

Evaluation of the interactions of HIV-1 integrase with small ubiquitin-like modifiers and their conjugation enzyme Ubc9

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Abstract. Human immunodeficiency virus type 1 (HIV-1) integrase mediates the integration of reverse-transcribed viral cDNA into the genome of the host for the stable maintenance of the viral genome and the persistence of HIV-1 infection. In this study, the relationships between HIV-1 integrase (HIV-1 IN) and three SUMO conjugation pathway proteins, as well as the effects of these associations, were investigated. The overexpression of SUMO1/SUMO2 and Ubc9 changed the intracellular localization of HIV-1 IN from a diffuse distribution to a punctate localization. SUMO1, SUMO2 and Ubc9 were shown to interact with HIV-1 IN. The SUMOylation of HIV-1 IN was verified. In addition, SUMO1, SUMO2 and Ubc9 were shown to influence the integration of both lentivirus and HIV-1. The overexpression of Ubc9 inhibited viral genome integration, and the upregulation of SUMO1 or SUMO2 enhanced the inhibitory effect of Ubc9. Knockdown of the endogenous levels of SUMO1, SUMO2 and Ubc9 increased the level of viral integration, while reverse transcription and the nuclear import of preintegration complex (PIC) were not affected. Our findings suggest that SUMO conjugation pathway proteins may act as cellular restriction factors and be detrimental to HIV-1 infection. These findings merit further investigation because of their potentially significant implications for the cellular antiviral response to HIV-1 infection.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of acquired immune deficiency syndrome. The HIV-1 genome encodes only nine genes, three structural genes and six

accessory genes. Because of its limited genetic capacity, the virus requires many cellular proteins and cellular pathways to complete its life cycle. Recent functional genome-scale RNAi screening and computational analysis have identified more than 300 host co-factors that are critical for HIV-1 replication in human cells (1-3). However, cells have evolved strategies to impede HIV-1 replication, and several innate cellular restriction factors have been found that may target a number of steps in the virus's replication cycle (4,5). This research suggests that intricate 'strike-counterstrike' protein interactions between the virus and the host cell govern the ultimate outcome of HIV-1 infection.

An essential step in the replication of HIV-1 is the integration of reverse-transcribed viral cDNA into the chromosome of the host cell. The key protein responsible for the integration process is the 32 kDa viral integrase. The catalytic function of the integrase is essential for the stable maintenance of the viral genome and the persistence of HIV-1 infection. For these reasons, the integrase has been the target of intensive pharmacological research (6). The integration event is a complex process that is aided by an ever-expanding list of cellular proteins (7). Intriguingly, few innate cellular restriction factors that target the integration process and restrict viral replication have been reported.

The small ubiquitin-related modifiers are small polypeptides of approximately 8-11 kDa that were identified as reversible post-translational protein modifiers. They are covalently linked as 93-97 amino acid polypeptides to specific lysine residues of various intracellular proteins (8,9). The process of SUMOylation is analogous to ubiquitin modification and occurs in three steps that are catalyzed by enzymatic machinery including the SUMO-activating enzyme E1, the conjugating E2 enzyme Ubc9 and various SUMO E3 ligases (10). SUMOylation regulates the function of the substrates mainly by altering their intracellular localization or protein-protein interactions or by affecting their ability to undergo other types of post-translational modifications. These changes, in turn, affect nuclear trafficking, gene expression, genomic stability, chromosomal integrity and signal transduction (11).

In the present study, we report that the overexpression of SUMO1/SUMO2 and Ubc9 changes the intracellular localization of HIV-1 integrase (HIV-1 IN). We also identified

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SUMO1, SUMO2 and Ubc9 as HIV-1 IN-binding proteins and evaluated the effects of these proteins on the integration of lentivirus and HIV-1.

Materials and methods

Cell culture and transfection. 293T cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS) at 37°C in a 5% CO₂ humidified atmosphere. Plasmid DNAs or siRNAs were transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. H9 cells and HIV-1/IIIB were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (USA). H9 cells were cultured in RPMI-1640 medium with 2 mM L-glutamine (Hyclone), 10% FBS and 100 U/ml of penicillin and streptomycin at 37°C in 5% CO₂ humidified atmosphere. Electroporation method was used for H9 cells.

Plasmid construction. The open reading frames (ORFs) of SUMO1, SUMO2 and Ubc9 were amplified by PCR from a human fetal brain cDNA library (Clontech Laboratories). The ORFs were inserted into the plasmid pCMV-HA (Clontech Laboratories) to generate the three expression plasmids HA-SUMO1, HA-SUMO2 and HA-Ubc9. Two truncation mutants, HA-SUMO1ΔC6 and HA-SUMO2ΔC4, were created by PCR-based C-terminal deletion of 6 amino acids from SUMO1 and 4 amino acids from SUMO2, respectively. For yeast-mating tests, pB42AD-SUMO1, pB42AD-SUMO2 and pB42AD-Ubc9 were generated by inserting the ORFs of SUMO1, SUMO2 and Ubc9, respectively, into the pB42AD plasmid (Clontech Laboratories). The plasmids pB42AD-SUMO1ΔC6, pB42AD-SUMO1ΔC4, pB42AD-SUMO2ΔC4 and pB42AD-SUMO2ΔC2 were constructed by PCR-based C-terminal deletion as mentioned above. The bait plasmid pLexA-HIV-1 IN has been described previously (12). To generate pDsRed-SUMO1, pDsRed-SUMO2 and pDsRed-Ubc9, the ORFs of SUMO1, SUMO2 and Ubc9 were subcloned into pDsRed-C1 (Clontech Laboratories). The pEGFP-HIV-1 IN has been described previously (13).

Intracellular localization assay. 293T cells were plated on coverslips in 6-well plates and transfected with the indicated plasmids. At 24 h post-transfection, cells were visualized using an Olympus LX70 microscope. The nuclei were stained with DAPI (4',6-diamidino-2-phenylindole).

