SUPPLEMENTARY DATA

Human immunodeficiency virus-1 Tat activates NF-κB *via* physical interaction with IκB-α and p65

Giuseppe Fiume[1], Eleonora Vecchio[1], Annamaria De Laurentiis[1], Francesca Trimboli[1], Camillo Palmieri[1], Antonio Pisano[1], Cristina Falcone[1], Marilena Pontoriero[1], Annalisa Rossi[1], Annarita Scialdone[1], Francesca Fasanella Masci[1], Giuseppe Scala[1] and Ileana Quinto[1,2].

[1]Department of Experimental and Clinical Medicine, University of Catanzaro "Magna Graecia", Viale Europa-Germaneto, 88100 Catanzaro, Italy; [2]Department of Biochemistry and Medical Biotechnology, University of Naples "Federico II", Via Sergio Pansini 5, 80131 Naples, Italy.

Supplementary Materials and Methods.

Plasmids

The plasmids pRc/CMV-3xHA-p65, pRc/CMV-3xHA-p65ΔC(1-318) and pRc/CMV-3xHA-p65ΔN(122-551) were generated by PCR-amplification of p65 cDNA from pRc/CMVp65, followed by ligation to the HindIII/XbaI-digested pCMV4-3xHA (Addgene, Cambridge, MA, USA). The plasmids p3xFLAG-CMV-Tat T,N(23,24)A and p3xFLAG-CMV-Tat K(50,51)A were generated by site-directed mutagenesis of p3xFLAG-CMV-Tat. The plasmids pGEX-2T-Tat T,N(23,24)A and pGEX-2T-Tat K(50,51)A were generated by PCR-amplification of Tat nucleotide sequence from p3xFLAG-CMV-Tat T,N(23,24)A and p3xFLAG-CMV-Tat K(50,51)A, followed by ligation to the BamHI/EcoRI-digested pGEX-2T (Clontech, Mountain View, CA, USA). To generate pNL4-3.FLAG-Tat.R-E-, the FLAG-Tat nucleotide sequence was PCR-amplified from p3xFLAG-CMV-Tat

followed by ligation to the NotI/XhoI-digested pNL4-3.Luc.R-E-, which replaced the *luciferase* gene with FLAG-Tat.

Primers for PCR amplification.

PCR-amplification of p65 for cloning in pCMV4-3xHA: wild type p65 (5'-CCCAAGCTTACCATGGACGAACTGTTCCCCC-3' and 5'-GCTCT AGATTAGGAGCTGATCTGACTCAGC-3'); p65ΔC (5'- CCCAAGCTTACCATG GACGAACTGTTCCCCC-3' and 5'-GCTCTAGATTAGAAAGGACTCTTCTTC ATGAT-3'); p65ΔN (5'- CCCAAGCTTACCAAGAAGCGGGACCTGGAG-3' and 5'-GCTCTAGATTAGGAGCTGATCTGACTCAGC-3').

Site-directed mutagenesis of p3xFLAG-CMV-Tat: Tat TN(23,24)A (5'-CAGCCTAAAACTGCTTGTGCCGCTTGCTATTGTAAAAAGT GTTGC-3' and 5'-ACAAGCAGTTTTAGGCTGACTTCCTGG-3'); Tat K(50,51)A (5'-GCATCTCCTATGGCAGGGCGGCGCGGAGACAGCGAAGAGCT-3' and 5'-CCTGCCATAGGAGATGGCTAAGGCTTTTGTC-3').

PCR amplification of Tat for cloning in pGEX-2T: 5'-GGCGGATCCATGGAGCCAGTAGATCCTAG-3' and 5'-CGCGAATTCCTATT CCTTCGGGCCTGTCGG-3'.

PCR amplification of FLAG-Tat for cloning in pNL4-3.Luc.R-E-: 5'-GCGGCCGCAATGGACTACAAAGACCAT-3' and 5'-CCGGCTCGAGTCATT CCTTCGGGCCTGTCGG-3'.

