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Regulation of Tat Acetylation and Transactivation Activity by the Microtubule-associated Deacetylase HDAC6*[S]

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Reversible acetylation of Tat is critical for its transactivation activity toward HIV-1 transcription. However, the enzymes involved in the acetylation/deacetylation cycles have not been fully characterized. In this study, by yeast two-hybrid assay, we have discovered the histone deacetylase HDAC6 to be a binding partner of Tat. Our data show that HDAC6 interacts with Tat in the cytoplasm in a microtubule-dependent manner. In addition, HDAC6 deacetylates Tat at Lys-28 and thereby suppresses Tat-mediated transactivation of the HIV-1 promoter. Inactivation of HDAC6 promotes the interaction of Tat with cyclin T1 and leads to an increase in Tat transactivation activity. These findings establish HDAC6 as a Tat deacetylase and support a model in which Lys-28 deacetylation decreases Tat transactivation activity through affecting the ability of Tat to form a ribonucleoprotein complex with cyclin T1 and the transactivation-responsive RNA.

The transactivator protein Tat is essential for HIV-1 transcription. Tat enhances HIV-1 transcription elongation through binding to the transactivation-responsive RNA (TAR)[2] structure, a stem-loop formed at the 5′-end of viral transcripts (1, 2). The interaction of Tat with TAR is in concert with cyclin T1 (CycT1), a component of positive transcription elongation factor b. CycT1 associates with both Tat and TAR to form a ternary complex and recruits Cdk9 (cyclin-dependent kinase 9), another component of positive transcription elongation factor b, to the vicinity of the HIV-1 LTR (3). Cdk9 then phosphorylates the C-terminal domain of RNA polymerase II, resulting in increased efficiency of HIV-1 transcription elongation (4).

The transactivation activity of Tat is regulated by acetylation (5). There are two well defined acetylation sites in Tat, Lys-28 in the activation domain and Lys-50 in the RNA-binding domain. Tat is acetylated at Lys-28 by p300/CBP-associated factor (PCAF), and the acetylation enhances HIV-1 transcription elongation by strengthening assembly of the Tat-CycT1-TAR complex (5, 6). In contrast, Tat is acetylated at Lys-50 by p300 and Gcn5 (5, 7, 8). Lys-50 acetylation disrupts the Tat-CycT1-TAR complex, releasing Tat from TAR. Tat then recruits PCAF to the elongating RNA polymerase II to facilitate chromatin remodeling (9–11).

Tat acetylation is a reversible process. Lys-50 in Tat is deacetylated by the class III protein deacetylase SIRT1 (sirtuin 1), which allows Tat to be recycled to TAR for subsequent rounds of HIV-1 transcription (12). In addition, SIRT2 and SIRT3, two other deacetylases in the class III family, have been shown to deacetylate Tat at Lys-50 (12). The deacetylase that mediates Lys-28 deacetylation has not yet been identified. Trichostatin A, a pan-inhibitor of class I and II histone deacetylases (HDACs), has been shown to enhance Tat acetylation, and this action of trichostatin A is preserved when Lys-50 is mutated to an unacetylatable residue (5), suggesting that lysines other than Lys-50, particularly Lys-28, might be deacetylated by a class I or II HDAC. In this study, we provide the first evidence that HDAC6, a member of the class II HDAC family, is a Tat deacetylase. Our data reveal that HDAC6 binds Tat and deacetylates Tat at Lys-28 in a microtubule-dependent manner and thereby regulates Tat transactivation activity.

EXPERIMENTAL PROCEDURES

Materials—Antibodies against HA, His, FLAG, GST, and α-tubulin (Sigma-Aldrich); GFP, Ack, and CycT1 (Cell Signaling); HDAC6, lamin B, and SIN3A (Santa Cruz Biotechnology); and Tat (Covance) were purchased from the indicated sources. Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. Fluorescein- and rhodamine-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. The mammalian expression plasmids pEGFPC1-Tat, pcDNA3-FLAG-HDAC6, pCMV-HA-HDAC6, and pcDNA3-FLAG-CycT1 were generated by PCR, and pCMV-FLAG-Tat has been described previously (13). The CMV promoter-driven luciferase plasmid was generated by cloning luciferase cDNA into the pCMV plasmid, and the HIV-1 LTR-driven luciferase plasmid has been described

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[2] The abbreviations used are: TAR, transactivation-responsive RNA; CycT1, cyclin T1; PCAF, p300/CBP-associated factor; HDAC, histone deacetylase; MEF, mouse embryonic fibroblast; VSV-G, vesicular stomatitis virus glycoprotein.

previously (12). GST fusion proteins were purified with glutathione-Sepharose beads (Amersham Biosciences), and His fusion proteins were purified with the nickel-nitrilotriacetic acid Superflow system (Qiagen).

