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# Histone Deacetylase 6 Regulates Human Immunodeficiency Virus Type 1 Infection

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**Efficient human immunodeficiency virus (HIV)-1 infection depends on multiple interactions between the viral gp41/gp120 envelope (Env) proteins and cell surface receptors. However, cytoskeleton-associated proteins that modify membrane dynamics may also regulate the formation of the HIV-mediated fusion pore and hence viral infection. Because the effects of HDAC6-tubulin deacetylase on cortical $\alpha$-tubulin regulate cell migration and immune synapse organization, we explored the possible role of HDAC6 in HIV-1-envelope-mediated cell fusion and infection. The binding of the gp120 protein to $CD4^{+}$-permissive cells increased the level of acetylated $\alpha$-tubulin in a CD4-dependent manner. Furthermore, overexpression of active HDAC6 inhibited the acetylation of $\alpha$-tubulin, and remarkably, prevented HIV-1 envelope-dependent cell fusion and infection without affecting the expression and codistribution of HIV-1 receptors. In contrast, knockdown of HDAC6 expression or inhibition of its tubulin deacetylase activity strongly enhanced HIV-1 infection and syncytia formation. These results demonstrate that HDAC6 plays a significant role in regulating HIV-1 infection and Env-mediated syncytia formation.**

## INTRODUCTION

Human immunodeficiency virus (HIV) infection is initiated by the virus binding to the cell surface after CD4 engagement and HIV-1 fusion with the plasma membrane (Stein *et al.*, 1987; Maddon *et al.*, 1988; McClure *et al.*, 1988; Moore *et al.*, 1997). The main coreceptors for HIV-1 infection have been shown to be CXCR4 and CCR5, which represented a major advance in understanding the mechanism of the HIV-1 infection (Cocchi *et al.*, 1995; Alkhatib *et al.*, 1996; Bleul *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996). As a result, it has been proposed that the sequential binding of gp120 viral protein to CD4 and to one coreceptor induces specific conformational changes in gp41, facilitating viral fusion with cell membrane (Clapham and McKnight, 2002).

The cell cytoskeleton is involved in the early events of viral infection, regulating viral penetration and genome uncoating, the movement of viral capsids, and integration of the viral genome. Accordingly, actin and microtubules are required for the efficient entry of herpes simplex type 1 and simian virus 40, respectively (Pelkmans *et al.*, 2002; Marozin *et al.*, 2004). It has been shown that disrupting the actin network can inhibit infection of HIV-1 and fusion with the host cell (Iyengar *et al.*, 1998; Jernigan *et al.*, 2000), presumably by disrupting the colocalization of CD4 and CXCR4 (Iyengar *et al.*, 1998). In addition, the actin cytoskeleton seems to be necessary for activation of the reverse transcription complex (Bukrinskaya *et al.*, 1998). However, little is known about the role of cytoskeleton-related enzymes in the control of HIV fusion and infection.

Histone deacetylase 6 (HDAC6) is exclusively located in the cytoplasm, and it regulates the acetylation of $\alpha$-tubulin (Hubbert *et al.*, 2002; Matsuyama *et al.*, 2002; Haggarty *et al.*, 2003; Zhang *et al.*, 2003). Acetylated $\alpha$-tubulin is involved in stabilizing microtubules (MTs) (Piperno *et al.*, 1987), and stable MTs are known to regulate actin-dependent ruffle formation at the plasma membrane and cell motility through the activation of Rac-1 or Rho (Kaverina *et al.*, 1999; Waterman-Storer and Salmon, 1999). Moreover, it has recently been shown that by acting on HDAC6 and mDia2, Rho-GTPase can influence the levels of acetylated MTs and hence actin organization, thereby interfering with osteoclast maturation (Destaing *et al.*, 2005). Another substrate that can be deacetylated and inactivated by HDAC6 is the heat shock protein 90, a chaperon that regulates structural maturation and the assembly of certain protein complexes (Bali *et al.*, 2005; Kovacs *et al.*, 2005). HDAC6 associates with MTs and colocalizes with the microtubule motor complex (Smith *et al.*, 2000; Hubbert *et al.*, 2002; Matsuyama *et al.*, 2002; Zhang *et al.*, 2003). Thus, it is a cytoplasmic enzyme that promotes cell migration (Hubbert *et al.*, 2002; Palazzo *et al.*, 2003; Saji *et al.*, 2005) and controls the membrane organization of CD3 and LFA-1, the translocation of the microtubule organizer center (MTOC), and T-cell activation (Serrador *et al.*, 2004).

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Abbreviations used: HDAC6, histone deacetylase 6; NaBut, sodium butyrate; PBL, peripheral blood lymphocyte; siRNA, short interference RNA; TSA, trichostatin A.

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HDAC6 is found at the leading edge of migrating cells, and it is associated with dynamic MTs involved in the modulation of cell motility (Wadsworth, 1999; Hubbert *et al*., 2002).

Thus, because HDAC6 is clearly an important regulator of membrane dynamics, we hypothesized that it might modulate HIV-envelope (Env)-mediated cell fusion and infection. Here, we have studied the effect of inhibiting or of overexpressing HDAC6 on the fusion activity of gp41/gp120 and on the capacity of HIV-1 to infect cells. Our data show that this cytoplasmic deacetylase exerts an important regulatory effect on these processes.

## MATERIALS AND METHODS

### Antibodies and Reagents

The anti-CD4 HP2/6 monoclonal antibody (mAb) (mAb; IgG1) that inhibits the gp120/CD4 interaction has been described previously (Carrera *et al*., 1987), and the anti-CD4 v4-phycoerythrin (PE) mAb (IgG1), which does not react with the gp120 binding site, was obtained from BD Biosciences (San Jose, CA). The PE-labeled anti-CXCR4 or -CCR5 mAb were from BD Biosciences, and the biotinylated mAbs against human CXCR4 and CCR5 were obtained from R&D (Marina del Rey, CA). The sc-5255 anti-HDAC6 polyclonal antiserum was from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-α-tubulin B-5-1-2, anti-acetylated α-tubulin 6-11B-1, and anti-vimentin mAbs were from Sigma-Aldrich (St. Louis, MO). Trichostatin A (TSA) and sodium butyrate (NaBut) were supplied by Calbiochem (San Diego, CA), whereas stromal cell-derived factor-1α (SDF-1α) and regulated on activation normal T-cell expressed and secreted were obtained from R&D. The fluorescent cell tracers Calcein-AM, CMTMR, and CMAC were from Invitrogen (Carlsbad, CA).

### Cells

The human T-cell Jurkat J77 and MT-2 lines and the Jurkat cell line expressing HIV-1-Env-Hxbc2 (Jurkat Hxbc2) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). HeLa P5 cells, stably transfected with human CD4 and CCR5 cDNA and with an HIV-LTR-driven β-galactosidase (β-Gal) reporter gene (Pleskoff *et al*., 1997), were provided by Dr. M. Alizon (Hôpital Cochin, Paris, France) as well as the HeLa P4 cells stably transfected with human CD4. HeLa 243 and HeLa ADA cells coexpressing the Tat and HIV-1-Env proteins were also provided by Dr. M. Alizon (Schwartz *et al*., 1994; Pleskoff *et al*., 1997). Human peripheral blood lymphocytes (PBLs) were isolated from healthy donors using Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The PBLs were activated over 3 d with 1 μg/ml phytohemagglutinin (Murex Diagnostics, Norcross, GA) and then cultured with interleukin-2 (6 U/ml).

