# Chromatin Regulation and the Histone Code in HIV Latency

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The formation of a latent reservoir of Human Immunodeficiency Virus (HIV†) infection hidden from immune clearance remains a significant obstacle to approaches to eradicate HIV infection. Towards an understanding of the mechanisms of HIV persistence, there is a growing body of work implicating epigenetic regulation of chromatin in establishment and maintenance of this latent reservoir. Here we discuss recent advances in the field of chromatin regulation, specifically in our understanding of the histone code, and how these discoveries relate to our current knowledge of the chromatin mechanisms linked to HIV transcriptional repression and the reversal of latency. We also examine mechanisms unexplored in the context of HIV latency and briefly discuss current therapies aimed at the induction of proviral expression within latently infected cells. We aim to emphasize that a greater understanding of the epigenetic mechanisms which govern HIV latency could lead to new therapeutic targets for latency reversal and clearance cure strategies.

## INTRODUCTION

Over 30 years after the identification of Human Immunodeficiency Virus (HIV) and an intense focus on prevention research, there are still over 2 million new infections every year across the world [1]. Improved public health and education outreach programs, increased STD testing, easier access to antiretroviral drugs, and recent studies showing the effectiveness of pre-exposure prophylaxis (PrEP) have all been significant contributors to the decline in new HIV cases since the peak of the epidemic, however, a preventative vaccine remains elusive. Despite these improvements and the high efficacy of antiretroviral therapy (ART) in diverse clinical settings, it is estimat-

ed only 46 percent of persons living with HIV were on ART by the end of 2015 [1]. There continues to be a disproportionally high burden of infection in Sub-Saharan Africa, which accounts for two-thirds of new infections [1]. Even here in the United States, at the end of 2012 1 in 8 people are unaware they were living with HIV [2]. These statistics reaffirm that despite effective treatment and increasingly effective prevention methods, without a change in the status quo of tools used to respond to the HIV pandemic, the virus is and will continue to be a persistent human pathogen.

While there are still public health issues which result in the chronic HIV burden worldwide, diagnosis combined with ART compliance have resulted in the ability of HIV-infected individuals to live a normal life-span [3]. As such, the field of HIV research has taken a significant shift towards identification of a functional or sterilizing cure, defined as control of infection without need for ART or clearance of all virus from an individual respectively [4]. If achieved and implemented in combination with current prevention strategies, the potential exists to eradicate HIV globally. A significant obstacle to cure research is the latent reservoir of HIV. Established very early in infection in resting CD4+ T-cells, these latent cells are indistinguishable from healthy cells and are one source of viral rebound upon halting ART. One strategy that has emerged from new research towards an HIV cure has been popularly labeled "shock and kill" [5]. A two component system, this strategy envisions the reversal of latency to reveal the latent virus reservoir, followed by clearance of these cells by a native or engineered immune response within infected individuals [5].

The latency reversal ("shock") portion of this cure strategy has focused on inducing expression of HIV to a level detectable by immune clearance mechanisms via the use of host-targeted therapies termed latency reversal agents (LRAs) [6]. Reversal of HIV latency has focused on the two main mechanisms of transcriptional repression, restriction of critical host factors and epigenetic repression of the integrated provirus. There is a significant understanding of the host factors and mechanisms that govern successful transcription of HIV [7]. However, there is still work to be done in understanding the epigenetic mechanisms that repress the viral DNA, how the modulation of these restrictions impacts viral transcription, and if successful therapeutic induction of viral transcription via targeting of these epigenetic blocks might lead to recognition and clearance of latent cells. Here we will focus primarily on the role of the histone code in transcriptional activation and repression of HIV latency and highlight new discoveries in the field of epigenetics which may have functional relevance in transcriptional silencing. We will also briefly examine current LRAs, their mechanisms, and the effectiveness of these treat-

ments. Ultimately, we hope to emphasize that a greater understanding of the molecular mechanisms which govern HIV latency could lead to new targets for epigenetic-based LRAs for latency reversal and clearance cure strategies.

## TRANSCRIPTIONAL ACTIVATION OF HIV

The events which govern successful transcriptional activation of HIV are a well characterized cascade of events featuring both major and minor players. For the purpose of this review, we will highlight the critical factors but aim to emphasize one point: successful reactivation of latent HIV requires both the release of host factors restricted in resting CD4+ T-cell in combination with a change in the repressive chromatin structure of the integrated virus. In resting CD4+ T-cells, various host proteins critical to driving HIV transcription such as NF-kB, NFAT, and P-TEFb are sequestered or are present at low levels [8]. T-cell activation via TCR signaling can remove these restrictions to allow recruitment to the viral promoter. While only NF-kB and Sp1 are required for activation of the LTR, binding of these and other non-essential host transcription factors results in minimal but sufficient transcriptional initiation and elongation by RNA polymerase II (RNAPII) to produce the multi-spliced Tat transcript. Minimal activation of the LTR also results in unproductive RNAPII transcripts approximately 60 nucleotides in length which encode the viral non-coding RNA TAR. TAR forms into a stem-loop structure that is recognized and bound by Tat. The Tat/TAR interaction helps to initiate a positive feedback loop at the LTR via Tat-mediated recruitment of various proteins such as P-TEFb and histone acetyltransferases which aid to drive sustained productive elongation from the viral promoter. As such, successful production of Tat is a critical driver in latency reactivation [9]. For those interested, the mechanisms of viral activation have been reviewed in far greater detail in the following [9-13].

