Epigenetic displacement of HP1 from heterochromatin by HIV-1 Vpr causes premature sister chromatid separation

Mari Shimura,1 Yusuke Toyoda,2 Kenta Iijima,1 Masanobu Kinomoto,3 Kenzo Tokunaga,4 Kinya Yoda,4 Mitsuhiro Yanagida,2 Tetsutaro Sata,3 and Yukihito Ishizaka1

1Department of Intractable Diseases, Research Institute, National Center for Global Health and Medicine, Shinjuku, Tokyo 162-8655, Japan
2Department of Gene Mechanisms, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
3Department of Pathology, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan
4Bioscience Biotechnology Center, Nagoya University, Nagoya 464-8601, Japan

Although pericentromeric heterochromatin is essential for chromosome segregation, its role in humans remains controversial. Dissecting the function of HIV-1-encoded Vpr, we unraveled important properties of heterochromatin during chromosome segregation. In Vpr-expressing cells, hRad21, hSgo1, and hMis12, which are crucial for proper chromosome segregation, were displaced from the centromeres of mitotic chromosomes, resulting in premature chromatid separation (PCS). Interestingly, Vpr displaced heterochromatin protein 1-α (HP1-α) and HP1-γ from chromatin. RNA interference (RNAi) experiments revealed that down-regulation of HP1-α and/or HP1-γ induced PCS, concomitant with the displacement of hRad21. Notably, Vpr stimulated the acetylation of histone H3, whereas p300 RNAi attenuated the Vpr-induced displacement of HP1-α and PCS. Furthermore, Vpr bound to p300 that was present in insoluble regions of the nucleus, suggesting that Vpr aberrantly recruits the histone acetyltransferase activity of p300 to chromatin, displaces HP1-α, and causes chromatid cohesion defects. Our study reveals for the first time centromere cohesion impairment resulting from epigenetic disruption of higher-order structures of heterochromatin by a viral pathogen.

Introduction

The scheduled separation of chromosomes is crucial for balanced chromosome segregation. A cohesin complex keeps sister chromatids held together until the onset of anaphase (Nasmyth, 2002; Yanagida, 2005). If centromeric cohesion is impaired, sister chromatids separate before anaphase, resulting in premature chromatid separation (PCS; Kitajima et al., 2006; Toyoda and Yanagida, 2006). We previously reported that PCS occurs in the peripheral blood lymphocytes (PBLs) of HIV-1-infected individuals (Shimura et al., 2005). Strikingly, in vitro HIV-1 infection induced PCS in PBLs isolated from healthy humans, strongly suggesting that a viral factor was responsible for PCS. As PCS has been associated with aneuploidy, it is important to identify the mechanisms involved (Thompson et al., 1993; Zhu et al., 1995; Kajii et al., 2001).

Centromere cohesion is regulated by a cohesin complex, which consists of four evolutionarily conserved subunits: the structural maintenance of chromosome (SMC) proteins Smc1 and Smc3 and the non-SMC proteins Scc3/SA and Scc1/Rad21/kleisin (Hirano, 2005). During mitosis, cohesin complexes at the chromosome arm are released non-proteolytically in a process mediated by Aurora B (AurB) and Pololike kinase 1 (Losada et al., 2002; Sumara et al., 2004). In contrast, centromeric...
HIV-1 consists of nine heterochromatin protein 1 (HP1), 2-3 heterochromatin. Are susceptible to disruption by the epigenetic modification of chromatin. Of chromatin. Are susceptible to disruption by the epigenetic modification of chromatin. The molecular mechanisms behind these pathological conditions are caused by mutations in genes essential for chromatid cohesion, including Bub1 (Kajii et al., 2001). PCS is also observed in malignant cancers (Thompson et al., 1993; Zhu et al., 1995). Although the molecular mechanisms behind these pathological conditions remain unclear, our data indicate that centromere proteins are susceptible to disruption by the epigenetic modification of chromatin.

High rates of PCS have been reported in human diseases caused by mutations in genes essential for chromatin cohesion, including ESCO2 and NIPBL/Adherin, which are responsible for Roberts syndrome and Cornelia de Lange syndrome, respectively (Kaur et al., 2005; Vega et al., 2005; Dorsett, 2007), and are involved in the spindle assembly checkpoint, including Bub1 (Kajii et al., 2001). PCS is also observed in malignant cancers (Thompson et al., 1993; Zhu et al., 1995). Although the molecular mechanisms behind these pathological conditions remain unclear, our data indicate that centromere proteins are susceptible to disruption by the epigenetic modification of chromatin.

Results

Vpr is responsible for PCS caused by HIV-1

To identify the HIV-1 gene responsible for PCS, we infected human PBLs with wild-type (wt) or mutant viruses (Δvpu, Δvpr, and Δvif; Fig. 1 A, bottom). HIV-1 consists of nine ORFs containing three structural genes, gag, pol, and env, plus the regulatory genes rev and tat, together with four different accessory genes, vif, vpr, vpu, and nef (Fig. 1 A, top). In this study, we used vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped viruses lacking the nef gene to perform a single-round replication assay (Kinomoto et al., 2007) in which we introduced mutations only into accessory genes with no influence on the rate of infection (Malim and Emerman, 2008). As shown in Fig. 1 B, PCS was observed in cells infected with wt, Δvpu, and Δvif viruses but not by Δvpr virus (Fig. 1 C, left). We carefully examined >300 chromosome spreads in each experiment. A chromosome spread was considered PCS positive if more than three chromosomes lacked linkages between sister chromatids. We obtained similar results in two additional independent experiments (Fig. S1 A). Infection rates with a multiplicity of infection of 0.007 were comparable, as assessed by luciferase reporter activity (Fig. 1 C, right), suggesting that vpr is responsible for HIV-1–associated PCS. Next, we examined PCS in MIT-23 cells, which stably express Vpr under the control of a tetracycline-inducible promoter (Shimura et al., 1999). On day 2 after the addition of doxycycline (DOX), PCS was clearly detected in >40% of treated cells (Fig. 1 D, right) but not in control cells (Fig. 1 D, left). To characterize the dosage of Vpr in MIT-23 cells, its expression was compared with HIV-1–infected cells. The level of Vpr in DOX-treated MIT-23 cells was less than one seventh of that in virus-producing U1 cells, which are latently infected and carry two copies of provirus HIV-1 DNA (Fig. S1 B and C; Folks et al., 1987). Based on these data, we conclude that Vpr is responsible for the PCS induced by HIV-1 infection.

