Impairment of Human Immunodeficiency Virus Type-1 Integrase SUMOylation correlates with an early replication defect

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HIV-1 integrase (IN)\textsuperscript{1} orchestrates the integration of the reverse transcribed viral cDNA into the host cell genome and participates also in other steps of HIV-1 replication. Cellular and viral factors assist IN in performing its multiple functions, and post-translational modifications contribute to modulate its activities.

Here, we show that HIV-1 IN is modified by SUMO proteins and that phylogenetically conserved SUMOylation consensus motifs represent major SUMO-acceptor sites. Viruses harboring SUMOylation-site IN mutants displayed a replication defect that was mapped during the early stages of infection, before integration but after reverse transcription. Since SUMOylation-defective IN mutants retained WT catalytic activity, we hypothesize that SUMOylation might regulate the affinity of IN for co-factors, contributing to efficient HIV-1 replication.

HIV-1 IN is a 288-amino acid protein consisting of three functionally independent domains. The N-terminal domain (NTD) harbors a highly conserved HHCC zinc binding motif that contributes to IN multimerization and enzymatic activities. The central core domain (CCD) contains the catalytic DDE motif that is conserved in all retroviral and retrotransposon INs, and in certain bacterial transposases. The C-terminal domain (CTD) is the least conserved among retroviral IN and binds DNA non specifically (for review see (1-2)). The best characterized activity of HIV-1 IN is the catalysis of integration, which is crucial for HIV-1 replication (3). This reaction can be reproduced \textit{in vitro} in the presence of recombinant IN alone and synthetic DNA species mimicking the viral LTR ends and an acceptor substrate (4-5). However, other components of the preintegration complex (PIC) contribute to the specificity and efficiency of integration \textit{in vivo} (6-8). Independently of its enzymatic activity, HIV-1 IN plays additional roles during the viral life cycle that are still ill defined. Indeed, many catalytically-active IN mutants have pleiotropic effects impairing reverse transcription and/or uncoating (9-17), PIC nuclear import (18-19) and virion protein composition and/or morphology (16, 20-21).

Post-translational modifications contribute to the regulation of IN activities. HIV-1 IN interacts with and is acetylated by both HAT p300 and GCN5 on C-terminal lysine (K) residues (22-23). Acetylation increases IN affinity for the viral cDNA, enhances its strand transfer activity \textit{in vitro} and might regulate the interaction between IN and cellular factors (24). However, the role of this modification during HIV-1 replication is still controversial (25). HIV-1 IN is also ubiquitinated and subsequently degraded by the proteasome (26-28). In the viral context, IN degradation seems to occur after integration and to be required for correct gap repair (29-30) and viral genes expression (26). Recently,
phosphorylation of HIV-1 IN by cellular JNK has also been proposed to modulate its stability and to be necessary for efficient integration (31). SUMOylation consists of the covalent attachment of small ubiquitin like modifier (SUMO) peptides to a K residue within the consensus motif (ΨKxe/D, where Ψ is a large hydrophobic residue, x any amino acid and E/D glutamic or aspartic acid) of a substrate protein. SUMO-conjugation is mediated by SUMO-specific E1-activating, E2-conjugating and E3-ligating enzymes and is reversed by SUMO-specific proteases (reviewed in (32)). In mammals, three major forms of SUMO proteins are expressed. SUMO-1 has approximately 45% amino acid sequence homology to SUMO-2 and SUMO-3, which are 96% identical to each other. SUMO modification is implicated in numerous cellular processes including signal transduction, protein stability and localization, transcriptional regulation, chromatin structure and genome stability (32). It is also well established that viruses interfere with and/or hijack the cellular SUMOylation machinery to replicate (for review, see (33-34)). Interactions between murine leukemia virus capsid (CA) protein and components of the SUMOylation pathway are required for proper execution of the early steps of replication after reverse transcription, but before integration (35). SUMOylation events have also been implicated in the early phase of HIV-1 infection. Indeed SUMO-2 and RanBP2, a SUMO E3 ligase, were identified in genome-wide screens for cell factors that promote HIV-1 reverse transcription and PIC nuclear import, respectively (36-37). Interaction of HIV-1 or Mason-Pfizer monkey virus gag proteins with SUMO-1 and the E2-conjugating enzyme Ubc9 during the late phases of replication have also been reported and are likely involved in the production of fully infectious virions (38-41). Here, we show that HIV-1 IN is SUMOylated and that three K residues, which are found within conserved consensus motifs, represent the major SUMO acceptor sites. In the viral context, mutation of SUMO-acceptor residues in IN led to reduced infectivity and slower replication kinetics. Biogenesis, release and reverse transcription steps of mutant HIV-1 particles were not affected. However, cells infected with viruses harboring SUMOylation-defective IN mutants showed a significant decrease in integration events compared to HIV-1WT-infected cells. Since SUMOylation-site IN mutants retained WT catalytic activity, we inferred that modification by SUMO might participate in the modulation of HIV-1 IN interaction network by regulating its affinity for co-factors, which are required for the efficient execution of early events of HIV-1 replication.

EXPERIMENTAL PROCEDURES

Cell lines and reagents - HeLa and 293T cells were grown in DMEM (Invitrogen). CEM-GFP cells (AIDS Reagent Program), a human T cell line harboring the GFP reporter gene under the control of HIV-1 LTR (42), were grown in RPMI (Invitrogen). Media were supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml of penicillin and 100 µg/ml streptomycin. Antibodies used are: mouse anti-IN (IN-2), mouse anti-His, rabbit anti-SUMO1 (FL-101) (Santa-Cruz Biotechnology, Inc.), rabbit anti-IN 756 and 757 (AIDS Reagent Program), rabbit anti-SUMO-2/3 (Zymed), mouse anti-CA (Hybridolab), mouse anti-Flag M2 (Sigma-Aldrich), mouse anti-HA 12CA5 (Roche), rabbit anti-Acetyl-lysine (Abcam).

