, or S183E GFP-H1.1 constructs were expressed in C2C12 cells for 24 hours, then cells were placed in differentiation media for 24 hours. Myogenin, MCK, and MyHC mRNAs all showed a significant decrease compared to WT H1.1 when the S183A and T152A S183A mutants were expressed (Fig. 2A, 2B, and 2C). A phosphorylation mimic mutant, S183E GFP-H1.1, showed normal induction of these muscle marker genes.
**H1 is released in a P-TEFb-specific manner from muscle marker genes during differentiation**

Our previous work in HeLa and HeLa-MAGI cells suggested that H1 is released from genes during P-TEFb-regulated transcription, and that H1 phosphorylation makes it more likely to leave DNA. We wanted to test this hypothesis in the context of differentiation by performing H1 ChIP at muscle-specific marker genes in C2C12 cells. As before, we treated cells with flavopiridol overnight, then placed cells in differentiation media with flavopiridol. Cells were treated with formaldehyde, lysates were prepared, and H1 ChIP was performed. We quantified H1 binding to the coding regions of the myogenin, MCK, and MyHC genes by qPCR with specific primers. We found that when differentiation is induced with horse serum, there is a loss of H1 from all these marker genes (Fig. 3A). This effect is specific to induced genes, because H1 binding at the EF1-α gene remains unaffected. When cells are induced with horse serum and treated with flavopiridol, H1 binding is restored. As a control, we examined H3 binding with the same cellular conditions. Histone H3 binding, unlike H1, remains the same with horse serum or flavopiridol treatment (Fig. 3B).

**Flavopiridol inhibits C2C12 myotube formation**

Next, we wanted to examine the effect of P-TEFb activity on the morphology change that C2C12 cells undergo from myoblasts to myotubes. By staining for a late marker such as myosin, we could pinpoint cells that had undergone myotube
formation. Therefore, we performed immunofluorescence using an antibody directed against myosin heavy chain. By the time myosin heavy chain protein is expressed, cells are elongated into myotube form.

We also wanted to observe the effect of CDK9 inhibition on myotube formation. We treated C2C12 cells with flavopiridol and induced differentiation as described. 48 h after changing media to horse serum, we stained for MyHC. As expected, fewer flavopiridol-treated cells stained positive for MyHC, and the cells that did stain looked more like myoblasts than myotubes (Fig 4A-B).

**Discussion**

The timing of gene transcription during development and differentiation of tissues needs to be tightly controlled. To prevent the aberrant growth of different tissue types, gene expression must be activated specifically. Because of its role in elongation of transcripts with poised polymerases, we wanted to explore the role P-TEFb plays in the context of muscle development and regeneration.

We found that P-TEFb inhibition by flavopiridol treatment decreased both early and late transcription using three muscle marker genes in C2C12 cells. Furthermore, this same effect was seen when CDK9 was depleted using specific siRNAs for CDK9. After observing the dependence of muscle gene transcription on active P-TEFb, we wanted to examine the effects of P-TEFb inhibition on cell differentiation and morphology change. Flavopiridol treatment not only inhibited
the expression of MyHC in immunofluorescence experiments, but also prevented
the formation of elongated or multinucleate myotubes.

In our previous work, we characterized the phosphorylation of histone H1 by P-
TEFb [168, Chapter II]. We therefore examined H1 phosphorylation in C2C12
cells. In differentiating C2C12 cells, H1 phosphorylation is induced, but
flavopiridol treatment prevents this phosphorylation. These data suggest that,
along with activating muscle gene transcription, P-TEFb could also direct H1
phosphorylation during differentiation.

In Chapter II, we characterized a specific site on histone H1.1 that is
phosphorylated by P-TEFb. To further examine the role of H1 phosphorylation in
muscle gene transcription, we expressed GFP-WT, T152A, S183A, T152A
S183A, and S183E H1.1 in C2C12 cells. S183A and T152A S183A GFP-H1.1
expression prevented the induction of myogenin, MCK, and MyHC when cells
were placed in differentiation media. Thus, preventing H1 phosphorylation by P-
TEFb at S183 specifically inhibited transcription. H1 ChIP experiments also
showed that H1 is released from these genes during differentiation, while
flavopiridol treatment inhibits this release. Like our previous work, which studied
H1 phosphorylation at the c-fos and hsp70 genes and the HIV-1 LTR [146], we
find that H1 needs to be phosphorylated at P-TEFb-specific sites and dissociate
from DNA in order for muscle-specific transcription to proceed.

