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Inhibition of SIRT1 by HIV-1 viral protein Tat results in activation of p53 pathway

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Dedicated to Professor Robert C. Gallo on his 75th birthday as a token of our respect for his discoveries in the field of human retroviruses.

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ABSTRACT

Human immunodeficiency virus-1 (HIV-1) disease is characterized by a relentless decline in $CD4^+$ T cells, resulting in the development of AIDS. Extracellular Tat secreted from the HIV-1 infected cells, enters non-infected T cells to induce apoptosis. A number of mechanisms, none of which is mutually exclusive, have been attributed to the cell depletion property of Tat protein. In the present communication, we provide evidence that the cell-killing effect of Tat is mediated by the activation of p53 pathway via inhibition of SIRT1, an NAD^+-dependent deacetylase belonging to class III histone deacetylases. This evidence is based on the following experimental facts reported herein: (1) Overexpression of Tat protein decreases both the deacetylase and promoter activity of SIRT1, (2) SIRT1 inhibition by Tat involves increased levels of acetylated p53 and (3) The activation of p53 leads to subsequent increases in the expression of p53 target genes, *p21* and *BAX*.

1. Introduction

Transactivation of transcription (Tat) is a multifunctional protein encoded by the human immunodeficiency virus type 1 (HIV-1). Transactivation of the HIV-1 long terminal repeat promoter by the Tat protein is essential for both viral gene expression and virus replication [1]. Tat protein, released by the infected cells, enters non-infected cells and disrupts many host immune functions by activating a wide variety of genes regulated by specific viral and endogenous cellular promoters [2,3]. The HIV–Tat protein, in addition to transactivating HIV–LTR and other genes, potently stimulates T cells, infected and non-infected, to undergo apoptosis [4–6]. T-cell depletion by HIV-1 Tat protein has been attributed to a number of mechanisms, none of which are mutually exclusive, and for each of which there is experimental evidence [7]. The p53 pathway is regulated by SIRT1, a deacetylase requiring NAD^+ for its functional activity [8–10]. Inhibition of SIRT1 induces p53 activity in several cell lines leading to enhanced cell killing [11–13].

The recent observation that Tat protein binds to the active center of deacetylase domain of SIRT1 leading to inhibition of its enzymatic activity [14] has motivated us to look for the triangular relationship between Tat protein, SIRT1 and the tumor suppressor protein p53. In the current report, we provide evidence that among the other mechanism reported earlier [7,15–17], Tat mediated inhibition of SIRT1 results in the functional activation of p53 which might be responsible for the T-cell depletion in HIV-1 pathogenesis. Thus, SIRT1 provides a potential novel therapeutic target for disrupting the functions of Tat in the progression of HIV disease.

2. Materials and methods

2.1. Cell lines

HEK293T cells and HeLa $CD4^+$ cells were cultured in Dulbecco's modified Eagle's medium, high glucose 4.5 g/L, without L-Glu (PAA) supplemented with 10% fetal calf serum; 1% L-glutamine, 1% penicillin and 1% streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were split every 2 or 3 days 1:4 or 1:5 depending on the density. HeLa $CD4^+$ cells originally called as TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. (no. 8129) [22,23]. This is a genetically engineered HeLa cell clone that expresses CD4 and contains a Tat-responsive reporter gene for β-galactosidase under the control of an HIV-1 long terminal repeat.

2.2. Transient transfection, plasmids and reporter gene assay

Transient transfection was performed as mentioned previously [35]. The plasmid constructs WWP-Luc ($p21^{WAF1}$ promoter)

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#16451 and pTA-Luc SIRT1 promoter (-202) #10971 and pcDNA3 Tat HA (P#29) #14654 were purchased from ADDGENE. The plasmid construct pIRES2–DsRed2–Tat was kindly provided by James Foulke, Institute of Human Virology, Maryland University School of Medicine, Baltimore, USA. As transfection control pcDNA3.1 vector or pIRES2–DsRed2, were added to achieve the total amount of plasmid if less than 200 ng of Tat plasmid (96 well format for reporter gene assay), less than 1600 ng of Tat (24 well format for RNA isolation and total cell lysate) or less than 1000 ng of Tat (12 well format for RNA isolation and total cell lysate), were transfected. Reporter gene assays were performed as described previously [35].

