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Supplemental Data

Human Immunodeficiency Virus Type 1 Tat Protein Inhibits the SIRT1 Deacetylase and Induces T Cell Hyperactivation

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Supplemental Experimental Procedures

Infection with lentiviral vectors

Each lentiviral vector (LTR-GFP, LTR-Tat-GFP, EF1α-GFP, and EF1α-Tat-GFP) was cotransfected into 293T cells together with a packaging construct (pCMV-R8.91) that provides all HIV genes required for production of infective particles and a plasmid encoding the vesicular stomatitis virus envelope G protein (VSV-G) to produce pseudotyped viral particles with broad host range and high infectivity. Viral supernatant containing 1500 ng of p24 was used to infect 18×10^6 Jurkat T cells. Infections were carried out in 6-well plates at 2400 rpm in a Beckman-Coulter centrifuge for 2 h at 32°C in the presence of polybrene (1 μg/ml, Sigma). Jurkat T cells (10^6) were stimulated with plate bound α-CD3 (3 μg/ml) and soluble α-CD28 (1 μg/ml) antibodies 36 h after infection as previously described (Ott et al., 1998) or were preincubated with nicotinamide (10 mM, Sigma) for 1 h before treatment with α-CD3/28 antibodies. Tat expression was visualized by western blotting with polyclonal α-FLAG and α-β-actin (Sigma) antibodies.

Knockdown of SIRT1 in Jurkat T cells

Pooled siRNAs directed against the human *SIRT1* gene (2 μg) and a control siRNA pool (Dharmacon) were introduced into Jurkat T cells (5×10^6) by nucleofection (Nucleofector Kit R; program O28; AMAXA Biosystems). 48 h after nucleofection, expression levels of endogenous SIRT1 protein were examined by western blotting using α-SIRT1 antibody. At this time, cells (10^6) were stimulated with plate-bound α-CD3 (3 μg/ml) and soluble α-CD28 (1 μg /ml) antibodies for 2 h. Total RNA was isolated and processed for real-time RT-PCR analysis.

Generation of SIRT1- and Tat-expressing MEF cell lines

To obtain recombinant virus, 10 μg of each construct (MSCV-SIRT1-puromycin and MSCV-Tat-zeocin) or empty control vectors were transfected into BOSC23 cells, a retroviral packaging cell line derived from 293 cells. The supernatants were collected 48 h after transfection and filtered through a 0.45-μm membrane. SIRT1-/- MEF cells (2×10^5) were incubated with 2 ml of the supernatant containing SIRT1-expressing retrovirus

or control virus together with polybrene (8 μg/ml, Sigma). Cells were selected after 48 h with puromycin (2.5 μg/ml, Invitrogen). Polyclonal puromycin-resistant MEF cells were reinfected with Tat-expressing or empty control retroviruses and selected in the presence of both puromycin (2.5 μg/ml) and zeocin (400 μg/ml, Invitrogen).

Infection of SIRT1+/+ and SIRT1-/- MEF cells with a Tat-expressing murine retrovirus

SIRT1+/+ and SIRT1-/- MEF cells (1×10^6) were incubated with 5 ml of supernatant containing Tat-expressing murine stem cell virus (MSCV)-based retroviral vectors or control viral vectors in the presence of polybrene (8 μg/ml) overnight. Cells were incubated for 48 h with fresh complete medium and were then selected in the presence of zeocin (400 μg/ml) for 4 days. Polyclonal zeocin-resistant populations (2×10^5) were activated with TNFα for 2 h and 8 h. Total RNA was purified and processed for real-time RT-PCR analysis.

Real-time RT-PCR

Real-time RT-PCR was performed in duplicate on the ABI PRISM 7700 thermocycler (Applied Biosystems). Human IL-2 and murine E-selectin mRNAs were quantified by QuantiTect gene expression assays (Qiagen). Murine IκBα and murine/human GAPDH mRNA concentrations were determined with SYBR Green I master mix (MCLab). Primers were designed using Primer Express software (Applied Biosystems). Mouse IκBα (NM_010907); Forward: 367 TGGCCTTCCTCAACTTCCAG, Reverse: 487 TCTCGGAGCTCAGGATCACA. Mouse GAPDH (BC095932); Forward: 204 ACTCCACTCACGGCAAATTCA, Reverse: 324 GCCTCACCCCATTTGATGTT. Human GAPDH (BC001601); Forward: 344 AGTCCACTGGCGTCTTCACC, Reverse: 464 TGGTTCACACCCATGACGAA. Gene expression was analyzed quantitatively using a comparative *C*t method (Applied Biosystems). The expression of each gene was first normalized to GAPDH expression by subtraction of the GAPDH *C*t value to the one obtained for the gene at any condition tested (Δ*C*t values). The ΔΔ*C*t was then calculated as the difference between the Δ*C*t values from stimulated (α-CD3/28 antibodies or TNFα) and nonstimulated control samples. Relative expression ratios between stimulated and nonstimulated conditions were calculated by the equation, ratio = $2^{-\Delta\Delta Ct}$. To calculate mRNA copy numbers, ten-fold serial dilutions of the IκBα cDNA were prepared at concentrations of 1.2×10^{10} to 1.2×10^3 copies per reaction and used for real-time RT-PCR analysis along with RNA samples from MEF cells. Relative copies of IκBα RNA were determined based on these standard curves. Every real-time RT-PCR analysis was performed in triplicate on at least three independent samples. Fold induction by TNFα in SIRT1+/+ and SIRT1-/- MEF cells infected with Tat-expressing retroviral vectors was calculated by the equation, ratio = $2^{-\Delta\Delta Ct}$.

