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Suv39H1 and HP1γ are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency

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HIV-1 gene expression is the major determinant regulating the rate of virus replication and, consequently, AIDS progression. Following primary infection, most infected cells produce virus. However, a small population becomes latently infected and constitutes the viral reservoir. This stable viral reservoir seriously challenges the hope of complete viral eradication. Viewed in this context, it is critical to define the molecular mechanisms involved in the establishment of transcriptional latency and the reactivation of viral expression. We show that Suv39H1, HP1γ and histone H3Lys9 trimethylation play a major role in chromatin-mediated repression of integrated HIV-1 gene expression. Suv39H1, HP1γ and histone H3Lys9 trimethylation are reversibly associated with HIV-1 in a transcription-dependent manner. Finally, we show in different cellular models, including PBMCs from HIV-1-infected donors, that HIV-1 reactivation could be achieved after HP1γ RNA interference.

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Introduction

HIV-1 infection is a dynamic process involving continuous rounds of infection, replication and cell death. Continuous viral replication causes the loss of CD4+ T cells and, therefore, determines the rate of progression to immunodeficiency in infected individuals. The use of highly active anti-retroviral therapy (HAART) has recently raised the possibility of a cure for HIV-infected individuals (Finzi and Siliciano, 1998). Despite this success, there have been reports of viral rebound after interruption of HAART in patients in whom HIV plasma viremia was undetectable (Chun *et al*, 1999). Virus rebound is due to a small population of non-productively infected resting T cells that constitute the viral reservoir (Chun and Fauci, 1999). This latent reservoir of HIV, in the pool of resting CD4+ T cells, is established rapidly in primary HIV infection and can persist for an extended period of time (Chun *et al*, 1998a). Two forms of viral latency have been described (Bisgrove *et al*, 2005). Preintegration latency (unstable) is due to infected resting T cells in which HIV remains in the cytoplasm, whereas post-integration latency is due to cells containing integrated, transcriptionally inactive provirus. This latter latent state is stable as its half-life is same as that of infected cells and its progeny (Pomerantz, 2002). Thus, a detailed understanding of viral latency and reactivation is required to design new therapies that can eradicate HIV (Marcello, 2006).

Following integration into the host cell genome, the HIV-1 provirus is organized, similar to cellular genes into chromatin (Marcello *et al*, 2004). It has been proposed that the chromatin environment near the viral integration site may play a role in the transcriptional silencing of the HIV-1 genome (Jordan *et al*, 2003). Integrated HIV-1 provirus has positioned nucleosomes in its 5′ LTR and a correlation between HIV-1 gene expression and nucleosome remodeling has been reported. Activation of HIV-1 gene expression by histone deacetylase inhibitors, cytokines and the viral transactivator, Tat, is accompanied by the loss or rearrangement of a positioned nucleosome, nuc-1, near the viral mRNA start site (Verdin *et al*, 1993; Van Lint *et al*, 1996; El Kharroubi *et al*, 1998). Moreover, TSA strongly induces cell-free transcription of a chromatinized HIV-1 promoter, suggesting that HDAC activity may play a role in LTR repression (Sheridan *et al*, 1997). Collectively, these studies strongly suggest that chromatin is an important regulatory component of HIV-1 transcription. Relevant to the role of chromatin in HIV-1 gene expression, Tat has been shown to associate with histone acetyltransferases (HATs) p300/CBP, p300/CBP-associated factor (PCAF) and hGCN5 (Benkirane *et al*, 1998; Hottiger *et al*, 1998; Marzio *et al*, 1998). Tat-recruited HATs presumably acetylate histones in LTR-proximal nucleosomes to potentiate transcription (Benkirane *et al*, 1998). Indeed, in the absence of Tat, LTR-associated nucleosomes are hypoacetylated (Lusic *et al*, 2003). Upon activation by Tat, recruitment of Tat coactivators, PCAF, CBP and GCN5, was observed and correlated to nucleosomal hyperacetylation (Lusic *et al*, 2003). Knock-down of PCAF and p300 dramatically reduced Tat

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transactivation of an integrated HIV-1 promoter (Bres *et al*, 2002). Thus, a limiting step in Tat-mediated activation of the LTR is to relieve the chromatin repression mediated by positioned nucleosomes. Several reports have implicated HDAC activity in chromatin-mediated repression of HIV-1 promoter. First, HDAC inhibitors are potent activators of the HIV-1 LTR (Van Lint *et al*, 1996). Transcription factors such as YY1, NF-κB p50 homodimer and thyroid hormone receptor have been shown to mediate recruitment of HDAC1 to the LTR and consequently inhibit transcription from the viral promoter (Coull *et al*, 2000; He and Margolis, 2002; Hsia and Shi, 2002; Williams *et al*, 2006). However, TSA is also a potent activator of a non-integrated promoter (Kiernan *et al*, 1999; Quivy *et al*, 2002; Pagans *et al*, 2005). TSA cooperates with NF-κB in the activation of a transiently transfected LTR (Quivy *et al*, 2002). Additionally, several transcription factors involved in the activation of the HIV-1 LTR are regulated by reversible acetylation, including NF-κB and Sp1 (Chen *et al*, 2001; Kiernan *et al*, 2003; Huang *et al*, 2005). Thus, whether activation of the HIV-1 promoter by TSA is due to inhibition of HDAC or FDAC (factor deacetylase) activities or both is not clear.

In addition to histone hypoacetylation, trimethylation of histone H3 lysine 9 (H3 Lys9) is correlated with heterochromatin assembly and gene silencing (Grewal and Moazed, 2003). H3 Lys9 trimethylation is mediated by a conserved methyltransferase Su(var)3-9 in *Drosophila*, Suv39H1 in humans and Clr4 in fission yeast (Rea *et al*, 2000; Nakayama *et al*, 2001). Swi6 in fission yeast and HP1 (heterochromatin protein 1) in *Drosophila* and humans are conserved proteins found associated with H3Lys9 methyltransferases Suv39H1 and Clr4 (Aagaard *et al*, 1999). Additionally, HP1 and Swi6 bind specifically to H3 Lys9 trimethylated by Suv39H1 and Clr4, respectively (Bannister *et al*, 2001; Lachner *et al*, 2001). Thus, it has been proposed that H3Lys9 methyltransferases are required to initiate formation of heterorochromatin by recruiting HP1 (Cheutin *et al*, 2003), and that heterochromatin propagation and maintenance involves a 'self-sustaining' loop, in which methylated H3Lys9 binds to HP1, which, in turn, recruits more Suv39H1 (Grewal and Moazed, 2003; Maison and Almouzni, 2004). In humans, three isoforms of HP1 protein exist that differ by their localization (Maison and Almouzni, 2004). Whereas HP1α and β are concentrated at pericentric heterochromatin, HP1γ also localizes to euchromatic sites (Minc *et al*, 2000; Nielsen *et al*, 2001). A consequence of HP1 recruitment is the establishment of a chromatin repressive state that leads to gene silencing (Grewal and Moazed, 2003; Maison and Almouzni, 2004).

Although important progress has been made in our understanding of the molecular mechanism involved in viral promoter activation (Marcello *et al*, 2001; Ott *et al*, 2004), the identity of cellular chromatin modifiers and the mechanism involved in the repression of HIV-1 promoter is still unclear. We hypothesized that heterochromatin machinery and repressive histone marks may play a determining role in chromatin-mediated HIV-1 transcriptional silencing and consequently participate in the establishment of the viral reservoir. Here, we present evidence that Suv39H1, HP1γ and histone H3 lysine 9 trimethylation are a major cellular determinant responsible for chromatin-mediated transcriptional silencing of HIV-1.