2.3. Western blotting and antibodies

Western blotting was performed as described previously [35]. Rabbit polyclonal antibody to p53, rabbit polyclonal antibody to acetyl-p53-K382 and rabbit polyclonal antibody to SIRT1 were obtained from Cell Signaling.

2.4. Quantitative real-time PCR

Total RNA was reverse transcribed as described earlier [35]. The relative mRNA levels of target genes were measured as triplicates using SYBR Green Master Mix from Applied Biosystems and the oligonucleotide primers were purchased from (Qiagen) with following sequences: β-actin-sense, 5-TTCCTGGGCATGGAGTC-3 and β-actin-antisense, 5-AGGTCTTTGCGGATGTC-3; p21-sense, 5-ATGTGTCCTGGTTCCCGTCCT-3 and p21-antisense, 5-CATTGTGGGAGGAGCTGTGA-3; BAX sense 5-GTGTCT CAAGCGCATCGGGGAC-3 and BAX-antisense 5-GAGGAGTCTCACCCAACCACCCTG-3. Data were normalized to the β-actin signal using the ΔΔ Ct method and results are shown as mean ± standard error.

2.5. SIRT1 activity assays

Assay was performed with Cyclex SIR2 assay kit, MBL #CY-1151 according to manufacturer's protocol.

2.6. Statistical analysis

A two-tailed, unpaired student's *t*-test was applied to the replicates. Results were considered to be significant, if $p < 0.05$. Results were labeled with * if $p < 0.05$ and with ** if $p < 0.01$.

3. Results

3.1. HIV-1 Tat decreases both SIRT1 deacetylase and SIRT1 promoter activity

Recently, Kwon et al [14] have demonstrated that binding of HIV-1 Tat protein to SIRT1 inhibits its deacetylase activity, resulting in an increased acetylation of NF-κB leading to overexpression of NF-κB responsive genes. We asked whether Tat, in addition to inhibition of enzymatic activity of SIRT1, could regulate SIRT1 expression at transcriptional level. Therefore, we performed assays to check both, the SIRT1 deacetylase activity and SIRT1 promoter activity, in the presence, or absence of Tat. Similar to the findings of Kwon et al [14], we also observed that recombinant Tat protein inhibits the ability of SIRT1 to deacetylate the acetylated peptide in a dose-dependent manner (Fig. 1A). Interestingly, we observed that Tat toxoid [18], a chemically modified Tat without transactivating activity behaved in the similar way as Tat in inhibiting the functional activity of SIRT1 (data not shown). This indicates that the sequence required for the transactivation potential of Tat has no relevance to its inhibitory effect on SIRT1. Further, luciferase assay, performed using the promoter of SIRT1 in presence of varying concentrations of Tat, revealed a clear dose-dependent decrease in the SIRT1 promoter activity (Fig. 1B). These results suggest that HIV-1 Tat protein targets SIRT1 (1) by inhibiting the deacetylase activity of SIRT1 and (2) by inhibiting the promoter activity of SIRT1.

