# Cellular factors that interact with acetylated integrase: new insights in HIV-1 integration

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To my family and to Hassen

**Abstract**

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The viral protein integrase (IN) catalyzes the integration of the HIV-1 cDNA into the host cellular genome. We have recently demonstrated that IN is acetylated by a cellular histone acetyltransferase, p300, which modifies three lysines located in the C-terminus of the viral factor (Cereseto et al., 2005). This modification enhances the catalytic activity and the DNA affinity of IN, as demonstrated by *in vitro* assays (Cereseto et al., 2005). Consistently, mutations introduced in the targeted lysines greatly decrease the efficiency of HIV-1 integration (Cereseto et al., 2005; Terreni et al., 2010; Apolonia et al., 2007).
Acetylation was proven to regulate protein functions by modulating protein-protein interactions. HIV-1 to efficiently complete its replication steps, including the integration reaction, requires the interaction with numerous cellular factors. Therefore, we sought to investigate whether acetylation might modulate the interaction between IN and cellular factors. To this aim we performed a yeast two-hybrid screening that differs from the screenings so far performed for using as bait IN constitutively acetylated. From this analysis we have identified thirteen cellular factors (seven are nuclear and four are cytoplasmic) involved in transcription, chromatin remodeling, nuclear transport, RNA binding, protein synthesis regulation and microtubule organization. Binding assays showed that acetylation increases the association of IN with four identified factors (KAP1 (TRIM28), Exp2, eIF3h and RanBP9), while for three two-hybrid hits (BTF3b, THRAP3 and HMGN2), the acetylation does not modulate their binding with IN. KAP1, which belongs to the TRIM family of antiviral proteins, was investigated for its role in HIV-1 infection and for the determination of the molecular mechanisms that underlie its interaction with IN. We found that KAP1 binds preferentially acetylated IN and induces its deacetylation through the formation of a protein complex including the deacetylase HDAC1. Modulation of intracellular KAP1 levels in different cell types including T-cells, the primary HIV-1 target, revealed that KAP1 curtails viral infectivity by selectively affecting HIV-1 integration. KAP1-dependent viral inhibition was found to require HDAC1 activity since cells deficient for this deacetylase were insensitive to KAP1-induced resistance. This study reveals that KAP1 is a novel cellular factor restricting HIV-1 infection and underscores the relevance of IN acetylation as a crucial step in the viral infectious cycle.

## AIMS OF THE THESIS

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that belongs to the lentivirus genus (Coffin et al., 1997). HIV-1 infects mainly CD4+ human T lymphocytes and causes the acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Popovic et al., 1984). AIDS is a human infectious disease that causes a progressive profound state of immune suppression characterized by a drop of the circulating CD4+ T lymphocyte number and leaves infected individuals susceptible to opportunistic infections and prone to develop tumors (Barre-Sinoussi et al., 1983; Popovic et al., 1984).

An essential step in the retroviral life cycle is the integration of the viral DNA into the host cellular genome; a reaction catalyzed by the viral protein integrase (IN) (Coffin et al., 1997). Purified HIV-1 integrase displays 3' end processing and DNA strand transfer activities that are sufficient to catalyze the cDNA integration reaction *in vitro* (Lewinski and Bushman, 2005; Vandegraaff and Engelman, 2007). However, *in vivo* numerous cellular proteins are required for efficient integration. The host factors regulate integrase enzymatic functions by modulating its stability and by mediating nuclear import and access to specific regions of the chromatin (Busschots et al., 2009). We have recently demonstrated that p300, a histone acetyl transferase, binds integrase and acetylates three lysines (K264, K266, K273) located in its C-terminus leading to enhanced integrase activity and DNA binding affinity (Cereseto et al., 2005). Acetylable lysines are necessary for virus integration and thus for optimal replication, as demonstrated by the inefficient infectivity observed following their mutations into arginine residues (K264, 266, 273R) (Apolonia et al., 2007; Cereseto et al., 2005; Terreni et al., 2010). Since it has been demonstrated that acetylation modulates the activities of cellular and viral proteins by affecting protein-protein interactions (Bannister et al., 2000; Berro et al., 2006; Bres et al., 2002; Das and Kundu, 2005; Dorr et al., 2002; Glozak et al., 2005; Kouzarides, 2000; Mujtaba et al., 2002; Mujtaba et al., 2004; Polesskaya and Harel-Bellan, 2001; Spange et al., 2009; Sterner and Berger, 2000), the aim of this study is to investigate whether integrase acetylation could affect its interaction with cellular factors and to reveal the possible functional role of these interactions in the HIV-1 replication cycle.

To reach this aim, four research steps were involved:

1- The identification of cellular proteins binding acetylated integrase by employing the tethered catalysis two-hybrid system in yeast, a method previously reported to efficiently identify factors binding specifically to acetylated proteins (p53, histones H3 and H4) (Acharya et al., 2005; Guo et al., 2004).
2- The validation of the interactions between acetylated integrase and the candidate cellular factors, identified from the yeast two-hybrid screening, by *in vitro* pull down assays and *in vivo* co-immunoprecipitation experiments.
3- The study of one identified candidate (KAP1 or TRIM28) through the investigation of its role in HIV-1 replication and specifically at the level of integration.
4- The search of the molecular mechanisms that underlie the role of KAP1 in HIV-1 infection.

Identification and study of novel cellular proteins interacting with HIV-1 acetylated integrase would provide more knowledge on host factors that promote or inhibit HIV-1 infection. Such information might be useful for alternative HIV-1 therapies based on the inhibition of viral and cellular protein-protein interactions when the interactions enhance HIV-1 replication or on the stimulation of the viral and cellular protein associations when the interactions impair HIV-1 infectivity (Busschots et al., 2009). In fact, the current highly active antiretroviral therapy (HAART) for HIV-1 that is based on the combination of effective drugs that inhibit specifically the viral proteins (reverse transcriptase, protease and recently integrase) resulted in the emergence of HIV-1 resistant strains. This resistance is due to the virus short replication cycle and to the high error rate of its reverse transcriptase that allow the virus to mutate very quickly (Busschots et al., 2009). Therefore, targeting interactions between cellular and viral proteins is a novel therapy strategy that might decrease the risk of HIV-1 drug resistance induced by targeting directly viral proteins (Busschots et al., 2009; Christ et al., 2010).

# INTRODUCTION

## HIV-1 replication

HIV-1 was discovered on 1983 by Montagnier's group, two years after the first description of AIDS. In this study, HIV-1 was called LAV (lymphadenopathy associated virus) and was described to be involved in several infectious syndrome including AIDS (Barre-Sinoussi et al., 1983). In 1984, Gallo's group gave also evidence that HIV-1 is the causal agent of AIDS; HIV-1 was termed as HTLVIII (Popovic et al., 1984). In 1985 came the cloning and sequencing of HIV-1 genome with identification of new open reading frames specific for lentiviruses (Ratner et al., 1985; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985). In 1986, a second retrovirus strain, known as HIV-2 and having 40-60% homology with HIV-1, was isolated from West African patients with AIDS (Clavel et al., 1986). The majority of the studies reported that HIV-2 is less pathogenic than HIV-1 and causes AIDS only in the minority of infected individuals; HIV-2 infection is mainly asymptomatic (de Silva et al., 2008).

## 1- HIV-1 genome and structure

HIV-1 is a human retrovirus that is related most closely to other animal lentiviruses (Coffin et al., 1997). Lentiviruses are "complex" retroviruses that encode a number of regulatory and accessory proteins not encoded by the genome of prototypical "simple" retrovirus (Coffin et al., 1997).

The HIV-1 genome is approximately 9 Kb in length and encodes 15 distinct proteins (**Figure 1**) (Frankel and Young, 1998). From the 5'- to 3'- ends of the genome are found the *gag* (for group-specific antigen), *pol* (for polymerase) and *env* (for envelope glycoprotein) genes. The *gag* gene encodes a polyprotein precursor, $pr55^{Gag}$ that is cleaved by the viral protease (PR) to produce Gag proteins matrix (also known as MA or p17), capsid (CA or p24), nucleocapsid (NC or p7) and p6. Two spacer peptides p2 and p1 are also generated upon $pr55^{Gag}$ processing. The *pol* gene encodes HIV-1 enzymes, protease (PR), reverse transcriptase (RT), integrase (IN) which are cleaved from $pr160^{GagPol}$ precursor by the viral protease (Frankel and Young, 1998). The envelope (Env) glycoprotein is also synthesized as a polyprotein precursor (**Figure 1**). Unlike the Gag and Pol precursors which are cleaved by the viral protease the Env precursor, known as gp160, is processed by a cellular protease (furin or furin like enzyme) during Env trafficking to the

cell surface. gp160 processing results in the generation of the surface (SU) Env glycoprotein gp120 and the trans-membrane (TM) glycoprotein gp41 (Freed, 2001). gp120 contains the determinants that interact with target cell receptor and co-receptors, while, gp41 anchors the gp120/gp41 complex in the membrane and also contains domains that are critical for catalyzing the membrane fusion reaction between viral and host lipid bilayers during virus entry (Freed, 2001). Comparison of *env* sequences from large number of virus isolates revealed that gp120 is organized into five conserved regions (C1-C5) and five highly variable domains (V1-V5). gp41 is composed of three major domains: the ectodomain (which contains determinants essential for membrane fusion), the trans-membrane anchor sequence and the cytoplasmic tail (Freed, 2001).

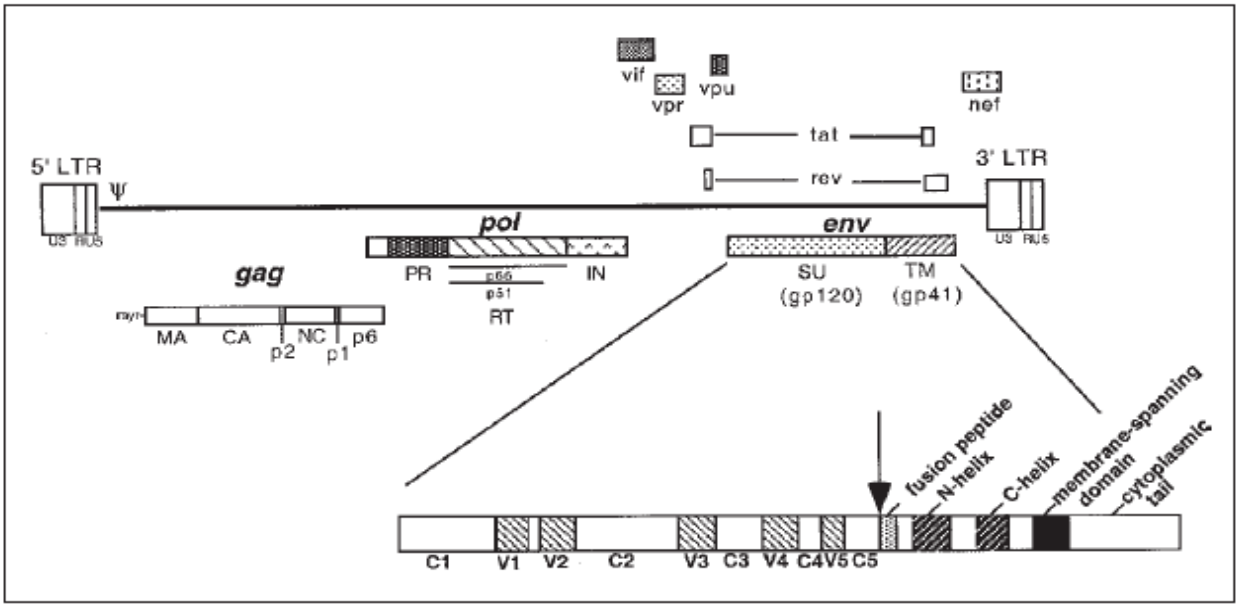


**Figure 1: .** Organization of the HIV-1 genome. The relative locations of the HIV-1 open reading frames *gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, *nef*, *tat*, and *rev* are indicated. The 5' and 3' long terminal repeats (LTRs) are shown, with U3, R, and U5 regions noted. The ψ indicates the position of the RNA packaging signal. The major Gag domains (MA, CA, NC, p6) and the Gag spacer peptides (p2 and p1) are shown under the *gag* gene. The site of Gag N-terminal myristylation is denoted as "myr". Under the *pol* gene are indicated the PR, RT (p66 and p51 sub-domains), and IN coding regions. The SU and TM Env glycoproteins (gp120 and gp41, respectively) are enlarged to show the position in gp120 of the major conserved (C1-C5) and variable (V1-V5) regions and in gp41 the location of the fusion peptide, the N- and C-helices, membrane-spanning domain, and the cytoplasmic tail (Freed, 2001).

In addition to *gag*, *pol* and *env* genes HIV-1 encodes two regulatory proteins: Tat and Rev and four accessory proteins: Vpu (viral protein u), Vpr (viral protein r), Vif (viral infectivity factor) and Nef (negative effector) (Frankel and Young, 1998) (**Figure 1**).

## 2- HIV-1 replication cycle

HIV-1 replication proceeds in a series of events that can be divided in infection phase and expression phase or early and late steps (**Figure 2**) (Freed, 2001; Peterlin and Trono, 2003).

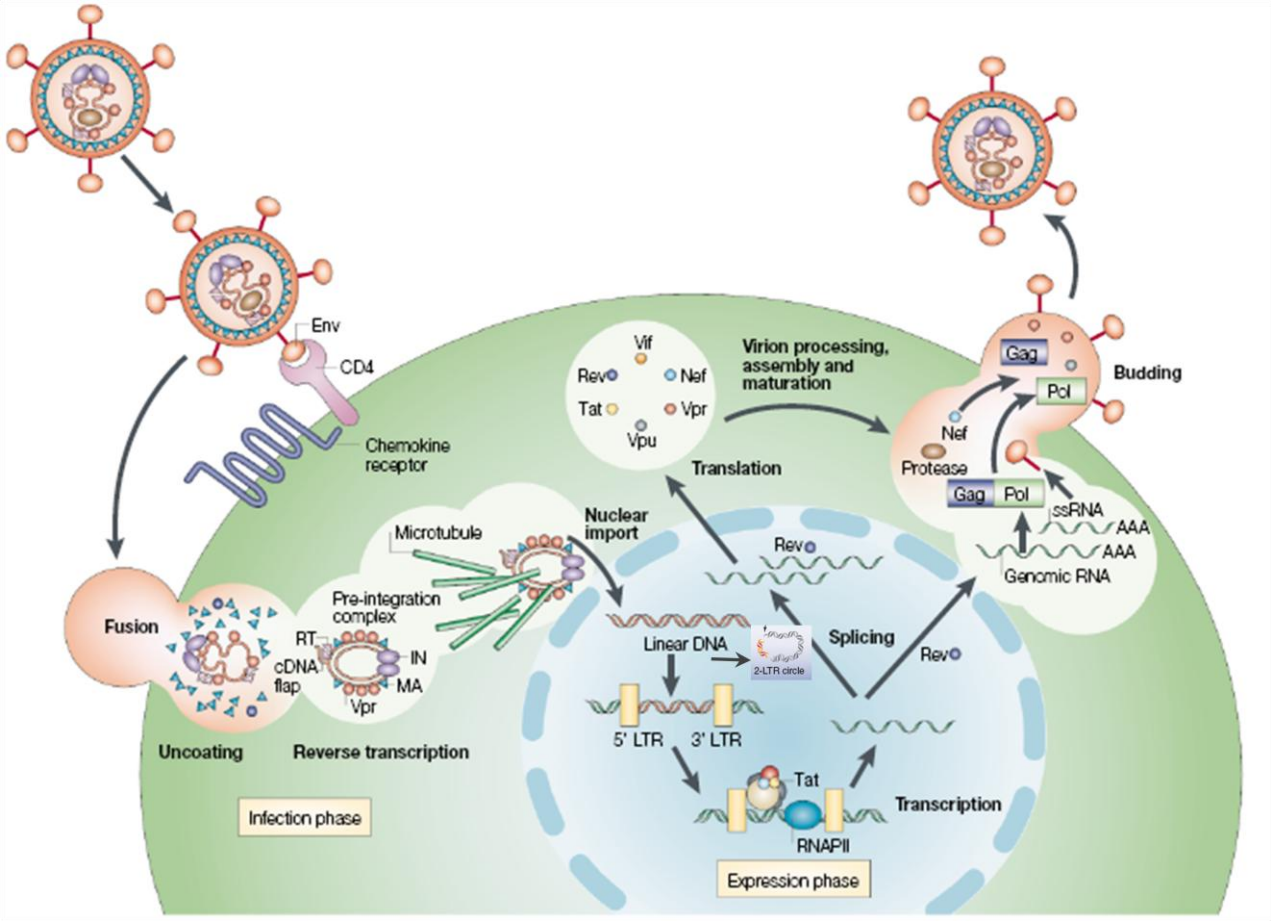


**Figure 2:** The replication cycle of HIV-1. The viral envelope protein (Env) of HIV-1 binds CD4 first, undergoes a conformational change, then binds one of two chemokine receptors — CCR5 (R5 strains) or CXCR4 (X4 strains) — and enters cells by fusion of the viral and cellular membranes. Reverse transcription begins, yielding double-stranded viral cDNA. The HIV-1 PIC enters the nucleus without cell division with the help of the viral and cellular proteins and the viral cDNA flap. Integrase (IN) catalyzes the insertion of the viral cDNA into the host geneome. The un-integrated viral cDNA is circularized in the nucleus into 1-LTR or 2-LTR circles. The provirus transcription, which is stimulated by Tat, yields the viral mRNAs. Rev transports partially spliced and unspliced genomic transcripts from the nucleus to the cytoplasm. Viral structural and enzymatic proteins are synthesized and transported to the plasma membrane, where they localize in lipid rafts. Late domains in group-specific antigen (Gag) then recruit components of multivesicular bodies to the site of budding, so that progeny virions are released from the infected cell (Peterlin and Trono, 2003).

### 2.1- Cellular entry

HIV-1 enters the body through the exchange of bodily fluids, and it infects mainly T helper cells, macrophages and to some extent microglial cells and dentritic cells (Peterlin and Trono, 2003). This tropism is determined at the level of viral entry by the use of CD4 as a

primary receptor and the use of co-receptors that are strain and target specific. R5 strains of HIV-1 use CC-chemokine receptor 5 (CCR5) as their co-receptor and can therefore enter macrophages, dentritic cells and T cells, whereas X4 strains of HIV-1 use CXCR4 co-receptors and infect only T cells (Doms and Trono, 2000). R5/X4 HIV-1 isolates have dual tropism and uses both CCR5 and CXCR4 co-receptors. The V3 loop of gp120 is demonstrated to play major role in determining HIV-1 tropism (O'Brien et al., 1990). HIV-1 entry in target cells was showed to occur mainly by viral and cellular membranes fusion that involve the following events: gp120 first binds CD4 receptors, a ternary complex composed of gp120, CD4 and co-receptor then forms, and finally conformational changes in gp41 ultimately trigger membrane fusion (Berger et al., 1999). Recent work entailing live cell imaging demonstrates that HIV-1 entry also occurs after virion endocytosis and shows that the cellular protein dynamin plays a pivotal role in this process (Miyauchi et al., 2009). Following entry, the viral core is delivered into the cytoplasm. The viral core is composed of a capsid (CA) protein shell that encapsidates the single stranded, dimeric viral RNA genome in complex with the viral nucleocapsid (NC) protein and the viral enzymes reverse transcriptase (RT) and integrase (IN) (Adamson and Freed, 2007; Ganser-Pornillos et al., 2008).

### 2.2- Reverse transcription

Following entry, the core is partially disassembled in a poorly understood process known as uncoating to form reverse transcription complex (RTC) and then pre-integration complex (PIC) (Adamson and Freed, 2007; Ganser-Pornillos et al., 2008). It should be noted that distinction between RTCs and PICs is somewhat arbitrary, since uncoating is believed to occur progressively, but PICs are usually defined as the integration competent complexes, whereas reverse transcription is incomplete in RTCs (Iordanskiy et al., 2006; Nisole and Saib, 2004). Reverse transcription, a hallmark step of retroviruses, which converts HIV-1 RNA genome into double stranded DNA, early post-infection is catalyzed by the reverse transcriptase enzyme (Coffin et al., 1997). The HIV-1 reverse transcriptase is a heterodimer of two subunits p66 and p51 domains (Freed, 2001). These two subunits are both derived from $Pr160^{GagPol}$ precursor protein; p51 is formed when the C-terminal, 15 KDa RNaseH domain of p66 is removed by viral protease. The HIV-1 reverse transcription is primed by the annealing of the cellular $tRNA^{Lys3}$ to the primer binding site (PBS) at the 5' end of the viral RNA downstream the LTR and involves "jumps" from one template to

another along the viral RNA (Kleiman, 2002). Reverse transcription generates a formation of a trimeric structure in the middle of HIV-1 cDNA called "central DNA flap" which has been demonstrated to play an important role in the PIC nuclear import and in HIV-1 replication (De Rijck and Debyser, 2006; Zennou et al., 2000). HIV-1 reverse transcriptase enzyme has a high mutation rate ($3 \times 10^{-5}$ per cycle of replication) resulting in highly heterogeneous HIV-1 sequence populations (Mansky and Temin, 1995). As a consequence, HIV-1 is able to rapidly evade host immune response and develop resistance to antiviral drugs.

### 2.3- Nuclear import

Unlike other retroviruses, HIV-1 does not require disintegration of the nuclear membrane during cell division to enter nucleus, thus it replicates efficiently in non-dividing cells such as differentiated macrophages and dentritic cells (Fassati, 2006). Moreover, HIV-1 was found to be able to infect cells arrested in cell cycle by treatments with aphidicolin or $\gamma$-irradiation and HIV-1 derived vectors infect hematopoietic stem cells and neurons (Fassati, 2006). The viral DNA is eventually transported to the nucleus as part of pre-integration complex (PIC). Nevertheless, the composition of the PIC and the mechanism by which the PIC translocates into the nucleus are still subject of debate (Fassati, 2006; Yamashita and Emerman, 2006). Matrix, Vpr and integrase were demonstrated to be components of the PIC and reported to contain several putative nuclear localization signals (NLSs) that showed to be important for the PIC nuclear import in non-dividing cells (Bukrinsky et al., 1993; Heinzinger et al., 1994; Popov et al., 1998; von Schwedler et al., 1994). However, there is no agreement in the existence of NLSs in HIV-1 matrix argued by the similar effect of HIV-1 mutant viruses in matrix putative NLS on nuclear import of the PICs in cycling and non-dividing cells (Depienne et al., 2000; Fouchier et al., 1997; Freed et al., 1995). The transferable NLSs on Vpr protein were extensively confirmed but their role in HIV-1 PIC nuclear import in non-dividing cells is under discussion since Vpr mutations in NLSs reduce mildly the ability of HIV-1 to replicate in macrophages (Fouchier et al., 1998; Le Rouzic and Benichou, 2005; Rey et al., 1998). The role of integrase in HIV-1 PIC nuclear import was also widely investigated and many reports attributed its role to its cellular interacting factors (see chapter HIV-1 replication section n°5).

## 2.4- Integration

Following nuclear import of the viral pre-integration complex, the 32 KDa integrase catalyzes the insertion of the linear double stranded viral DNA into the host cell chromosome preferentially (see chapter HIV-1 replication section n°3) (Lewinski and Bushman, 2005; Vandegraaff and Engelman, 2007).

In resting lymphocytes there are several barriers that preclude the completion of the early HIV-1 replication steps, described above, as an incomplete reverse transcription (Zack et al., 1990; Zack et al., 1992) or an inefficient nuclear import of the PICs (Bukrinsky et al., 1992). It has been also shown that in resting cells double stranded viral cDNA accumulate extra-chromosomally unable to integrate (Stevenson et al., 1990). Nevertheless, once they have been activated, even partially, T cells become fully permissive for HIV-1 infection. A recent study described that in activated T cells, HIV-1 integrase is phophorylated by c-jun kinase (JNK) and then stabilized by Pin1 isomerization which allow the virus to achieve an efficient nuclear import and integration (Manganaro et al., 2010). The lack of these integrase modifications in resting T cells, due to the dowregulated levels of JNK in these cells, contributes to the non-permessivness to HIV-1 infection.

## 2.5- Gene expression

Once integrated into the host genome, the provirus behaves like any human gene, with transcription being initiated at the 5' end and terminating at the 3' end. The 5' LTR contains enhancer and promoter sequences, with binding sites for several transcription factors and the 3' LTR contains a polyadenylation signal (Peterlin and Trono, 2003). Moving upstream from the transcription start site, the initiator, the TATA BOX and three SP1-binding sites are found (Peterlin and Trono, 2003). These elements position RNA polymerase II at the correct site for initiating transcription. In the absence of Tat, HIV-1 transcription begins but elongation is inefficient (Jones and Peterlin, 1994). Tat acts upon an RNA structure known as transactivation response region (TAR) which is found at the 5' end of all the viral transcripts (Berkhout et al., 1989). Tat binds TAR and recruits the positive elongation transcription factor b (P-TEFb) complex that contains cyclin T1 (CYCT1) and cyclin dependent kinase 9 (CDK9) heterodimer (Wei et al., 1998). The recruitment of P-TEFb to TAR results in the phosphorylation of the Carboxy-terminal domain of RNA polymerase II by CDK9 and a dramatic stimulation of transcription elongation (Wei et al., 1998). Memory CD4 lymphocytes have been demonstrated to be

involved in the establishment of a latent reservoir of infected cells harbouring a silent integrated provirus (Chun et al., 1995; Han et al., 2007). The mechanisms of HIV-1 post-integration latency are poorly understood, however, many studies suggested a main role of a transcription inhibition involving Tat and P-TEFb (Ghose et al., 2001; Kao et al., 1987; Marcello, 2006).

Transcription from HIV-1 LTR leads to the generation of more than 30 viral mRNAs (Freed, 2001). These fall into three major classes: 1) unspliced RNAs which function as the mRNAs for Gag and GagPol and are packaged into progeny as genomic RNA; 2) partially spliced mRNAs encoding to Env, Vif, Vpu and Vpr proteins and 3) multiply spliced mRNAs which are translated into Rev, Tat and Nef (Freed, 2001; Peterlin and Trono, 2003). To export the unspliced and partially spliced viral mRNAs from nucleus to cytoplasm, HIV-1 uses the Rev protein which acts on the *cis*-acting RNA element, the Rev responsive element (RRE) (Freed, 2001; Peterlin and Trono, 2003). RRE is a highly stem-looped RNA structure located in the *env* gene and is present in all unspliced and partially spliced HIV-1 mRNAs (Pollard and Malim, 1998). Rev binds RRE and forms a complex capable of the interaction with the cellular nuclear export machinery resulting in the export of HIV-1 mRNAs to the cytoplasm and then Rev shuttles back to the nucleus using its nuclear localization signal (Pollard and Malim, 1998).

### 2.6- Assembly and release

Following the synthesis of the full component of viral proteins using the cellular translation machinery, the assembly process begins within the plasma membrane (Adamson and Freed, 2007). Assembly is directed by Gag, which coordinates the incorporation of each of the viral components, together with the number of host cell factors, into the assembling particle (Adamson and Freed, 2007). The N-terminus domain of matrix is co-translationally modified by myristic acid; this fatty acid modification is essential for Gag-membrane binding. Capsid, the central domain of Gag, homo-oligomerizes in an ordered manner during assembly and is a critical determinant of particle morphology (Bieniasz, 2009). The GagPol precursor ($Pr160^{GagPol}$), which is synthesized as the result of a frame shifting event, is packaged into virions via its Gag domain, largely using the same Gag-Gag interactions that drive Gag assembly (Freed, 2001). The zing finger motifs and basic residues domain of nucleocapsid bind specifically the RNA packaging signal located in 5' of the *gag* initiation codon and encapsidates two positive single-stranded copies of the genomic RNA

into each viral particle (Freed, 2001). The Env glycoprotein precursor, gp160, is synthesized in the rough endoplasmic reticulum where gp120 domain is heavily glycosylated and oligomerized (Freed, 2001). gp160 is transported to the cell surface via the secretory pathway; during its trafficking through the Golgi, gp160 is cleaved by a host protease (furin or furin like enzyme) to generate the mature envelope glycoproteins, gp120 and gp41 (Freed, 2001). Although, the process by which the Env glycoproteins are incorporated into virions remains incompletely understood, a number of lines of evidence suggest that a direct or mediated interaction between the gp41 cytoplasmic tail and matrix domain of Gag recruits Env into virions (Lopez-Verges et al., 2006; Murakami and Freed, 2000). HIV-1 Gag particles are also able to incorporate heterologous glycoprotein envelopes such as amphotropic Env of murine leukemia virus (A-MLV), VSV-G of vesicular stomatitis virus (VSV) (Reiser et al., 1996), and many other viral envelopes (e.g. Ebola virus).

Virus particle production is completed upon budding of the nascent virion from the plasma membrane (Adamson and Freed, 2007). To facilitate virus release, the p6 domain of Gag hijacks components of the cellular endosomal sorting machinery complexes (ESCRTs), which normally function to promote the budding of vescicles into late endosomes to form multi-vescicular bodies (Bieniasz, 2009). The p6 domain of Gag contains two small peptides, called "late domains" PTAP and YPLTSL which bind Tsg101 (a component of ESCRTI) and ALIX (an ESCRT-I and ESCRT-III binding protein), respectively and trigger viral budding (Bieniasz, 2009). Concomitant with virus release, viral protease (PR) cleaves Gag and GagPol precursors into their respective protein domains leading to virion maturation. Following cleavage, capsid forms a conical shell around the RNA/protein complex within the core, a hallmark of mature HIV-1 virions (Freed, 2001).

### 2.7- Roles of accessory proteins in HIV-1 replication

In addition to the roles of HIV-1 structural (MA, CA, NC and Env), enzymatic (PR, RT and IN) and regulatory (Tat and Rev) proteins in the HIV-1 replication, HIV-1 accessory proteins (Vpu, Vif, Vpr and Nef) were shown to increase markedly HIV-1 infectivity and production. These proteins are known as "accessory" proteins because they are dispensable for virus growth in some cell culture. Nevertheless, they have essential roles in viral replication and progression to AIDS *in vivo* (Peterlin and Trono, 2003).

**Vpu** (viral protein u) is unique to HIV-1 except the highly related lentivirus chimpanzee simian immunodeficiency virus (SIVcpz). Vpu stimulates the viral release of budded particles from the plasma membrane and induces CD4 degradation in order to liberate gp160 from Env/CD4 complexes in the endoplasmic reticulum thereby increasing the amount of Env glycoprotein available for transport to cell surface (Freed, 2001). The virus particle release of HIV-1 viruses defective in Vpu is inhibited in certain human cells (such as HeLa) and is not affected in other human (for example HOS, HEK293T and HT1080) or in African green monkeys (COS-7) cell lines (Neil et al., 2006; Neil et al., 2007; Varthakavi et al., 2003).

**Vif** (viral infectivity factor) is conserved among lentiviruses except equine infectious anaemia virus (EIAV) (Freed, 2001). HIV-1 deleted in Vif is defective in viral cDNA synthesis (von Schwedler et al., 1993). The defective phenotype is cell-type dependent and is determined not by the target cells but by the virus producing cells (von Schwedler et al., 1993). For instance, HeLa, HEK293T, SupT1, CEMss and Jurkart cell lines are "permissive" for Vif deleted viruses; virus produced from these cell lines is fully infectious regardless of the target cell used (Gabuzda et al., 1992; Madani and Kabat, 1998; Simon et al., 1998). In contrast, CEM T cells, primary lymphocytes and macrophages are "non permissive" cells (Gabuzda et al., 1992; Madani and Kabat, 1998; Simon et al., 1998). The cell type dependency of the permissiveness to HIV-1 defective in Vpu or in Vif has suggested for long time the existence of specific cellular restriction factors that are counteracted by these accessory proteins. Recent studies have revealed new HIV-1 restriction factors tetherin (CD317/BST2) and APOBEC3G (CEM15) that are antagonized respectively by Vpu and Vif in the "non permissive" cells types (see chapter HIV-1 restriction factors) (Neil et al., 2008; Sheehy et al., 2002).

**Vpr** (viral protein r) is incorporated into the viral particles through its specific interaction with a Leucine rich motif located near the C-terminus of p6 (Freed, 2001). Vpr was found in the PIC and shown to promote HIV-1 nuclear import (Heinzinger et al., 1994). Vpr was reported to induce cell arrest in G2 phase probably to delay or to prevent apoptosis of infected cells (He et al., 1995).

**Nef** (negative effecter) is a membrane-associated protein that is expressed at high levels in infected cells and was shown to stimulate HIV-1 pathogenesis progression by stimulating the viral load in the infected individuals (Deacon et al., 1995). Nef was reported to down-regulate the cell-surface expression of the major histocompatibility

complex I (MHC-I) and of the CD4 chemokine receptors (Aiken et al., 1994; Collins et al., 1998). The correlation between Nef functions and the induction of the disease *in vivo* is not yet established, although CD4 down-regulation may prevent super-infection and impair the functions of T helper cells and MHC-I down-modulation may impair the cytotoxic T lymphocytes (CTLs) to detect and eliminate virus-expressing cells (Adamson and Freed, 2010).

In every step of its life cycle HIV-1 takes advantage of host cell factors and pathways to promote successful replication (Adamson and Freed, 2010; Al-Mawsawi and Neamati, 2007; Goff, 2007; Suzuki and Craigie, 2007; Van Maele et al., 2006; Vandegraaff and Engelman, 2007). However, it has become clear in recent years that the host cell has set up antiretroviral barriers in the form of restriction factors that impair specific steps in the replication cycle (chapter HIV-1 restriction factors) (Bieniasz, 2007; Goila-Gaur and Strebel, 2008; Huthoff and Towers, 2008; Malim, 2009; Nakayama and Shioda, 2010; Nisole et al., 2005; Strebel et al., 2009). In some cases, HIV-1 have responded by evolving counter-defense mechanisms to overcome these restriction factors (Neil et al., 2008; Sheehy et al., 2002).

## 3- HIV-1 integration

For an efficient production of progeny virions, the retroviral DNA must become covalently integrated into the host cell chromosome (Coffin et al., 1997). Some expression from un-integrated viral DNA can be detected but is not sufficient to sustain spreading infection (Engelman et al., 1995). The study of integrase mutants revealed that viral integrase (IN) encoded by the C-terminal *pol* gene is essential to catalyze retroviral cDNA insertion into the host genome (Panganiban and Temin, 1984).

### 3.1- Integration steps

Integration of HIV-1 cDNA into the host cell chromosome involves three main steps (**Figure 3**): 1) 3' processing of the viral DNA ends, 2) DNA strand transfer by joining the processed viral ends to the cellular target DNA and 3) the repair of the gaps of the recombination intermediate. The first two reactions are catalyzed by integrase, whereas the last is mediated by still undefined factors likely cellular DNA repair proteins (Lewinski and Bushman, 2005). Reverse transcription yields a copy of the long terminal repeat (LTR) at each end of the nascent reverse transcript. Integrase binds to short DNA sequences

called attachment sites at the U3 and U5 viral DNA ends of respectively 5′ and 3′ LTRs and cleaves two nucleotides from each 3′ termini end (Katzman and Katz, 1999; Li et al., 2006). Following terminal cleavage, a recessed hydroxyl group (3′-OH) is exposed that immediately follows a CA dinucleotide (**Figure 3a**). This CA motif is the more conserved among retroviruses and many related transposons and it showed fundamental for initial integrase-viral DNA binding (Esposito and Craigie, 1998; Lewinski and Bushman, 2005). The 3′ processing reaction is a defining moment in the formation of the pre-integration complex (PIC) (Vandegraaff et al., 2006). PICs are nucleoproteins complex isolated from acutely infected cells and able to catalyze endogenous retroviral cDNA integration inside a heterologous target DNA *in vitro* (Farnet and Haseltine, 1990). Only purified PICs containing viral cDNA correctly cleaved by integrase at the 3′ ends were demonstrated competent to integrate *in vitro* (Miller et al., 1997).

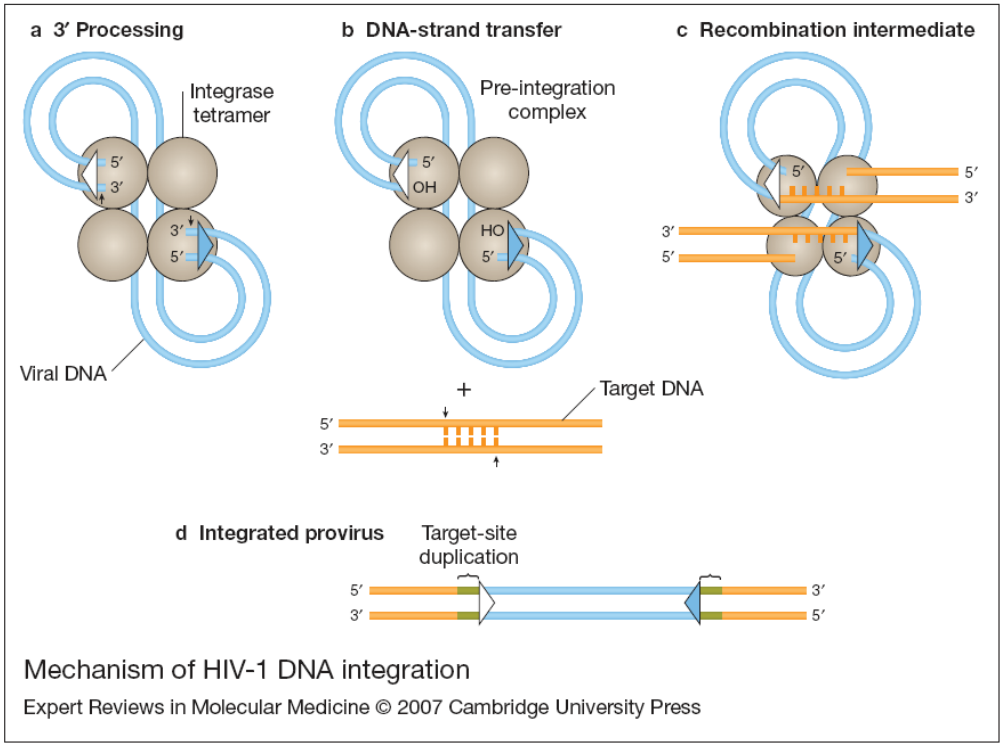


**Figure 3**: Mechanism of HIV-1 DNA integration. (**a**) A tetramer of integrase (grey circles) engages two ends of human immunodeficiency virus type 1 (HIV-1)DNA(blue lines) within a synaptic nucleoprotein complex. During 3′ processing, water is used by integrase to effect hydrolytic clips (vertical arrows), which trim off dinucleotides from both HIV-1 ends. Opened triangle, U3 DNA sequences in the upstream LTR recognised by integrase; closed triangle, downstream U5 sequences recognised by integrase. (**b**) After nuclear entry and locating a suitable target acceptor site within chromatin (orange lines), integrase uses the 3′-hydroxyl groups of the cleaved viral DNA to cut the target in a staggered fashion (vertical arrows), which concomitantly joins the viral 3′ ends to the 5′-phosphates of the cut. (**c**) The recombination intermediate formed by integrase's DNA-strand-transfer activity comprises joined viral 3′ends but free 5′ends. (**d**) Gap repair of the DNA recombination intermediate yields the integrated provirus flanked by a 5 bp duplication of target DNA (bracketed green lines). The sequence of the target-site duplication is defined by the sequence of the double-stranded staggered cut in panel (**b)** (Vandegraaff and Engelman, 2007).

In cells, PICs must access the nucleus to target chromosomes for integration. Unlike the "simple" gammaretroviruses, such as the Moloney murine leukemia virus (M-MLV), which infection is restricted to cycling cells (Roe et al., 1993), lentiviruses including HIV-1 infect cycling and non-dividing cells (Lewis et al., 1992). Although, several mechanisms were suggested about the ability of HIV-1 to infect non-dividing cells explaining the nuclear import of the PICs, the consensus mechanism is far from complete (Fassati, 2006; Yamashita and Emerman, 2006). The role of integrase and its cellular interacting factors in HIV-1 PIC nuclear import are discussed below in the section N° (5.4-) of this chapter. Once inside the nucleus, the PIC must engage a DNA sequence within chromatin for integrase to catalyse DNA-strand transfer. In this trans-esterification reaction integrase first uses the 3'-OH groups at the viral DNA ends to attack phosphodiester bonds on opposite strands of the target DNA at positions staggered by five nucleotide bases in the 5' direction and then uses the energy of the broken phosphodiester bonds to join covalently the recessed 3' ends of the viral cDNA to the 5' phosphates of the cleaved chromosomal DNA (**Figure 3b**) (Vink et al., 1990).

### 3.2- Post-integration repair

The product of DNA-strand transfer reaction is a recombination intermediate harboring single-stranded gaps flanking either side of the virus. Gap repair of the DNA recombination intermediate yields the integrated provirus flanked by a 5 bp duplication of target DNA (**Figures 3c** and **d**) (Vink et al., 1990). The gap repair consists on filling in the missing nucleotides, removing the short flap on the 5' ends of the viral DNA, ligation to newly synthesized 3' end of the host DNA and, likely, reconstitution of appropriate chromatin structure and composition. The mechanisms of this post-integration repair are not yet fully understood. Studies based on depletion of DNA repair proteins and monitoring of cell death following retroviral infection, as surrogate marker of defective DNA repair, suggested the involvement of components of non homologous end joining repair pathway (NHEJ) such as DNA-PK, Ku, XRCC4 and ligase IV (Daniel et al., 2004; Daniel et al., 1999). In addition, it has been also suggested that the gaps of the integration intermediate are sensed by ATM and ATR kinases, usually activated by DNA double-strand breaks (DSB), which in turn trigger the DNA repair response by activating the NHEJ pathway (Daniel et al., 2003; Daniel et al., 2001; Daniel et al., 2005; Lau et al., 2005). Nevertheless, the described roles of DNA-PK, Ku, ATM and ATR in HIV-1 replication were criticized in several

reports. On one hand, because the NHEJ associated proteins are known to repair DSBs and not single-stranded gaps and on the other hand because no effect on HIV-1 infectivity was shown following depletion of these proteins in different cell types (Ariumi et al., 2005; Baekelandt et al., 2000; Dehart et al., 2005). Another studies proposed that the activation of the NHEJ pathway is due to the un-integrated viral cDNA ends sensed by cells as DSB instead of the un-repaired proviruses (Jeanson et al., 2002; Kilzer et al., 2003; Li et al., 2001). In these reports it has been shown that NHEJ proteins, specifically Ku, XRCC4 and ligase IV, catalyze the ligation of the 5' and 3' LTRs leading to the formation of the 2-LTR circles (Jeanson et al., 2002; Kilzer et al., 2003; Li et al., 2001). The circularization of the viral cDNA was proposed as a way to prevent cell apoptosis induced by the un-integrated DNA (Kilzer et al., 2003; Li et al., 2001).

### 3.3- The un-integrated viral cDNA forms

Not all reverse transcribed retroviral cDNAs are integrated into host genome (Coffin et al., 1997). It has been estimated that about 5% and 15% of total HIV-1 reverse transcripts are integrated in HEK293T and in SupT1 cells, respectively (Butler et al., 2001). The un-integrated viral cDNA is usually circularized by non homologous end joining repair (NHEJ) to form 2-LTR circles or by homologous recombination between the 5' and 3' LTRs to form the 1-LTR circles (Kilzer et al., 2003; Li et al., 2001). A third circularized un-integrated viral cDNA, that does not require the cellular DNA repair machinery, is formed by the integration of the viral cDNA inside itself, yielding internally rearranged circular forms (Coffin et al., 1997). The formation of circularized viral cDNA is believed to be the result of failure in integration process since retroviruses carrying integrase mutants catalytically inactive are characterized by the nuclear accumulation of the 1-LTR and 2-LTRs circles (Coffin et al., 1997; Engelman, 1999; Engelman et al., 1995; Jurriaans et al., 1992). These circle un-integrated forms can be transiently expressed but they are unable to sustain a spreading infection (Engelman, 1999; Engelman et al., 1995). The exclusively nuclear location of the 1-LTR and 2-LTRs circles has been confirmed among diverse combinations of retroviruses and host cells, therefore, they are used as a surrogate marker for retroviruses nuclear import (Coffin et al., 1997).

### 3.4- Integration site selection

Retroviral DNA integration is not tightly sequence specific, however, integration site selection is not random. Genome-wide surveys of retroviral DNA-integration site selection revealed that retroviruses target the genome in different ways (Bushman et al., 2005). Lentiviruses like HIV-1 prefer to integrate into genes, displaying a greater propensity for active genes, whereas, gammaretroviruses such as M-MLV display marginal preference for genes and integrate preferentially within 5 Kb of either side of transcriptional start site (Mitchell et al., 2004; Schroder et al., 2002; Wu et al., 2003). Consistently with the preference of HIV-1 to integrate in transcription units, these studies showed that human endogenous retroviruses (HERVs), long interspersed nuclear elements (LINEs) and $\alpha$-satellite DNA, which are all genome DNA sequences repeats depleted from gene-rich regions and enriched in heterochromatin regions, strongly disfavor HIV-1 integration. Moreover, recent studies performed by sequence analysis using the ENCODE annotation (Wang et al., 2007b) and by a visualization analysis (Albanese et al., 2008) demonstrated that HIV-1 targets decondensed regions of the chromatin. All these lines of evidence suggest that one the mechanisms that define the HIV-1 integration site selection is the accessibility of the host DNA defined by the chromatin structure. HIV-1 integrase would also seem to mediate global access to specific regions of chromatin. An HIV-1-M-MLV chimera virus carrying gammaretroviral integrase targeted integration to regions nearby gene start sites similar to wild type M-MLV (Lewinski et al., 2006). LEDGF/p75, a cellular protein that binds tightly HIV-1 integrase, has been reported to be involved in HIV-1 targeting. LEDGF/p75 partial depletion from human cells diminished HIV-1 integration in transcription units without changing its preference to active genes (Ciuffi et al., 2005). More recent studies using *LEDGF* knockout mouse cells or more intensified LEDGF/p75 knockdown human cells showed a greater reduction of HIV-1 integration in transcription units and an increase of integration near transcription start sites, as M-MLV, and in CpG islands, normally disfavored for HIV-1 integration (Marshall et al., 2007; Shun et al., 2007). Although in both studies integration remained still favored in transcription units, the significant reduction of the integration events in genes in different cells types attributed a predominant role of LEDGF/p75 in lentiviral target site selection (Shun et al., 2007). Importantly, LEDGF/p75 demonstrated to bind exclusively lentiviral integrases and not integrases from other retrovirus *genera* (Busschots et al., 2005; Cherepanov, 2007; Llano et al., 2004b). Moreover, another integrase binding factor the histone acetyl

transferase p300 which is a broad transcriptional co-activator has been proposed to be a good candidate to target HIV-1 integration in active gene (Cereseto et al., 2005; Van Maele et al., 2006; Vandegraaff and Engelman, 2007). Integrase interactor 1 (INI1) is a component of SWI/SNF chromatin remodeling complex that interacts with integrase has been also hypothesized as integration targeting factor (Kalpana et al., 1994). However, INI1 binds only HIV-1 integrase and not integrases from related lentiviruses that integrate preferentially in active genes, such as SIV, which reduced its potentiality as integration site selection factor (Vandegraaff and Engelman, 2007; Yung et al., 2004).

## 4- HIV-1 integrase structure and functions

HIV-1 integrase catalyzes two essential reactions for the virus integration into host genome: the 3' end processing and DNA strand transfer (**Figure 3**). Purified HIV-1 integrase is able to catalyze *in vitro* the 3' processing and integration of recombinant DNA substrates that mimic the ends of the viral reverse transcripts (Bushman and Craigie, 1991). HIV-1 integrase belongs to a protein super-family of nucleotidyl transferases that include RNase H, Holliday junction resolvase (RuvC), bacterial Mu and Tn5 transposases and RAG1/2 recombinase (Dyda et al., 1994; Rice and Baker, 2001; van Gent et al., 1996). These enzymes break and/or join nucleic acids via their phosphodiester backbones. Moreover, they have active sites that harbor conserved amino acid residues aspartic acid (D) and glutamic acid (E) that coordinate $Mg^{2+}$ or $Mn^{2+}$ metal ions to catalyze bimolecular in-line nucleophilic substitution reactions (Sn2) at the scissile phosphodiester bond (Dyda et al., 1994).

### 4.1- Integrase domains

HIV-1 integrase comprises three protein domains (**Figure 4**): the N-terminal domain (NTD), the catalytic core domain (CCD) and the C-terminal domain (CTD), which function together to catalyze 3' processing and DNA strand transfer (Engelman et al., 1993; van Gent et al., 1993). The **NTD** (1-49 amino acid residues) adopts a helix-turn-helix protein fold and contains a pair of conserved histidine and cysteine residues (HHCC zinc binding motif) that coordinate a single zinc atom ($Zn^{2+}$) (Cai et al., 1997). Zinc binding contributes to proper integrase multimerization and catalytic function (Lee et al., 1997; Zheng et al., 1996).

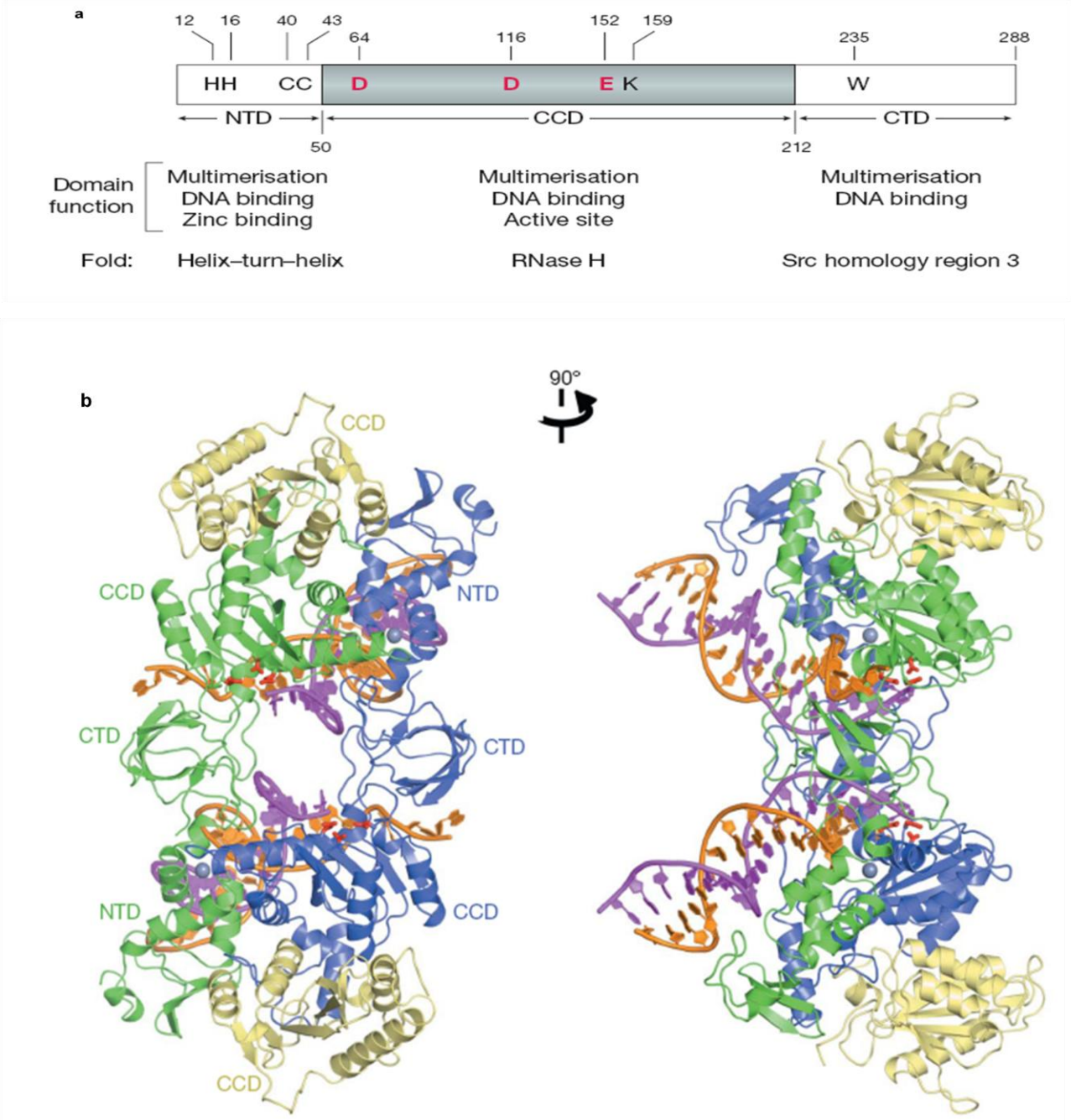


**Figure 4**: Retroviral integrase protein domains and structures. Domain organisation and amino acid residues conserved among retroviral integrase proteins. The enzyme comprises the N-terminal domain (NTD, HIV-1 residues 1–49), catalytic core domain (CCD, residues 50–212) and C-terminal domain (CTD, residues 213–288). The histidine (H) and cysteine (C) residues within the NTD are additionally conserved among retrotransposon integrase proteins. The aspartic acid (D) and glutamic acid (E) residues in the CCD form the DDE motif (red font) that likewise forms the catalytic centers of retrotransposon integrases and some bacterial transposases (Vandegraaff et al., 2006). (**b**) Structure of the prototype foamy virus integrase in complex with recombinant DNA mimicking processed LTR (PFV intasome). Views along (left) and perpendicular to (right) the crystallographic two-fold axis. The inner subunits of the IN tetramer, engaged with viral DNA, are blue and green; outer IN chains are yellow. The reactive and non-transferred DNA strands are magenta and orange, respectively. Side chains of D128, D185 and E221 active-site residues are red sticks; $Zn^{2+}$ atoms are grey spheres. Locations of the canonical IN domains (NTD, CCD and CTD) are indicated (Hare et al., 2010).