Plasmid construction and Mutagenesis - psPAX2, pWPI and pMD2.G (a gift from D. Trono), pNL4-3EnvFSGFP (a gift from D. Gabuzda), which contains a complete HIV-1 provirus with an env-inactivating mutation and enhanced GFP in the place of Nef (43) and INWT-Flag, which encodes a codon-optimized IN gene harboring an ATG initiation codon and a C-terminal Flag tag (44) have been already described. The cDNAs encoding WT IN, SUMO-1, -2 and -3, were amplified by PCR from IN-Flag and YFP-SUMO-1, -2 and -3 (45) and were subcloned in frame with an N-terminal 6xHis tag into the pcDNA3.1(-) vector (Invitrogen), yielding His-INWT, His-SUMO-1, -2 and -3. The cDNA encoding the C-terminal region of LEDGF/p75 (aa 325-530) was amplified by PCR from WT and D366N mutant HA-LEDGF/p75 (46). To produce IN mutants, changes were introduced by PCR into the IN sequence of the suitable plasmid using the Expand Long Templates PCR System (Roche). The entire recombinant IN coding fragment was confirmed by sequencing and swapped for the
corresponding WT fragment into the appropriate recipient vector.

**Purification of recombinant His-tagged IN and in vitro SUMOylation assay** - Bacterially-expressed His-tagged full-length IN, N-terminal (INΔN, encoding aa 50-288) or C-terminal (INΔC, encoding aa 1-213) truncation forms (47) or IN3KR mutant were purified as described previously (48). Next, recombinant His-tagged full-length or mutant IN proteins (200 nM) were used to perform an *in vitro* assay with the SUMOylation Kit (BIOMOL) according to the manufacturer's instructions.

**His-tagged protein purification on Ni-NTA beads in denaturing conditions** - 293T cells (3×10⁶) were seeded into 10-cm dishes and transfected 24 h later using a calcium phosphate precipitation technique with plasmids encoding Flag-tagged WT or mutant IN proteins, and vectors expressing Ubc9 and His-SUMO-1, -2 or -3 or an appropriate empty vector. After 40 h cells were lysed under denaturing conditions in buffer A (6M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM imidazole, pH 8.0) and sonicated (10 cycles, 40 sec pulse, 15 sec pause with the Bioruptor TM, Diagenode). Cell lysates were eluted by boiling in Laemmli buffer with 200 mM imidazole and resolved by SDS-PAGE. Tagged-proteins were probed for by Western blot.

**Analysis of HIV-1 IN proteins localization and half-life** - For indirect immunostaining, HeLa cells were grown on glass cover slips and transfected with Polyfect reagent (QIAGEN) according to the manufacturer’s instructions. After 48 h, cells were fixed with Phosphate Buffer Saline (PBS)-4% paraformaldehyde (10 min, 4°C), permeabilized with ice-cold methanol (5 min, 4°C) and incubated with primary antibodies ON at 4°C, followed by corresponding secondary antibodies conjugated to Alexa-Fluor488 (Jackson ImmunoResearch Laboratories, Inc.). Images were acquired on a laser-scanning confocal microscope (LSM510 Meta; Carl Zeiss) equipped with an Axiovert 200 M inverted microscope, using a Plan Apo 63/1.4-N oil immersion objective.

For fractionation experiments, 293T cells expressing Flag-tagged WT, 3KR or 3EQ IN were lysed in buffer C (10 mM Tris-Cl pH 7.4, 0.15 M NaCl, 1% CHAPS, EDTA-free complete protease inhibitors, Roche) (30 min, 4°C). Supernatant (cytosol) and pellet (nuclei) were separated by centrifugation (top speed, 5 min, 4°C). Nuclear content was extracted in buffer N (buffer C with 0.85 M NaCl final) (30 min, 4°C).

For IN stability studies, cycloheximide (100 μg/ml, Sigma) or MG132 (5μM, Calbiochem) were added to the culture medium 24 h after transfection. Next, cells were lysed in buffer N. Total protein content was measured with Bradford assay (Sigma). Proteins (25 μg/lane) were resolved by SDS-PAGE and detected by Western blot.

**Virus stock production and infectivity assay** - Single-round viruses were produced by cotransfection of 293T cells using a standard calcium phosphate precipitation technique with a plasmid encoding WT or mutant HIV-1-packaging DNA (psPAX2) and the genomic transfer vector encoding GFP (pWPI) or the pNL4-3EnvFsGFP vector and an expression vector for the glycoprotein G of vesicular stomatitis virus (VSVg) (pMD2.G). Replication-competent viruses were produced by transfecting the pNL4-3 plasmid that encodes a complete HIV-1 infectious provirus. Supernatants were collected 48 h post-transfection, clarified by low speed centrifugation, filtered through 0.45 μm-pore size filters and treated with 10U/ml Turbo DNAse (Ambion) (1h, RT). Viral particles were concentrated by ultracentrifugation (24 000 rpm, 1h 30 min, 4 °C) using a SW32 rotor (Beckman) on a 20% sucrose cushion. All viral stocks were normalized for the p24CA antigen content, as determined by ELISA (Zeptometrix) and used to infect target cells (6x10⁴ 293T or 1x10⁶ CEM-GFP cells). After 48 h, the percentage of GFP-expressing cells was measured by flow cytometry on a FACS-Calibur flow cytometer with CellQuest software (BD Biosciences).

**Western blot analysis of viral proteins** - Viral proteins associated with virions or with infected cells were analyzed by Western blot with anti-CA and anti-IN antibodies. For quantification of virion-associated CA and IN proteins, secondary antibodies coupled with IRDye near-infrared
dyes (IRDye800CW and IRDye680LT, Science Tec) were used. Proteins were visualized on an Odyssey infrared imager and quantified with Odyssey software (LI-COR Biosciences).

**Real-time PCR analysis** - Total genomic DNA was extracted using the Blood and body fluid kit (QIAGEN) from 293T cells (5x10^5) infected with single-round viruses. Full-length reverse transcripts, integrated HIV-1 DNA and 2-LTR circles were quantified using a previously described protocol (49). Parallel infections with heat-inactivated HIV-1_WT viruses were performed to control for residual levels of plasmid DNA that may have resisted DNAse treatment. Viral RNA was extracted with the RNeasy Mini kit (QIAGEN) and amplified with the HIV-1 real time RT PCR Kit (BioEvolution). Real-time PCR and RT-PCR were performed on a Lightcycler 1.0 (Roche Diagnostics).