Cell Culture, Transfection, and Infection—HEK293 and Jurkat cells were obtained from American Type Culture Collection. HDAC6$^{+/+}$ and HDAC6$^{-/-}$ mouse embryonic fibroblasts (MEFs) have been described previously (14). Jurkat cells in RPMI 1640 medium and other cells in Dulbecco's modified Eagle's medium were cultured in the indicated media supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. Plasmid transfections were performed using polyethyleneimine reagent (Sigma). siRNAs were synthesized by RiboBio and transfected into cells with Lipofectamine 2000 reagent (Invitrogen). HIV-1 viruses pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) were generated by cotransfection of HEK293 cells with the pSG3ΔEnv proviral vector and pCMV-VSV-G, and virus-containing supernatants were used to infect cells.

Yeast Two-hybrid Assay—Yeast two-hybrid assays were performed using the Matchmaker Gal4 two-hybrid system following the manufacturer's protocol (Clontech). The yeast strain AH109 was transformed with the bait plasmid pGBT9-Tat, which encodes Tat fused to the DNA-binding domain of Ga14, and a pACT2 vector-based human leukocyte cDNA library encoding proteins fused to the activation domain of Ga14. The activity of β-galactosidase was measured by standard protocols.

Immunofluorescence Microscopy—Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were blocked with 2% bovine serum albumin in phosphate-buffered saline and incubated with antibodies against FLAG and HDAC6 and then with fluorescein- and rhodamine-conjugated secondary antibodies, followed by staining with the nuclear dye 4′,6-diamidino-2-phenylindole for 5 min. Coverslips were mounted with 90% glycerol in phosphate-buffered saline and examined with a TCS SP5 confocal microscope (Leica).

Immunoprecipitation and GST Pulldown—For immunoprecipitation, cell lysates were incubated with antibody-coated agarose beads at 4 °C for 2 h. For GST pulldown, GST fusion proteins immobilized on Sepharose beads were incubated with purified His fusion proteins at 4 °C for 2 h. Proteins present on the beads of immunocomplexes or pulldown preparations were detected by SDS-PAGE and immunoblotting.

Immunoblotting—Samples were transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked and incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized with enhanced chemiluminescence detection reagent (Millipore).

In Vitro Tat Deacetylation Assay—Lys-28- or Lys-50-acetylated Tat was synthesized by Invitrogen and immobilized on anti-Tat antibody-coated agarose beads. The beads were incubated with purified His-HDAC6 in the absence or presence of preformed microtubules and immunoblotted with anti-Ack and anti-Tat antibodies.

Quantitative Real-time RT-PCR—RNA was isolated from HIV-1-infected Jurkat cells using RNAwiz reagent (Ambion).

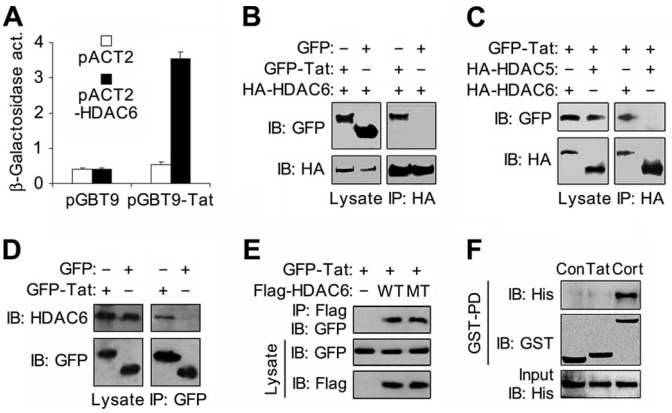

FIGURE 1. **Tat interacts with HDAC6 in yeast and mammalian cells.** *A*, yeast cells were transformed with the indicated plasmids, and β-galactosidase activity (*act.*) was measured. Data are represented as means ± S.D. of three independent experiments. *B* and *C*, HEK293 cells were transfected with plasmids expressing the indicated proteins. Cell lysates and anti-HA immunoprecipitates (*IP*) were immunoblotted (*IB*) with anti-GFP and anti-HA antibodies. *D*, cells were transfected with pEGFPC1-Tat or pEGFPC1. Cell lysates and anti-GFP immunoprecipitates were immunoblotted with anti-HDAC6 and anti-GFP antibodies. *E*, cells were transfected with pEGFPC1-Tat and pcDNA3-FLAG-HDAC6-WT or pcDNA3-FLAG-HDAC6-H216A/H611A (mutant (*MT*)). Cell lysates and anti-FLAG immunoprecipitates were immunoblotted with the indicated antibodies. *F*, purified GST (control (*Con*)), GST-Tat, and GST-cortactin (*Cort*) immobilized on Sepharose beads were incubated with purified His-HDAC6. GST pulldown (*PD*) preparations and input His-HDAC6 were immunoblotted with the indicated antibodies.

HIV-1 transcripts were then quantified by real-time RT-PCR with the QuantiTect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions.

Luciferase Reporter Assay—Cells were transfected with the HIV-1 LTR-driven luciferase plasmid or the CMV promoter-driven luciferase plasmid and a β-galactosidase-expressing plasmid. The luciferase activity was measured using an FB12 luminometer (Berthold Detection Systems) and normalized to β-galactosidase activity. To measure the transactivation activity of Tat, cells were transfected with the GFP-Tat expression plasmid or empty vector in addition to the above plasmids, and the extent of transactivation -fold) was determined.