The **CCD** (50-212 amino acids residues) is composed of mixed alpha helix and beta sheets and harbors three acidic residues D64, D116 and E152 known as DDE active site responsible for coordination of a pair of $Mg^{2+}$ ions for Sn2 chemistry (Dyda et al., 1994; Engelman and Craigie, 1992; Engelman et al., 1995; van Gent et al., 1992). Site-directed mutagenesis of conserved amino acids (DDE) in the catalytic core resulted in integrase catalytically inactive in 3' end processing and DNA strand transfer (Engelman and Craigie, 1992; van Gent et al., 1992). HIV-1 viruses carrying mutations in the DDE active site, called class I IN mutants, are integration defective and characterized by the accumulation of the dead end products (1-LTR and 2-LTRs circles) (Engelman, 1999). In addition to catalysis of the integration reactions, CCD contains conserved amino acid residues tyrosine 143 (Y143) and glutamine (Q148 ) that showed responsible for the binding to the viral DNA ends *in vitro* (Esposito and Craigie, 1998). Moreover, lysines 156 and 159 (K156 and K159), were suggested to be essential for the interaction between integrase and viral LTRs (Jenkins et al., 1997). HIV-1 virus containing mutations in K156 and K159 residues is integration defective (Jenkins et al., 1997). Furthermore, the serine 119 (S119) of CCD was shown to critical for the integrase-cellular DNA interaction and target site selection (Harper et al., 2001).

The **CTD** (213-288 amino acid residues) which is the least conserved among the three integrase domains, adopts a Src homology region 3 (SH3) protein fold (Vandegraaff and Engelman, 2007). SH3 domains are small (approximately 60 amino acids) structures that are involved in protein-protein interactions and in signal transduction pathways (Mayer, 2001). The CTD binds the viral cDNA ends and might also contribute to binding of chromosomal DNA during integration (Engelman et al., 1994). The CTD has strong but nonspecific DNA binding activity and thus has been called DNA binding domain (Engelman et al., 1994). The minimal region of CTD required for DNA binding comprises residues from 220 to 270 and mutations in K264 showed a strong reduction of integrase DNA binding and catalytic activities *in vitro* (Lutzke et al., 1994). In addition to K264, the CTD domain contains other amino acid residues, that have been identified important for DNA binding by protein-DNA cross-linking assays: E246, K258, P261, R262 and with some weaker involvement : S230 and R231 (Gao et al., 2001). Moreover, mutations in leucines 241 and 242 (L241 and L242) along CTD dimer interface have been shown to disrupt integrase tetramerization and to reduce its catalytic activity *in vitro* (Lutzke and Plasterk, 1998). Interestingly, lysines K264, K266 and K273 in the CTD are acetylated by the

histone acetyl transferase p300 (Cereseto et al., 2005). Moreover, GCN5 HAT acetylates these lysines and also K258 (Terreni et al., 2010). Acetylation of the CTD enhances DNA binding affinity and catalytic activity of integrase (Cereseto et al., 2005; Terreni et al., 2010).

### 4.2- Integrase structure

The three-dimensional structure of full length HIV-1 integrase either separately or in complex with viral DNA is still not determined due to the insolubility of the protein which hampered crystallization assays. The introduction of mutation points inside the integrase protein, that increase protein solubility, led to the resolution of single domains or fused domains crystals. The isolated NTD, CCD, CTD, NTD-CCD and CCD-CTD showed self association proprieties usually in dimers (Jaskolski et al., 2009). Integrase has been shown to function in multimeric form since mixing deleted mutants, each individually inactive, was sufficient to catalyze 3' end processing and DNA strand transfer *in vitro* (Engelman et al., 1993). Furthermore, a catalytically inactive integrase, mutant in DDE motif, could be complemented by an inactive integrase truncated at its C-terminal domain (van Gent et al., 1993). Such functional complementation was also reported in virions (Fletcher et al., 1997). Based on biochemical, mutagenesis and atomic force microscopy assays, the most suggested functional form of HIV-1 integrase is a tetramer (Faure et al., 2005; Li et al., 2006; Ren et al., 2007). Moreover, a recent study that resolved the crystal structure of NTD-CCD in complex with the integrase binding domain (IBD) of LEDGF/p75 showed a tetrameric structure of integrase composed of two dimers that were flexibly stabilized inside the integrase tetramer via salt bridges and hydrophobic interactions involving residues of NTD and CCD from each dimer (Hare et al., 2009). NTD-CCD tetramer structure showed an important role of salt bridge interaction between residues E11 of NTD and K186 of CCD in maintaining dimer-dimer interface stability (Hare et al., 2009). Interestingly, single point mutations of these residues E11K and K186E separately or in the same protein abolished concerted integration *in vitro* and viral infectivity by disturbing the integrase tetramer structure (Hare et al., 2009). However, complementation assays by mixing equal quantities of each integrase mutant or by introducing E11K and K186E mutations in the same protein restored the salt bridge interaction of the dimer-dimer interface and thus restored the concerted integration and the viral infectivity (Hare et al., 2009). In the same study, the addition of the IBD LEDGF/p75 stabilized the integrase

tetramer and rescued some activities of E11K and K186E IN mutants *in vitro*. A more recent study succeed to report for the first time a crystal structure of a full length retroviral integrase of prototype foamy virus (PFV) in complex with its cognate pre-processed viral DNA; all the structure was called PFV intasome (**Figure 4b**) (Hare et al., 2010). The integrase-DNA complex structure revealed a tetramer of integrase associated with a pair of viral DNA ends (Hare et al., 2010). As previously reported for HIV-1 NTD-CCD structures (Hare et al., 2009), the integrase tetramer was formed by a pair of dimers stabilized by intermolecular NTD-CCD interactions, however, the dimer-dimer interface was constrained and not flexible like in HIV-1 NTD-CCD structure. The inner subunits of the tetramer were responsible for all contacts involved in tetramerization and viral DNA binding. The CCDs of the outer subunits seemed to provide supporting function. In this intasome, the integrase-DNA interactions involved amino acid residues from each domain of the inner subunits, their interdomain linkers and 17 nucleotides from each viral cDNA end. The integrase-DNA intasome showed an intimate interaction between the pre-processed viral DNA end and the active site loop containing the DDE motif. Moreover, each DDE active site loop of the inner subunits coordinated two metal ions ($Mn^{2+}$); one ion was bound by the DD amino acid residues and the second ion was bound by the DE carboxylates of the same active site. This observation confirmed the expected two-metal binding mode of retroviral integrases suggested by their similarities to Tn5 transposase and RNAse H. In this model, the metal ions would serve as Lewis acids during Sn2 chemistry: the metal ion bound by DE would activate the 3'-OH group of the pre-processed viral end, whereas the other metal ion coordinated by DD would destabilize the scissile phosphodiester group in the target DNA; during the 3' end processing the DE bound metal ion would activate the nucleophile water.

In addition to its role in the integration reactions, integrase may play several roles in HIV-1 replication cycle. Indeed, several replication defective HIV-1 viruses carrying integrase mutations, different from the DDE active site residues, called class II mutants, have shown pleiotropic effects on reverse transcription, viral release and morphogenesis (Engelman, 1999).

## 5- HIV-1 integrase interacting host factors

Although purified HIV-1 integrase is sufficient to catalyze 3' end processing and DNA strand transfer *in vitro*, mounting evidence highlights important roles for host cell factors in enabling HIV-1 to accomplish integration in infected cells. Such factors might influence non catalytic aspects of pre-integration complex (PIC) biology such as stability, nuclear import and access to specific regions of chromatin and/or more directly influence integrase enzymatic function. Several host proteins have been shown to interact with HIV-1 integrase *in vivo*; however, so far the functional role of only few factors has been validated in HIV-1 integration process.

### 5.1- Lens Epithelium-derived Growth Factor: LEDGF/p75

Lens epithelium-derived growth factor/p75 (LEDGF/p75), a member of the hepatoma derived growth factor (HDGF) related protein (HRP) family, was initially implicated in lentiviral biology through its association with ectopically expressed Flag-tagged HIV-1 integrase in HEK 293T cells by immunoprecipitation assays (Cherepanov et al., 2003; Maertens et al., 2003). The association of HIV-1 integrase and LEDGF/p75 was independently confirmed by analyzing cellular proteins associated with integrase in HeLa cells (Turlure et al., 2004) and by yeast two hybrid screen for integrase interactors (Emiliani et al., 2005).

LEDGF/p75 was proven to bind specifically lentiviral integrases but not alpharetroviral, betaretroviral, gammaretroviral, deltaretroviral and spumaretroviral integrases (Busschots et al., 2005; Cherepanov, 2007; Llano et al., 2004b). LEDGF/p75 was found as component of purified HIV-1 pre-integration complexes (PICs) that were functional in the *in vitro* integration assays (Llano et al., 2004b). LEDGF/p75 is a ubiquitous nuclear protein tightly associated with chromatin throughout the cell cycle (Engelman and Cherepanov, 2008; Van Maele et al., 2006). LEDGF/p75 contains 530 amino acids and several functional domains (**Figure 5a**). In the N-terminal region of LEDGF/p75, a PWWP (proline-tryptophan-tryptophan-proline) domain is present and that functions as protein-protein interaction domain and/or chromatin binding domain. In the same region, a functional nuclear localization signal (NLS) and dual copy of the AT-hook DNA binding motif are present (Van Maele et al., 2006). The binding of LEDGF/p75 to DNA *in vitro* is mediated by the NLS and AT-hook motif, whereas the PWWP domain supplies a critical chromatin recognition function (Engelman and Cherepanov, 2008; Turlure et al., 2006).

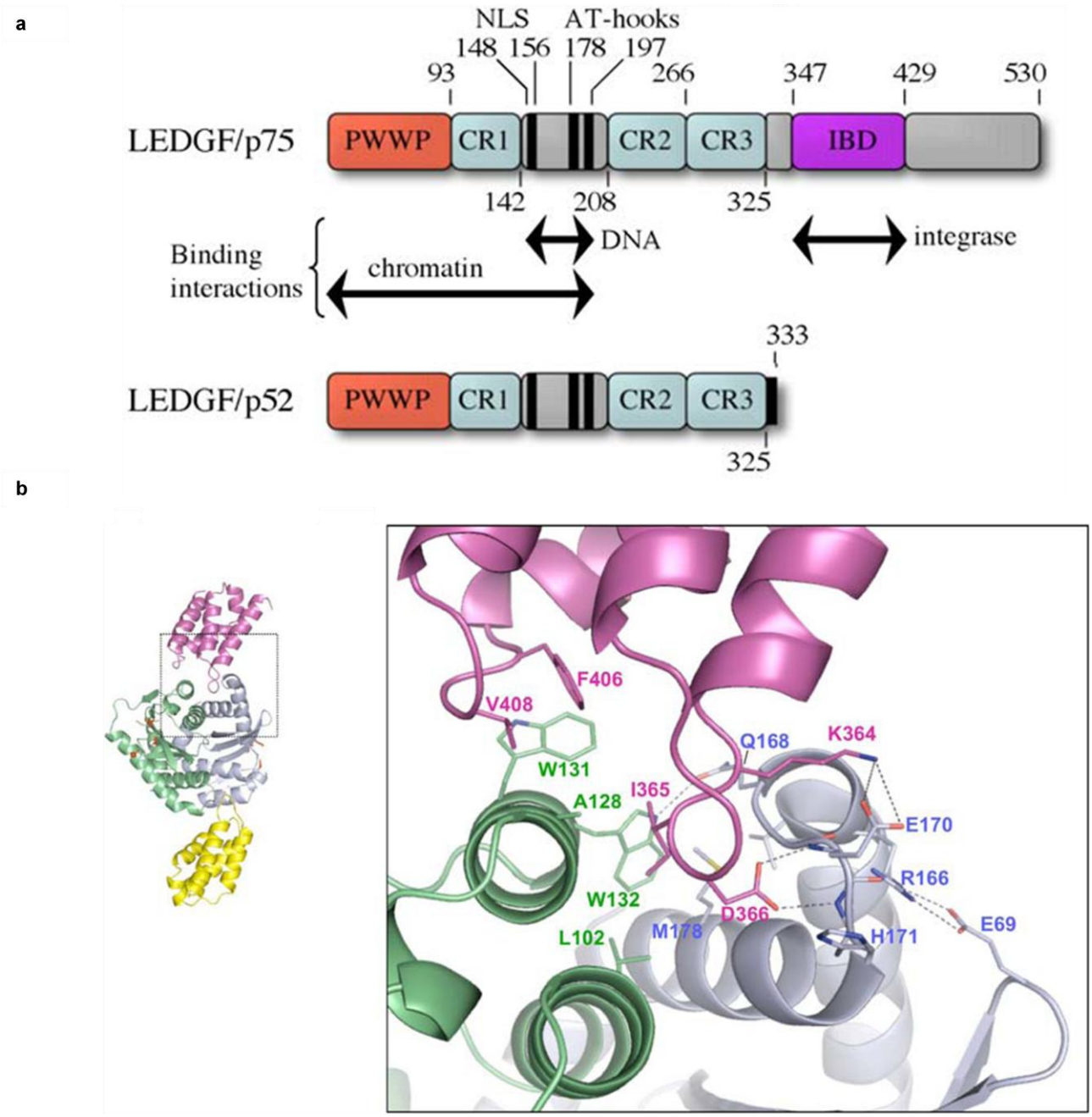


**Figure 5**: (**a**) Domain Organization of LEDGF/p75. The binding of LEDGF/p75 to DNA *in vitro* is mediated by the NLS and a nearby dual copy of the AT-hook DNA binding motif, whereas the N-terminal PWWP domain supplies a critical chromatin recognition function. Charged regions (CRs) 1–3 work in concert with the PWWP domain and AT-hooks to affect the wild type chromatin binding phenotype. The LEDGF/p75 IBD is critical for stimulation of HIV-1 IN function *in vitro* and for HIV-1 infection. The N-terminal 325 residues within LEDGF/p75 and LEDGF/p52 are identical, whereas the p52 isoform harbors a unique 8–amino acid residue tail. (**b**) Crystal structure of the LEDGF/p75-IN interaction. Left panel: cartoon representation of the CCD-IBD complex. IN CCD molecules are colored green and blue, whereas the LEDGF/p75 IBDs are magenta and yellow. The side chains of IN active site residues D64, D116, and E152 (Figure 5) are shown as red sticks. Right panel: details of the CCD-IBD interface. LEDGF/p75 hotspot residues I365 and D366, situated at the base of the loop between IBD helices 1 and 2, project into a pocket at the CCD dimer interface. I365 is buried into a hydrophobic pocket predominantly formed by IN residues (A128), W132, Leucine 102 (L102) and Methionine 178 (M178) if HIV-1 IN. Hydrogen bonds and salt bridges are shown as dotted lines (Engelman and Cherepanov, 2008).

Consistent with its ability to interact with integrase, an evolutionary conserved integrase-binding domain (IBD) (347-429 residues) was mapped on the LEDGF/p75 C-terminus (Cherepanov et al., 2004). LEDGF gene (*PSIP1*) encode two splice variants the p75 and p52 isoforms (**Figure 5a**). The p52 isoform shares the N-terminal 325 amino acid residues and lacks the IBD and consequently failed to bind integrase (Maertens et al., 2003).

In live cells, the N-terminal and the core catalytic domains (NTD and CCD) of integrase were shown involved in the binding to LEDGF/p75 (Maertens et al., 2003). CCD is the minimal domain required for this interaction, whereas NTD enhances the affinity of binding between integrase and LEDGF/p75 (Maertens et al., 2003). By site directed mutagenesis, isoleucine 365 (I365), aspartic acid 366 (D366) and phenylalanine 406 (F406) in the IBD region of LEDGF/p75 were revealed important for the interaction with integrase *in vitro* (Cherepanov et al., 2005b). Their principal involvement in the protein-protein interaction was confirmed through determination of the crystal structure of IBD in complex with the CCD (**Figure 5b**) (Cherepanov et al., 2005a). The complex of IBD with CCD consists of a dimer of CCD bound to two IBDs in a fully symmetric fashion. Each IBD burrows into a cleft created by the integrase dimer interface. The side chains carbonyl of LEDGF/p75 D366 forms a bidentate hydrogen bond with the backbone amides of residues glutamic acid 170 (E170) and histidine 171 (H171) from one integrase monomer, while I365 and F406 participate in multiple hydrophobic interactions with residues primarily donated from the other integase monomer. In particular, the side chain of I365 becomes buried within a hydrophobic pocket (Cherepanov et al., 2005a). Two regions within the CCD were identified to be indispensable for the interaction with LEDGF/p75 (Busschots et al., 2007). The first region centers around residues W131 and W132, while the second extends from I161 up to E170 amino acids of integrase (Busschots et al., 2007). A number of single amino acid substitutions to alanine (A) within these CCD two regions were shown to impair integrase-LEDGF/p75 interaction: W131A, I161A, arginine 166A (R166A), E170A (Busschots et al., 2007), glutamine 168 A (Q168A) (Busschots et al., 2007; Emiliani et al., 2005) and valine 165A (V165A) (Turlure et al., 2004). Interestingly, based on the graphical analysis of IBD-CCD crystal structure (**Figure 5b**), W131 forms hydrogen bond with IBD R405 and Q168 has electrostatic interaction with IBD lysine 402 (K402) (Busschots et al., 2007). Moreover, analysis of modeled mutant structures of W131A and Q168A CCD with IBD showed a disruption of the hydrogen and electrostatic interactions

that involve these residues and additionally an alteration of significant number of hydrophobic interactions between CCD and IBD (Busschots et al., 2007).
The purified recombinant LEDGF/p75 stimulated the DNA strand transfer activity of HIV-1 integrase *in vitro* (Cherepanov et al., 2003) and increased its affinity to DNA (Busschots et al., 2005). The LEDGF/p75 knockdown was shown to have three consequences on the feature of the ectopically expressed HIV-1 integrase: 1) re-distribution of integrase from the nucleus to the cell cytoplasm (Emiliani et al., 2005; Llano et al., 2004b; Maertens et al., 2003); 2) loosen of integrase chromosomal association (Emiliani et al., 2005; Llano et al., 2004b; Maertens et al., 2003) and 3) significant reductions in the steady-state levels of integrase (Emiliani et al., 2005; Llano et al., 2004a). Thus LEDGF/p75 tethers integrase to the chromatin and participates in its karyophilic proprieties, although no evident role of LEDGF/p75 in integrase or pre-integration complex (PIC) nuclear import was demonstrated. In addition, LEDGF/p75 protects integrase from proteasomal degradation (Llano et al., 2004a). The accumulation of integrase in the nucleus might be a consequence of the chromosomal tethering of integrase by LEDGF/p75 and the protection of integrase from proteasomal degradation. In fact, a mutant integrase (Q168A) that fails to bind LEDGF/p75 was unable to bind chromosomes (Emiliani et al., 2005) and proteasome inhibitor added to cells defective for LEDGF/p75 restored the nuclear accumulation of integrase (Llano et al., 2004b).

The role of LEDGF/p75 in HIV-1 infection was studied by three approaches: 1) using HIV-1 viruses carrying mutant integrases impaired for LEDGF/p75 interaction but catalytically active *in vitro*; 2) down-regulation of the endogenous LEDGF/p75 by knockdown assays or using *LEDGF* mouse knockout cells and 3) trans-dominant over-expression of the IBD. A Q168A integrase mutant HIV-1 virus is replication defective and showed a specific block at the integration step, whereas, reverse transcription and nuclear import were mildly hampered (Busschots et al., 2007; Emiliani et al., 2005). Furthermore, the HIV-1 virus harboring a mutant integrase (W131A) exhibited mildly impaired replication and integration (Busschots et al., 2007). The first studies performed partial LEDGF/p75 knockdowns and consequently failed to reveal an important role of the cellular factor in HIV-1 infection (Llano et al., 2004b; Vandegraaff et al., 2006; Zielske and Stevenson, 2006). Subsequently, efficient transient and stable LEDGF/p75 knockdowns resulted in a residual HIV-1 integration of 20-50% comparing to not silenced cells, without affecting

reverse transcription and nuclear import steps (Vandekerckhove et al., 2006). In the same study, HIV-1 infection was rescued upon back-complementation with a LEDGF/p75 shRNA-resistant expression vector. A more pronounced shRNA stable knockdown assuring the depletion of LEDGF/p75 chromatin fraction, which might be responsible for tethering integrase to chromosome, reduced HIV-1 integration to less than 10% of that observed in the normal LEDGF/p75 levels (Llano et al., 2006). Consistently, mouse embryo fibroblasts (MEFs) derived from *LEDGF* knockout animal supported 10% of HIV-1 integration comparing to control cells (Shun et al., 2007). In both cases HIV-1 infection was fully restored to LEDGF/p75-depleted cells by ectopic expression of the cell factor. Back-complementation studies in silenced cells highlighted two regions within LEDGF/p75, the IBD and the N-terminal PWWP/AT-hook chromatin and DNA binding motifs (**Figure 5a**), as crucial for HIV-1 infection (Llano et al., 2006; Shun et al., 2007). The requirement of the N-terminal elements suggested that LEDGF/p75 might primarily function to tether HIV-1 PICs to chromatin for integration (Engelman and Cherepanov, 2008). Importantly, over-expression of GFP-IBD fusion proteins inhibited HIV-1 integration while no inhibition was observed in cell over-expressing the integrase interaction deficient IBD (D366A) (De Rijck et al., 2006). These observations implicate the competitive role of the over-expressed IBD with endogenous LEDGF/p75 in binding viral integrase and consequently impairing its chromatin tethering (De Rijck et al., 2006). An HIV-1 mutant selected for its ability to replicate in MT4 T cell lines over-expressing the C-terminal portion of LEDGF/p75 (residues 326-530), that contains IBD, (**Figure 5a**) acquired two mutations in integrase: A128T and E170G (Hombrouck et al., 2007). Consistently with the IBD-CCD crystal structure (**Figure 5b**) (Cherepanov et al., 2005a) where E170 is involved in a salt bridge with K364 of IBD and A128 contributes to the hydrophobic pocket that buries LEDGF/p75 hotspot residue (I365), A128T and E170G mutations reduced the affinity of integrase interaction with LEDGF/p75 (Hombrouck et al., 2007; Rahman et al., 2007). Intriguingly, HIV-1 A128T/E170G was partially defective and its replication capacity was further reduced upon LEDGF/p75 knockdown suggesting that the integrase mutant still depended on LEDGF/p75 for integration (Hombrouck et al., 2007).

Since LEDGF/p75 is a lentivirus specific binding factor that tethers integrase to the chromatin, it has been hypothesized that LEDGF/p75 might contribute to the HIV-1 integration target site selection into the transcription units. LEDGF/p75 partial knockdown

in human cells showed a significant modest reduction of HIV-1 integration in transcription units, however, without shifting the bias toward these chromosomal regions (Ciuffi et al., 2005). More recent studies using *LEDGF* mouse knockout cells or more intensified LEDGF/p75 knockdown human cells showed a greater reduction of HIV-1 integration in transcription units (Marshall et al., 2007; Shun et al., 2007). Therefore, LEDGF/p75-dependent pathway is predominant for targeting lentiviral integration in transcription units.

Due to the crucial role of LEDGF/p75 in HIV-1 integration, the IBD-CCD interface represents a good target for molecules that might disturb the IN-LEDGF/p75 protein-protein interaction and induce a block of integration *in vivo*. A recent study described new small molecules 2-(quinolin-3-yl)acetic acid derivatives, known as LEDGIN compounds, as inhibitors of LEDGF/p75-IN interaction and HIV-1 replication in T cells (**Figure 6**) (Christ et al., 2010).

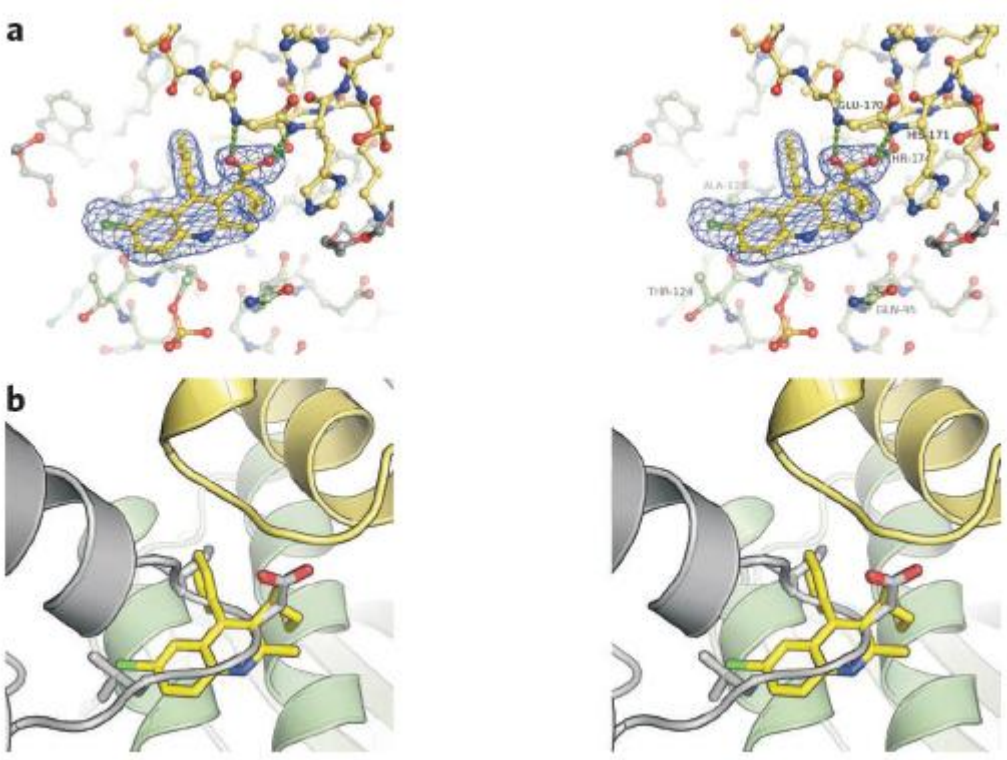


**Figure 6**: Crystal structure of LEDGIN compound 6 bound to the LEDGF/p75 binding pocket of the integrase catalytic core domain (CCD). (**a**) LEDGIN compound 6 (yellow) bound in the crystal structure of the CCD. Two chains of the IN dimer are shown in pale green and pale yellow. Hydrogen bonds made by the carboxyl moiety of LEDGIN compound 6 to the protein backbone near residues E170, H171 and T173 are shown as green dashed lines. (**b**) Cartoon representation of the IN CCD dimer (pale green and pale yellow) with the soaked LEDGIN compound (yellow stick) superimposed with the IBD-CCD complex structure reveals mimicry of the protein-protein interaction by the LEDGIN inhibitor. This validates the pharmacophore model, which was based on the interacting amino acids of the IBD such as the I365, D366 and the L368. These amino acids are clearly mimicked by the phenyl, acid and chlorine functions of the inhibitor, respectively (Christ et al., 2010).

These molecules may act by mimicking IBD residues and binding integrase in the same residues involved in LEDGF/p75 binding. Consistently, the same study resolved the CCD-LEDGIN compound 6 crystal structure (**Figure 6a**) showing that these molecules occupied the same space of the LEDGF/p75 binding pocket in the cleft between the two monomers of the CCD dimer. Interestingly, overlaying CCD-LEDGIN structure with the previously described CCD-IBD co-crystal structure (**Figure 5b**) (Cherepanov et al., 2005a) showed that the main chain nitrogen of residues E170 and H171 of integrase formed hydrogen bonds with carboxyl moiety of LEDGIN compound 6 (**Figure 6b**) (Christ et al., 2010). Therefore, this interaction might preclude the formation of the bidentate hydrogen bond between CCD E170 and H171 residues and the IBD D366 amino acid essential for IN-LEDGF/p75 interaction. Moreover, the CCD A128 residue that is important for CCD-IBD hydrophobic interactions is packed directly on the LEDGIN compound 6 (Christ et al., 2010).

### 5.2- Histone acetyl transferases: p300 and GCN5

**p300** is a histone acetyl transferase (HAT) protein that was demonstrated to directly bind and acetylate HIV-1 integrase *in vitro* and *in vivo* (Cereseto et al., 2005).

HAT family proteins functions enzymatically by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amine group of specific lysine residues within the histone basic N-tail region or the non-histone protein substrates (Roth et al., 2001). The HAT p300 is a transcriptional co-activator with a broad activity in gene transcription through its interaction with a number of transcription factors (Chan and La Thangue, 2001; Wang et al., 2008). HAT p300 has 2414 amino acid residues and contains several functional domains (**Figure 7a**): 1) three cysteine-histidine (CH)-rich domains (CH1, CH2 and CH3); 2) a KIX domain; the CH1, CH3 and the KIX domains bind a number of cellular and viral proteins and mediate protein-protein interactions; 3) the bromodomain, which is frequently found in mammalian HATs, recognizes and binds acetylated lysines; 4) the central HAT domain (1195-1673 amino acid residues) is the catalytic domain of p300 responsible for transferring acetyl group to target lysines and 5) the steroid receptor co-activator interaction domain (SID, also the SRC-1 interaction domain) (Chan and La Thangue, 2001; Wang et al., 2008).

HAT p300 binds HIV-1 integrase through the C-terminal domain (CTD) of integrase (Cereseto et al., 2005). Consistently, p300 acetylates *in vitro* and *in vivo* three lysine (K) residues in integrase CTD: K264, K266 and K273 (Cereseto et al., 2005; Topper et al., 2007). Interestingly, a modeled structure of HIV-1 integrase in complex with p300 predicted that the CTD tail between amino acids 271 and 288 due to its high flexibility, could easily adapt to the binding pocket of p300 (**Figure 7b**) (Di Fenza et al., 2009; Terreni et al., 2010). Thus, in this model, the K273 is expected to be the most prone for acetylation by p300 and the acetylation of the close lysines K264 and K266 might require more complex unfolding of their stable structure. The acetylation of K264, K266 and K273 by the recombinant p300 enhanced the binding affinity of integrase to a viral mimicking DNA *in vitro* (Cereseto et al., 2005). Interestingly, the CTD has been shown to have a strong but nonspecific DNA binding activity (Engelman et al., 1994). Importantly, it has been reported that the minimal region of CTD required for DNA binding is from 220 to 270 and mutations in K264 showed a strong reduction of integrase DNA binding and catalytic activity *in vitro* (Lutzke et al., 1994). Furthermore, a footprint analysis has revealed that K273 is also among the amino acids involved in DNA binding (Dirac and Kjems, 2001). In agreement with the increase of DNA binding affinity, acetylation enhanced integrase DNA strand transfer activity, while, 3' end joining activity was not affected (Cereseto et al., 2005). The role of integrase acetylation in HIV-1 infection was investigated by using a mutant HIV-1 virus carrying an integrase containing single amino acid substitutions of acetylation lysine sites to arginines (Cereseto et al., 2005). The replication of (K264,K266,K273R)-HIV-1 mutant virus was severely impaired in T cells comparing to the wild type virus. The impairment was specific to integration (95% reduction), while, reverse transcription and nuclear import were not affected (Cereseto et al., 2005). Importantly, the (K264,K266,K273R)-HIV-1 mutant virus formed more 2-LTR circles comparing to the wild type virus indicating that the inhibition in viral replication is due to an integration block and not to pleiotropic effect of integrase mutations (Cereseto et al., 2005).

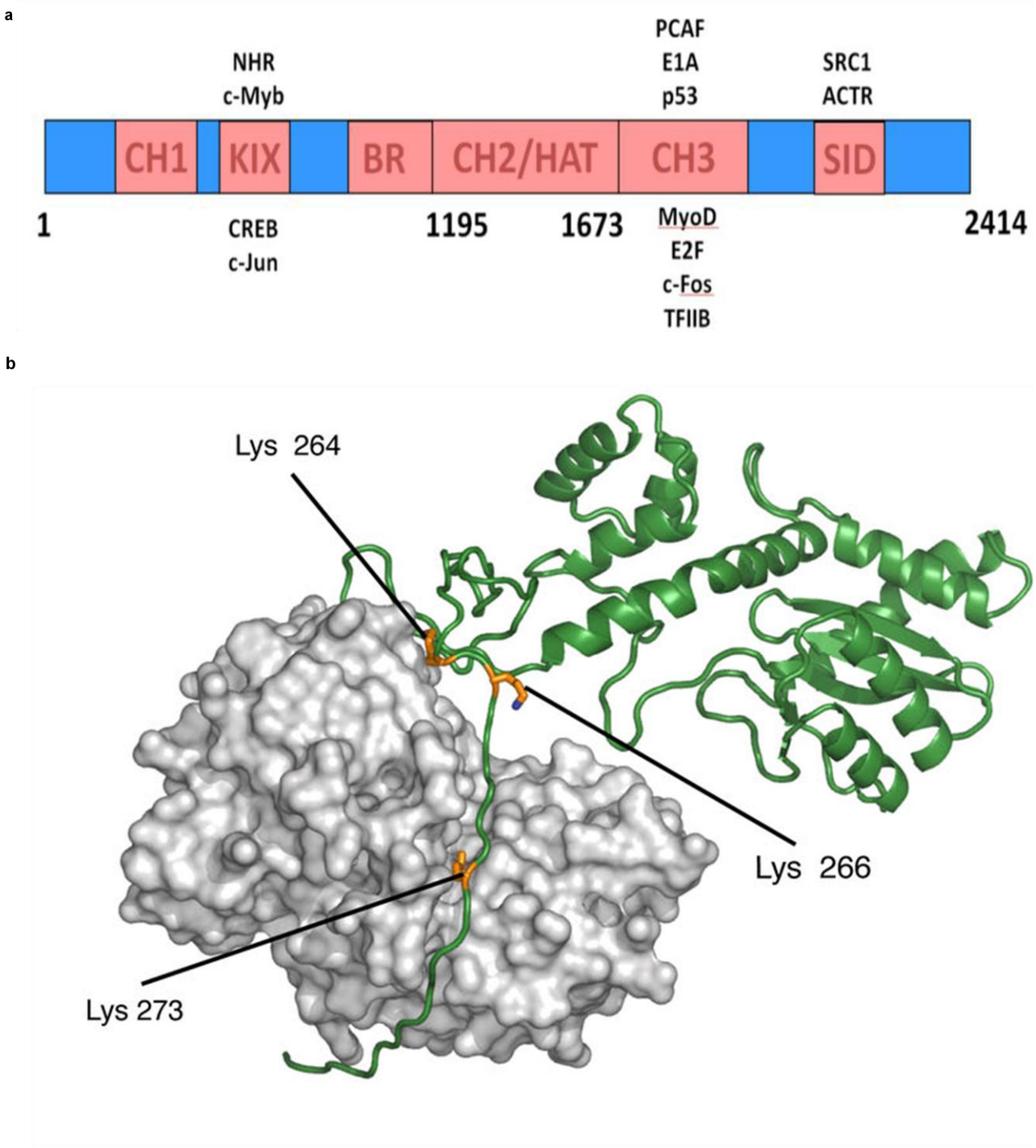


**Figure 7:** (**a**) Schematic representation of p300 domains. The functional domains in p300 (1-2414 amino acid residues) include the cysteine/histidine-rich domains CH1, CH2 and CH3, the KIX domain, the bromodomain (Br), the HAT domain (1195-1673 amino acids) and the steroid receptor coactivator interaction domain (SID, also the SRC-1 interaction domain). The regions that have been shown to bind to target proteins, together with the identity of the interacting proteins, are shown (Liu et al., 2008). (**b**) Three-dimensional model of IN complex with p300. IN is represented in green and p300 in light grey. The three lysine residues in the C-terminal domain of IN that are acetylated by p300 (Lys 264, Lys 266, and Lys 273) are shown in yellow. HAT p300 is rendered as surface, while IN as a cartoon to highlight the C-terminal unfolded portion which inserts in the binding pocket of HAT (Terreni et al., 2010).

The role of integrase acetylation lysines in HIV-1 infection was further investigated by three subsequent studies (Apolonia et al., 2007; Terreni et al., 2010; Topper et al., 2007). The first subsequent report (Topper et al., 2007) confirmed the p300 acetylation of integrase at the same described lysine sites (Cereseto et al., 2005), however, they attributed the severe integration impairment of the (K264,K266,K273R)-HIV-1 mutant virus (Cereseto et al., 2005) to the acidic Flag tag fused to the viral integrase CTD that in combination with the RRR mutations might disturb the functionality of the viral integrase *in vivo*. The same study (Topper et al., 2007) reported an untagged (K264,K266,K273R)-HIV-1 mutant virus that although was able to replicate in T-cells it gave a reproducible reduction (40%) in integration accompanied with a significant 4 fold increase in 2-LTR circles with respect to wild type virus. In the two other subsequent studies, the untagged triple mutant HIV-1 viruses gave a more pronounced significant decrease (80%) in HIV-1 infectivity and integration (Apolonia et al., 2007; Terreni et al., 2010). Moreover, an untagged (K264,K266,K273R)-HIV-1 mutant virus (Terreni et al., 2010) in a multiple-round replication showed a reduced virus production and a delay in the peak of infectivity comparing to the wild type virus. All these reports showed that integrase acetylation by p300 is not an absolute requirement for HIV-1 integration but it significantly participates to optimal integration.

HIV-1 integrase has been shown to be also acetylated by another HAT protein, **GCN5**, at the same lysine residues targeted by p300 (K264, K266 and K273) and at an additional CTD lysine K258 (Terreni et al., 2010). Similarly to p300, GCN5 demonstrated to bind integrase *in vitro* and *in vivo* and this interaction required the integrase C-terminal domain. Transient and stable knockdowns of GCN5 decreased HIV-1 integration by almost 50% consistent with an increase of the 2-LTR circles (Terreni et al., 2010). Moreover, an untagged quadruple mutant (K264,K266,K273,K258R)-HIV-1 virus exhibited the same integration deficiency (80% decrease) of the triple mutant HIV-1 virus (K264,K266,K273R) (Terreni et al., 2010). This study demonstrates that K264, K266 and K273 residues which are acetylated by both p300 and GCN5 are required for optimal viral integration, while, K258, acetylated exclusively by GCN5, does not appear to affect HIV-1 infection (Terreni et al., 2010).

### 5.3- Integrase interactor 1: INI1

INI1 is the first cellular factor that was identified to interact with HIV-1 integrase by yeast two hybrid screen (Kalpana et al., 1994). INI1 is the human homolog of yeast SNF5, transcriptional activator and component of the chromatin remodeling SWI/SNF complex. Likewise, INI1 was shown to be part of mammalian SWI/SNF complex that utilizes the energy of ATP hydrolysis to remodel chromatin (Wang et al., 1996). INI1 is a 385 amino acid protein with three conserved regions, including two direct imperfect repeats, repeat 1 (Rpt1) and repeat 2 (Rpt2), a C-terminal coiled-coiled domain and a homology region 3 (Morozov et al., 1998).

The interaction INI1-integrase is specific to HIV-1 and not other related lentiviruses (Yung et al., 2004). INI1 has been shown to be incorporated into the virions through integrase dependent manner (Yung et al., 2001; Yung et al., 2004) and also to be a component of the reverse transcription complex (Al-Mawsawi and Neamati, 2007). The minimal INI1 binding region of integrase spans amino acid residues 53-153, and lysine 71 (K71) is critical for INI1 binding (Al-Mawsawi and Neamati, 2007). The INI1 amino acid residues 183-243 are responsible for integrase binding (Maroun et al., 2006).

Recombinant INI1 as well as high molecular weight INI1 containing protein complexes partially purified from mammalian cells stimulated integrase DNA-strand transfer activity *in vitro* 10-20 fold as compared with reactions lacking INI1 (Kalpana et al., 1994). These results suggested a potential important role for INI1 during HIV-1 infection and specifically integration. However, no effect of INI1 on HIV-1 integration has been reported following potent down-regulation of endogenous INI1 by small RNA interference (siRNA) (Boese et al., 2004) or by short hairpin RNA (shRNA) (Ariumi et al., 2006). In contrast, another report has suggested that INI1 participates in an anti-viral cellular response by interfering with early steps of viral replication likely pre-integration complex (PIC) nuclear import since the transient knockdown of INI1 enhanced both 2-LTR circles and integrated viral cDNA (Maroun et al., 2006). This finding was in agreement with a previous observation that showed 30 min after HIV-1 infection INI1 as well as promyelocytic (PML) protein are exported from the nucleus to the cytoplasm to co-localize with the incoming PIC (Turelli et al., 2001).

The role of the incorporation of INI1 inside the HIV-1 virion particles was also investigated. HIV-1 particles lacking INI1 protein were shown to be defective for reverse transcription, indicating a potential role of INI1 in promoting HIV-1 infection in very early

steps of HIV-1 infection instead of integration (Sorin et al., 2006). Moreover, a critical role of INI1 in HIV-1 production and release has been also described based on the use of a peptide mimicking INI1 fragment able to bind integrase, called S6, that acted as trans-dominant inhibitor (Yung et al., 2004). The presence of S6 fragment in producer cells reduced the viral assembly and release dramatically, whereas, mutations in the S6 fragment that disturbed the integrase interaction abrogated the inhibitory effect on virus production (Yung et al., 2004). Furthermore, in the same study it has been demonstrated that the S6 inhibition was specific to integrase as part of Gag-Pol polyprotein precursor that is synthesized in producer cells and not to the free integrase protein separated from Gag-Pol during virus release and proteolytic maturation by the viral protease (Yung et al., 2004).

### 5.4- Host factors binding integrase and involved in HIV-1 nuclear import

#### a- Karyophilic properties of integrase and its association with importin factors

The tight association between integrase and the viral cDNA within the pre-integration complex (PIC) supported integrase as a good candidate to mediate HIV-1 nuclear import (Farnet and Haseltine, 1991). HIV-1 integrase is a karyophilic protein (Bouyac-Bertoia et al., 2001; Depienne et al., 2000; Devroe et al., 2003) and several putative nuclear localization signal (NLSs) have been mapped in its core catalytic domain (Armon-Omer et al., 2004; Bouyac-Bertoia et al., 2001) and in its C-terminal domain (Ao et al., 2005; Gallay et al., 1997). Since the reported integrase putative NLSs have been shown to interact with importin $\alpha$ (Armon-Omer et al., 2004; Gallay et al., 1997), importin $\beta$ (Hearps and Jans, 2006) and importin 7 (Ao et al., 2007), classical importin pathways have been proposed for integrase nuclear import. However, based on *in vitro* nuclear import assays, it has been shown that integrase was imported to the nucleus by a rapid, ATP-dependent saturable mechanism that did not require cytosolic factors and independent from the classical NLS import mechanisms (Depienne et al., 2001). Moreover, it has been reported that integrase might lack a transferable NLS and its tight association with the chromosomal DNA, mainly by LEDGF/p75, might facilitate its nuclear accumulation (Devroe et al., 2003; Emiliani et al., 2005). In the attempt to establish a correlation between the karyophilic proprieties of integrase and the HIV-1 PIC nuclear import during HIV-1 infection, several HIV-1 mutant viruses have been described. An HIV-1 mutant virus

in one putative integrase NLS (161-173 amino acid residues) showed a replication defect in cycling and non-dividing cells indicating that karyophilic proprieties of integrase are required to promote an efficient HIV-1 infectivity in host cells (Bouyac-Bertoia et al., 2001). Nevertheless, later reports demonstrated that the same HIV-1 mutant virus is replication defective owing to defective integrase catalytic activity and not to nuclear import block since same amounts of nuclear viral cDNA were detected with wild type and HIV-1 virus mutant in NLS integrase (Dvorin et al., 2002; Limon et al., 2002). Later on the same integrase NLS region (161-173 residues) has been shown to be a hotspot interface for IN-LEDGF/p75 interaction essential for HIV-1 integration *in vivo* (Busschots et al., 2007). Indeed, several integrase viral mutations have been shown to have pleiotropic effects often complicating viral phenotypes interpretations (Engelman, 1999; Engelman et al., 1995). Two studies (Bukrinsky et al., 1992; Riviere et al.) reported defective HIV-1 viruses deleted in integrase that were able to form 2-LTR circles, a surrogate marker of retroviral nuclear import (Coffin et al., 1997), as well as wild type virus suggesting that functional integrase is not necessary for HIV-1 nuclear import.

Among the importins binding integrase, the role of **importin 7** has been investigated by performing a transient knockdown by small interfering RNA (siRNA) in dividing cells and by testing an HIV-1 mutant virus carrying an integrase impaired for the importin 7 binding by single amino acid substitutions (Ao et al., 2007). In this report, HIV-1 infectivity was reduced by almost 50% in importin 7 knockdown cells comparing to not depleted cells and the mutant HIV-1 virus was replication defective due to a block in the nuclear import indicating an efficient role of IN-importin 7 interaction in the PIC nuclear translocation (Ao et al., 2007). However, in a previous study, it has been reported that a good importin 7 siRNA-mediated knockdown did not affect HIV-1 nuclear import, based on the formation of the 2-LTR circles, in dividing and non dividing cells (Zielske and Stevenson, 2005).

Several other studies have suggested and reported that HIV-1 PIC nuclear import is mediated by integrase binding cellular factors different from importin proteins. The first proposed candidate was **LEDGF/p75** because it binds tightly HIV-1 integrase (Cherepanov et al., 2003), it was found as a component of the HIV-1 PICs (Llano et al., 2004b) and it contains an N-terminal transferable NLS (Maertens et al., 2004). However, LEDGF/p75 depletion in macrophages or in dividing cells has been shown to affect only HIV-1 integration and based on the 2-LTR circles formation HIV-1 nuclear import was

unchanged (Llano et al., 2006; Shun et al., 2007; Vandekerckhove et al., 2006; Zielske and Stevenson, 2006).

**b- Transportin-SR2**

Recent study described a new integrase cellular binding factor, transportin-SR2 (TRN-SR2 or TNPO3), that has been identified by yeast two hybrid screen and has an important role in HIV-1 PIC nuclear import (Christ et al., 2008). TRN-SR2 is a member of karyopherin β protein family and shuttles essential splicing factors, the serine/arginine rich splicing factors (SR proteins), between the nucleus and the cytoplasm and therefore is involved in the regulation of the mRNA splicing. Importantly, transportin-SR2 was identified essential for HIV-1 early steps in two independent genome wide short interfering RNA (siRNA) screens for host factors required by HIV-1 for infection (Brass et al., 2008; Konig et al., 2008). The knockdown of transportin-SR2 in cells reduced HIV-1 replication by 80% (Christ et al., 2008). The analysis of viral cDNA species revealed that reverse transcription in transportin-SR2 knockdown cells was not affected while 2-LTR circles were decreased by 3 to 4 fold consistent with a severe decrease in HIV-1 integration and implicated an important role of transportin-SR2 in HIV-1 nuclear import (Christ et al., 2008). This role was corroborated by an *in vivo* HIV-1 PIC nuclear import assay (Albanese et al., 2008) which demonstrated a decreased number of visualized HIV-1 PIC in the nucleus when transportin-SR2 was depleted comparing to not silenced cells (Christ et al., 2008). The effect of transportin-SR2 on HIV-1 nuclear import was similar in dividing and non dividing cells (Christ et al., 2008). Later studies confirmed the requirement of transpotin-SR2 for HIV-1 infectivity and nuclear import, however, in capsid dependent manner (Krishnan et al., 2010; Lee et al., 2010). Indeed, it has been described an HIV-1 capsid mutant virus (N74D) pseudotyped with VSV-G envelope that was insensitive to transportin-SR2 knokdown (Lee et al., 2010). In contrast, a more recent study showed that the N74D mutant virus carrying the wild-type HIV-1 envelope is still dependent on transportin-SR2, which revealed a link between the viral entry of HIV-1 and its interaction with this cellular factor (Thys et al., 2011).

**c- JNK and Pin1**

C jun N-terminal kinase (JNK) is a member of MAP kinases that has been shown to bind and phosphorylate *in vitro* and *in vivo* HIV-1 integrase (Manganaro et al., 2010). The

phosphorylation occurs at serine 57 (S57) of the core catalytic domain (CCD) of integrase which is also the region required for the binding with JNK (Manganaro et al., 2010). This modification is required for integrase interaction with peptidyl-prolyl *cis-trans* isomerase Pin1 which in turn catalyzes conformational modification of integrase to increase its stability (Manganaro et al., 2010). Interestingly, in quiescent T cells non-permissive for HIV-1 infection JNK is low expressed and viral integrase was not detected at 8 hours after infection in contrast to activated T cells (Manganaro et al., 2010). Drug inhibition of JNK in activated T cells does not affect HIV-1 reverse transcription, however, decreases viral integration and 2-LTR circle formation indicating a defect in the nuclear import of viral cDNA (Manganaro et al., 2010). It has been proposed that in activated T cells integrase phophorylated by JNK is stabilized by Pin1 thus allowing efficient infection, conversely, in resting T cells lacking JNK activity integrase is unstable unable to sustain efficient nuclear import and integration (Manganaro et al., 2010).

**d- NUP153**

NUP153 is an essential nuclear pore complex (NPC) protein that binds directly HIV-1 integrase (Woodward et al., 2009). The C-terminal domain of NUP153 containing phenylalanine-glycine rich repeats (FG-repeats) is the region required for binding integrase (Woodward et al., 2009). The over-expression of NUP153 FG-repeats induced an inhibition of HIV-1 cDNA nuclear translocation suggesting that integrase-NUP153 interaction mediates the PIC nuclear import (Woodward et al., 2009).

**5.5- Gemin2**

Gemin2, has been identified as HIV-1 integrase interacting factor by yeast two hybrid screen (Hamamoto et al., 2006). Gemin2 is an integral protein component of the survival of motor neurons complex (SMN). The SMN complex is involved in many aspects of cellular RNA function. It acts as a macromolecular assembly complex for various ribonucleoproteins and has been implicated in fundamental cellular processes including transcription, RNA metabolism and mRNA or rRNA processing (Al-Mawsawi and Neamati, 2007). Gemin2-IN interaction was further confirmed *in vitro* and *in vivo* and required the C-terminal domain of integrase with partial contribution of the core catalytic domain (Hamamoto et al., 2006). A transient Gemin2 knockdown mediated by short interfering RNA (siRNA) in macrophages and in dividing cells reduced HIV-1 infectivity by almost 75%

comparing to not depleted cells (Hamamoto et al., 2006). The reduction of infectivity in Gemin2 knockdown cells was pinpointed at the level of reverse transcription (Hamamoto et al., 2006). Thus, although Gemin2 is an integrase interacting factor it contributes to HIV-1 infectivity at the early viral cDNA synthesis step (Hamamoto et al., 2006).

### 5.6- Human polycomb group EED protein

EED protein (embryonic ectoderm development) was identified as integrase binding factor by yeast two hybrid screen (Violot et al., 2003). EED is encoded by *eed* gene which is a member of the highly conserved *polycomb* group (Pc-G) of genes, whose products regulate and maintain the silent state of chromatin (Al-Mawsawi and Neamati, 2007). The EED-IN interaction was confirmed by *in vitro* binding assays (Violot et al., 2003). In addition, EED protein stimulated integrase catalytic activity *in vitro* (Violot et al., 2003). In the same study, EED and integrase were found to co-localize predominantly in the nucleus near to the nuclear pores six hours post-infection and at 24 hours post-infection no co-localization has been observed (Violot et al., 2003). The functional role of EED protein in HIV-1 infection is still not investigated. However, based on the nature of EED as a chromatin associated protein and on the IN-EED co-localization observations it has been suggested possible roles of EED in integration site selection and in HIV-1 nuclear import (Vandegraaff and Engelman, 2007; Violot et al., 2003).

### 5.7- Heat shock protein 60: HSP60

The yeast HSP60, which is the counterpart of human HSP60, was identified to bind HIV-1 integrase by *in vitro* interaction assays between the recombinant viral protein and the yeast cell protein extracts (Parissi et al., 2001). Heat shock proteins (HSP) bind and facilitate the proper folding of newly synthesized peptide chains. HSP60 functions in complex with HSP10 and drives the folding of newly synthesized peptides in an ATP-dependent manner (Parissi et al., 2001). The human HSP60 was also able to bind HIV-1 integrase *in vitro* and this interaction was shown to involve at least the catalytic core domain of integrase (Parissi et al., 2001). Moreover, integrase has been shown to be a specific substrate of HSP10-HSP60 chaperonin complex *in vitro* in an energy dependent manner (Parissi et al., 2001). The recombinant human HSP60 stimulated integrase 3' end processing and DNA strand transfer activities *in vitro* (Parissi et al., 2001). Finally, the lethal phenotype induced by HIV-1 integrase expression in yeast (Caumont et al., 1999)

was abolished in strains mutated in *HSP60* gene suggesting that HSP60-IN interaction might have a role on the functionality of integrase *in vivo* (Parissi et al., 2001). The role of IN-HSP60 interaction in HIV-1 replication is still not established.

### 5.8- Uracil DNA glycosylase isoform 2: UNG2

UNG2 was initially identified as cellular protein interacting with Vpr by yeast two hybrid screen (Bouhamdan et al., 1996) and a subsequent study revealed that UNG2 is incorporated into HIV-1 virions (Willetts et al., 1999). However, UNG2 is incorporated into virions not in Vpr-interaction dependent manner but through integrase (as domain of Gag-Pol precursor) interaction dependent manner (Willetts et al., 1999). UNG2 excises uracils present in G:U base mismatch of the DNA preventing the genome G to A mutations and resulting in abasic sites further repaired through the base excision repair pathway (Willetts et al., 1999). The UNG2 binding region of integrase was mapped on 170-180 amino acid residues (Willetts et al., 1999). There are conflicting results about the role of UNG2 in HIV-1 replication. In one study an HIV-1 mutant virus containing an integrase unable to incorporate UNG2 into the virions, by single point mutations in 170-180 amino acid region, was replication defective by a specific block at the integration step (Priet et al., 2003). In contrast, another report showed that the infectivity of HIV-1 virions produced in *UNG* knockout B cell lines was not affected (Kaiser and Emerman, 2006).