### HDAC6 Recombinant DNA Constructs and Lipotransfection

The wild-type (wt)-HDAC6-GFP and the dead mutant (dm)-HDAC6-GFP harboring a double point mutation (H216A/H611A) that causes deacetylase inactivation have been described previously (Grozinger *et al*., 1999; Serrador *et al*., 2004). These two expression vectors were transfected in HeLa P4 or P5 cells using Megafectin-20 (Qbiogene, Carlsbad, CA). Briefly, HeLa P4 or P5 cells were seeded in 75-$cm^2$ flasks at a density that would reach 50–80% confluence 24 h later. Then, HeLa cells were lipotransfected with Megafectin-20 liposomes bearing wt-HDAC6-GFP, dm-HDAC6-GFP, or green fluorescent protein (GFP) cDNA constructs (5 μg of cDNA/flask) for 4 h at 37°C in DMEM containing 3% FCS but without antibiotics. The HeLa cells were then trypsinized and cultured in fresh DMEM for 24 h (10% FCS and 1% antibiotics), and GFP expression was monitored by fluorescence. The HeLa P4 or P5 cells were assayed in Env-mediated cell fusion or HIV-1 infection experiments 24 h after Megafectin-20 transfection. In parallel, CD4, CXCR4, and CCR5 cell surface expression was analyzed by flow cytometry.

### HDAC6 Retroviral Transduction of T-Cells

MT-2 and Jurkat J77 T-cells expressing GFP, wt-, or dm-HDAC6-GFP were obtained by infection with a replication deficient Moloney murine leukemia virus. Briefly, GFP, wt-HDAC6-GFP, and dm-HDAC6-GFP constructs were cloned in the bicistronic pLZR-IRES/GHR vector (kindly provided by Dr. Antonio Bernad, National Centre for Biotechnology, Madrid, Spain), and then retroviral production and cellular transduction were performed as described previously (Abad *et al*., 2002).

### Immunofluorescence and Flow Cytometry

HeLa or T-cells were fixed and permeabilized for 3 min in 2% formaldehyde, 0.5% Triton X-100 in phosphate-buffered saline (PBS), and immunostained for acetylated α-tubulin, α-tubulin, or HDAC6 as described previously (Piperno *et al*., 1987; Serrador *et al*., 2004). The cells were visualized using a Leica DMR photomicroscope and a Leica TCS-SP confocal microscope (Leica, Heidelberg, Germany).

To assess the expression of the HIV-1 receptors (CD4, CXCR4, or CCR5), T- and HeLa cells that were untransfected or that were transfected with GFP and wt-HDAC6 or the dm-HDAC6-GFP were stained for CXCR4 or CCR5 using specific biotinylated mAbs that were visualized with allophycocyanin-labeled streptavidin. CD4 was recognized with a PE-labeled mAb against CD4. For drug treatments, untreated, TSA- or NaBut-treated cells were stained for CXCR4, CCR5, or CD4 using PE or fluorescein isothiocyanate-labeled mAbs. The cells were then analyzed in a FACScalibur flow cytometer (BD Biosciences, San Diego, CA). Irrelevant isotype-matched mAbs were used as controls.

### Western Blot Analysis of α-Tubulin Acetylation

To study gp120-induced α-tubulin acetylation, either untransfected or MT-2 cells transfected with wt- or dm-HDAC6-GFP were incubated with recombinant soluble $gp120_{IIIB}$ protein (5 μg/ml/1 × $10^6$ cells) for the time periods indicated. The cells were resuspended in 60 μl of MES buffer [10 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 7.4, 150 mM NaCl, 5 mM EGTA, and 5 mM $MgCl_2$] containing a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany), sonicated twice for 30 s at 4°C, and boiled in sample buffer containing 5% β-mercapto-ethanol for 1 min at 100°C. Cell lysates were immunoblotted and probed for α-tubulin and acetylated α-tubulin as described previously (Serrador *et al*., 2004). After treatment with TSA or NaBut, cells were resuspended in 60 μl of MES buffer, and protein samples were prepared as described above. Cell lysates were immunoblotted for HDAC6, α-tubulin, acetylated α-tubulin, and vimentin as described in Serrador *et al*., 2004. The protein bands were visualized and analyzed using the LAS-1000 charge-coupled device system and Image Gauge 3.4 software (Fuji Photo Film, Tokyo, Japan). The increase in α-tubulin acetylation was quantified and expressed as the ratio of the intensities of the acetylated α-tubulin and the total α-tubulin bands (Actub/tub).

### HDAC6 Knockdown

Three 20-nucleotide double-stranded short interference RNAs (siRNAs) were generated against the HDAC6 sequence spanning nucleotides 193–213, 217–237, and 284–304 (Eurogentec, Hampshire, United Kingdom) as described previously (Serrador *et al*., 2004). HeLa P4 or P5 cells were transfected with the siRNAs at a final concentration of 200 nM using Oligofectamine (Invitrogen). Mock transfection with a scrambled irrelevant double-stranded siRNA (Eurogentec) served as a negative control of mRNA interference. Cells were transfected twice over 3 d and collected 24 h later. To analyze HDAC6 silencing, whole cell lysates (40 μg) of transfected cells were analyzed by Western blot using a polyclonal antibody anti-HDAC6 and mAbs against acetylated α-tubulin and α-tubulin. HDAC6-silenced cells were used in Env-mediated cell fusion or HIV-1 infection experiments as described below.

### HIV-1-Env-mediated Cell–Cell Fusion

A β-galactosidase cell fusion assay was performed as described previously (Valenzuela-Fernandez *et al*., 2001). Briefly, HeLa 243 or HeLa ADA cells were mixed with untreated or treated HeLa P5 or P4 cells in 96-well plates in a 1:1 ratio and incubated for 16 h. The fused cells were washed, lysed, and the enzymatic activity was evaluated by chemiluminescence (β-Gal reporter gene assay; Roche Diagnostics). Syncytia formation was detected by fixing cells with 0.5% glutaraldehyde and staining with the β-galactosidase substrate X-Gal, as described previously (Pleskoff *et al*., 1997).

A dual fluorescence cell fusion assay was also performed as described previously (Valenzuela-Fernandez *et al*., 2001). Briefly, CMTMR-loaded Env+Jurkat-Hxbc2 cells were mixed with Calcein-AM-loaded parental or transfected J77 cells or with 2-d PHA-activated PBLs. The double-labeled cells were detected 6 or 12 h later by flow cytometry or fluorescence microscopy. When indicated, the target T-cells ($CD4^+$/CXCR4+) were preincubated with TSA or NaBut for 2 h at the concentrations indicated. The target cells were then washed extensively to remove the drugs before coculturing with Env-expressing cells. To assess the effects of Taxol or Nocodazole in Env-mediated fusion, these drugs were present throughout the experiment. Anti-CD4 mAb (5 μg/ml for 30 min at 37°C) was used as a control for the blockage of cell fusion. The extent of fusion was calculated as the percentage fusion = (number of bound cells positive for both dyes/number of bound cells positive for Calcein-labeled target cells) × 100.