**Vpr-integrase complementation** - Viral stocks generated by co-transfecting 293T cells with pNLX.Luc(R-Env-), pRL2P-Vpr-IN WT or mutant, and pNLX7 were used to infect Jurkat cells (2 × 10^6/ml, 5 × 10^5 RT cpm), as described (50). Cells were harvested 48 h after infection and lysed in passive lysis buffer (Promega). Frozen and thawed lysates were clarified by centrifugation (18,730×g, 15 min, 4°C), and supernatants were analyzed for luciferase activity in duplicate using the Promega luciferase assay system, an EG&G Berthold Microplate LB 96V luminometer, and a Microlite 1 flat-bottom microtiter plate (Thermo Labsystems). Luciferase activity was normalized to the protein concentration as determined by the Bio-Rad protein assay kit (Bio-Rad) and corrected for background levels from lysates of cells infected with Env-negative controls.

**Statistical analyses** - Pair-wise comparison between groups was performed using the Student’s t test. P<0.05 was set as a threshold for statistical significance.

**RESULTS**

**HIV-1 IN is covalently modified by the three SUMO paralogues in vitro** - By analyzing HIV-1 IN sequences, we identified three K residues at position 46, 136 and 244 (HXB2 numbering scheme) within canonical SUMOylation consensus motifs, which represent potential sites for modification by SUMO (SUMOplot, [http://www.abgent.com/tools/sumoplot](http://www.abgent.com/tools/sumoplot); SUMO sp 2.0 (51)). SUMO-consensus motifs harboring K46 and K244 are conserved in HIV-2, SIVcpz and SIVmac (fig. 1A and supporting information fig. S1), while the SUMO consensus harboring K136 is found in about one-third of HIV-1 strains (52).

The extent of conservation of these motifs prompted us to assess whether HIV-1 IN is post-translationally modified by SUMO proteins. To this aim we performed an in vitro SUMOylation assay. Slow migrating bands reactive to an antibody against IN were observed when purified recombinant IN bearing an N-terminal 6xHis tag was incubated with components of the SUMOylation machinery in the presence, but not in the absence, of ATP (fig. 1B and 1C, lanes 1 and 2). The appearance of several high-molecular-weight species indicates the addition of multiple SUMO moieties to IN. Of note, similar levels of modification, but slightly different patterns of conjugation by SUMO-1 or SUMO-2/3, were observed when IN was incubated in the presence of each SUMO protein separately (fig. 1B, lanes 3 to 5).

Since putative SUMO-attachment sites localize to each IN functional domain, we examined the modification of IN mutants in which the N-terminal or the C-terminal region was deleted. SUMOylated species were observed when INΔC was subject to the in vitro SUMOylation reaction (fig. 1C), while modified forms of INΔN could not be detected, likely due to technical limits (absence of the epitope or low affinity of the antibodies used) (data not shown).

Since putative SUMO-attachment sites localize to each IN functional domain, we examined the modification of IN mutants in which the N-terminal or the C-terminal region was deleted. SUMOylated species were observed when INΔC was subject to the in vitro SUMOylation reaction (fig. 1C), while modified forms of INΔN could not be detected, likely due to technical limits (absence of the epitope or low affinity of the antibodies used) (data not shown). To confirm that sites identified in silico are SUMOylated, we simultaneously replaced K residues at position 46, 136 and 244 by arginine (R), an amino acid with similar positive charge that cannot be modified by SUMO, into the His-IN plasmid. The resulting triple IN mutant...
(IN$_{3KR}$) was then tested in the *in vitro* SUMOylation assay. Under these settings, conjugation of SUMO to IN$_{3KR}$ was dramatically decreased compared to IN$_{WT}$ (fig. 1D). Altogether, these results show that HIV-1 IN is modified with similar efficiency, but with slightly different specificity, by the three SUMO paralogues on candidate consensus sites *in vitro*.

**SUMOylation of HIV-1 IN upon expression in the cell** - Since we found that HIV-1 IN is covalently modified by SUMO proteins *in vitro*, we next assessed SUMO conjugation in a cellular context. To this aim, 293T cells were co-transfected with a plasmid encoding HIV-1 IN bearing a C-terminal Flag tag (IN$_{WT}$-Flag), and expression vectors for Ubc9 and for N-terminal His-tagged SUMO-1, -2 or -3, or the appropriate control empty vector. Forty hours after transfection, purification by immobilized metal ion affinity chromatography in highly denaturing conditions was performed to ensure that only molecules covalently linked to IN were recovered and that the activity of SUMO proteases was blocked. While IN expression levels were comparable in all samples (fig. 2A, lower panel, lanes 1 to 4), modified species reactive to an anti-IN antibody and migrating at the size expected for IN conjugated to one (approximately 36+12=48 kDa) or several SUMO moieties, were observed when SUMO-1, -2 or -3 were expressed, but not in the control (fig. 2A, upper panel, lanes 1 to 4).

Since K-to-R changes at position 46, 136 and 244 lead to a drastic reduction of IN SUMOylation *in vitro*, the same mutations were introduced into the IN$_{WT}$-Flag plasmid. Candidate SUMOylation sites were disrupted either individually or in various combinations and modified IN derivatives were analyzed as described above. Single and double mutant IN proteins displayed SUMOylation profiles analogous to IN$_{WT}$ (data not shown and fig. S2). As expected, the enrichment of modified forms of IN$_{3KR}$, which was expressed at levels similar to IN$_{WT}$, was considerably diminished regardless of the SUMO protein expressed (fig. 2A, compare lanes 5 to 8 with lanes 1 to 4). We also generated an IN mutant in which the E residues at positions 48, 138 and 246 were substituted by glutamine (Q), yielding the IN$_{3EQ}$ mutant. Indeed, the acidic amino acid (E/D) at +2 of the SUMOylation consensus motifs is indispensable for SUMO conjugation to K (53). In agreement with the results obtained with IN$_{3KR}$, the SUMO-modified species of IN$_{3EQ}$ were much less abundant compared to that of IN$_{WT}$ (fig. 2A, compare lanes 9 to 12 with lanes 1 to 4).

To evaluate whether disruption of IN SUMOylation motifs affected other post-translational modifications targeting K residues, Flag-tagged WT or mutant IN proteins were expressed together with His-tagged ubiquitin in 293T cells and purification in denaturing conditions was performed. Both IN$_{3KR}$ and IN$_{3EQ}$ displayed ubiquitination profiles similar to IN$_{WT}$, demonstrating that K-to-R or E-to-Q changes specifically impaired SUMOylation, but not ubiquitination (fig. 2B).