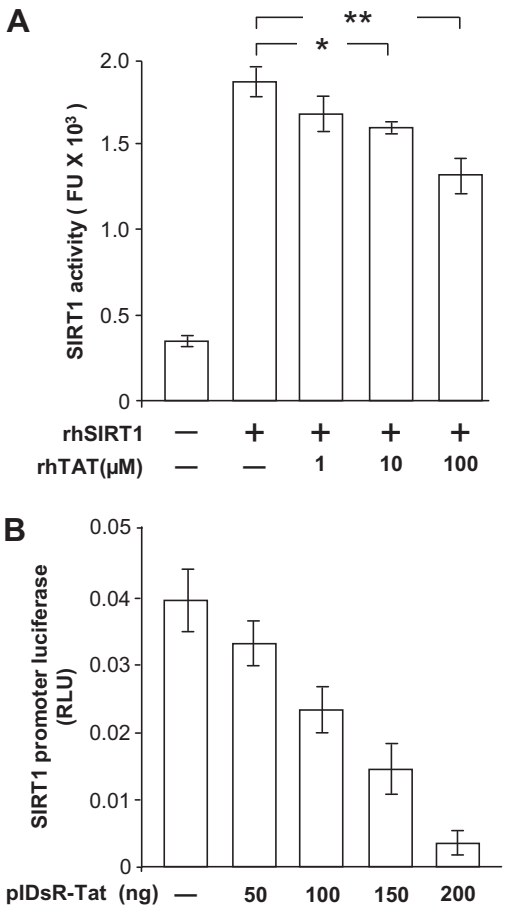

Fig. 1. HIV-1 Tat protein decreases both, deacetylase and the promoter activity of SIRT1. (A) Recombinant human SIRT1 and recombinant human Tat protein were added to HEK293T cells at indicated concentrations. After 24 h, cells were lysed and SIRT1 activity was measured. (B) Reporter gene assay was performed after HEK 293T cells were transfected with the indicated amounts of pIRES2–DsRed2–Tat plasmid (pIDsR–Tat) and with 50 ng of a SIRT1 promoter luciferase construct.

3.2. HIV-1 Tat decreases the SIRT1 mRNA and protein levels in HEK293T cells

Next we asked whether the decrease in SIRT1 promoter activity is associated with the decrease in mRNA and protein levels of SIRT1. To address this question, we overexpressed Tat in HEK293T cells and measured the mRNA and protein levels of SIRT1 in these cells. Indeed, we observed a dose dependent decrease in mRNA (Fig. 2A) and protein expression (Fig. 2B) of SIRT1 in HEK293T cells. Recently, it has been shown that Tat possesses the ability to directly inhibit NAMPT [19], an enzyme which is involved in the biosynthesis of NAD^+. Since NAD^+ is an essential cofactor required by SIRT1 to mediate its deacetylation reaction, inhibition of NAMPT by Tat can indirectly inhibit SIRT1 enzymatic activity. To find out whether Tat mediated inhibition of NAMPT contributes to the inhibition of SIRT1 deacetylase activity in HEK293T cells, we overexpressed Tat in HEK293T cells and measured the mRNA and protein levels of NAMPT in these cells. Similar to the previous report [19], we also found that overexpression of Tat reduced protein levels of NAMPT (data not shown).