### 5.9- Von Hippel-Lindau binding protein 1: VBP1

VBP1 has been identified to bind HIV-1 integrase by yeast two hybrid screen (Mousnier et al., 2007). VBP1 is a component of the cellular prefoldin chaperone. Mousnier et al (2007), showed that VBP1 binds integrase at 43-195 amino acid residues and bridges integrase with the cullin2-based von Hippel-Lindau ubiquitin ligase (cul2/VHL). This tethering induced integrase poly-ubiquitination and subsequently its proteasomal degradation. In this reported mechanism integrase degradation serves at a post-integration step to allow efficient transition from integration to viral transcription. Consistently, in VBP1 knockdown cells or in cul2/VHL mutant cells the HIV-1 gene expression was inhibited only when the viral genome had been integrated through an integrase dependent pathway and not following HIV-1 plasmid transfection (Mousnier et al., 2007).

### 5.10- TRAF and TNF receptor associated protein: TTRAP

The study from Zhang et al (2009) reports that TTRAP binds HIV-1 integrase in yeast two hybrid assay, *in vitro* and in *in vivo* in human cells (Zhang et al., 2009). TTRAP is a promyelocytic leukemia nuclear body (PML-NB) associated protein. In the same study, TTRAP knockdown or over-expression diminished or enhanced HIV-1 infectivity respectively. However, the molecular mechanism of TTRAP activity on HIV-1 infection has not been elucidated yet.

## HIV-1 restriction factors

In response to HIV-1 infection, cellular innate immunity has evolved proteins that interfere with several steps of HIV-1 replication, the restriction factors. The most described HIV-1 restriction factors are TRIM5$\alpha$, member of TRIM family proteins, APOBEC3G/F and the newly described tetherin (CD317/BST-2). These factors affect HIV-1 in cytoplasmic replication steps which are post-entry for TRIM5$\alpha$, reverse transcription for APOBECs and viral release for tetherin. TRIM22, another TRIM protein, has been suggested to inhibit HIV-1 transcription and recently has been shown to inhibit viral assembly. So far, few reports described inhibitor proteins that interfere with HIV-1 integration or nuclear pre-integration events ($p21^{Cip1/Waf1}$, RAD52, Rad18, XPB and XPD) with not yet establishment of molecular mechanisms.

## 1- TRIM family proteins

### 1.1- TRIM proteins: characteristics and implications in the innate immunity

The TRIM family is an expanding family of RING (really interesting new gene) proteins that contain an N-terminal tripartite motif called RBCC (**Figure 8**) (Reymond et al., 2001; Short and Cox, 2006). RBCC motif comprises a RING domain, one or two B-boxes and a predicted coiled-coil region (Reymond et al., 2001; Short and Cox, 2006). This RBCC motif is usually followed by either one or two C-terminal domains which are specific for each TRIM protein (Ozato et al., 2008; Short and Cox, 2006). The C-terminal domains determine the classification of the 72 human TRIM proteins into 11 subfamilies while one group remains unclassified due to the lack of the RING domain (**Figure 8**) (Ozato et al., 2008; Short and Cox, 2006). The architecture of TRIM proteins is conserved not only in human genes but also from other species, indicating that the RBCC is responsible for all common features of TRIM proteins (Ozato et al., 2008). The RING domain of some TRIM

proteins (such as TRIM5α, TRIM21, TRIM22 and TRIM25) possesses an E3 ubiquitin ligase activity that catalyses the addition of ubiquitin to substrate proteins targeting them for proteasomal degradation (Kajaste-Rudnitski et al., 2010; Ozato et al., 2008). The B-boxes and coiled coil domains are believed to participate in protein-protein interaction, oligomerization and formation of macromolecular complexes (Nisole et al., 2005; Ozato et al., 2008). The C-terminal sequences most commonly found in TRIM proteins are the PRY and SPRY domains (**Figure 8**) (Nisole et al., 2005; Ozato et al., 2008). The SPRY domain is found in 39 human TRIM family members including TRIM22, and is fused to the PRY domain to form PRYSPRY domain (also called B30.2) in 24 family members including TRIM5α and TRIM25 (Ozato et al., 2008). The cellular functions of the PRYSPRY domain are still unclear but it has been proposed that PRYSPRY domain has been evolved only in vertebrate under selective pressures for immune defense (Ozato et al., 2008). The C-terminus of TRIM family can also contain various other domains (**Figure 8**). For instance, the C-VI TRIM subfamily proteins that include TRIM24, TRIM28 (KAP1 or TIF1β) and TRIM33 are characterized by a C-terminus containing a plant homeodomain (PHD) zinc finger always followed by a bromodomain (BROMO) (**Figure 8**) (Ozato et al., 2008). The PHDs are found in nuclear proteins and are thought to be involved in transcriptional regulation and in binding histone methylated lysines, while, bromodomains are functional motifs that recognize acetylated lysine residues of histone and non histone proteins (Mujtaba et al., 2007; Zeng and Zhou, 2002). In regard to their structure, TRIM proteins form high-molecular-mass complexes that localize to specific sub-cellular compartments present either in the cytoplasm or in the nucleus (Reymond et al., 2001). For example, TRIM19, also known as promyelocytic leukemia protein (PML), exists in sub-nuclear structures known as "PML nuclear bodies", whereas TRIM5α is normally located in distinct cytoplasmic aggregates designated as "cytoplasmic bodies" (Ozato et al., 2008).

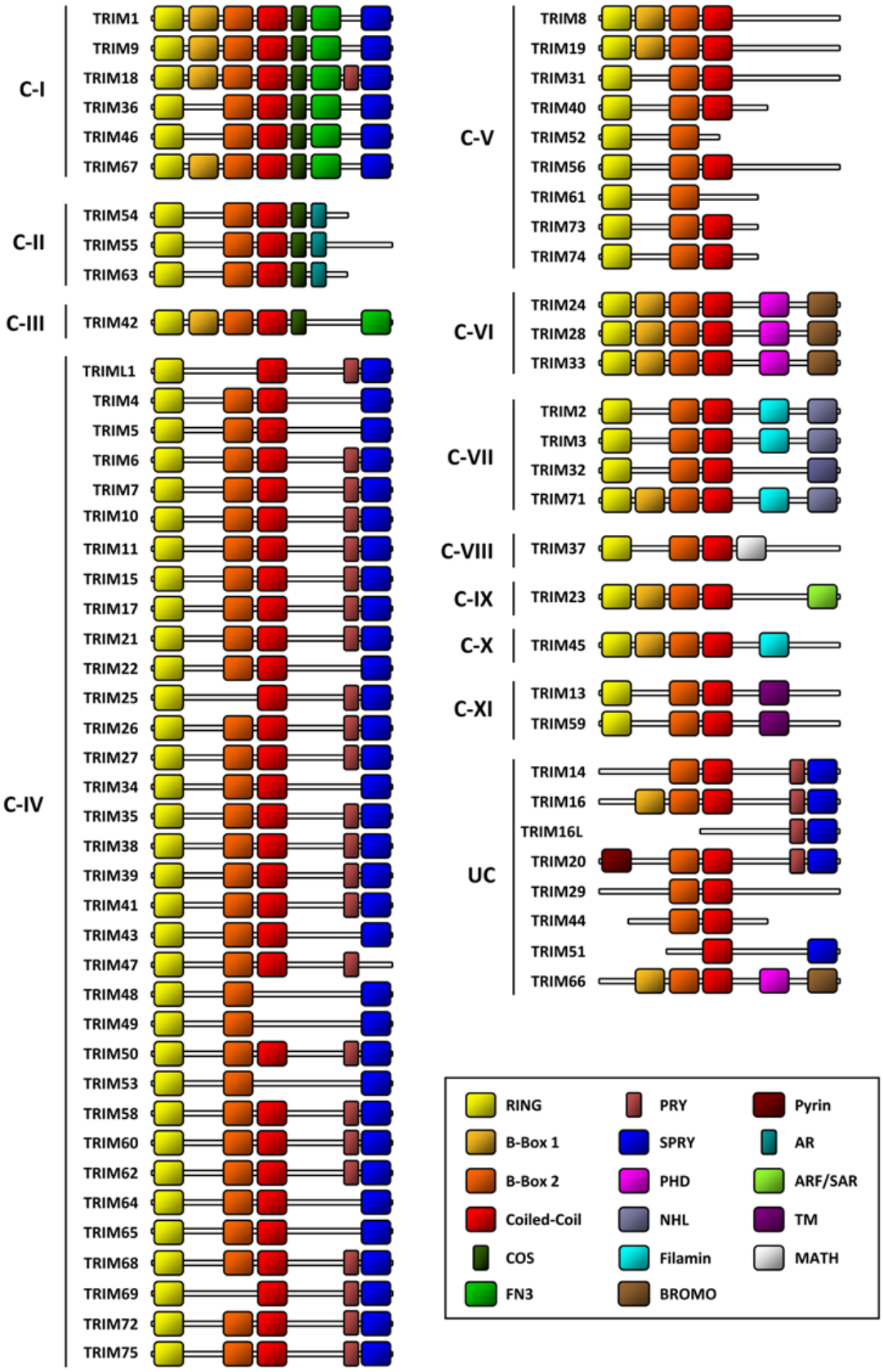


**Figure 8**: Human TRIM proteins. Classification of human TRIM proteins based on the nature of their C-terminal domains(s) (Ozato et al., 2008; Short and Cox, 2006). The TRIM protein family is composed of 11 sub-families, from C-I to C-XI, whereas some TRIM proteins remain unclassified (UC), since they do not have a RING finger domain as "true" TRIM proteins. NHL, NHL repeats; COS, COS box motif; FN3, fibronectin type III motif; PHD, plant homeodomain; BROMO, bromodomain; MATH, meprin and TRAF homology domain; TM, transmembrane domain; AR, acid-rich region (Carthagena et al., 2009).

TRIM family proteins have been involved in a variety of cellular processes, such as signal transduction, transcriptional regulation, cell proliferation, oncogenesis, and apoptosis (Nisole et al., 2005; Ozato et al., 2008). Furthermore, mounting evidence highlights the important role of TRIM proteins in the innate immunity. Indeed, in the recent years an increasing number of TRIM proteins have been found to display antiviral activities or to be involved in processes associated with immune response (Nisole et al., 2005; Ozato et al., 2008). For example, among the 72 TRIM human genes, 27 have been found to be up-regulated in response to interferons, the main mediators of the innate immunity against viral infection (Carthagena et al., 2009). Consistently, TRIM25 has been shown to control the retinoic acid inducible gene I protein (RIG-I) which is the cytosolic receptor of viral RNA that induces type I interferon–mediated host protective innate immunity against viral infection (Gack et al., 2007). It has been demonstrated that TRIM25 E3 ubiquitin ligase catalyzes the ubiquitination of RIG-I which is an essential modification for triggering the downstream immune signaling pathway upon viral infection (Gack et al., 2007). Moreover, TRIM19 (PML) has been shown to be involved in the cellular antiviral response against a wide range of RNA and DNA viruses including HIV-1, adenoviruses, herpes simplex virus (HSV) and cytomegalovirus (CMV) (Everett and Chelbi-Alix, 2007). Importantly, many indications suggest the important role of TRIM family proteins in the inhibition of retroviruses (Nisole et al., 2005). First, because TRIM5$\alpha$ from different primate species (humans, Old and New World monkeys) showed crucial role in the species-specific resistance to retroviruses (Huthoff and Towers, 2008; Nakayama and Shioda, 2010). Moreover, the resistance to HIV-1 and feline immunodeficiency virus (FIV) in the New World owl monkeys and in the Old World pig tailed monkeys, respectively, is mediated by a TRIMCyp protein that conserves the N-terminal RBCC tripartite motif of TRIM5$\alpha$ (Luban, 2007; Sokolskaja and Luban, 2006; Wilson et al., 2008). In addition, a wide screen performing an exogenous over-expression of 55 TRIM proteins from human and murine origins identified 20 TRIM proteins that affect viral entry and release of HIV-1, murine leukemia virus (MLV) and avian leukosis virus (ALV) (Uchil et al., 2008). Recently, TRIM28 (also known as KAP1 or TIF1$\beta$) has been shown to restrict MLV, visna, spuma and Mason-Pfizer monkey retroviruses in embryonic stem cells (Wolf and Goff, 2007; Wolf et al., 2008b). Interestingly, eight human TRIM genes (TRIM10, TRIM15, TRIM26, TRIM27, TRIM31, TRIM38, TRIM39 and TRIM40) are located in the major histocompatibility complex region of chromosome 6 which has protective impact on HIV-1 progression

(Carthagena et al., 2009; Fellay et al., 2007). The most described TRIM proteins that inhibit HIV-1 are the rhesus macaque TRIM5α (Stremlau et al., 2004) and the owl monkey TRIMCyp (Sayah et al., 2004) proteins that inhibit HIV-1 at early infection steps. The human TRIM22, also known as Staf50, has been described to inhibit HIV-1 in the later viral steps of transcription (Bouazzaoui et al., 2006) and viral assembly and release (Barr et al., 2008).

### 1.2-TRIM5α and TRIMCyp

The TRIM5α was identified as restriction factor of HIV-1 in the Old Word rhesus monkey cells in 2004 (Stremlau et al., 2004). Before that time TRIM5α was referred in the reports as the not yet identified proteins Ref1, that restricts N-tropic MLV (N-MLV) in human cells, and Lv1 that restricts HIV-1 in monkey cells (Hatziioannou et al., 2004; Huthoff and Towers, 2008; Nakayama and Shioda, 2010; Yap et al., 2004). The mechanism by which TRIM5α exerts its antiretroviral effect is not fully understood. However, it is known that TRIM5α targets intact or partially uncoated incoming viral cores via a pattern recognition function that identifies the structure formed by the capsid hexameric lattice (Luban, 2007; Sokolskaja and Luban, 2006; Towers, 2007). The block of replication appears to occur at pre-reverse transcription step impairing the efficient completion of the viral cDNA synthesis (Sokolskaja and Luban, 2006; Stremlau et al., 2004; Towers, 2007). Indeed, it has been suggested that TRIM5α blocks retroviral replication either by causing cores to undergo rapid or premature disassembly (Stremlau et al., 2004; Stremlau et al., 2006) and/or by recruiting cellular proteasome degradation machinery to the viral core-TRIM5α complex (Anderson et al., 2006; Campbell et al., 2008). In addition, to pre-reverse transcription inhibition, it has been suggested that TRIM5α blocks HIV-1 reverse transcription and nuclear import. This hypothesis was suggested since it has been observed that following the inhibition of proteasomes, TRIM5α does not inhibit HIV-1 post entry leading to complete cDNA synthesis, however, the nascent reverse transcripts fail to sustain an efficient infection (Luban, 2007; Wu et al., 2006; Yap et al., 2006). To explain this subsequent block of HIV-1 infection in primate cells, it was also suggested that in the absence of proteasome activity the incoming viral core remains stably sequestered in complex with TRIM5α without degradation which allows HIV-1 to accomplish reverse transcription but not to complete the infection (Campbell et al., 2008; Huthoff and Towers, 2008; Towers, 2007). Importantly, the antiretroviral effects of TRIM5α are exerted in a

species–specific manner (Sokolskaja and Luban, 2006; Stremlau et al., 2004; Towers, 2007). For example, HIV-1 replication is potently blocked by TRIM5α from the non human primate rhesus macaque (Stremlau et al., 2004). In contrast, human TRIM5α is less effective on HIV-1 but it inhibits the gammaretrovirus N-MLV and also the lentiretrovirus equine infectious anemia virus (EIAV) in different cell types (Hatziioannou et al., 2004; Huthoff and Towers, 2008). The specificity of retroviral restriction correlates with the ability of the TRIM5α to recognize the incoming viral core by its PRYSPRY (or B30.2) C-terminal domain (Sokolskaja and Luban, 2006; Towers, 2007); (Nakayama and Shioda, 2010). The PRYSPRY domain is the main determinant of core recognition by its specific binding to the retroviral capsid (Stremlau et al., 2004; Stremlau et al., 2006; Stremlau et al., 2005). The shorter isoforms of TRIM5, TRIM5γ and TRIM5δ, that lack PRYSPRY domain and contain alternative C-terminus did not exhibit antiviral activity and when over-expressed acted as dominant negative of TRIM5α restriction (Stremlau et al., 2004). The degree of capsid binding and restriction potency correlates with the amino-acid variations in the PRYSPRY domain and, remarkably, a single amino acid substitution (R332P) in the PRYSPRY domain is sufficient to enable human TRIM5α to restrict HIV-1 (Stremlau et al., 2005). Conversely, mutations in capsid modulate the susceptibility of incoming core to TRIM5α restriction (Stremlau et al., 2006).

Interestingly, in the New Word owl monkeys, the PRYSPRY domain has been replaced by cyclophilin A (CypA) by a LINE-1-mediated retrotransposition event to generate TRIMCyp protein that restricts HIV-1 (Sayah et al., 2004). TRIMCyp protein was also discovered in Old World pig tailed monkeys that restricts HIV-2 and FIV and not HIV-1 (Wilson et al., 2008). The CypA portion of TRIMCyp binds capsid and disruption of this interaction abolishes the HIV-1 restriction activity of TRIMCyp (Diaz-Griffero et al., 2006; Towers, 2007). Indeed, fusion of CypA to the C-termini of non restricting TRIM proteins such as TRIM1, TRIM18 or TRIM19 generated functional HIV-1 restriction factors (Yap et al., 2006). The CypA portion of TRIMCyp therefore replaces the capsid-binding function of the PRYSPRY domain in TRIM-mediated retroviral restriction. CypA is an ubiquitously expressed peptidyl prolyl isomerase that was earlier shown to interact with HIV-1 capsid and catalyses its *cis-trans* isomerization in a conserved prolyl peptide bond (Towers, 2007). The disruption of the interaction between CypA and HIV-1 capsid by Cyclosporine A competitive inhibitors or by point mutations in the viral protein inhibited HIV-1 replication (Luban, 2007; Towers, 2007). CypA is required in the released HIV-1 virions for

an efficient completion of reverse transcription in target cells (Braaten and Luban, 2001). It has been also shown that CypA confers protection to the incoming HIV-1 virus from cellular restriction factors (Towers et al., 2003). However, in non-human primate cells the disruption of capsid-CypA interaction or the down-regulation of CypA reduced the susceptibility of HIV-1 to the rhesus macaque TRIM5$\alpha$ restriction and rescued HIV-1 infectivity (Towers, 2007; Towers et al., 2003). Thus, CypA functions in an opposite manner in non-human primate as compared to human cells by promoting restriction rather than replication (Luban, 2007; Towers, 2007; Towers et al., 2003).

Although PRYSPRY domain is sufficient for capsid binding, TRIM5$\alpha$ restriction activity requires the contribution of all three domains of the N-terminal tripartite RBCC motif (Li and Sodroski, 2008; Luban, 2007; Perez-Caballero et al., 2005a). The primary function of the coiled-coil domain is to mediate oligomerization to increase capsid binding (Rold and Aiken, 2008). The B-box domain has been shown to induce higher-order self-association of TRIM5$\alpha$ oligomers to promote cooperative binding to the multimeric retroviral capsid (Li and Sodroski, 2008). The RING domain possesses E3 ubiquitin ligase activity *in vivo* and *in vitro* and was proposed to participate in HIV-1 restriction by recruiting proteasomal degradation machinery to the capsid-TRIM5$\alpha$ complex (Anderson et al., 2008; Campbell et al., 2008; Rold and Aiken, 2008). However, these findings have been debated due to the use of protease inhibitors that might affect several cellular and viral mechanisms complicating the interpretation of the true role of the RING E3 ubiquitin ligase in TRIM5$\alpha$ restriction activity (Huthoff and Towers, 2008; Luban, 2007). Moreover, TRIM5$\alpha$ is itself polyubiquitinated and its half-life is increased by the protease inhibitors (Luban, 2007). Finally, the formation of TRIM5$\alpha$ cytoplasmic bodies has been shown not to be required for retroviral restriction (Perez-Caballero et al., 2005a; Perez-Caballero et al., 2005b).

### 1.3- TRIM22

TRIM22, previously named "Stimulated Trans-acting Factor of 50 kDa (Staf50)", is located on chromosome 11 adjacent to TRIM5. TRIM22 was identified and cloned as an interferon-inducible gene and has been shown to inhibit LTR-mediated expression of a reporter gene suggesting a potential role of TRIM22 in controlling HIV-1 transcription (Tissot and Mechti, 1995). In another study, the over-expression of TRIM22 in macrophages inhibited HIV-1 infectivity suggesting an effect on LTR-HIV-1 transcription (Bouazzaoui et al., 2006). Interference of TRIM22 on HIV-1 viral production has been also

proposed based on the observation that p24 Gag antigen levels were decreased in the supernatant of infected macrophages following TRIM22 over-expression (Bouazzaoui et al., 2006).

A subsequent study showed that interferon induction of TRIM22 inhibits HIV-1 virion assembly in various human cell lines by interfering with Gag trafficking (Barr et al., 2008). Human TRIM22 binds and inhibits selectively HIV-1 Gag and not Gags from murine leukemia virus (MLV) or equine infectious anemia virus (EIAV) (Barr et al., 2008). TRIM22 interferes with Gag plasma-membrane accumulation, required for virion assembly, and hence induces Gag cytoplasmic diffusion without affecting Gag expression (Barr et al., 2008). The RING domain E3 ubiquitin ligase activity of TRIM22 is required for viral inhibition but not for Gag ubiquitination and degradation, suggesting that TRIM22 disturbs Gag cytoplasm trafficking by acting on another cellular or viral protein substrates (Barr et al., 2008).

A recent study showed that in primary blood mononuclear lymphocytes (PBMCs) from HIV-1 infected persons, the expression level of TRIM22 is higher than in healthy cells (Singh et al., 2011). The increased levels of TRIM22 were negatively correlated to the viral load which suggested a role of this TRIM protein in the progression of AIDS.

**2- APOBECs (A3G and A3F)**

APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3G or A3G, initially known as CEM-15) acts at the level of HIV-1 reverse transcription. APOBEC3G was first identified as the cellular factor that renders human cells non permissive for infection by HIV-1 viruses lacking a *Vif* gene (HIV-1$\Delta$Vif) (Sheehy et al., 2002). APOBEC3G is a member of a family of cytidine deaminases that catalyses the hydrolysis of cytidines (C) to uridines (U) (Goila-Gaur and Strebel, 2008). APOBEC3G targets cytidines in the negative sense single-stranded DNA that is generated during HIV-1 reverse transcription, a process also called as DNA editing or hyper-mutation. The C to U editing leads to guanine (G) to adenine (A) substitutions in the positive sense DNA strand of the reverse transcripts (Malim, 2009). These G to A hypermutations in the newly synthesized viral cDNA potentially leads to its instability and might inactivate viral gene products or regulatory genetic elements (Huthoff and Towers, 2008; Malim, 2009). The APOBEC3G is incorporated into virus particles budding from the infected or producer cells and editing occurs during reverse transcription upon infection of the new target cell (Huthoff and

Towers, 2008). The HIV-1 encoded Vif protein (viral infectivity factor) counteracts APOBEC3G by targeting it for ubiquitination, likely mediated cullin-5 E3 ubiquitin ligase complex, and subsequent degradation by the proteasome (Goila-Gaur and Strebel, 2008). The direct effect of Vif-induced APOBEC3G degradation is to prevent APOBEC3G incorporation into the virions (Malim, 2009). Therefore, the permissiveness to HIV-1ΔVif is dependent on producer cells regardless the target cells used. APOBEC3G is not ubiquitously expressed in all cell types which determine the permissiveness to HIV-1ΔVif viruses (Sheehy et al., 2002). For instance, APOBEC3G is expressed in non-permissive CEM, H9 cell lines and also in primary blood lymphocytes and not in CEMss, HeLa and HEK293T permissive cells (Sheehy et al., 2002). In addition to APOBEC3G, several other members of the APOBEC3 protein family have been shown to act against a wide range of viruses and transposable elements (Goila-Gaur and Strebel, 2008). Human APOBEC3F (A3F) also inhibits HIV-1 in a Vif dependent manner (Malim, 2009). Some studies described also a role of APOBECs in conferring intrinsic resistance to HIV-1 in dentritic cells and macrophages as target cells (Peng et al., 2007; Pion et al., 2006). It has been also shown that APOBEC3G and APOBEC3F interact with HIV-1 integrase and inhibit HIV-1 integration (Luo et al., 2007).

**3-Tetherin (CD317/BST-2)**

Tetherin, also known as CD317 or bone marrow stromal cell antigen 2 (BST-2), is a recent identified HIV-1 inhibitor factor that functions at the level of virus particle release from the cell surface (Neil et al., 2008; Van Damme et al., 2008). Tetherin is an interferon-inducible trans-membrane protein that retains virus particles of Vpu deleted HIV-1 viruses (HIV-1ΔVpu) at the cell surface after they have budded from the plasma membrane (Neil et al., 2008; Van Damme et al., 2008). Tetherin is not an ubiquitously expressed protein which determines the permissiveness to HIV-1ΔVpu viruses. Tetherin inhibits HIV-1 ΔVpu release in non-permissive cells such as HeLa cells, while, in permissive cells such as HEK239T cells HIV-1 ΔVpu release is unaffected (Neil et al., 2008; Van Damme et al., 2008). Tetherin inhibition is counteracted by Vpu in non permissive cells (Neil et al., 2008; Van Damme et al., 2008). The mechanism by which tetherin retains virus particles on the cell surface is still not defined (Bieniasz, 2009; Evans et al., 2010). Tetherin contains two trans-membrane anchors that might allow it to tether virions on the cell surface (Bieniasz, 2009; Evans et al., 2010). The mechanism by which Vpu is counteracting tetherin during HIV-1

infection is also not yet clear (Bieniasz, 2009; Evans et al., 2010). The initial reports did not detect significant reductions in tetherin levels upon Vpu expression (Neil et al., 2008) or a down-regulation of tetherin from the cell surface induced by Vpu (Van Damme et al., 2008). Subsequent studies, in contrast, were able to measure a significant reduction in total tetherin level following Vpu expression. It has been proposed that Vpu removes tetherin from the cell surface and induces its degradation in the lysosomes and/or in the proteasomes by serving as an adaptor between tetherin and the β-TrCP/SCF E3 ubiquitin ligase complex (Bieniasz, 2009; Evans et al., 2010). It has been also proposed that Vpu induces the endocytosis and the sequestering of tetherin inside the endosomes avoiding its accumulation at the cell surface (Bieniasz, 2009; Evans et al., 2010). Importantly, because the Vpu expression is limited to HIV-1 and a small number of closely related simian immunodeficiency viruses (such as SIVcpz), other lentiviruses have likely evolved distinct approaches to counteract tetherin. For example, the Env glycoprotein of some strains of HIV-2 possess Vpu-like activity and the Nef proteins of several SIVs antagonize tetherin (Bieniasz, 2009; Evans et al., 2010).

## 4- Factors inhibiting HIV-1 integration and nuclear pre-integration events

### 4.1- p21$^{CIP1/Waf1}$

So far p21$^{CIP1/Waf1}$ (p21) is the only described factor that targets specifically the HIV-1 integration step (Zhang et al., 2005; Zhang et al., 2007). Notably, p21 is the principal mediator of cell cycle arrest in response to DNA damage by inhibiting G1-phase cyclin-dependent kinases (CDKs) (Cazzalini et al., 2010). The inhibition of HIV-1 infectivity and integration by p21 was shown to occur in various cell types and to contribute to the intrinsic resistance of CD34+ hematopoietic stem cells to HIV-1 (Zhang et al., 2005; Zhang et al., 2007). Indeed, hematopoietic stem cells, the progenitor of either the myeloid or the lymphoid lineages, are one of a few cell types that resist HIV-1 infection despite the presence of functional HIV-1 CD4 and CXCR4 receptors (Weichold et al., 1998). A previous report indicated that a major block to HIV-1 infection in these cells is at virus entry, since infection can be achieved using pseudotyped HIV-1 virions that carry a heterologous envelope (VSV-G) (Shen et al., 1999). However, later work also suggested that a second block to HIV-1 replication is imposed by p21 (Zhang et al., 2005). Indeed, p21 knockdown using small interfering RNA (siRNA) enhanced the infectivity of pseudotyped HIV-1 vectors

about 3 fold (by measuring a reporter gene) and 7 fold (by measuring integrated viral cDNA) (Zhang et al., 2005). A subsequent study pinpointed the inhibition of HIV-1 infection by p21 at the level of integration (Zhang et al., 2007). In this report, it has been shown that p21 knockdown in primary CD34+ hematopoietic stem cells or in CMK cell lines, a p53-deficient human megakaryoblastic cells, did not affect reverse transcription, while proviral DNA was increased correlated with a decreased 2-LTR circles. These results suggest that p21 acts specifically at the level of integration. The mechanism by which p21 inhibits HIV-1 integration was not established, however, the same study demonstrates that the inhibition of integration was correlated with the presence of p21 in the purified HIV-1 pre-integration complex (PIC) from the restrictive hematopoietic stem cells and not from non restrictive T cell lines. Based on its viral phenotype and cellular functions, it has been suggested that p21 might recognize the incoming PIC and prevent HIV-1 integration by acting on the chromatin modifications at the DNA target site or on the DNA repair mechanisms.

### 4.2- RAD52

RAD52 is a member of RAD52 epistasis group involved in DNA repair of double strand break by homologous recombination (Symington, 2002). RAD52 has been shown to inhibit HIV-1 infectivity and provirus formation and was proposed to act by targeting the incoming viral cDNA within the pre-integration complex (Lau et al., 2004). In this report it has been shown that in *rad52* knockout mouse embryonic stem cells or following RAD52 transient knockdown in HeLa cells, HIV-1 infectivity and integration were increased by 10 fold in knockout cells and by 2 fold in knockdown cells. In *rad52* knockout cells, 2-LTR circles were also increased of almost 5 fold as compared to wild type cells (Lau et al., 2004). In this study, no information has been provided about reverse transcription or nuclear import although RAD52 showed to affect the amounts of the 2-LTR circles. The homologous recombination activity of RAD52 was not required for HIV-1 inhibition, however, the RAD52 DNA-binding activity was needed (Lau et al., 2004). Moreover, the same study showed that RAD52 competes with Ku in binding to HIV-1 LTRs *in vivo*. Ku is a cellular protein found in the PIC and required for the formation of the 2-LTR circles by non homologous end joining recombination (NHEJ) (Li et al., 2001). This observation suggested that RAD52 reduced the formation of the 2-LTR circles by displacing Ku from the viral ends of the un-integrated cDNA. However, in HeLa cells over-expressing RAD52,

there was no cell death (Lau et al., 2004) typically induced by the accumulation of the un-integrated viral cDNA in the absence of Ku (Li et al., 2001). Thus, the authors suggested a model independent from the requirement of Ku and the NHEJ repair for the circularization of the un-integrated viral cDNA and the prevention of cell death (Lau et al., 2004). They hypothesized that RAD52 inhibits specifically HIV-1 integration step by displacing from the PIC, integrase or other viral or cellular co-factors required for integration. This displacement might occur through the direct competition of RAD52 with the PIC proteins in binding to viral cDNA ends or indirectly by removing Ku that might be essential for the recruitment of the PIC proteins.

### 4.3- Rad18

Rad18 is a post replication/transletion DNA repair protein. Rad18 has been reported to interact with HIV-1 integrase *in vitro* and *in vivo* (Mulder et al., 2002). The same study shows that Rad18 stabilizes an ectopically expressed integrase and co-localizes with it in nuclear structures. In the subsequent report investigating the role of Rad18 during viral replication, it has been described that Rad18 suppresses the HIV-1 infection independently from integration likely by targeting the incoming viral cDNA inside the nucleus (Lloyd et al., 2006). Indeed, in *rad18* knockout murine embryonic fibroblasts, the infectivity of wild type HIV-1 or of mutant virus carrying an integrase catalytically inactive (D116A) was increased (based on the reporter gene) by 5 fold correlating with an accumulation of the HIV-1 late reverse transcripts that became evident after the eight hours of infection (Lloyd et al., 2006). Since Rad18 is nuclear, it has been suggested that the inhibitory effect of Rad18 might be by targeting linear viral cDNA, after has been crossed the nucleus, by inducing its degradation or its circularization (Lloyd et al., 2006).

### 4.4- XPB and XPD

Report from Yoder et al (2006) showed that XPB and XPD reduce HIV-1 integration efficiency by inducing the degradation of the viral cDNA available for integration (Yoder et al., 2006). XPB and XPD are DNA helicases with opposing polarity that function as integral components of the TFIIH protein complex. The helicase activity of TFIIH complex is required to separate DNA strands at promoters during transcription or at DNA damage sites during nucleotide excision repair (NER). HIV-1 infectivity was increased in either XPB or XPD mutant cell lines, susceptible for UV irradiation, comparing to the mutant cells

complemented with the expression of wild type genes, that restored an efficient DNA repair activity. The increase of infectivity in XPB and XPD mutant cells was correlated with an increase about 2,5 fold of the total viral cDNA, integrated proviruses and 2-LTR circles as compared to complemented cells. In XPB and XPD mutant cells the accumulation of the wild type HIV-1 cDNA was similar to a mutant virus encoding an integrase catalytically inactive. Moreover, upon treatment of cells with reverse transcriptase inhibitors the remaining viral cDNA was more accumulated in XPB and XPD mutant cells than in complemented cells. Thus, it has been concluded that XPB and XPD inhibit HIV-1 not by interfering with reverse transcription or integration efficiencies but by enhancing the degradation of the HIV-1 cDNA available for integration. Since XPB and XPD are exclusively nuclear, the Yoder et al (2006) suggested that this mechanism of defense is nuclear at pre-integration step.

## KAP1 (TRIM28)

### 1- KAP1 structure and functions

KRAB domain-associated protein 1 (KAP1), also called TRIM28, TIF1$\beta$ or KRIP1, is an ubiquitous nuclear protein belonging to TRIM protein family (chapter II.3) (Reymond et al., 2001). KAP1 has been identified to bind directly to the highly conserved Kruppel associated box (KRAB) repression domain of the KRAB zing finger proteins and so far KAP1 is their universal co-repressor (Friedman et al., 1996; Urrutia, 2003). The KRAB domain is present in the N-terminal region of more than 220 zinc finger proteins and mediate their binding to the co-repressor, whereas the $C_2H_2$ zinc finger C-terminal motif serves to bind DNA including RNA polymerase I, II and III promoters (Abrink et al., 2001; Peng et al., 2000; Urrutia, 2003).

KAP1 has 835 amino acid residues and contains on its N-terminus the RBCC motif composed of RING, two B-boxes (B1 and B2) and a coiled-coil domains and on its C-terminus a tandem of plant homeodomain (PHD) and bromodomain (PHD-BROMO) characteristic of subfamily C-VI of TRIM proteins (**Figures 8** and **9a**) (Reymond et al., 2001; Short and Cox, 2006). The RBCC motif is involved in KAP1 oligomerization, likely in trimer, and in protein-protein interactions (Peng et al., 2000). Notably, RBCC motif is responsible for KAP1 direct binding with KRAB domain (Peng et al., 2000). The PHD and BROMO domains act cooperatively for interaction with CHD3 (Mi2$\alpha$), which is a subunit of

NuRD histone deacetylase complex (Schultz et al., 2001), and with SETDB1 histone H3-K9 methyl transferase (Schultz et al., 2002). The crystal structure of PHD and BROMO domains showed a difference from p300 PHD and BROMO structure in which two domains cooperate to bind acetylated lysines of histone and non-histone proteins (Ragvin et al., 2004; Zeng et al., 2008). Indeed, PHD and BROMO domains of KAP1 failed to bind acetylated histone peptides *in vitro* (Zeng et al., 2008). The central domain of KAP1 contains an heterochromatin 1 (HP1) binding box domain (HP1BD) carrying a PxVxL motif that binds directly the chromoshadow domain of HP1 (Lechner et al., 2000). KRAB zing finger proteins and KAP1 have been shown to co-localize in nucleoplasmic foci called KAKA foci that are adjacent to PML bodies and have been proposed to contain the sumoylated KAP1 (Briers et al., 2009).

KAP1 has been shown to be post-translationally modified by either sumoylation or by phosphorylation which regulate its cellular functions in transcription and in DNA damage response. KAP1 PHD domain binds to ubiquitin conjugating enzyme (Ubc9) and directs SUMO conjugation of the adjacent BROMO domain at two main lysines K779 and K804 (Ivanov et al., 2007). KAP1 sumoylation has been shown important for the interaction with SETDB1 and CHD3, that both contain specific SUMO interacting motifs (SIM) (Ivanov et al., 2007). KAP1 has been reported to be phosphorylated by ATM kinase upon DNA damage at its C-terminal domain at the serine 824 (S824) (**Figure 9a**) (White et al., 2006; Ziv et al., 2006).

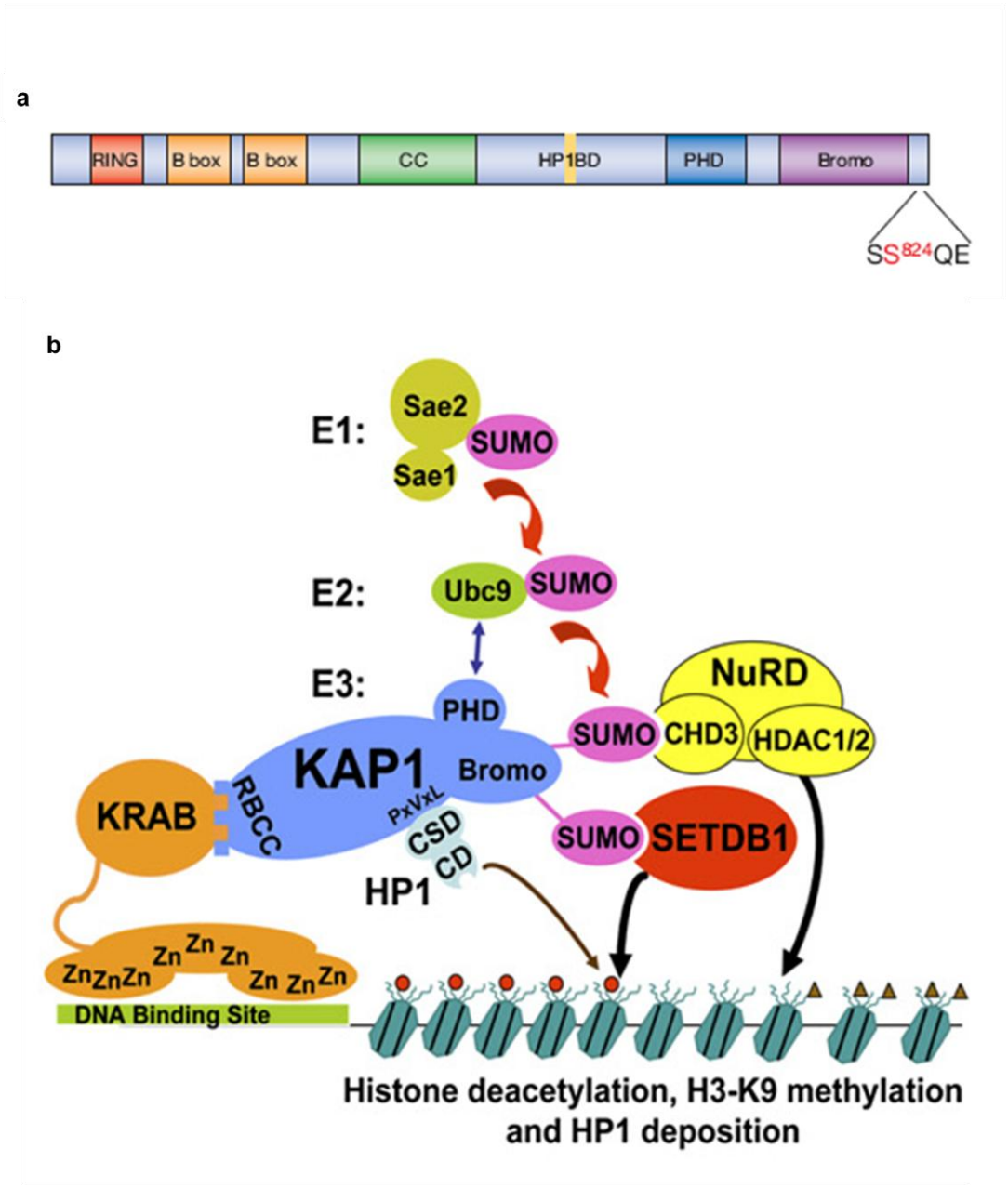


**Figure 9**: (**a**) Schematic representation of KAP1 domains. RBCC motif: RING, B boxes and CC (coiled–coil domains); HP1BD: HP1-binding domain, the PHD: plant homeodomain and BROMO: bromodomain (Ziv et al., 2006). (**b**) A schematic model for KRAB-KAP1-mediated repression. Following the interaction between the KRAB domain of the zinc finger protein with RBCC KAP1 domain at the target DNA to be repressed. The PHD domain of KAP1 catalyzes the sumoylation of the adjacent bromodomain mainly at lysines: K779 and K804. SUMO family proteins are conjugated to target lysines via a cascade of E1-activating, E2 transfer, and E3 ligase enzymes. The PHD-Ubc9 interaction is required for the Bromo sumoylation. Once modified by SUMO, the KAP1 bromodomain serves as a scaffold and recruits repression machinery through the recognition of the conjugated SUMO moieties by the SIM motifs of CHD3 (Mi2$\alpha$) and SETDB1 and their associated proteins. The KAP1 scaffold is further exploited through recruitment of HP1, which recognizes the H3-K9 methyl mark and establishes a repressive chromatin state. Ultimately, the concerted action of these effector molecules leads to chromatin reorganization at the promoter region resulting in silent chromatin (Ivanov et al., 2007).

KAP1 expression is important for normal development in mice (Cammas et al., 2000). Germ line homozygous knockout of *KAP1* gene results in embryonic lethality at early

implementation step (E5.5) (Cammas et al., 2000). KAP1 has been mainly described as transcriptional co-repressor through KRAB zing finger proteins by inducing formation of hetrochromatin at DNA targeted for silencing (Friedman et al., 1996; Lechner et al., 2000; Nielsen et al., 1999; Schultz et al., 2002; Schultz et al., 2001; Sripathy et al., 2006). Moreover, KAP1 has been also described to tether histone deacetylases (HDACs) to non-histone proteins such as p53, E2F1 and STAT3 to induce their deacetylation and consequently their inactivation (Tian et al., 2009; Tsuruma et al., 2008; Wang et al., 2005a; Wang et al., 2007a). In addition, KAP1 has been also reported as DNA damage response factor (White et al., 2006; Ziv et al., 2006) and as restriction factor of murine leukemia virus (MLV), visna, spuma and Mason-Pfizer monkey retroviruses in embryonic stem cells (Wolf and Goff, 2007; Wolf et al., 2008b).

## 2- Transcriptional co-repressor activity of KAP1

KAP1 is a hallmark scaffold protein that recruits and coordinates complexes and components of heterochromatin formation (**Figure 9b**) (Lechner et al., 2000; Schultz et al., 2002; Schultz et al., 2001; Sripathy et al., 2006). KAP1 is recruited to target promoters by KRAB zinc finger proteins through a direct interaction between its RBCC motif with KRAB domain (Ayyanathan et al., 2003; Groner et al., 2010; Sripathy et al., 2006). The PHD domain then binds Ubc9 and catalyzes the sumoylation of the adjacent bromodomain mainly at lysines K779 and K804 (Ivanov et al., 2007; Lee et al., 2007). Sumoylated KAP1 has been demonstrated to be the active form in KRAB-KAP1-mediated transcription repression (Ivanov et al., 2007; Lee et al., 2007; Li et al., 2007). Indeed, once modified by SUMO, KAP1 bromodomain serves as a scaffold and recruits repression machinery through the recognition of the conjugated SUMO moieties by the SIM motifs of CHD3 and SETDB1 (Ivanov et al., 2007). CHD3, also called Mi2$\alpha$, is a subunit of NurD histone deacetylase complex that contains also HDAC1 and HDAC2 (Grozinger and Schreiber, 2002). CHD3-KAP1 interaction is thus suggested to mediate the recruitment of NurD complex to catalyze histone deacetylation creating favorable environment for heterochromatin formation (Schultz et al., 2001; Sripathy et al., 2006). SETDB1 is a histone methyl transferase that catalyzes specifically the methylation of lysine 9 of histone 3 (H3K9) (Schultz et al., 2002; Sripathy et al., 2006). The function of KAP1 as scaffold is further exploited through the recruitment, by its PxVxL motif, of HP1 that binds the tri-methylated H3K9 mark and establish a repressive chromatin state or also called facultative

heterochromatin (Ayyanathan et al., 2003; Groner et al., 2010; Sripathy et al., 2006). An artificial tethering of KRAB-KAP1 to target DNA has been shown to induce the formation of heterochromatin along several tens of kilobases away from their binding DNA site (Groner et al., 2010). The direct tethering of KAP1 to promoters through heterologous DNA binding domain (Gal4-DBD) also induced repressive state through chromatin modification (Nielsen et al., 1999; Sripathy et al., 2006), however, weaker than in association with KRAB zinc finger proteins (Sripathy et al., 2006). Although KRAB-KAP1 repression mechanisms are well established, the mechanisms of target DNA choice to induce the formation of the heterochromatin are poorly understood. Intriguingly, a wide genome analysis of KAP1 binding sites by chromatin immunoprecipitation showed an enrichment of KAP1 in the promoter regions of KRAB zing finger genes which suggested that zinc finger proteins auto-regulate their transcription by recruiting KAP1 (O'Geen et al., 2007).

The first evidence of the role of histone deacetylase in KAP1 repression activity was demonstrated using a KAP1 deleted in HP1 binding box that was proven unable to completely relieve transcription repression only following cell treatment with HDAC inhibitor (trichostatin A or TSA) (Nielsen et al., 1999). Subsequently, the PHD-BROMO domain of KAP1 has been demonstrated to bind the CHD3 (Mi2$\alpha$), a component of NuRD histone deacetylase complex, by yeast two hybrid screening (Schultz et al., 2001). Consistently, the KAP1 full length co-immunoprecipitated *in vivo* with CHD3, HDAC1 and RbAp48, all components of NuRD complex (Schultz et al., 2001). Moreover, a KAP1 deletion mutant lacking PHD and BROMO domains was unable to retain CHD3 *in vivo*, however, no data has been shown regarding the association of this mutant with HDAC1 or RbAp48 (Schultz et al., 2001). Data sustaining the role of HDACs in transcription repression showed that the PHD-BROMO mutant domains inactive in transcription repression were unable to interact with CHD3 (Schultz et al., 2001). In addition, the expression of dominant negative protein corresponding to binding region of CHD3 with KAP1 or treatment with TSA relieved the transcription repression activity of the PHD and BROMO domains (Schultz et al., 2001). Therefore, it has been suggested that PHD and BROMO domains following their tethering to promoters recruit NuRD histone deacetylase complex to induce histone deacetylation (Schultz et al., 2001). Moreover, KAP1 tethered to promoter by an heterologous DNA binding domain (Gal4-DBD) has been shown to induce concomitant deacetylation of lysines 9 and 14 of histone 3 (H3 K9/K14) (Sripathy et al., 2006).

Further evidence in support of HDAC role in KAP1 activities derive from the complex formation of KAP1 with the nuclear receptor core-repressor (NcoR) complex 1 (N-CoR-1) identified by mass spectrometry analysis (Underhill et al., 2000). In fact, NCoR-1 is a histone deacetylase and chromatin remodeling repressive complex that contains HDAC3 and components of SWI/SNF complex: BRG-1, BAF 170, BAF 155 and BAF 47/INI1. KAP1 has been shown to co-localize with N-CoR in nucleus, however, no role of KAP1 in N-CoR-1 mediated transcription repression has been reported or the region of KAP1 involved in its association with this complex (Underhill et al., 2000).

Finally, KAP1 has been shown to inhibit c-Myc transcription activation by binding MM-1, a c-Myc co-repressor, and recruiting Sin3 histone deacetylase complex (Satou et al., 2001). In this study KAP1 associates with HDAC1 and Sin3A, components of Sin3 complex, however, no region of KAP1 has been mapped for the binding with HDAC1 or Sin3A (Satou et al., 2001).

### 3- Involvement of KAP1 in the deacetylation of non-histone proteins

KAP1 has been also described to tether histone deacetylases (HDACs) to non-histone proteins such as p53, E2F1 and STAT3 to induce their deacetylation and consequently their inactivation (Tian et al., 2009; Tsuruma et al., 2008; Wang et al., 2005a; Wang et al., 2007a).

**p53** was the first non-histone protein known to be regulated by acetylation (Gu and Roeder, 1997) and deacetylation (Luo et al., 2000). The p53 is a key component of a regulatory circuit that monitors signaling pathways from diverse sources, including DNA damage responses, abnormal oncogenic events, and normal cellular processes. The acetylation of p53 at several lysines in its DNA binding motif and on its C-terminal domain is catalyzed by histone acetyl transferases: p300, CBP/p300 and Tip60 (Ito et al., 2001; Tang et al., 2008). In response to DNA damage, the levels of p53 acetylation are enhanced which are correlated with an enhancement of its stabilization and activation (Ito et al., 2001; Tang et al., 2008). Although mutation of some acetylation sites is compensated by the modification of other target lysines, mutation of all acetylated lysines abolished completely the ability of p53 to activate p21 and to induce cell growth arrest in response to DNA damage (Tang et al., 2008). Phosphorylation of p53 failed to relieve and to compensate this inactivation (Tang et al., 2008). The p53 acetylation has been shown to increase its DNA binding affinity *in vitro* (Gu and Roeder, 1997), stabilization (Ito et al.,

2002; Ito et al., 2001; Luo et al., 2000), transcription activity (Tang et al., 2008) and protein-protein interaction by enhancing its association with transcription co-activators such as CBP/p300 (Mujtaba et al., 2004) and by blocking its interaction with its repressors MDM2 and MDMX (Tang et al., 2008). In unstressed cells, p53 is maintained inactivated by MDM2 E3 ubiquitin ligase which binds p53 and induces its deacetylation by recruiting HDAC1 (Ito et al., 2002). Most of the deacetylated lysines are ubiquitinated by MDM2 and p53 is targeted for degradation (Ito et al., 2002). MDM2 does not bind directly HDAC1 and requires cellular factors to recruit HDAC1 to the acetylated p53 (Ito et al., 2002). KAP1 has been demonstrated to cooperate with MDM2 in bridging HDAC1 to p53 (Wang et al., 2005a). KAP1 stimulates p53-HDAC1 complex formation and consequently deacetylation, degradation and inactivation of p53 (Wang et al., 2005a).

KAP1 has been demonstrated to link p53 to DNA damage response through a phophorylation process involving the acetylation and deacetylation of p53 (Tian et al., 2009). This pathway involves p53 binding to APAK, a KRAB zing finger protein, which bridges KAP1-HDAC1 complex to the acetylated p53 (**Figure 10**). This association maintains p53 deacetylated and inactivated in the absence of DNA damage. In response to DNA damage, ATM phosphorylates APAK and KAP1 causing their dissociations from p53 and consequently re-acetylation of p53 leading to the transactivation of apoptotic genes (bax, noxa and puma).

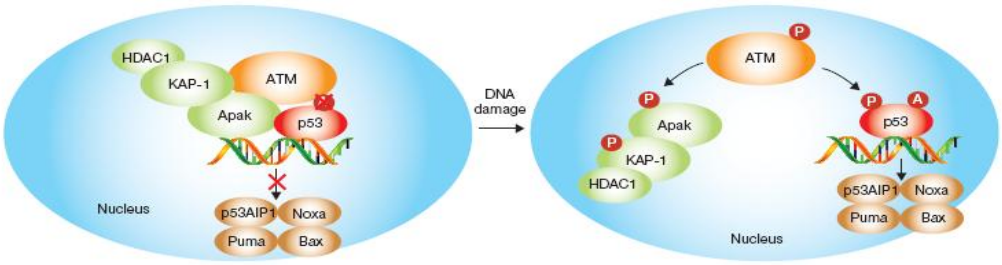


**Figure 10**. A model of the regulation of p53 acetylation mediated by KAP1 in the DNA damage response. Left panel: In the absence of DNA damage, APAK, a KRAB zinc finger protein, bridges KAP1-HDAC1 complex to p53 to maintain p53 deacetylated and inactivated. Right panel: In response to DNA damage, ATM phosphorylates APAK and KAP1 causing their dissociations from p53 and thus leading to the re-acetylation of p53 which becomes able to transactivate apoptotic genes (bax, noxa and puma) (Tian et al., 2009).

**E2F1** is a member of E2F family of transcription factors that play a key role in the regulation of expression of genes essential for DNA replication and cell cycle progression as well as in the surveillance of genomic integrity (Ren et al., 2002). In addition, E2F1 is activated following DNA damage by phosphorylation and acetylation modifications (Martinez-Balbas et al., 2000; Marzio et al., 2000; Ren et al., 2002). E2F1 acetylation is

mediated by p300/CBP and PCAF histone acetyl transferases and occurs at three specific lysines in the N-terminal portion of this factor, adjacent to its DNA binding domain (Martinez-Balbas et al., 2000; Marzio et al., 2000). Acetylation stabilizes E2F1 and enhances its DNA binding and transcriptional activities in order to up-regulate pro-apoptotic genes in response to DNA damage (Martinez-Balbas et al., 2000; Marzio et al., 2000). KAP1 has been reported to contribute to the functional regulation of E2F1 by reversing its acetylation state (Wang et al., 2007a). Indeed, it has been shown that KAP1 binds E2F1 and stimulates E2F1-HDAC1 complex formation leading to its deacetylation and inactivation (Wang et al., 2007a). In this study, E2F1 transcriptional activity was inhibited by KAP1 full length over-expression in cells, while, a KAP1 deletion mutant lacking PHD and BROMO domains was transcriptionally ineffective (Wang et al., 2007a). It has been presumed, based on a previous report describing the association of PHD and BROMO domains with NuRD histone deacetylase complex (Schultz et al., 2001), that this deletion mutant fails to bind HDAC1 and consequently does not induce the deacetylation of E2F1 (Wang et al., 2007a).