RESULTS

Tat Interacts with HDAC6 in Yeast and Mammalian Cells—To identify Tat-interacting proteins, we screened a human leukocyte cDNA library using the yeast two-hybrid system with Tat as bait. We obtained three positive clones containing HDAC6 cDNA fragments of different length. Yeast cells transformed with HDAC6 showed strong β-galactosidase activity in a Tat-dependent pattern (Fig. 1*A*), indicating an interaction of HDAC6 with Tat in yeast.

Immunoprecipitation assays revealed that GFP-Tat interacted with HA-HDAC6 but not with HA-HDAC5 in mammalian cells (Fig. 1, *B* and *C*), demonstrating specificity of the Tat-HDAC6 interaction. We further found that GFP-Tat interacted with endogenous HDAC6 in mammalian cells (Fig. 1*D*). In addition, GFP-Tat interacted with FLAG-tagged H216A/H611A, an inactive mutant of HDAC6, in a manner similar to its interaction with wild-type HDAC6 (Fig. 1*E*), indicating that the Tat-HDAC6 interaction is independent of the deacetylase activity of HDAC6. By GST pulldown assay, we further found that purified His-HDAC6 did not interact with purified GST-

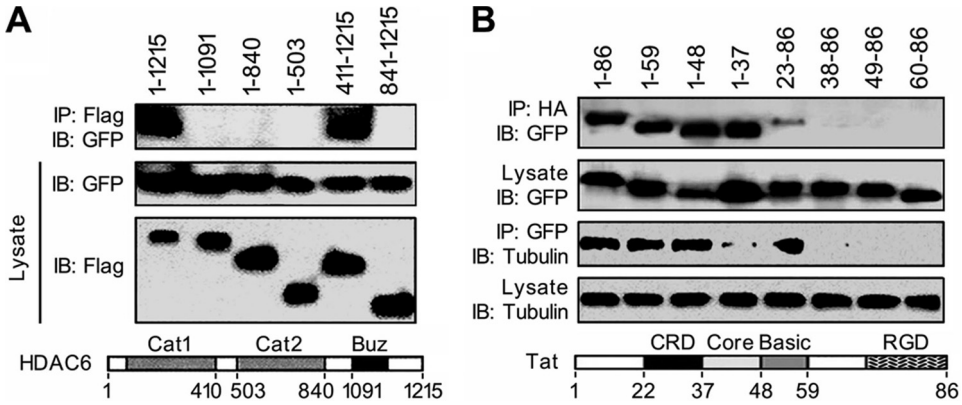

FIGURE 2. **Identification of Tat-HDAC6 interaction domains.** *A*, cells were transfected with pEGFPC1-Tat and pcDNA3-FLAG-HDAC6 (WT or deletion mutants). Immunoprecipitation (*IP*) and immunoblotting (*IB*) were then performed. A schematic diagram of HDAC6 is shown below. *Cat1*, first catalytic domain; *Cat2*, second catalytic domain. Buz is the ubiquitin-binding zinc finger. *B*, cells were transfected with pCMV-HA-HDAC6 and pEGFPC1-Tat (WT or deletion mutants). Immunoprecipitation and immunoblotting were then performed. A schematic diagram of Tat is shown below. *CRD*, cysteine-rich domain; *Core*, conserved core region; *Basic*, region of basic amino acids; *RGD*, region of Arg-Gly-Asp sequence.

Tat, although it could interact with purified GST-cortactin as described previously (Fig. 1*F*) (15). This finding suggests that the interaction between Tat and HDAC6 in cells is mediated by an indirect mechanism.

To identify the Tat interaction domain on HDAC6, cells were transfected with GFP-Tat and FLAG-tagged wild-type HDAC6 or deletion mutants. Immunoprecipitation assays showed that Tat interacted with sequence 411–1215 of HDAC6, similar to its interaction with wild-type HDAC6 (Fig. 2*A*). In contrast, Tat did not interact with sequence 1–1091 (lacking the ubiquitin-binding zinc finger, Buz), 1–840, 1–503, or 841–1215 of HDAC6. These data indicate that both the second catalytic domain and the Buz domain of HDAC6 are critical for its interaction with Tat. Similarly, by deletion mutant analysis, we found that the cysteine-rich domain of Tat is important for HDAC6 binding (Fig. 2*B*).

Tat and HDAC6 Interact in the Cytoplasm in a Microtubule-dependent Manner—To identify the cellular location of the Tat-HDAC6 interaction, we performed immunoprecipitation assays using cytoplasmic and nuclear lysates. We found that HDAC6 interacted with Tat in the cytoplasm but not in the nucleus (Fig. 3*A*). In contrast, in agreement with previous findings (16–18), CycT1, lamin B, SIN3A, and human NAP-1 were seen in the nuclear immunoprecipitates of Tat (Fig. 3*B*). Immunofluorescence confocal microscopy further revealed a significant colocalization of cytoplasmic Tat and HDAC6 (Fig. 3*C*), providing additional support for their interaction in the cytoplasm.