## EPIGENETIC CONTROL OF HIV TRANSCRIPTION

Concomitant with the binding of host transcription factors and the production of Tat, a sequence of events must also occur to change the local chromatin environment from a repressive to a transcriptionally permissive state. Epigenetic modifications which alter chromatin structure and transcriptional activity include DNA methylation and histone modifications. In the context of HIV, a role for DNA methylation has been long debated but looks not to be involved in transcriptional repression of HIV [14] whereas histone modifications appear to have a more functional role. A nucleosome consists of 146 base

pairs of DNA wrapped around an octamer of four histone proteins – H2A, H2B, H3, and H4 – and acts to structurally organize DNA. Histone H1, which binds linker DNA between nucleosomes, is involved in spatial compaction of nucleosomes to form higher order chromatin structure (reviewed in [15]). The role of H1 in HIV latency has been little characterized, although H1 appears to be present at the latent LTR and acts to repress Tat-mediated transcription [16,17], implicating some level of nucleosome compaction in latency. Each histone protein within the nucleosome has an unstructured tail which can be modified by various post-translational modifications (PTMs), the combination of which results in the histone code which governs the chromatin structure and transcriptional accessibility of a region of DNA [18]. PTMs found at histones tails include phosphorylation of serine and threonine and ubiquitination of lysine; however, the most studied modifications in relation to transcription are methylation and acetylation of lysine residues [19-21].

Acetylation and methylation are thought to be important in the regulation of transcription due to their effect on nucleosome stability. The basic charge of the core histone proteins can be neutralized by acetylation of lysine residues on the histone tails, resulting in destabilization of the DNA/histone interactions, increased accessibility of the local DNA, and decreased structural stability of the overall nucleosome (reviewed in [22]). Methylation of histone tails does not change the overall charge of the proteins and certain methyl marks can act to recruit chromatin regulators which further stabilize and compact the chromatin structure. Upon integration, the HIV LTR is structured by the placement of well-defined nucleosomes. Nuc-0 is positioned at the beginning of the U3 region, followed by an unbound and nuclease sensitive region (DNase hypersensitivity region 1 or DHS1) around 250bp in size which includes the critical transcription factor binding sites for NF-kB and SP1 [23,24]. Nuc-1 is located immediately after the TSS, followed by a second nuclease sensitive region (DHS2) and nuc-2 approximately 400bp downstream [23,24].

A study of global histone methylation and acetylation patterns in CD4$^+$ T-cells identified a histone "backbone," a group of 17 histone marks which individually associated with transcriptional activation, highly associated with each other, and when in combination were associated with higher gene expression [21]. These 17 marks include H2A.Z, H2BK5ac, H2BK12ac, H2BK20ac, H2BK120ac, H3K4ac, H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K9me1, H3K18ac, H3K27ac, H3K36ac, H4K5ac, H4K8ac, and H4K91ac [21]. Examination of 39 total histone lysine methylation and acetylation modifications resulted in the observation that the majority of histone lysine and acetylation marks associated with transcriptionally active euchromatin while only five marks associated

with repressive chromatin – H3K27me2, H3K27me3, H3K9me2, H3K9me3, and H4K20me3 [21].

## HISTONES MARKS AND TRANSCRIPTIONAL ACTIVATION OF HIV

### H3/H4 Acetylation

Consistent with the role of histone acetylation in the histone backbone which marks active transcription [21], one of the earliest events at the viral LTR upon activation is the recruitment of histone acetyltransferases (HATs). Proteins with known and characterized HAT activity including CBP, GCN5, and P/CAF have been observed at the LTR upon reactivation [25-28], however only CBP recruitment was shown to be Tat-independent [29]. Indeed, CBP and the closely related p300 are transcriptional coactivators of NF-kB, suggesting binding of NF-kB recruits the initial factors needed to initiate chromatin remodeling of the integrated provirus [29,30]. Recruitment of HATs has been linked to increases in global H3 and H4 acetylation at all three nucleosomes surrounding the LTR, as well as increases of the specific marks H3K9ac, H3K-14ac, H4K5ac, H4K8ac, and H4K16ac [25-29]. HATs have also been demonstrated to modulate Tat function by acetylation of Tat itself [26,31].

The importance of acetylation of lysine residues on histone tails at the LTR is emphasized by the fact that inhibitors of histone deacetylases (HDACs), the proteins responsible for removal of acetylation, have been shown to enhance reactivation from latency and are currently being tested in the clinic as LRAs [32-34]. The role of HDACs in HIV latency was first identified when HDAC1 was found in complex with transcriptional repressors YY1 and LSF at the LTR [35-37], with subsequent work identifying HDAC recruitment and the resulting histone deacetylation as the primary mechanism of transcriptional repression by this complex [38]. Since then, a variety of LTR-binding repressive proteins have been linked to HDAC recruitment [39-42]. There are currently 18 known HDAC proteins in humans divided amongst four classes based on sequence similarity (reviewed in [43]). While the members of Class I have been primarily linked to the deacetylation of histones at the LTR [44], a role for a Class II HDAC has been proposed in integration [45] and a Class III in Tat deacetylation [46], suggesting diverse roles throughout the HIV lifecycle. Studies have also described an apparent lack of significant substrate specificity amongst the HDACs, although this may depend on interacting proteins and other factors which are not apparent in biochemical studies of purified protein [43]. However, the ability to act to deacetylate multiple substrates indicates a degree of redundancy in the system which may suggest why pan-HDAC inhibitors have been one of the most effective epigenetic-targeted LRAs.

