JOURNAL OF VIROLOGY, Sept. 2011, p. 9078–9089 Vol. 85, No. 17
0022-538X/11/$12.00 doi:10.1128/JVI.00836-11

Epigenetic Silencing of HIV-1 by the Histone H3 Lysine 27 Methyltransferase Enhancer of Zeste 2[▽]

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Received 23 April 2011/Accepted 22 June 2011

Latent HIV proviruses are silenced as the result of deacetylation and methylation of histones located at the viral long terminal repeat (LTR). Inhibition of histone deacetylases (HDACs) leads to the reemergence of HIV-1 from latency, but the contribution of histone lysine methyltransferases (HKMTs) to maintaining HIV latency remains uncertain. Chromatin immunoprecipitation experiments using latently infected Jurkat T-cell lines demonstrated that the HKMT enhancer of Zeste 2 (EZH2) was present at high levels at the LTR of silenced HIV proviruses and was rapidly displaced following proviral reactivation. Knockdown of EZH2, a key component of the Polycomb repressive complex 2 (PRC2) silencing machinery, and the enzyme which is required for trimethyl histone lysine 27 (H3K27me3) synthesis induced up to 40% of the latent HIV proviruses. In contrast, there was less than 5% induction of latent proviruses following knockdown of SUV39H1, which is required for H3K9me3 synthesis. Knockdown of EZH2 also sensitized latent proviruses to external stimuli, such as T-cell receptor stimulation, and slowed the reversion of reactivated proviruses to latency. Similarly, cell populations that responded poorly to external stimuli carried HIV proviruses that were enriched in H3K27me3 and relatively depleted in H3K9me3. Treating latently infected cells with the HKMT inhibitor 3-deazaneplanocin A, which targets EZH2, led to the reactivation of silenced proviruses, whereas chaetocin and BIX01294 showed only minimal reactivation activities. These findings suggest that PRC2-mediated silencing is an important feature of HIV latency and that inhibitors of histone methylation may play a useful role in induction strategies designed to eradicate latent HIV pools.

Current highly active antiretroviral therapies (HAARTs) for HIV infection rely on cocktails of potent antiviral drugs to reduce virus in the peripheral circulation to below detectable levels (74). Unfortunately, this regimen fails to eradicate the virus. Even after decades of effective HAART, high levels of virus replication invariably resume when antiretroviral treatment is interrupted (13, 17). The viral rebound appears to be due to reactivation of virus from a long-lived pool of latently infected cells (21). Genetic evidence strongly suggests that the latent virus resides primarily in the pool of resting memory $CD4^+$ T cells, since both the residual virus recovered from treated patients (7) and the rebounding virus recovered during the short treatment interruptions (31) have much greater sequence homogeneity than would be expected for a viral population replicating at low levels. Eliminating the latent reservoir is particularly challenging since the reservoir is established early during infection (12), is extremely stable, with an estimated half-life of 44 months (64), and can be replenished during episodes of viremia (14) or by homeostatic replacement of latently infected cells (11). Since intensification of antiviral regimens has essentially no impact on eradicating the latent pool from the infected host (18), there is a pressing need to develop entirely novel forms of therapy that purge the pool of latent proviruses (62, 69).

Latent HIV infections arise when the expression of the viral *trans*-activator protein, Tat, which stimulates transcriptional elongation, is restricted leading to ineffective HIV transcription (reviewed in reference 35). The formation of chromatin blocks to HIV transcription are believed to be one of the primary events leading to Tat restriction and the silencing of HIV proviruses (reviewed in references 27 and 54). Although most latent HIV-1 proviruses are found integrated into actively transcribed genes (28, 48), latent HIV-1 proviruses characteristically acquire heterochromatic structures. Typically, the long terminal repeats (LTRs) of latent proviruses accumulate high levels of histone deacetylases (HDACs) and deacetylated histones (71, 75, 79), as well as methylated histones (19, 53, 60, 72). In addition to histone methylation, hypermethylation of CpG islands near the HIV-1 promoter correlates with silencing of HIV-1 transcription in both Jurkat cells and primary isolates from HIV-1-positive aviremic patients (4, 36). Treatment of these cells with the DNA methylation inhibitor 5-aza-deoxycytidine (5-aza-CdR) leads to enhanced reactivation and outgrowth of silenced proviruses (4, 36). Additional mechanisms that can contribute to HIV-1 silencing include transcriptional interference when the viruses have integrated into actively transcribed genes (29, 47). However, even in these circumstances the latent proviruses also acquire restrictive chromatin structures that are essential for the maintenance of HIV-1 latency (36, 60). Thus, epigenetic silencing of HIV-1 proviruses appears to be a general feature of all HIV-1 infections, which

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▽ Published ahead of print on 29 June 2011.

has been documented in transformed cells (32, 43, 60), *ex vivo* primary cell models for HIV-1 latency (4, 72), and latently infected cells obtained from patients (79).

Additional blocks to HIV-1 transcription initiation and elongation found in resting $CD4^+$ T cells ensure that latent proviruses remain transcriptionally silenced for long periods. Crucially, in resting cells the HIV-1 transcription initiation factors NF-κB (36, 76) and NFAT (5, 39) are sequestered in the cytoplasm, while the essential Tat cofactor, P-TEFb, is largely sequestrated into an inactive RNP complex (58, 78).

Despite these multiple restrictions, stimulation of cellular replication by drugs, cytokines, or by T-cell receptor activation provides a powerful signal leading to the resumption of HIV-1 transcription, virus production, and spread. Typically, proviral reactivation is dependent upon association of NF-κB and/or NFAT with the viral LTR. These transcription initiation factors act by directing recruitment of the histone acetyltransferases (HATs) p300, CBP-associated factor (PCAF), and hGCN5 to the HIV-1 LTR, which acetylatse histones near the promoter (51). The acetylated histones provide a signal for the recruitment of the chromatin remodeling complex BAF, which activates transcription by displacing the restrictive nucleosome 1 (Nuc-1) positioned immediately downstream from the transcriptional start site (52). Remarkably, the requirement for NF-κB and/or NFAT can be bypassed by treating latently infected cells with histone deacetylase inhibitors (HDACi), such as trichostatin A (TSA), valproic acid, and suberoylanilide hydroxamic acid (SAHA) (1, 16).

Previous reports have shown that latent HIV-1 proviruses carry methylated histone H3 that has been trimethylated on either lysine 9 (H3K9me3) or lysine 27 (H3K27me3) (19, 53, 60) or dimethylated on lysine 9 (H3K9me2) (30). Each of these modified histones are considered repressive marks (40). SUV39H1, which is the histone lysine methyltransferase (HKMT) responsible for synthesizing H3K9me3, has been implicated in maintaining HIV-1 latency in microglial cells because of its interactions with CTIP-2 and HP1γ (19, 53). In these systems, knockdown of either CTIP-2 or HP1γ proteins led to activation of HIV-1. Similarly, Imai et al. (30) proposed that the HKMT G9a, which is responsible for creating dimethyl H3K9, can also contribute to the maintenance of HIV-1 latency.

The HKMTs SUV39H1 and G9a are both associated with the formation of constitutive heterochromatin during development (reviewed in reference 23). In contrast, most inducible genes and many viral promoters are subject to silencing through the formation of facultative heterochromatin (reviewed in reference 68). Facultative heterochromatin is typically created by the Polycomb repressor complex 2 (PCR2) (8, 55), which mediates gene silencing through the posttranslational modification (PTM) of histones. The PRC2 complex is responsible for the methylation (di- and tri-) of Lys 27 of histone H3 (H3K27me2/3) through its subunits EZH1 and EZH2. We therefore hypothesized that PRC2, and specifically the enzymatically active EZH2 subunit, might also make a significant contribution to HIV-1 latency. Here we demonstrate that EZH2 is found at the promoter of latent HIV-1 proviruses in T cells together with the corresponding H3K27me3. Knockdown of EZH2 with shRNA, or inhibition of EZH2 with chemical inhibitors, efficiently reactivates a significant portion of silenced proviruses, indicating that silencing induced by PCR2/EZH2 is one of the commonly used mechanisms to restrict HIV-1 transcription during latency.