Consistent with studies linking PCS with aberrant segregation of sister chromatids (Babu et al., 2003; Sotillo et al., 2007; Zhang et al., 2008), numerous Vpr-expressing G/M phase cells displayed altered ploidy (Fig. 1 E). Time-lapse analysis revealed that mitosis was prolonged in Vpr-expressing cells (Fig. S1 D, right). Furthermore, >50% of the mitotic Vpr-expressing cells examined displayed abnormal mitosis (Fig. S1 D and Videos 1–3). Interestingly, Vpr-expressing cells suffered no apparent cellular crisis, as judged by FACS analysis of the sub-G1 population (Fig. 1 E) and increased aneuploidy (Fig. 1 E, arrow), which is consistent with our previous finding (Shimura et al., 1999).

Vpr causes cohesion defects primarily in mitosis

Because chromosome segregation is tightly controlled by centromeric cohesion (Nonaka et al., 2002; Pidoux and Allshire, 2004), we first investigated the expression of the cohesin subunits Smc1, Smc3, and Sccl/Rad21 in Vpr-expressing MIT-23 cells. 2 d after the induction of Vpr expression, the chromatin-enriched
insoluble fraction was isolated using a reported method (Todorov et al., 1995), and protein expression was assayed by immunoblotting. The amount of Smc1, Smc3, and Sccl/hRad21 proteins in the isolated chromatin decreased by 79, 58, and 53%, respectively (Fig. 2 A, top). In striking contrast, the levels of these proteins in whole-cell extracts remained stable (Fig. 2 A, bottom), suggesting that Vpr altered their subcellular localization. To focus on cohesin localizing to condensed chromosomes, we prepared chromosome spreads from Vpr-expressing cells and stained them with an antibody against hRad21 (Hoque and Ishikawa, 2002). As shown in Fig. 2 B, only small amounts of hRad21 were observed in the spreads, in which sister chromatids were aligned loosely as a result of PCS (Fig. 2 B, right). In contrast, intense hRad21 signals were detected in control cells, especially at the interface of sister chromatids within the centromere, as has been previously described (Fig. 2 B, left; Waizenegger et al., 2000).

Next, to study the effects of Vpr expression on the chromatin localization of hRad21 in interphase, MIT-23 cells were immunostained for chromatin-bound hRad21, and hRad21 intensities and the nuclear sizes were analyzed (Fig. S2, A and B). Chromatin-associated hRad21 levels were not reduced in regularly sized Vpr-expressing cells during interphase, though they were greatly reduced in mitotic cells (Fig. S2 A). Interestingly, the cells with larger nuclei had statistically less chromatin-bound hRad21 (Fisher’s exact test, P = 0.002; Fig. S2 B, bottom). As this population of cells could be produced via defective chromosome segregation (Figs. 1 E and S1 D), the results suggested that the diminished association of hRad21 to the interphase chromatin was observed after hRad21 mislocalization at mitosis.

Cohesin loading onto the interphase chromatin depends on NIPBL/delaning in human cells (Toyoda and Yanagida, 2006). This encouraged us to examine the involvement of NIPBL/delaning in the Vpr-induced PCS. RNAi depletion of NIPBL caused specific down-regulation of delanin/NIPBL (Fig. S2 C). The introduction of NIPBL RNAi decreased the hRad21 signal during interphase remarkably (Fig. S2 C, bottom right), whereas chromatin-associated hRad21 was observed in control cells (Fig. S2 C, bottom left). Transfection of MIT-23 cells with NIPBL RNAi did not alter the frequency of PCS (Fig. S2 D). In contrast, and in line with a previous study (Kaur et al., 2005), it induced PCS in 8.3% of control cells (Fig. S2 D). These data suggest that NIPBL is not a primary Vpr target and that Vpr-induced PCS results from cohesin defects that first become apparent in mitosis. Therefore, we then focused on the mitotic phenotypes to clarify the mechanisms underlying Vpr-induced PCS.

Vpr causes failure in protecting cohesin on the mitotic chromatin

Next, we addressed whether Vpr-induced mislocalization of hRad21 was a result of the displacement of the guardian protein hSgo1 from the mitotic centromere chromatin. MIT-23 cells and their chromosome spreads were immunostained for hSgo1 (Fig. 2 C). Upon Vpr expression, hSgo1 was undetectable on spread chromosomes and prometaphase cells (Fig. 2 C, bottom).
Vpr expression displaces HP1 and hMis12 from chromatin

In fission yeast, Swi6, an HP1 homologue, functions as a key regulator of centromere cohesion during mitosis (Nonaka et al., 2002; Pidoux and Allshire, 2004). HP1-α also regulates the localization of hSgo1 (Yamagishi et al., 2008). Therefore, we analyzed HP1 in Vpr-expressing cells. Western blot analysis using antibodies against three subtypes of human HP1 (HP1-α, HP1-β, and HP1-γ; Hayakawa et al., 2003) revealed that Vpr decreased the amounts of HP1-α and HP1-γ in the isolated chromatin (see Materials and methods; Remboutsika et al., 1999) by >90 and 20%, respectively (Fig. 3 A). Comparable results were obtained upon immunostaining chromatin-bound HP1-α.
Figure 3. Altered localization of HP1-α, HP1-β, and hMis12 proteins in Vpr-expressing cells. (A) HP1-α, HP1-β, and HP1-γ expression in DOX-treated MIT-23 and ∆Vpr cells. Loading control (Cont): a major band of the chromosomal fraction corresponding to 15 kD by Coomassie brilliant blue staining. (B) Chronological changes in HP1-α and hRad21 in the chromosomal (Ch) versus whole-cell (Wh) fractions after DOX addition (days 0–3). βTub, β-tubulin; Vpr, Vpr expression. Data are representative of three independent experiments. (C) Cells were preextracted with PBS containing Triton X-100 and stained with anti-HP1-α antibody and Hoechst 33342. In the bottom right, the pictured cells are in interphase (top cell) and undergoing mitosis (bottom cell). Similar results were obtained in at least three independent experiments. (D) Chromosome spreads were immunostained for HP1 subtypes and CENP-H. Similar results were obtained in three independent experiments. (E) Mitotic cells were immunostained for hMis12 and CENP-A. (F) Chromosome spreads prepared from DOX-induced cells (48 h) were immunostained for hMis12 and CENP-A. (G, left) DOX-induced cells (48 h) were immunostained for hMis12 and CENP-A. (inset) Magnifications of the centromere. (right) The hMis12/CENP-A signal intensity ratio was measured during prophase and prometaphase. Gray bars, ∆Vpr cells; black bars, MIT-23 cells. Values represent the mean ± SD (three experiments) of data generated using the cells shown on the left. *, P = 0.0008 versus Vpr-expressing cells in prophase. (H) Chromosome spreads immunostained with CENP-A and CENP-H antibodies and ACA antisera. Blue, DNA; red, CENP-A, ACA, or CENP-H. Vpr (−), DOX-treated ∆Vpr cells at 48 h; Vpr (+), DOX-treated MIT-23 cells at 48 h. Bars: (C, E, and G) 10 µm; (D, F, and H) 5 µm; (G, insets) 0.5 µm.
and HP1-γ (Fig. S2 E). In striking contrast, no apparent decrease in HP1-β was detected (Fig. 3 A, middle row). Time course analysis of HP1-α and hRad21 proteins revealed that HP1-α from the isolated chromatin (Remboutsika et al., 1999) was undetectable within 48 h of the induction of Vpr expression (Fig. 3 B, top). The hRad21 levels from the chromatin isolated using a reported protocol (Todorov et al., 1995) also decreased over a 72-h period. In addition, the total amount of each protein in whole-cell extracts remained unchanged when Vpr was expressed (Fig. 3 B), suggesting that Vpr alters their subcellular localization without modulating their overall expression. Next, we characterized the chromatin-associated HP1-α levels immunohistochemically and found that HP1-α became undetectable under Vpr expression within 48 h in both mitotic and interphase cells (Fig. 3 C, top right). In contrast, it was clearly detected in control cells (Fig. 3 C, top left). Notably, chromatin-associated HP1-α loss was observed in most of the Vpr-expressing interphase population (Fig. S2 E). However, the chromatin-associated hRad21 loss was not major in regularly sized nuclei but was observed significantly in large nuclear cells (Fig. S2 B). These observations support the idea that a defect in HP1-α precedes the alteration in hRad21.