## H3 Methylation

While acetylation of both histones and Tat are critical to HIV reactivation, studies are limited with regards to other highly studied and traditionally activating marks. H3K4me at the promoter and H3K36me and K3K79me within the gene body are strongly associated with transcriptionally active genes. Interestingly, H3K4me3, a mark well associated with active transcription but mutually exclusive to H3K4ac [19,21,47] has been linked to both repression and activation of the virus. H3K4me3 was observed in association with H3K9me3 and the bispecific H3K9/H3K4 demethylase LSD1 in microglial cells and was lost upon activation [48]. Conversely, an early study of P-TEFb observed H3K4me3 levels increased upon TNF-α activation, however this was observed well within the body of Gag and not examined at the LTR [49]. H3K79me3 marks active transcription, is linked to transcriptional elongation, and methylation is dependent on initial ubiquitination of H2BK123, a modification linked to disruption of chromatin compaction [50]. Knockdown of the only known H3K79 methyltransferase DOT1L has been linked to activation of the LTR [51], suggesting this mark may be inhibitory to viral expression. To date, no work has significantly examined a role for H3K36 methylation in HIV regulation, a mark implicated exon-intron demarcation, splicing, and repression of cryptic intragenic transcription [52-55]. H3K36me2/3 has been observed within the coding region of HIV [49,56] and is proposed to be deposited via the recruitment of the H3K36 methyltransferase SETD2 by IWS1, a protein which interacts with histone chaperone SUPT6H (Spt6) [56]. It would be interesting to determine if these methylation marks conform to established mechanisms in the context of HIV, as in the case of H3K4me3 and H3K79me3 alternate functions could be exploited for latency reversal strategies.

## HISTONE MARKS AND TRANSCRIPTIONAL REPRESSION OF HIV

Of the histone marks linked to repressive chromatin, HIV latency has been associated with both H3K27 and H3K9 methylation. These two pathways of chromatin repression have long been thought to be mutually exclusive of each other. Methylation of H3K9 has traditionally been associated with the idea of constitutive heterochromatin [57-60], a term that describes the highly stable and compact chromatin found at the pericentromeric regions, telomeres, repetitive elements, repressed endogenous retrovirus elements, and transposons. These regions tend to be gene-poor and are consistently silenced areas in all differentiated cell types. In contrast, regions marked by H3K27 are termed facultative heterochromatin [57-60]. Repression by H3K27 is considered less fixed, a feature allowing plasticity and differential gene expression in ter-

minally differentiated cells. Yet, recent evidence suggests crosstalk exists between these pathways [61-63] and transcriptional control of HIV could be a potential example of this paradigm shift.

### H3K27 Methylation and Polycomb

The Polycomb group (PcG) proteins were first identified as repressors of development-related genes in *Drosophila melanogaster* (reviewed in [64]). A highly conserved mechanism also required for temporal regulation of genes during mammalian development, PcG-mediated repression is carried out by two complexes, PRC1 and PRC2. Polycomb Repressive Complex 2 (PRC2) is the sole complex which catalyzes H3K27 mono-, di-, and tri-methylation in mammalian cells (reviewed in [64,65]). PRC2 requires three core proteins to mediate H3K27 methylation – SUZ12, the chromatin reader EED, and the methyltransferase EZH2 – and can also associate with other non-essential PcG proteins which enhance enzymatic activity or targeting of the complex [64,65]. The H3K27 mark has been linked to both active and repressive chromatin. Presence of H3K27me1 in concert with H3K36me3 within a gene body is associated with transcription and refractory to further H3K27 di- and tri-methylation [66]. H3K27me2/3 are strongly linked with repressive chromatin and reduced gene expression [21,65,67]. H3K27me3 at PRC2 targeted loci is maintained via binding and reading of existing H3K27me3 by the chromodomain of EED, initiating a reinforcing loop which is proposed to help maintain the repressive mark during DNA replication [64,65]. PRC2 is also intimately linked with Polycomb Repressive Complex 1 (PRC1) to modulate chromatin structure and maintain transcriptional silencing.

PRC1 is comprised of a CBX, PCGF, RING, and PHC protein of which there are multiple homologues for each, resulting in PRC1 complexes which differ among cell types (reviewed in [68]). Regardless of composition, PRC1 functions to monoubiquitinate H2A lysine 119 (H2AK119ub1) via the ubiquitin E3 ligase activity of the RING1A/B proteins [64,65]. H2AK119ub1 has been proposed to block RNAPolII transcription, leaving it in a 'poised' state at PRC regulated genes [69]. Early mechanistic characterization in *D. melanogaster* and in mammalian cells identified a canonical recruitment pathway by which PRC2-mediated H3K27me3 is recognized by the chromodomain of the CBX reader proteins, leading to recruitment of PRC1 and ubiquitination at H2AK119 at chromatin domains already marked by H3K27me3 (reviewed in [67,68,70]). However in recent years, research has emerged suggesting the relationship between PRC1 and PRC2 recruitment appears far more complex [67,68,70]. PRC1 can also mediate chromatin compaction through a mechanism independent of histone tails,