**STAT3** belongs to the STAT protein family which are latent transcription factors in cytoplasm used by most cytokine receptors to rapidly turn on gene expression in the nuclei (Darnell, 1997). Following cell stimulation by interleukins or by interferons, STAT3 becomes phosphorylated by tyrosine kinases and also acetylated by p300 or p300/CBP at one lysine residue (K685) (Wang et al., 2005b; Yuan et al., 2005). Acetylation stabilizes STAT3 dimers which are the required forms for STAT3 nuclear translocation and for DNA binding of target promoters (Wang et al., 2005b; Yuan et al., 2005). STAT3 acetylation is reversed by HDAC3 and with lesser extent by HDAC1 and HDAC2 to determine the transcriptionally inactive state of the protein in the absence of stimulus (Yuan et al., 2005). It has been shown in a following study that KAP1 binds selectively the phosphorylated STAT3, the transcriptionally active form, and tethers HDAC3 to induce its deacetylation and inactivation (Tsuruma et al., 2008).

### 4- Involvement of KAP1 in DNA damage response

KAP1 was demonstrated to be phosphorylated by members of phosphatidylinositol-3 kinase-like family of kinases, ATM, ATR and DNA-PK, following DNA double strand breaks (DSBs) induced by either neocarzinostatin radiometric drug or ionizing radiation (White et

al., 2006; Ziv et al., 2006). KAP1 phosphorylation occurs at serine 824 (S824) located at its C-terminus domain adjacent to bromodomain (White et al., 2006; Ziv et al., 2006). Phosphorylation of KAP1 is an early and transient event in the DNA damage response (White et al., 2006; Ziv et al., 2006). Indeed, it has been shown that phosphorylation happens five minutes after genotoxic treatment of cells, it is at its maximum thirteen minutes and persists up to two hours (White et al., 2006; Ziv et al., 2006). KAP1 phosphorylation catalyzed by ATM was detected exclusively at the DNA damage sites, from which the phosphorylated protein spreads rapidly throughout the nucleus (Ziv et al., 2006). The displacement of phophorylated KAP1 from DSB sites induces transient chromatin relaxation allowing the recruitment of DNA repair machinery (Ziv et al., 2006). In fact, two later studies underlined the role of ATM in DNA damage repair within heterochromatic regions through the phophorylation of KAP1 which permits an increase in nucleosome accessibility for the DNA repair proteins in these regions (Goodarzi et al., 2008; Goodarzi et al., 2009). Consistently, knockdowns of KAP1, HDAC1 or HP1, the KAP1 downstream silencing effectors, alleviated the DSB repair defects in *ATM* knockout cells (Goodarzi et al., 2008; Goodarzi et al., 2009).

Phosphorylation and sumoylation were reported to be antagonizing modifications that regulate KAP1 activity in response to genotoxic stress as well as in the control of cell cycle arrest and apoptosis (Lee et al., 2007; Li et al., 2007). In unstressed cells, sumoylated KAP1 is the transcription active form that establishes repression of genes involved in cell cycle arrest and apoptosis such as p21, Gadd45$\alpha$, bax, puma and noxa. Conversely, phosphorylated KAP1, activated in response to DNA damage, determines the de-repression of these genes (Lee et al., 2007; Li et al., 2007).

### 5- Involvement of KAP1 in retroviral inhibition

KAP1 (TRIM28) from murine origin was shown to restrict MLV replication by promoting gene silencing (Wolf and Goff, 2007). KAP1-mediated MLV restriction occurs in embryonic stem cells through KAP1 association with the nuclear repressor complex that binds to the retroviral "repressor binding site" (RBS) (Wolf and Goff, 2007). The RBS element comprises 17 base pairs that overlap closely with the 18 base pairs of primer binding site (PBS) of MLV. The MLV PBS has a sequence complementary to the cellular proline tRNA ($tRNA^{pro}$) that primes minus-strand DNA synthesis during reverse transcription (Wolf and Goff, 2007). Notably, differentiation of F9 murine embryonic stem cells by retinoic acid

leads to the relief of MLV restriction which is correlated with dissociation of nuclear repressor complex from RBS and a decrease of KAP1 expression levels (Wolf and Goff, 2007). KAP1-mediated restriction in embryonic cells is also effective on PBS complementary to lysine 1 and 2 tRNA ($tRNA^{Lys-1,2}$) found in visna, spuma and Mason-Pfizer monkey retroviruses (Wolf et al., 2008b). The KAP1 interaction with HP1 is required for PBS-dependent restriction of MLV in embryonic cells indicating the relevance of heterochromatin formation in this inhibition (Wolf et al., 2008a). KAP1 does not bind directly to PBS and a novel KRAB zinc finger protein, ZFP809, was identified as a recognition molecule bridging the MLV integrated proviral DNA with KAP1 (Wolf and Goff, 2009). Expression of ZFP809 is sufficient to render differentiated cells, such as HEK 293T cells, resistant to MLV infection, however, this restriction is always mediated by KAP1 (Wolf and Goff, 2009). Consistently, the expression levels of ZFP809 in MLV non-restrictive NIH3T3 cells is lower than embryonic stem cells (Wolf and Goff, 2009). The role of ZFP809 was investigated for other viruses showing that ZFP809 potently inhibits transcription from DNA constructs of the human T cell lymphotropic virus-1 (HTLV-1) that utilizes tRNA ($tRNA^{pro}$) as MLV, however, non effect has been found on transcription of HIV-1 that utilizes $tRNA^{Lys3}$ (Kleiman, 2002; Wolf and Goff, 2009). Artificial zinc finger proteins that contain KRAB domains engineered to bind specifically HIV-1 PBS were able to induce transcriptional silencing of an integrated HIV-1 provirus (Eberhardy et al., 2006; Pengue et al., 1995). KAP1 has been also shown to silence endogenous retroviruses particularly intracisternal A-type particles (IAP) in mouse embryonic stem cells by inducing the heterochromatinization of 5' un-translated regions (5'UTR) of various IAPs without interfering with their PBS (Rowe et al., 2010). In inducible *KAP1* knockout mouse embryonic stem cells, IAP repression was relieved and correlated with a decrease in tri-methyl H3K9 and an increase in H4 acetylation (Rowe et al., 2010). In conclusion repression of endogenous or exogenous retroviruses in embryonic stem cells is crucial for protecting genome integrity during early embryogenesis which underlies the relevance of the cellular role of KAP1.

**Acetylation and deacetylation of histone and non-histone proteins**

Acetylation and deacetylation of histone and non-histone proteins is a dynamic process controlled by the antagonistic actions of two large families of enzymes: the histone acetyl transferases (HAT) and the histone deacetylases (HDACs). The balance between the

actions of these enzymes serves as a key regulatory mechanism for gene expression, stability and activities of the proteins and also modulates protein-protein, DNA-protein and RNA-protein interactions (Glozak et al., 2005; Sadoul et al., 2008; Spange et al., 2009; Sterner and Berger, 2000). Moreover, HAT and HDACs regulate cellular functions of the proteins by acetylation and deacetylation cycles which in most cases correspond respectively to activation and inactivation. Reversible acetylation modulates the activities of proteins involved in several cellular processes such as transcription, DNA replication and DNA repair (**Figure 11**) (Yang and Seto, 2007).

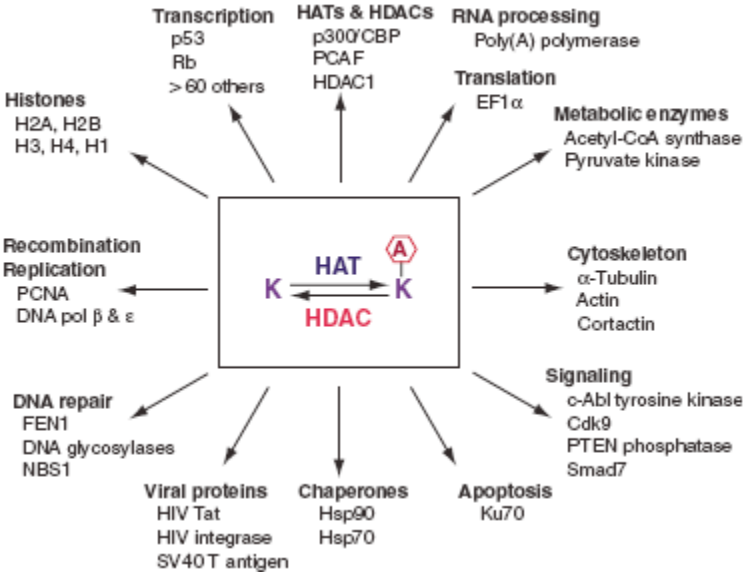


**Figure 11:** Schematic illustration of the prevalence of reversible lysine (K) acetylation in diverse cellular processes. The hexagon with the letter A refers to acetylation. For each process, only representative proteins are listed. In particular, acetylation of acetyl-CoA synthase is a key regulatory mechanism conserved from bacteria to humans. Cdk9, cyclin-dependent kinase 9; FEN1, Flap endonuclease 1; NBS1, Nijmegen breakage syndrome protein 1; PCNA, proliferating cell nuclear antigen; PTEN, phosphatase and tensin homolog; Rb, retinoblastoma suppressor protein (Yang and Seto, 2007).

### 1-Acetylation mediated by HATs

Histone acetyl transferase family proteins (**HATs**) transfer, through their HAT catalytic domain, an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amine group of specific lysine residues within the histone basic N-tail region (Roth et al., 2001). The addition of an acetyl group on lysines neutralizes their positive charges which has an impact on the electrostatic proprieties of the proteins. Histone acetylation facilitates the access of transcription machinery to DNA by loosening the interactions between both adjacent nucleosomes and DNA, as well as by serving as recognition site for the recruitment of accessory regulatory factors (Strahl and Allis, 2000). In addition to histones, HATs have been described to acetylate cellular and viral proteins mainly involved

in transcription such as E2F1, p53, STAT3, MyoD, HIV-1 Tat and adenovirus E1A (Sterner and Berger, 2000). HIV-1 integrase is also a substrate for histone acetyl transferases p300 and GCN5 (Cereseto et al., 2005; Terreni et al., 2010). Moreover, non-histone chromatin associated proteins such as HMG I(Y), HMG14 and HMG17 and cytoplasmic proteins like $\alpha$-tubilin are also substrates for HAT acetylation (Sterner and Berger, 2000). The acetylation of the non-histone proteins has been shown to modulate their activities by affecting their interactions with other proteins, DNA or RNA and also by altering their cellular localization (Glozak et al., 2005; Sadoul et al., 2008; Spange et al., 2009; Sterner and Berger, 2000). HATs are evolutionary conserved from yeast to man and are grouped into two general classes: nuclear A- and cytoplasmic B-type HATs (Kimura et al., 2005; Roth et al., 2001; Sterner and Berger, 2000). Nuclear A-type HATs are further grouped into five families: GNAT, MYST, p300/CBP, basal/general transcription factors and nuclear receptor cofactors (Kimura et al., 2005; Roth et al., 2001; Sterner and Berger, 2000). The A-type HATs acetylate nucleosomal histones within the chromatin and are potently linked to transcription by activating transcription factors through acetylation or by acting as co-activators at the level of gene promoters (Roth et al., 2001; Sterner and Berger, 2000). The cytoplasmic B-type HATs acetylate de novo synthesized free histones, promoting their nuclear localization and deposition onto newly synthesized DNA (Sterner and Berger, 2000). Although most HATs are able to acetylate free histones *in vitro* when assayed as a single polypeptide, in cells are usually found in conserved, cooperatively acting high-molecular-weight complexes such as GCN5/PCAF (human homologue of yeast SAGA complex), Tip60, HBO1 and MOZ/MORF complexes that contain several transcription regulators and chromatin binding proteins (Grant and Berger, 1999; Lee and Workman, 2007; Sterner and Berger, 2000). Many HATs such as p300 and CBP/p300 have an evolutionarily conserved bromodomain that recognizes specifically acetylated lysines and also directs chromatin associated proteins to acetylated histones (Lee and Workman, 2007; Mujtaba et al., 2007). Notably, p300 seems to have the broadest substrate acceptance for histones and non-histone proteins (Kimura et al., 2005).

Acetylation of numerous transcription factors such as p53, E2F1, STAT3, NFkB, p73 and MyoD has been shown to enhance their DNA binding affinity, transcription activities and/or stability (Glozak et al., 2005; Spange et al., 2009). For some transcription factors such as YY1, HMG-A1 and HMG-N2 acetylation induces their dissociation from DNA or chromatin to allow the accessibility of transcription factors for target genes (Glozak et al., 2005; Spange

et al., 2009). Notably, acetylation of HIV-1 integrase enhances its DNA binding affinity and its catalytic activity (Cereseto et al., 2005; Terreni et al., 2010). Integrase lysines targeted for acetylation by p300 and GCN5 (K264, K266 and K273) have been shown important to achieve optimal HIV-1 infection specifically at the level of integration (Apolonia et al., 2007; Cereseto et al., 2005; Terreni et al., 2010). Another HIV-1 viral protein, Tat, is modified by acetylation which promotes its transactivation functions. Tat acetylation at lysine 50 (K50) by p300 induces its dissociation from TAR RNA during early transcription elongation which allows the recruitment of PCAF to the elongating RNA polymerase II (Kiernan et al., 1999). Subsequently, PCAF acetylates Tat at lysine 28 (K28) which enhances its binding to CDK9 of P-TEFb complex in order to promote transcription elongation (Kiernan et al., 1999). Acetylated Tat at lysines K50 and K51 has been shown to bind with more affinity p32, a cellular splicing regulator, rather than the unmodified Tat (Berro et al., 2006). It has been suggested that this interaction recruits p32 at the LTR promoter to inhibit HIV-1 splicing needed for the production of full-length transcripts (Berro et al., 2006). Acetylation modulates protein-protein interaction of other several proteins. For instance, acetylated lysine 382 (K382) at the C-terminal domain of p53 is recognized specifically by CBP/p300 bromodomain (Mujtaba et al., 2004). That recognition enhances p53-CBP/p300 association which stimulates p53 transcriptional activity (Mujtaba et al., 2004). Similarly, bromodomains of either CBP/p300 or p300 recognize MyoD acetylated lysines (K99 and K102) enhancing the binding between these proteins (Polesskaya et al., 2001). This interaction serves to recruit CBP/p300 and p300 by MyoD at the target promoters to induce histone acetylation and transcription activation (Polesskaya et al., 2001). Moreover, acetylation also modulates interactions between proteins that do not have bromodomains. Indeed, importin $\alpha$ acetylation enhances its heterodimerization with importin $\beta$ and promotes the nuclear import of cargo proteins implicated in mRNA regulation such as HuR (Glozak et al., 2005; Spange et al., 2009). Acetylation of the tumor suppressor retinoblastoma (Rb) inhibits its phosphorylation, leading to cell cycle arrest, and enhances its interaction with MDM2 ubiquitin ligase (Glozak et al., 2005; Spange et al., 2009). Erythroid kruppel like factor (EKLF) acetylation enhances its association with SWI/SNF complex resulting in an open chromatin at the $\beta$-globin gene promoter (Glozak et al., 2005; Spange et al., 2009). Acetylation can also induce the dissociation between interacting proteins. For example, in unstressed cells, unmodified Ku70 has been shown to sequester bax apoptotic protein in cytoplasm (Cohen et al., 2004). In response to

apoptosis inducing agent, Ku70 becomes acetylated by p300 allowing the release of bax from its sequestration and enabling bax translocation to the mitochondria to initiate apoptotic cascade (Cohen et al., 2004). Acetylation of the adenoviral transcriptional activator, E1A, by p300 and PCAF was found to disturb its interaction with its co-repressor protein CtBP and with importin 3$\alpha$ which obstructs its nuclear import (Das and Kundu, 2005; Glozak et al., 2005; Spange et al., 2009).

**2- Deacetylation mediated by HDACs**

The mechanism of action of histone deacetylase enzymes (**HDACs**) involves removing of the acetyl group from histone and non-histone protein substrates (de Ruijter et al., 2003; Yang and Seto, 2007). Hypo-acetylation of histones results in a decrease in the space between nucleosomes and the DNA that is wrapped around it (Strahl and Allis, 2000). Tighter wrapping of the DNA diminishes accessibility for transcription factors leading to transcription repression (Strahl and Allis, 2000). Eighteen mammalian HDACs have been identified to date and are grouped into two families: the classical family of $Zn^{2+}$-dependent HDACs that includes class I, II and IV HDACs and the second one consists of the $NAD^{+}$-dependent HDACs that includes class III sirtiuns HDACs (Haberland et al., 2009; Spange et al., 2009). HADC classes are subdivided based on their homology to yeast HDACs. Class I HDACs (HDAC1, 2, 3 and 8) show homology to the yeast protein RPD3, are usually detected in the nucleus, and show ubiquitous expression in various mammalian cell lines and tissues (de Ruijter et al., 2003; Spange et al., 2009). Class II HDACs (HDAC4, 5, 6, 7, 9 and 10) have a high degree of homology to the yeast Hda1 protein and can shuttle between the nucleus and the cytoplasm (de Ruijter et al., 2003). Class III HDACs are homologous to the yeast Sir2 HDACs and HDAC11 is the unique member of the class IV HDACs (de Ruijter et al., 2003; Spange et al., 2009). HDACs lack intrinsic DNA binding activity and are recruited to target genes via their direct association with transcription activators and repressors as well as their incorporation into multi-protein transcription complexes (Sengupta and Seto, 2004).

**HDAC1** has a nuclear localization signal (NLS) and is an ubiquitous exclusively nuclear protein due to the lack of nuclear export signal (NES) (de Ruijter et al., 2003). HDAC1 contains 482 amino acid residues in which the deacetylase catalytic domain on its N-terminus forms the major part of the protein (390 amino acids) (de Ruijter et al., 2003; Haberland et al., 2009). HDAC1 is inactive when produced by recombinant techniques and

*in vitro*, displays activity only in the presence of cellular complexes and proteins (de Ruijter et al., 2003; Sengupta and Seto, 2004). These complexes consist of proteins necessary for modulating its deacetylase activity and its recruitment to target genes or non-histone proteins (de Ruijter et al., 2003). Indeed, HDAC1 together with the nearly identical HDAC2 (82% of sequence identity) are integral components of at least four histone deacetylase complexes (Sin3, NuRD, CoREST and NcoR). The Sin3 complex comprises HDAC1, HDAC2, RbAp48, RbAp46, mSin3A and Sin3-associated proteins 18 and 30 (SAP18 and SAP30) and interacts with DNA binding transcription factors, including MAD, Ikaros, REST and nuclear hormone receptors (de Ruijter et al., 2003; Grozinger and Schreiber, 2002). RbAp46 and RbAp48 serve for binding histones, SAP18 and SAP30 stabilize protein associations and mSin3A acts as scaffold for the assembly of the complex (Grozinger and Schreiber, 2002). The nucleosome remodeling and deacetylating (NRD or NuRD) complex includes HDAC1, HDAC2, RbAp48, RbAp46, CHD3 (Mi2$\alpha$) and CHD4 (Mi2$\beta$), MBD3 and MTA2 (de Ruijter et al., 2003; Grozinger and Schreiber, 2002). CHD3 and CHD4 possess DNA helicase/ATPase domains found in SWI/SNF family of chromatin remodeling proteins (Sengupta and Seto, 2004). MBD3 and MTA2 are necessary for histone deacetylase stimulation (Zhang et al., 1999). The NurD complex combines deacetylation by HDAC proteins with ATP-dependent nucleosome remodeling to affect transcription (Sengupta and Seto, 2004). HDAC1 and HDAC2 are also component of CoREST complex that contain MTA1 and MTA2 and p110. MTA1 and MTA2 serve to stimulate catalytic activity of HDAC1 and HDAC2 (de Ruijter et al., 2003; Sengupta and Seto, 2004). HDAC1 and HDAC2 are also part of NcoR-2 complex that contains NcoR, mSin3A and SAP30 (Underhill et al., 2000). In addition to functioning through these complexes HDAC1 can also binds directly to target proteins such as YY-1, MyoD or to DNA binding proteins such as Rb-binding protein 1 and Sp1 (de Ruijter et al., 2003; Sengupta and Seto, 2004). HDAC1 has been shown to be modulated by post-translational modifications at its C-terminal domain mainly by phosphorylation which promotes its enzymatic activity and complex formation (Galasinski et al., 2002; Pflum et al., 2001) or by sumoylation which affects its ability to induce transcription repression (David et al., 2002).

The acetylation of p53, E2F1, STAT3, Tat, Ku70, EKLF, YY1 and MyoD is reversed by HDACs leading to reversion of the acetylation protein impacts discussed in the previous section (Das and Kundu, 2005; Glozak et al., 2005; Spange et al., 2009). HDACs can bind their substrates directly or mediated adaptor proteins that bridge HDACs to the target

proteins (Das and Kundu, 2005; Glozak et al., 2005; Spange et al., 2009). KAP1 has been shown to tether HDACs to non-histone proteins and to stimulate their deacetylation and therefore their inactivation.

## MATERIALS AND METHODS

### 1- Vectors and constructs

pASK-IN-HATw and pASK-IN-HATm to express and purify IN-HATw and IN-HATm in bacteria were constructed by cloning integrase codon optimized (IN-CO) in frame with the HAT domain of p300 (a.a. 1195-1673) wild-type or mutated (D1395Y) in the pASK-IBA37 plus vector (IBA GmbH) containing at 5' of the MCS a 6xHis tag. During the cloning procedure a 3' HA tag and a Tobacco Etch Virus (TEV) protease cleavage site between IN and HAT were introduced by PCR. From the pASK-IBA37-IN-HATw/m vectors the IN-HATw/m, HATw/m and IN were PCR amplified and cloned in frame with the Gal4 DNA Binding Domain (GBD) in the pBD-Gal4 vector (Stratagene) for expression in yeast cells. HATw-HA was cloned in pASK-IBA37 plus vector by PCR starting from pASK-IBA37-IN-HATw. BTF3b, THRAP3, HMGN2, Exp2, RanBP9, eIF3h and KAP1 cDNAs were cloned by PCR in pFlag-CMV2 vectors starting from their truncated cDNAs isolated in the two-hybrid screening (RESULTS, **Table 1**). 6xHis-IN, GST-IN and GST-IN truncated domains (IN-Nt, IN-Cat and IN-Ct) have been previously described (Cereseto et al., 2005). pFlag-IN-CO was kindly provided by Alan Engelman (Dana-Farber Cancer Institute, Boston, MA). pcDNA3.1-HA expressing IN-CO was obtained by PCR cloning starting from the pFlag IN-CO. pFlag-IN-CO containing K264,266,273R mutations was constructed using recombinant PCR starting from the pFlag-IN-CO vector. Untagged INw and INm were cloned by PCR in pCDNA3.0 vector starting from their respective pFlag IN-CO constructs. Full-length KAP1 cDNA, in pOTB7 non expression vector, was purchased from Open Biosystems and cloned by PCR in pcDNA3.0, pcDNA3-HA and pFlag-CMV2 vectors. HA-KAP1 full length was cloned by PCR in pAIP lentiviral vector which was kindly provided by Jeremy Luban (Geneve University, Switzerland). KAP1 and KAP1 deletion mutants (1-381, 1-616 and 617-835) cDNAs were cloned by PCR in pCDNA3.1-HA. HA tagged KAP1 and deletion mutants cDNAs were cloned by PCR in pAIP lentiviral vector. HA-KAP1 (S824A) and HA-KAP1 (S824D) were cloned in pAIP by PCR starting from constructs obtained from Yossi Shiloh (Tel Aviv University, Ramat Aviv, Israel). pFlag-HDAC1 and pFlag-HDAC3 have been previously described (Sabo et al., 2008). pFlag-Luciferase (Flag-Luc) was purchased from Stratagene. pNL4.3.Luc.R-E- and pD64E were obtained from the NIH AIDS Research and Reference Reagent Program. pNL4.3-Luc-3mut (K264,266,273R) was previously described (Terreni et al., 2010). pGIPZ-shMM was obtained by cloning in pGIPZ lentiviral vector

(Open Biosystems) pre-synthesized oligonucleotides containing four mismatched mutations inside sh-1-KAP1. Lentiviral packaging plasmid p8.91, MLV transfer gene pLNC-CMV-eGFPT2A-fLuc and MLV packaging plasmid pCMVintronMLVgag-pol were kind of gifts from Zeger Debyser and Jan De Rijck (KU Leuven University, Belgium).

## 2- Yeast assays

### 2.1- Immunoprecipitations from yeast cells

AH109 yeast cells were transformed with pBD-Gal4-IN-HATw-HA, pBD-Gal4-IN-HATm-HA or pBD-Gal4 vector according to LiAc/ssDNA/PEG protocol described by (Gietz et al., 1995). For expression, one transformed colony was inoculated in 200 ml (-Trp) selective medium for 48 hours at 30°C. Yeast cells were then harvested and resuspend in 500 µl of lysis buffer (300 mM sorbitol, 10 mM Tris HCl pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$ and 5 mM EDTA) provided before use with 1mM PMSF and complete,mini,EDTA-free protease inhibitor cocktail tablets (Roche). Next, 1 g of glass beads, acid washed (425-600 µM diameter) (Sigma) was added to the lysates that were vortexed four times for 1 min each and 1 min of break chilled on ice. Lysed yeasts were then clarified at 4°C for 5 min at maximum, and 1% of Triton X-100 was added to the collected supernatant. After vortexing for 30 s, lysates were re-clarified at 4°C for 15 min at maximum. For immunoprecipitation, 200 µg of total yeast protein extracts were incubated with 0.8 µg of Rat anti-HA monoclonal antibodies (Clone 3F10) (Roche) for 2 hours at 4°C on rotating wheel and then 30 µl of protein G immobilized on trisacryl beads (Pierce) were added for additional 2 hours. Beads were then washed three times, for 10 min each, with 1 ml binding buffer (50mM Tris-HCl pH 7.4, 250 mM NaCl, 50 mM NaF and 0.1% NP-40). Immunoprecipitates were eluted by boiling beads in 15 µl of Leammli buffer (2% SDS, 10% glycerol, 5% Beta 2-mercaptoethanol, 0.002% bromophenol blue, 0.0625 M Tris HCl pH 6.8) for 10 min at 100°C and analyzed by Western blot.

### 2.2- Yeast two hybrid screen

In order to identify cellular factors interacting with constitutively acetylated HIV-1 integrase, we used GBD-IN-HATw-HA as "bait" in yeast two hybrid screen of human lymphocytes cDNA library (BD biosciences Clontech) expressed as Gal4 Activating Domain (GAD) fusion in pACT yeast expression vector. Amplification and purification of the cDNA

library were performed following manufacturer's instructions. The two hybrid screen was performed in AH109 yeast strain (James et al., 1996) which has as reporter genes *ADE2*, *HIS3* and *MEL1* under the control of distinct Gal4 upstream activating sequences (UASs) and TATA boxes. Library transformation and screening with GBD-IN-HATw-HA bait were performed following the protocols described in Matchmaker GAL4 two hybrid system 3 and libraries user manual of Clontech (http://www.clontech.com/images/pt/PT3247-1.pdf). The GAD prey cDNAs (Results, **Table 1**) and GBD and HA hybrid cDNAs: IN-HATw, IN-HATm, IN, HATw, HATm were co-transformed in AH109 yeast cells to check the specificities of interactions. The co-transformants were let to grow either in the two hybrid selective medium (-Trp, -Leu, -Ade and -His) or in the transformation selective medium (-Trp, -Leu) for at least 5 days at 30°C. Co-transformants of GAD-KAP1 with the GBD and HA hybrids cDNAs were streaked in -Trp, -Leu medium and then transferred on the nitrocellulose filters for the X-alpha-gal lift assay. Streaked colonies immobilized on the nitrocellulose filters were lysed by freezing them in liquid nitrogen and thawing at room temperature three times. The filters carrying lysed yeast were then deposed upon wattman paper pre-soaked in petri dish with 5 ml of X-alpha-gal solution containing 84 µl X-alpha-gal stock solution (20mg/ml) (Clontech) and 5 ml of Z buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2.PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM Beta 2-mercaptoethanol, the final pH was adjusted to 7). The filters were incubated at 30°C for a maximum of 8 hours.

**3- Recombinant IN-HATw, IN-HATm and HATw purifications and TEV digestion**

pASK-IN-HATw/m and pASK-HATw encoding for 6xHis-IN-TEV-HATw/m and HATw were transformed in *E. coli* Arctic Express RIL competent cells (Stratagene) and induction of protein expression was performed using 43 mM anhydrotetracycline hydrochloride (AHT) for 24 hours at 13 °C. Bacteria culture was lysed in binding buffer (1M NaCl, 20 mM Tris HCl pH 7.9 and 0.5% Triton X-100) containing 1mM PMSF and protease inhibitor cocktail (Roche). TALON Metal Affinity Resin (BD Biosciences) incubated for 2 hours at 4°C was used to recover the 6xHis-IN-HATw/m proteins. Following two washes in binding buffer containing 5 mM imidazole, proteins were eluted using binding buffer containing 200 mM imidazole and dialyzed in buffer containing 150 mM NaCl, 50 mM Tris HCl pH 8, 10% glycerol and 0.5 mM EDTA. For TEV digestion 20 µg of 6xHis-IN-HATw/m purified proteins were incubated with 30 units of AcTEV protease (Invitrogen) in 120 mM NaCl, 50

mM Tris-HCl pH 8, 0.5 mM EDTA and 1 mM of DTT in 250 µl total volume. To recover 6xHis-IN from the digested product the TEV treated samples were adjusted to 1 M NaCl and incubated with Ni-NTA agarose resin (Qiagen) for 2 hours at 4°C. Following washes in binding buffer containing 5 mM imidazole, 6xHis-IN was eluted using the binding buffer containing 250 mM imidazole and dialyzed in 1 M NaCl, 20 mM Tris-HCl pH 7.5, 1 mM DTT and 10% glycerol.

### 4- *In vitro* binding assays

*In vitro* translated $^{35}$S-Met-KAP1 was produced using TNT T7 Reticulocyte Lysate System (Promega) and using as a template pcDNA3-KAP1. GST-IN and its truncated domains (N-terminus (Nt), Core (Cat) and C-terminus (Ct)) were prepared as already described (Cereseto et al., 2005). For binding assays, 2 µg of recombinant purified GST proteins immobilized on S-glutathione beads were treated for 1 hour in rotating wheel at room temperature with DNase I (0.02U/µl) and RNase H (0,02 U/µl), to remove contaminant bacterial nucleic acids, in 1 ml buffer containing 50 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 25 mM $CaCl_2$, 5% glycerol and 1 mM DTT. GST treated proteins were then mixed with 1000 c.p.m of $^{35}$S-Met-KAP1 in 50 µl total volume in NETN buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT and 1 mM PMSF) which is supplemented with 0.1 mg/ml ethidium bromide to inhibit aspecific interactions between residual DNA and proteins. Binding reactions were incubated 1 hour in the shaker at 4 °C, and then bound complexes were washed 5 times with 300 µl of NETN buffer containing ethidium bromide (0.1 mg/ml) and resolved by SDS-PAGE electrophoresis. Radioactive proteins were visualized by phosphoimaging (Cyclone).

For *In vitro* binding between IN-HATw/m and $^{35}$S-Met-KAP1, 100 ng of either 6x-His-IN-HATw-HA or 6xHis-IN-HATm-HA purified proteins, pre-treated with DNase I and RNase H, as described above, were incubated with 1000 c.p.m of $^{35}$S-Met-KAP1, 20 µg of BSA and 15 µl of Ni-NTA agarose beads (settled beads volume) in 1000 µl of pull down buffer (150 mM NaCl , 2 mM $MgCl_2$, 25 mM imidazole, 0.1 % NP-40, 25 mM imidazole and 50 mM Tris-HCl pH 7.4) for 5 hours at 4 °C on rotating wheel. The beads were then washed trice with 1 ml pull down buffer for 10 min each. Finally beads were boiled in 20 µl Leammli buffer. Eluted proteins were separated by SDS-PAGE electrophoresis and radioactive $^{35}$S-Met-KAP1 was detected by phosphoimaging (Cyclone).

## 5- Cell lines and purification of primary blood lymphocytes (PBLs), Naïve (CD45RA+) and Memory (CD45RO+) CD4+ T cells

HEK293T and HeLa cells were maintained in standard growth medium: Dulbecco's Modified Eagle's Medium (DMEM) low glucose (Euroclone), supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 µg/ml Streptomycin. CEMss T cells were maintained in RPMI medium supplemented with Glutamax (GIBCO) and with 10% FBS and 100 U/ml Penicillin and 100 µg/ml Streptomycin.

Primary blood lymphocytes (PBLs) were isolated from buffy coats of healthy donors using Ficoll Histopaque gradient (Sigma). 25 ml of buffy coat, pre-diluted twice in RPMI medium, were overlayed on 12.5 ml Ficoll solution in 50 ml Falcon tubes. The gradient was obtained by centrifugation at 950 g with the break off for 15 min. Primary blood monocyte cells (PBMCs) were collected with Pasteur pipette at the interface between Ficoll and plasma-medium layers. PBMCs were washed twice with the RPMI medium, stained with trypan blue solution and counted. $200x10^6$ PBMCs were resuspend in 3 ml RPMI medium, overlayed onto 10 ml of hyper-osmotic percoll solution (48.5 % Percoll (Sigma) and 160 mM NaCl) and centrifuged for 15 min at 580 g with break off. Monocytes fraction at the interface was discarded and the pellet of lymphocytes was washed with RPMI medium. PBLs were stimulated for 24 hours with 2 µg/ml of phytohaemagglutinin (PHA-P) (Sigma) and then PBLs were washed and maintained in complete RPMI medium supplemented with 25 U/ml of human interleukin-2 (hIL-2) (Roche). The medium was replaced every two days and activated PBLs were cultured for 5 to 6 days before nucleofection with siRNAs.

Resting CD4+ T cells were isolated from purified PBLs by negative selection using CD4+ T cells isolation kit (Miltenyi Biotec). Naïve (CD45RA+) and Memory (CD45RO+) T cells were isolated from the CD4+ population using respectively CD45RA and CD45RO antibodies immobilized on microbeads (Miltenyi Biotec) and then were lysed in NEHN buffer (300 mM NaCl, 50 mM Hepes pH 7.5, 0.5% NP-40, 20% glycerol and 1 mM EDTA) and analyzed by Western bolt.

All cell types were cultured at 37°C with 5% $CO_2$ in an humidified atmosphere.

## 6- Anti-Flag beads pull down assays

HEK293T cells expressing Flag-proteins (KAP1, BTF3b, THRAP3, HMGN2, Exp2, eIF3h and Flag-Luc) were lysed in 50 mM Hepes pH 7.4, 150 mM NaCl and 0.5% NP-40 provided with 1 mM PMSF and protease inhibitor cocktail tablets. 250 µg lysate was mixed with 250

ng of recombinant proteins (6xHis-IN-HATw-HA, 6xHis-IN-HATm-HA, 6xHis-HATwt-HA, 6xHis-IN or 6xHis-IN TEV digested from IN-HATw/m: IN-Ac+/-) together with 5 µM of Lys-CoA (synthesized at the ICGEB Peptide Synthesis Core Faculty, Trieste, Italy) in 500 µl total volume with lysis buffer. Following 1 hour incubation at 4 °C, 20 µl of monoclonal anti-Flag M2 antibodies immobilized on agarose beads (Sigma) were added and incubated for 1 hour. Following three washes in 1 ml lysis buffer, 10 min each, samples were eluted in 15 µl of Leammli buffer analyzed by Western blot using polyclonal anti-HA (Santa Cruz) or monoclonal anti-IN (Santa Cruz) and polyclonal anti-Flag (Sigma) antibodies.

**7- Co-mmunoprecipitation experiments**

HEK293T cells were transfected with different constructs using standard calcium phosphate ($CaPO_4$) co-precipitation procedure or with polyethylenimine (PEI) (Sigma) or polyfect (Qiagen) transfectant reagents. HeLa cells were transfected exclusively with polyfect reagent.

The expression time for the majority of constructs was 36 hours. When Flag-tagged or untagged INw and INm were expressed for co-immunoprecipitations with endogenous KAP1, cells were harvested 24 hours post-transfection and pre-treated for 8 hours with 1 µM Trichostatin A (TSA) (Sigma). Induction of endogenous phosphorylated KAP1 was performed by treating cells, half hour prior harvest, with 200 ng/ml neocarzinostatin (Sigma), while ATM inhibitors (KU55933) were used at 20 µM for 2 hours. For the co-immunoprecipitation between Flag-IN and KAP1 phosphomutants (S824A and S824D), $1{,}5x10^6$ HEK293T cells were transduced simultaneously with 45 µg p24 of LKO.1-sh-3'KAP1 and 30µg p24 AIP-HA-S824A or AIP-HA-S824D. Cells were transfected with pFlag-IN at 48 hours post-transduction and harvested at 72 hours post-transduction.

Following construct expressions of Flag-tagged proteins (INw, INm, HDAC1 or HDAC3) alone or with HA-IN, CMV-INw or CMV-KAP1, HEK293T cells or HeLa transfected cells were harvested and lysed in NEHN buffer containing 300 mM NaCl, 50 mM Hepes pH 7.5, 0.5% NP-40, 20% glycerol and 1 mM EDTA and provided with 1 mM PMSF, 1 mM $Na_3VO_4$, 20 mM NaF and protease inhibitor cocktail tablets. Then 500 to 2000 µg of lysates expressing Flag-proteins were incubated with 30 µl Anti-Flag M2 agarose beads (Sigma) in 1 ml total volume, for 8 hours at 4°C on rotating wheel. Beads immobilizing immunoprecipitates were washed five times with 1 ml NEHN buffer for 10 min each at 4°C and one last washing with 1 ml NEHN buffer containing 600 mM instead of 300 mM. The

anti-Flag beads were resuspend in 15 µl Leammli buffer and boiled for 10 min at 100°C. Eluted immuno-complexes were analyzed by Western blot.

HEK239T cells expressing untagged INw or INm were lysed in NEHN buffer and 2000 µg of lysates were incubated for 8 hours at 4°C with 2.5 µg of polyclonal anti-KAP1 antibodies (Bethyl) in 1 ml total volume. 30 µl protein A immobilized on trisacryl beads were added for additional 4 hours incubation. Beads bound immuno-complexes were washed three times with 1 ml NEHN buffer at 4 °C, 10 min each, and then boiled in 15 µl Leammli buffer. Eluted immunoprecipitates were analyzed by Western blot.

Cells co-expressing Flag-HDAC1 or Flag-Luc with either HA-KAP1 or HA-KAP1 deletion mutants (1-381, 1-616 and 617-835) in HEK239T cells were lysed in RIPA buffer (1% deoxycholic acid, 0.1 % SDS, 150 mM NaCl and 50 mM Tris HCl pH 7.5). 500 µg lysates were incubated with 20 µl anti-Flag M2 agarose beads for 5 hours at 4°C in 1 ml total volume. Beads bound immuno-complexes were washed three times with 1 ml RIPA buffer, 10 min each, and then boiled in 15 µl Leammli buffer at 100°C for 10 min. Eluted immunoprecipitates were analyzed by Western blot.

**8- Western blot analysis and antibodies**

Whole cell extracts and immunoprecipitates were separated by 10% or 8% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) and electro-blotted onto nitrocellulose membranes Hybond-C-Extra (Amersham Biosciences). Membranes were blocked with 5% skimmed milk powder in TBS containing 0.1% Tween 20, and detection was carried out using specific primary antibodies. Primary antibodies for Western blot analyses were: monoclonal HIV-1 IN antibody (8G4) (NIH AIDS Research and Reference Reagent Program), polyclonal anti-HA (Y-11) (Santa Cruz Biotechnology), polyclonal anti-HDAC1 (Upstate). Antibodies anti-acetylated IN were previously described (Terreni et al., 2010). Polyclonal anti-KAP1 recognizing the N-terminal domain of KAP1 and polyclonal anti-phospho-KAP1 (γKAP1) recognizing phosphorylated serine 824 were purchased from Bethyl. Polyclonal anti-Flag, monoclonal anti-Flag M2 (Clone M2), monoclonal anti-Tubulin (CloneB-5-1-2) were all purchased from Sigma. Secondary antibodies HRP-conjugated anti-mouse or anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. Untagged INw and INm were detected with a mouse monoclonal IN antibody IN2 (Santa Cruz).

### 9- *In vitro* HDAC assay

Quantification of HDAC activity of Flag and Flag-IN immunoprecipitates (2 mg lystaes each) was measured using HDAC Fluorometric Activity Assay kit (Upstate) following manufacturer's instructions. The HDAC Activity assays were measured with an excitation and emission wavelength set to 355 and 460, respectively.

### 10- Virus and vector productions

NL4.3-Luc, D64E, NL4.3-Luc-3mut HIV-1 viruses pseudotyped with the Vesicular Stomatitis Virus-G (VSV-G) envelope were produced by transfecting $5x10^6$ HEK293T cells (in 100-mm diameter culture dish) with 20 μg pNL4.3.Luc.R-E-, pD64E or pNL4.3-Luc-3mut respectively together with 5 μg p-MDG-VSV-G using 150 nM polyethylenimine (PEI) reagent (Sigma). Lentiviral vectors GIPZ, AIP or LKO.1 empty or encoding for KAP1 shRNAs (GIPZ-sh-1-KAP1, GIPZ-shMM, LKO.1-sh-5-KAP1, LKO.1-sh3'KAP1) or cDNAs (AIP-HA-KAP1, AIP-HA-(1-381), AIP-HA-(1-616), AIP-HA-(617-835), AIP-HA-S824A, AIP-HA-S824D) were produced by transfecting $5x10^6$ HEK293T cells (in 100-mm diameter culture dish) with 20 μg lentiviral transfer gene, 10 μg lentiviral packaging plasmid (p8.91) and 5 μg p-MDG-VSV-G using 180 nM PEI. Transfections of lentiviral viruses and vectors were performed in 5 ml OPTIMEM supplemented with Glutamax (GIBCO) and antibiotic free medium, while viral productions were obtained by replacing transfection medium 24 hours post-transfection with 10 ml OPTIMEM medium containing antibiotics (100 U/ml Penicillin and 100 μg/ml Streptomycin). Viral supernatants were collected 72 hours post-transfection, quantified for their HIV-1 p24 antigen content using Innotest HIV Antigen mAb kit (INNOGENETICS).

The MLV production conditions were identical to those of lentiviral vectors. It has been used 20 μg MLV transfer gene pLNC-CMV-eGFP-T2A-fLuc, 10 μg MLV packaging plasmid pCMVintronMLVgag-pol and 5 μg p-MDG-VSV-G for the transfection of $5x10^6$ HEK239T cells with 180 nM PEI in 100-mm dish. The quantification of MLV transduction units (TU) was performed by serial infections of HeLa cells and detection of GFP positive cells at 48 hours post-infection using the Fluorescence-activating cell sorter (FACS).

### 11- Transient and stable knockdowns and back-complementation experiments

Transient knockdowns mediated by siRNAs were performed using si-Genome smart pool siRNAs pre-designed by Dharmacon. $1x10^5$ HeLa cells were treated with 200 nM KAP1

siRNAs or 150 nM HDAC1 siRNAs and equivalent amounts of control siRNAs (ON-TARGET plus Non-targeting Pool) using the Gene Silencer siRNA transfection reagent (Gene Therapy System). $3x10^5$ HEK293T cells were transfected with 100 nM HDAC1 siRNAs using Gene Silencer reagents. Five to six days activated PBLs ($5x10^6$) were transfected with 600 nM KAP1 siRNAs or control siRNAs by electroporation using the nucleofector II Amaxa biosystems instrument (Lonza), program number T23, in 100 μl T-cell Nucleofector solution (Lonza). The KAP1 pool contains the following four siRNAs: 1) 5'-GACCAAACCUGUGCUUAUG-3'; 2) 5'-GAUGAUCCCUACUCAAGUG-3'; 3) 5'-GCGAUCUGGUUAUGUGCAA-3'; 4) 5'- AGAAUUAUUUCAUGCGUGA-3'. The HDAC1 pool contains the following four siRNAs: 1) 5'-CUAAUGAGCUUCCAUACAA-3'; 2) 5'-GAAAGUCUGUUACUACUAC-3'; 3) 5'-GGACAUCGCUGUGAAUUGG-3'; 4) 5'-CCGGUCAUGUCCAAAGUAA-3'.

CEMss cells were transiently knocked down by transducing a pool of 5 LKO.1 lentiviral vectors each expressing a shRNAmir against the KAP1 gene (sh-5-KAP1) (Open Biosystem): 1) 5'-CCTGGCTCTGTTCTCTGTCCT-3' (sh-3'KAP1); 2) 5'-GAGAATTATTTCATGCGTGAT-3'; 3) 5'-GAGGACTACAACCTTATTGTT-3'; 4) 5'-CTGAGACCAAACCTGTGCTTA-3'; 5) 5'-GACCACCAGTACCAGTTCTTA-3'. CEMss cells ($1x10^5$) were transduced for 2 hours by spinoculation at 1200 g using 400 ng p24 from each LKO.1-shRNA (a total of 2 μg p24). Control cells were transduced with 2 μg p24 GIPZ lentiviral vector.

For back-complementation experiments, $1x10^5$ HeLa, CEMss or HEK239T cells were transiently knocked down by transducing 3 μg p24 of LKO.1-sh3'KAP1 (5'-CCTGGCTCTGTTCTCTGTCCT-3') targeting the 3' untranslated region (3'UTR) of KAP1 gene and HA-KAP1, HA-(1-381), HA-(1-616), HA-(617-835), HA-S824A, HA-S824D), containing only KAP1 coding region, were expressed using 2 μg p24 AIP-HA-KAP1,AIP-HA-KAP1, AIP-HA-(1-381), AIP-HA-(1-616), AIP-HA-(617-835) and 4 μg p24 AIP-HA-S824A and AIP-HA-S824D. Control cells were transduced with 5 μg p24 of GIPZ or AIP lentiviral vectors. The above described LKO.1, AIP and GIPZ lentiviral vectors were transduced simultaneously for 2 hours by spinoculation at 1200 g.

Stable KAP1 knockdown cells were obtained by expressing a shRNAmir (sh-1-KAP1: 5'-CCACTGAGGACTACAACCTTA-3') (Open Biosystem) through a lentiviral vector system (GIPZ-sh-1-KAP1). As control a mismatch oligonucleotide (sh-MM: 5'-CCACTGAG*CTGA*ACAACCTTA-3') containing four mutations in sh-1-KAP1 sequence was

expressed through the GIPZ lentiviral vector (GIPZ-shMM). $5x10^4$ HEK293T or HeLa cells were seeded in 10-cm diameter dish and transduced by 500 ng p24 of lentiviral vector in 10 ml total volume. After 24 hours incubation, the supernatant was removed and replaced by 10 ml DMEM medium. 48 hours post transduction, 10 ml DMEM medium supplemented with puromycin (2 µg/ml) were added. After 10 days of selection, changing fresh selection medium every 2 days, single colonies were picked and expanded to form monoclonal clones. Pools were formed by mixing at least 10 single colonies. The control cells were a pool of transduced cells with GIPZ-shMM. Expanded pools and monoclonal clones cells were maintained in DMEM medium containing 10 µg /ml puromycin.
Transient and stable knockdowns were controlled by Western blot analysis performed from the same cell populations used for infections.

**12- Cell infections and measurement of HIV-1 infectivity**

NL4.3-Luc, D64E and NL4.3-Luc-3mut HIV-1 viruses pseudotyped with VSV-G envelope and quantified for p24 antigen levels, were treated with 80 U/ml of DNAse I (Applied Biosystems) for 1 hour at 37°C prior to infections. Infections were performed for 2 hours at 37°C in 0.5 to 1ml total volume in well plates or in Flacons.
For the transiently knocked down cells the following infection conditions were used: 100 ng p24 for $6x10^5$ HeLa cells 48 hours after siRNA transfection; 250 ng p24 for $5x10^5$ CEMss cells 60 hours post-transduction with KAP1 LKO.1-shRNAs lentiviral vectors; 500 ng p24 for $2x10^5$ of activated PBLs 24 hours after siRNA transfection.
For back-complementation experiments, $5x10^5$ HeLa, HEK239T or CEMss cells were infected 60 hours post LKO.1-sh3'KAP1 and AIP-HA-KAP1, AIP-HA-(1-381), AIP-HA-(1-616), AIP-HA-(617-835), AIP-HA-S824A or AIP-HA-S824D transductions using 250 ng p24.
Infections of stable KAP1 knockdown HeLa clones cells ($3x10^5$) were performed using 300-1200 ng p24. KAP1 stable knockdown HEK293T clones and pools cells ($2.5x10^5$) were infected using 25 ng p24.
Infections of HEK293T cells transfected 24 hours in advance with pCDNA3-KAP1, pCDNA3HA-KAP1, pCDNA3HA-(1-381), pCDNA3HA-(1-616) or pCDNA3HA-(617-835) (6.25 µg of DNA for $1.2x10^6$ cells with PEI) were performed using 125 ng p24 for $2.5x10^5$ cells. Same conditions were used for HEK239T transfected 48 hours prior infections with HDAC1 siRNAs and 24 hours prior infection with pcDNA3-KAP1.

$5x10^5$ HeLa cells were treated with 800 nM trichostatin A (TSA) (Sigma) or with equal volume of DMSO (Sigma) for 2 hours previous infection, during 2 hours infection (200 ng p24) and till 24 hours post-infection.

Infectivity of NL4.3-Luc, D64E and NL4.3-Luc-3mut HIV-1 viruses was measured 48 hours post infection by luciferase activity quantification using the Luciferase Assay System Kit (Promega) and normalized to total protein concentrations.

HeLa cells ($2x10^5$) were infected with MLV at 48 hours post siRNAs transfection at a MOI 0.1 based on the transduction units obtained by FACS.

$3x10^5$ HEK293T or CEMss cells were infected with 60 μg p24 NL4.3-Luc and harvest at different time points post infection (1, 2, 3 and 4 hours) and were then lyzed in NEHN buffer supplemented with 1 mM $Na_3VO_4$ and 20 mM NaF phosphatase inhibitors to be analyzed by Western blot for phophorylated KAP1. Cells were treated 2 hours prior infections with 20 μM ATM inhibitors KU-55933.

## 13- Quantifications of retroviral cDNA species by real time quantitative PCR (Q-PCR) and their statistical analysis

Genomic DNA extraction from HeLa, HEK293T, CEMss and PBLs cells was performed using DNeasy Blood and Tissue DNA purification Kit (Qiagen). Amplification reactions were performed with the Light cycler 480 instrument (Roche Diagnostics). HIV-1 late reverse transcripts were analyzed using primers and PCR conditions previously described (Butler et al., 2001). Integrated HIV-1 copies by Alu-PCR and 2 LTRs circles were analyzed by Q-PCR following protocols previously described (Brussel and Sonigo, 2003).

HIV-1 total and integrated DNA quantifications in cells carrying GIP-Z, LKO.1 and AIP lentiviral DNAs were performed by using primers and probes specific for the luciferase gene in NL4.3-Luc or NL4.3-Luc-3mut 24 hours and 15 days post infection respectively to avoid cross reactivity between LTRs. Quantifications of MLV total and integrated viral DNA at 24 hours and 15 days post infection respectively were also performed using primers and probes specific to the luciferase gene in LNC-CMV-eGFP-T2A-fLuc MLV viral vector. Primers and probes specific for the luciferase gene and PCR conditions were previously described (Terreni et al., 2010). Briefly, primers and probe sequences are as follows: forward primer, LucFw: 5′-GAAGAGATACGCCCTGGTTCC-3′; reverse primer, LucRev: 5′-TGTGATTTGTATTCAGCCCATATCG-3′; and probe, LucProbe: 5′-FAM-TTCATAGCTTCTGCCAACCGAACGGACA-3′-BlackBerry Quencher. Reaction mixtures

contained 500-1500 ng of total genomic DNA, 1x Light Cycler 480 Probe Master (Roche Diagnostics), 300 nM each forward and reverse primers and 200 nM probe in a total volume of 20 μl. After incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out at 15 s at 95 °C followed by 1 min at 60 °C. A kinetic PCR assay for human beta-globin DNA was also carried out as endogenous control of analyzed samples. The primers and probes used have been previously described (Tan et al., 2006). Reaction mixture contained 100-1500 ng DNA template, 1x Light Cycler 480 Probe Master (Roche Diagnostics), 300 nM each forward (BGF) and reverse (BGR) primers and 100 nM probe (BGX-P ) in a total volume of 20 μl. After an initial denaturation step (95°C for 10 min), the cycling profile was 45 cycles consisting of 95°C for 10 s and 65°C for 10 s.
Statistical analyses of HIV-1 late reverse transcripts, 2-LTR circles and integrated DNA were performed using two-tailed Student's t-tests assuming equal variance between samples to determine differences at the 5% level of significance.

**14- HIV-1 LTR transcription assays in KAP1 knockdown HeLa and J-lat A1 cells**

HeLa KAP1 knockdown and control cells ($4x10^5$) were transfected with 200 ng pNL4.3.Luc.R-E- and 200 ng CMV-*Renella* (for normalization of transfection efficiencies) using polyfect (Qiagen). Luciferase activities were measured 48 hours post transfection using the DualGlo Luciferase assay Kit (Promega). J-lat A1 cells (Jordan et al., 2003) ($1x10^5$) untreated or TPA treated (10 nM TPA (Sigma), for 24 hours) were transduced with 2 μg p24 of LKO.1-sh-5-KAP1 lentiviral vectors (as described above for CEMss cells) or AIP lentiviral vector as control. Western blot analysis and percentage of GFP positive cells (analyzed by FACS) were performed 60 hours post LKO.1-sh-5-KAP1 transduction.