Our data provide an additional role for P-TEFb in differentiation. Other groups
had previously characterized P-TEFb as a necessary factor for muscle
differentiation [105, 106, 181], and our work identifies H1 phosphorylation by P-TEFb as a specific mechanistic step in muscle gene transcription. Original studies in C2C12 showed that overexpression of dominant negative CDK9 prevents myotube formation [106]. Similarly, we report that inhibition of CDK9 by flavopiridol results in the same phenotype. We additionally show that P-TEFb inhibition prevents the expression of genes specific to myotube formation and contractile function, and that H1 phosphorylation is required for the transcription of these genes.

The major muscle regulatory factor MyoD interacts with and recruits P-TEFb to the promoters of muscle-specific genes [105, 106]. The complex also interacts with the MEF2 family of transcription factors, providing additional activation of muscle structural genes [182]. Like the HIV-1 protein Tat, these transcription factors could draw P-TEFb out of its inhibitory complex with 7SK snRNA and Clp-1, the murine HEXIM1 homolog. Once these factors recruit P-TEFb to promoters, it is then able to phosphorylate RNA Pol II and promote transcription elongation. Our data suggest that H1 also needs to be phosphorylated to promote the transcription of these genes. Because the timing of gene expression during muscle differentiation is tightly regulated, we hypothesize that H1 phosphorylation is an important regulatory stage involved in this process.

P-TEFb has also been implicated in muscle regeneration. In experiments using skeletal muscle tissue from mice, CDK9 mRNA and protein expression is induced in satellite cells when muscle is damaged by cardiotoxin [181]. While we
have not examined muscle regeneration in this study, we would hypothesize that H1 phosphorylation is induced during regeneration after damage as well as initial tissue differentiation.

As a chromatin structural protein, histone H1 has proven essential for proper development in mammals. As pluripotent cells differentiate, the total amount of H1 in cells increases [136, 185]. Likewise, higher order packing of chromatin occurs, determining the accessibility of genes for transcription. Temporary changes in H1 binding would be beneficial for the expression of specific genes. Releasing H1 from chromatin by phosphorylation therefore would be one way to control the timing of transcription. H1 phosphorylation would help make muscle genes more accessible to chromatin binding factors and remodeling machinery, facilitating Pol II transcription through chromatin.

P-TEFb has been suggested to play a role in the differentiation cell types such as adipose tissue and hematopoiesis [170, 186, 187]. In this study we have shown the dependence of myotube differentiation on H1 phosphorylation by P-TEFb during transcription.
**Figure 1.** Treatment of C2C12 cells with flavopiridol inhibits early and late muscle marker expression.  
A. qPCR for Myogenin and MCK expression. Cells were incubated overnight in DMEM with 10% FBS with or without 25 nM flavopiridol. For siCDK9 and siMyogenin samples, 50 nM siRNA was transfected using Lipofectamine 2000. Cells were incubated with transfection mixes overnight, and the media was replaced with differentiation media (DMEM + 2% horse serum) and flavopiridol was added again (25 nM). RNA samples were isolated 24 hr later for qPCR.  
B. qPCR for CDK9 expression. C2C12 cells were mock transfected or transfected overnight with 50 nM CDK9 siRNA. Cells were then placed in differentiation media for 24 hr.  
C. Western blot for H1 phosphorylation, myogenin, MCK, and myosin heavy chain (MyHC). Cells were differentiated and treated with Flavopiridol, and total cell extracts were harvested. 10 µg total protein samples were run on 4-12% SDS PAGE.
**Figure 2.** Mutation of S183 of histone H1.1 inhibits early and late muscle marker expression. C2C12 cells were transfected with WT, T152A, S183A, T152A/S183A, or S183E GFP-H1.1 constructs and were allowed to express overnight. The following day, media was changed to DMEM + 2% horse serum, and RNAs were collected 24 hr later. 