**Table 1. Potential mechanisms for polycomb recruitment to integrated HIV.**

| Mechanisms of Polycomb Recruitment | |
|---|---|
| **CpG Islands** | Both PRC1 and PRC2 are known to co-localize with CpG islands [70]. CpG islands associate with promoter elements of active genes and remain methylation-free, in contrast to lone CpG dinucleotides which are highly subject to cytosine methylation (reviewed in [148]). The percentage of CpG sites in the HIV LTR does not meet the canonical definition of a CpG island, however studies of latent HIV in resting CD4+ T-cells suggests they are not subject to methylation [14,149,150] and furthermore that methylation of the LTR is highly repressive [151,152]. While the role for DNA methylation itself in latency is greatly debated [14,149-154], these elements may act as a marker for an intragenic promoter [155] and play a role in recruitment of PRC2. |
| **ncRNAs** | Noncoding RNAs have been identified as major regulators of gene silencing during development. Two of the most studied ncRNA mechanisms, X-chromosome inactivation and HOX gene silencing by their cognate ncRNAs XIST and HOTAIR, have defined PRC2 binding and recruitment by these ncRNAs as critical for establishment of transcriptional repression (reviewed in [156]). PRC2 core proteins EZH2, SUZ12 and accessory protein JARID2 have all been demonstrated to have RNA binding domains critical to this recruitment mechanism [157,158]. Knockdown of an HIV-expressed antisense RNA has been shown to decrease EZH2 at the LTR and increase transcriptional activation, however they were unable to establish a definitive role in a primary CD4+T-cell model [159]. |
| **H3K36 and the PCL Proteins** | Sub-stoichiometric components of the PRC2 complex, the PCL proteins recognize H3K36me3, a mark traditionally found within the gene bodies of transcriptionally active genes. The PCL proteins were shown to simultaneously recruit the H3K36 demethylase KDM2B (NO66), the H3K4 demethylase KDM5A (JARID1A), and PRC2 to drive heterochromatin formation [160-162]. This mechanism could have implications in repression of cryptic transcripts and may play a role in maintenance of latency when provirus is integrated into active genes. |

suggesting that while PTMs may aid in localization of PRC1, they are not necessary for this activity [71].

Presence of H3K27me3 as a marker of HIV latency at the promoter and the requirement for EZH2 has been observed in both cell culture and primary cell models of latency [72-75]. In the first study to characterize the role of PRC2 in HIV latency, shRNA-mediated knockdown of EZH2 strongly reactivated HIV in Jurkat-based models of latency and synergized with known T-cell activators [72]. They also demonstrated heterogeneity in the levels of H3K27me3 at the LTR was directly related to the ability of TNF-α to reactivate the virus [72], supporting the idea that different integration sites may be differentially regulated at the chromatin level which could impact the reactivation potential. Treatment of both Jurkat cells and a primary cell model of latency with the selective EZH2 methyltransferase inhibitor GSK-343 in combination with other LRAs including the HDAC inhibitor SAHA or the bromodomain inhibitor JQ1 increased levels of reactivation when compared to the individual compounds alone, suggesting H3K27 and EZH2 are active in maintenance of latency and that loss of H3K27me3 primed the LTR for reactivation [74]. This work also demonstrated for the first time that components of the PRC1 are pres-ent at the LTR during latency [74]. There have been no further studies to clearly link PRC1 and H2AK119ub1 to HIV latency; however, the presence of both Polycomb complexes at the LTR suggests the symbiotic relationship between these complexes may be relevant in maintaining latency and another potential target for development of LRAs.

Another interesting and open question is the mechanism by which PRC1/2 are recruited to the LTR. Knockdown of PRC2 components EZH2 and SUZ12 as well as pre-treatment of Jurkat cells with EZH2 inhibitors resulted in a decrease in establishment of latency using a reporter virus construct, implicating PRC2 in the earliest stages of chromatin repression of HIV [75]. In *D. melanogaster*, PcG recruitment is mediated by recognition of specific DNA sequences called polycomb repressive elements. Similar elements have not been found in mammalian cells and there is still significant debate as to the mechanisms which drive initial recruitment of PRC1/2 to target loci (reviewed in [67,68,70]). Furthermore, the preferential integration of HIV into active gene bodies [76] leads to an association of epigenetic marks of active transcription [77] which are traditionally refractory to PRC2, suggesting an active mechanism for PRC2

recruitment to the LTR. CpG islands, non-coding RNAs (ncRNAs), and H3K36 methylation have all been implicated as mechanisms for PRC2 recruitment and all have the potential to be functionally relevant in the context of latency establishment (see Table 1).