### Detection of α-Tubulin Acetylation at Env+ Cell-to-Target Cell Contact Areas

For the HeLa-based cell–cell fusion model, HeLa P5 and R5-Env+ HeLa ADA cells were detached with PBS-EDTA (5 mM), washed, and cocultured on sterile glass coverslips at a 1:1 ratio in fresh DMEM for 3 h at 37°C. It is important to note that HeLa cells kept a spherical morphology during this short time in culture. After 3 h, the cells were fixed and permeabilized for 3 min in 2% formaldehyde, 0.5% Triton X-100 in PBS and immunostained for acetylated α-tubulin as described above. For the T-cell-based fusion model,

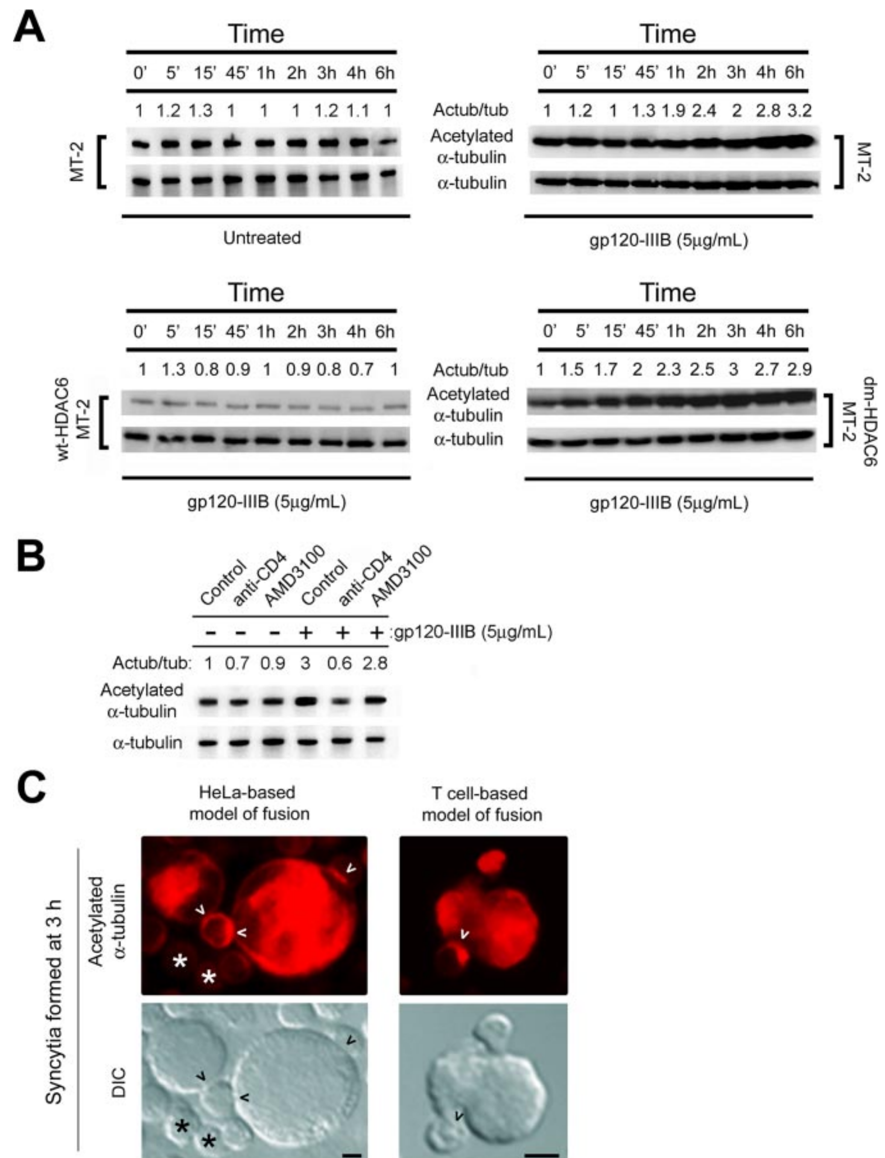


**Figure 1.** Effect of gp120 protein and HDAC6 overexpression on α-tubulin acetylation. (A) Kinetic analysis of gp120-induced α-tubulin acetylation in parental, dm-, and wt-HDAC6-transfected MT-2 cells (gp120-IIIB) compared with untreated cells. Lys40 α-tubulin acetylation was quantified and presented in reference to the total α-tubulin (Actub/tub). (B) Involvement of CD4 in gp120-mediated α-tubulin acetylation. A neutralizing anti-CD4 mAb impairs gp120-IIIB-mediated α-tubulin acetylation, whereas the CXCR4 antagonist AMD3100 has no effect. Control lines indicate untreated and $gp120_{III}$-treated MT-2 cells, respectively. To block CD4 or CXCR4/$gp120_{III}$ binding and signaling, MT-2 cells were pretreated with mAb anti-CD4 (5 μg/ml) or AMD3100 (50 μM) during 30 min at 37°C, before $gp120_{III}$ addition to the cells for 4 h at 37°C. (C) Redistribution of acetylated α-tubulin (red fluorescence, white arrowheads) at cell–cell contact areas within the syncytia. Cells not in contact with the syncytia (asterisks) did not accumulate acetylated α-tubulin. Black bars in differential interference contrast (DIC) images indicate 10 μm. Data are representative of three independent experiments.

Env+-Hxbc2 cells were cocultured with J77 cells in suspension at a 1:1 ratio for 3 h at 37°C. The cells were then fixed and permeabilized for 3 min in 2% formaldehyde, 0.5% Triton X-100 in PBS, and the acetylated α-tubulin present was assessed by immunostaining as described above. In both cell-to-cell fusion models, accumulation of acetylated α-tubulin was monitored in individual target cells in contact with Env+-formed syncytia. Cells were visualized using a Leica DMR photomicroscope and a Leica TCS-SP confocal microscope (Leica).

### *HIV-1 Viral Preparation*

Highly infectious preparations of HIV-$1_{NL4.3}$ and HIV-$1_{BaL}$ virus strains were generated by several consecutive passages of the original HIV-1 isolates in peripheral blood mononuclear cells (PBMCs; see Valenzuela *et al.*, 1997). Briefly, PBMCs were infected with one synchronous dose of HIV-$1_{NL4.3}$ or HIV-$1_{BaL}$, and culture supernatants were recovered 3 d later and stored at −70°C. Freshly thawed aliquots were filtered through 0.22-μm filters before use.

### *HIV-1 Entry and Infection*

HIV-$1_{NL4.3}$ entry and multiplicity of infection (MOI, 1) was assayed in PHA-activated PBLs or MT-2 T-cells over 90 min. When indicated, T-cells were pretreated with anti-CD4 mAbs (5 μg/ml), SDF-1α (300 nM), 3′-azido-2′,3′-dideoxythymidine (AZT) (5 μg/ml), TSA (dose response), or NaBut (500 μM). HIV-1 entry into parental or transfected MT-2 cells was monitored 12 h after HIV-1 infection by measuring proviral *gag* HIV-1 DNA by PCR (Zhao *et al.*, 2002). HIV-1 infection (MOI, 0.1) of PHA-activated PBLs was assessed every 48 h by measuring the concentration of p24 in the culture supernatant by enzyme-linked immunosorbent assay (INNOTEST HIV-1 antigen mAb; Innogenetic, Ghent, Belgium). HIV-$1_{NL4.3}$ or HIV-$1_{BaL}$ infection (MOI, 1) of HeLa P5 cells was performed in 96-well plates for 5 h at 37°C before the cells were washed and cultured for 52 h. Finally, the cells were lysed, and the HIV-1 infection was assessed by β-galactosidase measurement. Where indicated, cells were pretreated with a mAb anti-CD4 before HIV-1 infection.