Yeast two-hybrid assay. The MatchMarker LexA two-hybrid system was purchased from Clontech Laboratories. Interactions between HIV-1 IN and SUMO1, SUMO2, Ubc9 and their derivatives were detected using pB42AD-SUMO1, pB42AD-SUMO1ΔC6, pB42AD-SUMO1ΔC4, pB42AD-SUMO2, pB42AD-SUMO2ΔC4, pB42AD-SUMO2ΔC2 and pB42AD-Ubc9-transferred EGY48 (p8opLacZ) and pLexA-HIV-1 IN-transferred YM4271 according to the standard yeast-mating protocol provided by the manufacturer.

Immunoblotting, co-immunoprecipitation and antibodies. To prepare whole cell extracts, transfected 293T cells were lysed

with ice-cold RIPA buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with PMSF, a protease inhibitor cocktail (Sigma) and N-ethylmaleimide (Sigma). Lysates were clarified by centrifugation and stored at -80°C. For co-immunoprecipitation, cell lysates were prepared in lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 0.5 mM PMSF and 0.5% protease inhibitor cocktail). The lysates were incubated with the indicated antibodies for 2 h at 4°C. Pre-washed (25 μl) protein A/G Sepharose beads (Santa Cruz Biotechnology, Inc.) were added to each extract, and the mixtures were shaken overnight. The beads were washed three times with lysis buffer and boiled in 2X loading buffer. Protein samples were then separated on SDS-PAGE and transferred to a nitrocellulose membrane; the membrane was blocked in 5% skim milk in TBST and probed with the indicated antibodies. EGFP mouse monoclonal antibodies and rabbit polyclonal anti-HA antibodies were purchased from Santa Cruz Biotechnology, Inc.; HRP-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Sigma.

Virus stock production and infectivity assay. Lentiviral particles were produced by co-transfection of 293T cells using a three-plasmid system as previously described (12). Virus-containing cell supernatants were harvested at 48 h post-transfection, clarified by low-speed centrifugation, filtered through 0.45 μm pore size filters and treated with DNase I (0.2 U/μl) for 1 h at 37°C. The viral stocks were normalized for p24^{CA} antigen content by ELISA using the HIV p24 Lentivirus Titer kit (Cell Biolabs, Inc., USA) following the manufacturer's instructions. Percentages of EGFP-expressing cells, which represented the integration rate of the virus, were determined at 2 days post-infection using a flow cytometer (FACS Vantage SE).

Replication competent HIV-1/IIIB viruses were obtained from the NIH AIDS Research and Reference Reagent Program. For transfection, 10⁷ H9 cells were electroporated with 20 μg plasmid DNA or 2 μM siRNA, using a Bio-Rad gene pulser with a voltage of 300 V, a capacitance of 250 μF (14,15). After 24 h (for transfection of plasmid DNA) or 48 h (for transfection of siRNA), cells were washed with PBS and used for infection with HIV-1/IIIB at moi of 0.01 and 0.1.

Real-time PCR assay. To measure relative levels of lentiviral and spreading HIV-1 integration, genomic DNA was quantified by Alu-LTR real-time nested PCR array using a SYBR-Green-based detection kit (Toyobo code no. QPK-201, 201T) (16). At 48 h post-infection, cellular genomic DNA was extracted with a genomic DNA purification kit (Qiagen). The primers used for the first round of PCR were 5'-GGCTAACTAGGGAACCCAC TG-3', 5'-TCCCAGCTACTGGGGAGGCTGAGG-3' and 5'-GCCTCCCAAAGTGCTGGGATTACAG-3'. After an initial denaturation at 95°C for 8 min, 12 cycles of denaturing (95°C, 30 sec), annealing (55°C, 30 sec) and extension (72°C, 170 sec) were carried out. One aliquot (1/50) of the initial PCR product was subjected to a second round of PCR amplification. The primers used were 5'-GCTAGAGATTTTCCACACT GACTAA-3' and 5'-GGCTAACTAGGGAACCCACTG-3', and a 100 bp fragment was obtained. A pair of primers for β-actin, 5'-ACGAGGCCAGAGCAAGAG-3' and 5'-TCTC CATGTCGTCCCAGTTG-3', was used as a control. Ct values

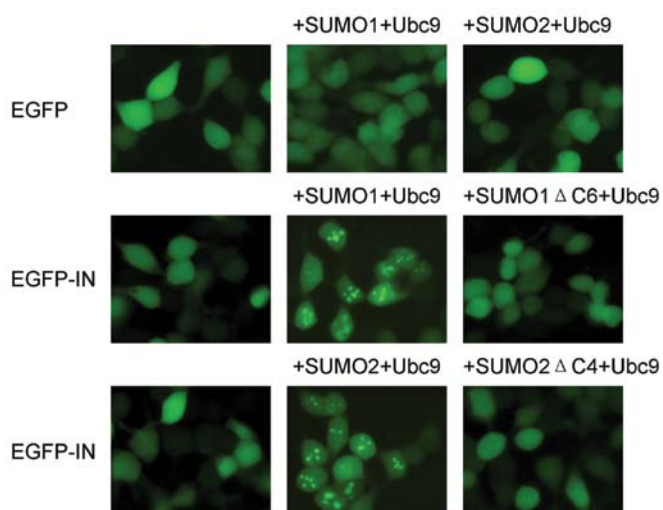


Figure 1. Intracellular localization changes of HIV-1 IN. Fluorescence microscopy analyses showing the intracellular localization of HIV-1 IN when co-expressed with SUMO1/SUMO1 Δ C6 and Ubc9 (second line) or SUMO2/SUMO2 Δ C4 and Ubc9 (third line). EGFP protein was used as the negative control (first line).

were collected, and the relative viral integration levels of the samples were calculated.

To measure relative levels of total viral cDNA synthesis and 2-LTR circle formation, total genomic DNA was extracted using the urea lysis method (17) and quantified by real-time PCR using previously described primer sets. Primers M667/M661 (18) were used to amplify full-length reverse transcripts, and primers 9600/515 (19) were used to amplify 2-LTR circles.