To identify which subtypes of HP1 proteins were affected for the chromosome localization by Vpr, we investigated the localization of HP1 during mitosis immunohistochemically (Fig. 3 D). In control cells, HP1-α localized to pericentromere regions and the inner centromere, consistent with a previous study (Kiyomitsu et al., 2010). HP1-β localized to the chromosome arm, and HP1-γ localized to both the chromosome arm and centromeric regions (Fig. 3 D, left). Morphological analysis of chromosome spreads from Vpr-expressing cells detected PCS in those lacking a centromeric HP1-α or HP1-γ signal but not those lacking a HP1-β signal (Fig. 3 D, right). CENP-H, a component of the inner kinetochoore, was unaffected in Vpr-expressing cells (Fig. 3 D, right). These data suggest that HP1-α is the HP1 subtype that is most strongly influenced by Vpr expression during mitosis.

HP1-α and HP1-γ physically interact with hMis12, a kinetochoore protein essential for chromosome segregation (Goshima et al., 2003; Obuse et al., 2004). Thus, hMis12 targeting in Vpr-expressing cells was analyzed. Immunohistochemical analysis revealed that hMis12 was undetectable at mitotic kinetochores in Vpr-expressing cells (Fig. 3 E), whereas the centromere-specific histone H3 variant CENP-A was unaffected (Fig. 3 E). Simultaneous morphological analysis of chromosome spreads detected PCS in spreads that lacked hMis12 signals (Fig. 3 F, bottom). In addition, centromere proteins detected by an anti-centromere antibody (ACA) that primarily binds to CENP-B were also unaffected by Vpr, similar to CENP-A and CENP-H (Fig. 3 H, right). We then characterized hMis12 localization during mitosis. In Vpr-expressing cells, hMis12 and CENP-A signals were both detectable at prophase (Fig. 3 G, top right). However, the hMis12 signal became nearly undetectable at prometaphase (Fig. 3 G, bottom right). CENP-A was detected in the same specimens on separated sister chromatids (Fig. 3 G, bottom right inset). To obtain more robust data, the relative signal intensities of hMis12 and CENP-A were measured in >400 sister kinetochores at prophase and prometaphase (see Materials and methods). In Vpr-expressing cells, the hMis12/CENP-A signal ratio was decreased significantly when cells entered prometaphase (P < 0.01; Fig. 3 G, black bar), whereas in control cells, it was comparable to that measured at prophase (Fig. 3 G, gray bar). These results suggest that Vpr interferes with the heterochromatin structure by displacing HP1-α/γ proteins in interphase and that their displacement affects the localization of the centromere chromatin proteins hMis12, hSgo1, and CPC (Fig. 2) after prophase.

**HP1-α and HP1-γ are required for chromatid cohesion during mitosis in human cells**

In an effort to obtain direct evidence indicating that the altered localization of HP1 is critical to cohesion defects caused by Vpr, the effects of down-regulating HP1-α and HP1-γ on centromere cohesion were investigated. Endogenous expression of HP1-α or HP1-γ was knocked down using RNAi, and specific knockdown was confirmed by immunoblotting (Fig. S3 B). RNAi-treated cells were arrested by nocodazole, and then Giemsa-stained chromosome spreads were examined (Fig. 4 A). Note that chromosomes from HP1-α and HP1-γ (HP1-αγ) RNAi cells appear swollen, with indistinct primary constrictions (Fig. 4 A), consistent with the morphological changes reported in hRad21-depleted cells (Toyoda and Yanagida, 2006). After transfection with HP1-α and HP1-γ siRNA, PCS was induced in 10.7 and 4.2% of cells (Fig. 4 B), respectively. When both HP1-α and HP1-γ were knocked down (HP1-αγ RNAi), PCS occurred more frequently (in 19.8% of the spreads examined).