## RESULTS

### *HIV-1-Env-mediated α-Tubulin Acetylation Is Abolished by HDAC6 Overexpression*

To study the possible role of HDAC6 in the control of HIV-1 infection, we first examined the capacity of the HIV-1-Env to induce the acetylation of α-tubulin. We found that $gp120_{IIIB}$, a X4-tropic Env protein, triggered acetylation of α-tubulin both in permissive parental cells and in MT-2 cells transfected with retroviral dm-HDAC6. The maximal threefold increase observed was detected within 4–6 h of exposure (Figure 1A, MT-2 and dm-HDAC6-MT-2, gp120-III treated). Likewise, the $gp120_{SF2}$ protein also triggered acetylation of α-tubulin (our unpublished data). In contrast, gp120-medi-

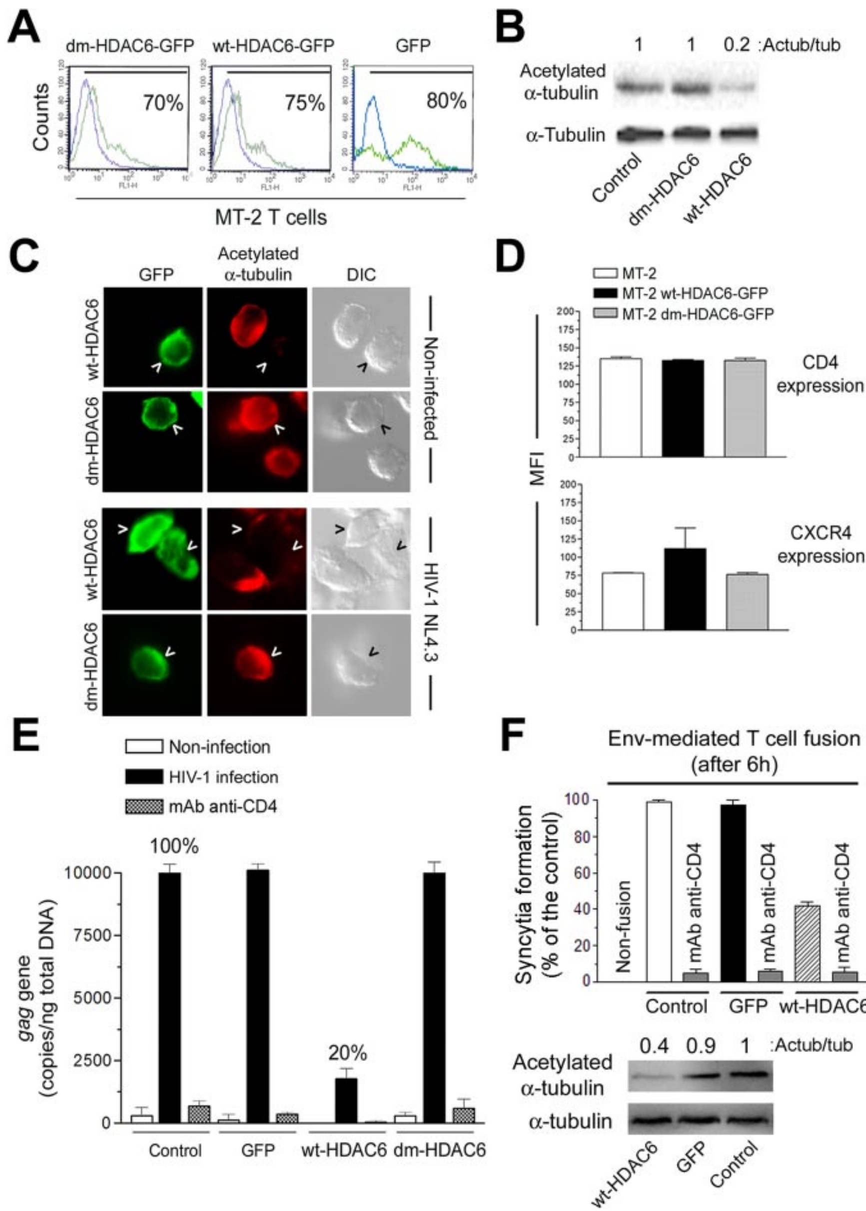


**Figure 2.** Overexpression of HDAC6 impairs HIV-1 infection and Env-mediated cell fusion. (A) Flow cytometry analysis of overexpression of wt- and dm-HDAC6-GFP in MT-2 cells. Top bars indicate the region gated to quantify construct expression. (B) Quantification of acetylated α-tubulin in parental, wt-, and dm-HDAC6-transfected MT-2 cells. (C) Expression and localization of endogenous acetylated α-tubulin (red) and transfected wt- or dm-HDAC-6-GFP molecules (green) in noninfected and HIV-$1_{NL4.3}$-infected MT-2 cells (1 h). Acetylated α-tubulin (red) was not readily detected in wt-HDAC6-GFP MT-2 cells (arrowhead-labeled cells), whereas it was strongly visible in untreated or dm-HDAC6-GFP-transfected cells and became reorganized during HIV-1 infection. (D) Flow cytometry analysis of CD4 and CXCR4 expression in untransfected, wt-HDAC6-, or dm-HDAC6-transfected MT-2 cells. (E) Analysis of HIV-1 entry and infection in MT-2 cells transfected with wt- or dm-HDAC6-GFP. Data correspond to the quantification of intracellular HIV-1-*gag* DNA. (F) Flow cytometry analysis of Env-mediated T-cell fusion after 6 h between parental, wt-, or dm-HDAC6-transfected J77 cells, and Hxbc2-Env+ T-cells. When indicated, syncytia formation was blocked with an anti-CD4 mAb. The Western blot analysis of α-tubulin acetylation levels in parental, GFP-, or wt-HDAC6-transfected J77 cells is shown at the bottom. Values indicate α-tubulin acetylation in relation to the total α-tubulin (Actub/tub). Data are representative of at least three independent experiments, and the values are means ± SEM; n = 3.

ated α-tubulin acetylation was not observed in MT-2 cells that overexpressed HDAC6 (Figure 1A, wt-HDAC6-MT-2, gp120-III treated). Untreated MT-2 cells presented stable levels of acetylated α-tubulin (Figure 1A). The wt-HDAC6-MT-2 cells contained ~20% of the acetylated α-tubulin detected in MT-2 cells (Figures 1A and 2B) and a normal cell surface expression of CD4 and CXCR4 was observed (Figure 2D). Therefore, the deacetylase activity of HDAC6 seems to affect the capacity of gp120 to induce α-tubulin acetylation.

Soluble HIV-1 gp120 protein can stimulate several signaling pathways due to the engagement of CD4 and CXCR4 or CCR5 receptors (reviewed in Perfettini *et al*., 2005). Therefore, it is conceivable that in MT-2 $CD4^+$/CXCR4+ cells, $gp120_{IIIB}$-mediated α-tubulin acetylation could occur through binding to either CD4 or CXCR4. To ascertain the role played by CD4 or CXCR4 in gp120-induced α-tubulin acetylation, parental MT-2 cells were pretreated with a neutralizing anti-CD4 mAb (5 μg/ml) or with the CXCR4 antagonist AMD3100 (50 μM) (reviewed in De Clercq, 2003) for 30 min at 37°C before adding $gp120_{IIIB}$. The $gp120_{IIIB}$-induced α-tubulin acetylation was only blocked by the neutralizing anti-CD4 mAb (Figure 1B), indicating that $gp120_{IIIB}$-dependent α-tubulin acetylation occurred through its interaction with CD4.

The localization of acetylated α-tubulin was assessed early in the process of HIV-1-Env-mediated cell fusion. Cocultures were established involving HeLa ADA (R5-tropic Env+ cells) or J77-Hxbc2 (X4-tropic Env+ cells) with their respective target cells ($CD4^+$/CXCR4+/CCR5+ HeLa P5 or $CD4^+$/CXCR4+ J77 T-cells). After 3 h, acetylated α-tubulin was mainly concentrated at cell–cell contacts formed between individual target cells and the Env+ syncytia (Figure 1C, arrowheads). Thus, in target cells that were not in contact with the Env+ cells acetylated α-tubulin did not concentrate at points of cell–cell contact (Figure 1C, see asterisk-labeled cells in HeLa-based model of fusion). Furthermore, acetylated α-tubulin did not accumulate at homotypic target cell–cell contacts, thereby indicating that the Env-CD4 inter-

action was required for the gp120-induced acetylation of α-tubulin. In addition, we found that HDAC6, acetylated α-tubulin, and the MTOC were colocalized in T-cell pseudopodia at early stages during infection by HIV-$1_{NL4.3}$ (our unpublished data), indicating that HDAC6 is recruited at HIV-1/cell contact areas.