A. Transcription of myogenin is inhibited by S183A and T152A/S183A GFP H1.1 expression. 

B. Transcription of MCK is inhibited by S183A and T152A/S183A GFP H1.1 expression. 

C. Transcription of myosin heavy chain is inhibited by S183A and T152A/S183A GFP H1.1 expression. 

D. Western blot for GFP-H1.1 expression in C2C12 cells.
**Figure 3.** Histone H1 is released from the coding regions of muscle marker genes during differentiation in a P-TEFb-specific manner.  A. H1 ChIP was performed with lysates from C2C12 cells growing in DMEM + 10% FBS, and DMEM + 2% horse serum with or without 25 nM flavopiridol. qPCR of the ChIP DNA samples was performed using primers for EF1-α, myogenin, MCK, and myosin heavy chain.  B. H3 ChIP was performed using lysates from C2C12 grown in FBS, horse serum, or horse serum with flavopiridol.
A

No Treatment

25 nM Flavo

B

% Cells expressing MyHC

No Treatment

Flavopiridol
Figure 4. Differentiation of C2C12 cells, measured by immunofluorescence of myosin heavy chain, is inhibited by flavopiridol. A. Immunofluorescence of C2C12 cells expressing MyHC. C2C12 cells were plated in DMEM + 10% FBS with or without 25 nM flavopiridol. The following day, media was changed to DMEM + 2% horse serum, and flavopiridol (25 nM) was added again. 48 hours later, immunofluorescence was performed using an antibody directed against myosin heavy chain. Images were taken using a Zeiss Axioskop fluorescence microscope at 40X magnification. B. Quantitation of MyHC-positive cells in untreated and flavopiridol-treated samples. Cells that stained positive for MyHC were counted and compared to total cells in each field to calculate percent of cells expressing MyHC. The values shown are based on the average of four independent immunofluorescence experiments.
CHAPTER IV: DISCUSSION
Discussion

The transcription of mRNA by Pol II is controlled at a number of different steps, including binding of specific and general transcription factors, Pol II recruitment, elongation factor binding, and chromatin modification and remodeling. If these requirements are not met, then transcription is blocked. The coordination of all of these processes is therefore necessary for proper gene expression. P-TEFb, like many other factors recruited to Pol II, has a multifaceted role in the regulation of Pol II transcription.

The initiation and elongation of RNA Pol II transcription is regulated by phosphorylation of the CTD of its largest subunit. At the many genes that are regulated at the step of elongation, P-TEFb is thought to be recruited to phosphorylate Ser2 of the Pol II CTD. Recent studies have suggested that other kinases, CDK12 or CDK13, can also phosphorylate Ser2 to promote elongation [15]. These kinases can work independently of P-TEFb, driving elongation by inducing changes in Pol II processivity. These recent discoveries highlight the concept that P-TEFb participates in elongation by phosphorylating additional substrates. P-TEFb is known to phosphorylate the negative elongation factor DSIF on its Spt5 subunit [44], and we have now demonstrated that P-TEFb also phosphorylates histone H1 during transcription [168].

We chose to study H1 phosphorylation by P-TEFb because of the relationship between P-TEFb and other chromatin binding and modifying factors. Brd4, a
bromodomain-containing protein that binds acetylated histone H3 and H4, is known to recruit P-TEFb to cellular genes to activate transcription [68-70]. Phosphorylation of Pol II Ser2 in mammals also recruits histone methyltransferases to methylate H3K36, marking regions of active elongation [113, 188]. P-TEFb activity precedes the action of the FACT (Facilitates Active Chromatin Transcription) complex, which is necessary for elongation through nucleosomes [189, 190]. Because phosphorylation of H1 causes its release from chromatin, it may be necessary for FACT and ATP-dependent remodeling machinery to access DNA. Since RNA Pol II needs each of these steps to proceed before moving forward, we hypothesized that histone H1 phosphorylation by P-TEFb is yet another mechanism to facilitate Pol II passage.