RNA interference. Sense sequences of siRNA duplexes specific for human SUMO1, SUMO2 and Ubc9 were 5'-CUGGGA AUGGAGGAAGAAG-3' (20), 5'-GUCAAUGAGGCAGA UCAGA-3' (21) and 5'-CAAAAAAUCCCGAUGGCAC-3' (22), respectively. A nontargeting siRNA was used as a negative control (NC). The siRNAs were synthesized by RiboBio Co., Ltd. (Guangzhou, China). The cells were used for viral infectivity assays 48 h after transfection.

Statistical analysis. Data are described using the mean and standard deviation of the mean where appropriate. Differences between the means of experimental groups were analyzed using a two-tailed Student's t-test. Differences with a p-value of ≤ 0.05 were considered significant.

Results

Overexpression of SUMOs and Ubc9 changes the intracellular localization of HIV-1 IN. To investigate the subcellular distribution of HIV-1 IN in the presence of SUMO-related proteins, pEGFP-HIV-1 IN, HA-SUMO1/SUMO2 and HA-Ubc9 were simultaneously introduced into 293T cells, and the intracellular localization of pEGFP-HIV-1 IN was observed by fluorescence microscopy. The truncated mutants SUMO1 Δ C6 and SUMO2 Δ C4, both of which lack SUMO conjugation activity, were tested in parallel. When expressed alone, the EGFP-HIV-1 IN fusion protein was diffusely

Table I. Yeast two-hybrid screening of HIV-1 IN and full-length or truncated mutants of SUMO1/SUMO2, Ubc9a.

GAL-BD fusions	GAL-AD fusions	HIV-1 IN binding
HIV-1 IN	SUMO1	+
	SUMO1 Δ C6	-
	SUMO1 Δ C4	+
	SUMO2	+
	SUMO2 Δ C4	-
	SUMO2 Δ C2	+
	Ubc9	+

+, Indicates interaction between the two proteins; -, indicates no interaction.

distributed throughout the cell (Fig. 1). However, when EGFP-HIV-1 IN was co-expressed with SUMO1/SUMO2 and Ubc9, its subcellular localization changed from diffuse to a distribution that was both diffuse and distinctly punctate. DAPI staining showed that the punctate staining associated with EGFP-HIV-1 IN was concentrated in the nuclei (data not shown). When SUMO1 Δ C6 or SUMO2 Δ C4 was co-expressed with EGFP-HIV-1 IN, EGFP-HIV-1 IN remained diffusely distributed. The EGFP-negative control showed no change in localization in the presence of SUMOs and Ubc9.

HIV-1 IN interacts with SUMO1, SUMO2 and Ubc9. The possible interactions between HIV-1 IN and SUMO-related proteins were investigated. Yeast two-hybrid assays were employed to identify SUMO-interacting proteins (23). SUMO1 Δ C4 and SUMO2 Δ C2, both of which retain the essential double glycine, were tested, and SUMO1 Δ C6 and SUMO2 Δ C4, which lack the double glycine essential for their conjugation activities, were used for comparison. The interaction between HIV-1 IN and SUMO1/SUMO2 in yeast cells depends absolutely upon the presence of the C-terminal double-glycine amino acid residues; when the conjugation-deficiency SUMO1 Δ C6 and SUMO2 Δ C4 were used, no interaction was detected (Table I). This result suggests that the interaction of HIV-1 IN with SUMO1/SUMO2 includes covalent conjugation of SUMO1/SUMO2 to integrase or, alternatively, that it requires the intact C-terminus of SUMO1/SUMO2 for protein-protein binding. Yeast-two-hybrid assays also showed robust binding of HIV-1 IN to the conjugation enzyme Ubc9.

To verify these interactions, co-immunoprecipitation assays were conducted. Human 293T cells were co-transfected with pEGFP-HIV-1 IN and HA-SUMO1/ HA-SUMO1 Δ C6, HA-SUMO2/HA-SUMO2 Δ C4 or HA-Ubc9, respectively. The rabbit anti-HA antibody precipitated EGFP-HIV-1 IN but not EGFP from extracts of these cells (Fig. 2A). The conjugation-deficiency forms of the SUMO proteins failed to co-immunoprecipitate with EGFP-HIV-1 IN (Fig. 2A).

We also examined the subcellular localizations of HIV-IN and SUMO1/SUMO2, Ubc9. The RFP-SUMO1/SUMO2 and RFP-Ubc9 fusion proteins were mainly found spread throughout the cells and were comparatively concentrated within the nucleus (Fig. 2B). EGFP-HIV-1 IN fusion protein