### *HDAC6 Overexpression Impairs HIV-1 Infection and Env-mediated Cell Fusion*

Because overexpression of HDAC6 prevented the HIV-1-gp120-mediated acetylation of α-tubulin (Figure 1A, wt-HDAC6-gp120-IIIB), we assessed the effect of HDAC6 deacetylase activity on HIV-1 infection and fusion. Permissive MT-2 cells were transduced by retroviral spinoculation with the wt or the dm form of HDAC6-GFP (Figure 2A, 70–75% of cells expressed HDAC6 and ~20% of cells at high levels). These proteins were localized in the cytoplasm, a similar distribution to that of the endogenous HDAC6 (Figure 2C; our unpublished data). Overexpression of functional HDAC6-GFP diminished the acetylated α-tubulin content of cells (Figure 2C, diminished red fluorescence in cells with arrowheads in wt-HDAC6 noninfected), whereas no changes in the levels of acetylated α-tubulin were seen in cells overexpressing inactive HDAC6-GFP (Figure 2C, arrowhead in dm-HDAC6 noninfected). After HIV-1 infection (1 h), acetylated α-tubulin was only redistributed in parental or in dm-HDAC6-MT-2 cells (Figure 2C, red fluorescent capping in the cell labeled with an arrowhead in dm-HDAC6 HIV-1 NL4.3; our unpublished data). In contrast, acetylated α-tubulin was not observed in MT-2 cells expressing wt-HDAC6-GFP (Figure 2C, lack of red fluorescence in cells labeled with an arrowhead in wt-HDAC6, HIV-1 NL4.3).

Remarkably, HIV-1 infection in MT-2 cells was strongly inhibited by overexpressing wt-HDAC6 but not in the presence of dm-HDAC6 (Figure 2E). Accordingly, wt-HDAC6 overexpression in J77 cells impaired Env-mediated cell–cell fusion with Env+ Hxbc2 cells (Figure 2F, 6 h of fusion). The low levels of acetylated α-tubulin detected after overexpression of wt-HDAC6 was correlated with the poor capacity of J77 cells in the fusion assays (Figure 2F). As expected, very little acetylated α-tubulin was detected in MT-2 and J77 cells expressing wt-HDAC6-GFP (Figure 2, B, C, and F, respectively). In contrast, MT-2 cells expressing the dm-HDAC6-GFP accumulated normal levels of acetylated α-tubulin (Figure 2, B and C). MT-2 cells expressing wt-HDAC6-GFP or dm-HDAC6-GFP did not undergo any significant morphological changes (Figure 2C), and the expression of CD4 and CXCR4 remained unaltered (Figure 2D).

Similar results were obtained when we assessed the effect of HDAC6 activity on the gp120/gp41 fusion capacity and on HIV-1 infection in HeLa P5 or P4 cells (Figure 3; our unpublished data). In these cells, wt-HDAC6-GFP and dm-HDAC6-GFP had a similar cytoplasmic distribution to the endogenous enzyme (Figures 3A and 4A). The increase in wt-HDAC6 expression induced a significant reduction in acetylated α-tubulin, which was not observed with dm-HDAC6 overexpression (Figure 3B). In agreement with the previous experiments, the expression of CD4, CXCR4, and CCR5 was not modified by the overexpression of HDAC6 in these cells (Figure 3C). Under these conditions, wt-HDAC6-GFP-transfected HeLa-permissive cells were much less susceptible to infection by R5-tropic (Figure 3D, 79% inhibition of infection in wt-HDAC6-transfected HeLa P5 cells) or X4-tropic viral strains (our unpublished data). Moreover, fusion with Env+ cells was inhibited by ~80% in wt-HDAC6-transfected HeLa P5 cells (Figure 3E, with R5-tropic+-HeLa ADA cells). These effects were not observed in dm-HDAC6-GFP-transfected cells (Figure 3, D and E).

These results indicate that the efficiency of HIV-1 infection and Env-mediated cell fusion is inversely related to HDAC6 activity and directly related to the acetylated α-tubulin cell content.

### *HDAC6 Knockdown Enhances HIV-1 Infection and Env-dependent Cell Fusion*

To further assess the role of HDAC6 on HIV-1 infection and Env-dependent cell fusion, we analyzed the effects of silencing HDAC6 expression. We found that a 50% inhibition of HDAC6 expression obtained with the 217 siRNA oligo (iRNA-217) in HeLa P5 cells induced a three- to fourfold increase in acetylated α-tubulin without affecting the levels of α-tubulin, vimentin, CXCR4, CCR5, and CD4 (Figure 4A; our unpublished data). Immunostaining of endogenous HDAC6 and acetylated tubulin in cells with impaired HDAC6 expression showed that the strong increase of α-tubulin acetylation was only observed in those cells where HDAC6 was knocked down (Figure 4A, see cells labeled with an arrowhead*)*. Thus, HDAC6 seems to regulate the cellular levels of acetylated α-tubulin.

In addition, HeLa P5 cells in which HDAC6 expression was silenced were more permissive to infection with R5- or X4-tropic HIV-1 viral particles (Figure 4B). Moreover, HDAC6 silencing generated more fusogenic HeLa P5 cells (Figure 4C), which formed larger syncytia (Figure 4, E and F, iRNA-217). To further corroborate the specificity of the functional knockdown of HDAC6 observed with the 20 nucleotides iRNA-oligo 217, we assayed two other 20-nucleotide oligos (iRNA-193 and iRNA-284) specific for HDAC6. When the effects of these oligos were compared with those of the iRNA-217 oligo, all three effectively silenced endogenous HDAC6 expression in HeLa P4 (Figure 4D) and P5 cells (our unpublished data), producing a three- to fourfold increase in the acetylation of α-tubulin (Figure 4D). HDAC6-silencing in HeLa P4 cells provoked an approximate increase of 80% in the fusion with Env+ HeLa 243 cells (Figure 4E, with iRNA-193, -217, and -284 oligos). Furthermore, larger syncytia were formed (Figure 4F, see large X-Gal-stained syncytia) compared with untreated cells or cells treated with a scrambled siRNA (controls).

These results confirm the role played by endogenous HDAC6 in regulating the capacity of target cells to fuse with and to be infected by HIV-1. Furthermore, they rule out a possible transport effect of Tat on syncytia formation after siRNA treatment, because Tat might alter β-Gal production but cannot affect the formation of large syncytia (Figure 4F). Although these data prompt us to suggest that HDAC6 silencing enhances gp41/gp120 fusion efficiency and HIV-1 infection, additional effects on postentry processes cannot be excluded.