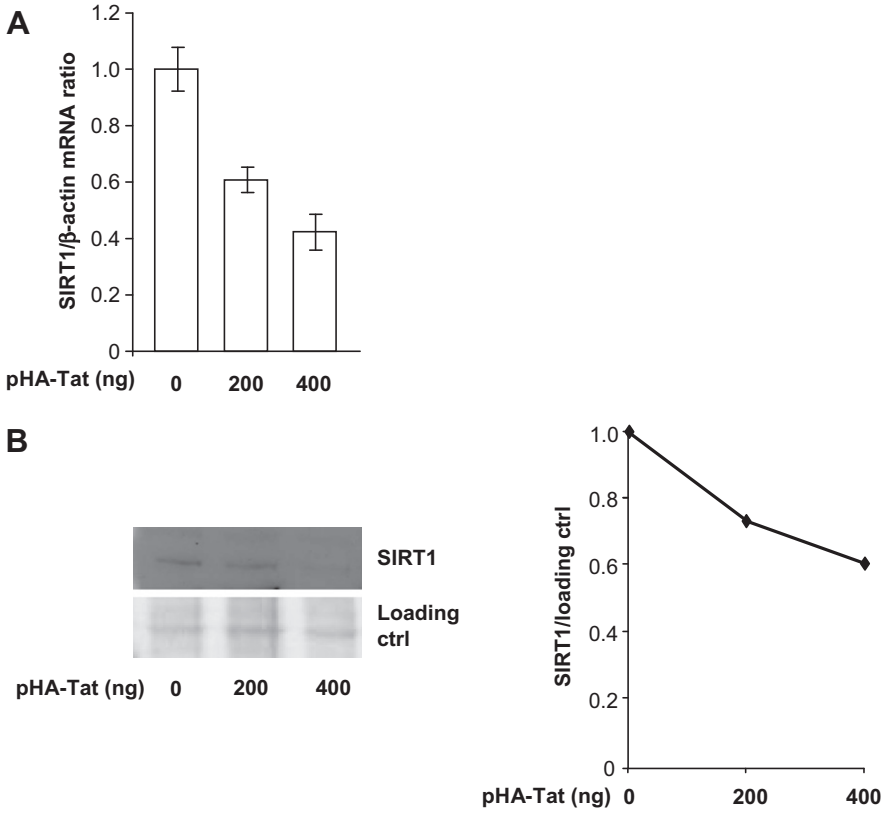

Fig. 2. Overexpression of HIV-1 Tat protein resulted in decrease in both the mRNA and protein levels of SIRT1. HEK 293T cells were transfected with the indicated amounts of pcDNA3 Tat HA (pHA–Tat) plasmid. (A) After RNA isolation the mRNA expression of SIRT1 was quantified with qRT PCR in untransfected control cells compared to Tat transfected cells. (B) Total cell lysates of transfected HEK 293T cells were applied to western blot using SIRT1 specific antibody (left). Quantification of the SIRT1 protein levels was accomplished by densitometric analysis of SIRT1 specific bands (right).

3.3. Inhibition of SIRT1 by HIV-1 Tat protein results in increased acetylation of p53 and activation of p53 target genes

From the previous findings [14,19] and our current results, it is quite clear that Tat imposes inhibitory effect on SIRT1. During the early phase of HIV-1 infection, Tat transactivates NF-κB dependent host genes essential for the survival of the virus [14]. However, in the late phase of the infection, Tat protein released by infected cells can enter non-infected cells and induce apoptosis, a mechanism important in the relentless depletion of the non-infected cells [20]. Tat has been shown to induce apoptosis also in HEK293T cells [21]. Since SIRT1 regulates a wide range of cellular processes, Tat mediated inhibition of SIRT1 can influence the global range of cellular targets. The tumor suppressor protein p53 is one of the major targets of SIRT1 [9]. Therefore, we asked whether inhibition of SIRT1 by Tat influences the acetylation levels of p53 protein. Indeed, we found that overexpression of Tat in HEK293T cells increases the acetylation levels of p53 at the lysine 382 residue, with a slight increase in the total amount of p53 (Fig. 3A). This suggests that Tat mediated inhibition of SIRT1 increases the acetylation of p53, which in turn partially increases the stability of p53 protein. We next checked whether Tat-mediated increased acetylation of p53 increases the functional ability of p53 to activate expression of *p21* and *BAX*, well-known target genes of p53. We observed that mRNA levels of both p21 (Fig. 3B) and BAX (Fig. 3C) were significantly increased in HEK293T cells overexpressing Tat. Further, we performed *p21* promoter luciferase assay to measure the influence of Tat on the ability of p53 to transcriptionally activate *p21*. We found that the promoter activity of p21 was significantly increased in presence of Tat when compared to mock transfected cells (Fig. 3D).

3.4. HIV-1 Tat inhibits SIRT1 and activates p53 acetylation in HeLa-CD4⁺ cells

Next, we took advantage of genetically engineered HeLa cell clone that expresses CD4 and contains a Tat-responsive reporter gene for β-galactosidase under the control of an HIV-1 long terminal repeat, and asked whether Tat/SIRT1 mediated activation of p53 is a relevant pathway in these cells [22,23]. Similar to the findings in HEK293T cells, we observed that overexpression of Tat in HeLa-CD4⁺ cells decreased the *SIRT1* promoter activity (Fig. 4A) and increased the *p21* promoter activity (Fig. 4B). Further in presence of Tat, the acetylation of p53 at lysine 382 was increased in HeLa-CD4⁺ cells, and similar to HEK293T cells the total amount of p53 was increased (Fig. 4C, left). Since the level of basal acetylated p53 was relatively low and required higher exposure time, we performed densitometry analysis of the acetylated p53 and normalized it to the loading control (Fig. 4C, right). Further, we could demonstrate in HeLa-CD4⁺ cells that the Tat-mediated inhibition of SIRT1 involves increased expression of p53 target genes, *p21* (Fig. 4D) and *BAX* (Fig. 4E).