Purification of recombinant SIRT1 protein

Overnight expression at 15°C in C41 (DE3) cells (Avidis) yields 6X histidine-tagged SIRT1. Cells were harvested and lysed by sonication in buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM imidazole, 10 mM β-mercaptoethanol and 0.1 mg/ml

PMSF. Lysate was centrifuged at 24,000g (RCF) for 45 min to remove cell debris. Supernatant was loaded onto Ni-NTA agarose (Qiagen) and washed with 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 30 mM imidazole, 10 mM β-mercaptoethanol and 5% glycerol. SIRT1 was then eluted with a 30 mM-600 mM imidazole gradient. Protein was further purified by Superdex 200 gel filtration to yield 80% pure full length SIRT1 in gel filtration buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 10 mM β-mercaptoethanol. Protein was concentrated to 3 mg/mL, frozen on dry ice and stored at -80°C until needed.

Fluorescent deacetylase assay

Other reaction components were 240 μM NAD^+ (Sigma) and assay buffer containing 25 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$. After 15 min, the reactions were stopped and developed with 10 mM nicotinamide (Sigma) and Fluor de Lys Developer II (BioMol). After 45 min, each well was read on a fluorescence plate reader with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. To calculate the IC_{50} value, the blank fluorescence was subtracted from the sample fluorescence, and the rate in μM/min for each Tat concentration was calculated using a standard curve. The data were then plotted in Prism with X equal to Log_{10}[Tat(μM)] and Y equal to rate. A nonlinear regression was done using the one-site competitive binding equation.

Radioactive HDAC assays

Recombinant p65 protein prepared from baculovirus-infected Sf9 insect cells (BD Biosciences) was acetylated by immunoprecipitated p300 overexpressed in 293 cells as previously described (Chen et al., 2005). Acetylated p65 protein was incubated with recombinant SIRT1 (1 μg/5U; Biomol) in SIRT1 deacetylase buffer (50 mM Tris-HCl pH 9, 4 mM $MgCl_2$, 0.2 mM DTT) in the presence of NAD^+ (1 mM; Sigma) for 3 h at 37˚C. Reactions containing synthetic Tat (0.1 μg) or nicotinamide (10 mM) were preincubated for 15 min at room temperature. Reactions were stopped by the addition of SDS loading buffer, boiled, and after brief centrifugation, analyzed by western blotting with rabbit α-AcK310 p65 (Chen et al., 2005) or mouse α-p65 antibodies (sc-8008, Santa Cruz).

Supplemental References

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Ott, M., Lovett, J.L., Mueller, L., and Verdin, E. (1998). Superinduction of IL-8 in T cells by HIV-1 Tat protein is mediated through NF-kappaB factors. J Immunol *160*, 2872-2880.

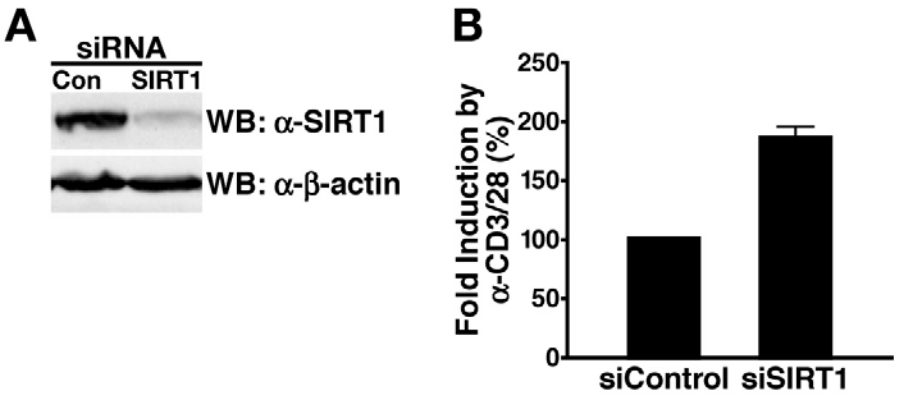

Figure S1. Knockdown of SIRT1 causes T-cell hyperactivation
(A) Western blot analysis of endogenous SIRT1 protein in Jurkat T cells transfected with siRNAs directed against SIRT1 or an irrelevant control siRNA (48 h).
(B) Real time RT-PCR analysis of IL-2 mRNA levels in Jurkat T cells after transfection with siRNAs (48 h) and activation with α-CD3/28 antibodies (2 h). Data represent the average of three independent experiments (±SEM) and are presented as fold induction by α-CD3/28 treatment relative to control siRNA-transfected cells (100%).