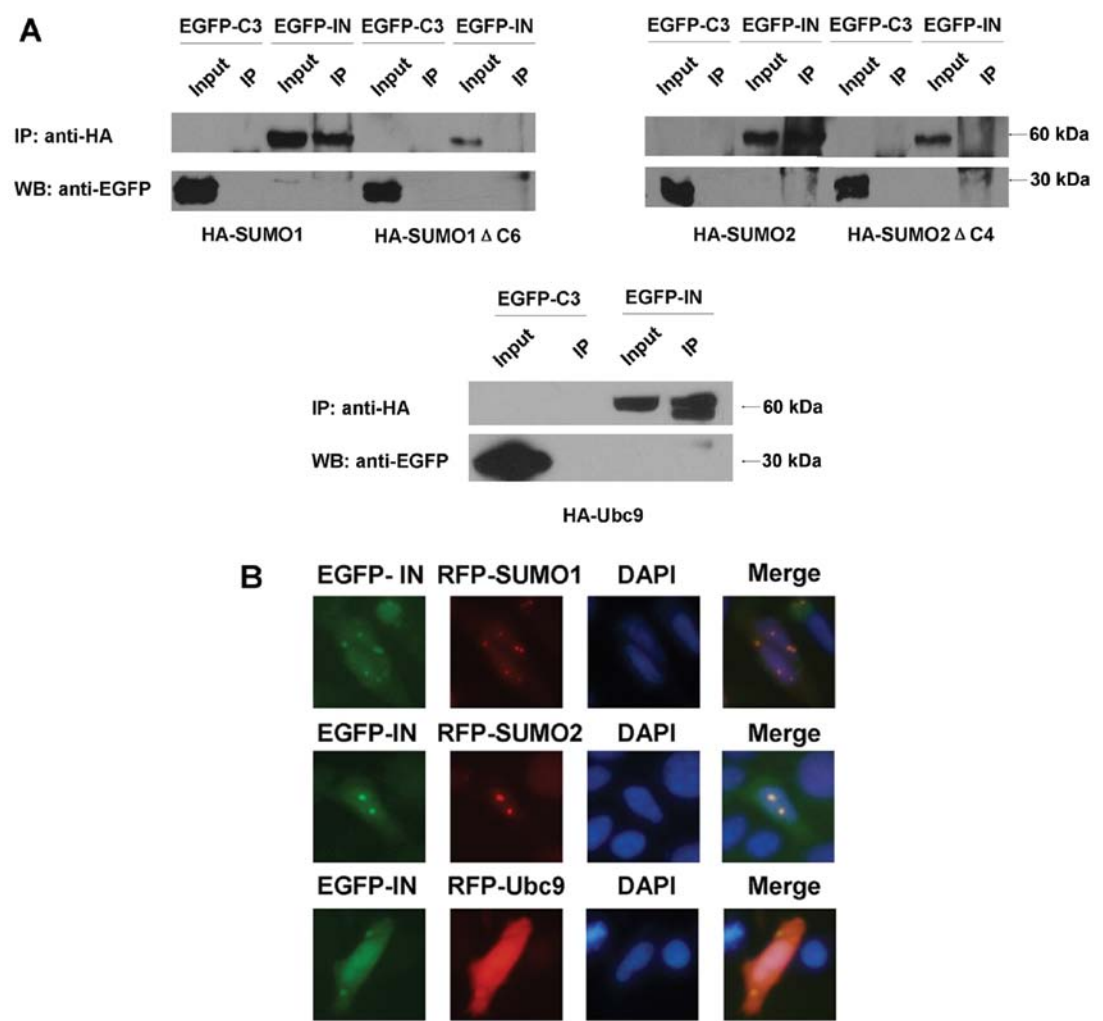


Figure 2. Interactions between HIV-1 IN and SUMO1/SUMO2, Ubc9. (A) Co-immunoprecipitation of HIV-1 IN and SUMO1/SUMO1ΔC6, SUMO2/SUMO2ΔC4, Ubc9. Cell lysates were incubated with a rabbit anti-HA antibody attached to A/G agarose beads. Samples were analyzed by immunoblotting with a mouse anti-EGFP antibody. (B) Subcellular localization of HIV-1 IN and SUMO1, SUMO2 and Ubc9. The pEGFP-HIV-1 IN and pDsRed-SUMO1/SUMO2/Ubc9 fusion protein constructs were co-transfected into 293T cells. The nuclei were stained with DAPI.

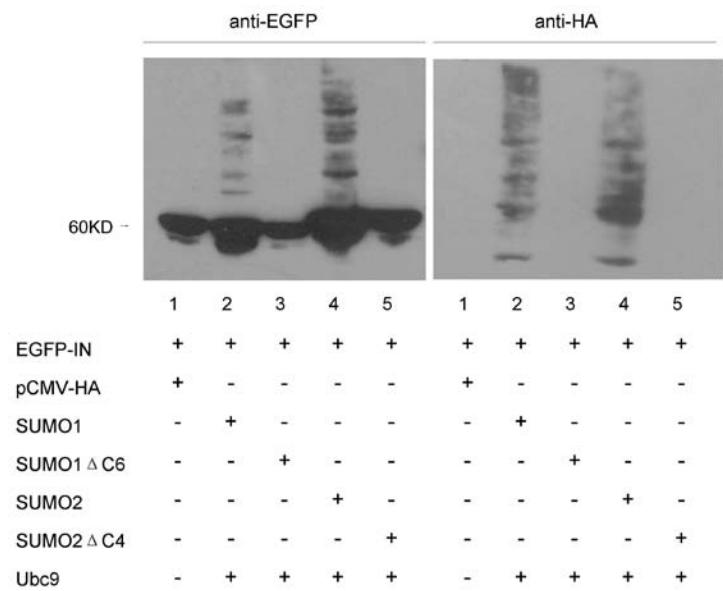


Figure 3. Covalent modification of HIV-1 IN by SUMO1 and SUMO2. Four main modified forms of EGFP-HIV-1 IN were detected above the 60 kDa primary band (lanes 2 and 4, left) in cells co-transfected with SUMO1/SUMO2 and Ubc9. The bands representing the modified forms were not detected in the absence of transfection with SUMO expression plasmids (lane 1, left) or in SUMO1ΔC6/SUMO2ΔC4 (lanes 3 and 5, left) co-transfected cells. Similar results were obtained when samples were probed with an anti-HA antibody.