Results

SUV39H1 and HP1γ mediate repression of the HIV-1 LTR in a chromatin-dependent manner

In order to evaluate the role of Suv39H1 and HP1 in chromatin-mediated repression of the HIV-1 promoter, we employed RNA interference in HeLa cells containing a single copy of an integrated LTR-*luciferase* reporter gene (HeLa LTR-luc) or HeLa cells transiently transfected with LTR-luc construct. The expression level of endogenous Suv39H1 was reduced using a specific small-interfering (siRNA) both in HeLa and HeLa LTR-luc. Knock-down of Suv39H1 was efficient, as measured by RT–PCR (Figure 1A, top panel). Knock-down expression of Suv39H1 in HeLa LTR-luc cells had no effect on basal expression, whereas it enhanced Tat-mediated activation by up to five-fold (Figure 1A, lower panel). Interestingly, no significant effect was observed when an LTR-luc construct was transiently transfected in HeLa cells (Figure 1A, lower panel). We next investigated the role of HP1 proteins in both basal LTR activity and Tat transactivation. We used specific siRNAs for each isoform of HP1 (Figure1B and Supplementary Figure S1). Whereas knock-down of HP1α and HP1β had only a minor effect on LTR basal activity (Supplementary Figure S1) and Tat-mediated transactivation (data not shown), knock-down of HP1γ both enhanced LTR basal expression level (up to 10-fold) and facilitated Tat transactivation of the HIV1 LTR (up to 100-fold) (Figure 1B, lower left panel). Knock-down of HP1γ had no effect on a transiently transfected LTR-luc reporter gene (Figure 1B, right panel). Taken together, these results show that Suv39H1 and HP1γ play a significant role in the chromatin-mediated repression of the viral promoter. Our results, showing that HP1γ but not HP1α or β is involved in LTR repression, are consistent with the recent findings that integrated HIV-1 (Schroder *et al*, 2002) and HP1γ are both localized in euchromatin, whereas HP1α and β are concentrated at pericentric heterochromatin (Minc *et al*, 2000; Nielsen *et al*, 2001). It should be noted that cells were transduced with GST-Tat, rather than transfected with a Tat expression plasmid (throughout our study), as we observed that knock-down of HP1 proteins affected the expression of genes from the CMV and TK promoters (Supplementary Figure S2 and data not shown). Enhanced LTR basal activity observed after HP1γ knock-down may result from general transcriptional derepression or from its direct involvement in the regulation of the viral promoter. Thus, we analyzed the consequence of HP1γ knock-down on the expression of cellular genes, such as IκBα, p53 and GAPDH, by RT–PCR. Whereas mRNAs corresponding to luciferase and IκBα were enhanced, no change in mRNA levels of p53 and GAPDH was observed (Figure 1C). Additionally, we found that knock-down of HP1γ did not affect expression of PCAF, p300 or CDK9 as monitored by Western blotting (Figure 4B).

Dynamic association of Suv39H1, HP1γ and H3TriMetK9 with the HIV-1 LTR

Next, we investigated the direct involvement of Suv39H1 and HP1γ in Tat-mediated activation of an integrated viral promoter by performing quantitative chromatin immunoprecipitation assay (Q-ChIP) (Figure 2). HeLa LTR-luc cells were treated for 4 h with GST or GST-Tat. We investigated, by RT–PCR using oligonucleotides specific for the luciferase

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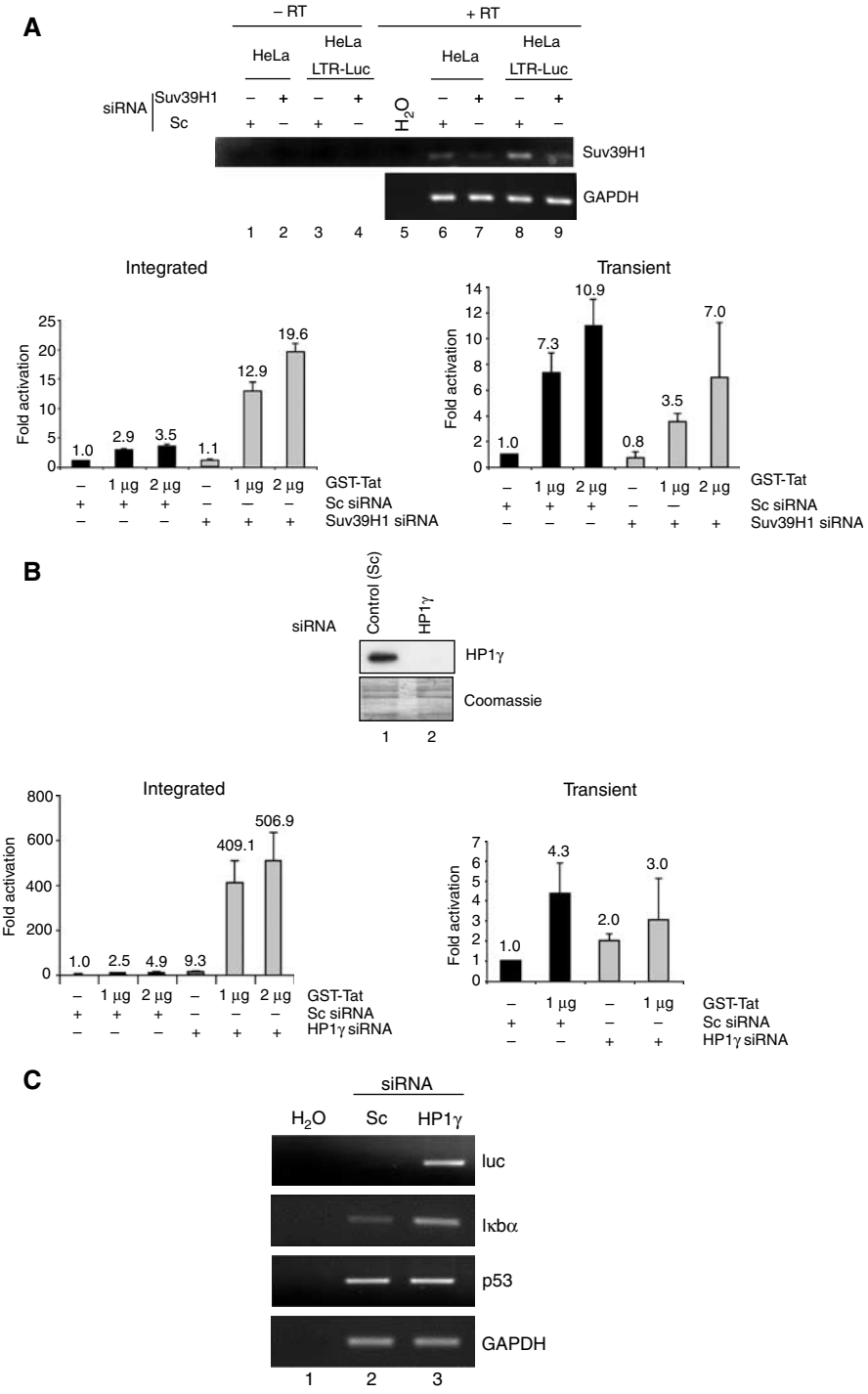

Figure 1 SUV39H1 and HP1γ mediate repression of the HIV-1 LTR in a chromatin-dependent manner. (**A**) HeLa LTR-Luc and HeLa cells were transfected twice with siRNA as indicated. For HeLa cells, LTR-Luc construct was cotransfected with siRNA in the second round of transfection. Twenty-four hours after the second round of transfection, cells were transduced with GST or GST-Tat as indicated. Expression of Suv39H1 and GAPDH mRNA was analyzed by RT–PCR using specific oligonucleotides (top panel). Twenty-four hours after transduction, extracts were prepared and luciferase activity was measured (lower panels). Results are presented as fold activation relative to GST-treated cells. The mean relative luciferase activities were obtained from three independent experiments. (**B**) Experiment was performed as in (A), except that siRNA specific for each isoform of HP1 was used. Expression levels of HP1α, β (Supplementary Figure S1) and γ were analyzed by Western blotting using specific antibodies (top panel). Results are presented as fold activation relative to GST-treated cells. The mean relative luciferase activities were obtained from three independent experiments. (**C**) Total RNA was prepared from cells transfected with control siRNA (Sc) or HP1γ-specific siRNA (experiment shown in (B)). RT–PCR was performed using oligonucleotides specific for the indicated genes. PCR products were analyzed on agarose gels and visualized by ethidium bromide.