Primers for Quantitative Real-Time PCR (RT-PCR)

RT-PCR of Tat and *MIP-1α* gene expression: Tat (5'-ATAGGCATAATTCGACAGAGGA-3' and 5'-TCGACCCAGATAATTGCTAAG AATC-3'); MIP-1α (5'-GCAACCAGTTCTCTGCATCA-3' and 5'-TGGCTGCTC GTCTCAAAGTA-3').

RT-PCR of CHIPs: MIP-1α NF-κB1 (5'CCCAGGGACCTATCACACAAA-3' and 5'-CCCTAAGCATGGAAAAAAATACAAA-3'); MIP-1α NF-κB2 (5'-

ATAAACGATGCTGGGTCAGG-3’ and 5’-GGTGGGTGTCAATAT GTCAGG-3’); MIP-1α NF-κB3 (5’-GCTGCCAAACATCTTGGTCT-3’ and 5’-CGTTTCGGAACCCTGTTTTTC-3’); GAPDH (5’-CCCATCACCATCTTCCAGG AG-3’ and 5’-GTTGTCATGGATGACCTTGGC-3’), ACTB (5’-GCCAGCTGCAA GCCTTGG-3’ and 5’-GCCACTGGGCCTCCATTC-3’).

Cell extracts

Whole cell extracts were obtained by incubating the cells in lysis buffer containing 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, Nonidet P-40 (NP-40) 1% and 1x Complete Protease Inhibitor (Roche Diagnostic GmbH) for 30 min on ice. Cells were centrifuged at 14,000 x *g* at 4°C for 15 min, and supernatants were collected as whole cell extracts. For nuclear and cytosolic extracts, cells ($5x10^7$) were harvested, washed in PBS twice and resuspended in a lysing buffer containing 10 mM N-2-hydroxyethylpiperazine-N’-2 sulfonic acid (HEPES) pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiotreithol (DTT) and 0.1% NP-40. Cells were lysed on ice for 2 min and checked for complete lysis by microscopy. Nuclei were centrifuged at 800 x *g* for 5 min and supernatant was collected as cytosolic extract. The nuclear pellet was washed with a buffer containing 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and lysed in a buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 0.45 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 1x Complete Protease Inhibitor; then nuclear lysates were clarified by centrifugation at 14,000 x *g* for 15 min and supernatant was collected as nuclear extract.

EMSA

NF-κB double-stranded oligonucleotide (Promega, E329A) (5 pmoles) was end-labeled with [γ-^{32}P] ATP (Perkin Elmer, Boston, MA, USA) using polynucleotide kinase T4 (Invitrogen), according to the manufacturer’s protocol. Recombinant p65 protein (100 ng; Active Motif Carlsbad, CA) was 5 min-incubated in 20 µL reaction mixture containing 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 2 µg

of poly[d(I-C)] (Roche Diagnostic GmbH) with or without *in vitro* translated FLAG-Tat and FLAG-Tat C(22, 25, 27)A (5 μL) on ice; then, 1 μL of γ-^{32}P-labeled double-stranded probe (5x10^4 cpm) was added to the mixture and the reaction was incubated at room temperature for 20 min. Competitions were performed with 1.25 up to 40-fold molar excess of unlabeled oligonucleotide. Protein/DNA complexes were resolved by electrophoresis on 6% acrylamide: bisacrylamide (30:1) gel in Tris-Borate-EDTA. Gel was dried and analysed by autoradiography. Competition of the p65 DNA-binding with cold NF-κB oligonucleotide was evaluated by densitometry of EMSA using NIH ImageJ software. The dissociation constant (Kd) of the p65/p65-DNA complex was measured as the concentration of cold competitor that halved the p65 DNA binding activity, using PRISM4-based statistical analysis (GraphPad Software Inc, La Jolla, CA, USA).