### H3K9 Methylation

Like the pattern of H3K27me, H3K9me1 is observed at the TSS of active genes while H3K9me2/3 strongly associate with constitutive heterochromatin [78]. H3K9 methylation is mediated by multiple HMTs which appear to have unique roles in the cell. SUV39H1 and SUV39H2 double knockouts show a severe impairment in H3K9me3 *in vivo* and loss of heterochromatin formation at pericentric regions, however *in vitro* methylation of unmodified H3K9 peptides suggests they can also mediate H3K9me1/me2 [79-81]. PRDM3 and PRDM16 have more recently been identified as mono-H3K9 methyltransferases which co-localize with SUV39 and constitutive heterochromatin regions [82]. In contrast, the H3K9me1/2/3 HMT SETDB1 and H3K9me1/2 HMTs G9a and GLP show distribution patterns over euchromatin regions of the genome [79,83-86]. Presence of these HMTs and addition of the H3K9me3 mark ultimately recruit heterochromatin protein 1 (HP1), of which there are three isoforms in mammalian cells, HP1α, HP1β, and HP1γ. HP1 is a chromatin reader which recognizes H3K9me3 and mediates chromatin compaction via homodimerization [87-90]. HP1α and HP1β associate with pericentric heterochromatin while HP1γ is found with euchromatin regions [91]. HP1 further helps to reinforce heterochromatin by recruiting DNA methyltransferases [92-94].

A role for H3K9 methylation in regulation of HIV was first identified when examining the role of transcriptional repressor CTIP2 in microglial cells. CTIP2 was found to repress transcription via sequestration of Tat in concert with HP1α [95]. In a follow-up study, the authors observed overexpression of CTIP2 in HEK293T cells containing an episomal LTR-luciferase reporter resulted in increased levels of H3K9me3, SUV39H1, and all three HP1 isoforms at the LTR reporter via chromatin immunoprecipitation (ChIP) [40]. In a more detailed study using integrated reporter constructs and Jurkat latency models, du Chéné et al. observed siRNA-mediated knockdown of SUV39H1 and HP1γ increased activation in TZB-bl cells, a HeLa-derived LTR-luciferase reporter line [96]. Interestingly, knockdown of HP1γ, but not SUV39H1, was able to reactivate the LTR in the absence of Tat and this activation was linked to recruitment of Sp1 [96], suggesting HP1γ likely acts to repress access to the DNA in this model. As knockdown of SUV39H1 resulted in significantly smaller levels of reactivation with Tat, this could suggest redundancy in the H3K9 pathway or that the SUV39 enzymes are not the primary H3K9

HMT in latency. A later study also demonstrated siRNA-mediated knockdown of G9a and treatment of latently infected Jurkats with the G9a/GLP specific inhibitor BIX01294 resulted in loss of G9a and H3K9me3 at the LTR via ChIP, increased transcription, and synergized with the HDAC inhibitor SAHA [97]. As G9a/GLP and HP1γ localize with euchromatin regions of DNA, it may be that SETDB1 plays a greater role as the mediator of H3K9me3 at the LTR than the SUV39 HMTs. However, a role for these epigenetic marks in primary lymphoid cells has yet to be demonstrated.

Position effect variegation (PEV), whereby introduction of a traditionally active gene into heterochromatin results in silencing of the active gene, is another phenomenon driven by repressive H3K9 methylation. Early Jurkat latency models in which some integration was found near heterochromatin may have been subject to PEV [98], however the relevance of this mechanism in primary cells with integration into active genes is debatable. Regardless, the field was further complicated by the discovery of the HUSH complex and the suggestion that this mechanism plays a role in HIV latency. The HUman Silencing Hub is comprised of H3K9me3 reader MPP8, TASOR, and periphilin and was identified by specifically searching for the proteins which were responsible for silencing of a lentiviral-GFP reporter via PEV [99]. HUSH recruits SETDB1 to mediate H3K9me3 and heterochromatin formation does not appear to be dependent on any of the HP1 proteins [99]. Knockdown of HUSH components MPP8, TASOR, and periphilin was shown to activate Jurkats transduced with an HIV-Tat-LTR reporter as well as activate four different J-Lat clones, Jurkat-based latency models [99]. Unfortunately, knockdown of SETDB1 was not tested or reported in the J-Lat lines, however SETDB1 knockdown in a latent line established in KBM7 cells of myeloid lineage did demonstrate a strong role for SETDB1 in LTR repression [99]. Further studies of the role of HUSH in HIV regulation are needed.

New work suggesting crosstalk between the H3K9 and H3K27 methylation pathways increases the potential complexity of HIV latency regulation by these pathways. While studies of this relationship are limited, one report observed PRC2 and H3K27me increased stability and binding of HP1 proteins to H3K9me3, however a direct interaction between HP1 and PRC2 components was not observed [61]. Another identified a direct interaction between PRC2 and G9a/GLP and demonstrated G9a deficient cells show impaired PRC2 recruitment at loci targeted by both PRC2 and G9a [62]. Interestingly, a recent study of the kinetics of HIV latency establishment observed viruses silenced within 3 days as measured by a fluorescent reporter showed increased H3K27me3 but no difference in H3K9me3 relative to the transcriptionally active population [75]. However, continued cultur-

ing of both the inactive and active populations for 60 days resulted in eventual silencing of 60 percent of the active population and increases in H3K27 and H3K9 in both populations [75]. The inactive population marked by early H3K27me3 and later H3K9me3 was observed to be harder to reactivate than the initial active population silenced with slower kinetics, implicating a mechanism involving both of these marks which drives the virus into a deeper latency [75]. Successful reactivation of this population may prove critical to latency reversal and clearance strategies and may depend on a more complete understanding of the interplay between H3K9 and H3K27 repression.