To provide further evidence that HP1-αγ depletion is linked directly to cohesion loss, we monitored the expression of exogenous myc8-tagged hRad21. HeLa cells were transfected with a plasmid encoding myc8 epitope–tagged hRad21 (phiRad21-myc8) with or without HP1-αγ RNAi. Subsequently, we examined the expression of hRad21-myc8 by detecting the myc tag (see Materials and methods). Approximately 70% of cells treated with control siRNA were positive for hRad21-myc8 (myc positive) within the pericentromeres (Fig. 4 C). In contrast, introduction of HP1-αγ RNAi reduced the number of myc-positive cells to 30% (Fig. 4 C). Of note, PCS was observed in myc-negative cells (Fig. 4 D, right), clearly indicating that the down-regulation of HP1-αγ induced cohesion defects that correlated with PCS. Our data support the idea that dissociation of HP1 from the heterochromatin is involved in Vpr-induced PCS. To further characterize Vpr-induced PCS, we transfected cells with hSgo1 RNAi and compared the phenotypic changes in chromosome spreads. Down-regulation of hSgo1 protein was confirmed by immunoblotting (Fig. S3 A). Representative chromosome spreads prepared from Vpr-expressing cells or HP1-αγ RNAi cells are shown in Fig. 4 E, and summarized results are also shown (Fig. 4, F and G). The effects of hSgo1 RNAi on the loss of cohesion caused by the down-regulation of HP1-αγ were monitored using phiRad21-myc8. Approximately 30% of the cells were myc negative with control RNAi, and the numbers of myc-negative cells were dramatically increased upon transfection with HP1-αγ RNAi (Fig. 4, H and I).
Figure 4. **Loss of cohesion from the mitotic centromere by HP1-α/γ RNAi**. (A) Giemsa-stained chromosomes from HeLa cells transfected with control (Cont), HP1-α, HP1-γ, or combined HP1-α + HP1-γ (HP1-α/γ) siRNA (48 h). (B) PCS frequencies. (C) Frequency of hRad21-myc8–positive cells among HH2B-EGFP–positive cells transfected with control or HP1-α/γ siRNA. (D) Control or HP1-α/γ siRNA–transfected HeLa cells were cotransfected with hRad21-myc8 and histone H2B-EGFP (HH2B-EGFP). Chromosome spreads were stained for c-myc (red) and EGFP (green). Similar results were obtained in three independent experiments. (E) Giemsa-stained chromosome spreads from cells transfected with no RNA (control) or with hSgo1 siRNA (48 h). ΔVpr cells + DOX (top), MIT-23 cells + DOX (top middle), HeLa cells and control RNAi (bottom middle), and HeLa cells and HP1-α/γ RNAi (bottom) are shown. (F) PCS frequencies in cells transfected with hSgo1 or control siRNA with or without Vpr expression. (G) PCS frequency in HeLa and HT1080 cells transfected with hSgo1 or control RNAi with or without HP1-α/γ RNAi. (H and I) Cells were transfected with control or hSgo1 siRNA with or without HP1-α/γ RNAi followed by hRad21-myc8 transfection (48 h). (H) Chromosome spreads stained for c-myc (red) and EGFP (green). (I) Frequency of myc-negative cells among HH2B-EGFP–positive cells. (H and I) Similar results were obtained in two independent experiments, and representative data are shown. (B, C, F, and G) Values represent the mean ± SD of three independent experiments. Bars: (A and E) 5 µm; (D and H) 10 µm.
hSgo1 RNAi increased PCS under any of the tested conditions (Fig. 4 G) and produced a remarkable increase in the myc-negative cell population (Fig. 4, H and I), suggesting that hSgo1 acts downstream of HP1-α in the maintenance of centromeric cohesin during mitosis. These observations were similar to those for Vpr-induced PCS (Fig. 4 F), lending support to the notion that Vpr-induced PCS depends on the disruption of HP1-α function.

Chromatin recruitment of p300 by Vpr and displacement of HP1 from chromatin

Given that Vpr interacts directly with p300/HAT (Kino et al., 2002), a HAT-regulating transcription (Boyés et al., 1998; Caron et al., 2003), and that HP1-α binds chromatin via histone H3 methylated at lysine 9 (H3K9; Jenuwein and Allis, 2001), we tested whether Vpr alters HP1-α localization by modifying the properties of this histone. First, we attempted to detect the acetylated form of H3K9 in condensed chromosomes, as Vpr-induced cohesin defects were specifically induced during mitosis. H3K9 associated with condensed chromosomes prepared from Vpr-expressing cells was highly acetylated (Fig. 5 A, bottom middle). Note that acetylated H3K9 was detected at sites of primary chromosome constriction (Fig. 5 B, bottom). In contrast, less acetylated H3K9 was detected at primary constriction sites in control cells (Fig. 5 B, top). Conversely, dimethylation on H3K9 decreased on mitotic chromosomes from Vpr-expressing cells (Fig. S5 A, bottom). Next, we examined p300 localization upon Vpr induction. Interestingly, we detected p300 in the condensed chromosomes of Vpr-expressing cells (Fig. 5 C, bottom). In marked contrast, p300 signals were not detected in control cells (Fig. 5 C, top). Furthermore, increased levels of chromatin-associated p300 were detected in interphase and mitotic Vpr-expressing cells (Fig. 5 D, bottom insets), whereas no signal was detected in control mitotic cells (Fig. 5 D, top). A detailed analysis of condensed chromosomes showed that the Vpr signal was localized to the chromosome arms and enriched at the primary constriction of chromosomes (Fig. 5 E). Moreover, simultaneous staining showed that p300 and Vpr colocalized on the chromosome (Fig. 5 F, right). Costaining with CENP-A or CENP-H showed that Vpr and p300 localized to the centromere and arm region (Fig. 5, G and H). These data suggest that Vpr actively mobilizes p300 to a range of chromatin regions including the centromeres, resulting in the displacement of HP1-α and PCS.

Figure 5. Forced association of p300 with chromatin in Vpr-expressing cells. Chromosome spreads were prepared from DOX-treated cells at 48 h. Vpr (-), ΔVpr cells; Vpr (+), MIT-23 cells. [A] Histone H3 lysine 9 is strongly acetylated in the chromosomes, including centromeric regions. Chromosome spreads were immunostained with antiacetylated H3K9 antibody. [B] Typical centromere regions shown at a higher magnification. H3K9 was highly acetylated along the whole chromosome, including the centromere region, in Vpr-expressing cells (arrowheads). However, only a weak signal was detected at the primary constrictions of the chromosomes in control cells (arrows). [C] Chromosome spreads stained for p300. [D] Preextracted cells stained for p300. Arrows, cells undergoing mitosis. Insets, mitotic cells expressing Vpr. ΔVpr cells + DOX (top) and MIT-23 cells + DOX (48 h; bottom) are shown. [E] Chromosome spreads stained for p300. [F] Chromosome spreads from DOX-treated MIT-23 cells stained for Vpr and p300. Arrows, signals at the centromere. Two pairs of sister chromatids are shown. [G and H] Chromosome spreads stained for CENP-A and Vpr [G] and CENP-H and p300 [H]. Similar results were obtained in three independent experiments. Bars: (C and E–H) 5 µm; (D) 10 µm.
To demonstrate the involvement of p300/HAT in Vpr-induced PCS, we further characterized p300 in Vpr-expressing cells. After first confirming that Vpr associated with p300 (Fig. 6 A), we subsequently showed that a Vpr immunoprecipitate demonstrated HAT activity against H3K9 (Fig. 6 B). This activity was partially inhibited by anacardic acid (AA), a p300/HAT inhibitor (Fig. S5 B), indicating that p300/HAT was present in the immunoprecipitate, although we cannot exclude a possibility of other HAT contamination in the Vpr immunoprecipitate.