The number of barriers to transcription hints at the danger of aberrant transcription of genes that are normally controlled at the step of elongation. P-TEFb is recruited to activate immediate early genes such as c-fos, c-myc, and c-jun, all of which act as oncogenes when their expression is deregulated [98, 148]. The formation of the inhibitory P-TEFb/7SK/HEXIM complex and the interaction of P-TEFb with specific transcription factors help to time transcription, preventing excessive cell growth or premature differentiation. Similarly, if P-TEFb activity is deregulated, unrestricted phosphorylation of H1 could lead to uncontrolled cell growth and replication.

At what step does P-TEFb phosphorylate H1 during transcription? Because our ChIP data showed a release of H1 from both transcription start sites and coding
regions, P-TEFb most likely phosphorylates H1 as it travels with Pol II. However, it remains unclear whether H1 phosphorylation occurs before or after elongation-coupled histone methylation events. Future studies using methylation site mutants of core histone proteins would clarify this timing of events. Because H1 binds to nucleosomes and the stretches of DNA that link them, phosphorylation of H1 may be necessary for FACT and other remodeling complexes to access nucleosomes and remodel them. Our work with phosphorylation site mutants of histone H1.1 showed that unphosphorylated H1 blocks transcription [168], and because H1 cannot be removed from DNA under these conditions, remodeling factors may not access nucleosomes and Pol II cannot transcribe. P-TEFb activity also results in the recruitment of RNA processing factors. Future experiments will determine whether H1 phosphorylation is necessary for the assembly of mRNA splicing and 3’ end processing factors on Pol II.

Another question that arose during our studies was whether H1 phosphorylation occurs as a result of transcription at all genes. When we examined global changes in phospho-H1, the changes we saw suggested that H1 could be phosphorylated and released from many genes simultaneously. Indeed global ChIP-Seq studies have shown lower levels of H1 bound to the coding regions of actively transcribed genes, while intragenic regions of DNA have more H1 [191]. H1 phosphorylation had been reported previously during the transcription of specific genes. When mouse mammary tumor virus (MMTV) transcription is activated, H1 is phosphorylated. Similarly, when transcription from the MMTV
promoter is inhibited, H1 is dephosphorylated and binds to chromatin once again [139, 140, 144]. A study of HTLV transcription also showed a loss of H1 from the HTLV LTR during Tax protein-mediated transactivation [145]. Importantly, these previous studies and ours show no dependence of housekeeping genes on H1 phosphorylation. Our work supports the role of H1 phosphorylation in the transcription of specific genes and implicates P-TEFb in this process.

The dependence of a particular gene's transcription on H1 phosphorylation also depends on the extent of H1 binding along the gene. If little H1 is bound, then it is a poor obstacle to Pol II passage. Genes that are constitutively expressed tend to have low levels of H1, whereas inducible genes contain more H1 [191]. Like the presence of core histone modifications, the amount of H1 bound to the coding regions of a particular gene could be an indicator of whether P-TEFb is necessary for its transcription.

Just as transcription patterns change during differentiation, so does the chromatin environment of the cell. Histone H1 isoforms are expressed at low levels in embryonic stem cells and at higher levels in terminally differentiated cells [113]. While H1 was originally thought to be a general repressor of transcription, studies in knockout mice have shown that embryonic cells can survive with up to three isoforms of histone H1 knocked out [136, 185]. With lower concentrations of H1 in the cell, nucleosomes move closer together, shortening the length of DNA between them and compensating for the lack of H1 [163, 185]. Loss of H1 thus does not result in much change in transcription.
Therefore, the temporary loss of H1 due to posttranslational modifications may tell us more about its role in gene expression than its complete loss.

P-TEFb is also thought to play a role in differentiation-related transcription. While CDK9 depletion does not affect the viability of terminally differentiated cells, CDK9 knockout flies die during metamorphosis and knockout mice die during embryogenesis [12, 100]. This data suggested that the timing of P-TEFb activity is important for proper development. Additionally, P-TEFb interacts with a number of tissue-specific transcription factors. We found that H1 phosphorylation was necessary for MyoD-activated transcription in a myoblast cell line (Chapter 3), and that H1 was released from differentiation marker genes in a P-TEFb-specific manner. Because P-TEFb is necessary for the differentiation of other tissues such as cardiac muscle, adipose tissue, erythroid cells, or macrophages, we anticipate that H1 is phosphorylated by P-TEFb as part of the differentiation program for a number of cell types.