### H4K20 Methylation

To date, there have been no published reports implicating H4K20 methylation in regulation of HIV latency. The H4K20 mark has been linked to chromatin stability, DNA replication, DNA damage, and transcriptional repression (reviewed in [100]). H4K20me1 is observed across euchromatin and the inactivated X-chromosome [101] while H4K20me2 is found throughout chromatin with approximately 80 percent of H4 in the cell marked either exclusively or in some combination with this mark [101,102]. Consistent with an association with transcriptionally repressive chromatin [21], H4K20me3 is found co-localized with H3K9me3 at constitutive heterochromatin including pericentric heterochromatin and telomeres [101,103]. The HMT PR-SET7 (SET8) is responsible for H4K20me1 and is necessary for subsequent H4K20me2/3 by the closely related SUV4-20H1/2 enzymes [104].

H4K20me3 at constitutive heterochromatin is dependent on placement of H3K9me3 and SUV4-20H2 interaction with HP1 [101,103]. Of note, H4K20me3 has been implicated in pausing of RNAPolII in a breast cancer model [105]. Epigenetic silencing of tumor suppressor TMS1 was maintained by H4K20me3 even after inhibition of accompanying H3K9 methylation and DNA methylation at the gene [105], suggesting H4K20me3 can maintain repression independently of other repressive marks or exist in parallel to reinforce transcriptional silencing. Early studies of H4K20me1/PR-SET7 also identified this mark to be associated with silenced chromatin [104]. H4K20me1 has since been implicated in transcriptional repression via recruitment of L3MBTL1, a methyl-lysine chromatin reader protein which can compact chromatin and interacts with HP1γ [106,107]. Based on localization of H4K20me1 to the inactive X chromosome, it has been proposed to be involved in facultative heterochromatin [106]. However, studies of global patterns of histone marks and gene expression have linked H4K20me1 to active transcription, a controversy reviewed in greater detail by Beck et al. [108]. While

H4K20me1 and H4K20me3 only represent a small fraction of total H4K20 methylation, a role in transcriptional repression and a link to pathways overlapping those already identified in HIV latency exist, suggesting these marks may be worth additional study in the context of HIV latency.

## NUCLEOSOME REMODELING/HISTONE CHAPERONES

The integration of HIV into genes which are transcriptionally active in resting CD4+ T-cells suggests repressive histone PTMs must be actively maintained in the context of elongating RNAPolII. ATP-dependent nucleosome remodeling complexes and histone chaperones are known to help maintain nucleosome positioning and histone PTMs during DNA replication and transcription.

ATP-dependent remodeling complexes use ATP to move, remove, or exchange nucleosomes and are thought to function to increase or decrease accessibility to DNA. Numerous complexes exist and many contain proteins which can recognize histone PTMs, a mechanism which has been implicated in how these complexes are recruited and differentially localized (reviewed in [109]). The biochemically distinct PBAF and BAF complexes are mammalian SWI/SNF-type remodelers which have been implicated in HIV latency. BAF has been linked to transcriptional repression via positioning of Nuc-1 at a less energetically favorable DNA sequence [110] while PBAF is recruited by acetylated Tat and promotes efficient transcription [111]. BAF has been observed to be highly refractory to co-localization and co-repression with PRC2 and H3K27me [109], an interesting observation in the context of HIV latency given the strong evidence for a role for PRC2. Regardless, small molecule inhibitors of the BAF complex show promise as a new class of LRAs in both cell lines and primary cell models of latency [112].

Histone chaperones remove and deposit nucleosomes around elongating RNA polymerase to repress spurious antisense and cryptic transcription within gene bodies [113,114]. While the HIV LEDGF/p75 complex is known for its role in directing integration, additional investigation has found a post-integration role in establishing and maintaining transcriptional repression in latently infected cells. LEDGF/p75 appears in complex with SUPT6H and IWS1 at the HIV LTR, whereby the H3 histone chaperone SUPT6H appears to maintain repressive nucleosomes across the HIV promoter and throughout the coding region [115]. Similar to the mechanism of SUPT6H, the FAcilitates Chromatin Transcription (FACT) complex acts to remodel nucleosomes by disrupting histones H2A-H2B ahead of elongating RNA polymerase II (RNAPII) [116]. Knockdown of SUPT16H and SSRP1, the components of FACT, have been demonstrated to reactivate

HIV transcription [117]. This work also observed knockdown of SUPT6H, the remodeler CHD1, and histone chaperones ASF1a and HIRA resulted in viral reactivation [117]. While these proteins are typically considered positive transcription factors in the context of normal mammalian transcription, they act to repress HIV transcription. This suggests a potential mechanism similar to transcriptional interference by which normal chromatin remodeling mechanisms represses access to the integrated viral LTR, potentially via maintenance of established repressive histone PTMs.