Finally, we analyzed conjugation of endogenous SUMO proteins to ectopically expressed IN. Thus, we generated plasmids encoding WT or 3KR IN with an N-terminal 6xHis tag that were used to transfect 293T cells, followed by affinity purification in denaturing conditions. Post-translational derivatives of IN enriched on Ni-NTA beads were detected with an anti-SUMO-2/3 antibody in the presence, but not in the absence, of IN$_{WT}$ expression (fig. 2C, upper panel, lane 2). Under the same settings, weak, but specific bands were also detected with an antibody against SUMO-1 (fig. 2C, middle panel, lane 2). However, while IN$_{3KR}$ was expressed at levels similar to IN$_{WT}$ (fig. 2C, lower panel, compare lane 2 and 3), corresponding SUMO-1- and SUMO-2/3-modified forms were significantly reduced (fig. 2C, upper and middle panels). Analysis of acetylation and ubiquitination of WT and 3KR IN under the same settings, showed that both proteins were modified to a similar extent (fig. 2D and data not shown).

Altogether, these results confirm that HIV-1 IN is modified by the SUMO paralogues *in vivo* and that conserved K residues at position 46, 136 and 244 are the principal sites of SUMOylation. Moreover, these data indicate that candidate SUMOylation sites are not targeted by ubiquitination or acetylation.

**Impairment of HIV-1 IN SUMOylation does not affect its subcellular localization or stability** - SUMOylation has been shown to regulate numerous cellular processes, including protein
localization and stability (32). First, the involvement of SUMO conjugation on HIV-1 IN subcellular distribution was studied by expressing Flag-tagged WT or mutant IN proteins in Hela cells, followed by immunofluorescence and confocal microscopy analysis. As already established, IN\textsubscript{WT} was enriched in the nucleus of transfected cells (fig. 3A). Likewise, IN\textsubscript{3KR} and IN\textsubscript{3EQ} concentrated in the nucleus (fig. 3A). A similar distribution was also observed upon analysis of the steady state nucleocytoplasmic partitioning of Flag-tagged IN\textsubscript{WT} or IN\textsubscript{3KR} or IN\textsubscript{3EQ} expressed in 293T cells by cell fractionation followed by Western blot with an anti-IN antibody. The proper separation of the nuclear and the cytoplasmic fraction was confirmed by immunoblotting with antibodies against the cytoplasmic protein LDH or the nuclear histone H2B, showing no detectable cross contamination (fig. 3B).

Second, to investigate the involvement of SUMOylation on IN stability, we studied the half-life of IN\textsubscript{WT}, IN\textsubscript{3KR} and IN\textsubscript{3EQ} overexpressed in 293T cells, by carrying out a chase analysis upon treatment with the protein synthesis inhibitor cycloheximide. While actin levels were not affected by addition of the drug to the culture medium, IN\textsubscript{WT} protein levels decreased over time, with an approximate half-life of 6 h (fig. 3C and 3D). Different experimental settings (ectopic expression of IN from a codon-optimized gene versus stable expression in a cell line) might account for discrepancies between this and earlier results, which reported shorter life-span for HIV-1 IN (26-27). Similar decay kinetics was also determined for IN\textsubscript{3KR} and IN\textsubscript{3EQ} (fig. 3C and 3D). In agreement with previous reports (26-28), treatment with MG132, an inhibitor of the ubiquitin-proteasome system, prevented the degradation of both WT and mutant IN proteins (fig. 3C), implicating that their degradation occurred mainly through the proteasomal pathway. Based on these results, we concluded that SUMOylation of K46, K136 and K244 neither significantly influences the subcellular distribution of HIV-1 IN nor the kinetics of its proteasomal-dependent degradation.

Mutation of key residues of IN SUMOylation consensus motifs impairs HIV-1 infectivity - Having established that HIV-1 IN is SUMOylated \textit{in vivo}, outside the viral context, we next addressed the role of SUMO conjugation to IN during viral replication. Identified SUMO-acceptor K residues were mutated either individually or simultaneously to R in an HIV-1-derived packaging construct. Lentiviral vectors, prepared by transfection of 293T cells with WT or mutant HIV-1-packaging DNA, a vector expressing the VSV\textsubscript{g} envelope and a genomic transfer vector encoding GFP, were harvested after 48 h and concentrated by ultracentrifugation through sucrose cushion. Next, viral preparations of equal p24\textsubscript{CA} content were used to challenge 293T cells, and the infectivity of WT and mutant viral particles was determined by measuring the amount of GFP-expressing cells by flow cytometry 48 h post-infection. While vectors harboring different single-point mutations of SUMO-acceptor K sites displayed moderate reductions in infectivity (between 25 and 59\% WT levels), an HIV-1-derived viral vector harboring IN\textsubscript{3KR} was 3\% as infectious as WT (fig. 4A). Although they can fully recapitulate the early phase of infection, lentiviral vectors lack both the ability to perform the late stages of HIV-1 replication and viral accessory proteins present in their parental counterpart, which are mostly dispensable for replication \textit{in vitro} but essential for viral pathogenesis \textit{in vivo}. Thus, we analyzed the effect of the disruption of the SUMOylation sites within IN also on the infectivity of authentic HIV-1 viruses. To this aim, K-to-R substitutions were introduced in a plasmid encoding full-length HIV-1 proviral DNA with a frame shift in Env and expressing the GFP reporter gene in the place of Nef (NL4-3Env\textsubscript{FsGFP}). Mutant virions harboring an IN protein bearing E-to-Q changes at identified SUMOylation consensus motifs were also generated to specifically disrupt SUMOylation without affecting other post-translational modification that target K residues. Single-round viruses were produced and used in infection experiments as described before. As shown in figure 4B, HIV-1 harboring IN\textsubscript{3KR} or IN\textsubscript{3EQ} were about 57\% and 33\% as infectious as HIV-1\textsubscript{WT}, respectively. The contribution of non structural viral proteins, absent from the lentivector background, might account for the differential viral infectivity of viruses bearing SUMOylation-deficient IN proteins under these
settings. Finally, to study the outcome of the impairment of IN SUMOylation on HIV-1 replication following entry via receptor-mediated fusion at the plasma membrane, K-to-R changes were introduced into the NL4-3 molecular clone of HIV-1. Human T-lymphoid CEM-GFP cells were infected with equal p24CA amounts of HIV-1WT or HIV-13KR and the percentage of GFP-positive cells was monitored by flow cytometry over time. Under these experimental conditions, the infectivity of HIV-13KR was 32% and 41% that of HIV-1WT at 48 and 96 h post-infection, respectively (fig. 4C). Additionally, HIV-13KR replicated with a delay of approximately 3 days compared to HIV-1WT, reaching peak growth at 7 days post-infection. Altogether these findings indicate that SUMO-modified K46, K136 and K244 play a role during HIV-1 replication.