Throughout our studies, our efforts were to design our experiments as relevant and specific to P-TEFb activity as possible. However, there is some possibility that CDK9 phosphorylation of H1 is not direct. P-TEFb may activate some other kinase that in turn phosphorylates H1 in a transcription-dependent manner. We addressed the specificity of the phosphorylation by examining H1 in both serum-starved and cycling cells. Specific depletion of CDK9 and CDK2 inhibited H1 phosphorylation in serum-starved and growing cells, respectively. We ruled out the activities of the CTD kinases CDK7 and CDK8, which are not inhibited by
flavopiridol at the concentrations used. While CDK7 and CDK8 contain proline-directed serine or threonine phosphorylation sites [11, 192], neither have been identified as P-TEFb substrates, nor have they been shown to phosphorylate H1. There is also a possibility that the newly characterized kinases CDK12 and CDK13 or some uncharacterized kinase phosphorylates H1 under these circumstances, and future work would have to determine those interactions.

The work reported in this thesis has led us to a model describing the role of histone H1 in P-TEFb-directed transcription (Fig.1). P-TEFb is released from the inhibitory complex with 7SK and HEXIM1/2 and then recruited to cellular genes by Brd4 or other cellular transcription factors. In the case of HIV infection, Tat binds P-TEFb, recruiting it to TAR RNA at the HIV LTR. In skeletal muscle, MyoD recruits P-TEFb to E-box containing genes for muscle differentiation. P-TEFb can then hyperphosphorylate Ser2 of the Pol II CTD and Spt5. These phosphorylation events reactivate Pol II transcription, but histone H1 blocks Pol II from transcribing. To alleviate this block, P-TEFb additionally phosphorylates H1, causing it to dissociate from DNA. Once H1 leaves chromatin, other chromatin modifying and remodeling factors such as methyltransferases or the FACT complex can access nucleosomes. Once nucleosomes are remodeled, then Pol II can effectively transcribe full length mRNAs.
Figure 1. Model for H1 phosphorylation by P-TEFb during transcription. At genes regulated at the step of transcription elongation, P-TEFb is recruited to RNA polymerase II by cellular transcription factors. P-TEFb then phosphorylates Ser2 of the Pol II CTD, promoting elongation. As Pol II transcribes, histone H1 acts as a barrier to its movement. P-TEFb phosphorylates H1 as Pol II moves, allowing H1 to leave chromatin and Pol II to continue transcription.
Appendix I: Protein Purification

P-TEFb purification from SF9 cells

The construct for baculoviral expression of CDK9 and Cyclin T1 had been characterized previously. SF9 cells (2 X 10^7) were seeded in 6 15cm cell culture dishes and incubated at 25°C overnight. The media (BD Baculogold) was replaced the following day with fresh media and 2 mL P-TEFb baculoviral stock. The cells were allowed to grow in the presence of the virus for 3 days, after which the cells were spun down at 1000rpm for 5min and snap frozen in liquid nitrogen. The cells were resuspended in BD insect cell lysis buffer (BD Biosciences) and incubated on ice for 45 min. The lysates were then spun at 12,000 g and 4°C for 45 min. Ni-NTA beads (Qiagen) were added to the supernatant incubated with rocking at 4°C for at least 1 hr. The lysate and beads were then applied to a small screening column, and the beads were washed with BD His Purification wash buffer (BD Biosciences) containing 50 mM imidazole. P-TEFb was then eluted from the beads using BD His Purification elution buffer (BD Biosciences) containing 100 mM, 200 mM, 400 mM, 500 mM, or 1 M imidazole. Elution fractions were evaluated for the presence of P-TEFb by running the fractions on 10% SDS-PAGE and staining with Coomassie blue. The fractions containing P-TEFb were pooled and dialyzed in dialysis buffer (15% glycerol, 0.1 mM NaCl, 10 mM Tris-HCl pH 8) overnight. P-TEFb was
quantitated by comparing CDK9 and Cyclin T1 bands to BSA standards of known concentration on a Coomassie-stained SDS PAGE gel.