4. Discussion

Tat is involved in almost every stage of the HIV-1 life cycle and its interaction with various critical host proteins makes this job possible [14,24–26]. The recent finding that Tat mediated inhibition of SIRT1 activity plays a critical role in the hyper activation of virus infected cells [14], further underscores the importance of Tat protein and its interaction with host proteins factors in the progression of AIDS. The knowledge on the interaction between Tat and SIRT1 opens treatment options for designing therapeutic

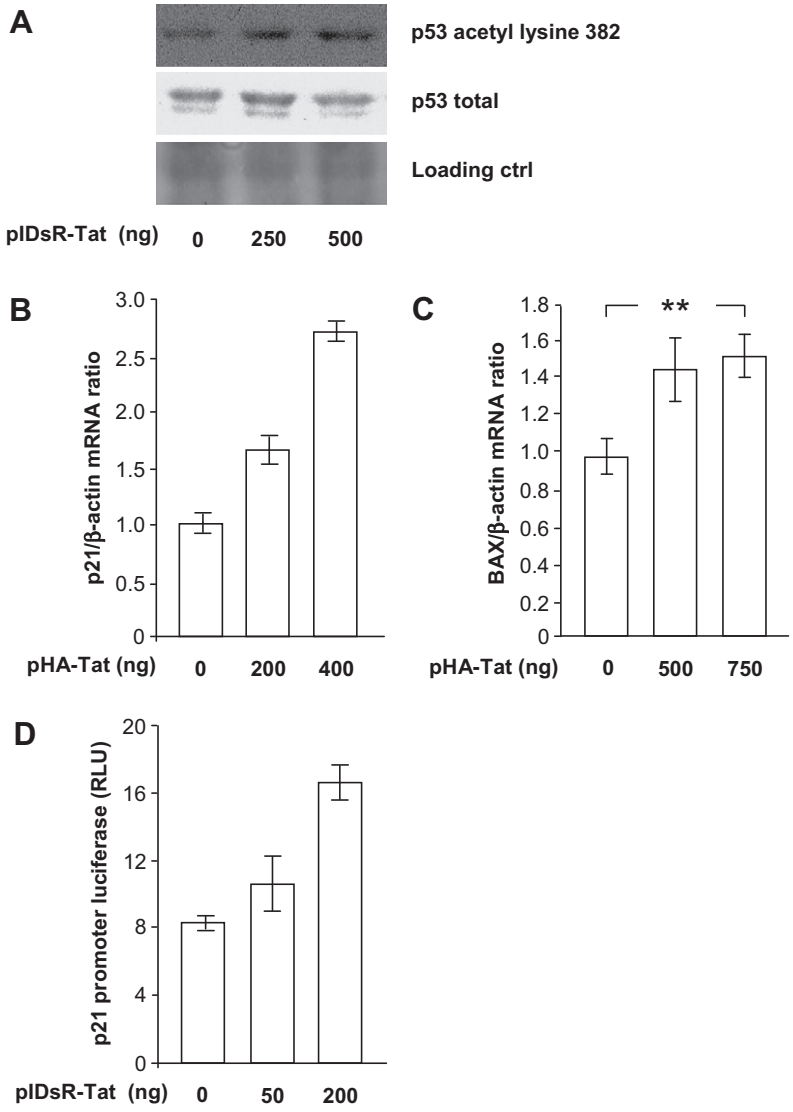

Fig. 3. HIV-1 Tat mediated inhibition of SIRT1 triggers acetylation of p53 and activates p53 target genes. HEK 293T cells were transfected with the indicated amounts of either pIRES2–DsRed2–Tat or pcDNA3 Tat HA plasmid. (A) Acetylation status of p53 lysine residue 382 and the amount of total p53 was investigated in western blot using antibodies specific for total p53 and p53 acetyl lysine 382. (B) After RNA isolation, the mRNA expression of p53 target genes $p21^{WAF1/CIP1}$ and. (C) BAX was quantified with qRT PCR. (D) Reporter gene assay was performed after HEK293T cells were transfected with the indicated amounts of pIRES2–DsRed2–Tat plasmid and with 50 ng of a $p21^{WAF1/CIP1}$-promoter luciferase construct.

agents which can specifically activate SIRT1 in HIV-1 infected cells, but it also raises an important question on the additional outcomes of Tat-SIRT1 interaction in the patients infected with HIV-1. Our current study suggests that interaction of Tat with SIRT1 plays a critical role in inducing p53-mediated cell cycle arrest and apoptotic pathway in HIV-1 infected cells. This is based on our results that Tat inhibits SIRT1 at both enzymatic and transcriptional levels, and that this inhibition of SIRT1 induces the acetylation of p53 with subsequent increases in the expression of *p21* and *BAX*.