MATERIALS AND METHODS

Cell lines and tissue culture reagents. Latently infected Jurkat E4 and G4 cells were described previously (58). Cells were maintained in HyClone RPMI medium with L-glutamine, 10% fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) in 5% CO_2 at 37°C. Cells used as the mixed population were obtained by infecting Jurkat clone E6 with lentivirus made in 293T cells by cotransfection with the G protein of vesicular stomatitis virus (VSV-G), pdR8.91, and a lentiviral vector containing Nef and d2EGFP (55). Cells were maintained for 1 month after infection, and decay of d2EGFP expression was followed by sequential fluorescence-activated cell sorter (FACS) analyses.

shRNA constructs and infections. Lentiviral vectors carrying shRNA inserted into the pLKO.1 backbone (negative [catalog number RHS4080], SUV39H1 [clone ID TRCN0000150622], and EZH2 [clone ID TRCN0000040074]) were obtained from Open Biosystems. Silenced cell populations carrying HIV-1 proviruses encoding a d2EGFP marker (i.e., less than 5% of cells in the unstimulated cell population had detectable d2EGFP expression) were superinfected with lentiviral vectors expressing control, SUV39H1, EZH2, or EZH2 shRNAs. For double infections with SUV39H1 and EZH2, a blasticidine marker was subcloned into the pLKO.1 vector in place of the puromycin marker. The blasticidine gene was amplified from the pcDNA6/V5-His ABC plasmid (catalog number V22020; Invitrogen) by using the primers BlastF (AGGTCGACATGG CCAAGCCTTTG), containing the restriction site HincII, and BlastR (ATGG TACCTTAGCCCTCCCACAC), with a KpnI restriction site. pLKO.1 EZH2 vector was cut with HincII and KpnI, and the backbone was religated with the blasticidine fragment.

A total of 1×10^6 Jurkat E4 cells were infected with VSV-G-pseudotyped lentiviral vectors expressing shRNAs. Three days after infection, drug-resistant cells were selected in medium containing either puromycin (2 μg/ml), blasticidine (10 μg/ml), or a combination of both for at least 7 days. Cell viability and d2EGFP expression were assessed via FACS.

Activations and shutdowns. Cells were activated overnight using tumor necrosis factor alpha (TNF-α; 10 ng/ml), anti-CD3 monoclonal antibody (MAb; 0.125 μg/ml), anti-CD3 MAb (0.125 μg/ml) plus anti-CD28 MAb (1 μg/ml), or stimulated overnight with TSA (500 nM), hexamethylbisacetamide (HMBA; 5 mM), or SAHA (5 mM). The histone methylation inhibitors 3-deazaneplanocin A (DZNep; (synthesized for this study), BIX01294 (B9311; Sigma), chaetocin (C9492; Sigma), or the DNA methylase inhibitor 5′-azacytidine (Fisher) were added to E4 cells plated at 1×10^6 cells/ml in a 12-well tissue culture plate for up to 3 days and analyzed for d2EGFP expression via FACS.

To monitor reversion of activated proviruses to a silenced state, cells were activated with 10 ng/ml of TNF-α for 21 h at 1 week after infection with the shRNA vectors. The cells were washed twice with phosphate-buffered saline (PBS) and resuspended in selective marker-containing medium. Samples for FACS analysis were taken every 3 h from 0 to 12 h and then every 24 h up to 72 h.

ChIP analysis. Latent E4 Jurkat clones were activated for 0 or 30 min with 10 ng/ml TNF-α at 2.5×10^6 cells/ml. After fixation of cells with formaldehyde (0.5%), DNA immunoprecipitates were prepared as previously described (37). It is important to note that during the preparation of the chromatin fragments, the DNA was sonicated to between 150 and 500 nucleotides (nt) in length. This permitted an average resolution of 200 nt in the chromatin immunoprecipitation (ChIP) experiments, which is sufficient to localize proteins to the Nuc-0, promoter, or Nuc-1 regions of the viral LTR.

The following antibodies were used: anti-histone H3, CT, pan, clone AS3 (05-928; Millipore), anti-EZH2 (39639; Active Motif), ChIP Ab plus H3K27me3 (17-622; Millipore), ChIP Ab plus H3K9me3 (17-625; Millipore), RNA polymerase II (sc-899; Santa Cruz), and anti-acetyl-histone H3 (06-599; Millipore). PCRs were performed in 25-μl reaction mixtures containing 5 μl of DNA, 12.5 μl of SYBR green master mix (Quanta), and 1 μl of each primer.

The following primer sets, numbered with respect to the transcription start site, were used: HIV-1 (Nuc-0) −390 F, ACA CAC AAG GCT ACT TCC CTG A, and −283 R,TCT ACC TTA TCT GGC TCA ACT GGT; HIV-1 (promoter) −116 F, AGC TTG CTA CAA GGG ACT TTC C, and +4 R, ACC CAG TAC AGG CAA AAA GCA G; HIV-1 (Nuc-1 position) +30 F, CTG GGA GCT CTC TGG CTA ACT A, and +134 R, TTA CCA GAG TCA CAC AAC AGA CG; HIV-1 (gag) +611 F, AGG CGT TAC TCG ACA GAG G, and +770 R, AGG CGT TAC TCG ACA GAG GA; d2EGFP +4078 F, AGC AGA AGA ACG GCA TCA AG, and +4277 R, CTC CAG CAG GAC CAT GTG AT; and

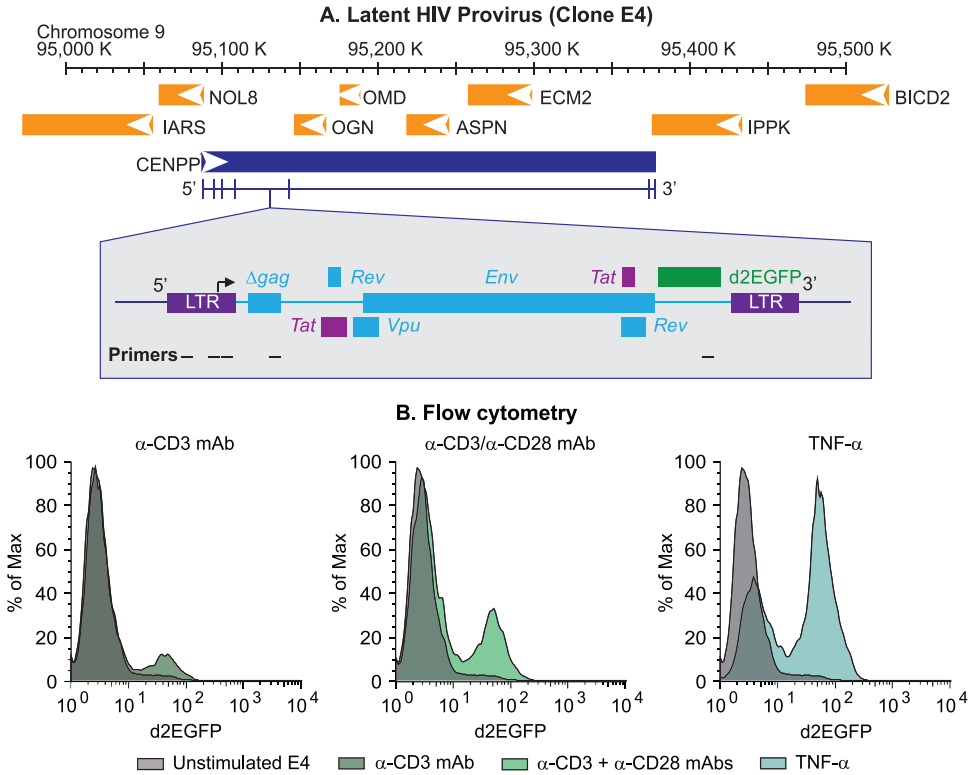

FIG. 1. The E4 clone model for HIV-1 latency and structure of the HIV-1 LTR. (A) Proviral insertion site in the latently infected Jurkat T-cell clone (E4). The provirus, derived from HIV-1_{NL4-3}, expresses the wild-type Tat protein and carries the fluorescent d2EGFP protein in place of the Nef gene as a marker of transcriptional activation. The provirus is integrated into the fourth exon of the centromere protein P gene (CENPP) on chromosome 9. (B) Induction of E4 cells by T-cell receptor stimulation or TNF-α activation. Cells were stimulated for 18 to 24 h with 0.125 μg/ml anti-CD3 MAb (left panel), 0.125 μg/ml anti-CD3 MAb plus 1 μg/ml anti-CD28 MAb (middle panel), or 10 ng/ml TNF-α (right panel). Flow cytometry was used to measure expression of d2EGFP, which provided an indirect measurement of HIV transcription.

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene −492 F, TGA GCA GAC CGG TGT CAC TA, and −348 R, AGG ACT TTG GGA ACG ACT GA. In the experimental descriptions we refer to PCR primers by the start site of the 5′ primer.