**GST-Pol II CTD Purification**

A GST-CTD-expressing construct was transformed into BL-21 *E. coli* cells and plated on LB-AMP plates overnight. One colony was taken from these plates and was used to inoculate a 5 mL LB-AMP culture. After an 8 hr incubation at 37°C, the 5 mL culture was used to inoculate a 100 mL culture of LB-AMP, and that culture was allowed to grow overnight. The 100 mL culture was then used to inoculate 2-500 ml LB-AMP cultures for induction. These cultures were grown to OD₆₀₀ ~0.6 at 30°C, induced with 0.1 mM IPTG, and grown for 8-10 hr. Cultures were then centrifuged at 5,000 rpm for 15 min at 4°C and snap frozen in liquid nitrogen. Pellets were thawed on ice and then resuspended in PI solution (50 μg/mL PMSF, 1 μg/mL Pepstatin, 2 mM EDTA, 1 mM DTT in PBS). The resuspended pellets were then sonicated and spun for 20 min at 12,000 rpm and 4°C. 10% Triton X-100 and DTT were added to the supernatant to a final concentration of 1% Triton and 1 mM DTT, and glutathione beads were added. The lysates were incubated with the glutathione beads with rocking at 4°C for at least 2 hr. The bead-lysate mix was then applied to a screening column and washed with wash buffer (1% Triton, 1 mM EDTA, 50 μg/ml PMSF, 1 mM DTT in PBS). The beads were then washed with TZ buffer (100 mM KCl, 0.5 mM EDTA, 50 mM Tris-HCl, pH 8.0, 12.5 mM MgCl₂, 1 mM DTT) and GST-CTD was eluted using 5 mM, 10 mM, 15 mM, and 20 mM glutathione in TZ buffer. Fractions were
run on 10% SDS-PAGE and Coomassie stained and presence of GST-CTD was evaluated. The fractions that contained GST-CTD were pooled and dialysed overnight in sterile dialysis buffer (15% glycerol, 0.1 mM NaCl, 10 mM Tris-HCl pH 8). GST-CTD solution was then concentrated in centricon tubes. Quantitation was performed by comparison to known quantities of BSA standard on Coomassie-stained SDS-PAGE.

**Purification of His-Histone H1**

We received the following GFP-Histone H1.1 constructs from Michael Hendzel: WT GFP-H1.1, T152A GFP-H1.1, and S183A GFP-H1.1. Using site-directed mutagenesis, we generated the T152A S183A GFP-H1.1 mutant. To examine these mutants *in vitro*, we subcloned these constructs into pQET-2, a bacterial expression vector. These plasmids were transformed into BL-21 cells and plated on LB-AMP plates. Resulting colonies were used to inoculate 5 mL LB-AMP cultures and were grown up for 8 hr at 37°C. These starter cultures were used to inoculate 100 mL cultures, which were then grown overnight. The 100 mL cultures were then added to two 500 mL of LB-AMP each and were grown to OD_{600} ~0.6 at 30°C. His-H1.1 expression was induced with 0.5 mM IPTG for 8 hr, and cultures were spun down at 5000 rpm for 15 min and snap frozen with liquid nitrogen. The pellets were thawed on ice and then resuspended in 50 mM Tris-Cl pH 7.5, 300 mM NaCl, and 1% Triton X-100. The lysates were incubated
on ice for 30 min and then sonicated using a microtip for 4 x 10 s on level 6. Lysates were then spun down for 12,000 g for 18 min, and NaCl was added to the supernatants to a final concentration of 750 mM. The supernatants were then rocked for 1 hr with Ni-NTA beads (Qiagen). The beads were then applied to a screening column and then washed with Wash Buffer A (10 mM Tris pH 7.8, 500 mM NaCl, 1% Triton X-100, 10 mM imidazole) followed by Wash Buffer B (10 mM Tris pH 7.8, 100 mM NaCl, 1% Triton X-100, 10 mM imidazole). The beads were then resuspended in Wash Buffer B without imidazole. The concentration of His-H1.1 was determined by running beads on SDS-PAGE along with BSA standards of known concentration and staining with Coomassie blue. Equal amounts of beads were used in kinase assays.