Besides transactivation and apoptotic functions, Tat protein is also involved in cellular transformations [27,28]. Our own published results show the association of Tat in Kaposi sarcoma associated malignancies [29–31]. The interaction between Tat and tumor suppressor protein p53 is supposed to play an important role in this process of cellular transformation [32,33]. The interaction of Tat with an acetylase, p300, inhibits acetylation of p53 leading to a decrease in p53 responsive transcription [34]. It is well known that SIRT1 mediated deacetylation of p53 inhibits its ability to transactivate genes relevant in cell cycle arrest and apoptosis [8,10]. The recent finding that Tat inhibits deacetylases activity of SIRT1 [14] together with our current finding that Tat induces p53 acetylation suggest for an additional mechanism by which interaction of Tat protein with SIRT1 can mediate apoptosis. The interaction of Tat with a variety of host cellular proteins fine-tunes the function of Tat under different cellular contexts. It is quite possible that during early phases of virus infection, when virus has to propagate, the Tat protein inhibits p300 and decreases the ability of p53 to induce apoptosis. The consequence of that might be the survival of malignant sub population of cells harboring multiple mutations. During the late phase of infection, which in most HIV cases is associated with depletion of HIV-1 infected and non-infected cells, Tat mediated inhibition of SIRT1 plays a critical role in inducing p53-dependent apoptosis.

Since SIRT1 subjects several cellular proteins to deacetylation, the inhibition of its activity by Tat can have global influence on the acetylation profiles of wide range of cellular proteins involved in various cellular functions. Identification and characterization of these proteins regulated by Tat–SIRT1 interaction is an interesting topic of future investigation. We have recently shown that the acetylation of FOXO3a is regulated by NAMPT/SIRT1 pathway