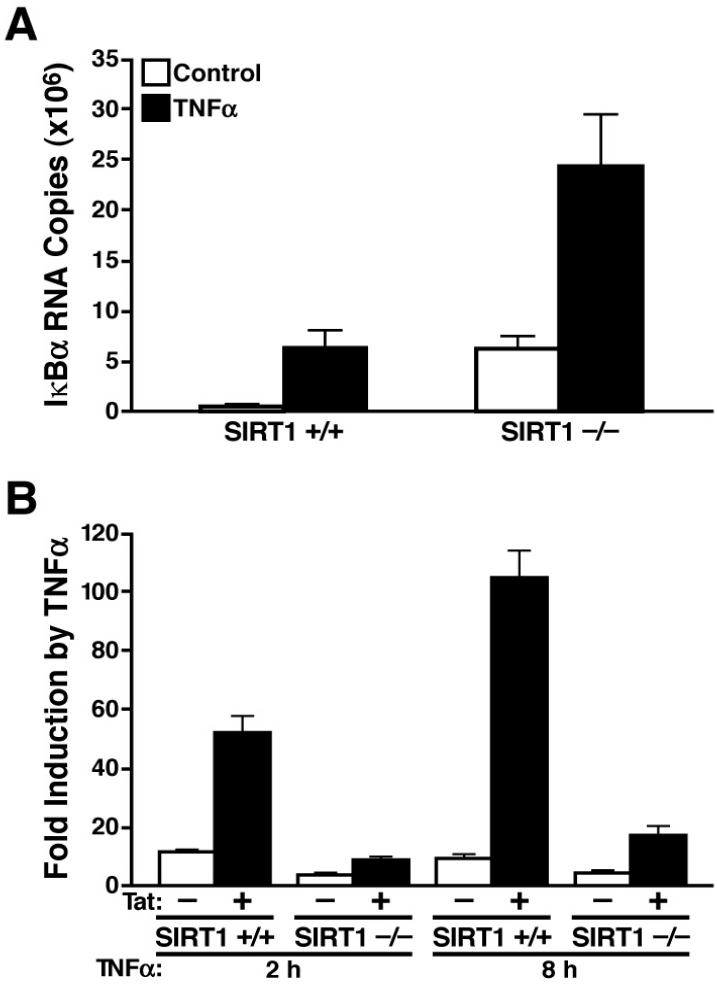

Figure S2. Analysis of IκBα gene expression in SIRT1+/+ and SIRT1 -/- MEF cells
(A) Real-time RT-PCR analysis of IκBα mRNA levels in SIRT1+/+ and SIRT1-/- MEF cells. Cells were treated with TNFα (20 ng/ml) for 2 h. Data are expressed as absolute copy numbers and represent the average of three independent experiments (± SEM). The copy numbers of IκBα mRNA in SIRT1-/- MEF cells are elevated under basal and activated conditions consistent with the model that SIRT1 suppresses expression of NF-κB-responsive genes.
(B) Real-time RT-PCR analysis of IκBα mRNA levels in SIRT1+/+ and SIRT1-/- MEF cells infected with a Tat-expressing or a control murine retrovirus. IκBα mRNA levels in the four polyclonal MEF populations were measured after incubation with TNFα for 2 h and 8 h. Data are presented as fold induction by TNFα treatment and show that Tat superinduces IκBα mRNA induction 5–11 fold in SIRT1+/+ MEFs while only a 2–3 fold increase is observed in SIRT1-/- MEFs. The average of three independent experiments (±SEM) is shown.