SUMOylation-site mutant HIV-1 is impaired at an early step(s) of replication preceding integration - We observed that simultaneous disruption of IN SUMOylation consensus motifs correlated with an impairment of HIV-1 infectivity. To establish what stage(s) of HIV-1 replication cycle was affected, we first asked whether the defects observed could be accounted by improper viral particle assembly, composition and/or release. Similar amounts of WT and mutant progeny virions were obtained following transfection of 293T cells with NL4-3EnvFsGFP plasmid encoding INWT, IN3KR or IN3EQ, in independent production experiments, as determined by p24CA ELISA (Table 1). The expression levels and patterns of protease-mediated cleavage of the Gag precursor were analyzed and found to be similar for WT and mutant HIV-1-producing cells (data not shown). Analysis of HIV-1WT, HIV-13KR and HIV-13EQ protein content by Western blot using near-infrared dye-conjugated antibodies followed by quantification of the corresponding emission signals on an Odyssey infrared imaging system, showed that WT and mutant viral particles displayed comparable amounts of IN protein relative to CA (fig. 5A and Table 1). Additionally, two bands reactive to an anti-IN antibody, with the apparent molecular mass of IN monomer and dimer molecules, were detected in all samples by analysis performed under non-reducing conditions (without β-Mercapto-ethanol), as previously described (54).

Quantification of the intensity of corresponding bands showed that HIV-13KR and HIV-13EQ displayed IN dimer-to-monomer ratio comparable to that of HIV-1WT (fig. 5B and Table 1). Finally, HIV-1WT, HIV-13KR and HIV-13EQ virions packaged similar amounts of viral genomic RNA, as established by real-time RT-PCR on total RNA extracted from viral stocks of equivalent p24 content (Table 1). These results demonstrated that viral particle biogenesis, release and maturation were mostly unaffected by the mutation of SUMO-acceptor sites within IN.

Since mutation of IN SUMOylation sites did not have an impact on the late events of the HIV-1 life cycle, we inferred that an early replication step might be affected. To address this question, we analyzed viral cDNA synthesis in cells infected with equivalent p24CA amounts of VSVg-pseudotyped single-round virions harboring INWT, IN3KR or IN3EQ by real-time PCR. While similar amounts of late reverse transcripts were synthesized over time (fig. 5C), the levels of integrated proviruses at 24 and 48 hpi were significantly diminished in cells infected with HIV-13KR or HIV-13EQ as compared with HIV-1WT-infected cells (fig. 5D). We also monitored the formation of 2-LTR circles, which are generally used as a marker of PIC nuclear import (reviewed in (55)), and found that the number of copies of 2-LTR circles formed upon infection with HIV-1 harboring WT or mutant IN proteins was similar (data not shown).

We next assessed whether mutation of SUMOylation sites of HIV-1 IN affected its enzymatic activity. For that purpose, we tested the function of SUMOylation site-mutant IN proteins in the viral context using a Vpr-IN complementation assay (56). WT or mutant IN proteins fused to Vpr were expressed together with an HIV-1 proviral vector encoding an active site IN mutant (HIV-1D64N/D116N). Vpr-mediated encapsidation of IN proteins, in which SUMOylated K residues were changed to R either individually or simultaneously, allowed recovery of viral infectivity comparable to that of Vpr-INWT (figure 5E and data not shown), suggesting that mutation of SUMO-acceptor
sites did not significantly affect IN catalytic activity under these infection conditions.

Finally, we asked whether impairment of provirus formation upon infection with HIV-13KR or HIV-13EQ could be accounted for by loss of interaction with LEDGF/p75, an extensively studied IN-interacting protein and an essential chromatin-docking factor for HIV-1 PIC (29, 57-58). To this aim, 293T cells were co-transfected with an expression vector for Flag-tagged INWT, IN3KR or IN3EQ and a plasmid encoding the C-terminal region (aa 325 to 530, encompassing the IN-binding domain) of LEDGF/p75 with an HA-tag (HA-LEDGF C-ter) or the corresponding D366N mutant, which has lost the ability to bind IN (46), or the appropriate control empty vector. Immunoprecipitation assays performed 40 h after transfection showed that both IN3KR and IN3EQ were able to bind an LEDGF/p75 C-ter in a manner similar to INWT (fig. 5F). As expected, INWT was not enriched on HA-matrix beads in the absence of WT LEDGF C-ter or in the presence of the D366N mutant (fig. 5F). Altogether, these results demonstrated that impairment of IN SUMOylation by substitution of residues K46, K136 and K244 or E48, E138 and E246, neither affected IN catalytic activity nor LEDGF/p75 binding, and correlates to an early replication defect occurring before proviral integration, but after reverse transcription.

DISCUSSION

HIV-1 IN is the viral enzyme that orchestrates the integration of the viral cDNA into cellular genome, a key event of retroviral replication and the target of novel anti-HIV therapeutic agents. Numerous studies have contributed to quite an extensive understanding of the molecular basis of IN catalytic functions. On the contrary, while it is well established that integrity of IN structure and/or its interaction network are critical for optimal execution of various steps of HIV-1 life cycle other than integration (59), the mechanisms by which IN exerts these additional functions are presently still elusive. To address this issue, IN protein-protein interactions have been widely explored (reviewed in (60)); however, information is scarce on IN post-translational modifications that represent a common, rapid and generally reversible mechanism for fine tuning of protein activities.

We repeatedly observed that numerous bands were detected in an anti-IN immunoblot following affinity purification of HIV-1 IN under denaturing conditions from either cellular or viral extracts, suggesting that it undergoes a high degree of post-translational modifications (data not shown). It has already been shown that IN is acetylated, ubiquitylated and phosphorylated (22-23, 25-29, 31). Since we had mapped putative sites of SUMO conjugation to K residues at position 46, 136 and 244, located within phylogenetically conserved canonical SUMOylation consensus motifs (32), we asked whether HIV-1 IN is also SUMOylated. We identified these amino acids by comparative analysis of IN sequences, which showed that K46 and K244 are found within SUMOylation consensus motifs common to HIV-1, HIV-2, SIVmac and SIVcpz (fig. 1A and SI fig. 1). K244 is also conserved among certain non-primate lentiviruses (61). Notably, analysis of naturally occurring variations within IN sequences of HIV-1 isolates, showed that K46 and K244 are not polymorphic (62-63). Conversely, K136 is conserved in about one third of HIV-1 strains and displays both inter- and intra-subtype substitutions to residues that cannot be modified by SUMO with a frequency rate > 0.5% (62-63). This extent of amino acid conservation suggests a role of these residues in preserved IN functions.