reporter gene that a 4-h treatment with GST-Tat is sufficient to induce transcription from the integrated LTR (Figure 2A). Consistent with transcriptional activation, GST-Tat was recruited to the viral promoter (Figure 2B). The amount of promoter immunoprecipitated using RNA polII, Sp1, p300 and CyclinT1 antibodies in the absence of GST-Tat was higher

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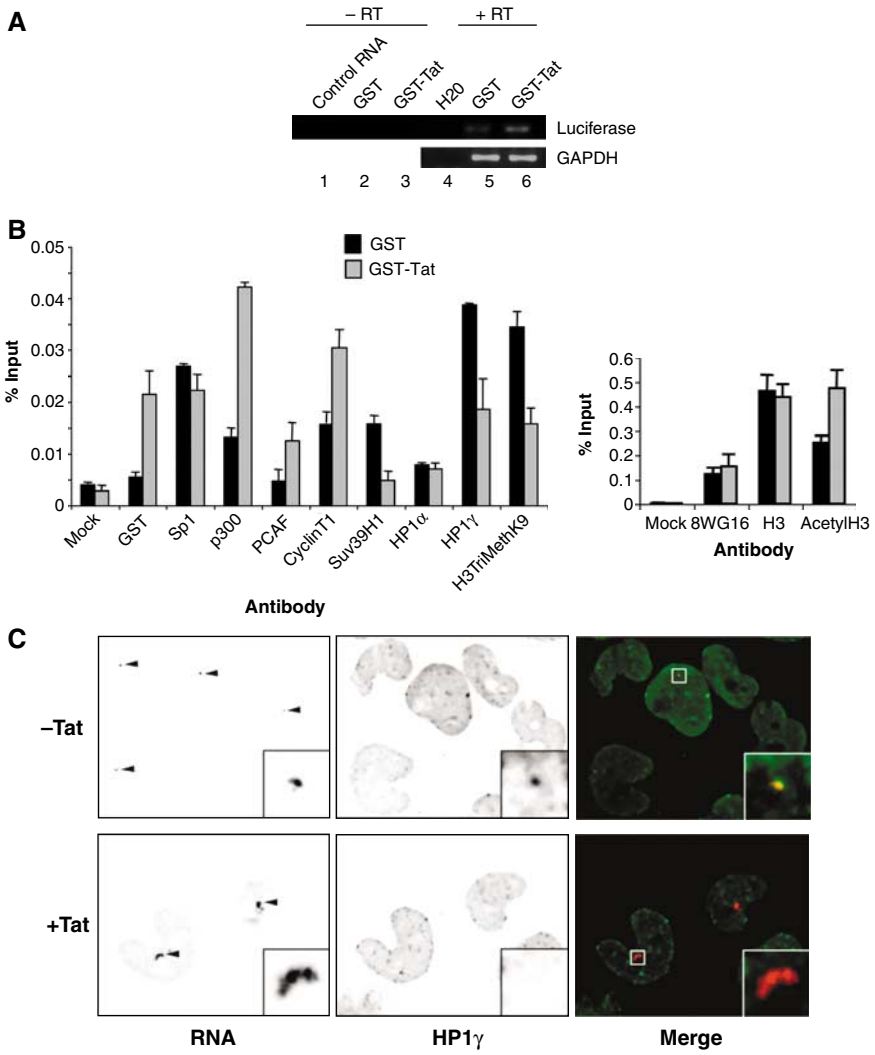

Figure 2 Tat-mediated activation of the HIV-1 LTR is associated with reduction of Suv39H1, HP1γ and H3TriMetK9 associated with the LTR and recruitment of coactivators. HeLa LTR-luc cells were treated for 4 hours with GST or GST-Tat. Tat-mediated LTR activation was measured by RT–PCR using luciferase-specific oligonucleotides and GAPDH oligonucleotide as control (**A**). Chromatin prepared from GST- and GST-Tat-treated cells was subjected to immunoprecipitation with the indicated antibodies (**B**). As a control, immunoprecipitation was performed in the absence of antibody (Mock). Input and immunoprecipitated DNAs were subjected to real-time PCR using LTR-specific oligonucleotides. The amount of immunoprecipitated material was normalized to the input DNA. Data are representative of three independent experiments. (**C**) HIV-1 promoters are associated with HP1γ foci in the absence of Tat. U2OS cells containing an integrated HIV-1 reporter were transfected with a Tat expression vector or a control plasmid. The HIV-1 transcription sites were visualized with a fluorescent oligonucleotide probe against the intronic part of the vector (left panels and red color in the merged image), and HP1γ was labelled with a specific antibody (middle panels and green color in the merged image). In the absence of Tat (upper panels), HIV-1 transcription sites colocalized with HP1γ foci with a high frequency (four out of four in the field shown, and 90% of randomly selected cells; $n = 60$). In contrast, in the presence of Tat (lower panels), HIV-1 transcription sites were located in regions containing low concentrations of HP1γ (two out of two in the field shown, and 95% of randomly selected cells; $n = 60$). Note that the contrasts in the RNA images were scaled differently with and without Tat to allow for their simultaneous visualization. Quantification of the signals in the deconvolved images showed that about 20 times more RNA accumulated at the transcription sites in the presence of Tat. Each field was 69×54 μm. Insets: zoom of the boxed region in the merged images (4.4×4.4 μm).

than that in the control immunoprecipitation sample (mock), suggesting that RNA polII, Sp1, p300 and CyclinT1 are associated with the promoter in the absence of Tat. GST-Tat treatment resulted in enhanced recruitment of p300 and CyclinT1, whereas the amounts of RNA polII and Sp1 were not affected. By contrast, PCAF was specifically recruited to the viral promoter after GST-Tat treatment. Interestingly, in the absence of GST-Tat, the amount of promoter region immunoprecipitated with Suv39H1, HP1γ and H3triMetK9 antibodies was higher than that in the control IP. Treatment of cells with GST-Tat resulted in promoter activation and reduction of promoter-associated Suv39H1 and HP1γ together with a reduced amount of H3Lys9 trimethylation mark. As a control, the amount of H3 was not affected by GST-Tat, whereas H3 acetylation was enhanced after GST-Tat treatment. Taken together, our results show that in the non-transcribing state, Suv39H1 and HP1γ are associated with the promoter and that H3 is trimethylated at K9. Tat-mediated activation of the LTR is accompanied by loss of Suv39H1 and HP1γ and recruitment of transcriptional coactivators such as PCAF, p300 and P-TEFb. At the level of histones, we found that loss of H3TriMetK9 after GST-Tat treatment was

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accompanied by H3 acetylation. To further analyze the dynamic association of HP1γ with the HIV-1 promoter, we performed *in situ* hybridization combined with immunofluoresence using anti-HP1γ-specific antibody. For this purpose, we used U2OS cells harboring an integrated HIV-1 vector containing the Rev responsive element (RRE) and Ψ located between the major splice donor and acceptor site 7, and an additional RNA tag inserted within this intron to facilitate its visualization by *in situ* hybridization. Previous experiments have shown that introns mostly accumulate at their transcription site, and control experiments where HIV-1 DNA and RNA were detected simultaneously showed that the foci detected with the intronic probe indeed corresponded to HIV-1 transcription sites (not shown). In the absence of transcription, integrated HIV-1 associated with HP1γ foci in the nucleus (Figure 2C, upper panels). Upon activation of transcription by Tat, HIV-1 was no longer associated with HP1γ (Figure 2C, lower panels). Taken together, these experiments clearly demonstrate that physical and functional interaction between HIV-1 and HP1γ is dynamic.