## RESULTS

### 1- Production of constitutive acetylated integrase fused to the HAT domain of p300

We have recently demonstrated that p300, a histone acetyltransferase, binds integrase and acetylates three lysines (K264, K266, K273) located in its C-terminus leading to enhanced enzymatic activity and DNA binding (Cereseto et al., 2005). Acetylable lysines are necessary for virus integration and thus for optimal replication, as demonstrated by the inefficient infectivity observed following their mutations into arginine residues (K264, 266, 273R) (Apolonia et al., 2007; Cereseto et al., 2005; Terreni et al., 2010). Since it has been demonstrated that acetylation modulates the activities of cellular and viral proteins by affecting protein-protein interactions (Bannister et al., 2000; Berro et al., 2006; Bres et al., 2002; Das and Kundu, 2005; Dorr et al., 2002; Glozak et al., 2005; Kouzarides, 2000; Mujtaba et al., 2002; Mujtaba et al., 2004; Polesskaya and Harel-Bellan, 2001; Spange et al., 2009; Sterner and Berger, 2000), in this study we investigated whether this protein modification could affect the interaction of integrase with cellular factors. To this aim we have employed the tethered catalysis two-hybrid system, a method previously reported to efficiently identify factors binding specifically to acetylated proteins (p53, histones H3 and H4) (Acharya et al., 2005; Guo et al., 2004). The tethered catalysis system allows obtaining a constitutively acetylated factor by exploiting the *cis* enzymatic activity of a HAT domain on a target factor within a single fusion protein.

To produce constitutively acetylated IN, a cDNA cassette was constructed containing a codon-optimized sequence for IN (IN-CO) fused to the acetyl transferase catalytic domain of p300 (IN-HATw). As control, IN was also fused to a sequence coding for a catalytically inactive HAT containing a D1395Y mutation (IN-HATm). The IN-HAT fusion proteins produced by these constructs contain at the N-terminus a histidine tag (6 x His) which allows affinity purification of the protein product by means of cobalt based resin columns (see Materials and Methods). In addition, the C-terminus of the chimera is in frame with a hemagglutinin (HA) epitope tag used to analyze the fusion protein by immunoblot with anti-HA antibodies. Finally, between the IN and the HAT domains a Tobacco Etch Virus (TEV) proteolytic cleavage site was introduced allowing IN separation from the HAT domain (**Figure 1a**). We initially produced and purified the chimera from bacteria by means of a 6xHis-tag fused to the N-terminus of the chimera (**Figure 1a**) to verify the

levels of acetylation. Western blot analysis using antibodies specific for acetylated integrase (Terreni et al., 2010), showed a high molecular size band corresponding to the full length chimera containing integrase fused to wild-type HAT catalytic domain (IN-HATw) (**Figure 1b, left panel**). Additional smaller molecular size products, positive for acetylation, are also visualized. Conversely, no signals are detected in the lane corresponding to integrase fused to a catalytically inactive HAT domain (IN-HATm) (**Figure 1b, left panel**) indicating a specific acetylation of the chimera IN-HATw. The same blot was then tested with antibodies against IN to check for protein loading (**Figure 1b, right panel**): a higher band corresponding to IN-HAT full length is clearly visible at similar amounts in both lanes corresponding to IN-HATw and IN-HATm. Additional smaller molecular size bands are also visible and are probably deriving from degradation of the single HAT domain expressed in bacteria.

We next sought to check whether integrase isolated from the HAT domain was positive for acetylation. To his aim the chimera was digested with the TEV protease and integrase separated from the HAT domain by means of the His-tag at the N-terminus of integrase (**Figure 1a**) using nickel columns. The purified digested product deriving from IN-HATw+ TEV showed by Western blot with antibodies specific for acetylated integrase, a main band at the same size of integrase, while no bands were visible in the samples containing integrase from IN-HATm+TEV (**Figure 1b, left panel**). Same amounts of the digested integrases were loaded (**Figure 1b, right panel**).

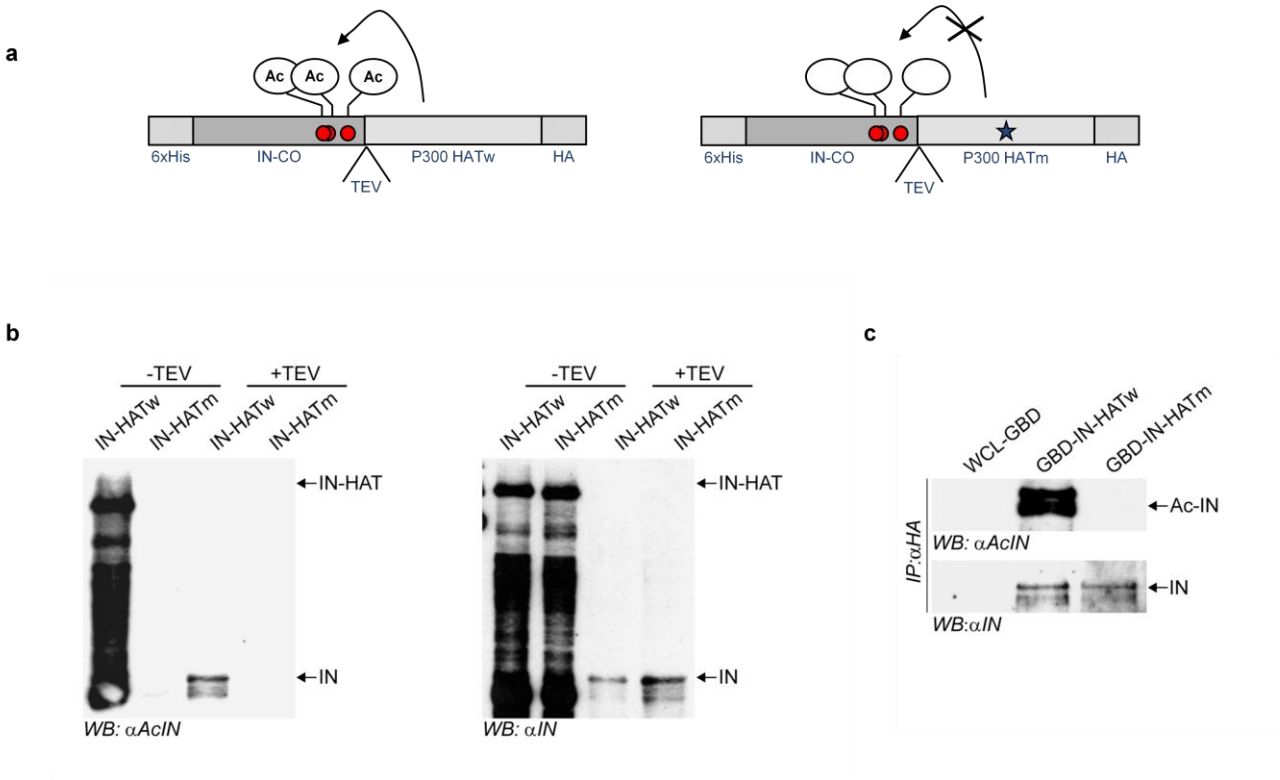


**Figure 1:** Constitutive acetylation of IN fused to the HAT domain of p300. (**a**) Schematic representation of IN-HATw (left) and IN-HATm (right) chimeras. IN codon optimized is fused to the p300 HAT domain either wild-type (HATw) or mutated (HATm). Between the IN and the HAT domains a TEV protease cleavage site is inserted to allow separation of the two domains. (**b**) IN from the IN-HATw chimera is highly acetylated. IN-HATw and IN-HATm fusion proteins undigested (-TEV) or TEV digested (+TEV) were immunoblotted with anti-acetylated IN specific antibodies (αAcIN) (left panel). The immunoblot was re-probed with αIN antibodies for total IN (right panel). (**c**) IN from the IN-HATw chimera is acetylated in yeast cells. The IN-HATw and IN-HATm chimeras fused to Gal4 DNA Binding Domain (GBD) were expressed in yeast cells (AH109), immunoprecipitated with αHA antibodies and immunoblotted with αAcIN antibodies to detect acetylated IN. The blot was re-probed with αIN antibodies for total IN.

To further verify the specific acetylation signal shown with IN-HAT wild type chimera (**Figure 1b, left panel**), the recombinant purified IN-HATw protein was sequenced and analyzed by mass spectrometry (MS). MS-MS analysis (MALDI-TOF/TOF) enabled the identification of three acetylated lysine residues on the C-terminal domain of integrase: K264, K266 and K273 (**Figure 2**). These identifications were supported by an adequate ladder of y (and b) ion family and by the presence of known marker ions for acetylated lysine: the acetylated lysine immonium ion at *m/z* 143.1 and the ion at *m/z* 126.1, which is a fragment ion induced by the loss of $NH_3$ from the ions at *m/z* 143.1 (Kim et al., 2002; Trelle and Jensen, 2008). The analysis confirmed the *in vivo* acetylation of lysines K264, K266 and K273 by p300 HAT previously identified by site-direct mutagenesis assays (Cereseto et al., 2005). Therefore, these data indicate that integrase is efficiently acetylated within the IN-HATw chimera, while no acetylation occurs on integrase derived

from the HAT-INm chimera. The IN-HAT cDNA cassette was then expressed in yeast cells in frame with the Gal4 DNA-binding domain (GBD) under the control of a yeast promoter (GBD-IN-HATw/m). Lysates from yeast cells expressing the control GBD, GBD-IN-HATw or GBD-IN-HATm were immunoprecipitated with anti-HA tag antibodies and the levels of integrase acetylation and expression were checked by immunoblot analysis. As shown in **Figure 1c** (**upper panel**) antibodies directed against acetylated integrase revealed a strong signal in the GBD-IN-HATw immunoprecipitates while no acetylation was detected either in GBD-IN-HATm or GBD control sample. Anti-integrase antibodies against the same membrane detected similar amounts of GDBD-IN-HATw and GDBD-IN-HATm immunoprecipitated with anti-HA antibodies (**Figure 1c, lower panel**). In conclusion, these results demonstrate that IN expressed as a fusion product with the HAT domain of p300 is highly acetylated in bacteria as well as in eukaryotic yeast cells.

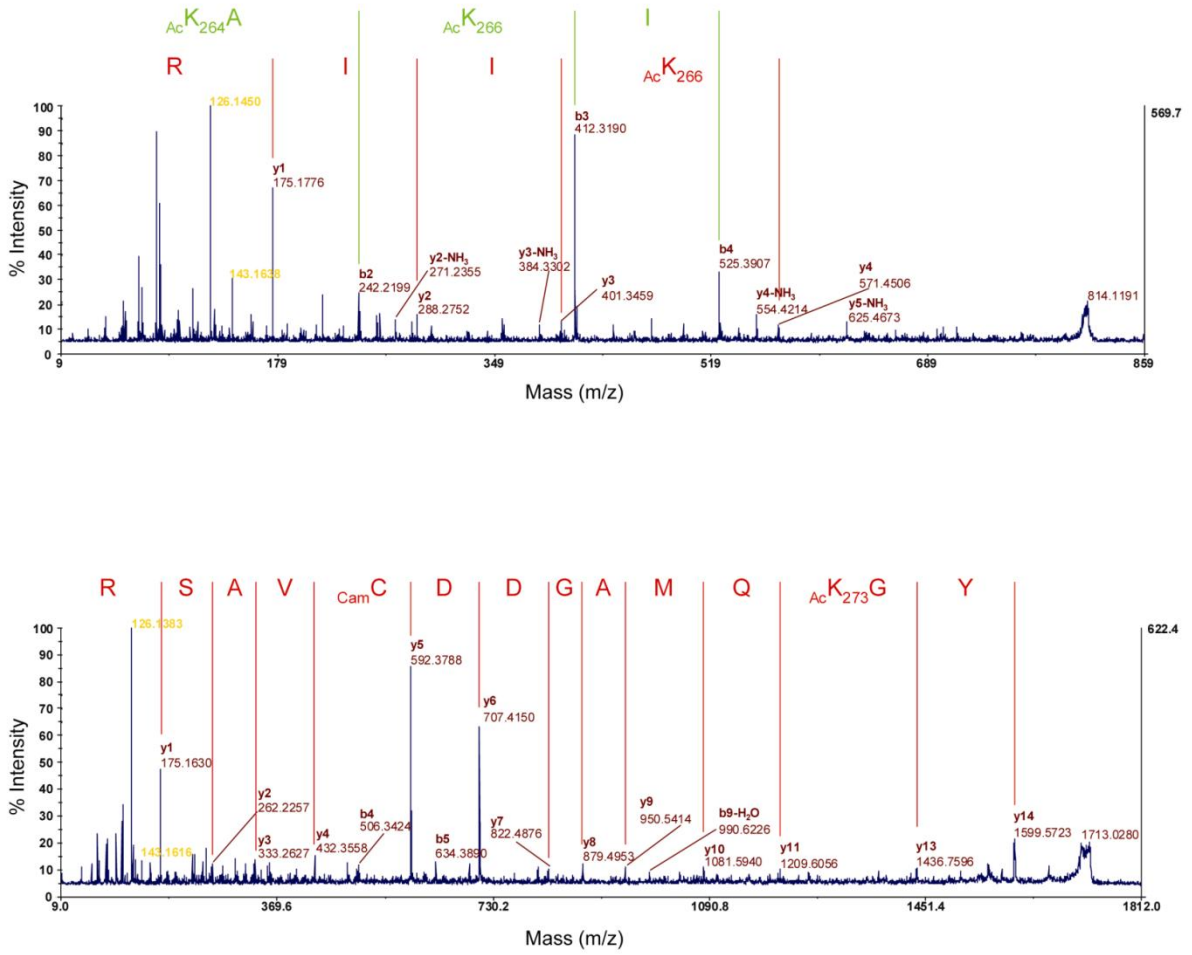


**Figure 2**: Identification of Ac-K264, Ac-K266 and Ac-K273 by MS-MS (MALDI-TOF/TOF) in the IN-HATw fusion recombinant protein. These identifications were supported by an adequate ladder of y (and b) ion family and by the presence of known marker ions for acetylated lysine: the acetylated lysine immonium ion at *m/z* 143.1 and the ion at *m/z* 126.1, which is a fragment ion induced by the loss of $NH_3$ from the ions at *m/z* 143.1(Kim et al., 2002; Trelle and Jensen, 2008). (**a**) Mass spectrum of AcK264 and AcK266 lysines. Fragmentation for bi-acetylated ion $_{Ac}$K264A$_{Ac}$K266IIR. (**b**) Mass spectrum of AcK273. Fragmentation for acetylated ion DYG$_{Ac}$K273QMAGDD$_{Cam}$CVASR. $_{Cam}$C stands for carbamidomethylated cysteine. In (a) and (b) $_{Ac}$K stands for acetylated lysines. Mapped y ion family sequence in red. Mapped b ion family sequence in green. Lysine acetylation marker ions marked in yellow.

## 2- Two-hybrid screening analysis using the constitutively acetylated integrase

To identify the cellular factors interacting with acetylated IN, we used the GBD-IN-HATw (**Figure 1c**) as a bait to screen a human lymphocytes cDNA library fused to the Gal4 activation domain (GAD). The two-hybrid screening was performed in the AH109 yeast strain. This strain contains *ADE2* and *HIS3* genes as reporters which allows the selection of positive clones using the selective medium (-Ade and -His). From almost 10.6 x $10^6$-screened transformants, 746 were positive clones encoding thirteen cellular proteins which are listed in **Table 1**.

**Table 1**: Cellular factors identified by yeast two-hybrid screening using constitutively acetylated IN (IN-HATw) as bait.

| Protein names (number of isolated clones) | Proposed function | Complete residues: peptides retrieved | GenBank Accession number | References |
|---|---|---|---|---|
| Lens epithelium-derived growth factor: **LEDGF/p75** (6 clones) | Transcription coactivator.<br>Factor interacting with lentiviral INs determining IN association to chromatin.<br>Putative tethering factor for HIV-1 integration. | 530: 344-530 | AF063020.1 | (Cherepanov et al., 2003; Emiliani et al., 2005; Ge et al., 1998 ; Maertens et al., 2003; Shun et al., 2007). |
| Krüppel-associated protein 1: **KAP1** (3 clones) | Transcription co-repressor.<br>damage response factor.<br>Regulator of acetylated non histone proteins.<br>Factor inhibiting infectivity of MLV in embryonic stem cells. | 835: 304-835 | HSU78773 | (Sripathy et al., 2006; Schultz et al., 2001; Tian et al., 2009; Wang et al., 2005a; Ziv et al., 2006; Tsuruma et al., 2008; Wang et al., 2007a; Wolf and Goff, 2007). |
| Basic transcription factor 3 isoform b: **BTF3b** (1 clone) | Component of the RNA polymerase II complex required for transcription initiation. | 162: 1-162 | NM_001207.4 | (Zheng et al., 1990). |
| Thyroid hormone receptor protein 3: **THRAP3** (1 clone) | Subunit of the large transcription mediator TRAP complex; positive regulator of RNA polymerase II promoters transcription. | 373: 1-361 | BC054046.1 | (Rachez and Freedman, 2001). |
| High-mobility group nucleosomal binding domain2: **HMGN2** (1 clone) | Component of the HMG non histone chromatin remodeling family of proteins.<br>Inducer of chromatin decondensation and transcription activity. | 90: 1-90 | BC014644.1 | (West, 2004). |
| Ran-binding protein 9: **RanBP9** (8 clones) | Ran binding protein involved in nuclear transport pathway NLS mediated.<br>A centrosomal protein that induces microtubule nucleation. | 729: 149-179 | BC063849.1 | (Gorlich, 1998; Nakamura et al., 1998; Yudin and Fainzilber, 2009). |
| Exportin 2 (synonyme : CAS): **Exp2** (1 clone) | Importin $\alpha$ binding protein.<br>Mediator of importin $\alpha$ nuclear export after NLS cargo release into the nucleus. | 971: 244-971 | BC108309.1 | (Kutay et al., 1997; Solsbacher et al., 1998). |
| Eukaryotic translation initiation factor 3 subunit H: **eIF3h** (726 clones) | Component of the eIF3 complex: promotes translation pre-initiation complex formation, mRNA recruitment and scanning for AUG recognition in the ribosomes. | 352:12-352; 172-352 | BC000386.2 | (Hinnebusch, 2006). |
| Elongation factor 1 alpha 1: **eEF1A-1** (1 clone) | Component of the alpha subunit of EF1 complex: promotes protein biosynthesis by delivering aminoacylated tRNA to ribosomes.<br>Binds HIV-1 matrix and nucleocapsid and stimulates HIV-1 transcription.<br>Its yeast homologue binds HIV-1 IN. | 46: 268-462 | BC082268.1 | (Calado et al., 2002; Cimarelli and Luban, 1999; Parissi et al., 2001). |
| Heterogenous nuclear ribonucleoprotein A2: **hnRNPA2** (1 clone) | RNA binding protein containing two RNA recognition motifs (RRM): involved in mRNA regulation (splicing and trafficking).<br>Regulator of HIV-1 RNA trafficking. | 341: 1-180 | NM_002137.2 | (Beriault et al., 2004; Shyu and Wilkinson, 2000). |
| Stathmin 1: **STMN1** (2 clones) | Tubulin binding protein: involved in microtubule depolymerization and signal transduction cascade. | 149: 1-149 | BC082228.1 | (Cassimeris, 2002). |
| Ribosomal protein L23: **RPL23** (1 clone) | Structural component of 60S subunit of ribosomes. Activates p53 by inhibiting MDM2. | 140: 18-140 | NM_000978.3 | (Berchtold and Berger, 1991). |
| Coiled-coil domain containing 32 isoform1: **CCDC32** (2 clones) | Unknown functions. | 185: 12-185 | BC001673.2 | (Ota et al., 2004). |

According to their properties and proposed functions, these cellular factors could be divided into three categories:

1- Transcription regulatory and chromatin remodeling factors: LEDGF/p75, KAP1, BTF3b, THRAP3 and HMGN2.

2- Translation regulatory and RNA binding proteins: eIF3h, eEF1A-1 and hnRNPA2.

3- Nuclear import-export proteins: Exp2 and RanBP9.

In addition to factors grouped in categories, we also identified RPL23, a structural ribosomal subunit, STMN1, a factor involved in microtubule organization and finally CCDC32, a protein with still unknown functions.

These newly identified factors, were subsequently tested by two-hybrid analysis with the IN-HATw chimera, as well as with each single domain (IN and HATw), to verify their association properties with the acetylated or unmodified IN and also with the HAT domain contained in the chimera. In addition, the analysis was performed with the GBD to check the bait specificity. Results shown in **Table 2** indicate that all identified factors associate with IN as a separate domain and that the majority do not bind the HAT domain except for THRAP3, HMGN2, RanBP9, eEF1A-1 and CCDC32.

**Table 2**: Interactions in yeast between GAD prey proteins and GBD hybrid baits (IN-HATw, IN and HATw).

| | **IN- HATw** | **IN** | **HATw** | **GBD** |
|---|---|---|---|---|
| **LEDGF/p75** | + | + | - | - |
| **KAP1** | + | + | - | - |
| **BTF3b** | + | + | - | - |
| **THRAP3** | + | + | + | - |
| **HMGN2** | + | + | - | - |
| **Exp2** | + | + | - | - |
| **RanBP9** | + | + | - | - |
| **EIF3h** | + | + | - | - |
| **EEF1A-1** | + | + | + | - |
| **HnRNPA2** | + | + | - | - |
| **STMN1** | + | + | + | - |
| **RPL23** | + | + | - | - |
| **CCDC32** | + | + | + | - |

+: interaction, -: no interaction

In conclusion, thirteen new factors binding to acetylated IN have been identified. These factors positively interact also with IN separated from the HAT domain, suggesting that acetylation modulates but is not an absolute requirement for virus cell interaction.

### 3-Analysis of binding interactions between two-hybrid factors and acetylated or un-modified integrase

To verify the interaction between IN and the cellular factors identified by two-hybrid screening (**Table 1**), pull down assays with the same factors expressed in human cells have been performed. Experiments were carried out with few selected factors based on their possible involvement in HIV-1 replication: transcription related proteins (BTF3b, THRAP3 and HMGN2) potentially involved in tethering viral integration in transcription units; a nuclear transport factors (Exp2 and RanBP9) possibly involved in nuclear-cytoplasmic translocation. In addition, eIF3h, a factor involved in protein synthesis, was tested due to its high frequency of identification (726 clones) in the two-hybrid screening. These factors were fused to a Flag tag and expressed in HEK293T cells. The derived cell lysates were incubated with either IN-HATw or IN-HATm recombinant proteins. Subsequently, the immunocomplexes were recovered with anti-Flag antibodies and analyzed by Western blot with anti-HA and anti-Flag antibodies. As an experimental control for binding specificity, the same analysis was performed using HEK293T cells expressing an unrelated control protein, luciferase, fused to the Flag tag (Flag-Luc). As shown in **Figure 3a, 3b** and **3c** higher amounts of IN-HATw than IN-HATm were found associated with Flag-Exp2, Flag-elF3h and Flag-RanBP9. Conversely, similar amounts of IN-HATw and IN-HATm bound Flag-BTF3b, Flag-THRAP3 and Flag-HMGN2 (**Figure 3d**, **3e** and **3f**). Finally, no specific binding was observed with the unrelated control protein, Flag-Luc (**Figure 3g**). As shown in lower panels of **Figure 3a-g**, incubation of the same filters with anti-Flag antibodies proved that similar amounts of Flag proteins were immunoprecipitated with either IN-HATw or IN-HATm. These results suggest that Exp2, eIF3h and RanBP9 bind with higher affinity the acetylated form of IN, whereas BTF3b, THRAP3 and HMGN2 show no preferential binding for either forms of the viral protein. In the next section we describe KAP1, another identified cellular factor (**Table 1**), that binds preferentially acetylated IN.

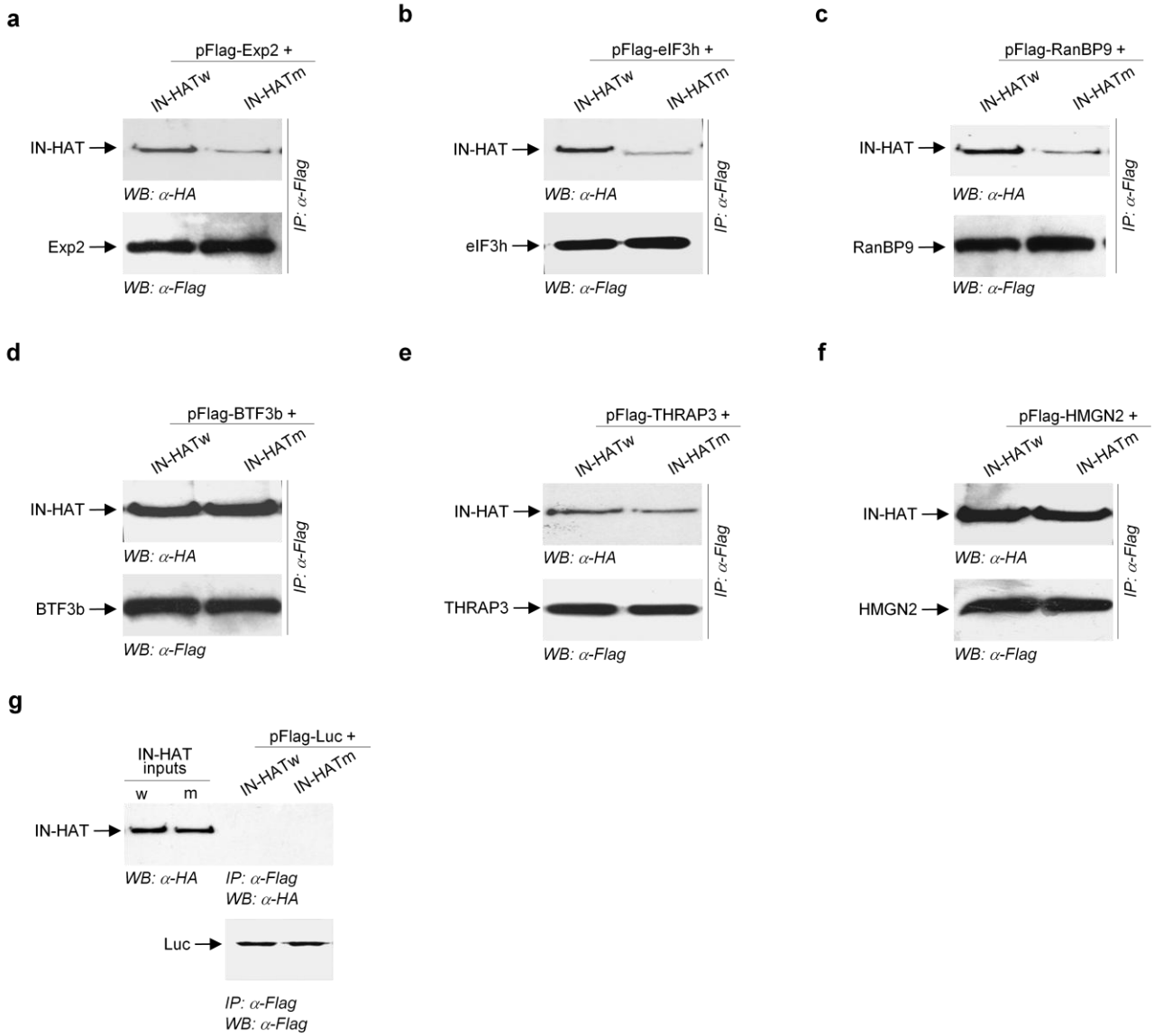


**Figure 3**: Binding analysis between acetylated IN and proteins identified by the yeast two-hybrid screening. (**a**) Lysates from HEK293T cells expressing Flag-Exp2 (**a**), Flag-eIF3h (**b**), Flag-RanBP9 (**c**), Flag-BTF3b (**d**), Flag-THRAP3 (**e**), Flag-HMGN2 (**f**) and FLAG-Luc (**g**) were incubated with recombinant IN-HATw or IN-HATm-HA and immunoprecipitated (IP) with monoclonal anti-Flag antibodies. Immunoprecipitates were then analyzed by Western blot with anti-HA antibodies (α-HA) (upper panels) and with polyclonal anti-Flag antibodies (α -Flag) (lower panels).

Next, the interaction between the two-hybrid hits and the single HAT and IN domains was verified using lysates of HEK293T cells expressing the Flag-tagged factors. No binding was observed with the HAT domain (**Figure 4a**, **upper panel**), even though high expression of two-hybrid Flag-tagged factors could be detected (**Figure 4a**, **lower panel**). Thus, these data demonstrate that the interaction between IN and the two-hybrid hits is specific and not mediated by the HAT domain. Moreover, this result suggests that the positive interactions observed by two-hybrid analysis with the HAT domain (**Table 2**), were likely a

result of the synergistic transactivation properties of these factors (THRAP3, HMGN2 and eEF1A-1) together with the HAT domain over the yeast promoter.

Finally, the unmodified form of IN (6xHis-IN) was verified by pull down assays with Flag-tagged two-hybrid hits expressed in HEK293T cells. High levels of IN was found associated with BTF3b, HMGN2 and THRAP3 (**Figure 4b**), while much lower amounts could be detected in complex with Exp2, eIF3h and KAP1.

Therefore, these data are in agreement with results in **Figure 3**, showing that BTF3b, HMGN2 and THRAP3 bind efficiently unmodified IN and do not require IN acetylation. Conversely, the low amounts of Exp2, eIF3h and KAP1 bound to IN is indicative that IN acetylation enhances binding affinity.

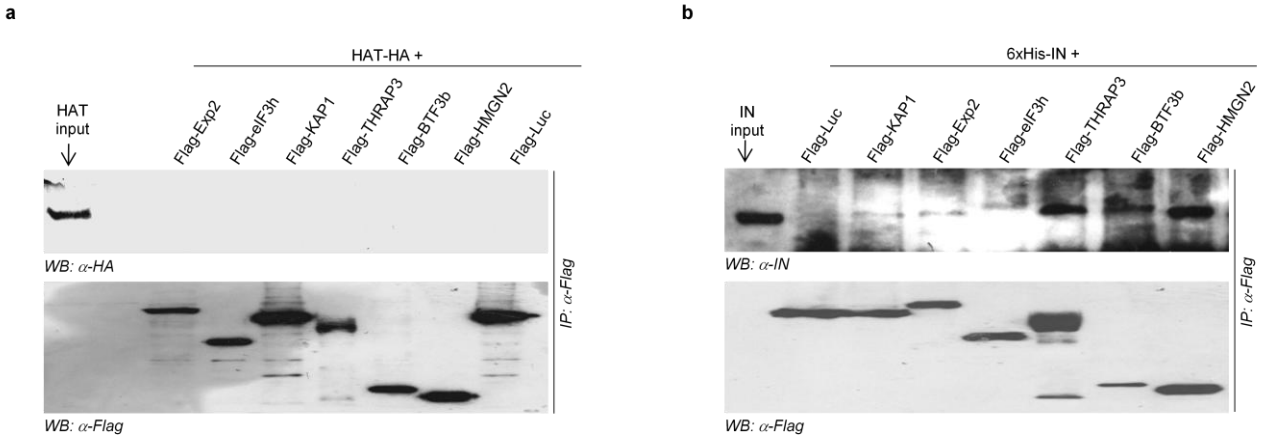


**Figure 4:** Binding between proteins identified by the two-hybrid screening and the HATw or IN domains. HEK293T cell lysates expressing Flag-eIF3h, Flag-BTF3b, Flag-THRAP3, Flag-HMGN2 or FLAG-Luc were incubated with either HATwt (**a**) or 6xHis-IN (**b**) recombinant proteins or immunoprecipitated (IP) with monoclonal anti-Flag antibodies. Immunoprecipitates were then analyzed by Western blot with anti-HA antibodies (α-HA) (**a,** upper panel) or with anti-IN antibodies (**b**, upper panel) and with polyclonal anti-Flag antibodies (α-Flag) (**a** and **b** lower panels).

## 4- Interaction between KAP1 and acetylated integrase

KAP1 was identified in the two hybrid screen to interact with acetylated integrase (**Table 1**). KAP1 is an ubiquitous nuclear protein that belongs to the TRIM family and it has been described to restrict MLV and other retroviruses in mouse embryonic stem cells (Wolf and Goff, 2007; Wolf et al., 2008b). Beside, KAP1 has been reported to regulate the activity of several acetylated non-histone proteins by modulating their acetylation levels through HDACs (Tian et al., 2009; Tsuruma et al., 2008; Wang et al., 2005a; Wang et al., 2007a). Due to the involvement of KAP1 in the retroviral replication and also in the regulation of the acetylated proteins, we decided to investigate the role of KAP1 and its interaction with

acetylated integrase in HIV-1 infection. The first experiments aimed to verify the specificity of binding of KAP1 with acetylated integrase. To reach this aim, comparative analysis was performed by co-transforming GAD-KAP1 in yeast with the following GBD/HA fusion proteins: IN-HATw, IN-HATm, HATw, HATm or GBD alone. A higher interaction between KAP1 and acetylated integrase (IN-HATw) was observed with respect to unmodified integrase (IN-HATm) or the individual domains (HATw, HATm and GBD) in two hybrid selective medium (-Ade,-His) (**Figure 5a, left panel**). This result was also confirmed by X–alpha-gal filter lift assay showing specific interaction of GAD-KAP1 with IN-HATw (**Figure 5a, right panel**). In the large screen, some clones carrying GAD-KAP1 and GBD-IN-HATm were positive, consistent with the interaction seen between KAP1 and GBD-IN (**Table 2**), indicating that KAP1 interacts even with unmodified integrase; however, as shown above (**Figure 5a**), this interaction is highly favored by integrase acetylation catalyzed by p300.

To validate the interaction occurring between the acetylated integrase and KAP1 the binding of the two proteins was further analyzed in a mammalian system. KAP1 fused to a Flag-tag was expressed in HEK293T cells and the lysate incubated with the IN-HATw and IN-HATm recombinant proteins. The binding complexes were then pulled down with anti-Flag antibodies immobilized on the agarose beads and analyzed by Western blot with antibodies anti-HA. Higher amounts of IN-HATw were shown to bind KAP1 as compared to IN-HATm (**Figure 5b, left-upper panel**). Incubation of the same membrane with antibodies anti Flag demonstrated that similar amounts of KAP1 protein were immunoprecipitated (**Figure 5b, left-lower panel**). The same experiment was performed using integrase isolated from the HATw or m domains following proteolytic cleavage of the recombinant chimeras with TEV protease (as schematized in **Figure 1a**). By incubating integrase derived from IN-HATw with lysates from cells expressing Flag-KAP1 a doublet at the same molecular size of integrase is clearly visible by anti-IN antibodies following anti-Flag immunoprecipetation (**Figure 5b, right-upper panel**). Conversely no bands were detected when the same reaction was performed with integrase derived from the IN-HATm chimera (**Figure 5b, right-upper panel**). The doublet observed in the IN-HATw immunoprecipitation is probably due to degradation of the integrase protein during purification procedures since similar doublets are also observed in **Figure 1b** following TEV digestion. In conclusion, these data clearly demonstrate that

constitutively acetylated integrase binds Flag-KAP1 with a higher affinity than the non-acetylated protein.

To further verify the integrase/KAP1 *in vivo* interaction, Flag-tagged integrase (Flag-INw) was expressed in HEK293T cells and the amount of complexed endogenous KAP1 was evaluated following anti-Flag immunoprecipitation and anti-KAP1 Western blot analysis. Endogenous KAP1 is co-immunoprecipated with Flag-integrase, while no bands appear in samples expressing the control Flag (**Figure 5c, upper panel**). To verify the role of lysines acetylated by p300 in the carboxy-terminus of integrase (Cereseto et al., 2005) on the interaction with KAP1, the same immunoprecipitation experiment was performed using a plasmid expressing Flag-integrase mutated in lysines targeted by p300 (K264,K266 and K273) (Flag-INm) (Cereseto et al., 2005). Lower amounts of endogenous KAP1 bound mutated integrase as compared to wild-type integrase (**Figure 5c, upper panel**). The level of integrase acetylation was verified by Western blot analysis of the same lysates used for the immunoprecipitation with antibodies specific for acetylated integrase (**Figure 5c, middle panel**). The total level of Flag-integrase expression was also verified by Western blot using anti-Falg antibodies (**Figure 5c, middle panel**). In order to further verify the preferential binding of endogenous KAP1 to acetylated integrase *in vivo,* reciprocal co-immunoprecipitation experiments were performed. Lysates from HEK293T cells expressing untagged integrase wild type (pCMV-INw) or mutated at the three acetylation sites (pCMV-INm) were immunoprecipitated using anti-KAP1 antibodies. Detection of binding complexes with anti-integrase antibodies showed higher amount of wild integrase (pCMV-INw) associated with endogenous KAP1 comparing to the mutated integrase (pCMV-INm), while, no signal was detected with control cells (pCDNA3) (**Figure 5d, upper panel**). Similar amounts of endogenous KAP1 were co-immunoprecipitated with pCMV-INw and pCMV-INm (**Figure 5d, second panel** from top). The expression of pCMV-INw and pCMV-INm in HEK293T cells detected by Western blot using anti-IN antibodies showed equal levels (**Figure 5d, third panel** from top). Finally, the endogenous levels of KAP1 in cells expressing either pCMV-INw or pCMV-INm were not modulated as compared to transfected control cells (pCDNA3) (**Figure 5d, lower panel**). Taken together (**Figure 5 c and d**), we conclude that endogenous KAP1 interacts with Flag-tagged and untagged integrases *in vivo*. In addition, a higher affinity of KAP1 was revealed with acetylated rather than the unmodified form of integrase.

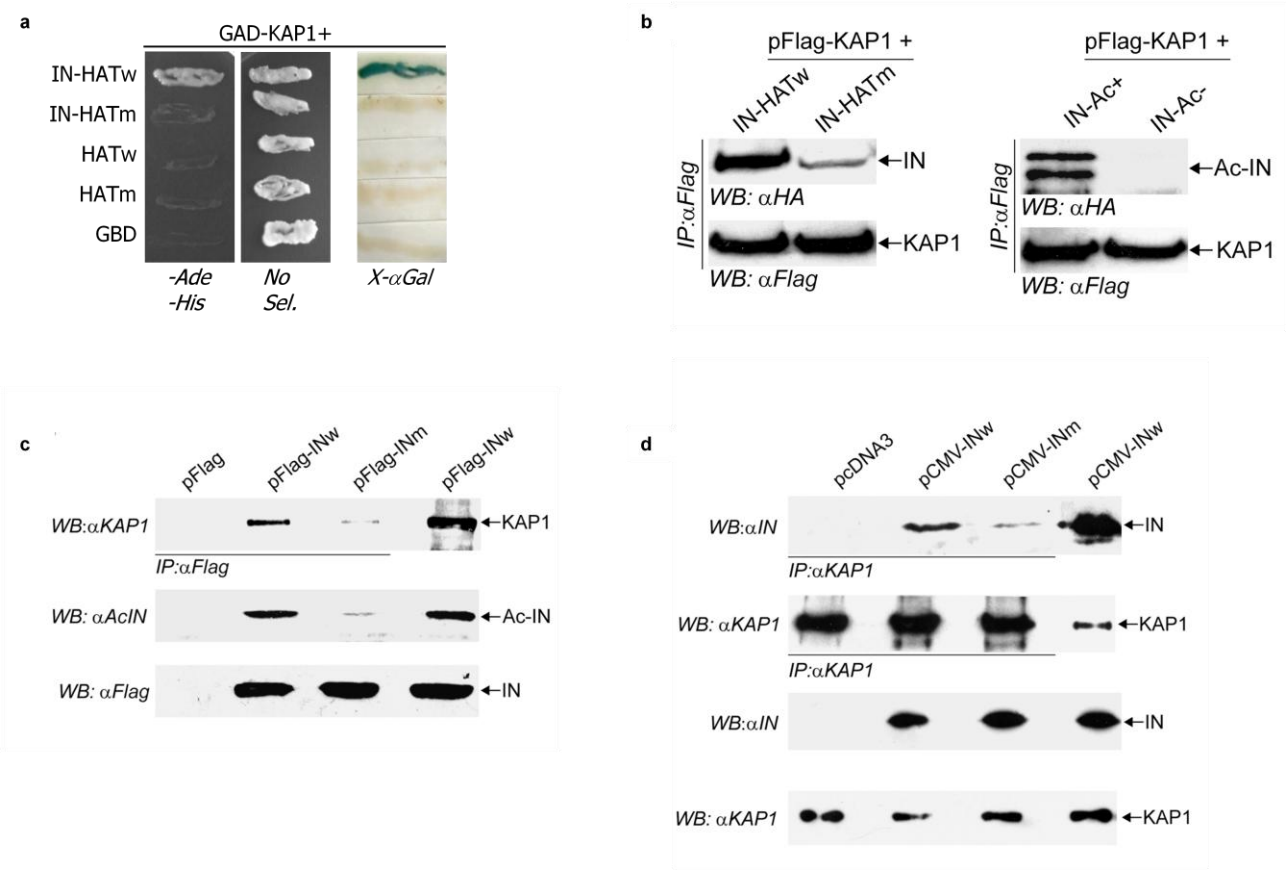


**Figure 5**: KAP1 interacts with acetylated integrase *in vivo* in yeast and in mammalian cells with higher affinity than non acetylated form. (**a**) Interaction efficiencies of the IN-HATw and IN-HATm chimeras and single domains (HAT, IN and GBD) with KAP1 in yeast two-hybrid on (–Ade,-His) selective two hybrid medium (left panel). Cotransformation efficiencies were controlled on no selective two hybrid medium (+Ade, +His) but lacking Trp and Leuc (-Trp, -Leuc) for the selection of GBD and GAD vectors, respectively (middle panel). All these cotransformants were subjected to X–alpha-gal filter lift assay assessing the efficiency of *MEL1* reporter gene to produce α-galactosidase in condition of positive interactions (right panel). (**b**) Flag-KAP1 binds acetylated IN with higher affinity than the non-acetylated form. HEK293T cell lysates expressing Flag-KAP1 were incubated with not digested IN-HATw/m recombinant chimeras (left panel) or with IN digested by TEV protease from wild type and mutated chimeras (IN-Ac+/-) (right panel); the binding complexes were pull downed with αFlag agarose beads. αFlag immunocomplexes were then blotted with αHA antibodies to detect bound IN-HAT chimeras (left panel) or with anti-integrase antibodies (αIN) to detect TEV digested integrases (INAc+/-)(right panel). The two membranes were re-probed with αFlag antibodies to check for KAP1 expression. (**c**) and (**d**) Endogenous KAP1 binds preferentially acetylated IN *in vivo*. (**c**) HEK293T cells expressing either Flag-IN wild type (Flag-INw) or mutated at lysine acetylation sites (K264, 266, 273R) (Flag-INm) were immunoprecipitated with αFlag antibodies and blotted using αKAP1 antibodies. The acetylation and expression levels of integrase wild type and mutated were detected by anti-Ac-IN (α-AcIN) and αFlag antibodies, respectively (**d**) HEK293T expressing either untagged IN wild type (pCMV-INw) or mutated (K264, 266, 273R) (pCMV-INm) were immunoprecipitated with α-KAP1 and blotted with α-IN and α-KAP1. Expression levels of IN and KAP1 were controlled with the indicated antibodies.

Since acetylated lysines are localized in the carboxy-terminus of integrase, we sought to investigate whether this domain regulates KAP1 binding. To this aim pull-down experiments were performed incubating recombinant integrase domains fused to glutathione-S-transferase (GST) with *in vitro* $^{35}$S-Met labelled KAP1. The protein complexes were pulled down with S-glutathione agarose beads. Almost 10% of $^{35}$S-KAP1 binds GST-

full length integrase (**Figure 6a, upper panel** and **graph**), while only 1.13% of the input was retained on the GST control demonstrating that integrase and KAP1 interact *in vitro*. The *in vitro* pull down assays were then performed with each single domain showing no interaction between KAP1 and either the N-terminus or the catalytic domain, while the C-terminus retained 12.41% of $^{35}$S-KAP1 input indicating that C-terminal domain of HIV-1 integrase is responsible for the IN/KAP1 complex formation *in vitro* (**Figure 6a, upper panel** and **graph**). Coomassie stained SDS-PAGE gel showed that similar amounts of GST-IN full length and single domains were used for the *in vitro* binding with $^{35}$S-KAP1 (**Figure 6a, lower panel**).

To investigate the relevance of the three acetylated lysines (K264, K266 and K273) of the C-terminal domain of integrase in IN/KAP1 *in vitro* binding, we incubated the $^{35}$S-Met labelled KAP1 with either 6xHis-IN-HATw or 6xHis-IN-HATm recombinant proteins (**Figure 1**). The protein complexes were pulled down using Ni-NTA columns. Integrase constitutively acetylated (IN-HATw) showed double efficiency to bind $^{35}$S-KAP1 (100%) as compared to unmodified integrase (IN-HATm) (48%) (**Figure 6b, upper panel** and **graph**), while no significant signal was shown in the control reaction ($^{35}$S-KAP1+ 6xHis). The amounts of IN-HATw and IN-HATm used in this *in vitro* binding, detected by Coomassie staining of the SDS-PAGE gel, were similar (**Figure 6b, lower panel**). Considering that K264, K266 and K273 lysine residues were found acetylated within the IN-HATw protein by mass spectrometry analysis (**Figure 2**), we conclude that the acetylation sites of integrase C-terminal domain are responsible for the enhancement of IN/KAP1 interaction *in vitro*. This result is in agreement with *in vivo* data where the integrase mutated at the lysine acetylation sites has lower affinity of binding with KAP1 than the wild type integrase (**Figures 5c** and **5d**).

In conclusion integrase and KAP1 interact both *in vivo* and *in vitro* and acetylation of integrase strongly enhances this complex formation.

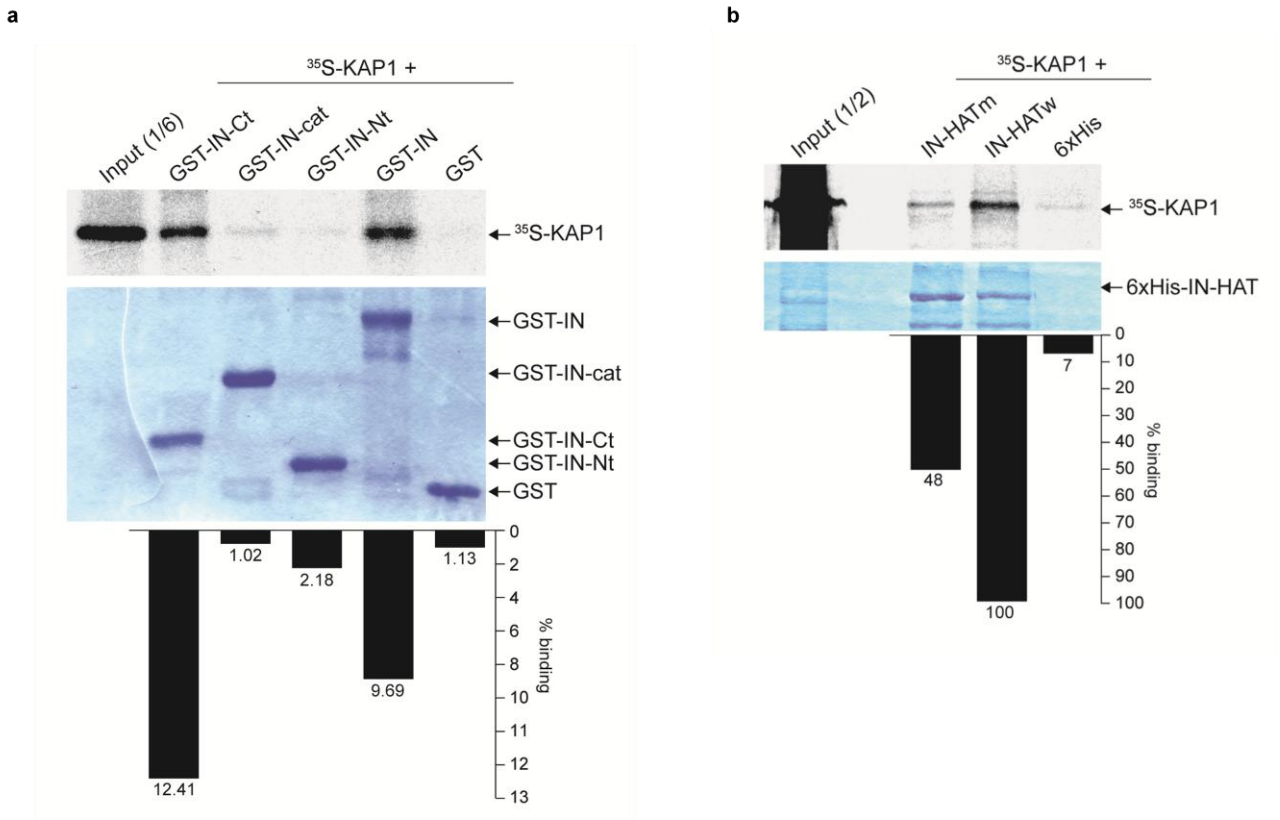


**Figure 6:** IN/KAP1 *in vitro* binding occurs through the IN C-terminal domain and is enhanced by IN acetylation. (**a**) the C-terminal domain of integrase responsible of the direct binding with KAP1. *In vitro* translated KAP1 labeled with $^{35}$S-Met ($^{35}$S-KAP1) was incubated with either recombinant full length IN fused to GST (GST-IN) or with each single recombinant IN domain fused to GST: N-terminus (GST-IN-Nt), catalytic (GST-IN-Cat) and C-terminus (GST-Ct). Pulled down complexes obtained using S-glutathione agarose beads were resolved on SDS-PAGE gel and analyzed by densitometry. The graph expresses the amounts of $^{35}$S-KAP1 bound protein as percentages of the input ($^{35}$S-KAP1). (**b**)IN/KAP1 direct binding is enhanced by integrase acetylation. *In vitro* translated KAP1 labeled with $^{35}$S-Met ($^{35}$S-KAP1) was incubated with 6xHis-IN-HATw or 6xHis-IN-HATm recombinant proteins and pulled down using Ni-NTA agarose beads. The graph express the amount $^{35}$S-KAP1 bound protein as percentages of 100% of $^{35}$S-KAP1 bound to IN-HATw. In (**a**) and (**b**) the upper panels show the gels exposed to Phosphoimaging (Cyclone) and the lower panels the Coomassie staining of the SDS-PAGE gels.

## 5- Transient knockdown of KAP1 increases HIV-1 infectivity and integration

To evaluate the role of KAP1 during HIV-1 infection, HeLa cells were transiently knocked down with a pool of small interfering RNAs (siRNA) directed against KAP1 gene and then infected with HIV-1 virus. Western blot analysis revealed that the expression of KAP1 was lowered in cells treated with KAP1 siRNAs (si-KAP1), as compared to cells untreated (UT) or transfected with a pool of control non targeting siRNAs (si-CTR) (**Figure 7a**). HeLa cells knocked down for KAP1 and control cells were infected, two days after siRNA treatment, by a single round HIV-1 virus clone carrying luciferase as reporter (NL4.3-Luc)

and pseudotyped with VSV-G envelope. Viral infectivity was measured by the levels of luciferase activity at 48 hours post-infection. In cells treated with KAP1 siRNA (si-KAP1) luciferase activities were three fold higher than cells treated with control siRNA (si-CTR) (**Figure 7b**). To better investigate which early viral replication step is affected by KAP1, quantitative real-time PCR (Q-PCR) have been performed to measure late reverse transcripts, 2-LTR circles and integrated viral DNA using primers that target specifically each viral cDNA form. The levels of late reverse transcripts quantified 24 hours post infection in KAP1 knockdown HeLa cells (si-KAP1) and in not silenced control cells (si-CTR) were not significantly different (**Figure 7c**) indicating that KAP1 does not affect reverse transcription. Two-LTR circles are viral cDNA molecules that translocate into the nucleus where they fail to integrate (Coffin et al., 1997; Engelman, 1999) and become circularized likely by the non homologous end joining (NHEJ) cellular repair (Li et al., 2001). Therefore, 2-LTR circles are a surrogate marker of retrovirus nuclear import (Coffin et al., 1997) and are indicative of an abortive integration (Engelman, 1999). The quantification of the 2-LTR circles by Q-PCR was performed 24 hours post infection. In HeLa cells knocked for KAP1 (si-KAP1) 2-LTR circles were significantly lower than in control not silenced cells of almost 2 fold (**Figure 7d**), indicating that KAP1 knockdown might increase HIV-1 integration efficiency or inhibit nuclear import of the viral cDNA. The detection of integrated HIV-1 proviruses by quantitative Alu-PCR at 48 hours post-infection showed that integrated viral DNA were significantly increased in KAP1 knockdown HeLa cells (si-KAP1) about 5 to 9 fold as compared to not silenced control cells (**Figure 7e**). Therefore, siRNA-mediated transient Knockdown of KAP1 in HeLa cells does not affect reverse transcription, decreases the formation of the 2-LTR circles and significantly increases HIV-1 integration. Since at similar amounts of late reverse transcripts, a decrease of the 2-LTR circles was correlated with an increase in the integration efficiency in the HeLa cells transiently knocked down for KAP1, we suggest that KAP1 affects the efficiency of HIV-1 integration.