Intriguingly, a Western blot analysis revealed that p300 RNAi restored chromatin retention of HP1-α in cells expressing Vpr (Fig. 6 D). Immunohistochemical analysis revealed p300/HAT is required for Vpr-induced displacement of HP1-α and PCS.
that p300 RNAi restored the chromatin levels of HP1-α in Vpr-expressing cells (Figs. 6 E and S4, A [right] and B). RNAi depletion of p300 specifically down-regulated the p300 levels (Figs. 6 E, S3 A, and S4 A [left]). These data strongly suggest that p300 was responsible for the displacement of HP1-α from chromatin.

To provide more convincing evidence that HAT/p300 contributes to Vpr-induced PCS, we performed additional experiments. First, we showed that AA reduced the rate of PCS (Fig. 6 F). Importantly, we confirmed that AA did not inhibit the chromatin recruitment of p300 in Vpr-expressing cells (Fig. S5, C and D). Second, we demonstrated that the down-regulation of p300 using RNAi also reduced the frequency of Vpr-induced PCS (Fig. 6 F). Moreover, although forced expression of p300 cDNA increased the frequency of Vpr-induced PCS (Fig. 7, A–C), neither PCS nor an increase in the level of chromatin-associated p300 was observed in the absence of Vpr expression (Figs. 7 [C–E] and S4). Forced expression of p300 cDNA lacking a catalytic domain (amino acids 1,472–1,522; ΔHAT-p300; Fig. 7 A) did not increase the frequency of Vpr-induced PCS (Fig. 7 C). We confirmed this finding through back transfection with an expression plasmid encoding an RNAi-resistant wt p300 cDNA (Fig. 7 F). Transfection with this plasmid increased the rate of Vpr-induced PCS, reversing the effects of p300 RNAi (Fig. 7, F–H). In contrast, a second plasmid encoding an RNAi-resistant form of ΔHAT-p300 did not affect the rate of PCS (P < 0.01; Fig. 7 H). We confirmed that both plasmids fostered similar levels of p300 expression (Fig. 7 G), with p300 being associated with chromatin in Vpr-expressing cells (Fig. 7, D and E). These data led us to conclude that the catalytic activity of p300 is crucial to Vpr-induced PCS and that Vpr aberrantly recruits p300 to chromatin, causing HP1-α to be displaced.

Discussion

HIV-1 infection has come to be considered a chronic infection since the introduction of antiretroviral therapy, which can be used to control HIV-1 production for extended periods, thereby improving patient prognosis (Mermin et al., 2008). However, integrated HIV-1 proviral copies cannot be eradicated from the host genome, and small numbers of progeny virions are still produced during therapy (Corbeau and Reynes, 2011). Therefore, it is essential to know how the virus influences host cells. Previously, we detected ~10 ng/ml Vpr in HIV-1–infected patient sera (Hoshino et al., 2007), and we showed that Vpr-expressing cells exhibited hyperploidy and aneuploidy (Shimura et al., 1999). We also found PCS, a hallmark of aneuploidy (Thompson et al., 1993; Kajii et al., 2001), in HIV-1–infected individuals (Shimura et al., 2005). In this study, we found that the vpr gene is responsible for HIV-1–associated PCS. Therefore, we focused on the molecular mechanisms of Vpr-induced PCS.

Vpr-expressing cells induce PCS, which is associated with reduced levels of cohesin, hSgo1, and HP1 and of the related proteins hMis12, AurB, and INCENP at chromatin. Among these proteins, immunofluorescence analyses indicated that HP1-α was mislocalized from insoluble nuclei first, whereas chromatin-bound hRad21 was likely diminished second, suggesting that HP1-α is a key factor in Vpr-induced PCS and that HP1 is essential for centromere cohesion during mitosis in human cells. Vpr displaced HP1, whose RNAi-mediated down-regulation induced PCS concomitant with the displacement of hRad21, consistent with a previous finding for hSgo1 (Yamagishi et al., 2008). Although the functional link between heterochromatin and centromere cohesion in humans remains controversial, Yamagishi et al. (2008) and our current observations suggest a strong functional connection between heterochromatin and centromere cohesion. We further suggest that pathogenic microorganisms can epigenetically impair the function of centromeric heterochromatin.

The present data support the idea that HP1-α plays a major role in centromere cohesion during mitosis and that centromere cohesion may be maintained by a network involving HP1-α and HP1-γ (Obuse et al., 2004). Consistent with our data, immunohistochemical analyses of metaphase spreads have shown that HP1-α associates with centromeres during mitosis (Mine et al., 1999; Hayakawa et al., 2003; Yamagishi et al., 2008; Kiyomitsu et al., 2010). On the other hand, all subtypes of HP1 are released from chromatin during M phase as a result of histone H3 phosphorylation, which is mediated by AurB kinase (Fischle et al., 2005; Hirotu et al., 2005). These findings suggest that most HP1 proteins are released during mitosis but that a small amount of HP1-α remains in the centromere region with hSgo1 to protect cohesin from removal until the onset of anaphase.

Notably, our observation suggests that the HP1-α–mediated regulation of cohesion is especially important during mitosis. Accordingly, AurB, INCENP, hSgo1, and human inner kinetochore protein (hMis12) were initially observed during prophase at the centromere, even in cells in which HP1-αγ was down-regulated by Vpr, but these proteins were displaced when the cells entered prometaphase. Given that the depletion of AurB or INCENP by RNAi resulted in altered hSgo1 localization during mitosis, whereas hSgo1 RNAi did not significantly affect AurB or INCENP localization to the inner centromeres (Kawashima et al., 2007; Pouwels et al., 2007), the effect of the loss of hSgo1 on Vpr expression may be mediated by the loss of the CPC from the inner centromere. These observations further suggest that cohesion is maintained by a network involving HP1-α and HP1-γ, especially during mitosis.

In Vpr-induced PCS, p300 might be responsible for the displacement of HP1-α from chromatin. In particular, the interaction of Vpr and p300 is essential for the displacement of HP1-α from chromatin, as mutant Vpr with a defective p300-binding leucine/arginine-rich (LR) domain (LR mutant; Kino et al., 2002) caused no apparent induction of PCS (Fig. S5 E). In our study, the artificial expression of p300 by itself resulted in neither the localization of p300 at insoluble nuclei nor PCS. In control cells, no association with p300 was detected in chromosomespreads, although an appropriate amount of p300 is essential for proper chromosome segregation (Ha et al., 2009). These data suggest that the stabilized association of p300 with chromatin as seen in Vpr-expressing cells is unusual. Interestingly, the abnormal association of p300 with chromatin through
Vpr was found to be both in interphase and M phase. The Vpr-mediated binding of p300 to chromatin could be essential for the displacement of HP1-α. However, the molecules required for the chromatin recruitment of Vpr remain to be clarified.