In vitro Translation

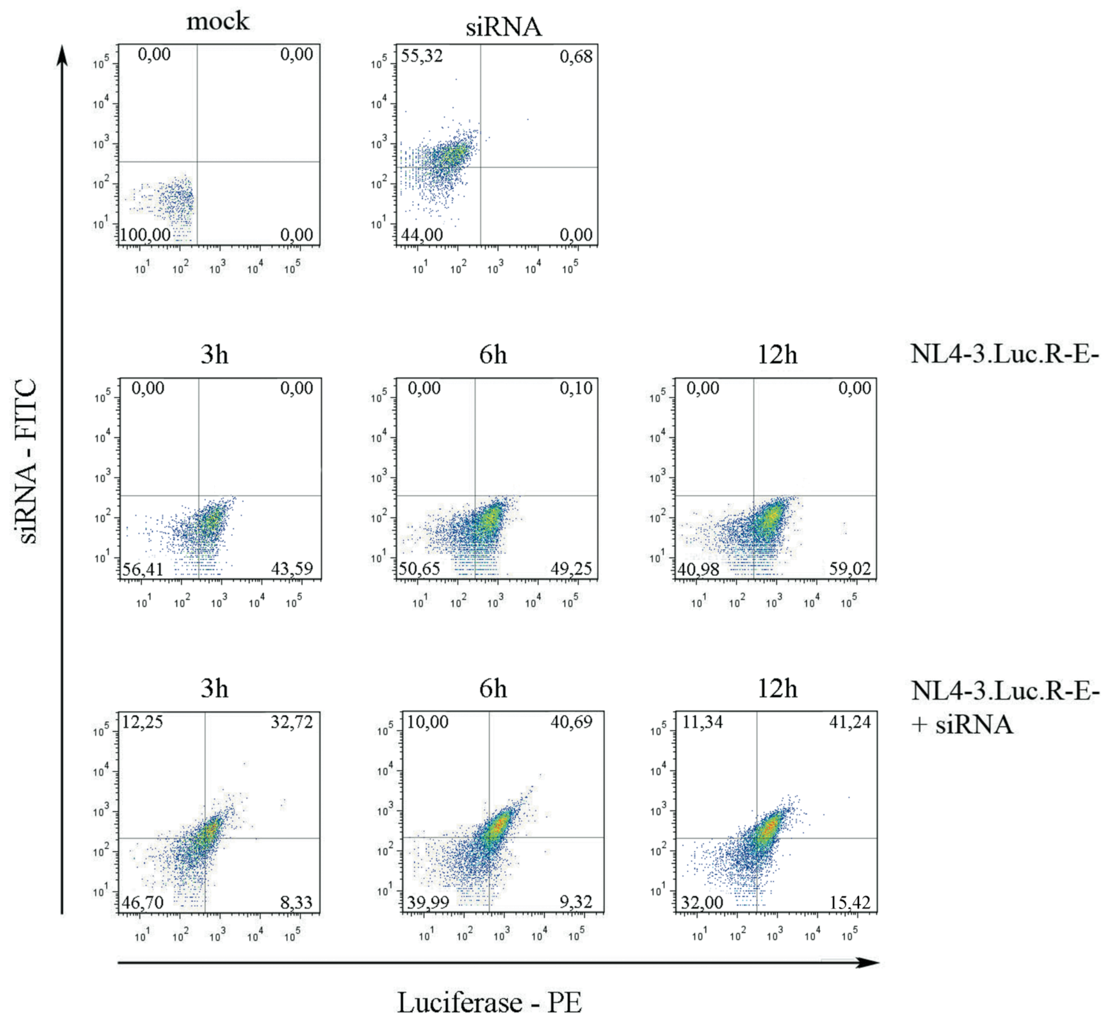
HA-IκB-α, p65 and [^{35}S]methionine-labelled p65 were expressed under the T7 promoter using pcDNA-HA-IκB-α and pRc/CMV-p65 as templates, and *in vitro* translated using the TnT Quick Coupled Transcription/Translation System (Promega), according to the manufacturer's protocol. Wild type and mutants Tat under the T7 promoter were PCR-amplified from the plasmids p3xFLAG-CMV-Tat, p3xFLAG-CMV-Tat T,N(23,24)A, p3XFLAG-CMV-Tat K(50,51)A, p3xFLAG-CMV-Tat R(49-57)A and p3xFLAG-CMV-Tat C(22, 25, 27)A using the primers 5'T7-FLAG, 5'-CGCCGGTAATACGACT CACTATAGGGACGCCACCATGGACTACAAAGACCATGAC-3' and 3'TAT, 5'-GCTCTAGACTATTCCTTCGGGCCTGTCG-3'; the PCR products were *in vitro* translated with the TnT Quick Coupled Transcription/Translation System (Promega).