We then investigated the molecular mechanism mediating the Tat-HDAC6 interaction. To this end, we first examined the possibility that these two proteins might interact in the cytoplasm through Hic, a protein known to contribute to the cytoplasmic localization of Tat (19). By immunoprecipitation assays, we found that the Tat-HDAC6 interaction was compromised by siRNAs against HDAC6 but not Hic (Fig. 3*D*), thus ruling out this possibility.

Another possible way for Tat and HDAC6 to interact lies in the microtubule cytoskeleton because both HDAC6 and Tat possess microtubule-binding capacity (20, 21), and tubulin association was detected for all the deletion mutants of Tat that

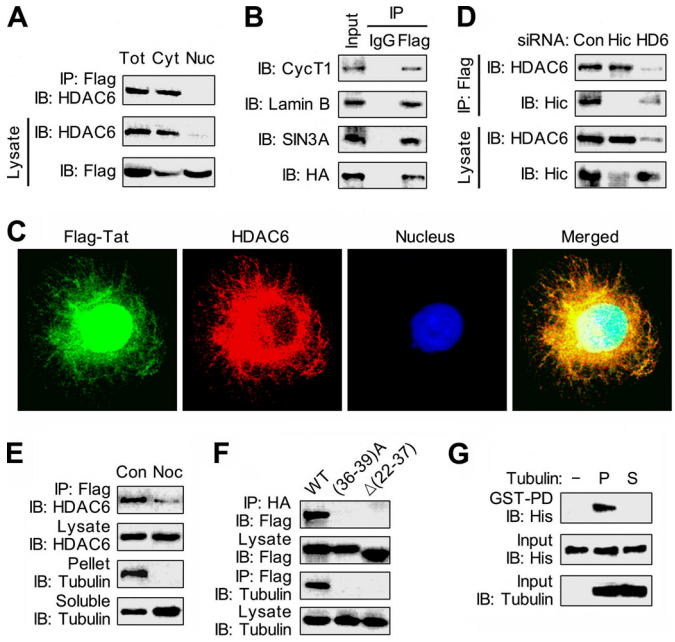

FIGURE 3. **Tat and HDAC6 interact in the cytoplasm in a microtubule-dependent manner.** *A*, total (*Tot*), cytoplasmic (*Cyt*), and nuclear (*Nuc*) lysates from HEK293 cells transfected with pCMV-FLAG-Tat were subjected to anti-FLAG immunoprecipitation (*IP*), followed by anti-HDAC6 immunoblotting (*IB*). *B*, nuclear lysates from cells transfected with pCMV-FLAG-Tat were immunoprecipitated with anti-FLAG antibody or IgG control and immunoblotted with the indicated antibodies. In the *lower panel*, cells were transfected with pCMV-HA-hNAP-1 together with pCMV-FLAG-Tat. *C*, cells were transfected with pCMV-FLAG-Tat, stained with anti-FLAG and anti-HDAC6 antibodies and the nuclear dye 4′,6-diamidino-2-phenylindole, and examined by confocal microscopy. *D*, cells were transfected with pCMV-FLAG-Tat and luciferase (control (*Con*)), Hic, or HDAC6 siRNAs. Cell lysates and anti-FLAG immunoprecipitates were immunoblotted with anti-HDAC6 and anti-Hic antibodies. *E*, cells were transfected with pCMV-FLAG-Tat and treated with nocodazole (*Noc*). Cell lysates and anti-FLAG immunoprecipitates were immunoblotted with anti-HDAC6 antibody. Polymeric/pellet and soluble tubulin fractions were immunoblotted with anti-α-tubulin antibody. *F*, cells were transfected with pCMV-HA-HDAC6 and pCMV-FLAG-Tat-WT, pCMV-FLAG-Tat(36–39)A, or pCMV-FLAG-TatΔ(22–37). Immunoprecipitation and immunoblotting were performed as indicated. *G*, purified GST-Tat immobilized on Sepharose beads was incubated with purified His-HDAC6 in the presence of polymeric (*P*) or soluble (*S*) tubulin. GST pulldown preparations and input His-HDAC6 and tubulin were examined by immunoblotting.

could interact with HDAC6 (Fig. 2*B*). We tested this possibility by treating cells with nocodazole, a microtubule-depolymerizing agent. We observed that nocodazole attenuated the inter-