Western blot assays. A total of 0.5×10^6 E4 cells/ml in 2 ml of RPMI medium were treated for 1 day with 50 nM chaetocin or 5 μM BIX01294 or for 3 days with equal amounts of dimethyl sulfoxide and 10 μM DZNep or 5 μM 5′-azacytidine (Fisher). Cells were washed twice with PBS, lysed in 250 μl of radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris [pH 7.5], 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), and sonicated in a Bioruptor XL for 10 min with 30-s cycles. DNA was removed through centrifugation at 13,000 rpm for 15 min. Protein was quantified using the Bradford assay, and 18 μg of total protein was analyzed by SDS-PAGE.

RESULTS

EZH2 and H3K27me3 are present at the LTR of latent HIV-1 proviruses. The experiments described were primarily performed using the E4 cell line, which is a latently infected Jurkat T-cell line. As shown in Fig. 1A, E4 cells carry a single integrated lentiviral vector that expresses the wild-type Tat gene, TAR RNA elements derived from HIV-1_{NL4-3}, and a short-lived green fluorescent protein (d2EGFP) reporter protein in place of the Nef gene (60). The HIV-1 provirus in the E4 clone is inserted into the fourth intron of the centromere protein P (CENPP) gene on chromosome 9 (60) and is orientated so that its promoter transcribes the same strand as the CENPP gene. The silenced provirus in the E4 clone is readily inducible by TNF-α and activation of the T-cell receptor with monoclonal antibodies (Fig. 1B) (38, 60).

ChIP assays were performed in order to measure changes in the chromatin structure at the provirus in E4 cells before and after induction of transcription by TNF-α (Fig. 2). As noted in numerous previous studies (37, 38, 60, 76), stimulation with TNF-α for 30 min resulted in RNA polymerase II (RNAP II) recruitment to the HIV-1 5′-LTR in the region of the promoter and nucleosome 1 (Nuc-1) (Fig. 2A). Because HIV transcription elongation is restricted at these early time points due to the absence of Tat, there is much less RNAP II detected downstream of the start site of transcription (detected using PCR primers beginning at +611 and +4078, where +1 denotes the start site of transcription) (37, 38, 60). RNAP II levels at the control GAPDH promoter remained constant under these conditions. Total histone H3 levels at the HIV-1 LTR and the control GAPDH gene were comparable and relatively constant before and after TNF-α induction (Fig. 2B). However, as noted previously, the HIV-1 LTR of latent proviruses is enriched in hypoacetylated histones (2, 60, 79). Acetylated histone H3 levels were 6- to 10-fold lower on the HIV LTR than on the GAPDH gene (Fig. 2C). Similarly, there was only a modest increase in histone H3 acetylation levels after activation by TNF-α.

Importantly, both EZH2 (Fig. 2D) and H3K27me3 (Fig. 2E)

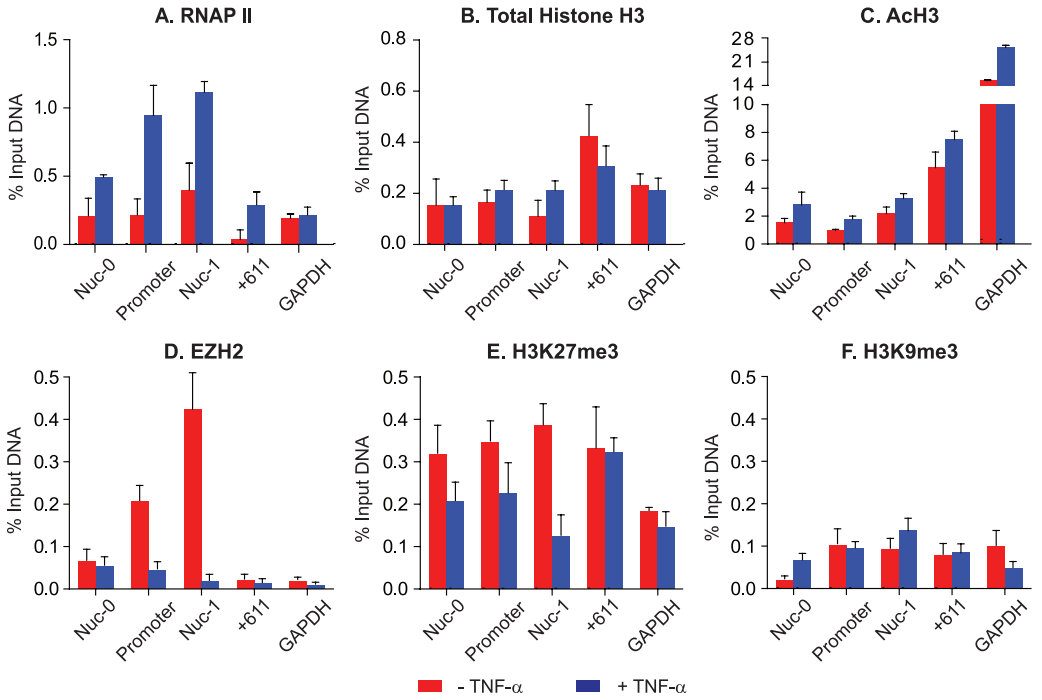

FIG. 2. Latent HIV-1 proviruses carry EZH2 and trimethylated histones at the viral LTR. ChIP assays were performed on unstimulated latently infected E4 cells and E4 cells stimulated for 30 min with 10 ng/ml TNF-α. Each sample was analyzed using a series of primers spanning the HIV-1 provirus (Fig. 1). The GAPDH promoter was used as a control. (A) RNAP II); (B) total histone H3; (C) total acetylated histone H3; (D) EZH2; (E) H3K27me3; (F) H3K9me3. Note that EZH2 is located in the Nuc-1 region of latent proviruses. After proviral induction by TNF-α, EZH2 is displaced and there is a concomitant loss of H3K27me3. Error bars represent the standard deviations of 4 separate real-time PCR measurements of the individual ChIP samples.

are found at the HIV-1 LTR prior to activation. EZH2 accumulates at the HIV-1 LTR in the promoter and Nuc-1 regions but is undetectable at the GAPDH promoter. Following TNF-α activation, there was a dramatic decrease in the EZH2 levels, suggesting that it is important for maintaining proviral silencing. The levels of H3K27me3 detected at the HIV-1 LTR followed a pattern similar to EZH2. H3K27me3 levels were more than twice as high on the HIV-1 LTR as on the GAPDH gene, and there was a 60% decrease in H3K27me3 levels at Nuc-1 following TNF-α treatment. In contrast, H3K9me3 (Fig. 2F) was present at only low levels at the HIV proviruses (less than 2-fold over background levels) and comparable to the levels detected at the GAPDH promoter. Furthermore, there was no measureable decrease in H3K9me3 levels following TNF-α treatment. Taken together, these data suggest that the presence of EZH2 and H3K27me3 correlates with silencing at the HIV-1 promoter and that the block imposed by H3K27me3 is removed following NF-κB activation by TNF-α.

Proviruses that reactivate poorly carry high levels of H3K27me3. Although there was a nearly 2-fold loss of H3K27me3 from the Nuc-1 region following TNF-α activation, some residual methylated histone remained. This could be either due to incomplete activation of individual proviruses under our experimental conditions or due to heterogeneity in the epigenetic histone marks within the E4 cell population. Although E4 cells are a clonal population that carries a single integrated provirus in every cell, individual cells might vary with respect to their complement of histone modifications and respond differentially to cellular signals. Consistent with this hypothesis, DNA methylation levels have recently been shown to vary within clonal populations of latent HIV proviruses (4, 36). We therefore treated cells with a suboptimal level of TNF-α and separated the cells that were fully activated (d2EGFP$^+$) from the smaller subset of cells that failed to respond to TNF-α (d2EGFP$^-$) by cell sorting (Fig. 3A). The sorted cells were expanded during the next 12 days. During this period the activated cells reverted to a silenced state and lost d2EGFP expression, with the cells arising from the subset of cells that were originally refractory to activation by TNF-α (d2EGFP$^-$) becoming more rapidly silenced than the cells arising from the activated population (d2EGFP$^+$). Control experiments have shown that the cells arising from the original d2EGF$^-$ cell population remain less responsive to external stimuli than the cells arising from the d2EGFP$^+$ population (data not shown). Thus, we were able to separate the subset proviruses that were highly restricted from the larger population that responded readily to TNF-α stimulation.