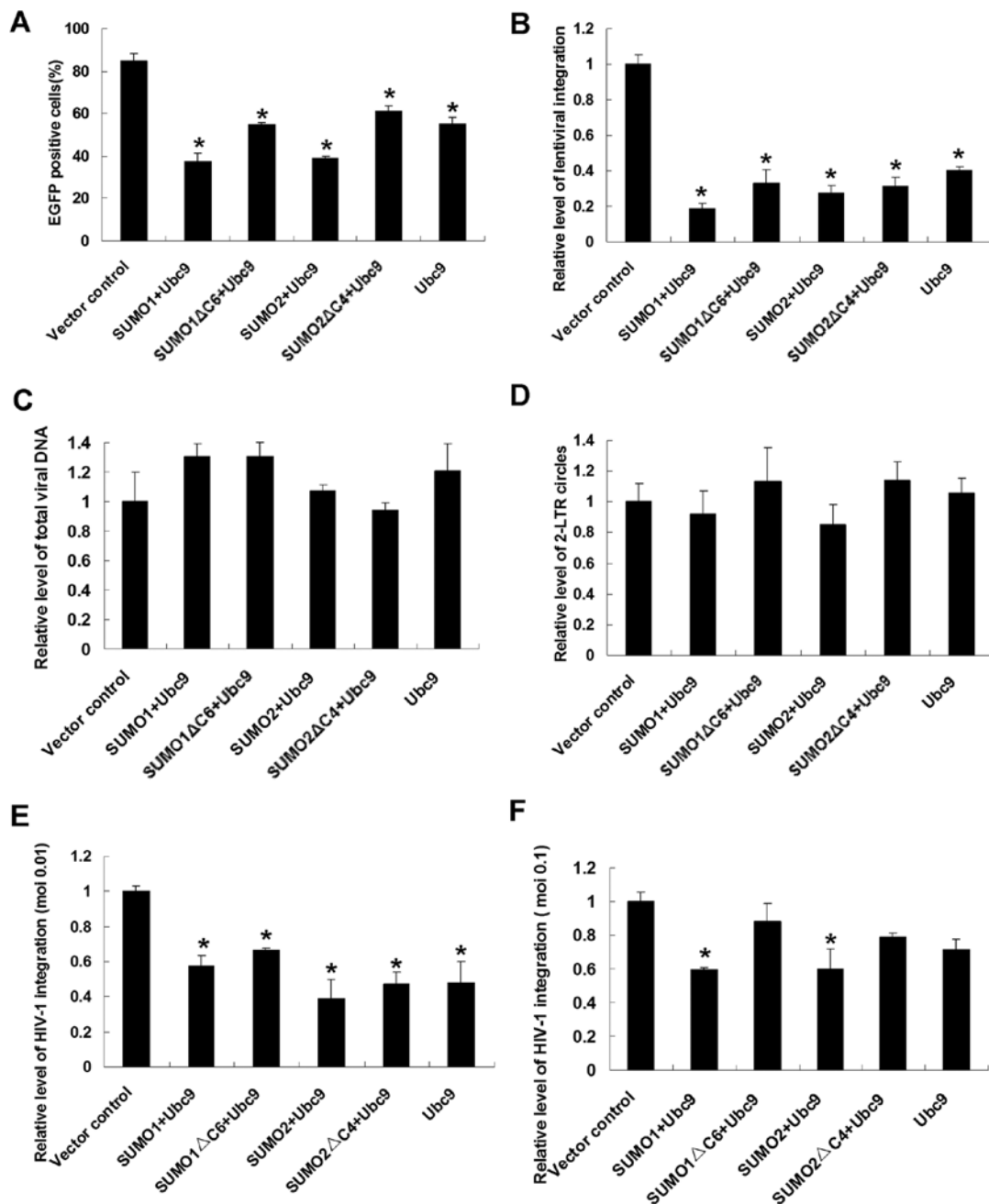


Figure 4. Upregulations of SUMO1/SUMO2 and Ubc9 inhibit lentivirus and HIV-1 integration. (A) Flow cytometry analysis of lentiviral EGFP reporter gene integration level in SUMO1/SUMO2 and Ubc9 upregulated 293T cells and control cells. (B) Alu-LTR real-time nested PCR analyses of the relative levels of lentiviral integration in SUMO1/SUMO2 and Ubc9 upregulated 293T cells and control cells. (C) Real-time PCR analyses of the relative level of total viral DNA in SUMO1/SUMO2 and Ubc9 upregulated 293T cells and control cells. (D) Real-time PCR analyses of the relative level of 2-LTR circles formation in SUMO1/SUMO2 and Ubc9 upregulated 293T cells and control cells. (E and F) Alu-LTR real-time nested PCR analyses of the relative levels of HIV-1 integration in SUMO1/SUMO2 and Ubc9 upregulated H9 cells and control cells (E) at moi of 0.01, (F) at moi of 0.1. * $p \leq 0.05$.

presented almost the same distributions as RFP-SUMO1/SUMO2 and RFP-Ubc9. These results indicated the co-localization of the corresponding proteins.

Covalent modification of HIV-1 IN by SUMO1 and SUMO2. To investigate SUMO conjugation of HIV-1 IN, 293T cells were co-transfected with pEGFP HIV-1 IN, HA-SUMO1/SUMO2 and HA-Ubc9. Forty-eight hours after transfection, cells were lysed with RIPA, proteins were electrophoretically separated on SDS-8% polyacrylamide gels and anti-EGFP antibody was used for HIV-1 IN immunoblot analysis. We detected

4 minor immunoreactive bands near the 60 kDa primary band (Fig. 3, lanes 2 and 4, left). These minor immunoreactive bands appeared to represent HIV-1 IN conjugated with various numbers of SUMO moieties. In control experiments with cells co-transfected with pEGFP-C3 and HA-SUMO1/SUMO2 or HA-Ubc9, no SUMO conjugation of EGFP was detected (data not shown). The minor immunoreactive bands were also not detected in cells that were not subjected to SUMO transfection (Fig. 3, lane 1, left) or in SUMO1ΔC6/SUMO2ΔC4 co-transfected cells (Fig. 3, lanes 3 and 5, left). When the same samples were probed with an anti-HA antibody, the four main