### *HDAC6 Inhibition in PHA-activated PBL Enhances HIV-1 Infection*

We also examined whether HDAC6 inhibition enhances HIV-1 infection of primary lymphocytes. Due to the difficulty of silencing HDAC6 and then activating PBLs before HIV-1 infection, we performed experiments with the chemical inhibitor of HDAC6, TSA, and with NaBut, an inhibitor of nuclear HDACs that does not effect HDAC6 (Hubbert *et al.*, 2002). We found that a 2-h exposure of PHA-activated PBLs to TSA (2 μM) enhanced HIV-$1_{NL-4.3}$ infection in a dose-dependent manner (Figure 5A). In contrast, high doses of NaBut had no effect on viral infection in these cells. Hence, increased TSA-mediated infection was correlated

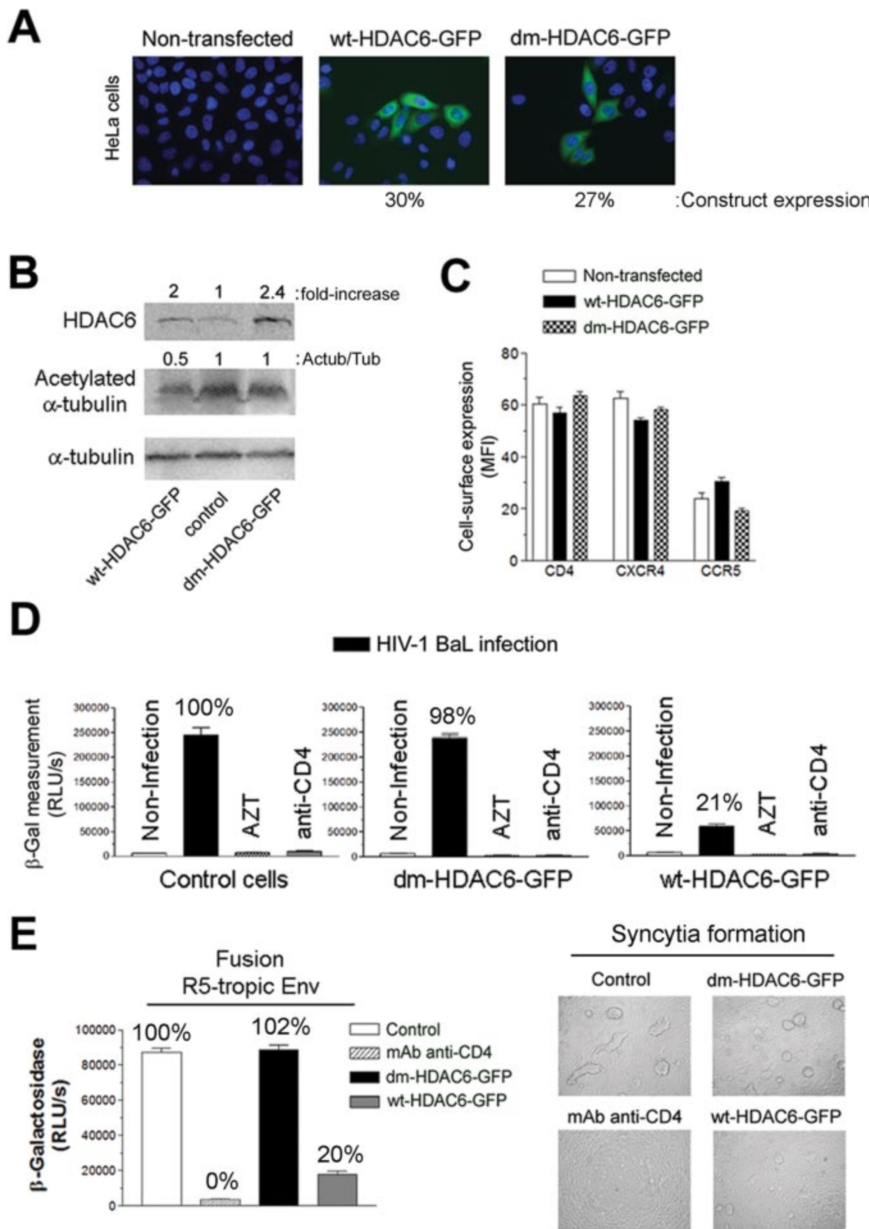


**Figure 3.** HDAC6 overexpression diminishes the susceptibility of permissive HeLa cells to fuse and to be infected by HIV-1. (A) Fluorescence images of wt- and dm-HDAC6-GFP-transfected and -nontransfected HeLa P5 cells with their nuclei counterstained with 4,6-diamidino-2-phenylindole. The proportion of construct expression is indicated. (B) Western blot analysis of wt- and dm-HDAC6-GFP expression and the effects on the levels of acetylated α-tubulin in transfected HeLa P5 cells. (C) Flow cytometry analysis of CD4, CXCR4, and CCR5 expression in nontransfected (open bars) or HeLa P5 cells transfected with wt- (solid bars) or dm-HDAC6-GFP (dotted bars). (D) HIV-1 infection of untransfected (control), wt-, or dm-HDAC6-GFP-transfected HeLa P5 cells with HIV-$1_{BaL}$ virus. The effect of an anti-CD4 mAb and AZT is also shown. (E) Quantification (β-Gal production) of Env-mediated cell fusion between HeLa ADA cells and HeLa P5 cells untransfected (control) or transfected with wt- or dm-HDAC6-GFP. Differential interference (DIC) contrast images of fused syncytia formed after 16 h are also shown. Western blot and DIC images are representative of three independent experiments, and the values are means ± SEM; n = 4.

with the higher amounts of α-tubulin acetylation stabilized by the drug (Figure 5, A–C, Western blot and immunofluorescence with 2 μM TSA). This enhancement was observed in assays where TSA was removed before the addition of HIV-1, whereas NaBut had no effect on viral infection under the same experimental conditions (Figure 5A). The short incubation times used here rule out a possible effect of TSA on transcription in HIV-1 replication (Quivy *et al*., 2002). This conclusion was further supported in an Env-mediated cell–cell fusion model where PHA-activated PBLs were cocultured with Env+-Hxbc2 cells for 12 h. For this purpose, PHA-activated PBLs were exposed to 2 μM TSA or 500 μM NaBut for 2 h, and the cells were then washed extensively to remove the drugs before coculturing for 12 h with Env+-Hxbc2 cells at a ratio of 1:1. TSA treatment strongly enhanced the capacity of PHA-activated PBLs to fuse with R4-tropic Env+ cells, producing a 94% increase in fusion after exposure to TSA (Figure 5D). In contrast, NaBut did not cause any change in Env-mediated fusion under the same experimental conditions (Figure 5D). The effect of TSA on the fusion capacity of PHA-activated PBLs correlated with the ability of TSA to stabilize acetylated α-tubulin (Figure 5E, 5-fold-increase), whereas NaBut did not induce α-tubulin acetylation (Figure 5E). As controls for the blockage of microtubule dynamics in Env-mediated fusion, Taxol and Nocodazole were used. Both drugs impaired the capacity of PHA-activated PBLs to fuse with Env+-Hxbc2 cells but not the effects on α-tubulin acetylation (Figure 5E). On the other hand, TSA and NaBut did not alter the expression of CD4 and CXCR4 in PHA-activated PBLs (our unpublished data).