Analysis performed in vitro showed that recombinant purified HIV-1 IN is covalently coupled by SUMO proteins. Under these experimental conditions, similar efficiency of modification, but slightly different patterns of conjugation by each paralogue were observed, pointing to the presence of both SUMO-1- and SUMO-2/3-specific attachment sites within IN. We further confirmed modification of IN by SUMO proteins in a cellular context, both in the presence of exogenous or endogenous expression levels of SUMO family members. In both cases SUMO-2/3-conjugated IN species were more abundant than SUMO-1-conjugated forms, likely reflecting the higher availability of SUMO-2/3 compared to SUMO-1 (64).

Finally, the formation of numerous high-molecular-weight species is consistent with the addition of multiple SUMO moieties to IN. The attachment of single SUMO molecules to
several K residues is supported by the fact that many SUMOylated IN species were detected when an in vitro reaction was conducted in the presence of SUMO-1 alone, which cannot form polySUMO chain. However, the conjugation of polymeric SUMO chains to one K residue of IN under different experimental settings cannot be ruled out. In light of emerging evidence suggesting that SUMO proteins display both common and specific target protein preferences and play both redundant and non-redundant cellular functions, these results might underlie functional differences of SUMO-1- and SUMO-2/3-conjugation to IN.

Simultaneous substitution of key residues within the three canonical SUMOylation consensus motifs (either K or E) was required to significantly decrease the occurrence of SUMOylated, but not ubiquitinated or acetylated, IN species. However, IN SUMOylation was not abolished indicating that K46, K136 and K244 represent the major, but not unique, SUMO-acceptor sites. In addition to the ΨKxE/D consensus, the K residue within the reverse E/DxKΨ signature can also be modified by SUMO (65). Concordantly, K residues at position 71 and 258, which are highly conserved among HIV-1 isolates, may represent additional sites of SUMOylation (fig. S1). Disruption of preferred modified sites might also result in the transfer or the enhancement of SUMO conjugation to other K residues, as reported for other proteins (66-67).

Mutation of identified SUMOylation sites did not significantly impact several IN properties such as subcellular distribution and stability of recombinant HIV-1 IN in human cells or IN oligomerization within the virion. Moreover, SUMOylation-defective IN mutants retained LEDGF/p75 binding and were catalytically active as demonstrated by their ability to rescue the replication defect of an IN active-site mutant virus when expressed as Vpr-fusion proteins in trans. On the basis of these observations, we deduced that K-to-R or E-to-Q changes did not considerably alter IN structure. In agreement, analysis of available 3D structures of HIV-1 IN (68-69) showed that key residues of identified SUMOylation consensus motifs (K46 and E48, K136 and E138 and K244 and E246) are located at the surface of IN multimers and do not seem to be engaged in contacts between IN protomers, suggesting that SUMOylation of these sites would not directly affect HIV-1 IN oligomerization (fig. S3A and S3B).

In the viral context, mutation of SUMO acceptor sites correlated with decreased infectivity and slower replication kinetics compared to HIV-1 WT, both in epithelial and T cell lines. Viruses harboring IN3KR displayed viral particle biogenesis and release similar to the WT counterpart, indicating that mutation of major SUMO-acceptor sites did not affect late stages of HIV-1 replication cycle. We next analyzed viral cDNA synthesis and provirus formation upon infection and found that SUMOylation-site mutant viruses displayed a defect at the integration step, while reverse transcription and PIC nuclear import were mostly unaffected. Altogether, these results show that integrity of identified consensus sites for SUMOylation is required both for optimal SUMOylation levels of IN and efficient proviral integration. Either concomitant or consecutive modification of K residues at position 46, 136 and 244 by SUMO might be required for a favorable outcome of HIV-1 replication. However, residual modification of non canonical SUMOylation sites within IN might compensate for the lack of conjugation to major SUMO-attachment sites and, thus, account for the moderate reduction of viral infectivity.

Interestingly, we obtained analogous results when studying HIV-1 3EQ, which bears an IN mutant in which E-to-Q changes were introduced at identified SUMOylation consensus motifs to specifically impair SUMO-conjugation but not other post-translational modifications that occur on K residues. The fact that viruses harboring either IN3KR or IN3EQ display an analogous phenotype supports a direct requirement for IN SUMOylation in the optimal execution of an event(s) following reverse transcription and nuclear entry, but before integration. Thus, our results are agreement with data from recent genome-wide studies indicating that components of the SUMOylation pathway promote HIV-1 replication (36-37).

To strengthen the role of IN SUMOylation during viral replication we asked whether revertant viruses would emerge following passages of HIV-1 3KR-infected cells. Total
genomic DNA was extracted at the pick of infection and used to amplify the IN fragment which was then entirely sequenced. Our preliminary results show that one out of 22 sequenced clones harbored an IN protein in which R residues at positions 136 and 244, but not 46, reverted simultaneously to K. This cloned harbored also a conservative L-to-I substitution at position 172.

We also attempted to establish the stage(s) of the HIV-1 life cycle during which IN SUMOylation could take place. We therefore generated 293T cell lines stably expressing His-SUMO proteins which were used to produce HIV-1 viral stocks. Bands migrating at the size expected for SUMO-conjugated IN or to Gag-Pol cleavage intermediates were detected following purification in denaturing conditions over NiNTA agarose beads of virus-producing cells, indicating that SUMOylation occurs during the late phases of HIV-1 infection (fig. S4). This result suggests also that SUMOylated IN might be incorporated within HIV-1 virions. However, SUMO-conjugated IN species were not detected upon lysis and purification of viral stocks (fig. S4). The fact that each HIV-1 virion encapsidates an estimated 250 molecules of IN (70) and that only a small fraction of it might be SUMOylated at steady-state, could at least in part explain these observations. Moreover, SUMOylation is reversible and efficient viral replication might rely on a dynamic process of conjugation-deconjugation of SUMO moieties to IN. We note also that HIV-1 p6 has been shown to be SUMOylated, but the incorporation of the SUMO-conjugated protein into virions has not been detected (38), despite p6 is about 20 times more abundant than IN.