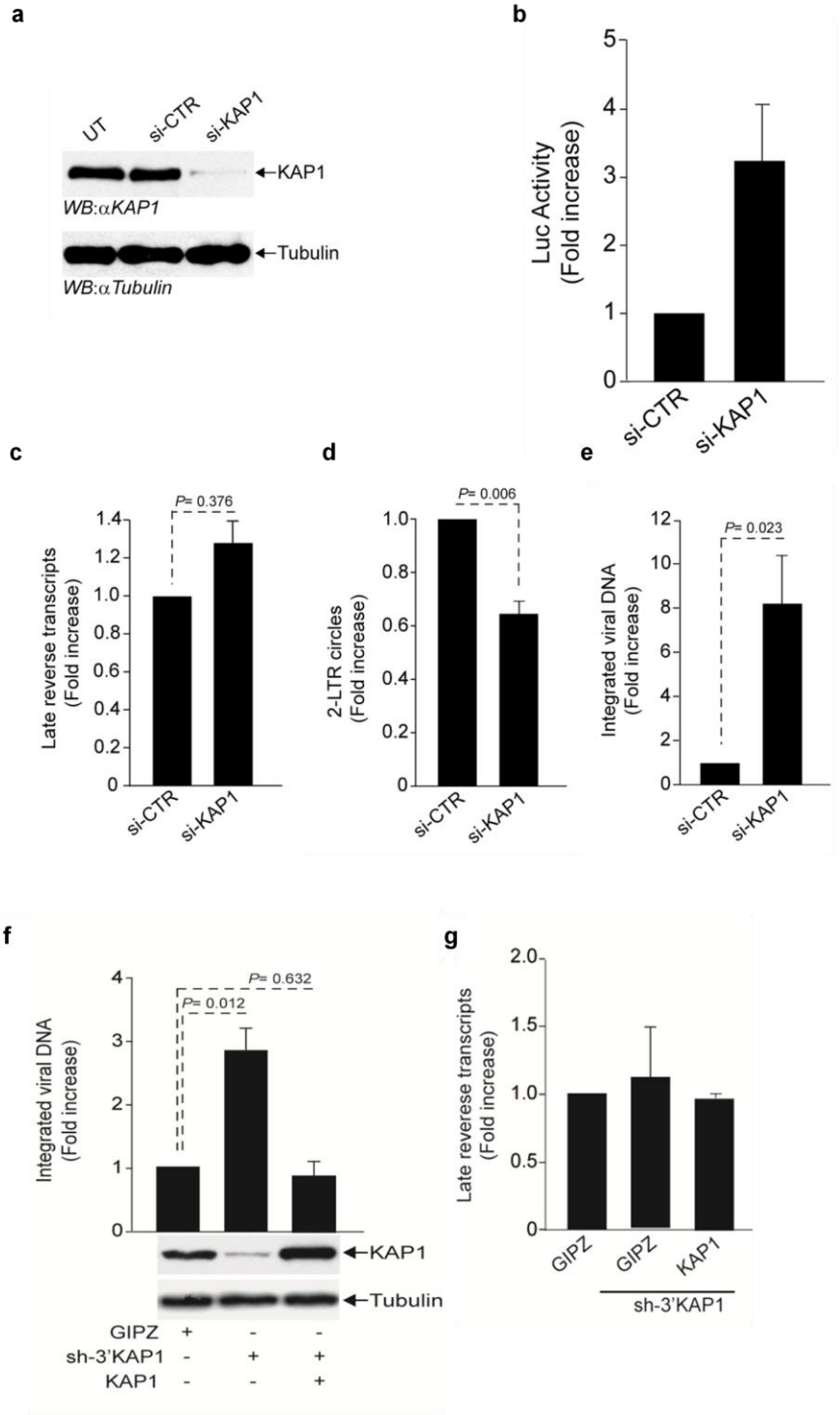


**Figure 7**: KAP1 transient knockdown increases HIV-1 integration in HeLa cells. (**a**) Expression of KAP1 in HeLa cells transiently knocked down with smart pool siRNAs (si-KAP1) and in control HeLa cells treated with a pool of non-targeting siRNAs (si-CTR). Immunoblot was performed using αKAP1 antibodies and re-probed with αTubulin antibodies to check for protein loading. (**b**-**e**) Transient KAP1 knockdown HeLa cells were infected with NL4.3-Luc (VSV-G) HIV-1 virus and analyzed for luciferase activity (Luc Activity) 48 hours post-infection (hpi) (**b**) and by Q-PCR for late reverse transcripts 24 hpi (**c**), 2-LTR DNA circles 24 hpi (**d**) and integrated HIV-1 DNA 48 hpi by Alu-PCR (e). (**f**-**g**) KAP1 knockdown HeLa cells (sh-3'KAP1), back-complementated (sh-3'KAP1 + HA-KAP1) and control treated (GIPZ) were infected with NL4.3-Luc (VSV-G) HIV-1 virus and analyzed by Q-PCR for integrated viral DNA at 15 days post infection (dpi) (**f**) and for total viral cDNA at 24 hpi (**g**). Expression of KAP1 was verified by immunoblot. All graphs are represented in fold increase with respect to control cells (± s.d. from at least two independent experiments).

To verify the specificity of the KAP1 knockdown on HIV-1 infection, HeLa cells were silenced transiently for KAP1 using a short hairpin RNA that recognizes the 3' untranslated region of KAP1 (sh-3'KAP1) and expressed from a lentiviral vector (LKO.1). These cells were back-complemented by the expression of KAP1 cDNA resistant to the sh-3'KAP1 and then were infected with NL4.3-Luc HIV-1 virus and analyzed by Q-PCR for total HIV-1 at 24 hours post infection and for integrated proviruses at 15 days post-infection. The control cells were treated with the GIPZ lentiviral vector. As shown in **Figure 7f**, the KAP1 knockdown mediated by the sh-3'KAP1 enhances significantly HIV-1 integration of almost three fold, while the KAP1 back-complemented cells (sh3'KAP1 + KAP1) were able to reverse integration to the basal levels of not silenced cells (GIPZ). In addition, in all these conditions total viral cDNA was not changed (**Figure 7g**), indicating the specificity of KAP1 knockdown on HIV-1 integration.

### 6- KAP1 stable knockdown increases HIV-1 infectivity and integration

In order to further confirm the inhibitory role of KAP1 on HIV-1 integration with a different knockdown approach, HeLa and HEK293T cells were stably knocked down for KAP1 using a short hairpin RNA targeting KAP1 gene (sh-KAP1) expressed from a lentiviral vector (GIPZ). Control cells were a pool of cells expressing a mismatched shRNA (sh-MM) containing four point mutations inside the sh-KAP1. Stable KAP1 knockdown cells were viable and did not display cytotoxic effects comparing to wild type cells or to cells stably expressing sh-MM, however, KAP1 endogenous levels were rescued 1 to 2 months following selection in culture even under stringent conditions, indicating the physiological relevance of this protein and that pronounced down-regulation could not be obtained. The limited levels of transient and stable knockdowns of KAP1 in cells have been previously reported (Wang et al., 2005a; Wang et al., 2007a; Wolf and Goff, 2007). Therefore, KAP1 expressions were checked by Western blot analysis simultaneously to each infectivity experiment (**Figures 8a** and **9a**). Following infection with NL4.3-Luc HIV-1 virus three KAP1 knockdown HeLa cell clones (H14a, H18 and H19)(**Figure 8a**) showed three fold more luciferase activity than the control cell pool (sh-MM) (**Figure 8b**). To investigate which step of viral infectivity is affected by KAP1, quantitative real time PCR (Q-PCR) were performed. HeLa KAP1-silenced and control cells were infected with NL4.3-Luc and at 24 hours post infection, a fraction of cells was analyzed for total viral cDNA and for 2-LTR

circles and remained cells were passed in culture for two weeks and analyzed for the integrated viral DNA. Measurement of late reverse transcripts and integrated viral DNA in the knockdown (sh-KAP1) or control (sh-MM) HeLa clones could not be performed by primers that recognize LTR due to cross detection of integrated lentivirus (GIPZ). Therefore, these viral DNA forms were analyzed by Q-PCR using primers that target the luciferase gene of NL4.3-Luc (Terreni et al., 2010). No significant difference in the total viral cDNA has been observed between HeLa cell silenced clones (H14a, H18 and H19) and control cell pool (sh-MM) (**Figure 8c**), indicating that KAP1 does not affect reverse transcription. The amounts of 2-LTR circles have been significantly decreased in KAP1 Knockdown HeLa cell clones of almost 2 to 10 fold with respect to not silenced cell pool (sh-MM) (**Figure 8d**). Consistently, integrated HIV-1 DNA in stable KAP1 knockdown HeLa cell clones at two weeks post infection were significantly increased by 2 to 3 fold as compared to not silenced cell pool (sh-MM) (**Figure 8e**).

The same infectivity experiments were performed with a different cell line, HEK293T, stably knocked down for KAP1 (sh-KAP1) and control not silenced pool (sh-MM) (**Figure 9a**). Infectivity of silenced HEK293T cell pool and clones (T-pool, T14 and T15) were increased about 4 to 6 fold with respect to not silenced pool (sh-MM) (**Figure 9b**). Total viral cDNA quantified by Q-PCR at 24 hours post infection was not changed in KAP1 knockdown HEK293T cell pool and clones comparing to not silenced pool (sh-MM) (**Figure 9c**). Whereas, 2-LTR circles at 24 hours post infection were decreased in KAP1 silenced HEK293T cells of almost 5 to 10 fold as compared to not knocked down pool (sh-MM) (**Figure 9d**). The observed decrease of the two-LTR circles was consistent with an enhancement of viral integration at two weeks post infection in KAP1 silenced HEK293T cell clones and pool about 2 to 3 fold with respect to not silenced cell pool (sh-MM) (**Figure 9e**).

In conclusion stable knockdown of KAP1 in HeLa and HEK293T cells, obtained by the use of short hairpin RNA enhances HIV-1 integration, decreases the 2-LTR circles while the reverse transcription remains unaffected. These data are consistent with those observed with KAP1 transient knockdown in HeLa cells (**Figure 7**), mediated by the small interfering RNAs, and suggest that KAP1 decreases HIV-1 infectivity by impairing specifically the integration step.

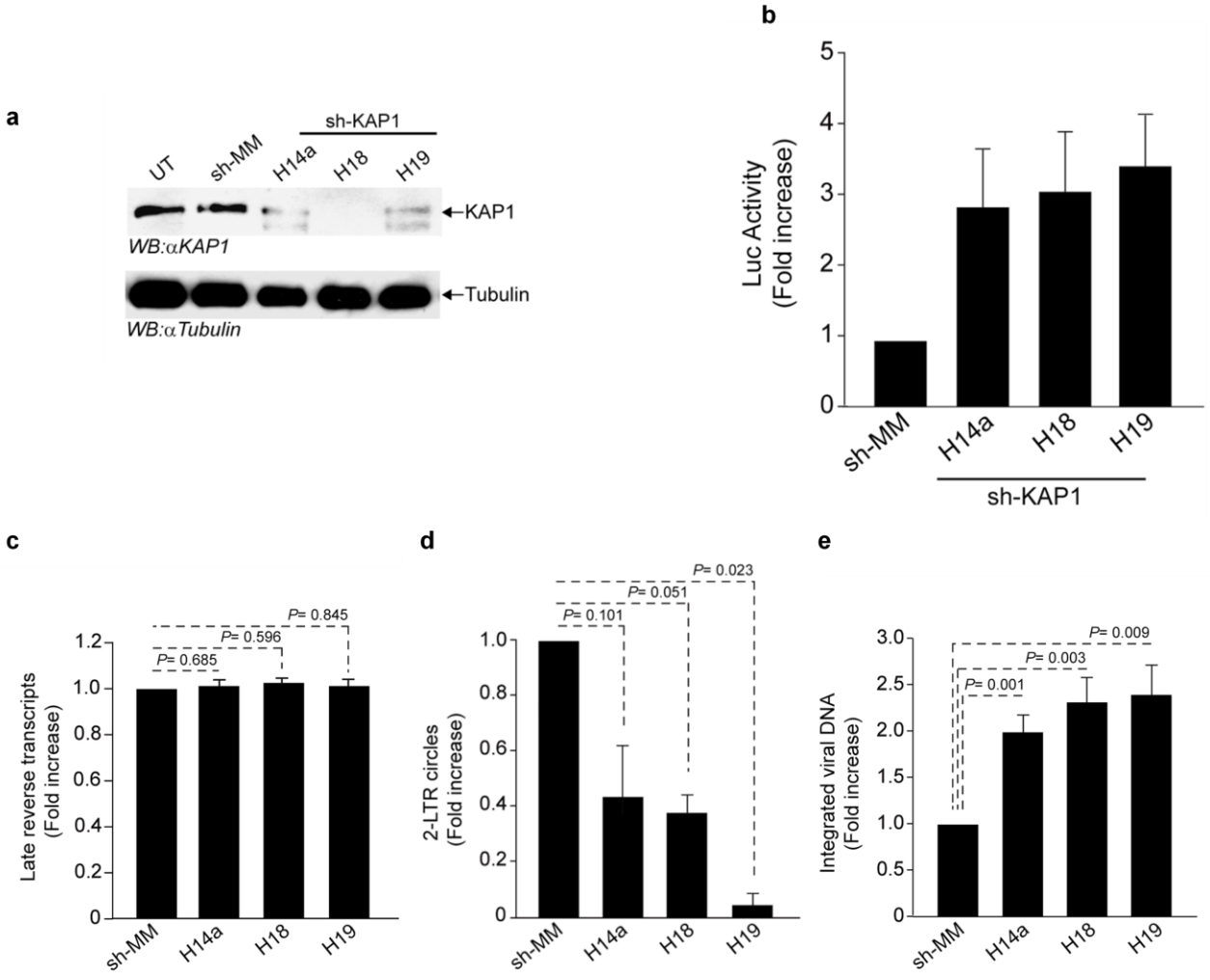


**Figure 8**: KAP1 stable knockdown increases HIV-1 integration in HeLa cells. (**a**) Expression of KAP1 in HeLa cells clones (H14a, H18 and H19) stably knocked down with KAP1 shRNA expressed by a lentiviral vector (GIPZ-sh-KAP1) and in control HeLa cells expressing a four point mutations mismatched KAP1-shRNA (GIPZ-sh-MM). Immunoblot was performed using αKAP1 antibodies and re-probed with αTubulin antibodies to check for protein loading. (**b-e**) HeLa cells stably silenced for KAP1 and control cells were infected with NL4.3-Luc HIV-1 virus (pseudotyped by VSV-G envelope) and analyzed for luciferase activity (Luc Activity) at 48 hours post-infection (hpi) (**b**) and by Q-PCR for late reverse transcripts at 24 hpi (**c**), 2-LTR circles at 24 hpi (**d**) and integrated HIV-1 DNA at 15 dpi (**e**). All graphs are represented in fold increase with respect to control cells (sh-MM) (± s.d. from at least two independent experiments).

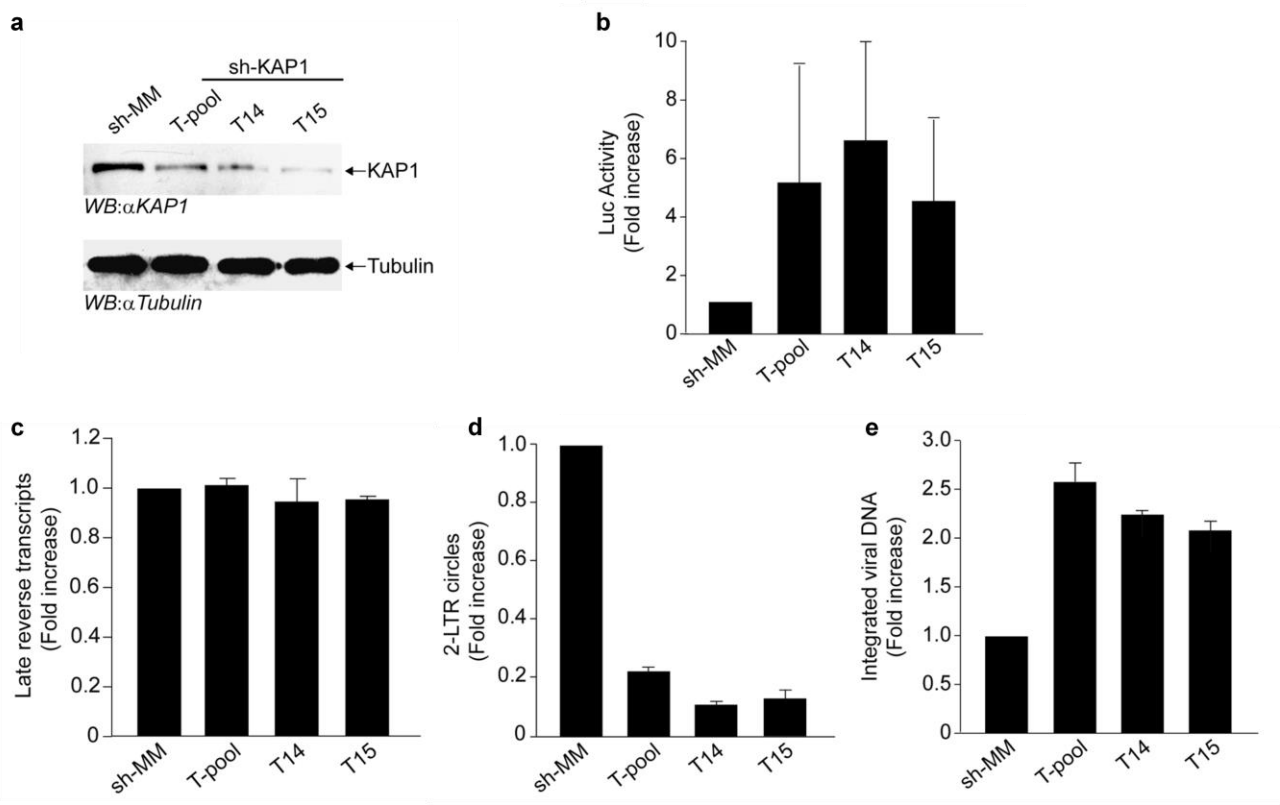


**Figure 9**: KAP1 stable knockdown increases HIV-1 integration in HEK293T cells. (**a**) Expression of KAP1 in HEK293T cells (T-pool) and cell clones (T14 and T15) stably knocked down with KAP1 shRNA expressed by a lentiviral vector (GIPZ-sh-KAP1) and in control HEK293T cells expressing four point mutations mismatched KAP1-shRNA (GIPZ-sh-MM). Immunoblot was performed using αKAP1 antibodies and re-probed with αTubulin antibodies to check for protein loading. (**b-e**) HEK293T cells stably silenced for KAP1 were infected with NL4.3-Luc HIV-1 virus (pseudotyped by VSV-G envelope) and analyzed for luciferase activity (Luc Activity) at 48 hours post-infection (hpi) (**b**) and by Q-PCR for late reverse transcripts at 24 hpi (**c**), 2-LTR circles at 24 hpi (**d**) and integrated HIV-1 DNA at 15 dpi (**e**). All graphs are represented in fold increase with respect to control cells (sh-MM) (± s.d. from at least two independent experiments).

## 7- KAP1 over-expression decreases HIV-1 infectivity and integration

The enhancement of viral integration in conditions of low KAP1 expression suggests that this factor negatively regulates viral infectivity through integration. To further verify this hypothesis the reciprocal experiment was performed by infecting with NL4.3-Luc HIV-1 virus cells transfected with a plasmid expressing KAP1. Cells over-expressing KAP1 (**Figure 10a**) showed almost 50% less luciferase activity as compared to cells transfected with an empty plasmid (**Figure 10b**), demonstrating that KAP1 negatively affect viral infectivity. To investigate which step of viral replication is affected by high KAP1 expression, Q-PCR were performed. Accordingly to luciferase results, quantitative Alu-PCR analysis revealed that proviral DNA is significantly reduced by almost 50% in cells over-expressing KAP1 as compared to control cells (**Figure 10c**), while total viral DNA analyzed

with late reverse transcript primers remained significantly unaltered in these conditions (**Figure 10d**).

In conclusion, KAP1 over-expression inhibits HIV-1 infectivity by affecting specifically viral integration. This confirms the reciprocal effects obtained by KAP1 knockdown experiments.

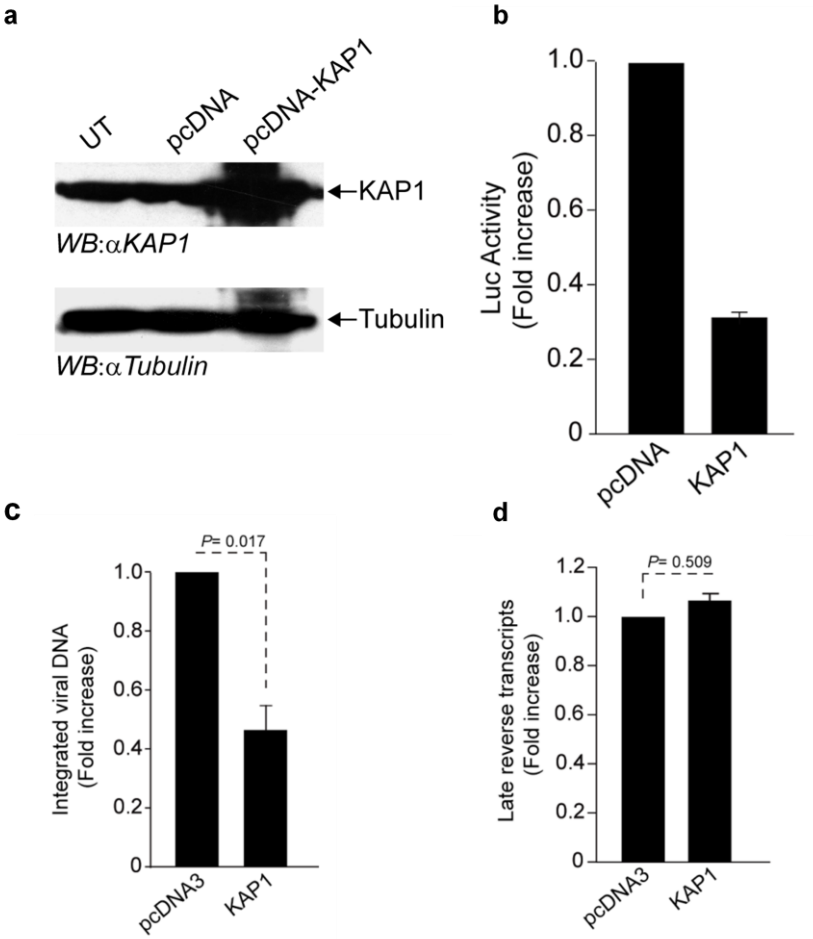


**Figure 10:** KAP1 over-expression decreases HIV-1 integration in HEK239T cells. (**a**) Expression of KAP1 in HEK293T cells transfected with KAP1 (pcDNA-KAP1), empty vector (pcDNA) and un-treated cells. Immunoblot was performed using αKAP1 antibodies and re-probed with αTubulin antibodies to check for protein loading. (**b-d**) HEK293T cells over-expressing KAP1 (pcDNA-KAP1) and control cells (pcDNA) were infected with NL4.3-Luc (VSV-G) HIV-1 virus and analyzed for luciferase activities (Luc Activity) 48 hpi (**b**), by Q-PCR for integrated HIV-1 DNA (Alu-PCR) at 48 hpi (**c**) and late reverse transcripts 24 hpi (**d**). All graphs are represented in fold increase with respect to control cells (± s.d. from at least two independent experiments).

## 8- KAP1 does not affect HIV-1 transcription

Since KAP1 is a transcriptional co-repressor (Sripathy et al., 2006), in order to assess its activity on viral events that do not depend on integrase activity, such as transcription, infectivity experiments were performed using a mutant HIV-1 viral clone containing an inactivating mutation in integrase gene (D64E) and a luciferase reporter gene under LTR

viral promoter. In fact, even though no proviruses originate from this viral clone, circular un-integrated viral cDNA forms could sustain gene expression from LTR (Engelman et al., 1995). HeLa cells stably knocked down for KAP1 (H14a, H18 and H19) (**Figure 8a**) as well as HEK239T stably silenced for the same gene (T-pool, T14 and 15) (**Figure 9a**) and their respective not silenced control cells (sh-MM) were infected with D64E HIV-1 virus pseudotyped by VSV-G envelope and infectivity was assessed two days post infection by measuring luciferase activities. The levels of luciferase activity remained unchanged in either HeLa or HEK293T KAP1 knockdown cells comparing to the not silenced cells (sh-MM) (**Figure 11a** and **b**), suggesting that KAP1 does not affect LTR transcription activity of HIV-1 at least in pre-integration steps. To further verify the independence of HIV-1 promoter from KAP1, pNL4.3-Luc was transfected in KAP1 Knockdown HeLa cells and luciferase produced exclusively upon viral promoter activation was evaluated. As shown in **Figure 11c** no differences in luciferase activity was observed in KAP1 silenced cells as compared to control cells, further proving the lack of activity of KAP1 on viral transcription even independently from virus infection.

Finally, in order to investigate whether KAP1 affects HIV-1 transcription following integration and chromatinization of HIV-1 DNA, cell lines containing a stably integrated virus (J-Lat A1 cells) were knocked down for KAP1 to control the viral gene expression. In fact, J-Lat A1 are jurkat T cells that carry a latent single copy of HIV-1 vector containing green fluorescent protein (GFP) as reporter gene under LTR control (Jordan et al., 2003). HIV-1 transcription could be activated in J-Lat A1 cells following treatment with phorbol esters (TPA) (Jordan et al., 2003). Transient KAP1 knockdown of latent or activated J-Lat A1 cells has been performed using a pool of lentiviral vectors (LKO.1) that express five different short hairpin RNAs targeting KAP1 gene (sh-5-KAP1). Not silenced control cells were untreated (-) or transduced with AIP lentiviral vector. Three days post-transduction with LKO.1-sh-5-KAP1 and AIP vectors, latent and activated J-Lat A1 cells were analyzed for KAP1 expression by Western blot analysis and for GFP expression detected by the Fluorescence-activating cell sorter (FACS) (**Figure 11d**). The percentage of GFP positive cells in non-activated J-Lat A1 cells knocked down for KAP1 (sh-5-KAP1) was as low as the value of not silenced cells (- or AIP) about 0.6% (**Figure 11d, graph, first three columns** from left), suggesting that KAP1 does not activate transcription of latent integrated HIV-1 virus. Moreover, in TPA activated not silenced J-Lat A1 cells (J-Lat A1+TPA), the percentage of GFP positive cells was increased by 60% compared to that of

not activated cells, however, even under these conditions KAP1 knockdown (sh-5-KAP1) did not vary the percentage of GFP expressing cells (**Figure 11d, graph, last three columns** from left), indicating that KAP1 does not affect the transcription of an active integrated HIV-1 virus. In conclusion KAP1 does not regulate HIV-1 transcription further sustaining its role specifically at the integration.

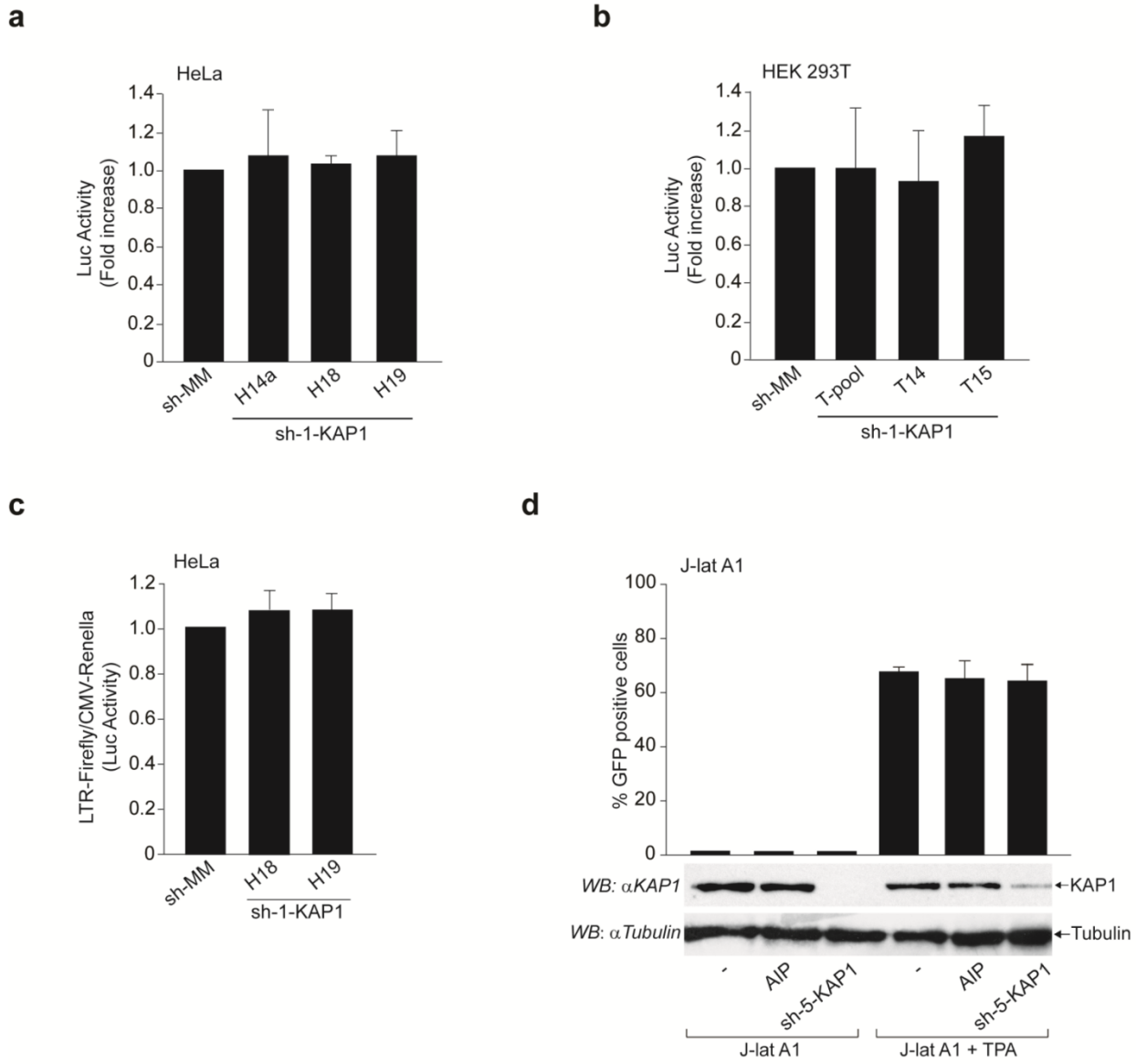


**Figure 11:** KAP1 does not regulate transcription activity of HIV-1 LTR promoter. (**a**) Stable KAP1 knockdown HeLa cell clones (H14a, H18 and H19) and control cells expressing a mismatched KAP1 shRNA (sh-MM) were infected with D64E HIV-1 virus. Luciferase activity was evaluated at 48 hpi (Luc Activity). (**b**) As in (**a**) using HEK293T cells (T-pool) and cell clones (T14 and T15) KAP1 stably knocked down and control cells expressing a mismatched KAP1-shRNA (shMM). In (**a**) and (**b**) graphs are represented in fold increase of infectivity of KAP1 knockdown cells with respect to control cells. (**c**) HeLa cells KAP1 stably knocked down (H14a, H18 and H19) and control cells (sh-MM) were transfected with pNL4.3.Luc.R-E- HIV-1 together with pCMV-*Renella* luciferase to normalize for transfection efficiency. Results are represented as a ratio of firefly luciferase (pNL4.3.Luc.R-E-) to *Renella* luciferase (pCMV-Renella). (**d**) Percentage of GFP positive J-lat A1 cells measured by FACS, non activated or TPA-activated following KAP1 knockdown (sh-5-KAP1). Expression of KAP1 in non activated or TPA-activated J-lat A1 cells following KAP1 knockdown (sh-5-KAP1) and in control cells (AIP) was verified by immunoblot using α-KAP1 antibodies. Loaded protein amounts were controlled by α-Tubilin antibodies. (± s.d. from at least two independent experiments).

## 9- Human KAP1 does not inhibit integration of murine leukemia virus (MLV)

In order to investigate whether human KAP1 could inhibit infection of other retroviruses, integration assays were performed with a gammaretrovirus, Moloney murine leukemia virus (MLV). HeLa cells were transiently transfected with a pool of si-RNAs against KAP1 gene (si-KAP1) and control cells were treated with a pool of non targeting si-RNAs (si-CTR). KAP1 expression was checked by Western blot analysis and showed a decrease in the levels of the protein following siRNA treatment (siKAP1) as compared to control cells (siCTR) (**Figure 12a**). At this time point cells were infected with either NL4.3-Luc HIV-1 or an MLV vector carrying luciferase reporter gene (MLV-Luc), both pseudotyped with VSV-G envelope. The luciferase activity, measured at 48 hours post infection, was increased about three fold in KAP1 knockdown HeLa cells (si-KAP1) infected with HIV-1 as compared to control cells (si-CTR) while the MLV infectivity was unchanged (**Figure 12b**). Total viral cDNA at 24 hours post infection and integrated viral DNA at 2 weeks post infection were measured by Q-PCR using primers that target luciferase gene of NL4.3-Luc and MLV-Luc. Consistently with the luciferase data, HIV-1 integrated DNA was increased in the KAP1 transiently knocked down HeLa cells (si-KAP1) of almost 4 fold as compared to not silenced cells (si-CTR), while MLV integrated DNA did not change following KAP1 knockdown, suggesting that human KAP1 does not inhibit MLV integration (**Figure 12c**). The total viral cDNA of HIV-1 and MLV were not varied in silenced cells as compared to their respective not silenced infected cells, indicating that KAP1 does not affect reverse transcription neither of HIV-1 nor of MLV (**Figure 12d**).

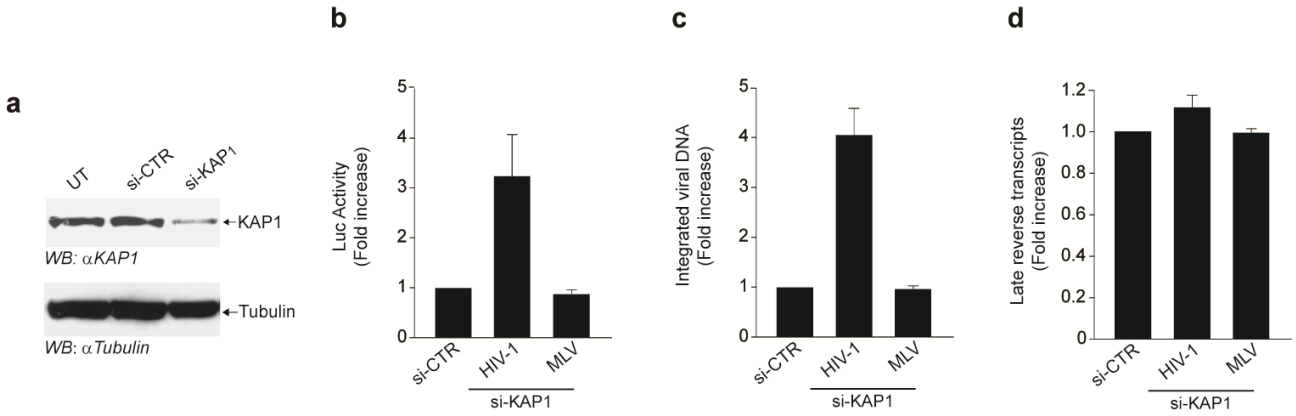


**Figure 12:** Human KAP1 does not affect MLV integration in HeLa cells. (**a**) Expression of KAP1 in HeLa cells knocked down with smart pool siRNAs (siKAP1) and control HeLa cells treated with a pool of non targeting siRNAs (siCTR). (**b**-**d**) HeLa KAP1 knockdown cells were infected with HIV-1 or with MLV viruses and analyzed for luciferase activity at 48 hpi (**b**) and analyzed by Q-PCR for integrated viral DNA at 15 dpi (**c**) and late reverse transcripts at 24 hpi (**d**). The si-CTR cells were infected separately with HIV-1 and with MLV, in the graph are represented in a unique column because both infections are normalized to one. (± s.d. from at least two independent experiments).

These data demonstrate that KAP1 specifically inhibits HIV-1 and not MLV at the integration level. The unchanged transcription activity of MLV in KAP1 Knockdown HeLa cells is perfectly in line with the previous report (Wolf and Goff, 2007) that showed that KAP1 does not affect MLV transcription in differentiated cells.

**10- KAP1 interaction decreases integrase acetylation**

Interaction assays reported in section n° 4 of RESULTS showed that KAP1 association with integrase is enhanced by acetylation of the viral protein. Therefore, in the attempt to delineate the molecular mechanism of KAP1 interference on viral infectivity, integrase acetylation has been analyzed relatively to KAP1 expression. To this aim integrase was expressed in HEK293T cells together with KAP1 and the acetylation levels were evaluated using antibodies against acetylated integrase in Western blot experiments. The over-expression of KAP1 significantly decreases the acetylation of integrase as compared to integrase expressed in the absence of exogenous KAP1 (**Figure 13a, upper panel**). The expression of KAP1 and integrase was controlled by blotting the same membrane with anti-KAP1 and anti-integrase antibodies respectively (**Figure 13a, middle** and **lower panels**). The level of integrase acetylation was then analyzed in HeLa cell clones stably knocked down for KAP1 (H14a, H18 and H19) (**Figure 8a**). Flag-integrase was expressed in silenced HeLa cell clones and control cells and then immunoprecipitated with $\alpha$-Flag antibodies and analyzed by Western blot with antibodies specific for acetylated integrase. The acetylation levels of Flag-integrase were significantly higher in KAP1 knockdown HeLa cells as compared to control cells expressing a mismatched KAP1 shRNA (sh-MM) (**Figure 13b, upper panel**). In this experiment total amounts of integrase were checked by blotting the same membrane with anti-Flag antibodies (**Figure 13b, lower panel**).
Therefore, these results demonstrate that KAP1 decreases the levels of integrase acteylation.

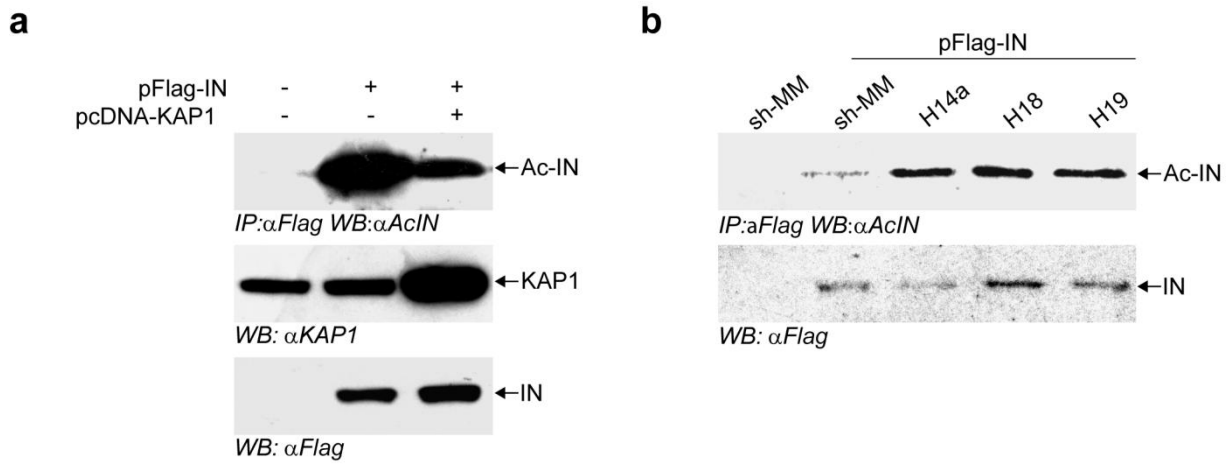


**Figure 13:** KAP1 decreases HIV-1 integrase acetylation. (**a**) Flag-integrase (Flag-IN) was expressed in HEK293T with or without exogenous KAP1 (pcDNA-KAP1). αFlag (IN) immunoprecipitates were blotted with anti-acetylated IN specific antibodies (αAcIN) to determine IN acetylation levels. Expression of KAP1 and total IN was determined by immunoblot using αKAP1 and αIN antibodies respectively. (**b**) KAP1 knockdown increases IN acetylation. Flag-IN was expressed in either HeLa cell clones stably knocked down for KAP1 by shRNA (H14a, H18 and H19) or in HeLa cells expressing mismatched KAP1-shRNA (sh-MM). αFlag (IN) immunoprecipitates were blotted with anti-acetylated IN antibodies (αAcIN) to detect acetylation levels; total IN was detected by re-probing with αFlag antibodies.

## 11- KAP1 mediates HDAC1 binding to integrase

KAP1 has been reported to tether HDACs to acetylated non-histones protein to induce their deacetylation such as p53 (Tian et al., 2009; Wang et al., 2005a), E2F1 (Wang et al., 2007a) and STAT3 (Tsuruma et al., 2008). Therefore, since we found that KAP1 down-regulates integrase acetylation we sought to investigate whether HDACs form a complex with integrase and KAP1. The initial investigations aimed to verify whether integrase acetylation is regulated by HDAC activity. To reach this aim, HEK293T cells expressing pCMV-IN were treated with an HDAC inhibitor, the trichostatin A (TSA). As shown in **Figure 14a,** the inhibition of cellular HDACs by TSA enhanced the acetylation level of integrase, indicating that this modification is regulated by the cellular deacetylases. To further verify this data, HEK293T lysates expressing Flag-IN were immunoprecipetated by anti-Flag antibodies and the deacetylase activity of these Flag-immunoprecipitates was detected using an *in vitro* fluorescence-based assay. **Figure 14b** shows that Flag-IN has a deacetylase activity indicating the association of HDACs with integrase *in vivo.* In addition, when the TSA was added to the *in vitro* deacetylase reaction, the Flag-IN deacetylase activity was inhibited further sustaining the specificity of the association of HDACs with integrase (**Figure 14b**). In order to explore the cellular HDACs associated with integrase *in vivo*, HEK293T cells were co-transfected with Flag-tagged HDAC1 or HDAC3 together with integrase HA-tagged. Similar amounts of HDAC1 and HDAC3 were

immunoprecipitated (**Figure 14c, third panel** from top) and high levels of integrase was found associated with HDAC1 and much less with HDAC3 (**Figure 14c, upper panel**). Interestingly, by blotting the same membrane with anti-KAP1 antibodies, endogenous KAP1 was detected in the HDAC1 complex but not with HDAC3 (**Figure 14c, third panel** from top). Lysates were finally controlled for total amounts of integrase expression (**Figure 14c, lower panel**). These data suggest that integrase forms a complex with HDAC1 and KAP1. To explore the hypothesis that the association of integrase with HDAC1 is mediated by KAP1, the binding of HDAC1 to integrase was checked in cells over-expressing KAP1. Higher levels of HA-integrase were associated with Flag-HDAC-1 in cells over-expressing KAP1 as compared to control cells (**Figure 14d**, **upper panel** and **second panel** from top respectively). The levels of HDAC1 and integrase expression were verified with anti-Flag and anti-HA antibodies respectively (**Figure 14d, third panel** from top and **lower panel**). Same co-immunoprecipitation experiments (**Figure 14d**) were performed using an untagged IN (pCMV-INw). As shown in **Figure 14e** (**upper panel**) Flag-HDAC1 was more associated with untagged integrase when KAP1 was over-expressed in HEK293T cells (**Figure 14e, second panel** from top). Same levels of Flag-HDAC1 and untagged integrase were detected with anti-Flag and anti-integrase antibodies, respectively, in cells over-expressing or not exogenous KAP1 (**Figure 14e, third panel** from top and **lower panel** respectively). These results strongly suggest that KAP1 plays a role in integrase/HDAC1 complex formation.

To further prove this hypothesis HeLa cells stably knocked down for KAP1 (H14a, H18 and H19) (**Figure 8a**) were transfected with both HA-integrase and Flag-HDAC1, and following HDAC1 immunoprecipitation the amounts of associated integrase were evaluated by Western blot. In conditions of low KAP1 expression the levels of integrase complexed with HDAC1 are strikingly lower than control cells expressing mismatched KAP1 shRNA (sh-MM) (**Figure 14f, upper panel**). The levels of integrase and HDAC1 expressed in control cells (sh-MM) and in HeLa cell silenced clones were similar (**Figure 14f, middle** and **lower panel** respectively). In conclusion these data clearly demonstrate that integrase binds HDAC1 and KAP1 mediates the complex formation.

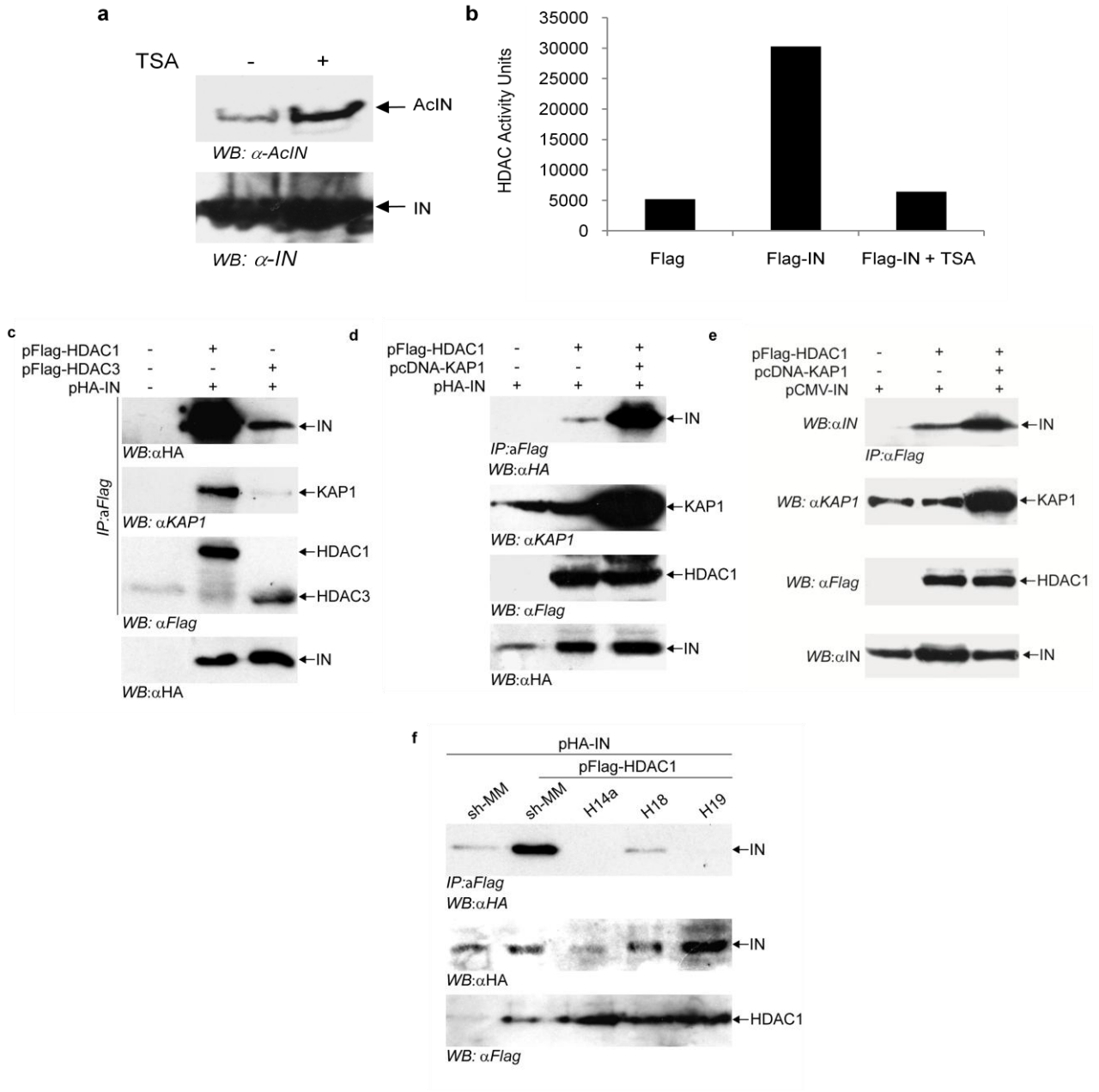


**Figure 14**: HDAC1 associates with integrase and KAP1 mediates IN/HDAC1 complex formation. (**a**) HEK293T cells expressing pCMV-IN were treated or not with TSA and the lysates were analyzed by western blot with α-AcIN and with α-IN. (**b**) Graphic representation of HDAC activity associated with Flag and Flag-IN immunoprecipitates treated or not with TSA determined using a fluorimetric-based assay. (**c**) HDAC1 is complexed with IN and KAP1. HA-IN was co-expressed with either Flag-HDAC1 or Flag-HDAC3 in HEK293T cells. αFlag immunoprecipitates were blotted with αHA (IN), αKAP1 and αFlag (HDAC1 and HDAC3) antibodies. Total IN was detected by αHA immunoblot. (**d**) KAP1 increases the association of HDAC1 with HA-IN. HA-IN and Flag-HDAC1 were coexpressed in HEK 293T cells with or without exogenous KAP1 (pcDNA-KAP1) and α-Flag (HDAC1) immunoprecipitates were blotted with α-HA (IN) antibodies. The expression levels of KAP1, HDAC1 and IN were controlled by immunoblot using the indicated antibodies. (**e**) KAP1 increases the association of HDAC1 with untagged**-**IN. IN and Flag-HDAC1 were co-expressed in HEK293T cells with or without exogenous KAP1 (pcDNA-KAP1). αFlag (HDAC1) immunoprecipitates were blotted with α-IN antibodies. The expression levels of KAP1, HDAC1 and IN were controlled by immunoblot using the indicated antibodies. (**f**) KAP1 knockdown decreases the HDAC1/IN complex formation. HA-IN and Flag-HDAC1 were co-expressed in KAP1 stably knocked down cell clones (H14a, H18 and H19) and in control mismatched shRNA HeLa cells (sh-MM). αFlag (HDAC1) immunoprecipitates were blotted with αHA (IN) antibodies. Expression levels of IN and HDAC1 were controlled by immunoblot using the indicated antibodies.

## 12- KAP1 inhibits HIV-1 integration by targeting acetylated lysines of integrase through HDAC1

Results here reported indicate that KAP1 lowers integrase acetylation through HDAC1 tethering. Since our previous report demonstrate that acetylation of integrase positively regulates viral integration (Cereseto et al., 2005; Terreni et al., 2010), we therefore hypothesized that in opposition HDAC1 mediated deacetylase activity might down-regulate HIV-1 integration. To explore this hypothesis, we initially investigated whether the deacetylase activity regulates HIV-1 integration. Therefore, HeLa cells were treated with the HDAC inhibitor trichostatin A (TSA), infected with NL4.3-Luc HIV-1 and analyzed by Q-PCR for the late reverse transcripts and for integrated viral cDNA (Alu-LTR) at 24 hours post infection. As shown in **Figure 15a**, the inhibition of cellular HDACs by TSA enhances HIV-1 integration about 3 fold as compared to control cells treated with DMSO, wihile the amounts of late reverse transcripts were unchanged (**Figure 15b**). This result demonstrates that the deacetylase activity inhibits viral integration. To verify the role of HDAC1 in this inhibition, HeLa cells were knocked down for HDAC1 and infected with HIV-1 in order to control integration in these cells. HeLa were treated transiently with a pool of siRNA directed against HDAC1 (si-HDAC1) and control not silenced cells were treated with a pool of non targeting siRNA (si-CTR) (**Figure 15c**). Two days post siRNA treatment cells were infected with NL4.3-Luc HIV-1 virus. The proviral DNA was evaluated by quantitative Alu-PCR at 48 hours post-infection showing that in conditions of low HDAC1 expression the integration was significantly increased of almost 4 fold (**Figure 15d**). Accordingly, the amount of un-integrated 2-LTR circles was almost 2 folds decreased (**Figure 15e**). To verify that HDAC1 specifically affects the integration reaction the total viral DNA was also quantified showing no major alteration of HIV-1 reverse transcription in cells silenced for HDAC1 (**Figure 15f**). Thus, similarly to KAP1 knockdown the silencing of HDAC1 increases HIV-1 integration.

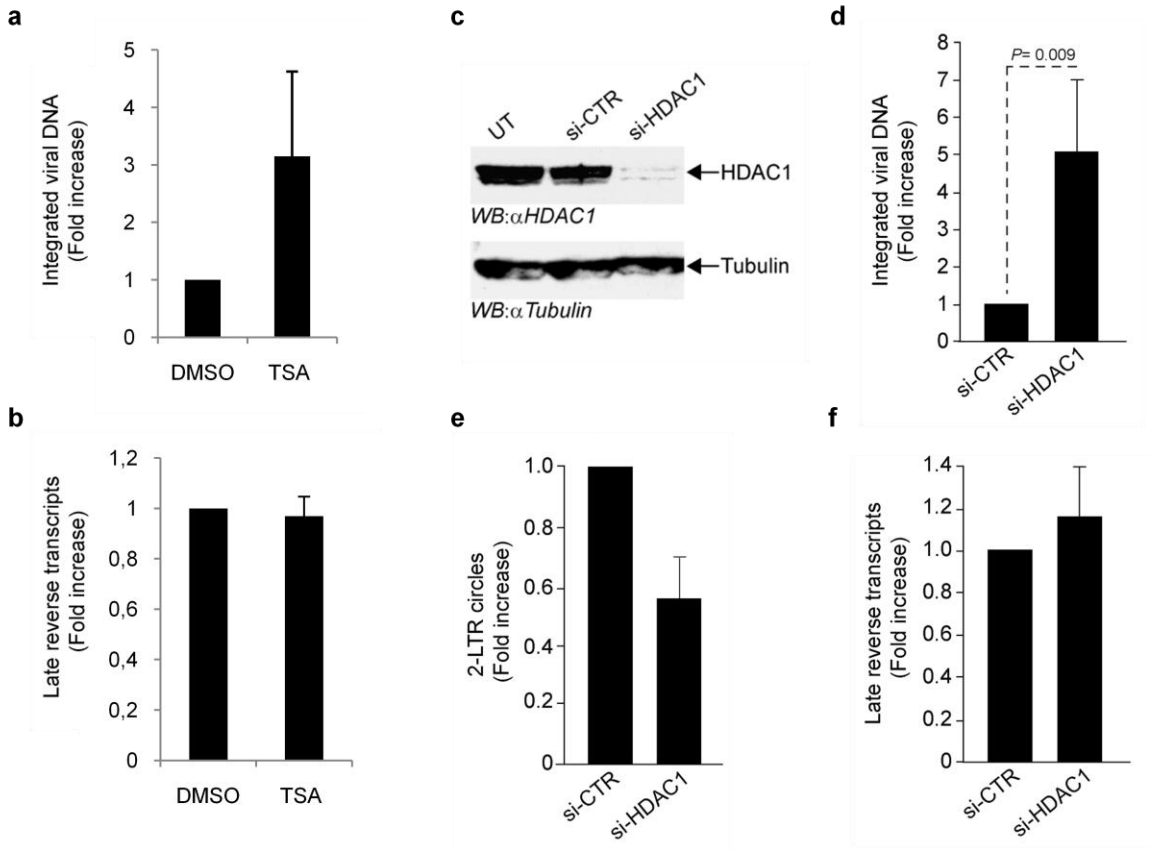


**Figure 15**: HDAC1 inhibits HIV-1 integration. (**a-b**) HeLa cells were treated with TSA or DMSO, infected with NL4.3-Luc (VSV-G) HIV-1 and analyzed by Q-PCR for integrated HIV-1 cDNA (Alu-LTR) at 24 hpi (**a**) and for late reverse transcripts at 24 hpi (**b**). (**c**) Expression of HDAC1 in HeLa cells transiently knocked down with smart pool siRNA (siHDAC1) and in control HeLa cells treated with a pool of non-targeting siRNA (si-CTR). Immunoblot was performed using αHDAC1 antibodies and re-probed with αTubulin antibodies to check for protein loading. (**d-f**) HeLa cells transiently knocked down for HDAC1 were infected with NL4.3-Luc (VSV-G) HIV-1 and analyzed by Q-PCR for integrated HIV-1 DNA (Alu-LTR) at 48 hpi (**e**) 2-LTR DNA circles at 24 hpi (**e**) and late reverse transcripts at 24 hpi (**f**). (± s.d. from at least two independent experiments).