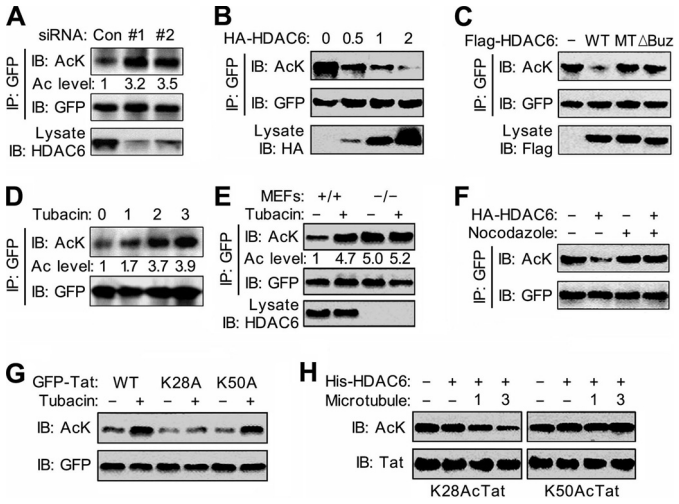

FIGURE 4. **HDAC6 deacetylates Tat at Lys-28.** *A*, HEK293 cells were transfected with pEGFPC1-Tat and control (*Con*) or HDAC6 siRNAs. Cell lysates and anti-GFP immunoprecipitates (*IP*) were immunoblotted (*IB*) with the indicated antibodies. Tat acetylation (*Ac*) levels were quantified by densitometric analysis of the blots. *B*, cells were transfected with pEGFPC1-Tat and 0, 0.5, 1, or 2 μg of pCMV-HA-HDAC6. Immunoprecipitation and immunoblotting were performed as indicated to examine Tat acetylation. *C*, cells were transfected with pEGFPC1-Tat and pcDNA3-FLAG-HDAC6-WT, pcDNA3-FLAG-HDAC6-H216A/H611A (mutant (*MT*)), or pcDNA3-FLAG-HDAC6ΔBuz. Immunoprecipitation and immunoblotting were then performed as indicated. *D*, cells were transfected with pEGFPC1-Tat in the presence of 0, 1, 2, or 3 μM tubacin. Anti-GFP immunoprecipitates were immunoblotted with anti-Ack and anti-GFP antibodies. Tat acetylation levels were quantified by densitometric analysis of the blots. *E*, HDAC6$^{+/+}$ and HDAC6$^{-/-}$ MEFs were transfected with pEGFPC1-Tat in the absence or presence of 3 μM tubacin. Cell lysates and anti-GFP immunoprecipitates were immunoblotted with the indicated antibodies. Tat acetylation levels were quantified by densitometric analysis of the blots. *F*, HEK293 cells were transfected with pEGFPC1-Tat and pCMV-HA-HDAC6 and treated with nocodazole. Anti-GFP immunoprecipitates were immunoblotted with anti-Ack and anti-GFP antibodies. *G*, cells were transfected with pEGFPC1-Tat-WT, pEGFPC1-Tat-K28A, or pEGFPC1-Tat-K50A in the absence or presence of tubacin. Anti-GFP immunoprecipitates were immunoblotted with anti-Ack and anti-GFP antibodies. *H*, synthetic Lys-28-acetylated (*K28AcTat*) or Lys-50-acetylated (*K50AcTat*) Tat protein was immobilized on agarose beads, incubated with purified His-HDAC6 in the presence of 1 or 3 μM preformed microtubules, and immunoblotted with anti-Ack and anti-Tat antibodies.

action of Tat with HDAC6 (Fig. 3*E*). We further found that Tat(36–39)A, a previously described Tat mutant that does not associate with microtubules/tubulin (21), failed to interact with HDAC6 (Fig. 3*F*). In addition, in agreement with previous results (22), TatΔ(22–37), a zinc binding-defective Tat mutant, did not interact with tubulin or HDAC6 (Fig. 3*F*). GST pull-down assay showed that Tat interacted with HDAC6 *in vitro* upon addition of preformed microtubules (Fig. 3*G*), providing additional evidence for a role of microtubules in mediating the Tat-HDAC6 interaction. Collectively, these data indicate that Tat and HDAC6 interact in the cytoplasm in a microtubule-dependent manner.

HDAC6 Deacetylates Tat at Lys-28—We then examined whether HDAC6 is a deacetylase of Tat. As shown in Fig. 4*A*, knockdown of HDAC6 increased the acetylation level of Tat without changing its expression. Conversely, overexpression of HA-HDAC6 or FLAG-HDAC6 reduced Tat acetylation (Fig. 4, *B* and *C*). In contrast, the ΔBuz mutant of HDAC6, which was unable to interact with Tat (Fig. 2*A*), did not affect Tat acetylation (Fig. 4*C*). In addition, the inactive mutant of HDAC6 did not alter Tat acetylation (Fig. 4*C*), although this mutant was able to interact with Tat (Fig. 1*E*).

To verify Tat deacetylation by HDAC6, cells were transfected with GFP-Tat in the presence of tubacin, a specific inhibitor of HDAC6 (23). We found that tubacin increased Tat acetylation in a dose-dependent manner without altering Tat expression (Fig. 4*D*). To further study the effect of HDAC6 on Tat acetylation, we used MEFs derived from HDAC6 wild-type and knock-out mice (14). HDAC6$^{-/-}$ MEFs showed higher Tat acetylation levels compared with HDAC6$^{+/+}$ MEFs, and tubacin increased Tat acetylation in HDAC6$^{+/+}$ MEFs but not in HDAC6$^{-/-}$ MEFs (Fig. 4*E*). In addition, Tat deacetylation by HDAC6 was blocked by nocodazole (Fig. 4*F*). Thus, microtubules are critical for Tat deacetylation by HDAC6.