Expression of components of the SUMOylation pathway, in particular SUMO-2 and the SUMO E3 ligase RanBP2, in target cells has recently been implicated in the completion of early events of HIV-1 infection (36-37). We are currently addressing the role of a potential functional interaction between RanBP2 and HIV-1 IN, which could take place during PIC-nuclear import. Preliminary experiments demonstrated also the existence of a physical and functional link between IN and SUMO E3 ligases of the PIAS family (Zamborlini A., Beaucclair G. unpublished results).

In conclusion, we hypothesize that SUMOylation might modulate the affinity of IN for co-factors required for efficient viral replication, as recently reported for IN acetylation (24). Further studies will be required to clarify the molecular mechanisms by which the cellular SUMOylation pathway participates in the control of HIV-1 IN functions during the early steps of viral replication.

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FOOTNOTES
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The abbreviations used are: IN, integrase; PIC, preintegration complex; HAT, histone acetyltransferases; SUMO, small ubiquitin-like modifier; RanBP2, Ran-binding protein 2; CA, capsid; VSVg, glycoprotein G of vesicular stomatitis virus; moi, multiplicity of infection; Ub, ubiquitin, WCL, whole cell lysate.

FIGURE LEGENDS
Fig 1. HIV-1 IN is modified by the three SUMO paralogues in vitro on conserved K residues. (A) Schematic representation of IN protein. Amino acid sequence alignment of candidate SUMOylation motifs of HIV-1 (HXB2 reference sequence), SIVcpz, HIV-2rod and SIVmac are shown. DDE: active site residues. (B) Purified recombinant His-IN (200 nM) was incubated with purified SUMO-specific E1 and E2 enzymes and the three SUMO proteins (S1, S2, S3) added simultaneously or individually. The reaction was conducted in the presence or in the absence of ATP/Mg\(^{2+}\), as indicated. Modified and unmodified forms of IN were visualized with an anti-IN antibody. (C) Incubation of purified His-tagged full-length and C-terminal truncation mutant IN (IN\(^{\Delta C}\)) or (D) WT and 3KR mutant IN proteins with the three SUMO proteins simultaneously was performed as in (B). Lower panels from B through D show shorter exposure times.

Fig 2. HIV-1 IN is SUMOylated by over-expressed or endogenous SUMO proteins in the cell (A) 293T cells were co-transfected with plasmids encoding Flag-tagged IN\(_{WT}\) or IN\(_{3KR}\) or IN\(_{3EQ}\), Ubc9 and His-tagged SUMO-1, SUMO-2 or SUMO-3 or an empty vector (mock) and, 40 h later, were lysed in denaturing conditions followed by purification on Ni-NTA beads. (B) 293T cells co-expressing WT or 3KR or 3EQ IN-Flag and His-tagged ubiquitin were treated as in (A). (C) 293T cells expressing His-IN\(_{WT}\) or His-IN\(_{3R}\) were treated as in (A) and modification by endogenous SUMO-1 or SUMO-2/3 was assessed. A longer exposure time of the inset enclosed in the black square is shown (middle panel). Arrows indicate SUMO1-conjugated IN forms. (D) Acetylated forms of His-IN\(_{WT}\) or His-IN\(_{3R}\) were analyzed following purification as in (A). Proteins expressed in the cells or enriched on NiNTA beads in panel A through D were visualized by Western blot with the indicated antibodies.

Fig 3. Subcellular distribution and stability of HIV-1 IN are not affected by mutation of SUMOylation consensus motifs. (A) Hela cells expressing WT or 3KR or 3EQ IN-Flag were fixed with
4% PFA and then stained with an antibody anti-Flag, followed by an Alexa488-conjugated secondary antibody. Cells were visualized on a confocal microscope, using a 63X magnification. Representative images are shown. (B) Twenty-four hours after transfection, 293T cells expressing Flag-tagged IN<sub>WT</sub> or IN<sub>3R</sub> or IN<sub>3Q</sub> were subject to cell fractionation followed by Western blot with anti-IN. Purity of the fractions was verified with a nuclear marker, H2B, and a cytoplasmic marker, LDH. Following quantification of ECL signals with ImageJ, the distribution of WT or mutant IN proteins in the nucleus and the cytoplasm was determined by dividing the intensity of IN signals for the corresponding H2B or LDH signals. The values for IN<sub>WT</sub> were arbitrarily set to 100. (C) Twenty-four hours after transfection 293T cells expressing Flag-tagged IN<sub>WT</sub> or IN<sub>3KR</sub> or IN<sub>3EQ</sub> were treated with cycloheximide (CHX) or MG132 or left untreated (NT). At the indicated times, cells were lysed and total proteins (25 µg/lane) were separated by SDS-PAGE followed by Western blot with anti-IN or anti-actin antibodies. (D) ECL signals from Western blots of figure 3B were quantified using ImageJ and values were plotted as the percentage of the signal at t = 0 (given an arbitrary value of 100%) remaining at indicated time points. Results shown are representative of three independent experiments.

Fig 4. HIV-1 virions harboring SUMOylation-site mutant IN display reduced infectivity. (A) Equivalent p24<sub>CA</sub> amounts (5 ng) of VSVg-pseudotyped lentiviral vectors harboring WT or SUMOylation-site mutant IN and encoding GFP were used to infect 293T cells. Infectivity of each viral stock, expressed as the percentage of GFP-positive cells at 48 hpi, was measured by flow cytometry. The infectivity of WT viruses was arbitrarily set to 100. (B) The infectivity of equal p24<sub>CA</sub> amounts of VSVg-pseudotyped HIV-1<sub>WT</sub> or HIV-1<sub>3KR</sub> or HIV-1<sub>3EQ</sub> (5 ng) was determined as described in (A). *, p<0.05 (paired t test). (C) For spreading infection experiments, CEM-GFP cells were infected with equal amounts of HIV-1<sub>WT</sub> or HIV-1<sub>3KR</sub> (20 ng of p24<sub>CA</sub>, an approximate moi of 0.15). Mock infections were performed using heat-inactivated WT viruses. Plotted values in panels A through C represent the mean ± S.D. from three independent experiments.