ChIP assays were used to measure RNAP II levels and chromatin modification at the latent proviruses in both cell populations once they had reverted to latency at 12 days after sorting (Fig. 3B). The d2EGFP$^-$ cells showed lower levels of RNAP II and acetylated histones than the d2EGFP$^+$ cells at both the promoter and Nuc-1 region of the HIV-1 provirus. As a control, total H3 levels remain unchanged in both cell populations. Consistent with a role for H3K27me3 in restricting HIV-1 transcription, the d2EGFP$^-$ cells also showed higher levels of H3K27me3 at the latent proviruses than the cells that arose from the d2EGFP$^+$ population. We were surprised to

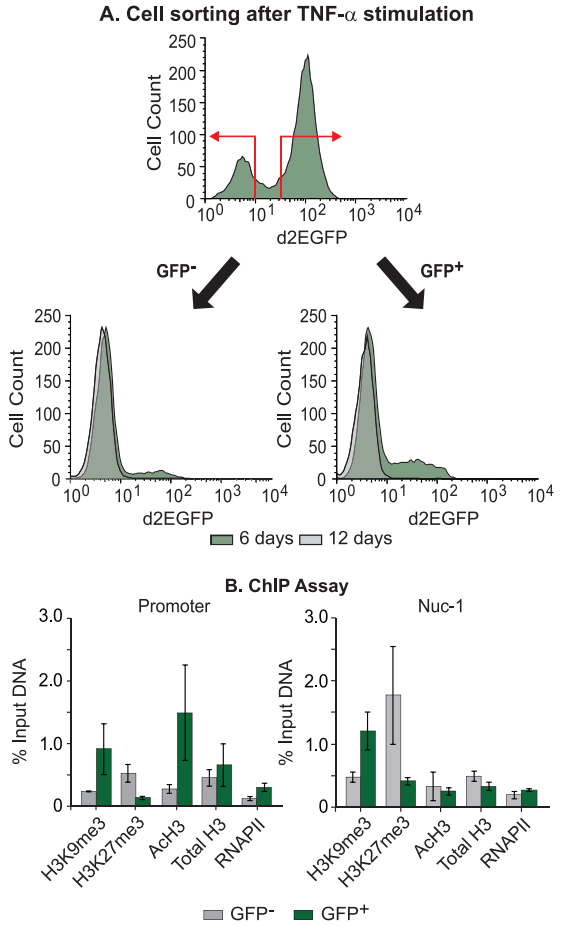

FIG. 3. Latent HIV-1 proviruses that respond poorly to cellular activation signals carry high levels of H3K27me3 at their promoters. (A) Experimental scheme. E4 cells were activated for 24 h with a suboptimal level of TNF-α (2 ng/ml) and then sorted into d2EGFP$^+$ and d2EGFP$^-$ populations. The sorted cells were then expanded over the next 12 days, and d2EGFP expression levels were monitored by flow cytometry. By 12 days both cell populations had reverted to a silenced state. (B) ChIP assays measuring the distribution of RNAP II and chromatin-associated proteins at the HIV promoter (−116 to +4; left panel) and Nuc-1 region (+4 to +134; right panel) in the cells grown from the sorted d2EGFP$^-$ and d2EGFP$^+$ populations. Error bars represent the standard deviations of 4 separate PCR measurements.

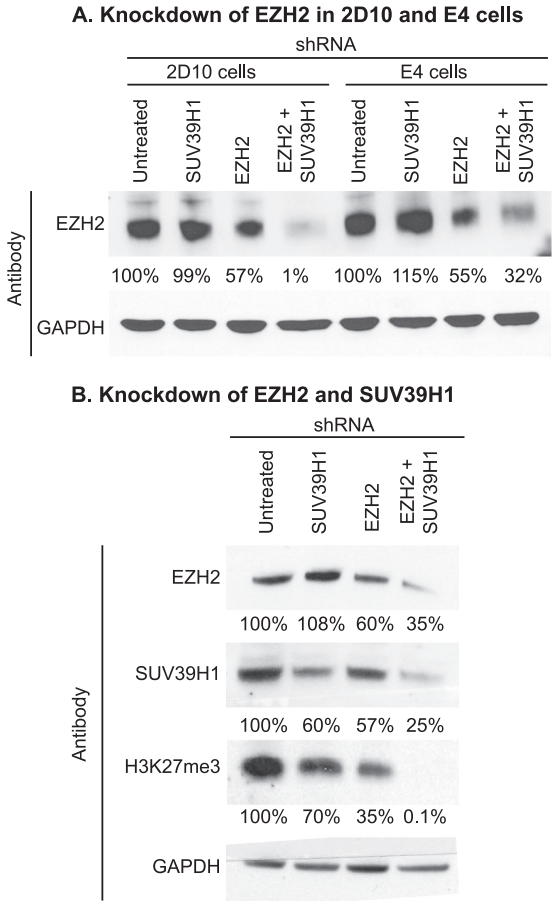

FIG. 4. Knockdown of EZH2 and SUV39H1 by shRNA. (A) Western blot of EZH2 levels following superinfection of 2D10 cells (left panels) or E4 cells (right panels) with lentiviruses expressing shRNA. Cells were infected with vectors carrying a scrambled control shRNA or shRNA against EZH2 or SUV39H1 or dually infected with a combination of EZH2 and SUV39H1. Whole-cell extracts from the E4 clone infected with the lentiviral vectors expressing the indicated shRNAs were used for Western blot assays. For each cell line, 18 μg of protein in the RIPA cell extract was probed with antibodies against EZH2. GAPDH was used as a loading control. The relative expression levels of EZH2 as estimated by densitometry of the gels are shown below each band. Protein levels were normalized to the GAPDH loading control. (B) Impact of EZH2 knockdown on H3K27me3 expression in E4 cells. Cell extracts were prepared as described above, and Western blots were probed with antibodies to EZH2, SUV39H1, H3K27me3, and GAPDH.

find that the H3K9me3 mark appeared to segregate from the H3K27me3 mark in this experiment. Although H3K9me3 is generally considered to be a repressive mark, the d2EGFP$^+$ cell population was strongly enriched in H3K9me3 compared to the d2EGFP$^-$ cells. The observation that proviruses from the GFP$^+$ population have reduced levels of H3K27me3 compared to cells derived from the GFP$^-$ population implies that although silencing can occur in the absence of the H3K27me3 restriction, proviruses that carry the H3K27me3 restriction are more difficult to reactivate.

Knockdown of EZH2 leads to reactivation of latent HIV proviruses. To determine if EZH2 is required to maintain transcriptional repression at the HIV-1 provirus, Jurkat T-cell clones were superinfected with lentiviral constructs carrying shRNA against EZH2, SUV39H1 (19, 53), and a combination of both shRNAs. Negative controls consisted of the pLKO.1 vector or scrambled shRNA sequences. The shRNA vectors carried either a puromycin or a blasticidine resistance gene, allowing cell populations expressing the various shRNAs to be selected.

As shown in Fig. 4A, expression of EZH2 shRNA in either 2D10 or E4 cells led to a 40% reduction of protein expression levels. There was no significant change in EZH2 expression following infection with vectors carrying shRNA against SUV39H1; however, use of a combination of shRNA against EZH2 and SUV39H1 led to a 70 to 99% reduction in EZH2 levels. Similar results were obtained in the experiment shown in Fig. 4B. Expression of the EZH2 shRNA led to a 37% reduction in total cellular EZH2 protein levels and a corre-

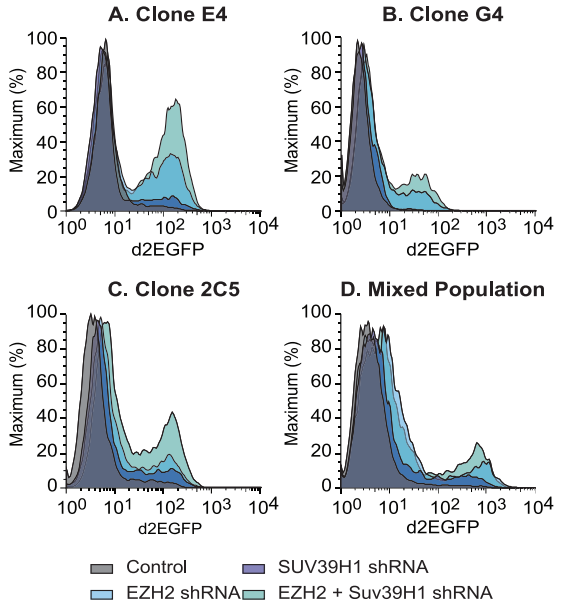

FIG. 5. Knockdown of EZH2 by shRNA induces HIV-1 transcription in latently infected cells. The cells were superinfected with lentiviral vectors carrying shRNA against SUV39H1, EZH2, or a combination of both vectors. An empty vector was used as a control. Superinfected cells were selected for 4 days using puromycin (for SUV39H1), blasticidine (for EZH2), or a combination of puromycin and blasticidine for the double infections. The FACs profiles were obtained 6 days postinfection. (A) The latently infected Jurkat T-cell clone E4; (B) G4 clone; (C) 2C5 clone, which carries a Nef$^+$ provirus; (D) a mixed population of latently infected Jurkat cells.