A variety of microorganisms express proteins that target p300/HAT and modulate its activity (Hottiger and Nabel, 2000; Caron et al., 2003). p300 functions as a critical regulator of transcription from integrated HIV-1 long terminal repeats (LTRs; Marzio et al., 1998). Vpr, meanwhile, has been shown to cooperate with p300 and to enhance the transcriptional activity of LTRs by up-regulating NF-κB (Felzien et al., 1998). Our results suggest that the enhanced LTR transcription may be mediated by Vpr-induced p300 retention as a result of binding to chromatin. Host cells suppress HIV-1 LTR activity by histone deacetylation, as HIV-1 enters latency through histone deacetylase (HDAC) recruitment to HIV-1 LTRs (Williams et al., 2006), and HDAC inhibitors increase HIV-1 expression (Matalon et al., 2011). HAT activity caused by the p300–Vpr interaction was found both in interphase and M phase. The Vpr-mediated binding of p300 to chromatin could be essential for the displacement of HP1-α. However, the molecules required for the chromatin recruitment of Vpr remain to be clarified.
complex at chromatin may contribute to viral transcription by overcoming host cell HDACs. Moreover, Vpr-mediated p300 binding to chromatin may provide an advantage for viral integration. It is notable that integration of the HIV-1 genome into that of the host cell occurs preferentially near transcriptionally active genes (Brady et al., 2009). The Vpr-induced association of p300 with chromatin may influence transcriptional activity and hence favor viral integration. Cereseto et al. (2005) reported that the HIV-1 protein integrase, which catalyzes HIV-1 integration (Chow et al., 1992), bound p300 directly and that the acetylated integrase showed increased affinity for DNA and stimulated viral integration. The stabilized association of p300 with chromatin caused by Vpr may facilitate the active recruitment of integrase to chromatin, thereby favoring efficient integration of the viral genome. We anticipate that the Vpr-induced stable association of p300 with chromatin may favor active viral production by affecting the functions of other nuclear proteins. It was recently proposed that p300/HAT antagonists could be useful in anti-AIDS therapies (Mantelingu et al., 2007). Merging the molecular interactions of Vpr and p300 could inhibit the recruitment of p300/HAT to chromatin and thus reduce viral replication. It could also reduce the genomic instability associated with PCS.

This is the first study to reveal centromere cohesion impairment via the epigenetic disruption of heterochromatin by a viral pathogen. The use of Vpr expression, RNAi, and specific inhibitors, in combination with appropriate functional assessments, revealed a new specific mechanism of chromosome cohesion. Vpr expression could therefore be considered a useful system for further study of the complex regulation of chromatin dynamics.

**Materials and methods**

**Viruses and infection**

VSV-G-pseudotyped viruses were produced by cotransfecting 293T cells with the infectious HIV-1 proviral clones pNL4-3L and pNL4-3 L (Harland et al., 1995), pNL4-3 L-F-Vif deficient; Kinomoto et al., 2007), pNL4-3 L-E R-Vpr deficient; Connor et al., 1995, and pNL4-3 L-E U (Vpu deficient; Iwazumi et al., 2009) using the VSVG expression vector pHIT/G (Fouchier et al., 1997) and FuGENE 6 transfection reagent (Roche). Levels of p24 antigen in culture supernatants were measured using an HIV-1 p24 antigen capture ELISA kit (ZeptoMetrix). PBLs were donated by healthy volunteers approved by the Institutional Ethics Committee of the National Center for Global Health and Medicine. 1.5 x 10⁶ PBLs were infected with 2 ng/ml p24 antigen from wt or mutant virus (multiplicity of infection 0.007) to p24 antigen from wt or mutant virus (multiplicity of infection 0.007). Viral replication was confirmed by ChIP assay (Thermo Fisher Scientific). Luciferase counts were normalized or Coomassie brilliant blue stain) was used to confirm equivalent loadings of p300 and stimulated viral integration. The stabilized association of p300 with chromatin may influence transcriptional activity and thereby favor integration. Cereto et al. (2005) reported that the HIV-1 protein integrase, which catalyzes HIV-1 integration (Chow et al., 1992), bound p300 directly and that the acetylated integrase showed increased affinity for DNA and stimulated viral integration. The stabilized association of p300 with chromatin caused by Vpr may facilitate the active recruitment of integrase to chromatin, thereby favoring efficient integration of the viral genome. We anticipate that the Vpr-induced stable association of p300 with chromatin may favor active viral production by affecting the functions of other nuclear proteins. It was recently proposed that p300/HAT antagonists could be useful in anti-AIDS therapies (Mantelingu et al., 2007). Merging the molecular interactions of Vpr and p300 could inhibit the recruitment of p300/HAT to chromatin and thus reduce viral replication. It could also reduce the genomic instability associated with PCS.

**DNA plasmids and transfection**

A plasmid encoding hRadd1-myc8 (hRadd1-myc8) was constructed by inserting the entire coding region of hRadd1 and eight tandem copies of the myc antigen in-frame into pcDNA3.1/V5His6 (Invitrogen), as previously described (Toyoda and Yanagida, 2006). The plasmid pH2BGFP encodes the chimeric protein histone H2B-GFP (Kanda et al., 1998). The rhRadd1-myc8 and pH2BGFP plasmids were cotransfected into HeLa cells at a 5:1 ratio using the Effectene Transfection kit (Qiagen). A DNA plasmid encoding Flag-tagged wt p300 (pFlag-p300wt) was generated using pFlag-p300HAT, together with Flag and p300wt, as previously described (Boyes et al., 1998). The 5' fragment of pFlag-p300HAT was excised using SnaB1 and Xba1, recovered, and ligated into the corresponding p300wt sites. To generate DNA expression plasmids encoding an siRNA-resistant form of p300, a 2.5-kb fragment (nucleotides 420–2,870 from the initiation codon) was first subcloned into a pBluescript KS II(+) vector (Agilent Technologies). An oligonucleotide cassette [5’-CCAGATGACCATGGGCACGCCCCCTATGTACCCGCCGAACTCAGACGAG-3’] (Tomita and Yanagida, 1997) and FuGENE 6 transfection reagent (Roche). Levels of p24 antigen in culture supernatants were measured using an HIV-1 p24 antigen capture ELISA kit (ZeptoMetrix). PBLs were donated by healthy volunteers approved by the Institutional Ethics Committee of the National Center for Global Health and Medicine. 1.5 x 10⁶ PBLs were infected with 2 ng/ml p24 antigen from wt or mutant virus (multiplicity of infection 0.007) to obtain a similar PCI frequency to that typically found in HIV-1 patients (Shimura et al., 2005) in the presence of 2% phytohemagglutinin-M form (Invitrogen). 82 h later, infected cells were analyzed for luciferase activity using PicoGene (Toyo Ink) and for overall protein content using a bicinchoninic acid assay (Thermo Fisher Scientific). Luciferase counts were normalized or Coomassie brilliant blue stain) was used to confirm equivalent loadings of p300 and stimulated viral integration. The stabilized association of p300 with chromatin may influence transcriptional activity and thereby favor integration. Cereto et al. (2005) reported that the HIV-1 protein integrase, which catalyzes HIV-1 integration (Chow et al., 1992), bound p300 directly and that the acetylated integrase showed increased affinity for DNA and stimulated viral integration. The stabilized association of p300 with chromatin caused by Vpr may facilitate the active recruitment of integrase to chromatin, thereby favoring efficient integration of the viral genome. We anticipate that the Vpr-induced stable association of p300 with chromatin may favor active viral production by affecting the functions of other nuclear proteins. It was recently proposed that p300/HAT antagonists could be useful in anti-AIDS therapies (Mantelingu et al., 2007). Merging the molecular interactions of Vpr and p300 could inhibit the recruitment of p300/HAT to chromatin and thus reduce viral replication. It could also reduce the genomic instability associated with PCS.