Fig 5. HIV-1 harboring SUMOylation-defective IN is impaired in provirus formation, but retains WT protein composition, catalytic activity and LEDGF/p75-binding. (A) Proteins contained in HIV-1<sub>WT</sub> or HIV-1<sub>3KR</sub> or HIV-1<sub>3EQ</sub> viral preparations (100 ng or 300 ng of p24<sub>CA</sub>) were separated by SDS-PAGE in reducing (+βME) or (B) non-reducing (-βME) conditions and were revealed by Western blot with anti-CA or anti-IN antibodies. Emission signal intensities relative to CA and IN were quantified by laser scanning of corresponding bands on an Odyssey infrared imaging system. (C) 293T cells were infected with the indicated single-round viruses (100 ng p24<sub>CA</sub>, corresponding to an moi of 0.5 for HIV-1<sub>WT</sub>) and, at the indicated time points, late reverse transcripts were quantified by real-time PCR and normalized for total DNA content. Signals detected in parallel infections with VSVg-minus viruses were subtracted from envelope-mediated infections to correct for input plasmid DNA carry over. (D) Integrated proviruses were quantified by Alu-PCR at 24 and 48 hpi. Results shown in panels A and B represent the mean ± S.D. of two independent experiments performed in duplicates. *, p<0.05 (paired t test) (E) Analysis of the ability of WT, D116A or 3KR IN expressed in trans as Vpr-fusion proteins to rescue the replication defect of integration-defective virions (NN), expressed as percentage of WT HIV-1<sub>NLX.Luc(R-)</sub> activity (56). (F) Lysates from 293T cells expressing Flag-tagged IN<sub>WT</sub> or IN<sub>3KR</sub> or IN<sub>3EQ</sub> and HA-tagged WT or D366N mutant LEDGF<sub>Cter</sub> were immunoprecipitated with an HA affinity matrix. Bound proteins were analyzed by Western blot with antibodies anti-Flag and anti-HA.

Table 1 Analysis of WT and SUMOylation-site mutant viruses release and composition a. the efficiency of viral particle release was established by p24<sub>CA</sub> ELISA on the supernatant of virus-producing cells. The values corresponding to WT viruses are arbitrarily set to 100; b. percentage of emission signals corresponding to virus-associated IN and CA measured on an Odyssey infrared imaging system; c. percentage of emission signals corresponding to virus-associated monomeric and dimeric IN, measured as in b; d. average copies viral genomic RNA per ng of p24<sub>CA</sub>. Results are the mean of three independent experiments.
Figure 1

### A

| HIV-1 IN | NTD 50 | CCD | 212 | CTD 288 |
|----------|--------|-----|-----|---------|
| HIV-1    | LKGE   | IKQE| WKGE|
| SIVcpz   | LKGE   | IQQE| WKGE|
| HIV-2    | QKGE   | IEQS| WKGE|
| SIVmac   | QKGE   | IEHT| WKGE|

### B

- **WB: α-IN**
  - S1 + S2 + S3
  - ATP/Mg²⁺
  - IN-SUMO conjugates
  - kDa: 72, 55, 36

### C

- **WB: α-IN**
  - IN
  - INΔC
  - kDa: 36, 27

### D

- **WB: α-IN**
  - IN
  - IN-SUMO conjugates
  - kDa: 36, 27

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**Legend:**
- WB: Western Blot
- α-IN: Antibody against IN
- ATP/Mg²⁺: ATP and Mg²⁺
- IN-SUMO conjugates: IN conjugated with SUMO
- kDa: Kilodaltons
Figure 2

A

| Protein | IN<sub>WT</sub>-Flag | IN<sub>3KR</sub>-Flag | IN<sub>SEQ</sub>-Flag |
|---------|---------------------|----------------------|----------------------|
| mock    | His-SUMO1          | His-SUMO2/3         | His-SUMO3           |
|        | IN-SUMO conjugates | IN-SUMO conjugates  | IN-SUMO conjugates  |

WCL:

WB: α-IN

SUMO conjugates

SUMO

WB: α-SUMO1 & α-SUMO2/3

WCL:

WB: α-IN

Ubc9

WB: α-Ubc9

B

| Protein | WT | 3KR | 3EQ |
|---------|----|-----|-----|
| IN-Flag |    |     |     |
| IN-Ub   |    |     |     |

purification on Ni-NTA beads

WCL:

WB: α-IN

IN

WB: α-Ub

UB

WB: α-Ub

C

| Protein | mock | WT | 3KR |
|---------|------|----|-----|
| IN-SUMO2/3 conjugates |

purification on Ni-NTA beads

WCL:

WB: α-SUMO2/3

longer exposure

IN-SUMO1 conjugates

WCL:

WB: α-SUMO1

AcK-IN

WCL:

WB: α-AcK

IN

WB: α-IN
**Figure 3**

**A**

WT 3KR 3EQ

**B**

Cytoplasmic Nuclear

|       | WT | 3KR | 3EQ |
|-------|----|-----|-----|
| WB: α-IN |    |     |     |
| WB: α-H2B |    |     |     |
| WB: α-LDH |    |     |     |

% IN/actin

% 100 106 109 100 94 91

**C**

|       | WT | 3KR | 3EQ |
|-------|----|-----|-----|
| WB: α-IN |    |     |     |
| WB: α-actin |    |     |     |
| WB: α-actin |    |     |     |

**D**

% remaining (IN/actin) vs time (h)

- WT
- 3KR
- 3EQ
Figure 4

A. Lentiviral vectors

B. NL4-3 + VSVg

C. NL4-3

% infected cells (U/μg p24)

| Time (days) | WT | 3KR | 3EO |
|-------------|----|-----|-----|
| 2           | 75 | 50  | 25  |
| 4           | 75 | 50  | 25  |
| 7           | 75 | 50  | 25  |
| 9           | 75 | 50  | 25  |
| 11          | 75 | 50  | 25  |
| 14          | 75 | 50  | 25  |
| 16          | 75 | 50  | 25  |

* Indicates significant difference
Figure 5

A

+ βME

WB: α-IN

WB: α-CA

- βME

WB: α-IN

B

WT 3KR 3EQ

WB: α-IN

IN

IN

C

total cDNA

copies/µg total DNA (1 E+04)

WT 3KR 3EQ

3 9 24 48

time (h)

D

integrated

copies/µg total DNA (1 E+04)

24 48

time (h)

E

% WT gene expression

NN NN + VprIN WT

IN-Flag

WT 3KR 3EQ

mock D366N

IP: α-HA matrix

WB: α-IN

HA-LEDGF_C-ter

WB: α-LEDGF_C-ter

WB: α-IN

WCL

WB: α-HA

LEDGF_C-ter