To identify the lysine residue in Tat that is deacetylated by HDAC6, we mutated individually Lys-28 and Lys-50 to alanine, which eliminates possible acetylation. Tubacin increased the acetylation of wild-type and K50A Tat to a similar extent (~4.4-fold) (Fig. 4*G*). In contrast, tubacin induced only a slight increase in the acetylation of K28A (1.5-fold). Furthermore, in the presence of microtubules, purified His-HDAC6 was able to deacetylate synthetic Lys-28-acetylated Tat but not Lys-50-acetylated Tat (Fig. 4*H*). Taken together, these results indicate that HDAC6 deacetylates Tat at Lys-28.

HDAC6 Inhibits Tat-mediated Transactivation—Using an HIV-1 LTR-driven luciferase plasmid, we found that HA-HDAC6 decreased the transactivation activity of Tat, and this effect of HDAC6 was blocked by nocodazole (Fig. 5*A*). We also found that HDAC6 could decrease the transactivation activity of Tat when cells were transfected with different amounts of GFP-Tat (Fig. 5*B*). Importantly, HDAC6 did not affect the transcription activity of the CMV promoter, a promoter used to drive the expression of GFP-Tat and HA-HDAC6 in these cotransfection experiments (Fig. 5*B*). These results demonstrate the specificity of the inhibitory effect of HDAC6 on Tat-mediated HIV-1 transcriptional transactivation. We also found that Tat transactivation activity was reduced by FLAG-tagged wild-type HDAC6 but not by the inactive mutant or the ΔBuz mutant (Fig. 5*C*). In addition, transfection of cells with HDAC6 siRNAs or treatment of cells with tubacin increased Tat transactivation activity (Fig. 5, *D* and *E*).

We next sought to examine the role of HDAC6 in HIV-1 infection. Jurkat T cells were transfected with HA-HDAC6 and then infected with VSV-G-pseudotyped HIV-1. The VSV-G envelope, instead of the HIV-1 envelope, was used to restrict analysis to a single infection cycle and to eliminate the negative effect of HDAC6 on HIV-1 entry as reported previously (24). Viral infection was determined by quantitative real-time RT-PCR analysis of HIV-1 transcripts. As shown in Fig. 5*F*, overexpression of HDAC6 in Jurkat cells reduced HIV-1 gene expression. Conversely, siRNA-mediated knockdown of HDAC6 expression or inhibition of HDAC6 activity by tubacin enhanced HIV-1 gene expression (Fig. 5, *G* and *H*). These results are consistent with the negative effect of HDAC6 on Tat transactivation activity.

We then used HDAC6$^{+/+}$ and HDAC6$^{-/-}$ MEFs to further examine the ability of HDAC6 to inhibit Tat transactivation activity. Human CycT1 was introduced into these cells because