Transcriptional activation of the HIV-1 LTR observed after HP1γ knock-down requires Sp1, P-TEFb and PCAF

Next, we investigated the mechanism involved in HP1γ-mediated repression of the LTR by performing ChIP assays to determine promoter occupancy by repressors and activators in cells expressing HP1γ compared to cells where HP1γ level was reduced using specific siRNA (Figure 3). Consistent with experiments shown in Figure 1B, knock-down of HP1γ resulted in a 10-fold increase in LTR basal activity (Figure 3A). By ChIP, the amount of promoter region immunoprecipitated using anti-HP1γ antibody was reduced in cells transfected with HP1γ-specific siRNA compared with control cells (Figure 3B). Interestingly, reduction of Suv39H1 and H3TriMetK9 was observed in HP1γ knock-down cells. Loss of H3TriMetK9 was accompanied by acetylation of H3. While the amount of RNA polII, Sp1 and p300 bound to the promoter was not affected by HP1γ knock-down, we observed a recruitment of PCAF and CyclinT1. These experiments suggest a role for PCAF and P-TEFb in transcriptional activation observed after HP1γ knock-down. As physical and functional interactions between Sp1 and cyclinT1 (Yedavalli *et al*, 2003), and Sp1 and PCAF have been reported (Chu *et al*, 2005), we decided to perform a double knock-down assay to address the requirement for Sp1, CyclinT1 and PCAF in LTR derepression (Figure 4). Knock-down of HP1γ resulted in 74-fold activation of the LTR (Figure 4A). This transcriptional activation was reduced by five-fold when both HP1γ and Sp1 levels were reduced in cells suggesting that the observed LTR activation is dependent on Sp1 transcription factor. Similarly, the enhanced expression from the LTR observed after HP1γ knock-down was dependent on the expression of CDK9 and PCAF (Figure 4B). Requirement for CTD kinase activity in the LTR activation observed after HP1γ knock-down was confirmed by using DRB, which is known to inhibit RNAPII CTD phosphorylation by CDK9, or by expressing a CDK9 dominant-negative mutant in HP1γ knock-down HeLa LTR-luc cells (data not shown). Consistent with ChIP assays, double knock-down of HP1γ and p300 had only a minor effect (two-fold reduction of LTR activity). Taken together, these experiments show that enhanced LTR basal activity after knock-down of HP1γ is mediated by Sp1, P-TEFb and PCAF.

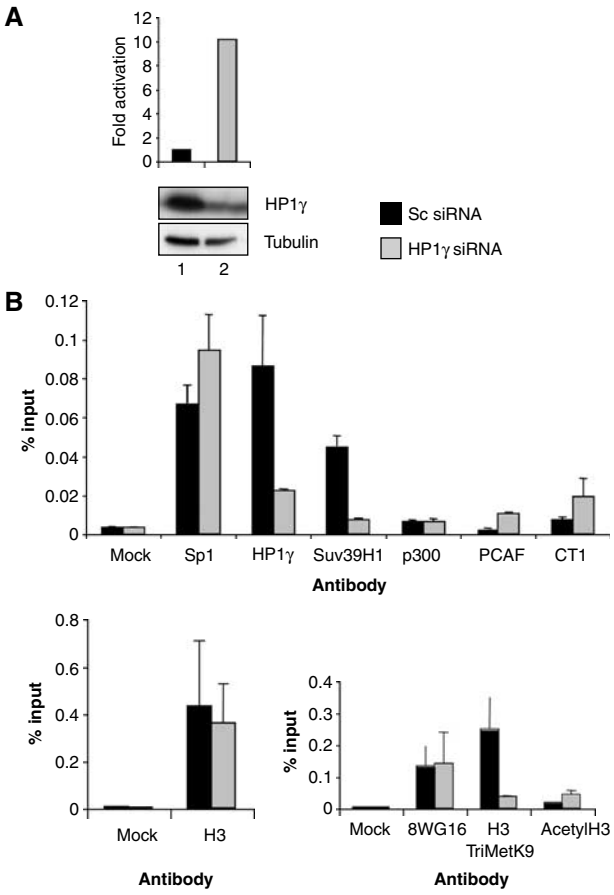

Figure 3 HP1γ knock-down results in reduction of Suv39H1 and H3TriMetK9 associated with the LTR and recruitment of PCAF and P-TEFb. HeLa LTR-Luc cells were transfected with control Sc siRNA (**A**, lane 1; **B**) or HP1γ-specific siRNA (A, lane 2; B). Forty-eight hours post-transfection, cells were harvested. An aliquot of cells was used to analyze luciferase activity (presented as fold activation relative to Sc siRNA-transfected cells) and HP1γ and tubulin expression by Western blotting (A). ChIP assay was performed as in Figure 2 using the indicated antibodies (B). Data are representative of three independent experiments.

Knock-down of HP1γ leads to HIV-1 reactivation in latently infected cells

Our data highlight the role of Suv39H1and HP1γ in chromatin-mediated repression of the HIV-1 promoter. We next evaluated the role of HP1γ in HIV-1 replication by employing HP1γ RNA interference. Recent findings have shown that stochastic fluctuations in Tat influence the commitment to viral latency decision (Weinberger *et al*, 2005). Low Tat expression or low Tat transcriptional activity (due to mutations in the gene or low expression of a coactivator) leads to phenotypic bifurcation and commitment toward latency. In this regard, latently infected U1 cells, which express mutant Tat unable to efficiently activate the LTR and consequently produce low amount of virus (Emiliani *et al*, 1998), represent an interesting model to study the involvement of HP1γ in HIV-1 transcriptional latency. Thus, we analyzed the consequence of HP1γ knock-down on HIV-1 production in U1 cells. As shown in Figure 5A, knock-down of HP1γ (left panel) results in increased HIV-1 production compared with control, HP1α or HP1β siRNA-transfected cells, suggesting a role of HP1γ in HIV-1 transcriptional latency in U1 cells. This experiment also confirms the lack of function of HP1α and

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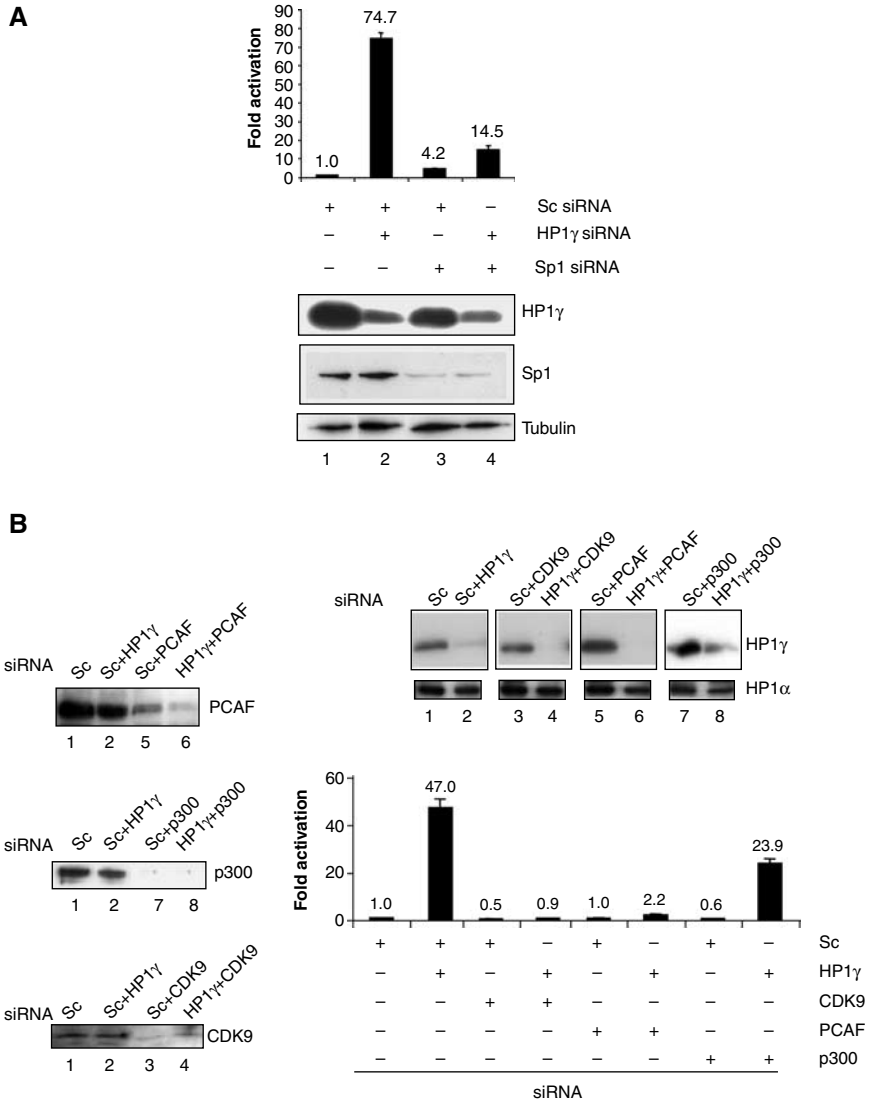

Figure 4 Transcriptional activation of the HIV-1 LTR observed after HP1γ knock-down requires Sp1, P-TEFb and PCAF. HeLa LTR-Luc cells were transfected with siRNAs specific for HP1γ, Sp1, CDK9, PCAF, p300 or control (Sc), as indicated. Luciferase activity was measured 48 h after transfection. Expression levels of HP1γ, Sp1, CDK9, PCAF, p300 and HP1α were determined by immunoblotting. Results are presented as fold activation relative to transfection of control siRNA. The mean relative luciferase activities were obtained from three independent experiments.