This is the first study to reveal centromere cohesion impairment via the epigenetic disruption of heterochromatin by a viral pathogen. The use of Vpr expression, RNAi, and specific inhibitors, in combination with appropriate functional assessments, revealed a new specific mechanism of chromosome cohesion. Vpr expression could therefore be considered a useful system for further study of the complex regulation of chromatin dynamics.

**RNAS**

siRNAs were used to block the expression of NIPBL, hSgo1, HP1-α, HP1-γ, and p300. The siRNA sequences used are as follows: NIPBL, 5’-GCUU-UUGAGCCUCUCAATTTC-3’ (Toyoda and Yanagida, 2006); hSgo1, 5’-CAUGAAGACCCUGCUAGAA-3’ (McGuinness et al., 2005); HP1-α, 5’-CUGAGAAGAACUUUGAUU-3’; HP1-γ, 5’-UUUUCUUGAUGUGAUU-3’; and p300, 5’-AACCCUCUCCUCCACAGA-3’ (Debes et al., 2002). A double-stranded RNA with the sequence 5’-UAAAGCCAUGAUAGAAGAC-3’ (siCONTROL nontargeting siRNA #2; Thermo Fisher Scientific) was used as a control. RNAi procedures were performed according to the instructions included in the Oligofectamine kit (Invitrogen) for HeLa cells and the Nucleofector kit (Lonza) or GenePORTER kit (Genlantis) for HT1080-based cell lines. In these transfections, siRNA was used at a concentration of 200 nM; in combined RNAi treatments, each siRNA was used at a concentration of 100 nM.

**FACS analysis**

A flow cytometer (FACSCalibur; BD) was used in cell cycle analyses, as previously described (Shimura et al., 1999). In brief, cells fixed in 70% ethanol at −20°C for at least 1 h were treated with 100 µg/ml RNase (Sigma-Aldrich) and stained with 5 µg/ml propidium iodide (Sigma-Aldrich) to allow visualization of the DNA.

**Cell extraction and immunoblotting**

Chromatin was isolated for HP1 immunoblotting using a combination of previously described methods (Remboutika et al., 1999). In brief, cells were washed twice in ice-cold PBS and then resuspended in buffer N (15 mM Tris pH 7.4, 60 mM KCl, 15 mM NaCl, 1 mM CaCl₂, 1 mM dithiothreitol, 2 mM sodium vanadate, 250 mM sucrose, protease inhibitor cocktail [Sigma-Aldrich], and 1 mM PMSF). An equal volume of buffer N containing 0.6% NP-40 was then added to the resuspended cells, and the resulting suspension was mixed gently and incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 7,500 × g for 5 min at 4°C. The nuclei were then washed once in Pipes buffer and resuspended in 10 mM Pipes buffer, pH 6.5, containing 10 mM EDTA, protease inhibitor cocktail, and 1 mM PMSF. The resulting chromatin pellets were washed once in Pipes buffer and resuspended in SDS-PAGE loading buffer after centrifugation at 6,000 g for 10 min at 4°C. Equivalent samples (in terms of initial DNA and the final protein) from the supernatant fractions were subjected to 10–15% SDS-PAGE. A control (α-tubulin, PSRAFT, HH3, or Coommassie brilliant blue stain) was used to confirm equivalent loadings
in each lane. Cellular extraction for cohesion analyses was performed as previously described (Todorov et al., 1995), with slight modifications. After washing in PBS and cytoskeleton (CSK) buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 300 mM sucrose, and 3 mM MgCl₂), cells were incubated for 5 min at RT in CSK extraction buffer (CSK buffer containing 0.5% Triton X-100, 0.5 mM PMSF, and protease inhibitor cocktail). Particles were pelleted by centrifugation at 700 g for 5 min at 4°C. The resulting pellets were resuspended in CSK extraction buffer and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% LDS, and protease inhibitor cocktail) and were then sonicated on ice. Equivalent amounts of protein were subjected to 10–15% gradient SDS-PAGE. In immunoblot analysis, cell extracts were transferred to nitrocellulose membranes after SDS-PAGE. After blocking in 10% BSA (Sigma-Aldrich), membranes were incubated with antibodies against SMC1 or SMC3 (provided by K. Kimura, Tsukuba University, Tsukuba, Japan), HP1α (Millipore), HP1β or HP1γ (provided by T. Haraguchi and T. Hayakawa, Kobe Advanced Information and Communications Technology Research Center; National Institute of Information and Communications Technology, Kobe, Japan), NIPBL/delangin (Toyoda and Yanagida, 2006), p300 (Millipore), Hrad21 (Abcam), or tubulin (Millipore). The anti-Vpr antibody BD1 (IgG2a) was raised by immunization with a full-length Vpr peptide (Peptide Institute, Inc.; Nakai-Murakami et al., 2007). Immune complexes were detected using anti–rabbit or –mouse IgG fragments (GE Healthcare) conjugated to HRP. Bound antibody was visualized using a Western blot detection system (ECL Plus; GE Healthcare). The intensity of the scanned blots was measured on ImageJ software (National Institutes of Health).