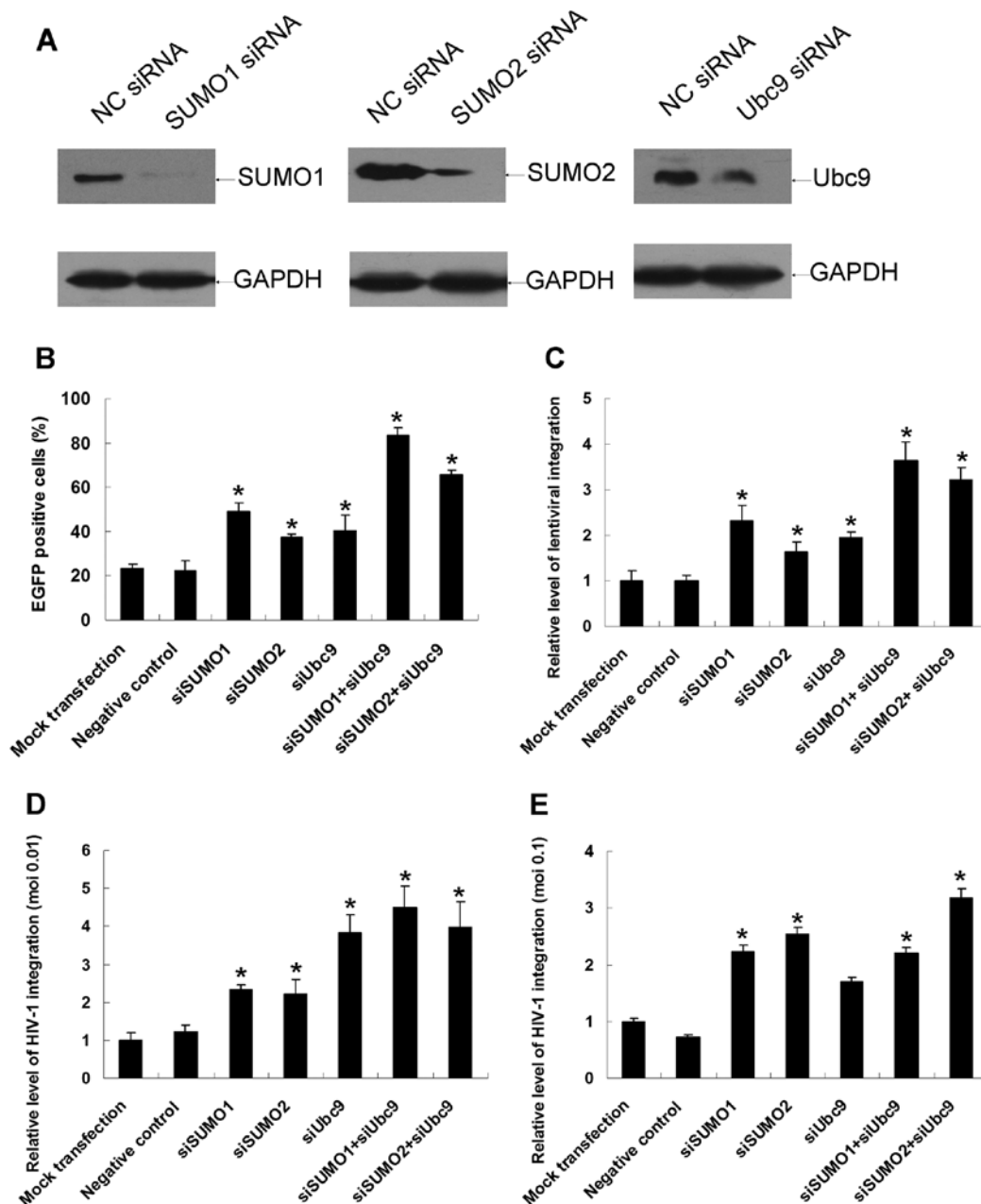


Figure 5. Downregulations of SUMO1/SUMO2 and Ubc9 restrict lentivirus and HIV-1 integration. (A) Expression of SUMO1, SUMO2 and Ubc9 were determined by western blotting analysis as described in Materials and methods. Total protein lysates were prepared 48 h after siRNA transfection. A human non-silencing duplex RNA (NC siRNA) was used as a negative control. The amount of GAPDH protein was analyzed as a loading control. (B) Flow cytometry analysis of the level of lentiviral EGFP reporter gene integration in SUMO1/SUMO2 and Ubc9 knockdown 293T cells. (C) Alu-LTR real-time nested PCR analyses of the relative level of lentiviral integration in SUMO1/SUMO2 and Ubc9 knockdown 293T cells. (D and E) Alu-LTR real-time nested PCR analyses of the relative levels of HIV-1 integration in SUMO1/SUMO2 and Ubc9 downregulated H9 cells and control cells (D) at moi of 0.01, (E) at moi of 0.1. * $p \leq 0.05$.

modified forms of HIV-1 IN were readily detected (Fig. 3, lanes 2 and 4, right). The additional bands detected with the anti-HA antibody presumably represent cellular proteins that were conjugated with HA-SUMO1/SUMO2 in the co-transfected cells. Consistent with the results of anti-EGFP antibody analysis, no bands were detected in the other three samples (Fig. 3, lanes 1, 3 and 5, right). These results affirmatively suggest that a fraction of overexpressed HIV-1 IN protein can be covalently modified by both SUMO1 and SUMO2.

Upregulations of SUMO1/SUMO2 and Ubc9 inhibit lentivirus and HIV-1 integration. Because we demonstrated that

HIV-1 IN can be covalently modified by SUMO1 and SUMO2, it was of interest to determine whether these modifications affect the fundamental function of the integrase. Firstly, an HIV-1-derived lentiviral vector system was used, and changes in the integration rate of the reporter gene (EGFP) were detected by flow cytometry. Transient transfection of prepared 293T cells with HA-SUMO1/SUMO2 and HA-Ubc9 resulted in upregulation of the cellular expression of SUMO1/SUMO2 and Ubc9. In Ubc9-overexpressing cells, there was a substantial decline in the percentage of EGFP-positive cells ($55.24 \pm 3.13\%$) compared with the vector control cell group ($84.76 \pm 3.51\%$) (Fig. 4A). Interestingly, in populations co-transfected with

HA-SUMO1 and HA-Ubc9, the percentage of EGFP-positive cells was much lower, $37.42 \pm 3.89\%$ ($p < 0.01$). This result indicates that the presence of SUMO1 and Ubc9 can inhibit the integration of lentivirus and that these two proteins exert an additive effect on lentiviral integration. When SUMO2 was co-expressed with Ubc9, the percentage of EGFP-positive cells declined to $39.02\% \pm 0.88\%$ ($p < 0.05$). However, when mutated SUMO1 or SUMO2 was co-expressed with Ubc9, the percentage of EGFP-positive cells was almost the same as in populations of Ubc9-overexpressing cells.