HP1β in the viral promoter activity observed in Supplementary Figure S1. Interestingly, TNFα-mediated activation of HIV-1 was enhanced two-fold in HP1γ knock-down U1 cells and not affected when HP1α and HP1β levels were reduced (Figure 5). Similarly, Knock-down of HP1γ, but not HP1α, induced viral LTR activity in Jurkat cells harboring a latent HIV-1 retroviral vector lacking the *tat* gene and in which *gfp* is under the control of the HIV-1 LTR (Jordan *et al*, 2003) (Supplementary Figure S3). Next, we analyzed the role of HP1γ in HIV-1 infection of Jurkat T-cell line. Jurkat cells that were transfected with control siRNA or HP1γ-specific siRNA (Figure 5B) were infected with HIV-1. Virus replication was monitored by measuring the production of p24 in the supernatant every 3 days post-infection. Comparison of HIV-1 replication reveals faster replication kinetics of HIV-1 in HP1γ knock-down Jurkat cells (Figure 5C). This effect was observed early post-infection (days 3, 7 and 10: Supplementary Figure S4). Given that the knock-down of HP1γ is only transient, the observed effect on virus replication suggests that HP1γ-mediated repression of viral gene expression, and consequently virus replication, takes place early during infection. To verify this hypothesis, we evaluated the effect of HP1γ knock-down in a single-round infection assay, where cells were infected with HIV-1 lacking its envelope gene, pseudotyped with vesicular stomatitis virus (VSV.G) envelope and harboring luciferase gene (HIV-1VSV-Luc). Interestingly, in a single-round infection assay, we observed that knock-down of HP1γ resulted in increased virus production compared with control siRNA-transfected Jurkat cells, as measured by luciferase activity (Figure 5D). As expected, knock-down of p300 resulted in reduction of virus production. Furthermore, we found that HP1γ knock-down did not affect reverse transcription or the number of HIV-1 integration events (Supplementary Figure S5). Taken together, these experiments show that HP1γ-mediated chromatin repression of HIV-1 gene expression is established early during infection at a post-integration step. Enhanced virus production observed after HP1γ knock-down is due to either enhanced transcription from integrated HIV-1 or an enhanced number of transcribing HIV-1-integrated proviruses. To further

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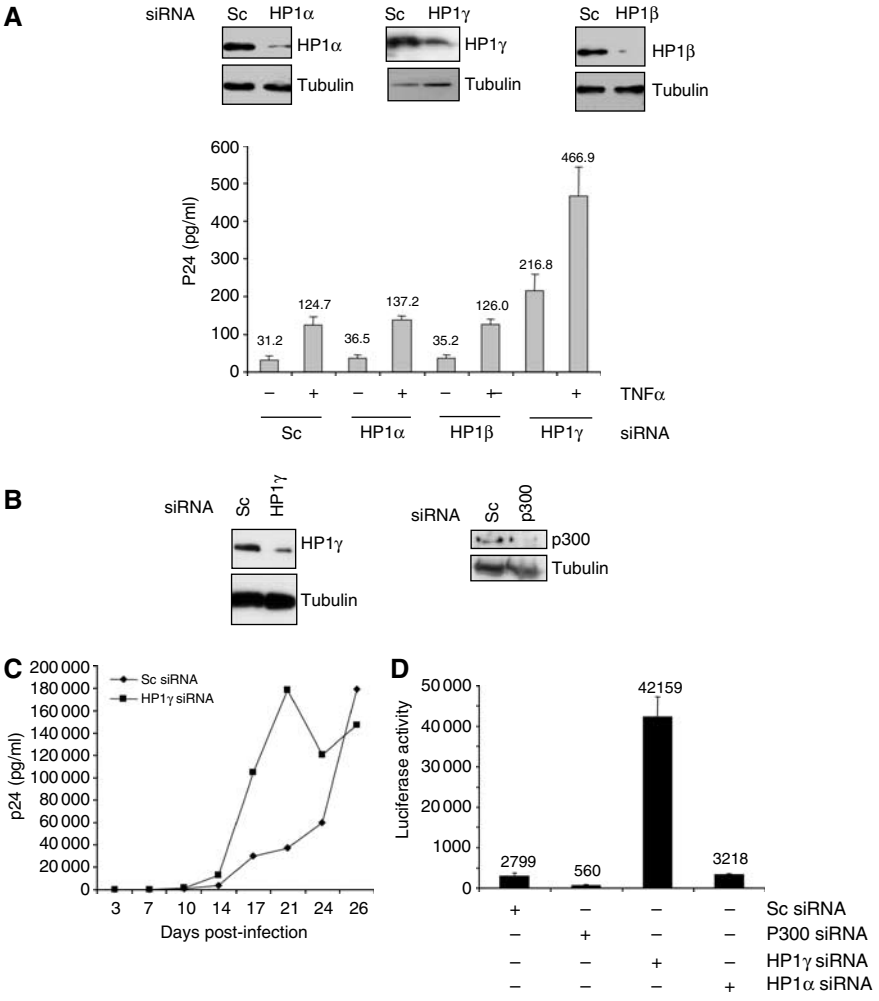

Figure 5 (**A**) Knock-down of HP1γ results in increased virus production in U1 cells, a cellular model for HIV-1 latency. U1 cells were transfected with siRNA as indicated in the figure. Efficiency of HP1α, HP1β and HP1γ knock-down was analyzed 48 h after transfection by Western blotting using a specific antibody. Virus production was monitored 48 h post-transfection by measuring p24 antigen in culture supernatant. Cells were treated with TNFα for 12 h when indicated. (**B–D**) HP1γ-mediated inhibition of HIV-1 replication is established early after virus infection. Jurkat cells were transfected with either control siRNA or HP1γ-, p300- or HP1α-specific siRNA. At 48 h post-transfection, cells were either (B) analyzed for expression levels of HP1γ, HP1α and p300 or (C) infected with HIV-1, and virus replication was monitored every 3 days after infection by measuring p24 viral antigen in culture supernatant or (D) infected with a single-round infectious virus (HIV-1VSV-luc) and virus production was monitored 48 h after infection by measuring luciferase activity in cell extracts.

characterize the involvement of HP1γ in chromatin-mediated HIV-1 repression in more physiological assays, we analyzed the effect of HP1γ knock-down on HIV-1 replication in PBMCs from HIV-1-infected untreated donors (Figure 6A). CD8+ T-cell-depleted PBMCs were transfected with control siRNA or HP1γ siRNA. As shown in Figure 6, knock-down of HP1γ in PBMCs from HIV-1-infected patients resulted in faster HIV-1 replication kinetics compared with control siRNA-transfected PBMCs. Interestingly, when PBMCs were isolated from HAART-treated patients with undetectable viremia, virus could be isolated from HP1γ siRNA-, but not from control siRNA-transfected PBMCs (Figure 6B). These results demonstrate the involvement of HP1γ in HIV-1 silencing in naturally infected cells.

Discussion

In this paper, we have shown that Suv39H1, HP1γ and H3Lys9 trimethylation plays a major role in chromatin-mediated repression of HIV-1 gene expression in several systems, including HeLa cells containing a single integrated copy of the HIV-1 LTR-luc reporter gene, an HIV-1-infected T-cell line and PBMCs from infected individuals. These findings further advance our understanding of Tat-mediated activation of viral gene expression and the mechanism involved in transcriptional latency, which leads to the establishment of a viral reservoir observed in HIV-infected patients.