Therefore, TSA seemed to enhance Env-mediated cell fusion and HIV-1 infection through the inhibition of HDAC6, as monitored by the increase in acetylated α-tubulin (Figure 5, B and E). It is important to note that the effect of siRNA or TSA on HDAC6 and HIV-1 infection could be inhibited by a neutralizing anti-CD4 mAb, by AZT, or with chemokines (Figures 4B and 5A), indicating that HDAC6 silencing or

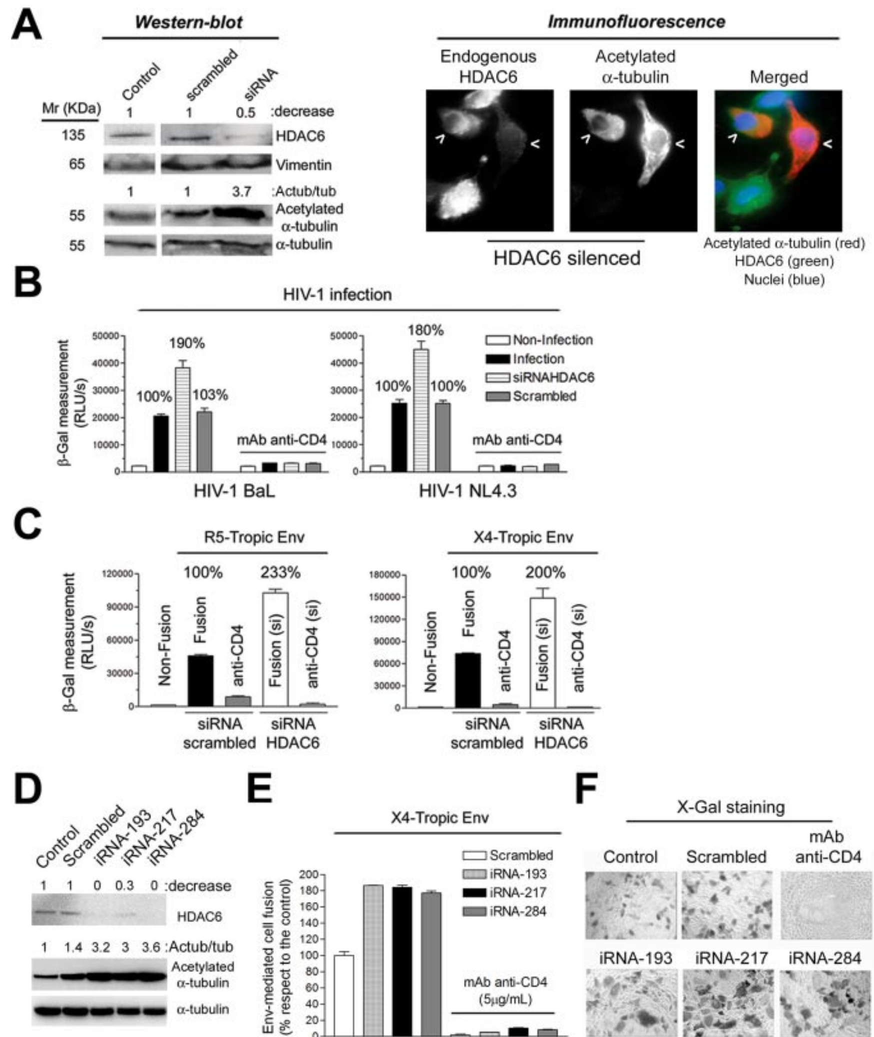


**Figure 4.** HDAC6 silencing enhances HIV-1 infection and Env-mediated cell fusion. (A) Western blot quantification of endogenous HDAC6 expression and acetylated α-tubulin in siRNA (iRNA-217)-HDAC6-treated HeLa P5 cells. Lys40 α-tubulin acetylation was quantified and expressed in reference to the total α-tubulin (Actub/tub). Fluorescence images show high levels of acetylated α-tubulin (red) in cells where endogenous HDAC6 (green) was silenced (white arrowheads). (B) Effect of specific HDAC6 silencing (iRNA-217) in HeLa P5 cells on HIV-1 infection. Infection was measured by β-Gal production, and the effects of transfection of a scrambled siRNA and of a neutralizing mAb are also shown. (C) β-Gal quantification of Env-mediated cell fusion of siRNA (iRNA-217)-HDAC6 (si)- or scrambled siRNA-treated HeLa P5 cells, with HeLa cells expressing R5-tropic (ADA) or X4-tropic (243) Env, in the presence or absence of an anti-CD4 mAb. (D) Western blot quantification of endogenous HDAC6 silencing and acetylated α-tubulin in HeLa P4 cells treated with three different siRNA-HDAC6 oligos (iRNA-193, -217, and -284). Lys40 α-tubulin acetylation was quantified and expressed in relation to the total α-tubulin (Actub/tub). (E) β-Gal quantification of Env-mediated cell fusion between HeLa P4 cells treated with iRNA-193, -217, and -284 or with scrambled oligos, and HeLa 243 cells expressing X4-tropic Env, in the presence or absence of an anti-CD4 mAb. (F) X-Gal staining of syncytia formed after 16 h between HeLa 243 cells fused with iRNA-193-, -217-, and -284-treated HeLa P4 cells or untreated cells (control) and HeLa P4 transfected with a scrambled siRNA control. The effect of an anti-CD4 mAb is also shown. The data are representative of three independent experiments, and the values are means ± SEM; n = 4.

inhibition did not affect the first Env/CD4/coreceptor, or ligand/coreceptor interactions. Together, these results show a clear correlation between the silencing or inhibition of HDAC6-tubulin deacetylase activity and an enhancement in the susceptibility of cells to fuse and to be infected by HIV-1.

## DISCUSSION

The molecular events underlying HIV-mediated cell fusion play a key role in the process of HIV-1 infection. Different reports suggest that each HIV-1-Env trimer interacts with multiple CD4 and CXCR4 or CCR5 molecules (Doms and Trono, 2000) and that multiple trimers are necessary for the formation of the fusion pore (Layne *et al.*, 1990). However, little information is available on the role of cytoskeleton in this process. Nevertheless, it has been reported that actin microfilaments regulate the clustering of CD4 and CXCR4 (Iyengar *et al.*, 1998) and that actin and tubulin are involved in HIV-1 transmission (Pearce-Pratt *et al.*, 1994; Jolly *et al.*, 2004) and secretion (Pearce-Pratt *et al.*, 1994).

How the dynamic state of the host plasma membrane regulates HIV-1 virus infection is poorly understood. Experimental evidence indicates that short cortical microtubules are directly involved in controlling membrane dynamics (Waterman-Storer and Salmon, 1999) and cell migration, which in turn are controlled by HDAC6-tubulin deacetylase activity (Hubbert *et al.*, 2002). Indeed, it has been shown that HADC6 regulates the redistribution of CD3 and LFA-1 during immune synapse formation (Serrador *et al.*, 2004). In the present study, we show for the first time that gp120 binding to CD4 in T lymphocytes induces acetylation of α-tubulin and that HDAC6 activity strongly influences the susceptibility of target cells to HIV-1 infection, without affecting CD4, CCR5, and CXCR4 cell surface expression or distribution. Quantitative analysis of α-tubulin acetylation together with an enhancement of HIV-1 fusion and infection when HDAC6 is silenced highlighted the inverse relationship between susceptibility to HIV-1 infection and HDAC6-tubulin deacetylase activity.