To confirm these results, a more precise quantitative method, Alu-LTR real-time nested PCR, was employed to verify changes in integration efficiency. The results were consistent with the results of flow cytometry analysis (Fig. 4B).

To explore the possible effects of SUMO conjugation pathway proteins on other stages of HIV-1 early events, including reverse transcription and preintegration complex (PIC) nuclear import, real-time PCR was performed to determine the relative level of total viral DNA synthesis and the formation of 2-LTR circles, which are used as a marker of PIC nuclear import (24). Similar amounts of late reverse transcripts and 2-LTR circles were detected in SUMO-overexpressing cells (Fig. 4C and D).

Having established that SUMO1/SUMO2 and Ubc9 are presented as cellular restriction factors of the lentivirus, we next addressed the effects of these SUMO pathway proteins on the infectivity of spreading HIV-1 viruses at moi of 0.01 and 0.1 and expanded our conclusion to authentic HIV-1 viruses. For this purpose, SUMO pathway proteins were overproduced in human T-lymphoid H9 cells and at 24 h post-transfection, treated cells were infected with equal amounts of replication competent HIV-1/IIIB viruses and the relative levels of virus integration were monitored by Alu-LTR real-time nested PCR. At moi of 0.01, SUMO pathway proteins overexpressed cells displayed moderate reductions in the HIV-1 integration rate (between 39 and 66% of control levels, $p < 0.05$) (Fig. 4E). Under the 0.1 virus titer condition, the integration levels of SUMO1/SUMO2 and Ubc9 overproducing cells exhibited significant reductions (Fig. 4F) ($p < 0.05$).

Additionally, the relative level of total viral DNA synthesis and the formation of 2-LTR circles were also monitored after HIV-1/IIIB viruses' infection. No significant changes were detected at moi of both 0.01 and 0.1 (data not shown). Altogether these findings indicate that SUMO pathway proteins play as innate cellular restriction factors during HIV-1 replication.

Downregulations of endogenous SUMO1/SUMO2 and Ubc9 increase lentivirus and HIV-1 integration. SUMO1-, SUMO2- and Ubc9-specific siRNAs were introduced into 293T cells to downregulate the corresponding proteins. Western blotting assays verified that the three siRNAs dramatically reduced the expression of endogenous SUMO conjugation pathway proteins in 293T cells 48 h after siRNA transfection (Fig. 5A). Cells were transfected with siRNAs for 48 h and then infected with equivalent amounts of virions. Two days after infection, the percentage of EGFP-expressing cells was measured by flow cytometry. The percentages of EGFP-positive cells in the mock transfection and negative siRNA groups were $23.24 \pm 1.95\%$ and $22.40 \pm 4.51\%$, respectively, whereas in SUMO1/SUMO2 and Ubc9 simultaneous knockdown cells,

the percentage of EGFP-positive cells increased dramatically to $83.29 \pm 3.61\%$ ($p < 0.01$) and $65.57 \pm 1.98\%$ ($p < 0.01$), respectively (Fig. 5B). There was also a noticeable increase in the percentage of EGFP-positive cells when single siRNAs were introduced into the cells. As measured by the Alu-LTR real-time nested PCR assay, the relative levels of lentiviral integration rose by as much as 2.63-fold when SUMO1 and Ubc9 were simultaneously downregulated (Fig. 5C). The relative level of total viral DNA synthesis and the formation of 2-LTR circles were also monitored by real-time PCR under downregulation conditions, and no significant differences were found (data not shown).

Spreading HIV-1 infection experiments were also carried out under downregulation conditions, the virus integration levels increased dramatically to 4.49- and 3.99-fold when SUMO1- and Ubc9-specific siRNAs or SUMO2- and Ubc9-specific siRNAs were co-introduced into H9 cells (Fig. 5D). At moi of 0.1, results kept high degree of consistency with those of lower virus titer except that downregulation of Ubc9 alone did not affect the virus integration (Fig. 5E). Besides, relative level of total HIV-1 DNA synthesis and the formation of 2-LTR circles were not affected by these SUMO-related proteins at neither virus titer (data not shown). In summary, downregulation of endogenous SUMO1, SUMO2 and Ubc9 is rather advantageous to HIV-1 infection.

Discussion

We investigated the relationship between HIV-1 IN and three SUMO-conjugation-related proteins and determined how these associations affect the gene transfer efficiency of HIV-1-derived lentivirus as well as the infection of authentic HIV-1 viruses. Firstly, we showed that HIV-1 IN subcellular localization changes to be punctate in the context of excessive SUMO1/Ubc9 or SUMO2/Ubc9. The interactions between SUMO1, SUMO2, Ubc9 and HIV-1 IN were verified by yeast two-hybrid, co-immunoprecipitation and subcellular localization assays. Further experimentation revealed that HIV-1 IN could be covalently modified by SUMO1 and SUMO2. Finally, overexpression of SUMO1/SUMO2 and Ubc9 inhibited viral integration in an additive manner and that RNAi-mediated downregulation of these proteins promoted viral integration.

In addition to orchestrating the integration of viral cDNA into the cellular genome, HIV-1 IN has also been shown to participate in various steps of the virus life cycle, including reverse transcription and nuclear localization (25). To rule out possible effects of SUMO-conjugation-related proteins on processes other than viral integration, viral cDNA synthesis and 2-LTR circle formation were monitored. No significant impact on either of these processes was detected under conditions of upregulation or downregulation of SUMO pathway proteins.