To verify whether KAP1 inhibition of viral integration might occur through HDAC1 deacetylase activity tethered to integrase, infections were performed in cells where the expression of both proteins was simultaneously modulated. HEK293T cells over-expressing KAP1, by transfecting an ectopic cDNA (pcDNA-KAP1), were siRNA-silenced or not for HDAC1 gene (**Figure 16a**) and subsequently infected with NL4.3-Luc HIV-1 virus. Control cells were transfected with both an empty expression vector and a pool of non targeting siRNAs. To verify the level of KAP1 and HDAC1 expression Western blot analysis was performed in the same cells used for infection. As shown in **Figure 16a** the levels of KAP1 is increased in both cells transfected with KAP1 regardless the anti HDAC1 siRNA treatment, while HDAC1 expression is specifically reduced in cells treated with specific

siRNAs. Accordingly to the data shown in **Figure 10c**, integration of viral cDNA, determined by quantitative Alu-PCR at 48 hours post infection, was reduced of almost 50% in cells over-expressing KAP1 as compared to control cells (**Figure 16b, first** and **second columns** from left). However, over-expression of KAP1 does not show any inhibitory effect in cells that were simultaneously silenced for HDAC1 (**Figure 16b, third column** from left). Under these experimental conditions the total viral cDNA did not varied (**Figure 16c**). Therefore, these data clearly demonstrate that KAP1 integration inhibitory effect occurs through HDAC1 activity.

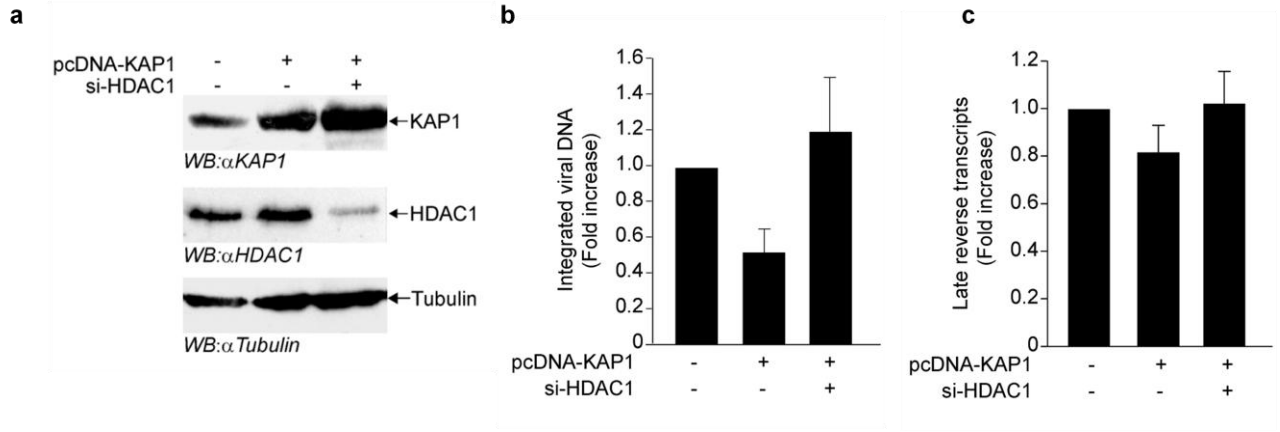


**Figure 16:** KAP1 inhibits HIV-1 integration through HDAC1. (**a**) Expression of KAP1 and HDAC1 in HEK293T cells transfected with pcDNA-KAP1 and transiently knocked down with si-HDAC1. Immunoblots were performed using the indicated antibodies. (**b-c**) KAP1 inhibition of HIV-1 integration is reversed by HDAC1 silencing. HEK 293T cells over-expressing KAP1 (pcDNA-KAP1) were co-treated or not with si-HDAC1, subsequently infected with NL4.3Luc (VSV-G) HIV-1 and analyzed by Q-PCR for integrated HIV-1 DNA (Alu-PCR) at 48 hpi (**b**) and for total HIV-1 DNA at 24 hpi (**c**). (± s.d. from at least two independent experiments).

In order to investigate the relevance of integrase lysine acetylation sites (K264, K266 and K273) in KAP1 inhibition during HIV-1 infection, HeLa cells stably knocked down for KAP1 (H14a and H18) and not silenced control cells (sh-MM) (**Figure 8a**) were infected with single-round HIV1 viruses either wild type NL4.3-Luc or mutated in integrase at three acetylation sites to arginine (NL4.3-Luc-3mut). As previously shown (Terreni et al., 2010), this virus was almost 5-fold less infectious than the parental NL4.3-Luc as measured by luciferase activity 48 hours post infection (**Figure 17a, first** and **third column** from left). However, while the wild-type virus showed increased infectivity in KAP1 knockdown HeLa cells of almost 2 to 3 fold, the mutant virus was unaffected (**Figure 17a**). Similarly, integrated viral DNA of wild type virus at 15 days post infection were significantly increased about 2 to 2,5 fold in HeLa knockdown cells (sh-KAP1), while, integrated NL4.3-

Luc3mut DNA were not significantly affected, comparing to not silenced control cells (sh-MM) (**Figure 17b**). Therefore, KAP1 inhibition of HIV-1 integration is dependent on the presence of integrase lysines targeted for acetylation by p300.

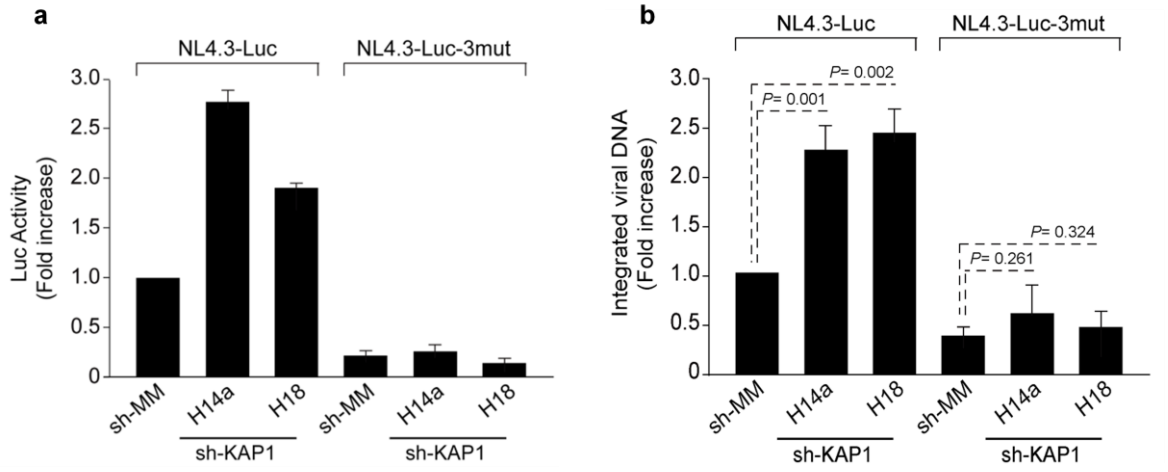


**Figure 17:** KAP1 does not affect integration of NL4.3-Luc-3mut carrying IN mutated at acetylable lysines (K264, 266, 273R). KAP1 knockdown (H14a and H18) and control (shMM) HeLa cell clones were infected with NL4.3-Luc or NL4.3-Luc-3mut and analyzed for luciferase activity at 48 hpi (**a**) and integrated HIV-1 DNA by qPCR (Alu-PCR) 48 hpi (**b**). All graphs are represented in fold increase with respect to control cells (± s.d. from at least two independent experiments).

## 13- Validation of KAP1 inhibition of HIV-1 integration in natural HIV-1 target T cells: CEMss and primary blood lymphocytes (PBLs)

In order to confirm KAP1 inhibition of HIV-1 integration in natural HIV-1 target cells, CEMss cell lines and primary blood lymphocytes (PBLs) from four healthy donors were knocked down for KAP1 and infected with either wild type NL4.3-Luc HIV-1 virus or with NL4.3-Luc-3mut mutated virus at the integrase lysine acetylation sites to arginine (K264, K266 and K273R). CEMss cells were silenced by a pool of five short hairpin RNAs (sh-5-KAP1) expressed from lentiviral vector (LKO.1) that target KAP1 gene and control cells were transduced with GIPZ lentiviral vector (**Figure 18a**). Three days post transduction cells were infected with NL4.3-Luc or NL4.3-Luc3mut viruses and analyzed by quantitative real time PCR (Q-PCR) for total viral cDNA at 24 hours post infection and for integrated viral DNA at two weeks post infection. Consistently with results observed with HeLa and HEK293T cells in sections n° 5 and 6 of RESULTS, knockdown of KAP1 in CEMss cells does not affect reverse transcription (**Figure 18c**) but increases significantly viral integration of

almost 3 fold of NL4.3-Luc HIV-1 virus while affects marginally integration of mutated virus at the three lysines targeted for acetylation by p300 (NL4.3-Luc3mut) (**Figure 18b**).

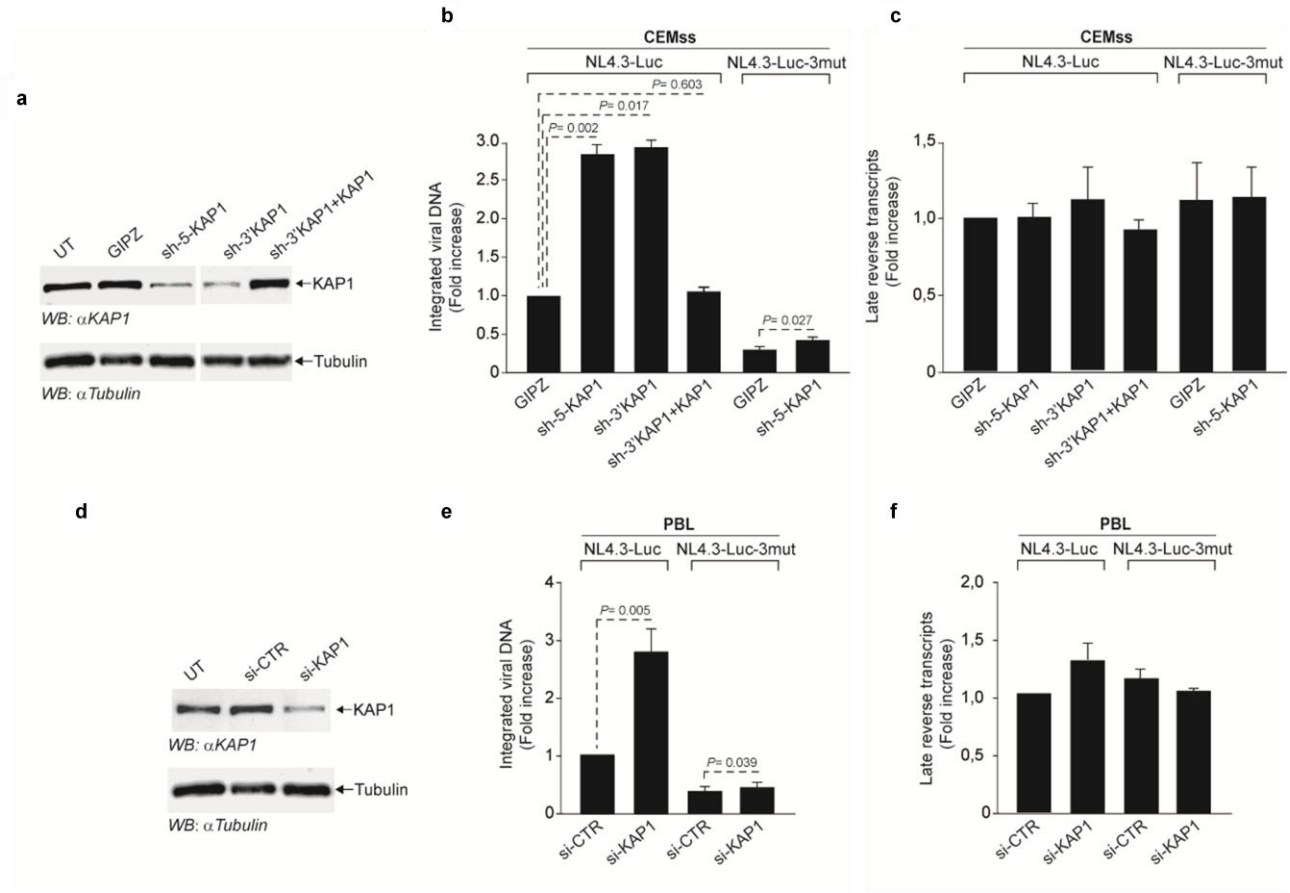


**Figure 18:** KAP1 inhibition of HIV-1 integration occurs in natural HIV-1 target cells: CEMss T cell lines and primary blood lymphocytes (PBLs). (**a**) Left panel: expression of KAP1 in a T-cell line (CEMss) transiently knocked down using a cocktail of lentiviral vectors (LKO.1) expressing five different shRNA sequences targeting the KAP1 gene (sh-5-KAP1). Right panel: expression of KAP1 in CEMss Tcells transiently knocked down for KAP1 using a shRNA that target 3'UTR of KAP1 gene (sh-3'KAP1) or back-complemented by expression of KAP1 cDNA resistant to sh-3'KAP1 from a lentiviral vector (AIP). Control not silenced cells were transduced with GIPZ lentiviral vector. (**b-c**) KAP1 knockdown (sh-5-KAP1), (sh-3'KAP1), back-complemented (sh-3'KAP1+ KAP1) and control (GIPZ) CEMss cells were infected with NL4.3-Luc (VSV-G) HIV-1 virus and analyzed by Q-PCR for integrated HIV-1 DNA 15 dpi (**b**) and for late reverse transcripts at 24 hpi (**c**). KAP1 knockdown (sh-5-KAP1) and control (GIPZ) CEMss cells were simultaneously infected with NL4.3-Luc (VSV-G) HIV-1 virus and analyzed for the integrated provirus (**b**) and for the total viral cDNA(**c**). (**d**) Expression of KAP1 in stimulated primary T-cells (PBLs) transiently knocked down with smart pool siRNA (si-KAP1 and si-CTR) by Amaxa transfection. (**e**-**f**) KAP1 knockdown (si-KAP1) and control (si-CTR) T-cells were infected with NL4.3-Luc or NL4.3-Luc-3mut (VSV-G) HIV-1 viruses and analyzed by Q-PCR for integrated HIV-1 DNA (Alu-PCR) 48 hpi (**e**) and for late reverse transcripts at 24 hpi (**f**). All graphs are represented in fold increase with respect to control cells (± s.d. from at least three independent experiments).

These results were further confirmed in stimulated PBLs from four healthy donors that were transiently silenced for KAP1 with a pool of siRNAs (siKAP1) (**Figure 18d**), infected with either NL4.3-Luc or NL4.3-Luc3mut viruses and analyzed by Q-PCR for late reverse transcripts at 24 hours post-infection and for integrated viral DNA (Alu-PCR) at 48 hours

post infection. The integration of wild type virus in silenced PBLs was significantly enhanced of almost 3 fold as compared to PBLs treated with control siRNAs (si-CTR), while, the integration of triple mutant virus was not affected (**Figure 18e**). Accordingly to cell lines KAP1 knockdown does not affect reverse transcription in stimulated PBLs (**Figure 18f**). Furthermore, as shown before with HeLa cells in section n° of 5 RESULTS, the expression of an exogenous KAP1 (AIP-HA-KAP1) resistant to sh3'KAP1 reversed the increase of integration in knockdown cells and showed no significant difference with basal levels of integration of not silenced cells (GIPZ) (**Figure 18b**), without affecting total viral cDNA (**Figure 18c**).

These results indicate that the inhibitory effect of KAP1 on HIV-1 integration occurs in the natural target cells of the virus and depends on the acetylable lysines of integrase.

## 14- Determination of the region of KAP1 required for the inhibition of HIV-1 integration

In order to determine which region of KAP1 is required for HIV-1 inhibition, different fragments of the cellular protein (schematized in **Figure 19a**) were tested in viral integration. HEK293T cells over-expressing the three KAP1 fragments fused to an HA tag, 1-381, 1-616 and 617-835 (**Figure 19b, left panel**) were tested for viral infectivity. As shown in **Figure 19b, right panel**, integration of HIV-1 was inibited at the same extent as full length by the 1-381 and 1-616 fragments, while the 617-835 fragment showed no decreased integration. Rather, cells expressing the 617-835 fragment showed increased integration efficiency as compared to baseline. To further verify the role of each KAP1 fragments in HIV-1 infectivity reciprocal experiments were performed in KAP1 knocked down CEMss cells expressing each single domain resistant to KAP1 shRNA (**Figure 19c**, **left panel**). Expression of 1-381, 1-616 restored viral integration to baseline at the same extent as full length KAP1, while the 617-835 fragment could not revert KAP1 knocked down integration phenotype (**Figure 19c**, **right panel**).

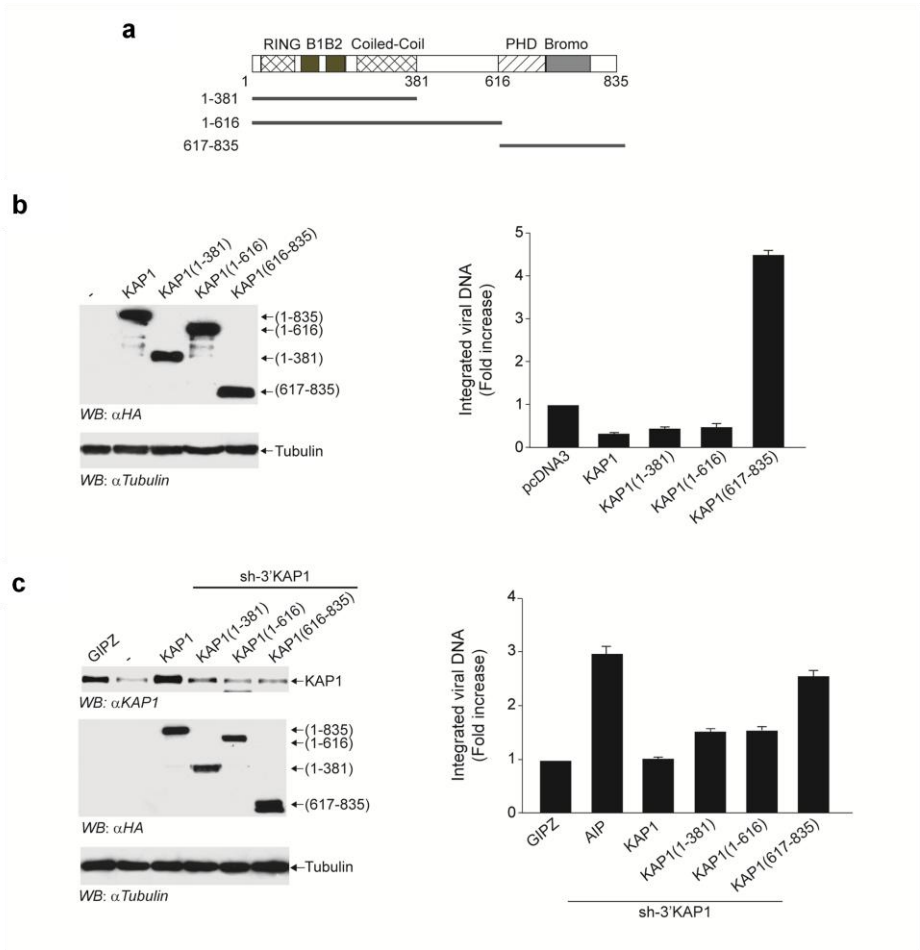


**Figure 19.** KAP1 domains required for HIV-1 inhibition. (**a**) Schematic representation of HA-tagged KAP1 FL (1-835) and deletion mutants (1-381, 1-616 and 617-835). (**b**) Integrated NL4.3-Luc HIV-1 DNA (Alu-LTR) 48 hpi of HEK293T cells expressing KAP1 full length (1-835) or KAP1 deletion mutants (1-381, 1-616 and 617-835). Protein expression was verified by immunoblot. (**c**) Integrated NL4.3-Luc HIV-1 DNA 25 dpi in CEMss cells knocked down for KAP1 (sh3'KAP1) and back-complemented with either KAP1 full length (1-835) or KAP1 deletion mutants (1-381, 1-616 and 617-835). Protein expression was verified by immunoblot. (± s.d. from at least two independent experiments).

Since our results indicate that KAP1 activity on HIV-1 integration occurs through HDAC1, we then evaluated the binding of each KAP1 fragment with HDAC1. HEK293T cells were co-transfected with Flag-HDAC1 together with each fragment analyzed for HIV-1 integration. As shown in **Figure 20a**, **left panel** HDAC1 co-precipitated with fragment 1-616 at the same extent as full length KAP1. Due to different migration conditions, a separate gel reported in the **right panel** of **Figure 20a**, shows that fragment 1-381 is positive for HDAC1 binding, while the 617-835 fragment is negative. Therefore, the associations of HDAC1 with the 1-381 and 1-616 correlate with the capacity of these fragments to inhibit viral integration and to reverse the KAP1 knockdown phenotype. On the contrary, fragment 617-835 unable to bind HDAC1 does not carry the inhibitory

activity of KAP1 both in over-expression and in knockdown experiments. In order, to further correlate the capacity of KAP1 fragments in the inhibition of HIV-1 integration, we also investigated the binding between KAP1 domains and HIV-1 integrase. For this aim, we performed *in vivo* co-immunoprecipitation experiments in HEK293T cells by using the KAP1 fragments (1-381, 1-616, 617-835) fused to HA and co-expressed with Flag-IN. As shown in **Figure 20b**, the fragment which corresponds to the RBCC domain (1-381) positively binds integrase. Conversely no binding was observed with the 617-835 fragment corresponding to the PHD-Bromo domain. To further investigate the specificity of 1-381 fragment in the interaction with the acetylated integrase, we performed co-immunoprecipitation experiments with integrase wild-type (Flag-INw) or mutated in the three acetylable lysines (K264R, K266R and K273R) (Flag-INm). These experiments showed that the 1-381 fragment binds preferentially the acetylated integrase (**Figure 20c**). Therefore, these results further sustain the role of the RBCC N-terminal region in the association between KAP1 and acetylated integrase. Moreover, these interaction data are consistent with the infectivity results (**Figure 19**) showing that the KAP1 domain responsible for inhibiting viral integration spans the 1-381 region (RBCC) while the 617-835 region (PHD-Bromo) which does not bind integrase had no effect on viral integration.
Collectively, these data clearly show that KAP1 mediated recruitment of HDAC1 to integrase and the association of KAP1 with the acetylated form of integrase are both necessary for the inhibition of viral integration. These results further sustain the conclusion that KAP1 inhibition occurs through deacetylation of integrase by HDAC1.

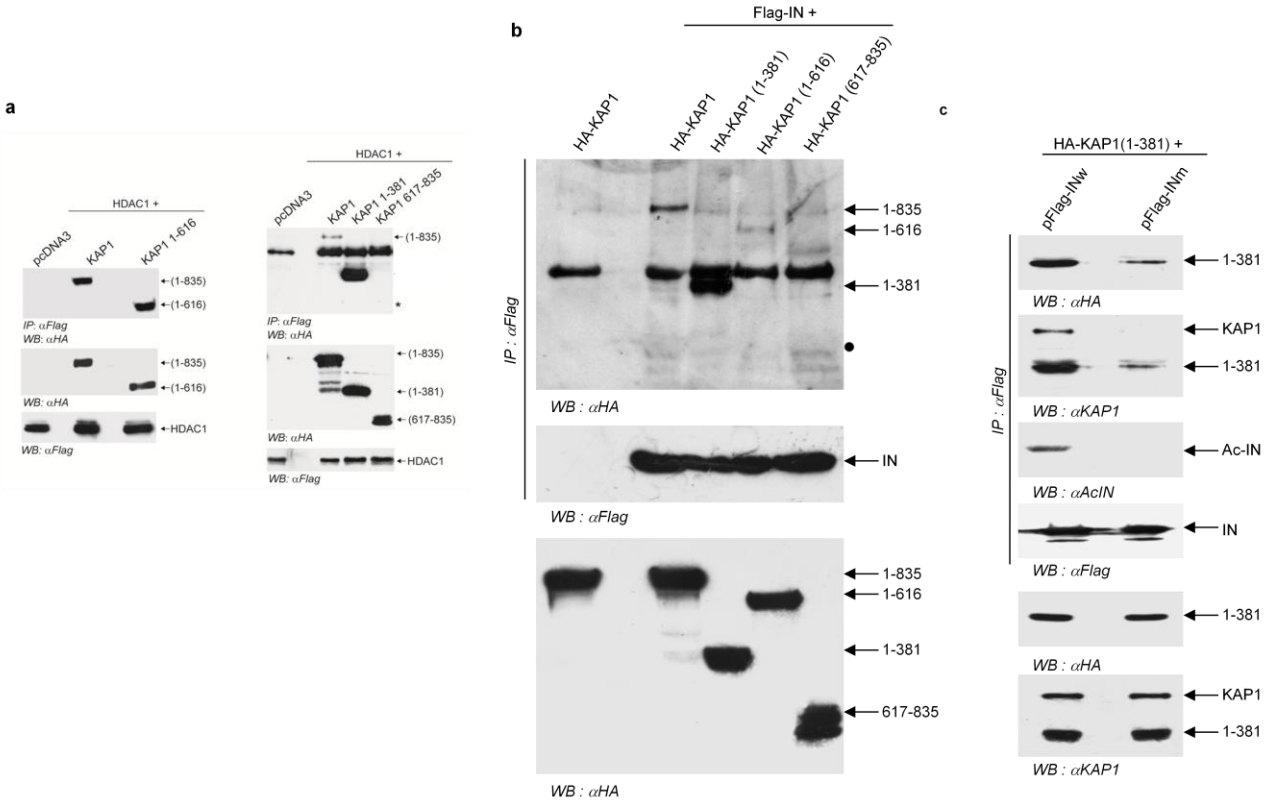


**Figure 20**. The minimal domain of KAP1 required for the inhibition of HIV-1 integration (1-381) binds HDAC1 and preferentially the acetylated form of integrase. (**a**) Lysates from HEK293T cells co-expressing Flag-HDAC1 with HA-KAP1 full length (1-835) or HA-KAP1 (1-616) (left panel) and lysates from HEK293T cell co-expressing Flag-HDAC1 with HA-KAP1 full length (1-835), HA-KAP1 (1-381) or HA-KAP1 (617-835) (right panel) were immunoprecipitated with α–Flag antibodies and blotted with α-HA antibodies. Expression levels of KAP1 full length or deletion mutants and HDAC1 were verified using the indicated antibodies. (**b**) Lysates from HEK293T cells co-expressing Flag-IN with HA-KAP1 full length (1-835), HA-KAP1 (1-381), HA-KAP1 (1-616) or HA-KAP1 (617-835) were immunoprecipitated with α–Flag antibodies and blotted with α-HA antibodies. Expression levels of KAP1 full length or deletion mutants and IN were verified using the indicated antibodies. (**c**) Lysates from HEK293T cells co-expressing Flag-INw or Flag-INm with HA-KAP1 (1-381) were immunoprecipitated with α–Flag antibodies and blotted with α-HA or α-KAP1 antibodies. Expression levels of KAP1 (1-381) and IN were verified using the indicated antibodies. The acetylation levels of IN were detected using anti-acetylated IN antibodies (α-AcIN).

## 15- Expressions of KAP1 and HDAC1 in cells relevant in HIV-1 pathogenesis

In order to investigate whether expression levels of KAP1 and HDAC1 vary in lymphocytes that play an important role in HIV-1 pre- or post-integration latencies, expressions of both proteins were checked in resting and activated primary blood lymphocytes (PBLs) and in CD45 RA+ naïve and CD45 RO+ memory CD4+ T cells, purified from healthy donors. Activated T lymphocytes are highly permissive to HIV-1 infection, whereas in resting T cells, despite the efficient entry of HIV-1 no viral progeny is produced. In resting lymphocytes there are several barriers that preclude the completion of the early steps as an incomplete reverse transcription or an inefficient nuclear import and integration (Bukrinsky et al., 1992; Zack et al., 1990; Zack et al., 1992). In addition, due to the lack

of c-jun kinase (JNK) activity in resting CD4 T cells, HIV-1 integrase does not become phosphorylated and then stabilized by Pin1 which unable the virus to achieve an efficient nuclear import and integration (Manganaro et al., 2010). Memory CD4 lymphocytes have been demonstrated to be involved in the establishment of a latent reservoir of infected cells harbouring a silent integrated provirus (Chun et al., 1995; Han et al., 2007). The mechanisms of HIV-1 post-integration latency are poorly understood, however, many studies suggested a main role of a transcription inhibition (Ganesh et al., 2003; Ghose et al., 2001; Kao et al., 1987). As shown in **Figure 21**, the amounts of KAP1 and HDAC1 were correlated to protein expressions of resting and activated PBLs, indicating no variation in their expression levels. Equal levels of KAP1 and HDAC1 were also detected in CD45RO+ memory and CD45RA+ naïve T cells (**Figure 21**). Therefore, these results indicate no variation in the expression levels of KAP1 and HDAC1 in HIV-1 permissive lymphocytes and in the main cells implicated in pre- and post-integration latencies.

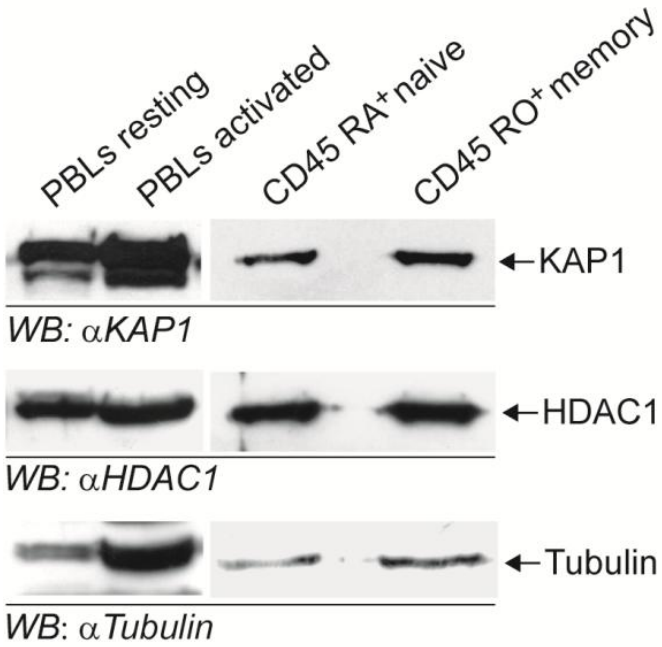


**Figure 21:** KAP1 and HDAC1 expressions in Resting or Activated PBLs and in Naive (CD45RA+) or Memory (CD45RO+) CD4+ T cells. Equal number of Resting or Activated PBLs (2 x $10^6$) was analyzed by western blot for KAP1 and HDAC1 expressions using the indicated antibodies (αKAP1 and αHDAC1). Protein loading was controlled by α-Tubilin antibodies. Equal amounts of proteins (60 μg) from CD45RA+ Naive or CD45RO+ Memory CD4+ T cells were analyzed by Western blot for KAP1 and HDAC1 expressions using the indicated antibodies (αKAP1 and αHDAC1).

**16- Could KAP1 phosphorylation during HIV-1 infection be a way for the virus to escape KAP1 inhibition?**

Recently several studies reported that in response to double strand breaks (DSB) DNA damage, KAP1 becomes phophorylated by ATM kinase at serine 824 (S824) of its C-terminal domain (Goodarzi et al., 2008; Goodarzi et al., 2009; White et al., 2006; Ziv et al., 2006). That modification has been demonstrated to displace KAP1 from DNA damage sites allowing chromatin relaxation and recruitment of DNA repair machinery to these sites (Ziv et al., 2006). Moreover, it has been shown that KAP1 phosphorylation serves to DSB repair in hetrochromatin region through the removal of KAP1 silencing effectors mainly HDAC1 and SETDB1 H3K9 methylase (Goodarzi et al., 2008; Goodarzi et al., 2009; Ziv et al., 2006). Interestingly, a recent study showed that in the absence of DNA damage KAP1-HDAC1 complex is recruited to p53 through APAK, a KRAB zinc finger protein, to maintain p53 deacetylated and inactivated, however, in response to DNA damage ATM phosphorylates KAP1 causing the dissociation of KAP1 complex from p53 and therefore allowing the re-acetylation and the activation of p53 (Tian et al., 2009). Viral cDNA ends of un-integrated HIV-1 virus has been shown to be sensed by cells as DSBs thus triggering DNA response signaling mainly by the activation of ATM kinase (Jeanson et al., 2002; Kilzer et al., 2003; Lau et al., 2005; Li et al., 2001). Based on these observations, we asked whether HIV-1 may induce KAP1 phosphorylation which in turn would remove KAP1 from the integrase complex. Detachment of KAP1 from integrase would then result in re-acetylation of integrase by p300 and enhanced integration efficiency. Based on this model (**Figure 22**), induction of KAP1 phosphorylation may represent a way for HIV-1 to escape KAP1 inhibition. In order to explore this hypothesis, we initially investigated whether KAP1 is phophorylated following HIV-1 infection. Therefore, HEK293T cells were infected with NL4.3-Luc HIV-1 virus and harvested at 2 and 4 hours post infection and then analyzed by Western blot using specific anti-phospho S824 of KAP1 ($\gamma$KAP1) antibodies. Western blot positive control for $\gamma$KAP1 antibodies was performed by treating HEK239T cells with neocarzinostatin (NCS) which is a radiometric drug that causes KAP1 phosphorylation by ATM kinase. As shown in **Figure 23a** (**upper panel**), no KAP1 phosphorylation signal has been detected (0 hour) in non infected cells, while, at 2 hours post infection KAP1 became phosphorylated and this signal was down-regulated at 4 hours post-infection. The levels of endogenous KAP1 were not modulated following HIV-1 infection and phosphorylation of the protein (**Figure 23a**, **second panel** from top). Viral integrase and its acetylated form

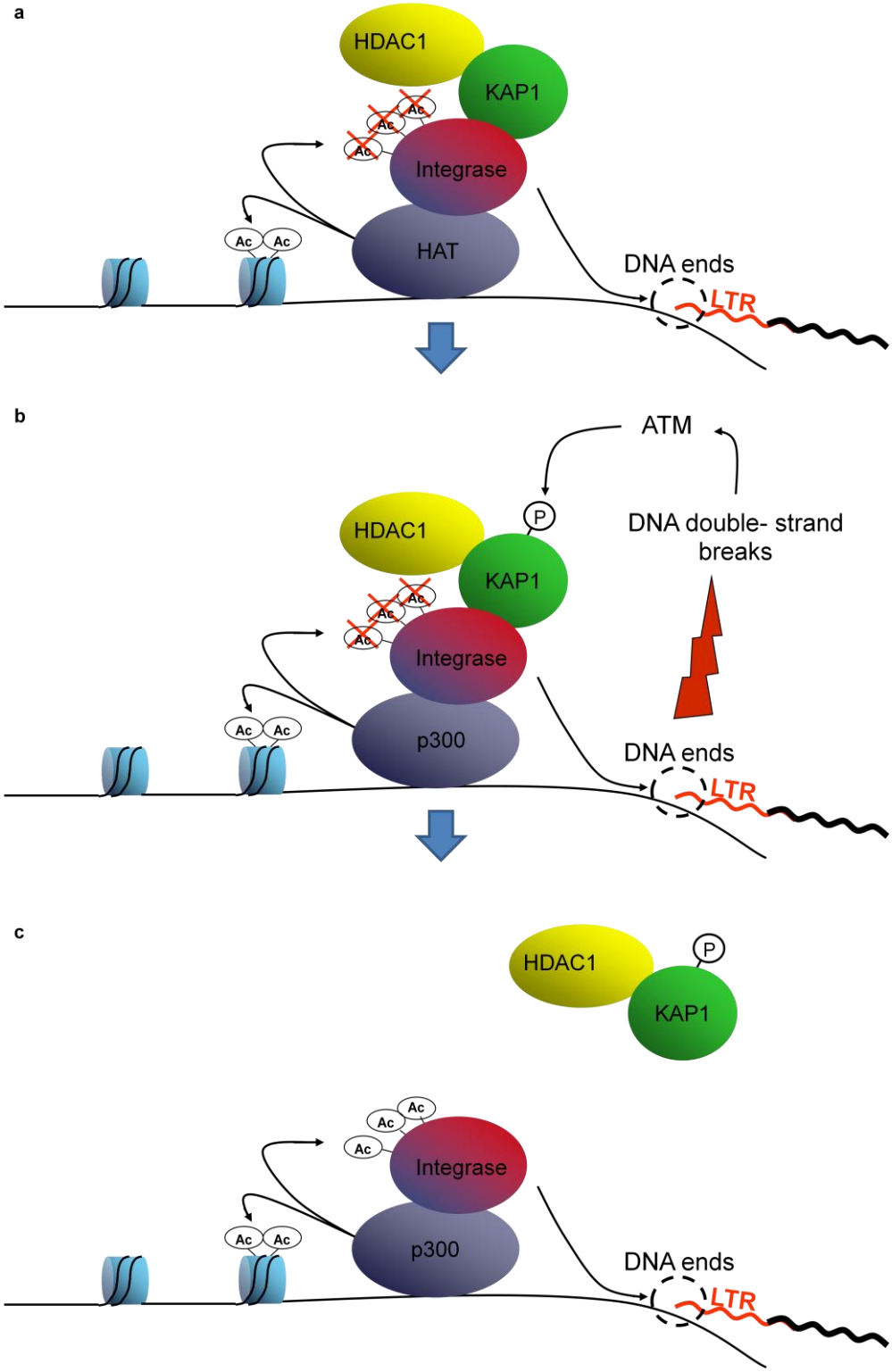


**Figure 22**. An hypothesized model for HIV1 escape from KAP1 inhibition. The results reported above demonstrate that KAP1 binds acetylated integrase, decreases integrase acetylation, mediates the binding of integrase with HDAC1 and inhibits HIV-1 integration through HDAC1 and by acting on the acetylable lysines of integrase (**a**). To escape this KAP1 inhibition, we hypothesized that HIV-1 through its DNA ends triggers double strand break response which activates ATM kinase that would phosphorylate KAP1 to lead to the dissociation of KAP1-HDAC1 complex from integrase (**b**). This dissociation would result in the re-acetylation of integrase by p300 and enhanced integration efficiency (**c**).

were barely detected in the infected HEK239T cells (**Figure 23a**, **third panel** from top and **lower panel** respectively). These data indicate that HIV-1 infection induces KAP1 phosphorylation. In order to further investigate whether KAP1 phophorylation could occur in T cells following HIV-1 infection and whether is mediated by ATM kinase, CEMss cells were infected by NL4.3-Luc HIV-1 virus and were harvested at 1, 2, 4 and 6 hours post infection. In addition, CEMss cells were treated with a specific ATM inhibitor (KU55933 or ATMi) and harvested 2 hours post infection. To control the specificity of ATM inhibition, KU55933 was added to cells treated by NCS.

Similarly to HEK293T cells, KAP1 phosphorylation was detected at 2 hours post-infection and no phosphorylation signal was revealed at 1, 4 or 6 hours post infection (**Figure 23b**). KAP1 phosphorylation was down-regulated in cells treated by both NCS and ATMi comparing to cells treated only by NCS, however, the signal of KAP1 phosphorylation at 2 hours post infection was not inhibited by ATMi (**Figure 23b**). This result suggests that KAP1 phosphorylation induced by HIV-1 is not mainly mediated by ATM kinase. In order to explore whether phosphorylation is somewhat related to KAP1 inhibition of HIV-1 integration, we investigated a possible differential binding of integrase to KAP1 phosphorylated and unmodified forms. HEK293T cells expressing Flag-IN were treated with the HDAC inhibitor Trichostatin A (TSA) in order to favor the *in vivo* acetylation of integrase and then were treated with NCS and KU55933. Following NCS treatment the phosphorylation levels of KAP1 were enhanced with respect to not treated cells or simultaneously treated with the ATM inhibitor (**Figure 23c**, **third panel** from top), while, the expression levels of KAP1 and Flag-IN were not varied under these conditions (**Figure 23c**, **fourth panel** from top and **lower panel** respectively). Flag-IN was then immunoprecipitated and analyzed by Western blot for either total or phosphorylated KAP1. As shown in **Figure 23c** (**upper panel**) the levels of total KAP1 associated with Flag-IN were not changed neither following induction of phosphorylation nor following its inhibition by ATM inhibitors, indicating that KAP1 phosphorylation does not modulate the KAP1/IN binding. However, same membrane re-probed with anti-phospho KAP1 antibodies showed that no phosphorylated KAP1 was retained by Flag-IN (**Figure 23c**, **second panel** from top). In order to better clarify the affinity of binding of phosphorylated KAP1 to integrase, we wanted to investigate the binding between integrase and KAP1 phosphomutant (S824A), that is unable to be phosphorylated, and KAP1 phosphomimic (S824D), that mimics a constitutive phosphorylated KAP1. For this aim, Flag-IN was expressed in

HEK293T cells that were silenced for KAP1 by the sh-3'KAP1 and back-complemented by the expression of either S824A or S824D phosphomutant cDNAs resistant to the sh-3'KAP1 in order to prevent the possible competition of binding to integrase by the endogenous KAP1. Flag-IN was then immunoprecipitated and analyzed by Western blot for KAP1. As shown in **Figure 23d**, **upper panel**, Flag-IN binds KAP1 phosphomutant (S824A) and KAP1 phosphomimic (S824D) with the same affinity. The expressions of Flag-IN (**Figure 23d**, **second panel** from top) and of S824A and S824D (**Figure 23d**, **lower panel**) were similar. These results further indicate that the KAP1 phosphorylation does not modulate the affinity of binding of KAP1 with integrase. Finally, we aimed to investigate the role of KAP1 phosphorylation in HIV-1 integration. To reach this aim, CEMss T cells were knocked down for KAP1 by the sh-3'KAP1 and back-complemented by the expression of the KAP1 wild type (KAP1), phosphomutant (S824A) and phosphomimic (S824D) resistant to the sh-3'KAP1 (**Figure 23e**). At this time point, cells were infected with NL4.3-Luc HIV-1 virus and analyzed by Q-PCR for total HIV-1 cDNA at 24 hours post-infection and for integrated proviruses at 15 days post-infection. **Figure 23f**, **left panel,** shows that KAP1 knockdown mediated by sh-3'KAP1 enhances HIV-1 integration of almost 2,5 fold and the KAP1 phosphomutant (S824A) was able to reverse HIV-1 integration to baseline as well as the wild type KAP1 (KAP1), while the KAP1 phosphomimic (S824D) does not have any effect on HIV-1 integration. The total viral cDNA did not vary under these conditions (**Figure 23f**, **right panel**). These results indicate that the phosphorylated form of KAP1 does not inhibit HIV-1 integration.

In conclusion, these data demonstrate that KAP1 is phosphorylated at two hours following HIV-1 infection and this phophorylation is not mediated mainly by ATM kinase. Although KAP1 phosphorylation does not modulate the binding of KAP1 with integrase, the KAP1 mutant (S824D) mimicking a constitutive phosphorylated KAP1 was not able to inhibit HIV-1 integration as the KAP1 wild type and phosphomutant (S824A).

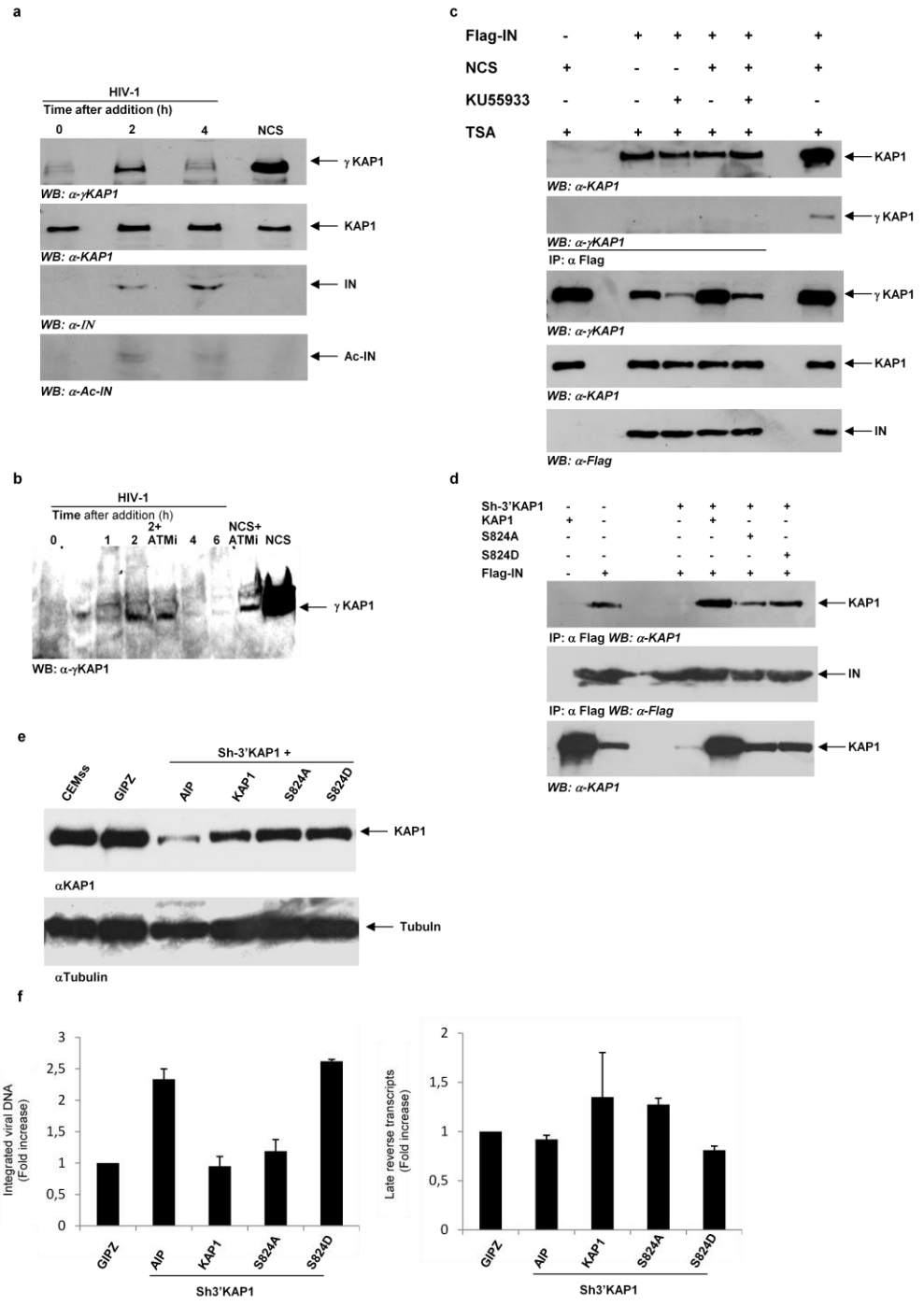


**Figure 23:** KAP1 phosphorylation in HIV-1 infection. (**a**) HEK293T cells were infected with NL4.3-Luc (VSV-G) HIV-1 virus and harvested at 2 and 4 hours post infection and analyzed by Western blot for phosphorylated KAP1 (γKAP1), total KAP1, integrase (IN) and acetylated IN (Ac-IN). (**b**) CEMss T cells were infected with NL4.3-Luc (VSV-G) HIV-1 virus and harvested at 1, 2, 4 and 6 hours post-infection; cells harvested at 2 hours were treated or not with ATMi (KU55933). (**c**) HEK239T cells expressing Flag-IN were treated or not with NCS and KU55933. α-Flag immunoprecipitates were analyzed with α-KAP1 and α-γKAP1 antibodies. The expressions of phosphorylated KAP1, total KAP1 and Flag-IN were checked by immunoblot. (**d**) HEK239T cells expressing Flag-IN silenced for KAP1 (sh-3'KAP1) were back-complemented with KAP1 wild type (KAP1), phosphomutant (S824A) and phosphomimic resistant to the sh-3'KAP1. α-Flag immunoprecipitates were analyzed with α-KAP1. The expressions of phosphorylated KAP1, total KAP1 and Flag-IN were analyzed by immunoblot. (**e**-**f**) CEMss T cells were knocked down for KAP1 (sh3'KAP1), back-complemented with KAP1 wild type (KAP1), phosphomutant (S824A) and phosphomimic resistant to the sh-3'KAP1 (**e**) were infected with NL4.3-Luc (VSV-G) HIV-1 virus and analyzed by Q-PCR for integrated HIV-1 cDNA at 15 dpi and for total HIV-1 DNA at 24 hpi (**e**). (± s.d. from at least two independent experiments).

## DISCUSSION

### 1- Potential roles in HIV-1 replication of the identified host factors binding acetylated integrase

This study exploits the tethered catalysis system (Guo et al., 2004) to produce an HIV-1 viral protein, integrase, constitutively acetylated by p300 (IN-HATw). Mass spectrometry analyses of the recombinant IN-HATw confirmed the *in vivo* acetylation of integrase at lysines K264, K266 and K273 by p300 HAT which are the same residues previously identified by site direct mutagenesis assays (Cereseto et al., 2005). The construct, verified to produce acetylated integrase in bacteria and in yeast, was used to screen by two-hybrid a human lymphocytes cDNA library. Most integrase interacting factors have been identified through the yeast two-hybrid approach (Al-Mawsawi and Neamati, 2007; Christ et al., 2008; Emiliani et al., 2005; Rain et al., 2009; Van Maele et al., 2006). However, conventional two-hybrid screening does not allow identifying interacting factors that may require post translational modifications, such as acetylation, in order to bind a protein. From this screening we have identified thirteen cellular factors, twelve of which have never been reported to interact with HIV-1 integrase. The binding analyses performed by two-hybrid and pull down assays revealed a basal association of all factors with the unmodified integrase while the acetylation of the viral factor variably affects the affinity with the two-hybrid hits. Interestingly, Exp2, eIF3h RanBP9 and KAP1 showed higher affinity to acetylated integrase than to unmodified form, while acetylation did not modulate the binding of BTF3b, THRAP3 and HMGN2 to integrase. These results confirmed the starting hypothesis of this study according to which integrase acetylation might affect its protein-protein interactions with cellular factors.

The newly identified factors interacting with acetylated or un-modified integrase showed no obvious simple sequence similarity. Nevertheless, it is plausible that integrase recognizes common elements present in these proteins. In fact, these factors can be grouped into three categories based on their functional properties: a) transcription regulatory and chromatin remodeling factors; b) translation regulatory and RNA binding proteins; c) nuclear import-export proteins.

Interestingly, LEDGF/p75, one of the factors identified in this screening, is one of the most described integrase interacting protein required for efficient HIV-1 integration (Cherepanov et al., 2003; Emiliani et al., 2005; Llano et al., 2006; Maertens et al., 2003;

Shun et al., 2007; Vandekerckhove et al., 2006). This result validates the system of analysis employed in this study and proves that fusion of IN to the HAT domain does not significantly alter the integrase structure. LEDGF/p75 binds the core catalytic domain of HIV-1 integrase. Nevertheless, the N-terminal domain of integrase was proven to enhance the binding affinity with this factor (Maertens et al., 2003). Therefore, the identification of LEDGF/p75 in our screening might suggest that also the C-terminus of integrase and its acetylation could also affect IN/LEDGF-p75 interaction. Recently, it has been shown that integrase C-terminus is protected within HIV-1 pre-integration complex by cellular factors and mainly by the presence of LEDGF/p75 further sustaining the role of this domain in LEDGF/IN complex formation (Benkhelifa-Ziyyat et al., 2010).

HIV-1 integration preferentially occurs in regions of the chromatin rich in transcriptionally active genes (Bushman et al., 2005; Mitchell et al., 2004; Schroder et al., 2002). Recent studies performed by sequence analysis using the ENCODE annotation (Wang et al., 2007b) and by a visualization analysis (Albanese et al., 2008) demonstrated that HIV-1 targets decondensed regions of the chromatin. It has been hypothesized that cellular factors interacting with integrase may tether the virus to appropriate sites for integration. Indeed, LEDGF/p75 knockdown and knockout cells show a significant reduction of integration frequency in transcription units (Bushman et al., 2005; Marshall et al., 2007; Shun et al., 2007). Nevertheless, since in the absence of LEDGF/p75 the virus still does not integrate randomly in the genome, additional factors may be required for integration specificity. The screening reported in this study, uncovered factors involved in transcription and chromatin structure regulation, thus good candidate proteins to tether HIV-1 integration. BTF3b and THRAP3, found in the two-hybrid screening, are positive regulators of gene transcription that act by associating with RNA polymerase II (Rachez and Freedman, 2001; Zheng et al., 1990). The HMNG2 protein, another two-hybrid hit, is involved in chromatin structure regulation by binding to nucleosomes in a DNA sequence independent manner (West, 2004). This factor induces chromatin decompaction, which in turn facilitates DNA transcription and replication (West, 2004). In fact, HMNG2 was found to localize in active transcription chromatin regions (Bustin, 2001; Hock et al., 1998). Finally, HMGA1, another HMG family member, was previously reported to stimulate HIV-1 integration by promoting the formation of IN/cDNA complexes (Hindmarsh et al., 1999) (Li et al., 2000).