Currently, it is not clear how transcriptional silencing of HIV-1 genes is established. HDAC1 has been implicated in chromatin-mediated LTR silencing. Both YY1 and the NF-κB p50 homodimer have been shown to recruit HDAC1 to the LTR to repress its basal transcription activity (Coull *et al*, 2000; He and Margolis, 2002; Williams *et al*, 2006). Knock-down of p50 stimulated basal LTR activity up to three-fold (Williams *et al*, 2006). We observed that knock-down of HDAC1 had a minor effect on LTR basal activity (two-fold: Supplementary Figure S6), suggesting that inhibition of HDAC1 activity is not sufficient for efficient LTR derepression. In contrast, the deacetylase inhibitor TSA induces high promoter activity despite the modest effect of HDAC1

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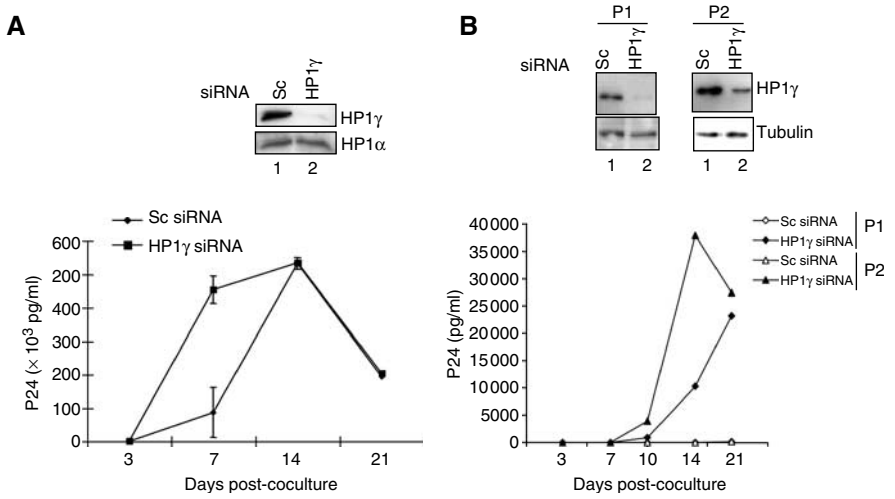

Figure 6 Knock-down of HP1γ results in faster HIV-1 replication in PBMCs from infected donors. CD4 + T cells purified from HIV-1-infected untreated (**A**) or HAART-treated (**B**) patients were transfected with control siRNA or HP1γ-specific siRNA using nucleofection. siRNA-transfected cells were cocultured with activated PBMCs from a healthy donor. Virus replication was monitored every 3 days after co-culture by measuring p24 viral antigen in culture supernatant (P1: patient 1; P2: patient 2). Knock-down of HP1γ was analyzed by Western blotting 48 h post-transfection in cells that were not subjected to coculture.

knock-down. One possibility is that HDAC-mediated repression of the viral promoter involves more than one HDAC and knock-down of one of the HDACs is not sufficient for full promoter de-repression. Alternatively, it has recently been shown that treatment with TSA reduces the amount of HP1 proteins, particularly HP1γ, in cells (Bartova *et al*, 2005). Thus, TSA, by reducing the expression level of HP1γ, mimics the effect of HP1γ-specific siRNA on LTR activity. In support of this, we found that TSA-mediated activation of an integrated LTR was maintained after HDAC1 knock-down, whereas it decreased five-fold when HP1γ expression level was reduced using specific siRNA (Supplementary Figure S6), suggesting that the effect of TSA is dependent on HP1γ. Further experiments are required to analyze the effect of HDAC1 knock-down and TSA treatment on HP1γ removal from the viral promoter. Recently, it has been shown that activation of HIV-1 from latently infected Jurkat cells by NF-κB requires the recruitment of TFIIH (Kim *et al*, 2006), suggesting that limitation in TFIIH may lead to transcriptional silencing and latency.

In microglial cells, which are targets of HIV infection within the central nervous system, COUP-TF interacting protein 2, CTIP2, represses LTR activity through its interaction with COUP-TF and Sp1 transcription factors (Marban *et al*, 2005). Whether CTIP2 plays a repressive role in T cells is not known. A surprising but interesting observation was that knock-down of Sp1 transcription factor enhanced basal LTR activity up to four-fold (Figure 4A). Thus, knock-down of Sp1 may lead to removal of CTIP2 from the viral promoter and consequently its de-repression. Further work is needed to evaluate the role of CTIP2 in LTR activity in T cells.

HP1γ is recruited to the HIV-1 promoter

Our experiments clearly show that HP1γ plays a major role in chromatin-mediated repression of viral gene expression. Up to 100-fold activation of basal LTR activity could be observed depending on the efficiency of HP1γ knock-down. How the heterochromatin complex is recruited to the HIV-1 promoter is unknown. In cells, it is known however that the repetitive nature of satellite repeats and transposons plays a role in the recruitment of heterochromatin machinery leading to silencing of nearby genes (Grewal and Moazed, 2003). Additionally, non-coding RNA molecules, including dsRNA, are known to play a role in heterochromatin formation presumably by a mechanism involving a direct interaction between chromodomain proteins and RNA (Grewal and Moazed, 2003). Whether Suv39H1 and/or HP1γ can bind to the HIV-1 short transcripts produced in the absence of Tat and thus be recruited to the viral promoter to establish a silent chromatin state is not known. It has been proposed that HP1, bound to methylated H3Lys9, mediates recruitment of Suv39H1 for heterochromatin propagation and maintenance in a 'self-sustaining' loop (Grewal and Moazed, 2003; Maison and Almouzni, 2004). Accordingly, when we analyzed the consequence of HP1γ knock-down on LTR occupancy (Figure 7), we found that Suv39H1 was displaced and the H3TriMetK9 repressive mark was reduced. In contrast, coactivators, such as PCAF and P-TEFb, were recruited. Interestingly, whereas knock-down of Suv39H1 did not affect basal expression from an integrated HIV-1 LTR (Figure 1A) or virus production in U1 cells (data not shown), HIV-1 replicated with faster kinetics when Jurkat cells were transfected with Suv39H1-specific siRNA and subsequently infected with HIV-1 (Supplementary Figure S7). This experiment suggests that Suv39H1 is required for the deposition of the repressive H3Lys9 methylation mark, which consequently leads to recruitment of HP1γ to maintain the repressive state. Derepression of the viral promoter observed after HP1γ knock-down is dependent on the presence of Sp1 transcription factor. Interestingly, it has recently been shown that Sp1 can recruit P-TEFb and PCAF to Sp1 target promoters. Sp1 transcriptional activity is dependent on the CTD-kinase activity of P-TEFb. Indeed, Sp1 interacts directly with the regulatory subunit of P-TEFb, CyclinT1 (Yedavalli *et al*, 2003). Activation of the TbetaRII promoter involves