## CURRENT AGENTS FOR LATENCY REVERSAL AND CLEARANCE

Current LRAs act either by allowing the release of host factor restriction, modulating HIV LTR chromatin structure, or perhaps in some case both, to favor transcription. Within the former is the large class of protein kinase C (PKC) agonists. Induction of the PKC signaling pathway in T-cells results in the activation of NF-kB and AP-1, both which bind the viral LTR and synergize to reactivate viral transcription [118]. Prostratin [119,120], Bryostatin 1 [121], and ingenol [122] are all small molecules which mimic diacylglycerol activation of PKC and are among the most potent LRAs. However, there are significant concerns regarding use of PKC agonist in patients. PKC agonists are non-specific, can activate the PKC pathway in multiple cell types, and can upregulate markers of T-cell activation (reviewed in [118,123]). A study in the 1990's of anti-CD3, a potent T-cell activator via activation of the T-cell receptor, demonstrated significant toxicity when used in patients [124]. This has led to a conservative approach in the clinic, and clinical experiments of this kind were not attempted until a recent study of single dose administration of Bryostatin 1 at two low concentrations showed tolerability and limited adverse effects, but failed to show PKC activation or reactivation of HIV [125].

HDAC inhibitors (HDACi) represent a class of LRAs which primarily act to modulate chromatin structure of the virus. Current HDACi are pan inhibitors which target class I and II HDACs and include three FDA approved molecules for treatment of T-cell lymphomas, Vorinostat (SAHA), Belinostat, and Romidepsin (reviewed in [126]). HDACi have been shown to induce HIV activation *in vitro* [33,38,127,128], however early clinical studies with valproic acid demonstrated no impact on the latent reservoir [129-131]. A clinical study of the more potent inhibitor Vorinostat showed induction of cell associated viral RNA [132], however recent results also demonstrate a failure to measurably decrease the viral reservoir [32]. Of note however, these clinical trials have reported minimal safety concerns regarding HDACi *in vivo*. A recent *in vitro* comparison of PKC agonists and HDACi on $CD4^+$ and $CD8^+$ T-cells reinforce these favorable results. PKC agonists Bryostatin 1 and Prostratin demonstrated high levels of T-cell activation and impaired $CD8^+$ function while HDACi showed minimal to no impact on these pathways [133]. Thus while able to modify the restrictive chromatin structure, HDACi are likely limited in the ability to alter host factor restriction and are thus less potent LRAs as compared to PKC agonists.

Bromodomain inhibitors (BETi), in clinical testing for oncology, are also being examined as LRAs and may act to both ease host factor and chromatin restrictions. Bromodomains are responsible for recognition of acetylated lysine residues and are found in a wide range of proteins including HATs, helicases, and transcriptional mediators, to name a few (reviewed in [134]). In the context of HIV, bromodomain-containing protein BRD4 is known to compete with Tat for binding of P-TEFb [135]. Testing of JQ1, a BETi with primary specificity for BRD4, but also for BRD2 and BRD3, has been shown to induce viral reactivation, presumably by removing BRD4 and increasing p-TEFb accessibility [136-139]. However, a study has also proposed JQ1 acts in a Tat-independent mechanism by inhibiting BRD2 and that BRD2 functions as a repressor of transcription at the LTR [140]. While the specific mechanisms for JQ1 activation of HIV as well as BRD2 repression remain unclear, BETi show promise as future LRAs which may move towards the clinic.

While clinical studies of single agent LRAs have not yet achieved convincing depletion of latent infection, results of such studies may be improved if LRAs are used in combination with specific viral clearance strategies [141]. However, increased LRA activity may also be needed. Mediators of NF-kB activation tested in combination with various HDACi or JQ1 *ex vivo* in resting $CD4^+$ T-cells isolated from stably suppressed individuals demonstrated increased viral reactivation as compared to single agents alone, even at suboptimal dosing of PKC agonists [142]. However, no combinations at full dose reached equivalent levels of reactivation observed via maximal PMA/Ionomycin stimulation and *in vitro* response varied between patients. In addition to these observations it has been demonstrated that maximal stimulation of resting CD4+ T-cells via small molecule agonists of T-cell activation *in vitro* fail to simultaneously reactivate all inducible proviruses [143]. They found no evidence to suggest these proviruses were integrated into a highly repressed chromatin region of the host genome, nor did they observe increases in repressive DNA methylation at the LTR, leading to the hypothesis that induction even under maximal stimulation is stochastic [143]. In this setting, the idea of stochastic reactivation is meant to mean that a given viral promoter may respond differently to the same stimuli at two moments in time, due to mo-

**Table 2.** Histone marks and the reader, writer, and eraser proteins implicated in control of HIV transcription and reactivation from latency.