Immunoprecipitation Assay and GST pull down

For immunoprecipitation, antibodies (5 μg) were incubated with 20 μL protein G-Sepharose (GE Healthcare) in 500 μL of modified RIPA buffer (20 mM Tris-HCl pH

7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM PMSF, 1x Complete Protease Inhibitor) overnight at 4°C on a rocking platform. Protein G-Sepharose-coupled antibodies were then incubated with whole cell extract (1 mg) or aliquots of *in vitro* translated proteins in 800 μL of RIPA buffer overnight at 4°C on a rocking platform. Immunocomplexes were collected by centrifugation at 700 x *g* for 5 min at 4°C, washed in RIPA buffer and resuspended in SDS gel loading buffer. Proteins were separated on 12% SDS-PAGE, transferred to PVDF membrane, and analysed by immunoblotting with antibodies or autoradiography.

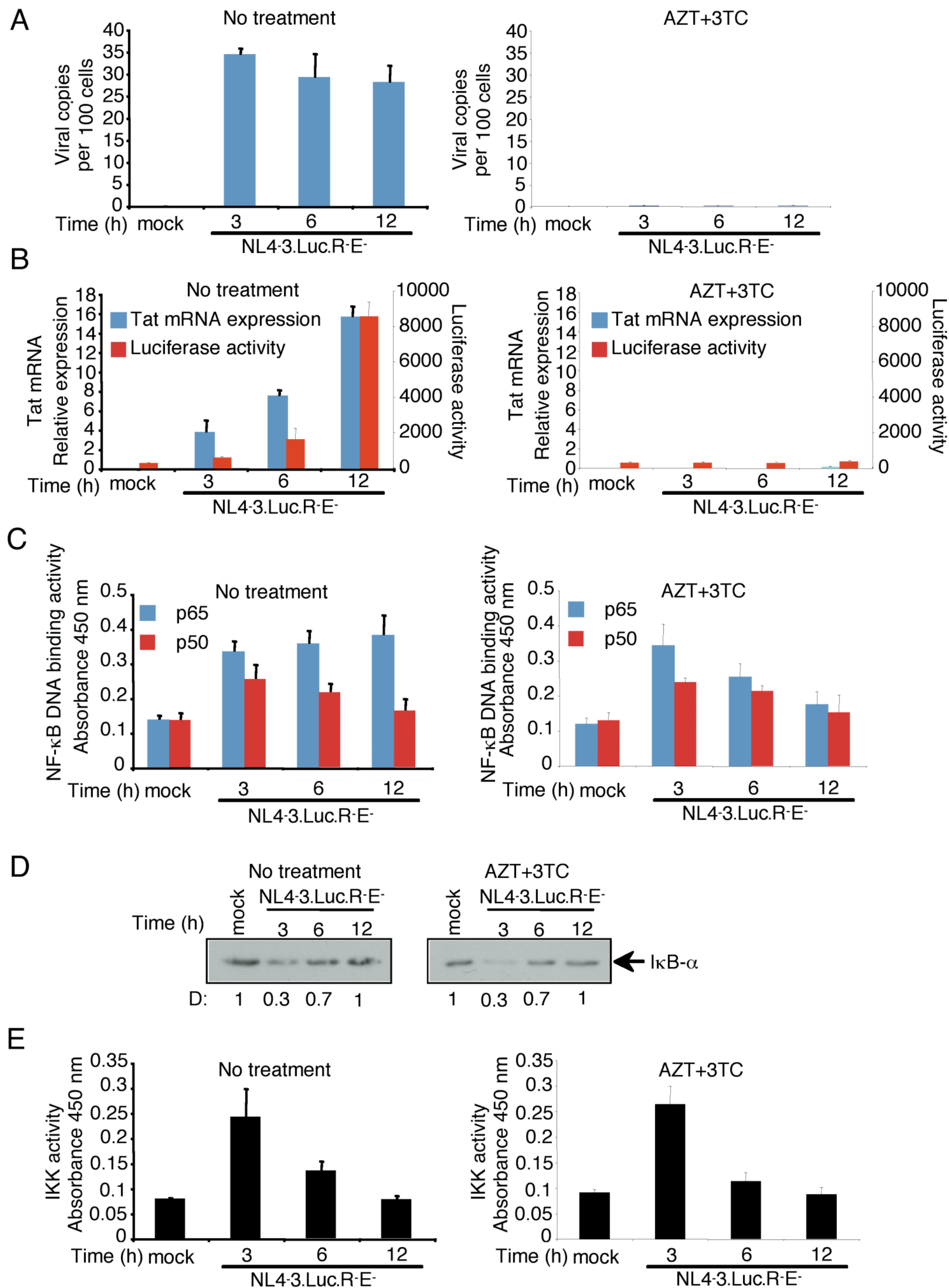
For GST-pull down, GST proteins (5–10 μg) were conjugated with Glutathione-Sepharose (30 μL) in 500 μL of modified RIPA buffer for 2 hr at 4°C, and collected by centrifugation at 700 x *g* for 5 min at 4 °C. Glutathione-Sepharose-conjugated GST proteins were incubated with *in vitro* translated p65 (5 μL) in 500 μL of modified RIPA buffer for 3 h at 4 °C on a rocking platform. Protein complexes were collected by centrifugation at 700 x *g* for 5 min at 4 °C, washed in modified RIPA buffer, and resuspended in loading buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 1% bromphenol blue, 10% β-mercaptoethanol, 25% glycerol). Proteins were resolved on 12% SDS-PAGE and analyzed by immunoblotting with anti-GST (B-14) or anti-p65 (C-20) purchased from Santa Cruz Biotechnology.