sponding 65% reduction in cellular H3K27me3 levels (Fig. 4B). Similarly, expression of the SUV39H1 shRNA led to a 48% reduction in SUV39H1 levels, while EZH2 levels were increased slightly to 108% of control levels. A 62% reduction of EZH2 protein levels was seen when cells were dually infected with vectors carrying shRNAs to EZH2 and SUV39H1, and this resulted in a >95% loss of H3K27me3. Thus, in multiple experiments the individual shRNAs against EZH2 and SUV39H1 were able to significantly and reproducibly reduce the levels of these proteins in Jurkat T-cell lines. Although neither protein was completely removed by the individual shRNAs, a combination of shRNAs to EZH2 and SUV39H1 was able to remove the majority of the SUV39H1, EZH2, and H3K27me3 from the cell.

The reactivation of latent proviruses following knockdown by SUV39H1 and EZH2 was measured by FACS analysis of d2EGFP induction. In E4 cells superinfected with the EZH2-shRNA vector (Fig. 5A), 40.5% of the proviral population was activated. In contrast, only 8.2% of the proviruses were induced in cells infected with the control vector, and only 16.0% of the cells infected with the SUV39H1 shRNA vector showed viral reactivation. In cells that were doubly infected with vectors expressing shRNA to EZH2 and SUV39H1, 51.0% of the latent proviruses became reactivated.

To demonstrate that the reactivation of the proviruses following knockdown of EZH2 reflected a general feature of HIV-1 silencing, rather than a specific effect observed in the E4 cell line, we tested the shRNAs in a variety of latently infected cell lines (Fig. 5). The G4 clone (Fig. 5B) carries a single integrated provirus similar in structure to the provirus in E4 cells, whereas the 2C5 clone (Fig. 5C) carries an integrated provirus expressing the HIV-1 Nef gene and carrying an attenuated H13L Tat gene (60). We also studied a mixed population of cells that became silenced after infection with the H13L Tat Nef$^+$ virus (Fig. 5D). In every cell line, knockdown of EZH2 led to a significant induction of proviral expression. Furthermore, knockdown of EZH2 was always at least 2-fold more effective at inducing proviral expression than knockdown of SUV39H1, and knockdown of both SUV39H1 and EZH2 had an additive effect. Thus, there is a strong correlation between the loss of EZH2 and H3K27me3 and spontaneous proviral induction. Knockdown of both SUV39H1 and EZH2 further enhanced proviral reactivation. We attribute this effect to the further reduction in EZH2 levels that we saw in the presence of the SUV39H1 shRNA, since SUV39H1 shRNA is a poor inducer on its own. However, we cannot exclude the possibility that SUV39H1 is also directly silencing a subset of the latent proviruses.

EZH2 knockdown sensitizes latent proviruses to cellular reactivation signals. Since HIV-1 silencing arises because of multiple epigenetic blocks, it seems likely that reductions in EZH2 that are insufficient to induce proviral reactivation might nonetheless make silenced proviruses more responsive to cellular activation pathways. We therefore compared the extent of proviral reactivation in the control E4 cell line and in the corresponding cell lines in which SUV39H1 and EZH2 were knocked down by shRNA (Fig. 6).

As shown in Fig. 6A and D, stimulation of the control E4 cells through the T-cell receptor (TCR) using anti-CD3 MAb (15% d2EGFP$^+$ cells) or a combination of anti-CD3 and anti-CD28 MAbs (29% d2EGFP$^+$ cells) resulted in only partial proviral reactivation. Knockdown of SUV39H1 did not appreciably enhance the responses of these cells to TCR signaling (Fig. 6B and D). In contrast, in cells where EZH2 was knocked down, the basal activation level increased to 30.1% d2EGFP$^+$ cells, and stimulation of the EZH2 knockout cells with anti-CD3 MAb resulted in activation of 75.1% of the latent proviruses. Stimulation with anti-CD3 and anti-CD28 MAbs resulted in activation of 85.1% of the latent proviruses.

Knockdown of EZH2 also potentiated proviral reactivation in cells treated with a wide variety of other stimuli (Fig. 6D). Activation of cells with TNF-α, which is typically more potent than activation through the TCR, resulted in 68.3% reactivation of the proviruses in control E4 cells and 91.6% and 93% reactivation of the proviruses in cells in which SUV39H1 and EZH2 were knocked down, respectively. Activation of E4 cells with HMBA, which is believed to activate HIV-1 transcription by enhancing the release of P-TEFb from the inactive 7SK RNP complex (15), resulted in activation of 21.8% of the silenced proviruses in E4 cells. Following knockdown of SUV39H1, only 33.6% of the proviruses were activated under these conditions. However, knockdown of EZH2 resulted in reactivation of 55.3% of the latent proviruses in response to HMBA. Finally, treatment of cells with the HDAC inhibitor TSA resulted in 56% proviral reactivation in E4 cells but more than 86.3% reactivation in cells where EZH2 was knocked down. Nearly identical patterns of sensitization of proviral reactivation following SUV39H1 and EZH2 knockdown were observed in the G4 cell line (data not shown).

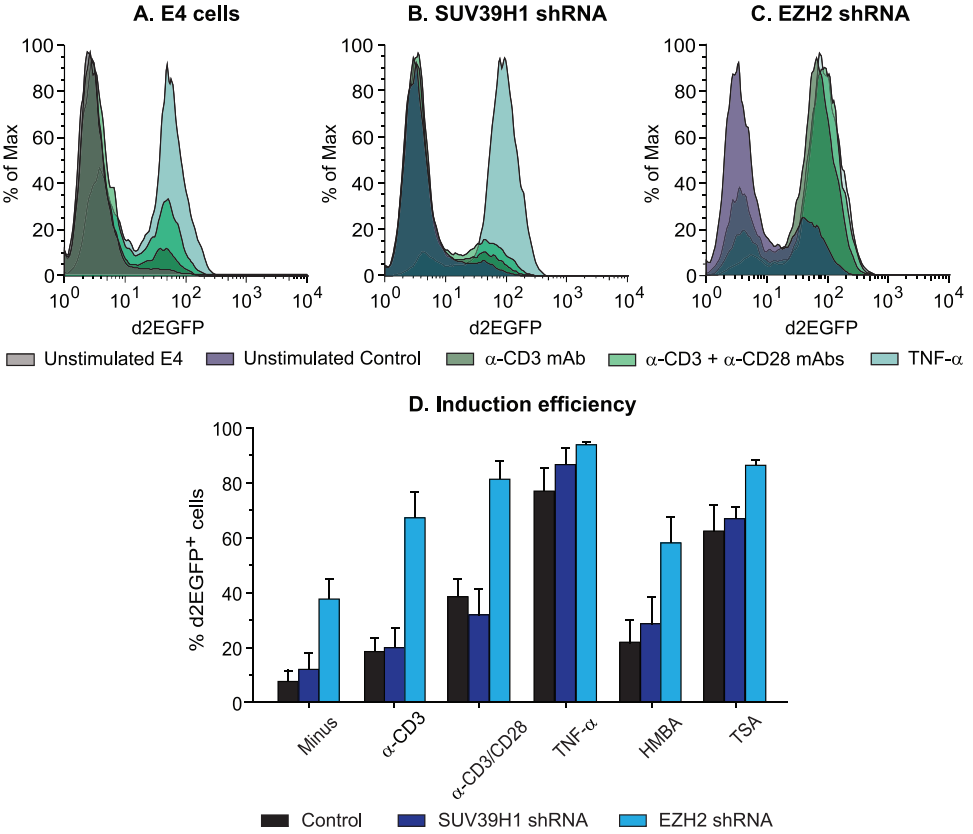

FIG. 6. Knockdown of EZH2 by shRNA potentiates HIV-1 emergence from latency. (A) Flow cytometric analysis of the latent E4 clone. The cells were stimulated for 16 h with 0.125 μg/ml anti-CD3 MAb, 0.125 μg/ml anti-CD3 MAb plus 1 μg/ml anti-CD28 MAb, or 10 ng/ml TNF-α. (B) E4 cells were superinfected with a lentiviral vector carrying SUV39H1 shRNA and then stimulated as described for panel A. (C) E4 cells were superinfected with a lentiviral vector carrying EZH2 shRNA and then stimulated as described for panel A. (D) Histogram summarizing flow cytometric analysis results for E4 cells superinfected by control vectors or vectors carrying SUV39H1 shRNA and/or EZH2 shRNA. Error bars represent the standard deviations of 4 separate experiments performed over a period of several months. The cells were either untreated (Minus) or stimulated for 16 h with 0.125 μg/ml anti-CD3 MAb, 0.125 μg/ml anti-CD3 MAb plus 1 μg/ml anti-CD28 MAb, or 10 ng/ml TNF-α, 5 mM HMBA, or 0.5 μM TSA. Data are expressed as the percentage of $EGFP^+$ cells (mean fluorescent intensity of $>2 \times 10^1$).