**Immunofluorescence microscopy**

Immunofluorescence staining was performed as previously described (Shimura et al., 1999). In brief, cells were washed in PBS, fixed in 2% PFA in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 10% goat serum or 1% BSA in PBS. To remove soluble proteins while leaving chromatin-bound proteins intact, cells were incubated in PBS or CSK buffer containing 0.2% Triton X-100 and 1% BSA for 5 min on ice and were then fixed in 2% PFA in PBS for 10 min. Primary antibodies against hMis12 (1:1,000; Goshima et al., 2003), AurB (1:200; BD), INCENP (1:200; Bethlehem Laboratories, Inc.), CENP-A (1:200; Abcam), CENP-H (1:200; provided by K. Yoda), Hrad21 (1:50; Toyoda and Yanagida, 2006), hSgo1 (1:1,000), Mxi1 (1:1,000), H3K9ac (1:200; Millipore), CENP-A (1:200; BD), INCENP (1:200; Bethyl Laboratories, Inc.), CENP-H (1:200; provided by K. Yoda), and CENP-A (1:200; BD) were added, and the resulting mixtures were incubated at 4°C overnight. The beads were then washed once with ice-cold buffer and boiled in sample buffer. The resulting immunocomplexes were analyzed using an anti-Vpr (BD1) mAb (1:1,000; Nakai-Murakami et al., 2007) or an anti-p300 pAb (1:100; Santa Cruz Biotechnology, Inc.), as well as mouse IgG (Sigma-Aldrich) or rabbit IgG (Sigma-Aldrich), were added to 1,000 µg cellular protein suspended in 250 µl lysis buffer. The resulting mixtures were incubated at 4°C overnight. The beads were then washed once with ice-cold buffer and boiled in sample buffer. The resulting immunocomplexes were analyzed using an anti-Vpr (BD1) mAb (1:1,000; Nakai-Murakami et al., 2007) or an anti-p300 pAb (1:100; N-5; Santa Cruz Biotechnology, Inc.). For HAT activity assays, Dynabeads coincubated with a mouse mAb against Flag (M2; Sigma-Aldrich) or a control mouse IgG were added to 1.0 µg lysed cellular proteins in 250 µl lysis buffer, and the resulting mixtures were incubated at 4°C overnight. The beads were then washed with ice-cold buffer and then with 1x HAT assay buffer [HAT assay kit, Millipore] according to the manufacturer’s instructions. Next, a HAT assay cocktail containing 10 µg histone H3 (Millipore) and 100 µM acetyl-CoA (Sigma-Aldrich) was added to the beads, which were then incubated at 30°C in a shaking incubator for 30 min. Acetylation of histone H3 was assessed by Western blotting, performed using an anti-acetylated histone H3 antibody (1:1,000; Cell Signaling Technology).

**Preparation of chromosome spreads**

Cells were treated with 0.1 µg/ml colcemid (Invitrogen) for 2 h or with 0.1 µg/ml nocodazole (Sigma-Aldrich) for 4 h at 37°C. Rounded cells and cells in mitotic arrest were collected, washed in PBS, and incubated in 75 mM KCl for 1 to 2 h at RT. Next, 1 ml of 3% acetic acid was added to the cell suspensions, which were then mixed gently. The Carnoy’s solution was replaced three times. Drops of cell suspension were prepared Carnoy’s solution (a 3:1 mixture of methanol and glacial acetic acid) was added to the cell suspensions, which were then mixed gently. The resulting mixtures were incubated at 4°C overnight. The beads were then washed once with ice-cold buffer and boiled in sample buffer. The resulting immunocomplexes were analyzed using an anti-Vpr (BD1) mAb (1:1,000; Nakai-Murakami et al., 2007) or an anti-p300 pAb (1:100; N-5; input, 10% of the protein used in immunoprecipitation; Santa Cruz Biotechnolgy, Inc.). For HAT activity assays, Dynabeads coincubated with a mouse mAb against Flag (M2; Sigma-Aldrich) or a control mouse IgG were added to 1.0 µg lysed cellular proteins in 250 µl lysis buffer, and the resulting mixtures were incubated at 4°C overnight. The beads were then washed with ice-cold buffer and then with 1x HAT assay buffer [HAT assay kit, Millipore] according to the manufacturer’s instructions. Next, a HAT assay cocktail containing 10 µg histone H3 (Millipore) and 100 µM acetyl-CoA (Sigma-Aldrich) was added to the beads, which were then incubated at 30°C in a shaking incubator for 30 min. Acetylation of histone H3 was assessed by Western blotting, performed using an anti-acetylated histone H3 antibody (1:1,000; Cell Signaling Technology).

**HAT activity**

293T cells were transfected with Flag-EGFPl, Flag-Vpr, or Flag-p300 (positive control). To capture immunocomplexes, Dynabeads conjugated with anti-Flag (M2) antibody (Sigma-Aldrich) or a control IgG (mouse mAb) were added, and the resulting mixtures were incubated in 250 µl lysis buffer, and the resulting mixtures were incubated at 4°C overnight. The beads were then washed with ice-cold buffer and then with 1x HAT assay buffer [HAT assay kit, Millipore] according to the manufacturer’s instructions. Next, a HAT assay cocktail containing 10 µg histone H3 (Millipore) and 100 µM acetyl-CoA (Sigma-Aldrich) was added to the beads, which were then incubated at 30°C in a shaking incubator for 30 min. Acetylation of histone H3 was assessed by Western blotting, performed using an anti-acetylated histone H3 antibody (1:1,000; Cell Signaling Technology).

**Statistical analysis**

Statistical analyses were performed using a two-tailed unpaired t test. P < 0.05 was considered statistically significant.

**Online supplemental material**

Fig. S1 shows a PCS assay in two additional PBL donors, Vpr expression levels, and abnormal chromosome segregation in Vpr-expressing cells accompanying Videos 1–3. Fig. S2 shows the effects of Vpr expression on the interphase chromatin localization of Hrad21 and HP1α and an assessment of NIPBL in Vpr-induced PCs. Fig. S3 shows the specific down-regulation

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of HsG01, p300, and HP1α/γ protein expression by RNAi. Fig. S4 shows image analyses of chromatin-bound p300 and HP1α in Vpr-expressing cells. Fig. S5 shows reduced dimethylation on histone H3 lysine 9 residue upon Vpr expression, the suppression of Vpr-associated HAT activity and localization of p300 in the presence of the HAT inhibitor, and the reduced frequency of PCS in cells expressing a Vpr protein carrying a mutated Lp (p300 binding) domain. Videos 1–3 show mitosis in control [Video I] and Vpr-expressing [Video II, Videos 2 and 3] cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201011188/DC1.

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