Supplementary Figure 1.

Jurkat cells ($5x10^6$) were transfected with a siRNA-FITC (Dharmacon) (200 picomoles), or left untrasfected. Twenty-four h post-transfection, cells were infected with NL4-3.Luc.R^-E^- virions (500 ng of p24). After infection, cells were collected at the indicated time by centrifugation (600 x *g,* 5 min) at 4°C, and fixed in fixation solution (Cytofix-Cytoperm kit, BD Biosciences) for 5 min. Cells were washed with permeabilization solution (Cytofix-Cytoperm kit), and incubated with anti-luciferase mouse antibody (Santa Crutz Biotechnology) (1:25) for 1 h. After washing in PBS, cells were incubated with phycoerythrin (PE)-labeled secondary anti-mouse antibody (Molecular Probes, Invitrogen) (1:1000) for 30 min. FITC- and PE-relative fluorescence intensities were measured with FACSCalibur™ dual-laser Flow

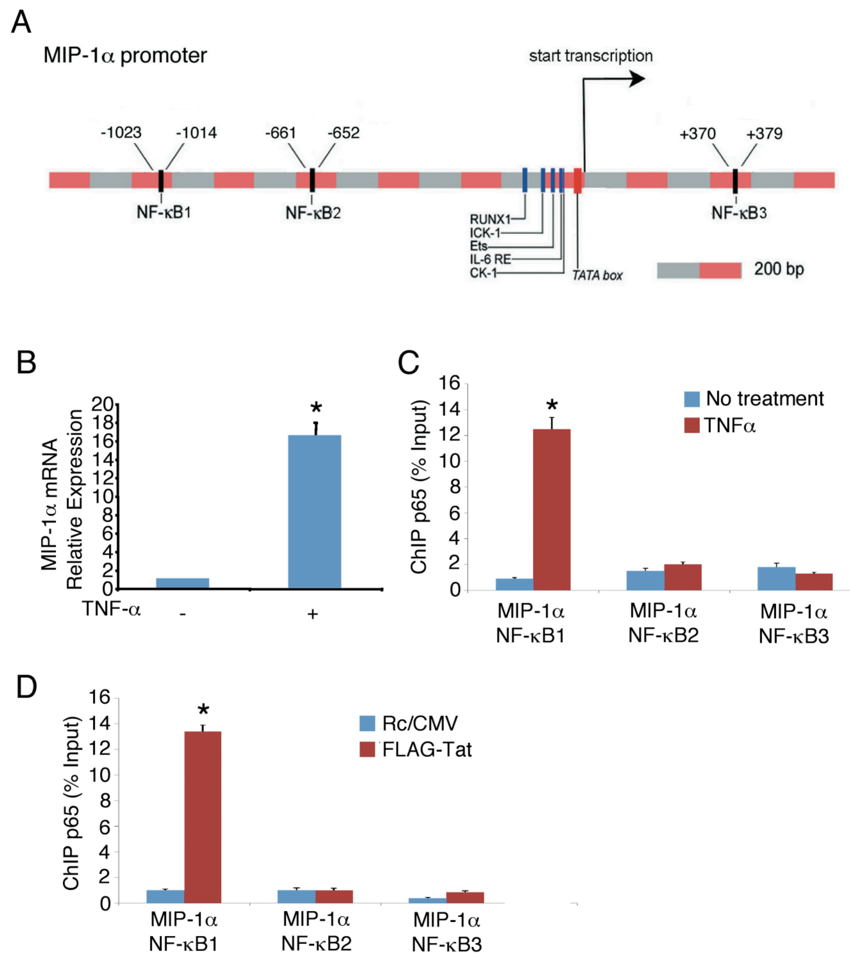
Cytometer (BD Bioscience) and analysed using FlowJo 7.6 software (FlowJo TreeStar).