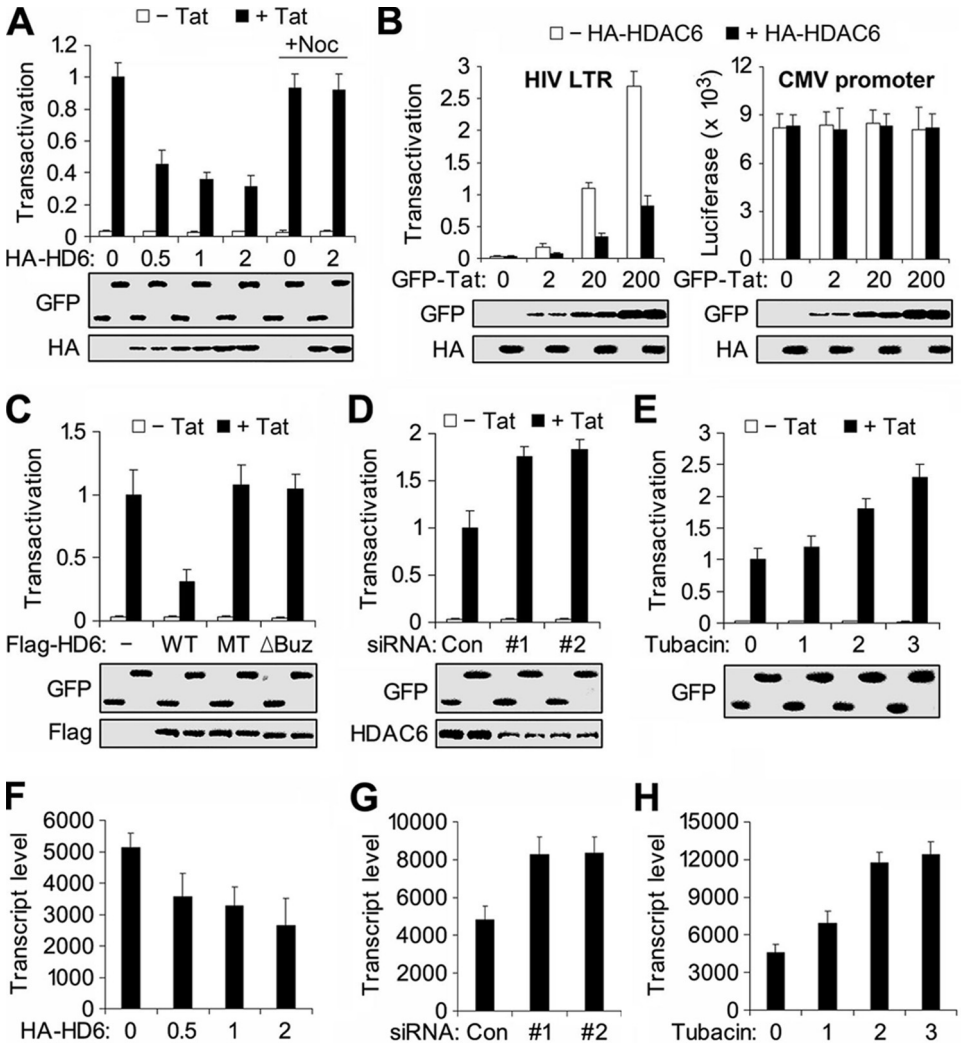

FIGURE 5. **HDAC6 inhibits Tat-mediated transactivation.** *A*, cells were transfected with pHIV-LTR-luciferase, pEGFPC1-Tat, or pEGFPC1 and 0, 0.5, 1, or 2 μg of pCMV-HA-HDAC6 and then treated with nocodazole (*Noc*). Luciferase activity was measured, and the extent of Tat-mediated transactivation (-fold) was determined. The levels of GFP, GFP-Tat, and HA-HDAC6 were examined by immunoblotting. Data in this and the following graphs are represented as means ± S.D. of three independent experiments. *B*, Tat-mediated transactivation in cells transfected with pHIV-LTR-luciferase, pCMV-HA-HDAC6, or pCMV-HA and 0, 2, 20, or 200 ng of pEGFPC1-Tat. In another group of experiments, pCMV-luciferase was used as a control for pHIV-LTR-luciferase. The levels of GFP-Tat and HA-HDAC6 were examined by immunoblotting. *C*, Tat-mediated transactivation in cells transfected with pHIV-LTR-luciferase, pEGFPC1-Tat, or pEGFPC1 and with pcDNA3-FLAG-HDAC6-WT, pcDNA3-FLAG-HDAC6-H216A/H611A (mutant (*MT*)), or pcDNA3-FLAG-HDAC6ΔBuz. The levels of GFP, GFP-Tat, and FLAG-HDAC6 were examined by immunoblotting. *D*, Tat-mediated transactivation in cells transfected with pHIV-LTR-luciferase, pEGFPC1-Tat, or pEGFPC1 and with control (*Con*) or HDAC6 siRNAs. The levels of GFP, GFP-Tat, and HA-HDAC6 were examined by immunoblotting. *E*, Tat-mediated transactivation in cells transfected with pHIV-LTR-luciferase and pEGFPC1-Tat or pEGFPC1 in the presence of 0, 1, 2, or 3 μM tubacin. The levels of GFP and GFP-Tat were examined by immunoblotting. *F*, real-time RT-PCR analysis of HIV-1 transcripts in Jurkat cells transfected with 0, 0.5, 1, or 2 μg of pCMV-HA-HDAC6 and infected with HIV-1. *G*, real-time RT-PCR analysis of HIV-1 transcripts in Jurkat cells transfected with control or HDAC6 siRNAs and infected with HIV-1. *H*, real-time RT-PCR analysis of HIV-1 transcripts in Jurkat cells infected with HIV-1 and treated with 0, 1, 2, or 3 μM tubacin.

Tat does not functionally interact with mouse CycT1 (25, 26). In the presence of human CycT1, Tat transactivation activity was significantly increased in HDAC6$^{-/-}$ MEFs compared with HDAC6$^{+/+}$ MEFs, and this effect was blocked by reconstitution of HDAC6 in HDAC6$^{-/-}$ MEFs (Fig. 6*A*). HDAC6$^{+/+}$ and HDAC6$^{-/-}$ MEFs did not exhibit an obvious difference in Tat transactivation activity when the K28A mutant of Tat was used (Fig. 6*B*). Together, these data provide further evidence for a role of HDAC6 in the negative regulation of Tat transactivation activity and implicate CycT1 in this action. By immunoprecipitation assay, we found that tubacin increased the interaction of Tat with CycT1, and this effect of tubacin was abolished when the K28A mutant of Tat was used (Fig. 6*C*). These data show additional evidence that HDAC6-mediated Lys-28 deacetylation inhibits Tat transactivation activity by affecting Tat interaction with CycT1.

DISCUSSION

HDAC6 is a member of the class II HDAC family with a predominant localization in the cytoplasm. It regulates cell motility by deacetylating α-tubulin and cortactin (15, 20). HDAC6 also acts as a deacetylase of Hsp90 and modulates Hsp90-dependent activation of the glucocorticoid receptor (27). In addition, HDAC6 plays a role in modulating cellular redox activities by deacetylating peroxiredoxins (28). In this study, we have identified, by yeast two-hybrid screening, HDAC6 as an interacting protein of the HIV-1 transactivator Tat. Importantly, we demonstrated that HDAC6 functions