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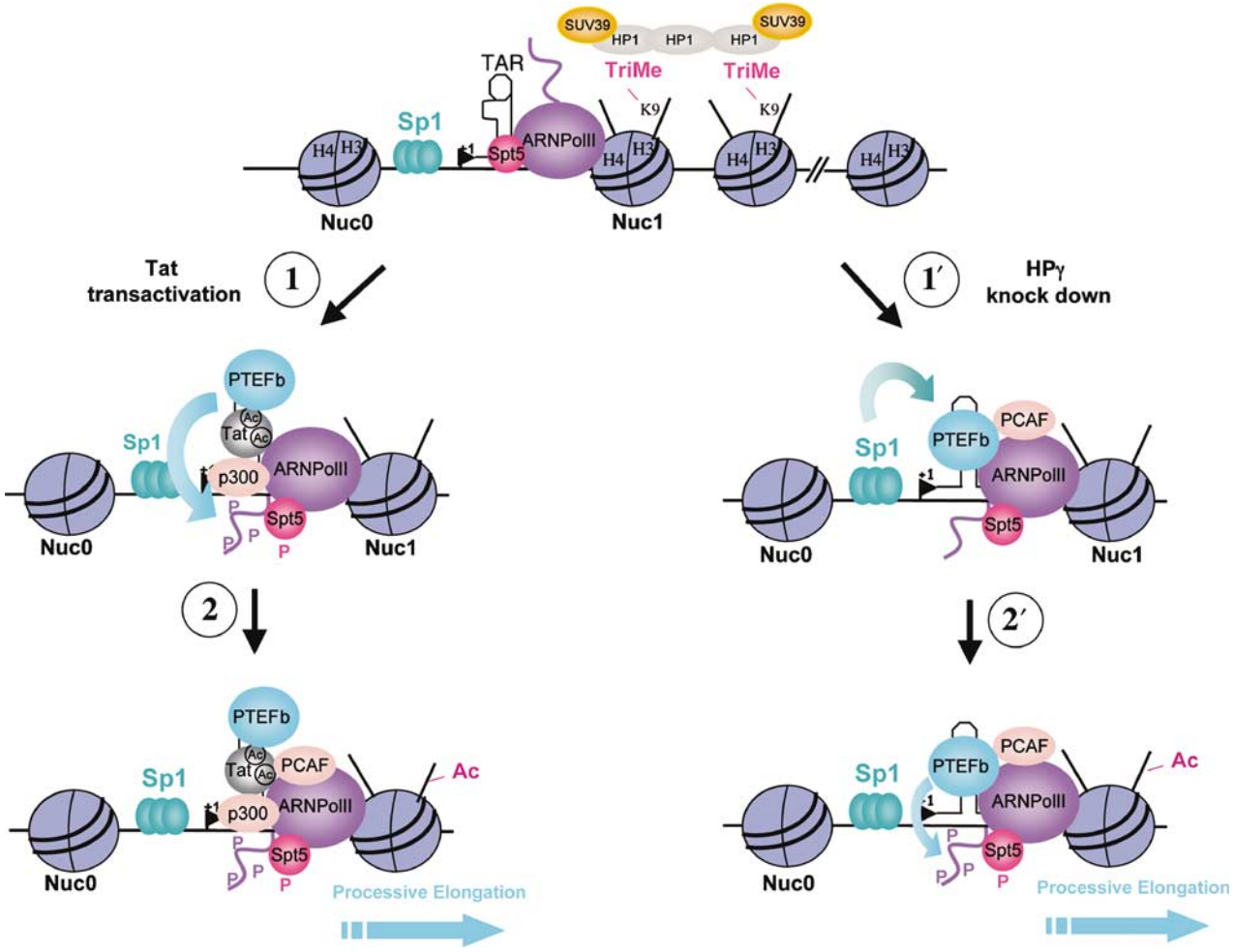

Figure 7 A proposed model for the involvement of chromatin in the regulation of HIV-1 promoter activity.

recruitment of PCAF and p300 into a complex containing Sp1/NF-Y/HDAC1 (Huang *et al*, 2005). Acetylation of Sp1 by PCAF leads to dissociation of HDAC1 from the Sp1/NF-Y complex (Huang *et al*, 2005). Consistent with this, we found that P-TEFb and PCAF are required for Sp1-mediated activation of the viral promoter after HP1γ knock-down, suggesting a similar mechanism. Alternatively, NF-κB can also recruit P-TEFb to the viral promoter (Barboric *et al*, 2001). However, knock-down of p65 did not affect promoter derepression observed after HP1γ knock-down (Supplementary Figure S8).

Tat-mediated transactivation of the HIV-1 promoter involves chromatin derepression and recruitment of coactivators

In addition to its effect on basal transcription from the LTR, we observed that Tat-dependent activation of the integrated LTR was also enhanced 100-fold after HP1γ knock-down, suggesting that knock-down of HP1γ also facilitates Tat-mediated transactivation. The increased Tat transcriptional activity was dependent on its binding to TAR RNA, as mutation of TAR RNA, which leads to loss of Tat binding, inhibited Tat transactivation of the LTR in both control and HP1γ knocked-down cells (Supplementary Figure S9). By contrast, activation of basal expression of the LTR observed after HP1γ knock-down was not affected by mutation of TAR (Supplementary Figure S9). Interestingly, Tat recruitment to the TAR element is accompanied by recruitment of coactivators such as P-TEFb, PCAF and p300 and concomitant reduction of Suv39H1, HP1γ and H3TriMetK9 associated with the promoter (Figure 7). Whereas HP1γ knock-down results in recruitment of PCAF and P-TEFb to the promoter, p300 was not recruited, unlike in cells treated with Tat. Thus, for optimal transactivation, Tat needs to relieve chromatin-mediated repression and recruit specific coactivators to the promoter (Figure 7). Whether p300 plays a role in Tat-specific de-repression of the LTR remains to be determined. Indeed, it is not clear how Tat relieves chromatin repression of the LTR. One possibility is that Tat associates with an enzymatic activity responsible for direct modification of H3TriMetK9 (Chen *et al*, 2006; Cloos *et al*, 2006; Klose *et al*, 2006; Yamane *et al*, 2006) or modification of local histones that destabilizes HP1γ binding (Fischle *et al*, 2005). Alternatively, a reported interaction between Tat and silent information regulator, SirT1 (Pagans *et al*, 2005), may contribute to the release of heterochromatin complex from the viral promoter. Indeed, Sir proteins are known to play a central role in the recruitment of heterochromatin complexes by inducing a local hypoacetylation of histones (Grewal and Moazed, 2003). Tat has been shown to be deacetylated by SirT1(Pagans *et al*, 2005). Thus, Tat may decoy the HDAC activity of SirT1, leading to a local hyperacetylation of histones and consequently the release of HP1γ and Suv39H1 from the LTR. Further work is needed to

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elucidate the mechanism involved in Tat-mediated chromatin derepression within the viral promoter.

Involvement of HP1γ in chromatin-mediated HIV-1 transcriptional silencing and latency

Transcriptional silencing of HIV-1 provirus in CD4 + T-cells is the major mechanism responsible for viral latency and establishment of a stable viral reservoir (Bisgrove *et al*, 2005). This reservoir is established early during primary infection. Results from several experiments presented in this paper suggest a role of HP1γ in HIV-1 transcriptional latency and the establishment of the viral reservoir. (1) HP1γ-mediated repression of HIV-1 replication was observed in PBMCs from infected donors and in U1 cells, a cellular model for HIV-1 transcriptional latency. (2) HP1γ-mediated repression of the viral promoter can be observed in a single-round infection assay, suggesting that promoter repression is established early during infection. (3) Transient knock-down of HP1γ results in faster HIV-1 replication kinetics in Jurkat cells. (4) HP1γ does not affect the number of integrated proviruses, but rather the rate of transcription from integrated HIV-1.

A challenge in AIDS treatment is the reactivation of the viral reservoir in combination with HAART to completely eradicate the virus (Pomerantz, 2002; Lassen *et al*, 2004). In this respect, several molecules have been used to reactivate viral reservoir, including prostratin (Kulkosky *et al*, 2001), valproic acid (Lehrman *et al*, 2005), IL-2 (Chun *et al*, 1998b), IL-7 (Brooks *et al*, 2003) and the viral transactivator Tat (Lin *et al*, 2003). How conceivable is the use of HP1γ knock-down in viral reservoir reactivation? Although long-term knock-down of HP1γ would certainly be cytotoxic, HIV-1 derepression requires only a transient knock-down of HP1γ, which may be tolerated. Indeed, HP1γ knock-down was not cytotoxic as measured by cell viability assay (data not shown). siRNA-based therapy has been successfully used to protect mice from lethal herpes simplex virus 2 infection (Palliser *et al*, 2006). Additionally, antagomirs were successfully used in mice to inhibit the function of a cellular microRNA. Thus, it will be interesting to evaluate the feasibility of a combinational therapy involving HP1γ-specific siRNA and HAART in eradicating the HIV-1 reservoir in infected individuals.