Thus, removal of epigenetic blocks at silenced HIV-1 proviruses by reduction in EZH2 levels with shRNA potentiates viral reactivation in response to a wide variety of stimuli. Although knockdown of SUV39H1 is able to sensitize the cells slightly, knockdown of EZH2 was uniformly more effective and resulted in dramatic increases in proviral reactivation by a wide variety of stimuli in each of the HIV latency models that we tested.

EZH2 accelerates HIV-1 silencing. Cell lines that carry latent HIV-1 proviruses that have been reactivated quickly revert to a quiescent state when the activation stimulus is removed (60). In order to determine if HKMTs contribute to the silencing of HIV-1 transcription, control E4 cells and cells carrying SUV39H1 and EZH2 shRNAs were activated with TNF-α for 21 h and monitored by FACS as they reverted to latency over the next 72 h in the absence of TNF-α (Fig. 7). As previously noted, following removal of TNF-α, there is a biphasic decline in d2EGFP expression (60). During the initial phase, which takes place over the first 12 h, there was a rapid loss of d2EGFP expression resulting from a decline in NF-κB levels, which in turn restricted HIV-1 transcription initiation and Tat expression (Fig. 7C). The decline in d2EGFP expression in this first phase was similar for each of the cell lines tested. During the second slower second phase, which takes place between 12 h and 72 h, there is a further decline in d2EGFP expression levels, but control cells showed a much more rapid decline than cells in which EZH2 and SUV39H1 have been ablated. Strikingly, in cells in which EZH2 was knocked down, d2EGFP expression levels were nearly the same at 12 h and 72 h after removal of TNF-α, and they were more than twice the levels seen in control cells. Knockdown of SUV39H1 somewhat reduced the rate at which cells reverted to latency, but the effect was much smaller than that seen with the EZH2 knockdowns. Similar results were observed in both the E4 and G4 cell lines (Fig. 7D). Thus, histone methylation stimulated by EZH2 appears to be essential in order to achieve full proviral silencing.

Histone lysine methyltransferase inhibitors are able to activate latent HIV-1 proviruses. To further evaluate the contributions of epigenetic modifications to the maintenance of HIV-1 latency, we examined whether a variety of chemical inhibitors of histone and DNA modifications were able to induce latent proviruses (Fig. 8). Recent reports have suggested that DNA methylation contributes to HIV-1 silencing

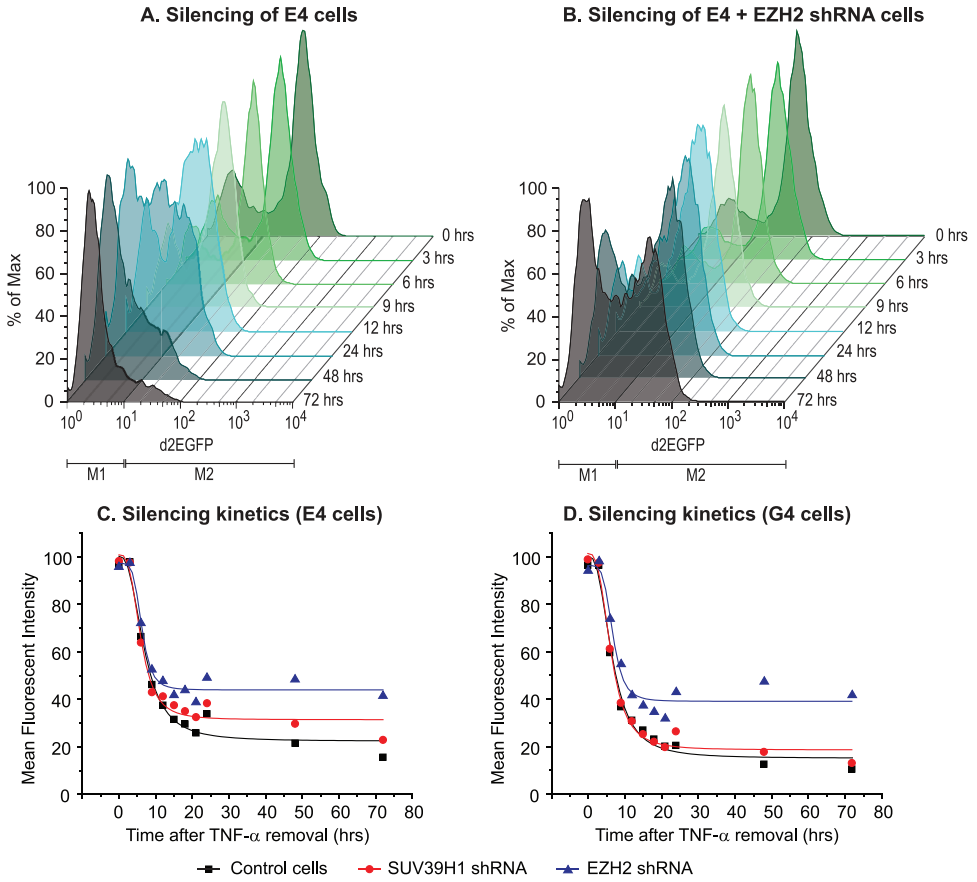

FIG. 7. Depletion of EZH2 by shRNA blocks the reversion of activated proviruses to a latent state. (A) Representative FACS profiles of the E4 cell populations during proviral shutdown. E4 cells were stimulated with 10 ng/ml TNF-α for 16 h, washed, and then monitored by flow cytometry at 0, 3, 6, 9, 12, 24, 48, and 72 h after removal of TNF-α. (B) Shutdown of E4 cells carrying EZH2 shRNA. Note the slower progression to latency compared to the control cells shown in panel A. (C) Silencing kinetics of E4 cells. The graph shows the mean fluorescent intensity of E4 cell populations treated as described for panel A. A biphasic curve was fitted to the samples. Note that proviral silencing was slowed by the knockdown of EZH2. (D) Silencing kinetics of G4 cells. The experimental protocol was identical to that described for panel C.

and that treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-Aza-CdR) leads to reemergence of virus (4, 36). We found that E4 cells treated for 3 days with 0.1 and 0.5 μM 5-Aza-CdR showed no measurable increase in proviral activation; however, there was a slight increase at 5 μM (11.9%) (Fig. 8A).

We also tested the HKMT inhibitor BIX01294, which is a selective inhibitor of the HKMT G9a, previously reported to induce latent proviruses (30). In our experiments, BIX01294 was a comparatively poor inducer of latent proviruses and was only able to induce 21.1% of the latent proviruses in the E4 cell line after overnight exposure to the drug (Fig. 8B). This level of induction is similar to the levels that were reported previously (30).

Chaetocin is a specific and potent inhibitor of SUV39H1 (25). As shown in Fig. 8C, chaetocin did not significantly activate latent HIV-1 proviruses in E4 cells at concentrations up to 200 nM. Because both chaetocin and BIX01294 were highly toxic to the cells, it was not possible to evaluate the effects of these drugs during time periods longer than an overnight exposure (data not shown).

The broad-spectrum HKMT inhibitor DZNep is capable of downregulating several cellular HKMTs and EZH2 (56). Treatment of E4 cells with 5 or 10 μM DZNep led to induction of 31.5% of the latent proviruses (Fig. 8D). The effects of DZNep are progressive, with maximal proviral induction seen after treatment for 3 days (data not shown). Western blot assays showed that, as previously reported (56), exposure of cells to 10 μM DZNep led to global reductions in H3K27me3 (44% reduction) and H3K9me2 (70% reduction) (data not shown). There was a slight reduction in EZH2 levels (21% reduction). Surprisingly, DZNep strongly induced SUV39H1, leading to a doubling of the cellular protein levels, but it did not increase global H3K9me3 levels.