**Kinase assays using P-TEFb and GST-CTD**

To measure the phosphorylation activity of P-TEFb *in vitro*, kinase assays were performed with purified P-TEFb and GST-CTD. 40 nM P-TEFb was incubated for 0 to 30 min at 30°C with 10nM GST-CTD in P-TEFb kinase buffer (20 mM HEPES (pH7.7), 50 mM NaCl, 10 mM MgCl2, 2 mM EDTA, 0.02% Triton-X 100) containing a mix of 50 μCi γ32P-labeled ATP in 3.5 μM cold ATP. Reactions were stopped using Laemllli buffer and run on 4-20% gradient SDS-PAGE. Gels were exposed to phosphor-storage screens, which were scanned using a Fujifilm phosphorimager. Quantitation was performed using ImageGauge software (FujiFilm).
Figure 1. Protein purification from insect cells and *E. coli*. A. P-TEFb purification fractions from insect cells. Human His-tagged Cyclin T1 and CDK9 were expressed from a baculovirus vector and purified from SF9 cells using Ni$^{2+}$ beads and eluting with increasing concentrations of imidazole. B. Purification of GST-CTD from *E. coli*. GST-tagged Pol II CTD was expressed and purified from *E. coli* using glutathione-conjugated agarose beads and eluted using reduced glutathione. C. Purification of His-Histone H1.1 from E.Coli. WT, T152A, S183A, and T152A S183A His-tagged histone H1.1 constructs were expressed in *E. coli* and purified using Ni$^{2+}$ beads and eluting with increasing concentrations of imidazole. D. In vitro kinase assay with purified P-TEFb and GST-CTD.
Appendix II. Pepchip Kinase Microarray

To identify novel kinase substrates of P-TEFb, we used the Pepchip Kinase Microarray™ system (Pepscan Systems). The Pepchip microarray was incubated with a mastermix of 1X optimal P-TEFb kinase buffer (20mM Tris-HCl pH 8.0, 5mM MgCl₂, 60mM NaCl), 10% glycerol, 0.01mg/mL BSA, 0.01% Brij-35, 10µM cold ATP, 300µCi/mL γ³²P-labeled ATP, and 500ng/mL purified P-TEFb. Fifty microliters of this mastermix was pipetted onto a 25x60mm coverslip. The Pepchip slide was then lowered onto the coverslip and carefully turned over. The slide was then transferred to a Petri dish containing wet Kimwipes™ (Kimberly-Clark Corporation and was incubated for 2 hours at 30°C. The slide was washed by placing it in a 50mL conical tube containing PBS with 0.1% Triton X-100 and shaking gently for 5 minutes. The slide was then washed with 2mM NaCl containing 0.1% Triton, followed by another two washes with sterile water. The Pepchip was allowed to dry fully, was exposed to a phosphor-storage screen overnight. The screen was then scanned using an Image Reader FLA-5000 Phosphorimager (FujiFilm). The microarray was repeated with an identical Pepchip, and spots corresponding to phosphorylated sites on both Pepchip experiments were collected and identified by superimposing the Pepchip evaluation grid orientation file on the scanned images.
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Figure 2. Pepchip Kinase slide. Phosphorimager scan of Pepchip slide incubated with purified P-TEFb and $\gamma^{33}$P-ATP. Black spots denote substrates phosphorylated by P-TEFb in vitro.
Appendix III: Classification of Flavopiridol Analogs

siRNA Transfections in HeLa

As controls for P-TEFb and CDK2 inhibition, we used siRNA to knock down the expression of CDK9 and CDK2, respectively. CDK9 was targeted by a custom designed siRNA (Dharmacon) (sense sequence 5'UGACGUCCAUGUUGAGUA 3'), while CDK2 was targeted using a previously designed and characterized siRNA (5'AGUUGUACCUCCCUGGAU 3') (Du, 2004). 50-100nM siRNA was transfected into HeLa cells using Lipofectamine (Invitrogen) and RNA was collected 24-48 hours later.