Remarkably, target cells that overexpress wt-HDAC6-GFP and that are less permissive to fusion and infection by HIV-1 display normal cell surface expression of CD4 and coreceptor preferentially localized in the HIV-1-induced pseudopodia. However, these cells contain reduced levels of acetylated α-tubulin. Therefore, it is feasible that the redistribution of HDAC6 to the CXCR4/CD4 capping regions diminishes the amount of cortical acetylated α-tubulin at HIV-1/cell contact areas, resulting in changes in plasma membrane dynamics (Hubbert *et al.*, 2002; Matsuyama *et al.*, 2002) and a diminished susceptibility to the changes induced

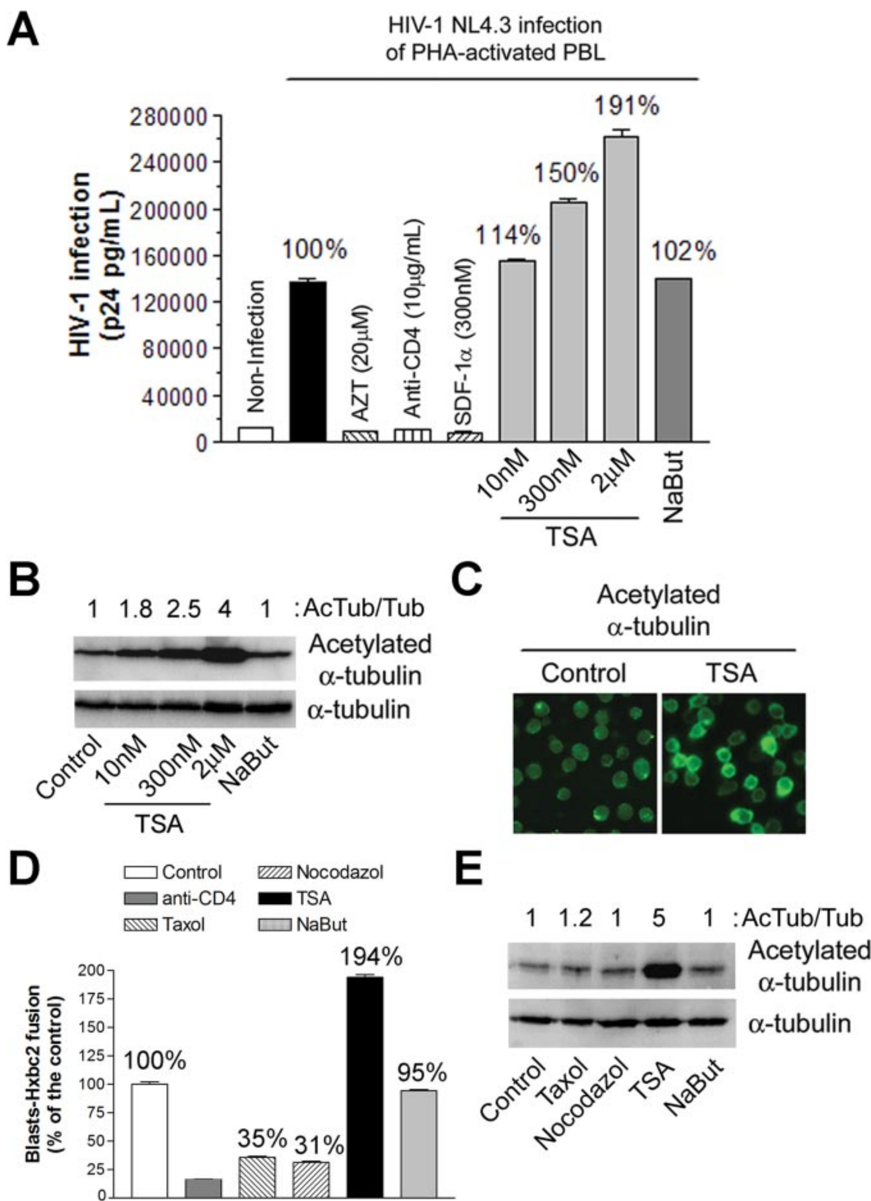


**Figure 5.** HDAC6 inhibition enhances HIV-1 infection and Env-mediated fusion in primary lymphocytes. (A) Viral production at 3 d in PHA-activated and HIV-1-infected PBLs pretreated with TSA or NaBut (500 $\mu$M) and in untreated cells. (B) Western blot shows the level of acetylated $\alpha$-tubulin in PHA-activated PBLs after a 2-h exposure to TSA (10 nM, 300 nM, and 2 $\mu$M) or NaBut (500 $\mu$M) and the addition of the HIV-1 virus. Lys40 $\alpha$-tubulin acetylation was quantified and expressed in reference to the total $\alpha$-tubulin (Actub/tub). (C) Immunofluorescence images show the level of acetylated $\alpha$-tubulin in PHA-activated PBLs after a 2-h exposure to TSA (2 $\mu$M). (D) Flow cytometry analysis of Env-mediated PHA-activated PBL fusion after 12 h with Env+Hxbc2 T-cells. PHA-activated PBL were pretreated for 2 h with TSA (2 $\mu$M), NaBut (500 $\mu$M), and Taxol (5 $\mu$M). The cells were the cocultured with Hxbc2 cells for 12 h after washing out the drugs. Taxol and Nocodazole (5 $\mu$M) remained present in the culture medium during the 12-h fusion with Hxbc2 cells. Where indicated, syncytia formation was blocked with an anti-CD4 mAb. (E) Western blot analysis of $\alpha$-tubulin acetylation in PHA-activated PBLs treated with TSA (2 $\mu$M), NaBut (500 $\mu$M), Taxol (5 $\mu$M), and Nocodazole (5 $\mu$M). Lys40 $\alpha$-tubulin acetylation was quantified and expressed in relation to the total $\alpha$-tubulin (Actub/tub). The data are representative of at least three independent experiments, and the values are means ± SEM; n = 3.

by HIV-1. In this regard, acetylated $\alpha$-tubulin is a hydrophobic form of tubulin that predominantly associates with the membrane by directly interacting with the transport protein $Na^+,K^+$-ATPase (Nunez Fernandez *et al.*, 1997; Alonso *et al.*, 1998; Casale *et al.*, 2003). Hence, we propose that the association between the plasma membrane and acetylated $\alpha$-tubulin is strongly influenced by HDAC6, regulating the dynamic tension of the plasma membrane. This could conceivably be the mechanism by which HDAC6 regulates HIV-induced cell fusion and infection, affecting pore fusion formation by HIV-1-Env. In this respect, the binding of gp120 protein induces acetylation of $\alpha$-tubulin in permissive but not in wt-HDAC6-transfected cells in a CD4-dependent manner.

In contrast with other HDAC class II enzymes, HDAC6 is mainly located in the cytoplasm of eukaryotic cells (Verdel *et al.*, 2000; Hubbert *et al.*, 2002; Matsuyama *et al.*, 2002). Indeed, it has been reported that HDAC6 possesses a potent nuclear-specific export signal at its N terminus (Verdel *et al.*, 2000; Bertos *et al.*, 2004), making it very unlikely that HDAC6 participates in histone deacetylation (Verdel *et al.*, 2000; Bertos *et al.*, 2004). Hence, our data indicate that cell signals that promote HDAC6 transcription and its subsequent cytoplasmic export should induce a more protective phenotype to host cells against HIV-1 infection. Accordingly, the different cellular levels of HDAC6 could account for the differential susceptibility shown by some cell types to HIV-1 viral infection in vitro. However, it is also feasible that under certain conditions, HDAC6 could be located in the nucleus and participate in the control of HIV-1 replication by repressing *LTR* activation.

Cooperative interactions must take place among the viral envelope, cell receptors, and coreceptor molecules to permit viral entry (Doms, 2000). It has been shown that modifying the codistribution of CD4 and coreceptor molecules at the points of virus–cell contact may alter the process of pore fusion formation (Dimitrov *et al.*, 1999; Xiao *et al.*, 2000), thereby regulating HIV-1 infection. However, our results indicate that the effect of HDAC6 on Env-mediated cell

fusion and HIV-1 infection is not exerted through altering the expression or colocalization of CD4 and CXCR4.

Taking into account all the experimental evidence, we conclude that HDAC6 regulates HIV-1 infection, independently of the viral tropism. Although our data on Env-dependent cell fusion suggest a direct effect of HDAC6 on fusion pore formation, we cannot rule out any influence of HDAC6 on postentry events that regulate HIV-1 infection. Therefore, it will be important to determine what cell signals regulate HDAC6 expression and its cytoplasmic distribution as well as to discover the specific HDAC6-activating agents to design new anti-HIV-1 therapeutic strategies.

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