In summary, the drug data are consistent with the shRNA data and show that reductions in H3K27me3 levels can lead to significant proviral induction, whereas there are comparatively modest effects on proviral induction by blocking the formation of H3K9me3 and H3K9me2.

Blocking EZH2 enhances proviral reactivation by SAHA. The histone deacetylase inhibitor SAHA, currently FDA approved under the name Vorinostat, is one of the most efficient activators of transcription from latent HIV-1 proviruses that

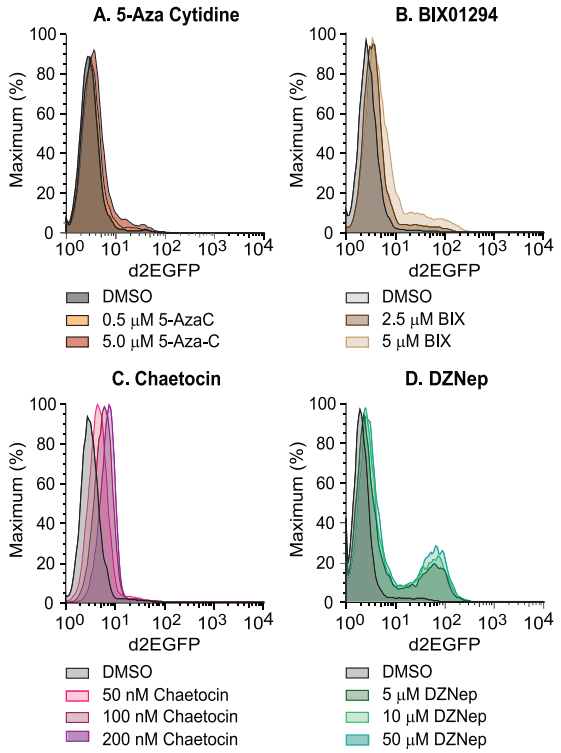

FIG. 8. The EZH2 inhibitor DZNep activates latent HIV-1 proviruses. (A) E4 cells stimulated with 5-Aza-CdR, a DNA methylase inhibitor, for 72 h. (B) E4 cells stimulated with BIX01294 (G9a inhibitor; 16 h). (C) E4 cells stimulated with chaetocin (SUV39H1 inhibitor; 16 h). (D) E4 cells stimulated with DZNep (EZH2 inhibitor; 72 h). Note that DZNep was the most potent compound for inducing latent proviruses.

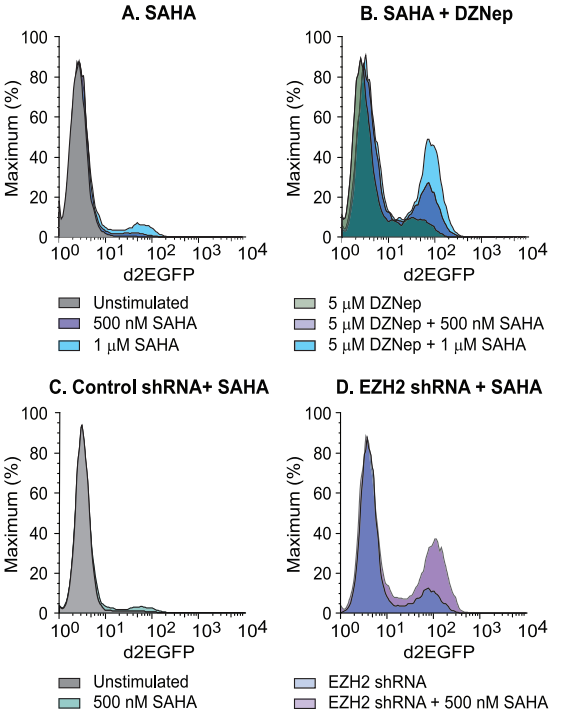

FIG. 9. Proviral reactivation by SAHA is enhanced by DZNep or treatment of cells with shRNA to EZH2. (A) E4 cells were treated with 0, 1, or 5 μM SAHA, and d2EGFP expression was measured by flow cytometry. (B) E4 cells were treated for 2 days with 5 μM DZNep and then treated overnight with 0, 1, or 5 μM SAHA. Note that at each SAHA concentration tested, DZNep was able to further stimulate proviral reactivation. (C) Activation of E4 cells exposed to 0 or 500 nM SAHA. There was less than 6% activation of the cells when using this low compound concentration. (D) Activation of E4 cells superinfected with a lentiviral vector expressing EZH2 shRNA and then exposed to 0 or 500 nM SAHA.

has been characterized (1). SAHA works primarily as a histone deacetylase inhibitor, although a recent report suggested that it may also enhance release of functional P-TEFb from the 7SK RNP complex (16). Since SAHA acts by a mechanism of action distinct from that of DZNep, we tested whether the two compounds could cooperate in the activation of latent HIV-1 proviruses. E4 cells were treated for 2 days with DZNep and then treated with increasing concentrations of SAHA (Fig. 9A). Synergistic effects were seen when we used a combination of 500 nM SAHA, which is a suboptimal concentration, and 5 μM DZNep; 500 nM SAHA induced only 4.2% of the latent proviruses and 5 μM DZNep induced 15.3% of the proviruses, whereas the two drugs combined were able to induce 29.3%.

Similar synergistic effects on proviral reactivation were seen when SAHA was used to activate proviruses in cells carrying EZH2 shRNA (Fig. 9D), but not in control cells (Fig. 9C) or in cells carrying shRNA to SUV39H1 (data not shown). Thus, reduction of H3K27me3 levels for HIV-1 proviruses, either by shRNA or by DZNep treatment, partially activates the silenced viral population, making them more sensitive to activation by the histone deacetylase inhibitor SAHA.

DISCUSSION

Epigenetic silencing of HIV-1 by histone methylation. Extensive genome-wide analyses have shown that histone methylation can lead to either the activation or repression of genes, depending on which histone lysine residues are modified and whether they are mono-, di-, or trimethylated. In general, transcriptionally active genes carry H3K4me3 and H3K36me3, whereas repressed genes found in heterochromatic structures contain H3K9me3, H3K27me3, and H4K20me3. Heterochromatin can be either constitutive or facultative. Constitutive heterochromatin is composed of genes that are permanently silenced and carry the H3K9me3 and H4K20me3 histone modifications and are enriched in the linker histone H1, whereas facultative heterochromatin carries temporarily silenced genes identified by the H3K27me3 mark (68).

PRC2 is the only moiety in mammalian cells that is able to catalyze the di- and trimethylation of H3K27 (55). The data presented here demonstrate that EZH2, the enzymatic component of PRC2 which is responsible for the formation of H3K27me3 (44), contributes to the establishment and maintenance of transcriptional silencing of HIV-1. Knockdown of EZH2 not only induces latent HIV-1 proviruses, but it also sensitizes latent proviruses to stimulation by exogenous signals and limits transcriptional silencing. EZH2 are potent inducers of facultative heterochromatin that have been functionally linked to Hox gene silencing, X inactivation, maintenance of stem cell pluripotency, and cancer (8). In T cells, EZH2 has

been found to contribute to T-cell differentiation and maintaining silencing of the interleukin-4 (IL-4)–IL-13 gene locus in T_H1 primed cells (41, 61).

There is increasing evidence that epigenetic silencing, mediated by PRC2/EZH2 and leading to the creation of facultative heterochromatin, is an important feature of intrinsic immunity to a wide range of viral infections. Recent work has demonstrated that epigenetic silencing is critical for the induction of latency in Kaposi sarcoma-associated herpesvirus (KSHV)-infected cells (26, 50, 67). Soon after infection, there is widespread deposition of H3K27me3 across the KSHV genome, but the H3K9-me3 mark is largely absent. Subsequently, additional silencing due to DNA methylation was observed (26). Induction of latency of herpes simplex virus 1 is also associated with the deposition H3K27me3, which is used to restrict the synthesis of the latency-associated transcript (LAT) (45). We propose by analogy to well-documented examples of herpesvirus silencing (26, 50, 67) that heterochromatic silencing of HIV-1 is mediated by EZH2 recruitment and H3K27me3 deposition.

We also attempted to demonstrate a role for EZH2 in the maintenance of latency in infected resting memory T cells obtained from patients on HAART by using small interfering RNA (siRNA) against EZH2 to induce viral outgrowth. We found that siRNA against EZH2 was able to stimulate viral outgrowth over the levels seen in cells treated with the control luciferase siRNA in 4 of the 5 patient samples, with the induction of proviruses following EZH2 knockdown most easily detected in the cells of patients with the highest initial levels of resting cell infection. Unfortunately, these results were not statistically significant both because there was large interpatient variability in the frequency of viral outgrowth and because the nucleofection procedure we used to introduce the siRNA weakly activated the patient T cells. We are continuing to investigate the contribution of EZH2 to HIV latency in patients and using improved methods.