Quantitative PCR

To measure P-TEFb and CDK2 activity in the presence of flavopiridol analogs, we used quantitative PCR (qPCR) to detect mRNA levels of both P-TEFb and S-phase (CDK2) activated genes. To monitor P-TEFb activity, we examined c-Fos, hsp70, and mcl-1 transcription in HeLa cells with 10nM Flavopiridol, 12i, or 12d treatment overnight. To monitor CDK2 activity, we examined cdc2 and Cyclin A transcription with 200nM Flavopiridol, 12d or 12i treatment overnight. As controls, we also measured these genes after HeLa cells were treated with 10nM or 200nM Flavopiridol, CDK9 siRNA, and CDK2 siRNA. HeLa RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer’s protocol, and 1ug of
each RNA sample was then reverse transcribed using Superscript II (Invitrogen) with random hexamer primers. The resulting cDNA samples were diluted 1:5 with nuclease free water and Sybr Green (Abgene) qPCR reactions were performed using an MJ Research PTC-200 thermocycler. Expression levels of mRNAs of interest were calculated by normalizing to GAPDH using the \( \Delta \Delta CT \) method.

The following primer sets were used to detect P-TEFb and CDK2-controlled transcripts:

**GAPDH:** forward, 5' GAAGGTGAAGGTCGGAGTC 3'; reverse 5' GAAGATGGTGATGGGATTTCC

**c-Fos:** forward, 5'GTCTCCAGTGCCAACTTCATT 3'; reverse 5' GGTCCGTGCAAGATCCTG 3'

**hsp70:** forward, 5' CATCGCCTATGGGCTGGAC 3'; reverse, 5' GTCAATGGAGAACCGACAC 3'

**mcl-1:** forward, 5' TGCTTCGAAAATGGGACATCA 3'; reverse 5' TAGCCACAAAGGCACCAAAAG 3'

**cdc2:** forward, 5' TCCACCAGATCGACTTGATCG 3'; reverse, 5' GCCCTTTCTTTAGGGTGATGC 3'

**Cyclin A:** forward, 5' CGCTGGCGGTACTGAAGTC 3'; reverse 5' AAGGAGGAACGGTGACATGC 3'
CDK9: forward, 5' AACCACGACTTTCTTCTGGTCCGA 3'; reverse 5' ATTGCAGACTCTGTTGTTGGACT 3'

CDK2: forward, 5' TGGATGAAGATGGACGGAGCTTGT 3'; reverse 5' TGGCTTGTCACATCCTGGAAGAA 3'
Figure 3. Chemical structures of flavopiridol and its analogs.
Figure 4. Flavopiridol analogue 12d specifically and potently inhibits P-TEFb-regulated genes. P-TEFb activity was examined by using qPCR to measure relative levels of c-fos, hsp70, and mcl-1 mRNA, whereas CDK2 activity was assessed by measuring relative levels of cdc2 and cyclin A mRNA. HeLa cells were treated, overnight, with 10 nm flavopiridol, 12d, or 12i, or transfected with cdk9 or cdk2 siRNAs. RNA was harvested and reverse transcribed, and c-fos, hsp70, mcl-1, cdc2, and cyclin A transcript levels were measured by qPCR. Expression levels were calculated by normalizing to GAPDH. A) The cdk9 siRNA specifically inhibited c-fos, hsp70, and mcl-1 genes with no effect on expression of cdk2, cdc2 and cyclin A; control=GAPDH. B) Flavopiridol (FP) and analogue 12d specifically inhibited the P-TEFb-regulated genes c-fos, hsp70, and mcl-1,
but had no effect on $\textit{cdc2}$ and $\textit{cyclin A}$ expression. Compound 12i did not inhibit CDK9 or CDK2 in the cells. C) Flavopiridol at high concentration significantly reduced expression of $\textit{cdc2}$ and $\textit{cyclin A}$, while 12d and 12i had no effect.
REFERENCES

31. Homo Sapiens Cyclin T1.


92. Biglione, S., et al., Inhibition of HIV-1 replication by P-TEFb inhibitors DRB, seliciclib and flavopiridol correlates with release of free P-TEFb from the large, inactive form of the complex. Retrovirology, 2007. 4: p. 47.