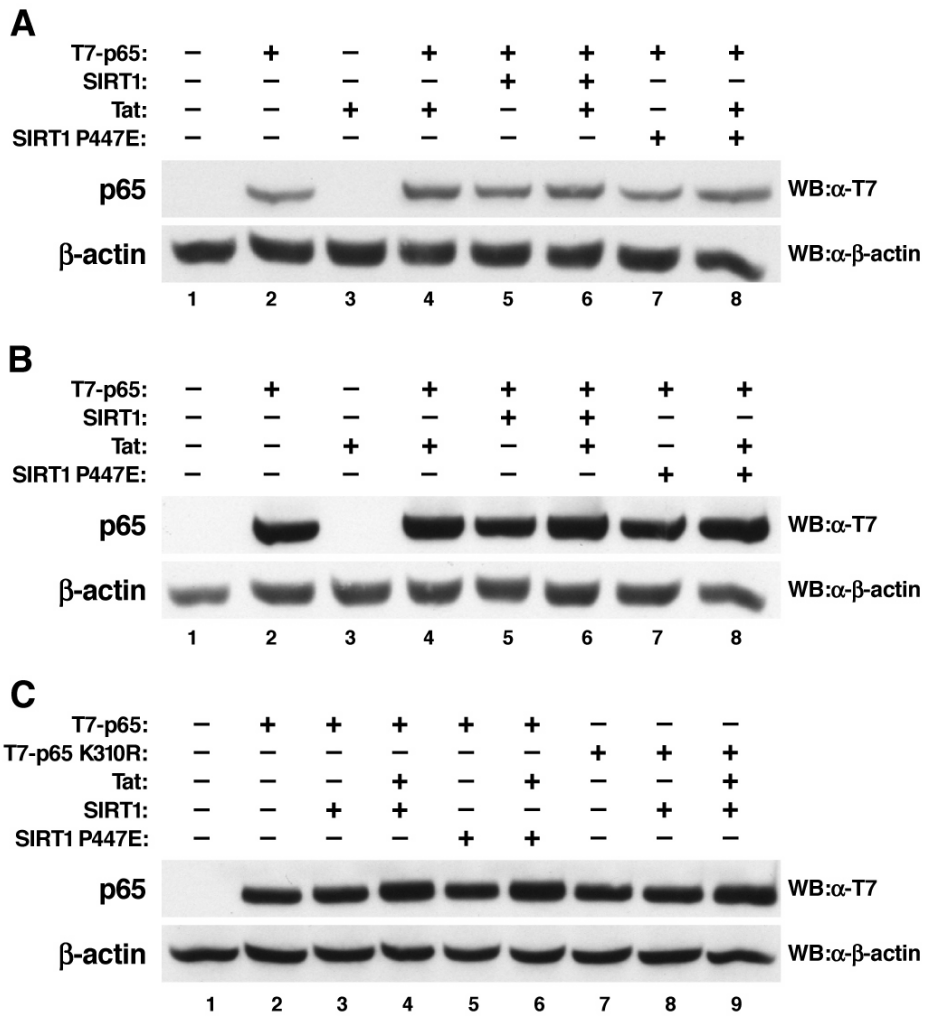

Figure S3. Equivalent expression of p65 in luciferase reporter assays

Western blot analysis of T7-p65 proteins in HeLa cells transfected with (A) the IκBα, (B) the E-selectin, and (C) the 3X-κB luciferase reporter construct together with expression constructs for SIRT1 proteins and Tat (see Figure 4). This result shows that the modulation in p65 activity observed in the presence of SIRT1 and Tat is not due to changes in p65 expression.

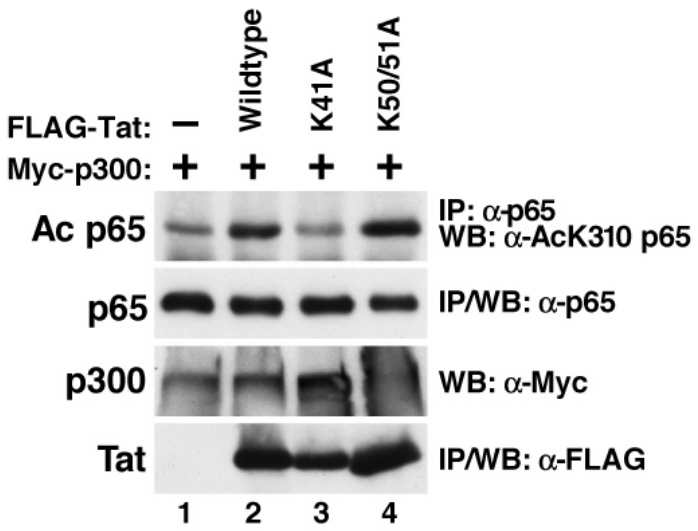

Figure S4. Induction of endogenous p65 hyperacetylation by wild type and mutant Tat.

293 cells were cotransfected with expression vectors encoding FLAG-tagged Tat (0.5 μg) and Myc-tagged p300 (10 μg) as indicated and treated with TNFα (20 ng/ml) and trichostatin (TSA; 400 nM) over night. Immunoprecipitations were performed with α-p65 or α-FLAG to isolate endogenous p65 or Tat followed by western blotting with α-AcK310 p65 and α-p65, or α-FLAG antibodies. Western blotting for p300 was performed with α-Myc antibodies. This result shows that K41A Tat fails to induce hyperacetylation of endogenous p65 protein while K50/51A Tat induces hyperacetylation as efficiently as wild type Tat.