The SUMO conjugation pathway has been implicated in a variety of cellular processes and is proving to be as important a system as ubiquitination. Viruses could manipulate the cellular SUMOylation system to facilitate viral infection; the system could also function as an antiviral host response to inhibit viral functions (26). The precise biological role of SUMOylation in viral fitness is complicated and remains to be fully characterized.

A report on a similar topic was published and somewhat different results were obtained (27). However, we repeated our experiments in both the lentiviral vector transduction system and the authentic HIV-1 viruses and verified our data and conclusion. Further investigations may be needed for more detailed clarification.

Our study suggests that SUMO-related proteins function as cellular restriction factors that are detrimental to HIV-1 infection. These findings merit further investigation for their potentially significant implications in the cellular antiviral response to HIV-1 infection.

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References

- Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, Lieberman J and Elledge SJ: Identification of host proteins required for HIV infection through a functional genomic screen. *Science* 319: 921-926, 2008.
- Tastan O, Qi Y, Carbonell JG and Klein-Seetharaman J: Prediction of interactions between HIV-1 and human proteins by information integration. *Pac Symp Biocomput* 14: 516-527, 2009.
- Zhou H, Xu M, Huang Q, Gates AT, Zhang XD, Castle JC, Stec E, Ferrer M, Strulovici B, Hazuda DJ and Espeseth AS: Genome-scale RNAi screen for host factors required for HIV replication. *Cell Host Microbe* 4: 495-504, 2008.
- Strebel K, Luban J and Jeang KT: Human cellular restriction factors that target HIV-1 replication. *BMC Med* 7: 48, 2009.
- Wolf D and Goff SP: Host restriction factors blocking retroviral replication. *Annu Rev Genet* 42: 143-163, 2008.
- Nair V: HIV integrase as a target for antiviral chemotherapy. *Rev Med Virol* 12: 179-193, 2002.
- Van Maele B, Busschots K, Vandekerckhove L, Christ F and Debyser Z: Cellular co-factors of HIV-1 integration. *Trends Biochem Sci* 31: 98-105, 2006.
- Hay RT: SUMO: a history of modification. *Mol Cell* 18: 1-12, 2005.
- Johnson ES: Protein modification by SUMO. *Annu Rev Biochem* 73: 355-382, 2004.
- Gill G: SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev* 18: 2046-2059, 2004.
- Zhao J: Sumoylation regulates diverse biological processes. *Cell Mol Life Sci* 64: 3017-3033, 2007.
- Huang L, Xu GL, Zhang JQ, Tian L, Xue JL, Chen JZ and Jia W: Daxx interacts with HIV-1 integrase and inhibits lentiviral gene expression. *Biochem Biophys Res Commun* 373: 241-245, 2008.
- Zhang JQ, Wang JJ, Li WJ, Huang L, Tian L, Xue JL, Chen JZ and Jia W: Cellular protein TTRAP interacts with HIV-1 integrase to facilitate viral integration. *Biochem Biophys Res Commun* 387: 256-260, 2009.
- Fraga MF, Berdasco M, Ballestar E, Ropero S, Lopez-Nieva P, Lopez-Serra L, Martin-Subero JI, Calasanz MJ, Lopez de Silanes I, Setien F, *et al*: Epigenetic inactivation of the Groucho homologue gene TLE1 in hematologic malignancies. *Cancer Res* 68: 4116-4122, 2008.
- Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P and Sharp PA: siRNA-directed inhibition of HIV-1 infection. *Nat Med* 8: 681-686, 2002.
- Brussel A, Delelis O and Sonigo P: Alu-LTR real-time nested PCR assay for quantifying integrated HIV-1 DNA. *Methods Mol Biol* 304: 139-154, 2005.
- Ikeda T, Nishitsuji H, Zhou X, Nara N, Ohashi T, Kannagi M and Masuda T: Evaluation of the functional involvement of human immunodeficiency virus type 1 integrase in nuclear import of viral cDNA during acute infection. *J Virol* 78: 11563-11573, 2004.
- Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A and Chen IS: HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* 61: 213-222, 1990.
- Lusi EA, Guarascio P, Presutti C, Villani R, Pellicelli A and Soccorsi F: One-step nested PCR for detection of 2 LTR circles in PBMCs of HIV-1 infected patients with no detectable plasma HIV RNA. *J Virol Methods* 125: 11-13, 2005.
- Dorval V, Mazzella MJ, Mathews PM, Hay RT and Fraser PE: Modulation of Abeta generation by small ubiquitin-like modifiers does not require conjugation to target proteins. *Biochem J* 404: 309-316, 2007.
- Guo WZ, Sugaya S, Satoh M, Tomonaga T, Nomura F, Hiwasa T, Takiguchi M, Kita K and Suzuki N: Nm23-H1 is responsible for SUMO-2-involved DNA synthesis induction after X-ray irradiation in human cells. *Arch Biochem Biophys* 486: 81-87, 2009.
- Lin X, Liang M, Liang YY, Brunicardi FC and Feng XH: SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4. *J Biol Chem* 278: 31043-31048, 2003.
- Kroetz MB and Hochstrasser M: Identification of SUMO-interacting proteins by yeast two-hybrid analysis. *Methods Mol Biol* 497: 107-120, 2009.
- Goff SP: Retroviridae: The retroviruses and their replication. In: *Fields Virology*. Knipe DM and Howley PM (eds), 4th edition, Lippincott Williams and Wilkins, Philadelphia, PA, pp1871-1939, 2001.
- Engelman A, Englund G, Orenstein JM, Martin MA and Craigie R: Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. *J Virol* 69: 2729-2736, 1995.
- Boggio R and Chiocca S: Viruses and sumoylation: recent highlights. *Curr Opin Microbiol* 9: 430-436, 2006.
- Zamborlini A, Coiffic A, Beauclair G, Delelis O, Paris J, Koh Y, Magne F, Giron ML, Tobaly-Tapiero J, Deprez E, *et al*: Impairment of human immunodeficiency virus type-1 integrase SUMOylation correlates with an early replication defect. *J Biol Chem* 286: 21013-21022, 2011.