| Histone Mark/Chromatin Regulator | Observed Effect | Latency Model | Reference |
|---|---|---|---|
| **Positive Epigenetic Regulators of HIV Transcription** | | | |
| HATs (p300, CBP, P/CAF, hGCN5) | Tat recruits transcriptional coactivators with HAT domains which are important in HIV activation. | LTR-CAT reporter +/- Tat in HeLa, Jurkat, and 293 cell lines (integrated and non-integrated) | [25-28] |
| H3K9ac, H3K14ac, H4K5ac, H34K8ac, H4K16ac, various HATs, total H3ac, total H4ac | Study of TPA-induced LTR activation and recruitment of the listed marks/HATs over time via ChIP. | LTR-CAT HeLa reporter (HL3T1, integrated) and U1 cell lines | [29] |
| H3K36me2 | Observed H3K36me2 in coding region upon TNF-α activation of LTR via ChIP. Implied positive regulator. | OM-10.1 cell line | [49] |
| H3K36me3 | Observed in coding region via ChIP. Implied positive regulator. | HLM107 cell line | [56] |
| pBAF | pBAF is important in Tat-mediated transcriptional activation of viral LTR. | TZM-bl, productively infected PMBCs | [111] |
| **Negative Epigenetic Regulators of HIV Transcription** | | | |
| H3K4me3, H3K9me, LSD1 | Decreased LSD1, H3K9me3, and H3K4me3 associated with increase in viral transcription. | Human microglial cells and U1 cell line | [48] |
| Total H3ac, Total H4ac, HDACs | HDAC recruitment and loss of H3/H4 acetylation results in transcriptional repression. | Various, including primary resting CD4+ T-cells from durably suppressed donors | [32,33, 35-37,44] |
| H3K79me2, DOT1L | siRNA knockdown of H3K79 methyltransferase DOT1L and decreased H3K97me2 associated with increase in LTR-driven transcription. | HeLa cells with integrated LTR-Luciferase reporter | [51] |
| H3K27me3, PRC2 | Decrease in H3K27 methylation via siRNA and small molecule targeting of PRC2 components results in increased viral transcription. | Various Jurkat-derived latency reporter cell lines, primary T-cell models | [72-75] |
| PRC1 | Observed PRC1 components at LTR during latency. Implied negative regulator. | Jurkat-derived 2D10 reporter line, primary T-cell model | [74] |
| H3K9me3, SUV39H1, HP1α/β/γ, CTIP2, HDAC1/2 | Repressor CTIP2 recruits HDAC1/2, SUV39H1, and HP1 to the viral LTR, resulting in increased H3K9me3. | Microglial, 293T, and HeLa lines with integrated or episomal LTR-Luciferase reporter, U1 cell line | [40,95] |
| H3K9me3, SUV39H1, HP1γ | siRNA knockdown of H3K9 methyltransferase SUV39H1 and reader HP1γ results in decreased H3K9me3 and increased H3ac and viral transcription. | LTR-Luciferase reporter in HeLa (integrated and non-integrated/transient) | [96] |
| H3K9me3, G9a | siRNA and small molecule targeting of G9a results in loss of G9a and H3K9me3 at LTR and increased viral transcription. | LTR-Luciferase reporter, Ach2, OM-10.1 cells lines | [97] |
| HUSH Complex, SETDB1 | Knockdown of HUSH complex (H3K9me3-mediated PEV) components results in viral reactivation. | Jurkat LTR-Tat-GFP reporter, J-Lat models, and myeloid latency model | [99] |
| Histone Chaperones (SUPT6H, FACT, CHD1, ASF1a, HIRA) | Knockdown of various histone chaperones promotes viral reactivation. | J-Lat models | [115,117] |
| BAF | Knockdown of BAF complex components promotes viral reactivation. | J-Lat models and LTR-Luciferase reporter | [110] |

lecular, temporal fluctuations in precise biochemical and biophysical state of the promoter. However, such precise observations as to the state of the local chromatin and the repression of the DNA structure surrounding an individual integrated provirus cannot yet be assessed in cells from infected individuals due to rarity of such cells *in vivo*, and the difficulties with generating a truly representative *in vitro* model of latency [144].

## CONCLUSIONS

The proposal of the histone code came 16 years after the identification of HIV as the causal agent of AIDS, yet this field of research has become integral towards understanding of the mechanisms which govern HIV latency (see Table 2). New advances in the understanding of chromatin regulation must force us to constantly re-examine these pathways in the context of HIV latency. The code of histone PTMs that recruit chromatin readers, writers, and erasers to modulate transcriptional activity has grown more complex as the interplay and crosstalk between these pathways becomes apparent. Furthermore, the nature of our understanding of chromatin structure and how it relates to transcription is also changing, as recent studies suggest the highly compact 30nm fiber previously thought to characterize heterochromatin does not exist *in vivo* but is rather formed by irregular folding of 10nm fibers (reviewed in [145,146]). There is also evidence that the 3D position of integrated HIV DNA with respect to heterochromatic regions on neighboring chromosomes may also impact reactivation potential [147]. Rather than view chromatin in a binary open or closed fashion, this suggests a more dynamic state for chromatin which is in large part governed by the histone code.

In the context of HIV latency, histone PTMs may act individually or in concert to pause RNA Pol II or mediate some level of nucleosome compaction to repress access to the integrated provirus. Presence of multiple histone marks such as H3K27me3, H3K9me3, H2AK119ub1, and/or H4K20me3 may act to layer repressive signals, each of which may need to be removed in order to lower the activation barrier for productive transcription. This would be critical for production of viral antigen needed for the majority of clearance strategies. There is also the possibility of a temporal component, whereby the long-term latency in the context of a stably ART-suppressed individual may reinforce the repressive chromatin structure and drive the integrated provirus into a state which is harder to reactivate. For each of these potential mechanisms, there also exists the likelihood of significant heterogeneity between cells within an individual, a factor which may account for the inability to fully reactivate the latent reservoir. Continued work towards understanding the main epigenetic drivers of HIV latency and how they fit into the context of normal host cell regulation will provide greater insight into the relationship between chromatin and transcriptional activity and will allow a targeted approach for LRA development. In the future this may allow for rational design of treatment regimens to unmask the latent reservoir and result in effective viral clearance towards an HIV cure.

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