Supplementary Figure 2

PBMCs ($1x10^7$) were infected with NL4-3.Luc.R$^-$E$^-$ virions (500 ng of p24) or left

uninfected. At 1 hour post-infection, cells were treated with or without AZT (1 μM; Sigma-Aldrich) and 3TC (20 μM; Sigma-Aldrich), harvested at the indicated time, and washed extensively with PBS. (**A**) Real-Time PCR of genomic DNA measured the HIV-1 integration. Viral DNA was normalized to cellular genomic GAPDH. Primers were as follows: MH531, 5'-TGTGTGCCCGTCTGTTGTGT-3' and MH532, 5'-GAGTCCTGCGTCGAGAGA GC-3', for HIV-1; GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and GAPDH reverse, 5'-GAAGATGGTGATGGGATTTC-3', for GAPDH. (**B**) Real-Time PCR of Tat in total RNA, and luciferase activity in cell extracts measured the viral expression. (**C**) The NF-κB DNA binding activity was measured in nuclear extracts (20 μg) using the NF-κB Transcription factor ELISA assay kit (Cayman). (**D**) IκB-α was detected in cytosolic extracts (30 μg) upon 12% SDS-PAGE and western blotting with anti-IκB-α (Santa Cruz Biotechnology); densitometry (D) of blots was performed with NIH ImageJ Software. (**E**) IKK activity was measured in cytosolic extracts (100 μg) using the HTScan IKK kinase assay kit (Cell Signaling). Values (mean ± SE, n = 3) are shown.



Supplementary Figure 3

(**A**) Schematic representation of the *MIP-1α* promoter. The nucleotide positions of three putative NF-κB enhancers are indicated, as identified by Jaspar-based analysis. (**B**) HeLa cells ($5x10^6$) were treated with TNF-α (20 ng/mL), or left untreated, for 30 min. Total RNA was extracted and analysed for *MIP-1α* expression by Real-Time PCR. (**C**) HeLa cells ($5x10^6$) were treated with TNF-α (20 ng/mL), or left untreated, for 30 min. Chromatin was immunoprecipitated with anti-p65 or IgG (Santa Cruz Biotechnology); ChIPs eluates were analysed by Real-Time PCR using appropriate primers for the three putative NF-κB enhancers of *MIP-1α*. (**D**) HeLa cells ($5x10^6$) were transfected with p3xFLAG-Tat, or p3xFLAG empty vector (10 µg). Forty-eight h later, chromatin was immunoprecipitated with anti-p65 or IgG (Santa Cruz Biotechnology). CHIPs eluates were analysed by Real-Time PCR using appropriate primers for the three putative NF-κB enhancers of *MIP-1α*. ChIP values were

normalized to input DNA for each sample, and reported as % of Input over the rabbit IgG control. Values (mean ± SE, n = 3) are shown. The asterisks indicate statistically significant differences ($p < 0.05$) as compared to untreated cells, or empty vector.