Materials and methods

Cell culture, transfection and transduction

HeLa and HeLa cells containing a single copy of integrated HIV-1LTR-*luciferase* reporter construct (HeLa LTR-Luc) (Treand *et al*, 2006) were propagated in DMEM with 10% FBS. Jurkat, U1 and PBMCs were propagated in RPMI containing 10% FBS. Oligofectamine was used for siRNA transfection into HeLa cells. Jurkat, U1 and PBMCs were transfected using nucleofection technology according to the manufacturer's protocol (Amaxa). Transduction with GST and GST-Tat was carried out as previously described (Bres *et al*, 2005). Luciferase activity was measured 48 h after transduction according to the manufacturer's protocol (Promega). Luciferase activity was normalized to protein concentration using Bradford (Biorad).

The HeLa LTR_{HIV-1}wt-Luc and HeLa LTR_{HIV-1}TARm-Luc cells are derived from HeLa cells in which one copy of a *luciferase* gene under the control of the entire HIV-1 BRU promoter (LTR from −454 to +338) wt or ΔTAR (deletion of the 3-nt bulge of TAR, nt 476–478) has been inserted by recombination using the 'Flp-In™ system' kit (Invitrogen) according to the manufacturer's protocol (see Supplementary data).

Plasmids

The LTR-Luc reporter gene construct was previously described (Kiernan *et al*, 1999).

Small-interfering RNAs

RNA oligonucleotides corresponding to SUV39H1 (forward: 5′-ACCUCUUUGACCUGGACUATT-3′; reverse: 5′-UAGUCCAGGUCAAA GAGGUTT-3′), HP1α (forward: 5′-GCUUUGAGAGAGGACUG GAACTT-3′; reverse: 5′-GUUCCAGUCCUCUCUCAAAGCTT-3′), HP1β (forward: 5′-GACUCCAGUGGAGAGCUCAUGTT-3′; reverse: 5′-CAUGAGCUCUCCACUGGAGUCTT-3′), HP1γ (forward: 5′-GAGG CAGAGCCUGAAGAAUTT-3′; reverse: 5′-AUUCUUCAGGCUCUGC CUCTT-3′), Sp1 (forward: 5′-UGCAUGGUGUUGAGAAGAATT-3′; reverse: 5′-UUCUUCUCAACACCAUCCATT-3′), CDK9 (forward: 5′-CCAAAGCUUCCCCCUAUAATT-3′; reverse: 5′-UUAUAGGGGGAAG CUUUGGTT-3′), PCAF (forward: 5′-UCGCCGUGAAGAAAGCG CATT-3′; reverse: 5′-UGCGCUUUCUUCACGGCGATT-3′) and p300 (forward: 5′-CAGAGCAGUCCUGGAUUAGTT-3′; reverse: 5′-CUAAUCCAGGACUGCUCUUGTT-3′) were synthesized (MWG Biotech, Ebersberg, Germany). Control Scramble II siRNAs were purchased from MWG Biotech.

Western blot analysis

Western blot analysis was performed as previously described (Kiernan *et al*, 1999). Proteins were visualized by chemiluminescence (Pierce). Primary antibodies used were anti-HP1α, anti-HP1β, anti-HP1γ (Euromedex), anti-PCAF (Santa Cruz), anti-p300 (Pharmingen), anti-Sp1 (Santa Cruz) and monoclonal anti-tubulin (Sigma).

Chromatin immunoprecipitation

See Supplementary data.

RT–PCR

Total RNA was extracted from HeLa cells using the NucleoSpin RNA kit (Machery Nagel, Duren, Germany) and reverse-transcribed using SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen). RT products were PCR-amplified using oligonucleotides specific for luciferase (forward: 5′-AAG AGA TAC GCC CTG GTT CCT G-3′; reverse: 5′-CGG TAG GCT GCG AAA TGT TCA-3′), GAPDH (forward: 5′-GTA TTG GGC GCC TGG TCA CC-3′; reverse: 5′-CGCTCC TGG AAG ATG GTG ATG G-3′), p53 (forward: 5′-CAG CCA AGT CTG TGA CTT GCA CGT AC-3′; reverse: 5′-CTA TGT CGA AAA GTG TTT CTG TCA TC-3′), IκBα (forward: 5′-GCA GCA GCT CAC CGA GGA CG-3′; reverse: 5′-CAG GAC TGA GTC AGG ACT CC-3′) and Suv39H1 (forward: 5′-AGGACTTAGAAAGGGAGCTGC-3′; reverse: 5′-TACAGTGATGCGTCCCAGATG-3′). PCR products were resolved on 1% agarose/TAE gels containing ethidium bromide.

In situ hybridization

Human U2OS cells were cotransfected with a hygro marker and a previously described HIV-1 vector containing the LTRs, RRE and Ψ (Zufferey *et al*, 1998). The RRE and Ψ were located between the major splice donor and acceptor site 7 and an additional RNA tag was inserted within this intron to facilitate its visualization by *in situ* hybridization, as described previously (Fusco *et al*, 2003). Stable clones were then selected by hygromycin and individual clones were analyzed for their ability to induce transcription when transfected with a Tat-expressing vector. Clone 2E-11 showed a strong response to Tat and was used in further experiments.

Clone 2E-11 was transfected by Tat or an empty vector and cells were fixed 24 h later. *In situ* hybridization was performed as previously described, with a Cy3-labeled oligonucleotide probe specific for the tagged intron (Fusco *et al*, 2003). For HP1γ detection, cells were then re-permeabilized in 0.1% Triton/PBS for 10 min at room temperature and incubated with HP1γ-specific antibody. Cells were imaged on a DMRA microscope (Leica) equipped with a Cool-snap Fx camera (Roper scientific). Image stacks were collected at 0.2 μm intervals and deconvolved using Huygens2 (Bitplane).

PBMC isolation and CD8+ T-cell depletion

Peripheral blood mononuclear cells of HIV-1-infected, untreated patients from University Hospital of Montpellier were isolated by Ficoll–Paque (Eurobio) density centrifugation. PBMCs (10^6 cells/ml) were then incubated for 1 h at 4°C with anti-CD8 monoclonal antibody B9–11 (1 μg/ml; Beckman-Coulter) in PBS supplemented

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with 0.1% FCS. Cells were washed twice and incubated for an additional hour at 4°C with 100 µl of magnetic beads coated with anti-mouse Ig (CELLection™ Pan Mouse IgG kit, Dynal Biotech). The mixture was placed in a magnet and cells that were not bound to beads were collected. The collected cells contained less than 5% CD8+ T cells.

Coculture assay for viral production

PBMCs from healthy donors were preactivated using 5 µg/ml PHA (phytohemagglutinin-P; DIFCO)/10 U/ml IL-2 (interleukin-2, Roche) for 72 h. They were then washed once with PBS and once with RPMI medium before coculture assay. The siRNA-electroporated HIV-positive PBMCs (10^6 cells/ml) were cocultured with preactivated PBMCs (10^6 cells/ml) from healthy donors in the presence of 10 U/ml IL-2. The culture medium was collected every 3 or 4 days. Fresh preactivated healthy PBMC were added to the culture every 7 days. Viral production was measured by quantifying the amount of p24 in the culture medium using an ELISA kit (Beckman-Coulter).

Infection

Jurkat cells were infected with HIV-1 for 2 h at 37°C, washed and resuspended in RPMI containing 10% FCS. Virus production was monitored in culture supernatant every 3 days by measuring p24 antigen (Beckman Coulter).

Pseudotyped virion production and single-round infection

The plasmid pNL4.3-env$^-$-Luc$^+$, harboring a *luciferase* gene (obtained from AIDSreagent Program), was cotransfected with the envelope plasmid pMD.2G encoding the G protein of VSV.G into human embryonic kidney cells 293T. The virions, named HIV-1VSV-Luc, were collected and filtered (0.45-µm filters) 48 h post-transfection.

For single-round infection, Jurkat cells were transfected with siRNA and 48 h later were incubated with the VSV-Luc virions or heat-inactivated virions (56°C, 30 min) at an MOI of 0.01 for 18 h. They were then washed twice with PBS and cultured in RPMI-1640 medium containing 10% FCS. Luciferase activity was measured 48 h post-infection using a luminometer. Total DNA was also extracted from a portion of infected cells for real-time PCR assay.

Integration *(Brussel and Sonigo 2003)*

See Supplementary materials.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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