Heterogeneous epigenetic silencing in clonal cell populations. In contrast to developmentally regulated cellular genes, epigenetic silencing of HIV-1 results in complex and heterogeneous patterns of histone modifications and DNA methylation (4, 36, 60, 80). Heterogeneity of epigenetic markers exists both between individual clones and, more surprisingly, within clonal populations that carry identical integrated proviruses. As shown here, proviral populations that respond poorly to activation signals are enriched in H3K27me3 compared to proviruses that are more easily reactivated (Fig. 3). Similarly, Verdin and his colleagues reported that latently infected cell lines have heterogenous levels of proviral DNA methylation (4, 36).

Epigenetic variation provides an explanation for why certain subsets of silenced proviruses fail to be reactivated when cells are stimulated with exogenous signals. Because of the Tat feedback mechanism, when latently infected cells are partially activated, intermediate viral expression levels are rarely observed. Instead, the subset of cells that is able to produce Tat becomes fully activated, while the subset of cells that fail to achieve threshold levels of Tat revert to a silenced state. Thus, when the epigenetic restrictions imposed by histone methylation are removed either by shRNA, siRNA, or chemical treatments, only a subset of cells becomes activated, while the remainder of the population becomes sensitized to additional activation stimuli.

Role of Polycomb repressive complex 2 in silencing of HIV proviruses. In addition to its enzymatic activity, EZH2 acts as a structural component of PRC2. PRC2 can serve as a binding platform for multiple histone-modifying and DNA-modifying enzymes, including DNA methyltransferase 1 (DNMT1) (73), the SWI/SNF component bromo-domain-containing protein Brd7 (66), and histone deacetylases (9). It seems likely that these additional components of the silencing machinery also contribute to the development of HIV latency. For example, recruitment of DNMT1 by PRC2 could mediate methylation of the HIV LTR and further enhance HIV-1 latency (4, 36, 73). Consistent with this idea, we found that the relief of DNA methylation in the E4 clone by 5-Aza-CdR treatment led to reactivation of a small subset of viruses (Fig. 8A) (73).

Two other intriguing parallels between mechanisms associated with PRC2 silencing of genes and HIV transcriptional control deserve mention. First, two recent reports have demonstrated that PRC2 characteristically targets genes that carry paused RNAP II and generate short RNA transcripts, analogous to the HIV provirus (20, 34). In addition, PRC2 targets genes frequently contain domains of "bivalent" chromatin (simultaneously enriched for the active histone markers H3K4me3 and H3K27me3) (3, 42). Similarly, we recently found H3K4me3 is also associated with the HIV provirus, especially in the downstream Nuc-2 region (data not shown).

The mechanisms used to recruit PRC2 to mammalian promoters, which are presumably similar to the mechanisms used to silence HIV, are still ill-defined. Margueron and Reinberg (55) proposed that PRC recruitment is achieved by establishing a series of relatively low-affinity interactions near the promoters of target genes. PRC2 is typically found at genes that are enriched in CpG islands, although these sequences do not comprise a consensus PRC2 response element (42). HIV also carries CpG islands in its LTR that are subject to DNA methylation (4) and could contribute to PRC2 recruitment. A strong candidate for a DNA-binding protein that mediates PRC2 recruitment to genes is the Jumonji and ARID-domain-containing protein (JARID2). Recently Pasini et al. (59) demonstrated that JARID2 is able to recruit PRC2 proteins to a heterologous promoter and that inhibition of JARID2 expression leads to a major loss of PRC2 binding and to a reduction of H3K27me3 levels on target genes. Similarly, Li et al. (49) found that JARID2 associates with PRC2 and stimulates the enzymatic activity of EZH2 *in vitro*. Finally, there is increasing evidence that noncoding RNAs can help recruit PRC2 to target genes (33, 70). It is tempting to speculate that HIV RNA transcripts may also participate in the recruitment of PRC2 to the LTR.

EZH2 and SUV39H1 play unique roles in the silencing of HIV-1. Previous studies found that SUV39H1 and HP1 help to maintain a transcriptionally repressed provirus in microglial cells and in fibroblasts (10, 19, 53). Our results confirm that SUV39H1 can make a contribution to HIV-1 latency in T cells, but it appears to be much less effective than EZH2 in the T-cell clones that we have studied. For instance, we found that knockdown of SUV39H1 only induced 5% of the latent proviruses and only produced a subtle delay in reversion to latency

upon removal of exogenous stimuli, whereas knockdown of EZH2 can induce up to 40% of latent proviruses. These findings are consistent with the idea that H3K27me3 is the dominant repressive histone mark on silenced HIV-1 proviruses in the Jurkat cell system.

Gene silencing by SUV39H1 involves a distinct Polycomb silencing complex, PRC1. The PRC1 complex, which mediates ubiquination of histone H2A and chromatin compaction, selectively interacts with SUV39H1 both *in vitro* and *in vivo* (63). During the formation of constitutive heterochromatin, recruitment of SUV39H1 induces a self-reinforcing cycle involving the recruitment of the adaptor molecule HP1, DNMTs, and additional HDACs (24), and it seems likely that an analogous process occurs during proviral silencing by SUV39H1.

Although distinct regions of the genome are targeted by PRC1 and PRC2, there are also numerous examples of silenced genes in embryonic stem cells that are occupied by both the PRC1 and PRC2 complexes (6). In these cases, H3K27 methylation by EZH2 aids the initial recruitment of PRC1 to target genes, and disruption of PRC2 leads to loss of PRC1 from chromatin targets (6). It seems likely that similar events take place at the HIV LTR, since we have observed that the knockdown of either EZH2 or SUV39H1 by shRNA reduces the levels of both proteins and induces a disproportionate loss of H3K27me3. As might be expected, HIV-1 proviral reactivation is enhanced when both EZH2 and SUV39H1 are knocked down by shRNA. Since SUV39H1 is a highly specific enzyme that can only generate H3K9me3, the loss of the H3K27me3 marker suggests that the stability of EZH2 on the HIV provirus is reduced when SUV39H1 is removed.

Therapeutic implications. Strategies designed to purge the latent proviral pool require nontoxic activator molecules to induce transcription of latent HIV-1 proviruses and target their host cells for destruction. The strongest existing candidate molecules for this role are HDAC inhibitors, such as SAHA and valproic acid (1, 46, 79). Unfortunately, HDAC inhibitors are relatively nonspecific and can activate multiple cellular genes. Here we have demonstrated that silencing of HIV-1 proviruses is highly dependent on formation of the repressive histone mark H3K27me3 by the HKMT EZH2. This suggests that targeting H3K27me3 formation might prove to be a more selective method to induce latent proviruses. In support of this idea we have demonstrated here that the broad-spectrum histone methyltransferase inhibitor DZNep can activate transcription from latent HIV-1 proviruses. In comparison to 5-Aza-CdR, which is an inhibitor of DNA methylation, chaetocin, an inhibitor of the HKMT SUV39H1, and BIX12094, an inhibitor of the HKMT G9a, DZNep shows increased potency. Unfortunately, DZNep is cytotoxic at concentrations needed to activate latent HIV proviruses. However, DZNep can act cooperatively with the HDAC inhibitor to activate HIV transcription suggesting that, as has been proposed in the context of cancer therapy, the selective combination of DZNep with an HDAC inhibitor provides the most effective means to reverse epigenetic gene silencing (22, 65, 77). Finally, our studies suggest that inhibitors of PRC2 activity and H3K27me3 formation have great potentials as selective inducers of latent HIV-1 proviruses.

ACKNOWLEDGMENTS

We thank Derek Mann as well as past and present members of the Karn laboratory: Richard Pearson, Julian Wong, Mudit Tyagi, Kara Lassen, Hongxia Mao, Uri Mbonye, Michael Greenberg, Amy Graham, Curtis Dobrowolski, and Julie Jadlowsky for gifts of materials, help, and useful discussions.

This work was supported by grants from the National Institutes of Health: R01-AI067093 and DP1-DA028869 to J.K., R01-AI025899 to C.K.C., and U19-AI082608 to D.M. J.F. was supported in part by the Cell and Molecular Biology training grant (T32-GM08056). We also thank the CWRU/UH Center for AIDS Research (P30-AI036219) for provision of flow cytometry services.

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