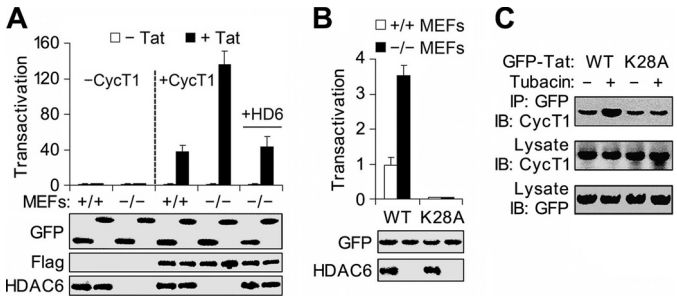

FIGURE 6. **HDAC6 regulates Tat transactivation activity by modulating Tat interaction with CycT1.** *A*, Tat-mediated transactivation in HDAC6$^{+/+}$ and HDAC6$^{-/-}$ MEFs transfected with pHIV-LTR-luciferase, pEGFPC1-Tat, or pEGFPC1 and with pcDNA3-FLAG-CycT1 or pcDNA3-FLAG. In another group of experiments, HDAC6$^{-/-}$ MEFs were reconstituted with pCMV-HA-HDAC6. The levels of GFP, GFP-Tat, FLAG-CycT1, and HDAC6 (*HD6*) were examined by immunoblotting. *B*, Tat-mediated transactivation in HDAC6$^{+/+}$ and HDAC6$^{-/-}$ MEFs transfected with pHIV-LTR-luciferase, pEGFPC1-Tat-WT or pEGFPC1-Tat-K28A, and pcDNA3-FLAG-CycT1. The levels of GFP-Tat and HDAC6 were examined by immunoblotting. *C*, HEK293 cells were transfected with pEGFPC1-Tat-WT or pEGFPC1-Tat-K28A in the absence or presence of tubacin. Cell lysates and anti-GFP immunoprecipitates (*IP*) were immunoblotted (*IB*) with the indicated antibodies.

as a Tat deacetylase. Tat thus joins a growing list of HDAC6 substrates and represents the first exogenous pathogenic protein identified to be deacetylated by HDAC6.

Lys-28 in Tat is essential for its transactivation activity, and Lys-28 acetylation by PCAF stimulates HIV-1 transcription by promoting Tat-CycT1-TAR complex assembly (5, 6). It has been proposed that a balance between Lys-28 acetylation and deacetylation exists to allow for fine-tuning of HIV-1 transcription (6). Using multiple approaches, we have shown that HDAC6 deacetylates Tat at Lys-28. Especially, inactivation of HDAC6 by its specific inhibitor tubacin markedly increased the acetylation of wild-type Tat but not the K28A mutant. Unlike trichostatin A, the pan-inhibitor of class I/II HDACs, tubacin enhanced Tat acetylation and transactivation activity without altering Tat expression. Our results thus help solve the long-standing ambiguity as to whether Tat acetylation is under the control of a class I or II HDAC.

Despite the primary location and function of Tat in the nucleus, our data show that Tat interacts with and is deacetylated by HDAC6 in the cytoplasm. At present, the source of cytoplasmic Lys-28-acetylated Tat is unclear. Our data reveal that Lys-28-acetylated Tat undergoes nucleocytoplasmic trafficking similarly to unacetylated Tat (supplemental Fig. S1). Therefore, it is possible that cytoplasmic Lys-28-acetylated Tat may derive from the export of Lys-28-acetylated Tat from the nucleus. It is also possible that the acetyltransferase PCAF may simply acetylate Tat at Lys-28 in the cytoplasm, in a manner similar to its acetylation of Tat in the nucleus. This notion is supported by the recent observation that PCAF localizes in both the nucleus and the cytoplasm (15). Additional studies are warranted to examine the source of cytoplasmic Lys-28-acetylated Tat and to elucidate how the nucleocytoplasmic trafficking pattern of Lys-28-acetylated and unacetylated forms of Tat affects its transactivation activity.

Our results show that microtubules are crucial for HDAC6 to interact with Tat and for the subsequent actions of HDAC6 to deacetylate Tat and to suppress HIV-1 transactivation. It should be noted, however, that the binding of microtubules/tubulin to Tat does not entirely mirror the binding of HDAC6 to Tat. Whereas the binding of microtubules/tubulin involves primarily the core domain of Tat, the binding of HDAC6 involves more the cysteine-rich domain, with an overlap of both binding domains at residue 36 and possibly residue 37. Although the precise mechanism of how microtubules are involved in the Tat-HDAC6 interaction remains to be investigated, our results suggest a model for a scaffolding role of microtubules. In this model, the outer surface of microtubules may function as a platform to facilitate the interaction between HDAC6 and Tat. One protein, either HDAC6 or Tat, may acquire conformational changes upon binding to microtubules, generating a binding pocket for the other protein.

This study reveals that HDAC6-mediated Tat deacetylation leads to an inhibition of Tat transactivation activity toward HIV-1 transcription. However, the connection between HDAC6 and HIV-1 is not unprecedented. HDAC6 has been reported to inhibit HIV-1 entry into cells by suppressing the viral envelope-mediated cell fusion process (24). Those findings, together with ours, suggest HDAC6 as a potential therapeutic target for HIV-1 management. The identification of agents such as chemical compounds or small peptides that are able to enhance HDAC6 expression or activity would be instrumental in the design of HDAC6-based therapeutics against HIV-1.

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