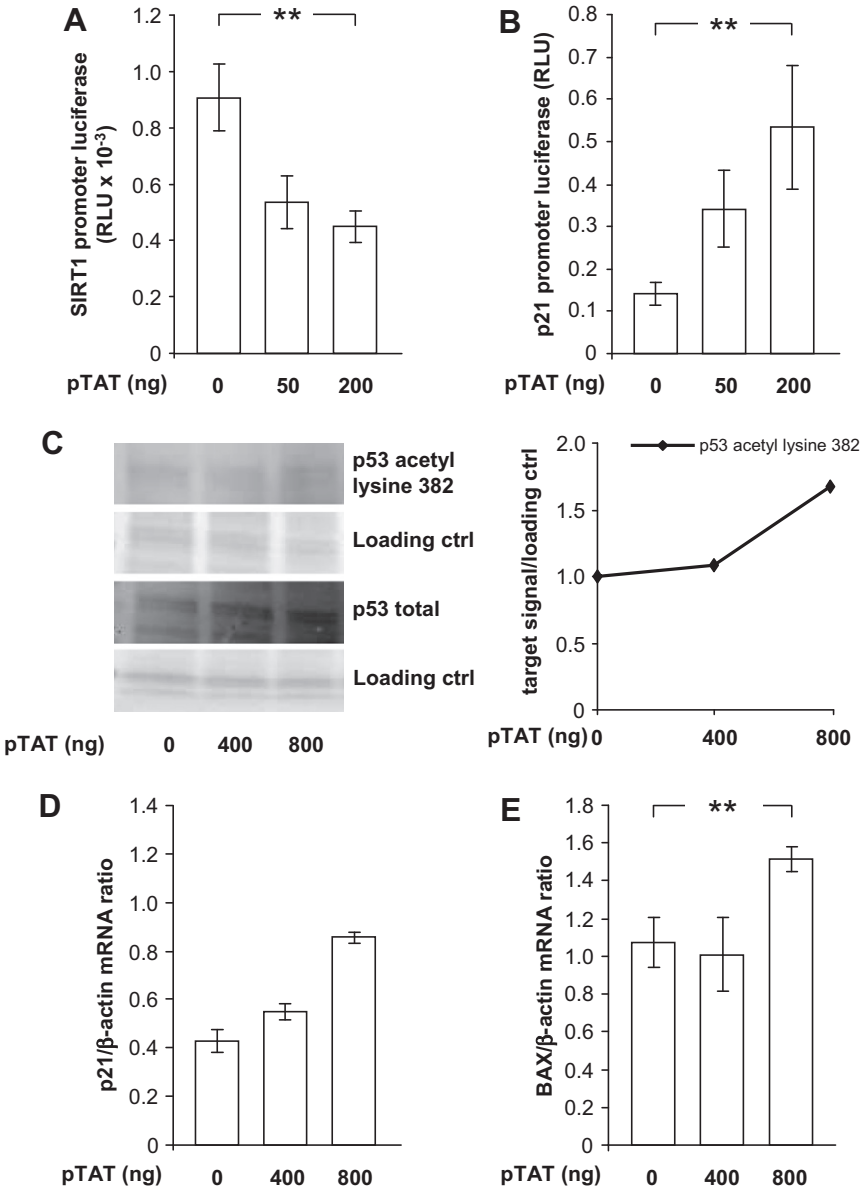

Fig. 4. HIV-1 Tat inhibits SIRT1 and activates p53 acetylation in HeLa CD4$^+$ cells. HeLa CD4$^+$ cells were transfected with the indicated amounts of pcDNA3 Tat HA plasmid. (A) After co-transfection with 50 ng of SIRT1 promoter luciferase construct reporter gene assay in pcDNA3 Tat HA transfected cells were assessed. (B) After co-transfection with 50 ng of p21$^{WAF1/CIP1}$-promoter luciferases construct with indicated amounts of pcDNA3 Tat HA plasmid reporter gene assay was performed. (C) Acetylation status of p53 lysine residue 382 and the amount of total p53 was investigated in western blot using antibodies specific for total p53 and p53 acetyl lysine 382 (left). Further quantification of the acetylation levels was accomplished by densitometric analysis of the specific bands (right). (D) After RNA isolation the mRNA expression of p53 target genes *p21$^{WAF1/CIP1}$* and (E) *BAX* was quantified with qRT PCR.

[35]. Previous findings suggest the role of FOXO3a in Tat mediated apoptosis of CD4$^+$ T cells [36]. Since both, p53 and FOXO3a are known to induce apoptosis in target cells it will be interesting to find whether acetylation of FOXO3a plays a role in Tat–FOXO3a-apoptosis axis in HIV-1 infected cells.

The involvement of Tat in a wide range of cellular processes raises an interesting question that how a single viral protein is capable to mediate such diverse cellular functions. This can be explained, in part, by the fact that (1) Tat protein undergoes multiple post translational modifications [37] and (2) the levels of Tat proteins might change during the early and late phases of viral infection [38]. Earlier report suggests that acetylation of Tat leads to different cellular fate under similar context of HIV-1 infection [14,24]. On the one hand, Tat transactivates HIV-1 transcription [24], but on the other hand when acetylated, Tat inhibits SIRT1 deacetylase activity, which further inhibits transcription by blocking the interaction between SIRT1 and NF-κB [14]. Our results, showing that the Tat-mediated inhibition of SIRT1 activates the function of p53, further adds complexity to the functions of Tat. Because p53 plays a key role in regulating cell cycle and apoptosis, it will be interesting to study the flux of Tat–p53 interaction during the course of HIV-1 infection. Further studies to correlate the anti-Tat IgG serology in HIV-1 infected patients [18,30,39] with the interaction between Tat and SIRT1 are in progress. Again the change in the concentration of Tat protein might determine the fate of infected cells towards transformation or apoptosis. It has been proposed that Tat–p53 heterodimer complex attenuates the ability of p53 to efficiently bind to its target DNA, which could favor cellular transformation [40]. Alternatively, Tat mediated p53 dimer formation could in turn favors the fate of the infected cell towards apoptosis. It would be interesting to study the role played by SIRT1 in the functional switch regulated by Tat–p53 circuit.

The previous findings that Tat is involved in malignancies associated with AIDS, taken together with our finding that Tat increases the functional activity of p53, make it quite tricky to target HIV-1. Since p53 is a strong tumor suppressor, pharmacological inhibition of p53 pathway to prevent apoptosis of HIV-1 infected and non-infected cells, might lead to malignant transformation of cells, harboring Tat protein. We speculate that partial inhibition of p53 might overcome the apoptosis associated with Tat mediated activation of p53, but can still maintain the basal activity of p53 to prevent malignant cellular transformation. Alternatively, partial activation of SIRT1 might attenuate the Tat mediated activation of p53 activity by inducing its deacetylation. In conclusion, the data presented here offer options to design new therapeutic approaches to overcome Tat mediated apoptotic response in HIV-1 patients.

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