HIV-1 nuclear import occurs through still incompletely understood mechanisms (Fassati, 2006; Yamashita and Emerman, 2006). One of the viral factors hypothesized to be involved in nuclear translocation is integrase which is a karyophilic protein that contains several putative nuclear localization signals (NLSs) (Ao et al., 2005; Armon-Omer et al., 2004; Bouyac-Bertoia et al., 2001; Depienne et al., 2000; Devroe et al., 2003; Gallay et al., 1997). In our study, two proteins that regulate the nuclear importin pathway Exp2 (CAS) and RanBP9 have been identified (Gorlich, 1998; Kutay et al., 1997; Solsbacher et al., 1998; Yudin and Fainzilber, 2009). These factors may trigger the nuclear import of integrase by importin $\alpha$ and β complex. However, the implications of importin factors and pathways have been explored for HIV-1 infectivity leading to contradictory results (Ao et al., 2007; Bouyac-Bertoia et al., 2001; Dvorin et al., 2002; Limon et al., 2002; Zielske and Stevenson, 2005). Indeed, it has been reported that integrase may lack functional NLSs (Depienne et al., 2001; Devroe et al., 2003) and the nuclear accumulation of integrase was attributed to LEDGF/p75 by tethering the viral protein to the chromatin and preventing its proteasomal degradation (Devroe et al., 2003; Emiliani et al., 2005; Llano et al., 2004b; Maertens et al., 2003). Although no role of LEDGF/p75 in pre-integration complex nuclear import has been found, a recent identified integrase interactor foctor, transportin SR2 (TNPO3) has been shown to mediate the transport of HIV-1 to the nucleus (Christ et al., 2008). RanBP9 is also a protein of microtubule organizing center (MTOC) that induces microtubule nucleation (Nakamura et al., 1998; Yudin and Fainzilber, 2009). Interestingly, visualization of HIV-1 particles in the cytoplasm showed that HIV-1 accumulates near to the MTOC and associates with dynein and the microtubules network to migrate toward the nucleus (McDonald et al., 2002). In our two hybrid screening, we identified another protein that regulates microtubule organization, STMN1, through binding tightly tubulin and inducing microtubule destabilization (Cassimeris, 2002; Howell et al., 1999). These observations are in line with previous reports showing that HIV-1 integrase binds microtubule-associated proteins such as the yeast STU2p, a centrosomal protein, and Dyn2p (dynein light chain protein) (de Soultrait et al., 2002; Desfarges et al., 2009). It has been hypothesized that integrase interaction with these factors may be responsible for its nuclear import (de Soultrait et al., 2002; Desfarges et al., 2009).

A recent two-hybrid screening performed with integrase of another retrovirus, the Moloney Murine Leukemia Virus (MoMLV), identified the murine eIFs2, a subunit part of the translation initiation factor 3 (eIF3) complex (Studamire and Goff, 2008). Moreover, in this

study it was demonstrated that HIV-1 integrase does not interact with eIFs2. The human eIF3f, another component of eIF3 complex, was reported to inhibit HIV-1 replication at post-integration step by interfering with the 3' end processing of HIV-1 mRNAs (Valente et al., 2009). From our screening we identified eIF3h, another subunit belonging to the eIF3 complex, as a factor interacting with acetylated HIV-1 integrase. Thus, since numerous studies report the association of retroviruses with the eIF3, this protein complex presumably plays an important function in the viral replication cycle, even though the detailed molecular mechanism has not yet been unraveled.

One of the two-hybrid hits was eEF1A-1 factor which has been previously reported to be involved in HIV-1 biology. Most interestingly, in agreement with our results, a former study using a yeast expression experimental system suggested that integrase interacts with eEF1A-1 (Parissi et al., 2001). Moreover, eEF1A-1 was identified to be involved in HIV-1 replication by binding with the viral gag polyproteins (matrix and nucleocapsid) (Cimarelli and Luban, 1999) and also by activating the viral promoter (Wu-Baer et al., 1996). All these reports imply the multi-roles of eEF1A-1 in HIV-1 replication cycle.

One identified factor from the screening is hnRNPA2 involved in mRNA splicing and trafficking regulation and it has also been described to regulate HIV-1 RNA trafficking (Beriault et al., 2004; Shyu and Wilkinson, 2000). In line with this observation, HIV-1 integrase has been shown to bind another RNA regulator protein Gemin2 that has been demonstrated to promote reverse transcription (Hamamoto et al., 2006).

## 2- KAP1 targets acetylated integrase and inhibits HIV-1 integration through HDAC1

One of the factors identified by the two hybrid screening, employed in this study, is KAP1. KAP1 is an ubiquitously expressed nuclear protein that belongs to TRIM family proteins recently implied in the innate immunity against viral infections (Kajaste-Rudnitski et al., 2010; Nisole et al., 2005; Ozato et al., 2008; Reymond et al., 2001). The higher association of KAP1 with acetylated integrase as compared to the unmodified form has been confirmed *in vivo* by co-immunoprecipitation experiments in HEK293T cells, and *in vitro* by pull down assays. Interestingly, KAP1 binds the C-terminal domain of integrase that contains the three lysines targeted for acetylation by p300 and involved in the *in vitro* binding with p300 (Cereseto et al., 2005). Investigation on the role of KAP1 during viral replication cycle showed that KAP1 down-regulation enhances viral infectivity due to

specific increase in viral integration. Indeed, transient and stable knockdowns of KAP1 in HeLa and HEK239T cells did not alter HIV-1 reverse transcription, however, decreased two-LTR circles consistently with an increase of the integrated proviruses. Similar effects on HIV-1 viral cDNA forms have been reported following knockdown of another cellular factor, $p21^{Cip1/Waf1}$, which inhibits specifically HIV-1 integration (Zhang et al., 2005; Zhang et al., 2007). The inhibition of HIV-1 integration was further confirmed by reciprocal experiments showing that KAP1 over-expression reduces provirus formation. While seeking to explore the mechanisms by which KAP1 inhibits HIV-1 integration, we found that this factor down-regulates integrase acetylation and mediates the association of HDAC1 to integrase. In conformity with these results, previous reports showed that KAP1 decreases acetylation of p53 (Tian et al., 2009; Wang et al., 2005a), E2F1 (Wang et al., 2007a) and STAT3 (Tsuruma et al., 2008) by tethering HDACs. The KAP1-mediated deacetylation of these cellular factors involved in DNA damage response induces their inactivation. Similarly to KAP1, the knockdown of HDAC1 enhanced HIV-1 integration. Interestingly, the inhibitory effect of the over-expressed KAP1 on HIV-1 integration was relieved when HDAC1 was knocked down. Consistently, KAP1 inhibition does not affect an HIV-1 viral clone carrying an integrase mutated at the three lysines targeted for acetylation by p300 (K264, K266 and K273) to arginines. The dependence of KAP1 inhibition on HDAC1 and on integrase acetylable lysines suggests that KAP1 affects integration efficiency by inducing integrase deacetylation through HDAC1. The specificity of KAP1 inhibition was further confirmed in HEK293T and HeLa cells knocked down for KAP1 and back-complemented by the exogenous expression of KAP1 cDNA resistant to the shRNA. These cells were able to restore HIV-1 infectivity and integration to the basal levels of not silenced cells. KAP1-mediated inhibition of integration was also confirmed in HIV-1 natural target cells, CEMss T cells and primary blood lymphocytes (PBLs). Similarly to the effect observed in HeLa and HEK293T cells lines, KAP1 knockdown in CEMss cells and PBLs enhances HIV-1 integration and its back-complementation by resistant cDNAs reverts this enhancement. Furthermore, the dependence of KAP1 inhibition on the presence of the three acetylable lysines (K264, K266 and K273) of integrase was also confirmed in T cells.

Investigations on the regions of KAP1 required for the inhibition of HIV-1 integration determined that the N-terminal region (1-381), that corresponds to the RBCC domain, acts on integration as the full length protein. Moreover, the RBCC domain was able to retain

HDAC1 as the full length KAP1 further sustaining the relevance of KAP1-HDAC1 interplay in HIV-1 inhibition. These investigations also showed that the C-terminal region of KAP1 (617-835), that corresponds to the PHD-BROMO domain, was not able to inhibit HIV-1 integration. Consistently, the PHD-BROMO domain fails to bind HDAC1. The RBCC domain of KAP1 has been mainly described for its role in transcription repression by specifically recognizing the KRAB domain of KRAB zing finger proteins that bind the target promoters to be silenced (Ayyanathan et al., 2003; Groner et al., 2010; Sripathy et al., 2006). Our study reveals a new role of the RBCC domain in the inhibition of HIV-1 integration through HDAC1. The PHD-BROMO domain has also an important role in transcription repression. Indeed, following KAP1 recruitment to the target promoters by the KRAB zing finger protein, the PHD and BROMO regions act cooperatively for the interaction with CHD3 (Mi2$\alpha$), which is a subunit of NuRD histone deacetylase complex (Schultz et al., 2001), and with SETDB1 histone H3-K9 methyl transferase (Schultz et al., 2002) in order to induce the heterochromatinization of the targeted DNA (Ivanov et al., 2007; Sripathy et al., 2006). Since, NurD complex contains also HDAC1 and HDAC2 (Grozinger and Schreiber, 2002), it has been presumed that the PHD-BROMO domain is responsible for the association of KAP1 with HDAC1 (Schultz et al., 2001). Our data show that the KAP1 mutant deleted in the PHD-BROMO (1-616) is able to retain HDAC1 while the PHD-BROMO domain (617-835) fails to bind this HDAC. Notably, even though it has been experimentally shown that the PHD-BROMO regions bind the HDAC-containing NuRD complex, no report verified the capacity of the individual N-terminal RBCC domain (1-381) in associating with frees HDAC1 (or HDAC1 complexes different from the NurD). In fact, Schultz et al. (2001), reported that several mutations impair KAP1 association with Mi 2$\alpha$ (a PHD-BROMO 1-616 deletion mutant or single W664A in the PHD-BROMO domain); the same mutants were not verified for their binding capacity to HDAC1 (Schultz et al., 2001). Similarly, Ivanov et al. (2007), reported that a KAP1 mutant deleted in the bromodomain (dB) is unable to bind Mi2$\alpha$; again the binding capacity of this deletion mutant with HDAC1 was not tested (Ivanov et al., 2007). In addition, the absence of association between HDAC1 and the single PHD-BROMO domain (617-835) observed in our experiments is consistent with the kinetic model determining KAP1-mediated transcriptional repression (Groner et al., 2010; Ivanov et al., 2007; Sripathy et al., 2006). Based on this model the PHD-BROMO domain recruits the silencing machinery, including the HDAC1 containing NurD complex, following the tethering of KAP1 to the targeted DNA

by the KRAB zing finger proteins or by the heterologous DNA binding domains. Therefore, our data suggest that the molecular mechanisms leading to the recruitment of HDAC1 by KAP1 in the integration inhibition (RBCC) do not correspond to the mechanisms involved in the transcription repression (PHD-BROMO). Consistently with the capacity of the RBCC domain to inhibit HIV-1 integration at the same extent of the full length KAP1, we found that this region (1-381) is responsible for the binding with integrase and determines the preferential association of KAP1 with the acetylated form of the viral protein. These results clearly show that the KAP1 domain responsible for inhibiting viral integration spans the 1-381 region (RBCC) consistently with its ability to recognize preferentially acetylated integrase and to bind HDAC1 and further sustaining that KAP1 inhibits HIV-1 integration through the targeting of the acetylated integrase and the recruitment of HDAC1 to catalyze its deacetylation.

Our results showed that the PHD-Bromo domain of KAP1 does not bind integrase and does not determine the specificity of KAP1 in associating the acetylated form of integrase. This data seems perplexing because it is well defined that the bromodomains bind specifically acetylated lysines and the PHD domain is cooperating to stabilize this association (Mujtaba et al., 2007). Nevertheless, even though the bromodomain of KAP1 is structured in ZA and BC loops similarly to other bromodomain proteins (p300, PCAF, CBP or BPTF) the three acetyl lysine binding residues of these loops are not conserved in KAP1. In fact, the crystal structure of the PHD-BROMO tandem of KAP1 reported by Zeng et al. (2008) showed that the characteristic pocket structure between the ZA and BC loops in the bromodomain is distorted with comparison to the canonical bromodomain (Zeng et al., 2008). This renders the bromodomain of KAP1 atypical. Consistently with the crystallographic analysis and similarly to our results, the tandem PHD finger–bromodomain of KAP1 was shown unable to bind the lysine-acetylated peptides derived from histones H3 or H4 (Zeng et al., 2008).

Human KAP1 does not inhibit integration of another retrovirus murine leukemia virus (MLV). Thus, unlike TRIM5$\alpha$ and APOBEC3G, KAP1 does not prevent human cells from MLV integration (Harris et al., 2003; Hatziioannou et al., 2004). A similar result has been reported with another HIV-1 inhibitor factor, TRIM22, that interferes selectively with viral assembly of HIV-1 and does not affect assembly of either MLV or equine infectious anemia virus (EIAV) (Barr et al., 2008). Moreover, $p21^{CIP1/Waf1}$, that inhibits specifically HIV-1

integration, does not affect the replication of macaque simian immunodeficiency virus (SIVmac) (Bieniasz, 2007; Zhang et al., 2007).

### 3- KAP1 does not affect HIV-1 gene expression and likely is not involved in viral latency

Since KAP1 is a transcription repressor (Lechner et al., 2000; Schultz et al., 2002; Schultz et al., 2001; Sripathy et al., 2006) that has been described to inhibit transcription of MLV, visna, spuma and Mason-Pfizer monkey retroviruses as well as of endogenous retroviruses (IAPs) in embryonic stem cells (Rowe et al., 2010; Wolf and Goff, 2007; Wolf et al., 2008b), we investigated whether it could affect HIV-1 transcription. In KAP1 knockdown cells, transcription of either an integration defective (D64E) or an integrated HIV-1 viruses was not modulated. These results are in agreement with the observations that KAP1 affects transcription of retroviruses carrying primer binding sites (PBS) complementary to $tRNA^{pro}$ such as MLV and HTLV-1 (Wolf and Goff, 2007, 2009) or to $tRNA^{Lys-1,2}$ found in visna, spuma and Mason-Pfizer monkey viruses (Wolf et al., 2008b). Moreover, KAP1 retroviral repression of $PBS^{pro}$ is mediated by a KRAB zinc finger, ZFP809, that has been shown unable to affect HIV-1 transcription (Wolf and Goff, 2009).

Since HIV-1 latency of integrated HIV-1 is mainly explained by the transcription repression at the level of the LTR promoter (Marcello, 2006), a possible role of KAP1 in the latency could be suggested. However, the lack of the effect of KAP1 on HIV-1 transcription demonstrated by our results and by others (Wolf and Goff, 2009) and also our observations that KAP1 expression levels are unchanged in J-latA1 cells carrying a latent integrated HIV-1 or in memory (CD45 RO+) T cells involved in viral latency, disfavour this hypothesis.

### 4- KAP1 as a restriction factor for HIV-1 infectivity

The well described HIV-1 restriction factors are rhesus TRIM5$\alpha$, owl monkey TRIMCyp, APOBECs (A3G and A3F) and tetherin (CD317) (Bieniasz, 2009; Evans et al., 2010; Huthoff and Towers, 2008; Luban, 2007; Malim, 2009; Nakayama and Shioda, 2010; Neil et al., 2008; Sheehy et al., 2002; Towers, 2007; Van Damme et al., 2008). These restriction factors interfere with cytoplasmic steps of HIV-1 replication which are post-entry for TRIM5$\alpha$, reverse transcription for APOBECs and viral release for tetherin. So far, few reports described HIV-1 inhibitor proteins that interfere with HIV-1 integration or

nuclear pre-integration events ($p21^{Cip1/Waf1}$, RAD52, Rad18, XPB and XPD) with not yet establishment of molecular mechanisms (Lau et al., 2004; Lloyd et al., 2006; Yoder et al., 2006; Zhang et al., 2005; Zhang et al., 2007). It has been proposed that these factors inhibit HIV-1 infection affecting the nuclear viral cDNA available for integration by inducing its degradation or circularization (Rad18, XPB and XPD) or by competing with proteins of the PIC in the binding of the viral cDNA ends (RAD52). The only inhibitor factor that has been described to decrease infectivity by interfering with viral integration is $p21^{Cip1/Waf1}$. Since $p21^{Cip1/Waf1}$ is a cell cycle checkpoint protein playing a role in DNA damage response, it has been speculated that DNA damage pathways activated by the virus are responsible for $p21^{Cip1/Waf1}$ antiviral activity; however, the molecular mechanism underlying viral integration inhibition has not been delineated (Zhang et al., 2007).

The knockdown experiments performed in our study, showed that siRNA-mediated knockdowns of KAP1 enhanced HIV-1 integration (5-9 fold) higher than the shRNA-mediated knockdowns (2-4 fold) regardless they are transient or stable knockdowns. These differences of the magnitudes of the viral phenotype obtained by shRNAs and siRNAs was also observed with $p21^{Cip1/Waf1}$ (Zhang et al., 2005; Zhang et al., 2007). Severe depletion of KAP1 endogenous levels could not be obtained due the incompatibility with cellular conditions and cell viability (Wang et al., 2005a; Wang et al., 2007a; Wolf and Goff, 2007), which might hindered us to assess the exact fold inhibition of KAP1 on HIV-1 integration. Recently, it has been described an inducible *KAP1* knockout in murine stem cells (Rowe et al., 2010) that might be a useful model to study HIV-1 integration in transient severe depletion of KAP1. However, the data obtained in the murine model should be carefully interpreted due to the differences with the human system.

**5- Models for KAP1 inhibition of HIV-1 integration**

Since acetylation of HIV-1 integrase by p300 has been shown to increase its binding affinity to the viral DNA and to enhance its catalytic activity and also it has been shown that acetylable lysines (K264, K266 and K273) contribute to an optimal HIV-1 integration (Apolonia et al., 2007; Cereseto et al., 2005; Terreni et al., 2010), a simplest model can be proposed in which KAP1 recognizes acetylated integrase through a high affinity of interaction with the modified viral protein, recruits HDAC1 to acetylated integrase, thus, it induces integrase deacetylation which affects negatively integrase catalytic and DNA binding activities and consequently reduces HIV-1 integration efficiency.

Since KAP1 binds the C-terminal domain of integrase which is the substrate for acetylation and notably is the region required for integrase-p300 complex formation, we can also propose a model in which p300 and KAP1-bridging-HDAC1 compete for the binding to the C-terminal region of integrase resulting in cycles of acetylation and deacetylation of the lysines carried in this domain which corresponds to activation and inactivation of integrase, respectively. This model might also explain the achievement of optimal HIV-1 integration in several cell types where KAP1 is ubiquitously expressed.

A third model can be proposed based on the properties of KAP1 as co-repressor scaffold that binds DNA binding proteins, mainly KRAB zing fingers, recruits HDACs, SETDB1 histone methylase and HP1 to the chromatin regions to be silenced and induces heterochromatin formation (Ayyanathan et al., 2003; Groner et al., 2010; Sripathy et al., 2006). Therefore, it can be proposed that KAP1 interacting with integrase at the integration target site, it induces integrase deacetylation by HDAC1 which may reduce the viral protein binding to host and viral DNAs and also KAP1 might induce the transient hetrochromatinization of the target site to render it inaccessible for integrase to perform an efficient integration of the viral genome.

### 6- Could HIV-1 escape from KAP1 inhibition through the induction of KAP1 phosphorylation?

Recently, it has been demonstrated that following DNA double strand breaks damage the nuclear kinase ATM phosphorylates KAP1, diminishing KAP1 interaction with the chromatin (Goodarzi et al., 2008; Ziv et al., 2006). Perturbation of the chromatin by loss of the co-repressor KAP1, adaptor of HP1, SETDB1 methylase, and HDACs, determines chromatin relaxation to enable DNA double strand breaks repair (Goodarzi et al., 2008; Ziv et al., 2006). Notably, it has been suggested that un-integrated HIV-1 cDNA is a substrate for double-DNA strand break repair factors thus activating DNA damage response (Abrink et al., 2001; Baekelandt et al., 2000; Jeanson et al., 2002; Kilzer et al., 2003; Lloyd et al., 2006). ATM that predominantly detects DNA double strand breaks has been demonstrated to be activated following HIV-1 infection and its inhibition through a small molecule (KU-55933) drastically decreases cellular susceptibility to viral infection (Lau et al., 2005). Since KAP1 is ubiquitously expressed showing no major restriction of HIV-1 infectivity, we explored a hypothesis where ATM activated by un-integrated HIV-1 phosphorylates KAP1 leading to the disassembly of the integrase/HDAC1 complex (see Figure 22 of RESULTS).

This would ultimately reverse HDAC1 deacetylase activity associated to integrase, thus, allowing the reconstitution of integrase acetylation by p300 and restored levels of integration efficiency. Our initial investigations of this hypothesis revealed that KAP1 is phosphorylated at two hours following HIV-1 infection and at four hours post infections the phosphorylation is down-regulated. Interestingly, it has been shown that upon double strand breaks damage, the phosphorylation of KAP1 is an early and transient event that is peaked at thirty minutes post damage and persists only up to two hours (White et al., 2006; Ziv et al., 2006). Moreover, following infections with HIV-1, it has been shown that phosphorylation of p53 by ATM kinase persists up to eight hours post infection (Lau et al., 2005). The persistence of the p53 modification has been correlated with the post-integration repair of HIV-1. These observations suggest that KAP1 phosphorylation, as in DNA damage, is related to early HIV-1 infection events. Moreover, our results showed that the KAP1 phosphorylation induced by HIV-1 infection, was not inhibited by the ATM inhibitor (KU-55933) which suggest that, the phophorylation of KAP1 in HIV-1 infection is not mediated by ATM.

In our model, the phosphorylated KAP1 is displaced from the pre-integration complex and would not interact with integrase. However, the interaction assays that were performed in our study between integrase and KAP1, following induction of its phosphorylation by DNA damage, or between integrase and KAP1 phosphomutants showed that KAP1 phosphorylation does not modulate the KAP1/integrase complex formation. However, further interaction investigations are necessary to perform between phosphorylated KAP1 and the viral integrase in the context of the pre-integration complex during HIV-1 infection instead of an integrase expressed in cells by transfection. These investigations would be useful, also because we found that the KAP1 phosphomimic (S824D) was unable to inhibit HIV-1 integration as the KAP1 wild type or phosphomutant (S824A). Therefore, the study of the phosphorylated KAP1/integrase interactions during HIV-1 infection would address whether the KAP1 phosphomimic (S824D) is unable to inhibit HIV-1 integration because it is not associated with the integrase during HIV-1 integration. In fact, the inability of the KAP1 phospho-mimic (S824D) to inhibit HIV-1 integration could be also explained by the nuclear localization of this mutant, which is not associated with the chromatin (Ziv et al., 2006) where integration is occuring, that may consequently hamper its association with integrase during viral integration.

## CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, we report a list of novel cellular proteins that interact with acetylated HIV-1 integrase. These are new potential factors involved in HIV-1 replication by either inhibiting or favoring the virus at specific steps involving integrase activity. Further analyses are required to establish their roles in HIV-1 biology. Moreover, we identified a new HIV-1 inhibitor factor, KAP1, that interferes specifically with HIV-1 integration and thus joins the very short list of cellular factors that inhibits this viral step. Investigations on the molecular mechanisms by which KAP1 is acting on integration led us to reveal that KAP1 regulates negatively integrase by inducing its deacetylation through the recruitment of HDAC1. Further investigations are needed to understand whether the virus escape from KAP1 inhibition is mediated the induction of KAP1 phosphorylation or involved some other mechanisms.

KAP1 might be a good candidate for AIDS therapy strategy. Therefore, it would be useful to investigate whether the delivery of KAP1 protein or RBCC peptides, the region of KAP1 responsible for the association with acetylated integrase and with HDAC1, in infected T cells may delay and inhibit efficiently the HIV-1 replication. However, both strategies should be preceded by investigations on the potential of these molecules in the induction of resistant HIV-1 strains. Therefore, it would be worthy to study the HIV-1 replication in T cells stably expressing KAP1 and RBCC domain and to monitor and sequence the emergent HIV-1 resistant strains.

## References

**Abrink, M., Ortiz, J.A., Mark, C., Sanchez, C., Looman, C., Hellman, L., Chambon, P., and Losson, R. (2001).** Conserved interaction between distinct Kruppel-associated box domains and the transcriptional intermediary factor 1 beta. Proc Natl Acad Sci U S A *98*, 1422-1426.

**Acharya, A., Xu, X.J., Husain-Ponnampalam, R.D., Hoffmann-Benning, S., and Kuo, M.H. (2005).** Production of constitutively acetylated recombinant p53 from yeast and Escherichia coli by tethered catalysis. Protein expression and purification *41*, 417-425.

**Adamson, C.S., and Freed, E.O. (2007).** Human immunodeficiency virus type 1 assembly, release, and maturation. Adv Pharmacol *55*, 347-387.

**Adamson, C.S., and Freed, E.O. (2010).** Novel approaches to inhibiting HIV-1 replication. Antiviral Res *85*, 119-141.

**Aiken, C., Konner, J., Landau, N.R., Lenburg, M.E., and Trono, D. (1994).** Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell *76*, 853-864.

**Al-Mawsawi, L.Q., and Neamati, N. (2007).** Blocking interactions between HIV-1 integrase and cellular cofactors: an emerging anti-retroviral strategy. Trends Pharmacol Sci *28*, 526-535.

**Albanese, A., Arosio, D., Terreni, M., and Cereseto, A. (2008).** HIV-1 pre-integration complexes selectively target decondensed chromatin in the nuclear periphery. PLoS One *3*, e2413.

**Anderson, J.L., Campbell, E.M., Figueiredo, A., and Hope, T.J. (2008).** Heat shock perturbs TRIM5alpha restriction of human immunodeficiency virus type 1. J Virol *82*, 2575-2579.

**Anderson, J.L., Campbell, E.M., Wu, X., Vandegraaff, N., Engelman, A., and Hope, T.J. (2006).** Proteasome inhibition reveals that a functional preintegration complex intermediate can be generated during restriction by diverse TRIM5 proteins. J Virol *80*, 9754-9760.

**Ao, Z., Fowke, K.R., Cohen, E.A., and Yao, X. (2005).** Contribution of the C-terminal tri-lysine regions of human immunodeficiency virus type 1 integrase for efficient reverse transcription and viral DNA nuclear import. Retrovirology *2*, 62.

**Ao, Z., Huang, G., Yao, H., Xu, Z., Labine, M., Cochrane, A.W., and Yao, X. (2007).** Interaction of human immunodeficiency virus type 1 integrase with cellular nuclear import receptor importin 7 and its impact on viral replication. J Biol Chem *282*, 13456-13467.

**Apolonia, L., Waddington, S.N., Fernandes, C., Ward, N.J., Bouma, G., Blundell, M.P., Thrasher, A.J., Collins, M.K., and Philpott, N.J. (2007).** Stable gene transfer to muscle using non-integrating lentiviral vectors. Mol Ther *15*, 1947**-1954.**

**Ariumi, Y., Serhan, F., Turelli, P., Telenti, A., and Trono, D. (2006).** The integrase interactor 1 (INI1) proteins facilitate Tat-mediated human immunodeficiency virus type 1 transcription. Retrovirology *3*, 47.

**Ariumi, Y., Turelli, P., Masutani, M., and Trono, D. (2005).** DNA damage sensors ATM, ATR, DNA-PKcs, and PARP-1 are dispensable for human immunodeficiency virus type 1 integration. J Virol *79*, 2973-2978.

**Armon-Omer, A., Graessmann, A., and Loyter, A. (2004).** A synthetic peptide bearing the HIV-1 integrase 161-173 amino acid residues mediates active nuclear import and binding to importin alpha: characterization of a functional nuclear localization signal. J Mol Biol *336*, 1117-1128.

**Ayyanathan, K., Lechner, M.S., Bell, P., Maul, G.G., Schultz, D.C., Yamada, Y., Tanaka, K., Torigoe, K., and Rauscher, F.J., 3rd (2003).** Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. Genes Dev *17*, 1855-1869.

**Baekelandt, V., Claeys, A., Cherepanov, P., De Clercq, E., De Strooper, B., Nuttin, B., and Debyser, Z. (2000).** DNA-Dependent protein kinase is not required for efficient lentivirus integration. J Virol *74*, 11278-11285.

**Bannister, A.J., Miska, E.A., Gorlich, D., and Kouzarides, T. (2000).** Acetylation of importin-alpha nuclear import factors by CBP/p300. Curr Biol *10*, 467-470.

**Barr, S.D., Smiley, J.R., and Bushman, F.D. (2008).** The interferon response inhibits HIV particle production by induction of TRIM22. PLoS Pathog *4*, e1000007.

**Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C.*, et al.* (1983).** Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science *220*, 868-871.

**Benkhelifa-Ziyyat, S., Bucher, S., Zanta-Boussif, M.A., Pasquet, J., and Danos, O. (2010).** Changes in the accessibility of the HIV-1 Integrase C-terminus in the presence of cellular proteins. Retrovirology *7*, 27.

**Berchtold, M.W., and Berger, M.C. (1991).** Isolation and analysis of a human cDNA highly homologous to the yeast gene encoding L17A ribosomal protein. Gene *102*, 283-288.

**Berger, E.A., Murphy, P.M., and Farber, J.M. (1999).** Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol *17*, 657-700.

**Beriault, V., Clement, J.F., Levesque, K., Lebel, C., Yong, X., Chabot, B., Cohen, E.A., Cochrane, A.W., Rigby, W.F., and Mouland, A.J. (2004).** A late role for the association of hnRNP A2 with the HIV-1 hnRNP A2 response elements in genomic RNA, Gag, and Vpr localization. J Biol Chem *279*, 44141-44153.

**Berkhout, B., Silverman, R.H., and Jeang, K.T. (1989).** Tat trans-activates the human immunodeficiency virus through a nascent RNA target. Cell *59*, 273-282.

**Berro, R., Kehn, K., de la Fuente, C., Pumfery, A., Adair, R., Wade, J., Colberg-Poley, A.M., Hiscott, J., and Kashanchi, F. (2006).** Acetylated Tat regulates human immunodeficiency virus type 1 splicing through its interaction with the splicing regulator p32. J Virol *80*, 3189-3204.

**Bieniasz, P.D. (2007).** An intrinsic host defense against HIV-1 integration? J Clin Invest *117*, 302-304.

**Bieniasz, P.D. (2009).** The cell biology of HIV-1 virion genesis. Cell Host Microbe *5*, 550-558.

**Boese, A., Sommer, P., Gaussin, A., Reimann, A., and Nehrbass, U. (2004).** Ini1/hSNF5 is dispensable for retrovirus-induced cytoplasmic accumulation of PML and does not interfere with integration. FEBS Lett *578*, 291-296.

**Bouazzaoui, A., Kreutz, M., Eisert, V., Dinauer, N., Heinzelmann, A., Hallenberger, S., Strayle, J., Walker, R., Rubsamen-Waigmann, H., Andreesen, R.*, et al.* (2006).** Stimulated trans-acting factor of 50 kDa (Staf50) inhibits HIV-1 replication in human monocyte-derived macrophages. Virology *356*, 79-94.

**Bouhamdan, M., Benichou, S., Rey, F., Navarro, J.M., Agostini, I., Spire, B., Camonis, J., Slupphaug, G., Vigne, R., Benarous, R.*, et al.* (1996).** Human immunodeficiency virus type 1 Vpr protein binds to the uracil DNA glycosylase DNA repair enzyme. J Virol *70*, 697-704.

**Bouyac-Bertoia, M., Dvorin, J.D., Fouchier, R.A., Jenkins, Y., Meyer, B.E., Wu, L.I., Emerman, M., and Malim, M.H. (2001).** HIV-1 infection requires a functional integrase NLS. Mol Cell *7*, 1025-1035.

**Braaten, D., and Luban, J. (2001).** Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. EMBO J *20*, 1300-1309.

**Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J., and Elledge, S.J. (2008).** Identification of host proteins required for HIV infection through a functional genomic screen. Science *319*, 921-926.

**Bres, V., Kiernan, R., Emiliani, S., and Benkirane, M. (2002).** Tat acetyl-acceptor lysines are important for human immunodeficiency virus type-1 replication. J Biol Chem *277*, 22215-22221.

**Briers, S., Crawford, C., Bickmore, W.A., and Sutherland, H.G. (2009).** KRAB zinc-finger proteins localise to novel KAP1-containing foci that are adjacent to PML nuclear bodies. J Cell Sci *122*, 937-946.

**Brussel, A., and Sonigo, P. (2003).** Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J Virol *77*, 10119-10124.

**Bukrinsky, M.I., Haggerty, S., Dempsey, M.P., Sharova, N., Adzhubel, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., and Stevenson, M. (1993).** A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature *365*, 666-669.

**Bukrinsky, M.I., Sharova, N., Dempsey, M.P., Stanwick, T.L., Bukrinskaya, A.G., Haggerty, S., and Stevenson, M. (1992).** Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc Natl Acad Sci U S A *89*, 6580-6584.

**Bushman, F., Lewinski, M., Ciuffi, A., Barr, S., Leipzig, J., Hannenhalli, S., and Hoffmann, C. (2005).** Genome-wide analysis of retroviral DNA integration. Nat Rev Microbiol *3*, 848-858.

**Bushman, F.D., and Craigie, R. (1991).** Activities of human immunodeficiency virus (HIV) integration protein in vitro: specific cleavage and integration of HIV DNA. Proc Natl Acad Sci U S A *88*, 1339-1343.

**Busschots, K., De Rijck, J., Christ, F., and Debyser, Z. (2009).** In search of small molecules blocking interactions between HIV proteins and intracellular cofactors. Mol Biosyst *5*, 21-31.

**Busschots, K., Vercammen, J., Emiliani, S., Benarous, R., Engelborghs, Y., Christ, F., and Debyser, Z. (2005).** The interaction of LEDGF/p75 with integrase is lentivirus-specific and promotes DNA binding. J Biol Chem *280*, 17841-17847.

**Busschots, K., Voet, A., De Maeyer, M., Rain, J.C., Emiliani, S., Benarous, R., Desender, L., Debyser, Z., and Christ, F. (2007).** Identification of the LEDGF/p75 binding site in HIV-1 integrase. J Mol Biol *365*, 1480-1492.

**Bustin, M. (2001).** Chromatin unfolding and activation by HMGN(*) chromosomal proteins. Trends Biochem Sci *26*, 431-437.

**Butler, S.L., Hansen, M.S., and Bushman, F.D. (2001).** A quantitative assay for HIV DNA integration in vivo. Nat Med *7*, 631-634.

**Cai, M., Zheng, R., Caffrey, M., Craigie, R., Clore, G.M., and Gronenborn, A.M. (1997).** Solution structure of the N-terminal zinc binding domain of HIV-1 integrase. Nat Struct Biol *4*, 567-577.

**Calado, A., Treichel, N., Muller, E.C., Otto, A., and Kutay, U. (2002).** Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. EMBO J 21, 6216-6224.

**Cammas, F., Mark, M., Dolle, P., Dierich, A., Chambon, P., and Losson, R. (2000).** Mice lacking the transcriptional corepressor TIF1beta are defective in early postimplantation development. Development *127*, 2955-2963.

**Campbell, E.M., Perez, O., Anderson, J.L., and Hope, T.J. (2008).** Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha. J Cell Biol *180*, 549-561.

**Carthagena, L., Bergamaschi, A., Luna, J.M., David, A., Uchil, P.D., Margottin-Goguet, F., Mothes, W., Hazan, U., Transy, C., Pancino, G.*, et al.* (2009).** Human TRIM gene expression in response to interferons. PLoS One *4*, e4894.

**Cassimeris, L. (2002).** The oncoprotein 18/stathmin family of microtubule destabilizers. Curr Opin Cell Biol *14*, 18-24.

**Caumont, A., Jamieson, G., de Soultrait, V.R., Parissi, V., Fournier, M., Zakharova, O.D., Bayandin, R., Litvak, S., Tarrago-Litvak, L., and Nevinsky, G.A. (1999).** High affinity interaction of HIV-1 integrase with specific and non-specific single-stranded short oligonucleotides. FEBS Lett *455*, 154-158.

**Cazzalini, O., Scovassi, A.I., Savio, M., Stivala, L.A., and Prosperi, E. (2010).** Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. Mutat Res *704*, 12-20.

**Cereseto, A., Manganaro, L., Gutierrez, M.I., Terreni, M., Fittipaldi, A., Lusic, M., Marcello, A., and Giacca, M. (2005).** Acetylation of HIV-1 integrase by p300 regulates viral integration. EMBO J *24*, 3070-3081.

**Chan, H.M., and La Thangue, N.B. (2001).** p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J Cell Sci *114*, 2363-2373.

**Cherepanov, P. (2007).** LEDGF/p75 interacts with divergent lentiviral integrases and modulates their enzymatic activity in vitro. Nucleic Acids Res *35*, 113-124.

**Cherepanov, P., Ambrosio, A.L., Rahman, S., Ellenberger, T., and Engelman, A. (2005a).** Structural basis for the recognition between HIV-1 integrase and transcriptional coactivator p75. Proc Natl Acad Sci U S A *102*, 17308-17313.

**Cherepanov, P., Devroe, E., Silver, P.A., and Engelman, A. (2004).** Identification of an evolutionarily conserved domain in human lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) that binds HIV-1 integrase. J Biol Chem *279*, 48883-48892.

**Cherepanov, P., Maertens, G., Proost, P., Devreese, B., Van Beeumen, J., Engelborghs, Y., De Clercq, E., and Debyser, Z. (2003).** HIV-1 integrase forms

stable tetramers and associates with LEDGF/p75 protein in human cells. J Biol Chem *278*, 372-381.

**Cherepanov, P., Sun, Z.Y., Rahman, S., Maertens, G., Wagner, G., and Engelman, A. (2005b).** Solution structure of the HIV-1 integrase-binding domain in LEDGF/p75. Nat Struct Mol Biol *12*, 526-532.

**Christ, F., Thys, W., De Rijck, J., Gijsbers, R., Albanese, A., Arosio, D., Emiliani, S., Rain, J.C., Benarous, R., Cereseto, A.*, et al.* (2008).** Transportin-SR2 imports HIV into the nucleus. Curr Biol *18*, 1192-1202.

**Christ, F., Voet, A., Marchand, A., Nicolet, S., Desimmie, B.A., Marchand, D., Bardiot, D., Van der Veken, N.J., Van Remoortel, B., Strelkov, S.V.*, et al.* (2010).** Rational design of small-molecule inhibitors of the LEDGF/p75-integrase interaction and HIV replication. Nat Chem Biol *6*, 442-448.

**Chun, T.W., Finzi, D., Margolick, J., Chadwick, K., Schwartz, D., and Siliciano, R.F. (1995).** In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. Nat Med *1*, 1284-1290.

**Cimarelli, A., and Luban, J. (1999). T**ranslation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. J Virol *73*, 5388-5401.

**Ciuffi, A., Llano, M., Poeschla, E., Hoffmann, C., Leipzig, J., Shinn, P., Ecker, J.R., and Bushman, F. (2005). A role for LEDGF/p75 in targeting HIV DNA integration. Nat Med *11*, 1287-1289.**

**Clavel, F., Guyader, M., Guetard, D., Salle, M., Montagnier, L., and Alizon, M. (1986).** Molecular cloning and polymorphism of the human immune deficiency virus type 2. Nature *324*, 691-695.

**Coffin, J.M., Hughes, S.H., and Varmus, H.E. (1997).** Retroviruses.

**Cohen, H.Y., Lavu, S., Bitterman, K.J., Hekking, B., Imahiyerobo, T.A., Miller, C., Frye, R., Ploegh, H., Kessler, B.M., and Sinclair, D.A. (2004).** Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. Mol Cell *13*, 627-638.

**Collins, K.L., Chen, B.K., Kalams, S.A., Walker, B.D., and Baltimore, D. (1998).** HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature *391*, 397-401.

**Daniel, R., Greger, J.G., Katz, R.A., Taganov, K.D., Wu, X., Kappes, J.C., and Skalka, A.M. (2004).** Evidence that stable retroviral transduction and cell survival following DNA integration depend on components of the nonhomologous end joining repair pathway. J Virol *78*, 8573-8581.

**Daniel, R., Kao, G., Taganov, K., Greger, J.G., Favorova, O., Merkel, G., Yen, T.J., Katz, R.A., and Skalka, A.M. (2003).** Evidence that the retroviral DNA integration process triggers an ATR-dependent DNA damage response. Proc Natl Acad Sci U S A *100*, 4778-4783.

**Daniel, R., Katz, R.A., Merkel, G., Hittle, J.C., Yen, T.J., and Skalka, A.M. (2001).** Wortmannin potentiates integrase-mediated killing of lymphocytes and reduces the efficiency of stable transduction by retroviruses. Mol Cell Biol *21*, 1164-1172.

**Daniel, R., Katz, R.A., and Skalka, A.M. (1999).** A role for DNA-PK in retroviral DNA integration. Science *284*, 644-647.

**Daniel, R., Marusich, E., Argyris, E., Zhao, R.Y., Skalka, A.M., and Pomerantz, R.J. (2005).** Caffeine inhibits human immunodeficiency virus type 1 transduction of nondividing cells. J Virol *79*, 2058-2065.

**Darnell, J.E., Jr. (1997).** STATs and gene regulation. Science *277*, 1630-1635.

**Das, C., and Kundu, T.K. (2005).** Transcriptional regulation by the acetylation of nonhistone proteins in humans -- a new target for therapeutics. IUBMB Life *57*, 137-149.

**David, G., Neptune, M.A., and DePinho, R.A. (2002).** SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities. J Biol Chem *277*, 23658-23663.

**De Rijck, J., and Debyser, Z. (2006).** The central DNA flap of the human immunodeficiency virus type 1 is important for viral replication. Biochem Biophys Res Commun *349*, 1100-1110.

**De Rijck, J., Vandekerckhove, L., Gijsbers, R., Hombrouck, A., Hendrix, J., Vercammen, J., Engelborghs, Y., Christ, F., and Debyser, Z. (2006).** Overexpression of the lens epithelium-derived growth factor/p75 integrase binding domain inhibits human immunodeficiency virus replication. J Virol *80*, 11498-11509.

**de Ruijter, A.J., van Gennip, A.H., Caron, H.N., Kemp, S., and van Kuilenburg, A.B. (2003).** Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J *370*, 737-749.

**de Silva, T.I., Cotten, M., and Rowland-Jones, S.L. (2008).** HIV-2: the forgotten AIDS virus. Trends Microbiol *16*, 588-595.

**de Soultrait, V.R., Caumont, A., Durrens, P., Calmels, C., Parissi, V., Recordon, P., Bon, E., Desjobert, C., Tarrago-Litvak, L., and Fournier, M. (2002).** HIV-1 integrase interacts with yeast microtubule-associated proteins. Biochim Biophys Acta *1575*, 40-48.

**Deacon, N.J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellett, A., Chatfield, C.*, et al.* (1995).** Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science *270*, 988-991.

**Dehart, J.L., Andersen, J.L., Zimmerman, E.S., Ardon, O., An, D.S., Blackett, J., Kim, B., and Planelles, V. (2005).** The ataxia telangiectasia-mutated and Rad3-related protein is dispensable for retroviral integration. J Virol *79*, 1389-1396.

**Depienne, C., Mousnier, A., Leh, H., Le Rouzic, E., Dormont, D., Benichou, S., and Dargemont, C. (2001).** Characterization of the nuclear import pathway for HIV-1 integrase. J Biol Chem *276*, 18102-18107.

**Depienne, C., Roques, P., Creminon, C., Fritsch, L., Casseron, R., Dormont, D., Dargemont, C., and Benichou, S. (2000).** Cellular distribution and karyophilic properties of matrix, integrase, and Vpr proteins from the human and simian immunodeficiency viruses. Exp Cell Res *260*, 387-395.

**Desfarges, S., Salin, B., Calmels, C., Andreola, M.L., Parissi, V., and Fournier, M. (2009).** HIV-1 integrase trafficking in S. cerevisiae: a useful model to dissect the microtubule network involvement of viral protein nuclear import. Yeast *26*, 39-54.

**Devroe, E., Engelman, A., and Silver, P.A. (2003).** Intracellular transport of human immunodeficiency virus type 1 integrase. J Cell Sci *116*, 4401-4408.

**Di Fenza, A., Rocchia, W., and Tozzini, V. (2009).** Complexes of HIV-1 integrase with HAT proteins: multiscale models, dynamics, and hypotheses on allosteric sites of inhibition. Proteins *76*, 946-958.

**Diaz-Griffero, F., Vandegraaff, N., Li, Y., McGee-Estrada, K., Stremlau, M., Welikala, S., Si, Z., Engelman, A., and Sodroski, J. (2006).** Requirements for capsid-binding and an effector function in TRIMCyp-mediated restriction of HIV-1. Virology *351*, 404-419.

**Dirac, A.M., and Kjems, J. (2001).** Mapping DNA-binding sites of HIV-1 integrase by protein footprinting. Eur J Biochem *268*, 743-751.

**Doms, R.W., and Trono, D. (2000).** The plasma membrane as a combat zone in the HIV battlefield. Genes Dev *14*, 2677-2688.

**Dorr, A., Kiermer, V., Pedal, A., Rackwitz, H.R., Henklein, P., Schubert, U., Zhou, M.M., Verdin, E., and Ott, M. (2002).** Transcriptional synergy between Tat and PCAF is dependent on the binding of acetylated Tat to the PCAF bromodomain. EMBO J *21*, 2715-2723.

**Dvorin, J.D., Bell, P., Maul, G.G., Yamashita, M., Emerman, M., and Malim, M.H. (2002).** Reassessment of the roles of integrase and the central DNA flap in human immunodeficiency virus type 1 nuclear import. J Virol *76*, 12087-12096.

**Dyda, F., Hickman, A.B., Jenkins, T.M., Engelman, A., Craigie, R., and Davies, D.R. (1994).** Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases. Science *266*, 1981-1986.

**Eberhardy, S.R., Goncalves, J., Coelho, S., Segal, D.J., Berkhout, B., and Barbas, C.F., 3rd (2006).** Inhibition of human immunodeficiency virus type 1 replication with artificial transcription factors targeting the highly conserved primer-binding site. J Virol *80*, 2873-2883.

**Emiliani, S., Mousnier, A., Busschots, K., Maroun, M., Van Maele, B., Tempe, D., Vandekerckhove, L., Moisant, F., Ben-Slama, L., Witvrouw, M.*, et al.* (2005).** Integrase mutants defective for interaction with LEDGF/p75 are impaired in chromosome tethering and HIV-1 replication. J Biol Chem *280*, 25517-25523.

**Engelman, A. (1999).** In vivo analysis of retroviral integrase structure and function. Adv Virus Res *52*, 411-426.

**Engelman, A., Bushman, F.D., and Craigie, R. (1993).** Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex. EMBO J *12*, 3269-3275.

**Engelman, A., and Cherepanov, P. (2008).** The lentiviral integrase binding protein LEDGF/p75 and HIV-1 replication. PLoS Pathog *4*, e1000046.

**Engelman, A., and Craigie, R. (1992).** Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro. J Virol *66*, 6361-6369.

**Engelman, A., Englund, G., Orenstein, J.M., Martin, M.A., and Craigie, R. (1995).** Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. J Virol *69*, 2729-2736.

**Engelman, A., Hickman, A.B., and Craigie, R. (1994).** The core and carboxyl-terminal domains of the integrase protein of human immunodeficiency virus type 1 each contribute to nonspecific DNA binding. J Virol *68*, 5911-5917.

**Esposito, D., and Craigie, R. (1998).** Sequence specificity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein-DNA interaction. EMBO J *17*, 5832-5843.

**Evans, D.T., Serra-Moreno, R., Singh, R.K., and Guatelli, J.C. (2010).** BST-2/tetherin: a new component of the innate immune response to enveloped viruses. Trends Microbiol.

**Everett, R.D., and Chelbi-Alix, M.K. (2007).** PML and PML nuclear bodies: implications in antiviral defence. Biochimie *89*, 819-830.

**Farnet, C.M., and Haseltine, W.A. (1990).** Integration of human immunodeficiency virus type 1 DNA in vitro. Proc Natl Acad Sci U S A *87*, 4164-4168.

**Farnet, C.M., and Haseltine, W.A. (1991).** Determination of viral proteins present in the human immunodeficiency virus type 1 preintegration complex. J Virol *65*, 1910-1915.

**Fassati, A. (2006).** HIV infection of non-dividing cells: a divisive problem. Retrovirology *3*, 74.

**Faure, A., Calmels, C., Desjobert, C., Castroviejo, M., Caumont-Sarcos, A., Tarrago-Litvak, L., Litvak, S., and Parissi, V. (2005).** HIV-1 integrase crosslinked oligomers are active in vitro. Nucleic Acids Res *33*, 977-986.

**Fellay, J., Shianna, K.V., Ge, D., Colombo, S., Ledergerber, B., Weale, M., Zhang, K., Gumbs, C., Castagna, A., Cossarizza, A.*, et al.* (2007).** A whole-genome association study of major determinants for host control of HIV-1. Science *317*, 944-947.

**Fletcher, T.M., 3rd, Soares, M.A., McPhearson, S., Hui, H., Wiskerchen, M., Muesing, M.A., Shaw, G.M., Leavitt, A.D., Boeke, J.D., and Hahn, B.H. (1997).** Complementation of integrase function in HIV-1 virions. EMBO J *16*, 5123-5138.

**Fouchier, R.A., Meyer, B.E., Simon, J.H., Fischer, U., Albright, A.V., Gonzalez-Scarano, F., and Malim, M.H. (1998).** Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex. J Virol *72*, 6004-6013.

**Fouchier, R.A., Meyer, B.E., Simon, J.H., Fischer, U., and Malim, M.H. (1997).** HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. EMBO J *16*, 4531-4539.

**Frankel, A.D., and Young, J.A. (1998).** HIV-1: fifteen proteins and an RNA. Annu Rev Biochem *67*, 1-25.

**Freed, E.O. (2001).** HIV-1 replication. Somat Cell Mol Genet *26*, 13-33**.**

**Freed, E.O., Englund, G., and Martin, M.A. (1995).** Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. J Virol *69*, 3949-3954.

**Friedman, J.R., Fredericks, W.J., Jensen, D.E., Speicher, D.W., Huang, X.P., Neilson, E.G., and Rauscher, F.J., 3rd (1996).** KAP-1, a novel corepressor for the highly conserved KRAB repression domain. Genes Dev *10*, 2067-2078.

**Gabuzda, D.H., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Haseltine, W.A., and Sodroski, J. (1992).** Role of vif in replication of human immunodeficiency virus type 1 in CD4+ T lymphocytes. J Virol *66*, 6489-6495.

**Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S.*, et al.* (2007).** TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature *446*, 916-920.

**Galasinski, S.C., Resing, K.A., Goodrich, J.A., and Ahn, N.G. (2002).** Phosphatase inhibition leads to histone deacetylases 1 and 2 phosphorylation and disruption of corepressor interactions. J Biol Chem *277*, 19618-19626.

**Gallay, P., Hope, T., Chin, D., and Trono, D. (1997).** HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc Natl Acad Sci U S A *94*, 9825-9830.

**Ganesh, L., Burstein, E., Guha-Niyogi, A., Louder, M.K., Mascola, J.R., Klomp, L.W., Wijmenga, C., Duckett, C.S., and Nabel, G.J. (2003).** The gene product Murr1 restricts HIV-1 replication in resting CD4+ lymphocytes. Nature *426*, 853-857.

**Ganser-Pornillos, B.K., Yeager, M., and Sundquist, W.I. (2008).** The structural biology of HIV assembly. Curr Opin Struct Biol *18*, 203-217.

**Gao, K., Butler, S.L., and Bushman, F. (2001).** Human immunodeficiency virus type 1 integrase: arrangement of protein domains in active cDNA complexes. EMBO J *20*, 3565-3576.

**Ge, H., Si, Y., and Roeder, R.G. (1998).** Isolation of cDNAs encoding novel transcription coactivators p52 and p75 reveals an alternate regulatory mechanism of transcriptional activation. EMBO J *17*, 6723-6729.

**Ghose, R., Liou, L.Y., Herrmann, C.H., and Rice, A.P. (2001).** Induction of TAK (cyclin T1/P-TEFb) in purified resting CD4(+) T lymphocytes by combination of cytokines. J Virol *75*, 11336-11343.

**Gietz, R.D., Schiestl, R.H., Willems, A.R., and Woods, R.A. (1995).** Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast *11*, 355-360.

**Glozak, M.A., Sengupta, N., Zhang, X., and Seto, E. (2005).** Acetylation and deacetylation of non-histone proteins. Gene *363*, 15-23.

**Goff, S.P. (2007).** Host factors exploited by retroviruses. Nat Rev Microbiol *5*, 253-263.

**Goila-Gaur, R., and Strebel, K. (2008).** HIV-1 Vif, APOBEC, and intrinsic immunity. Retrovirology *5*, 51.

**Goodarzi, A.A., Noon, A.T., Deckbar, D., Ziv, Y., Shiloh, Y., Lobrich, M., and Jeggo, P.A. (2008).** ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. Mol Cell *31*, 167-177.

**Goodarzi, A.A., Noon, A.T., and Jeggo, P.A. (2009).** The impact of heterochromatin on DSB repair. Biochem Soc Trans *37*, 569-576.

**Gorlich, D. (1998).** Transport into and out of the cell nucleus. EMBO J *17*, 2721-2727.

**Grant, P.A., and Berger, S.L. (1999).** Histone acetyltransferase complexes. Semin Cell Dev Biol *10*, 169-177.

**Groner, A.C., Meylan, S., Ciuffi, A., Zangger, N., Ambrosini, G., Denervaud, N., Bucher, P., and Trono, D. (2010).** KRAB-zinc finger proteins and KAP1 can mediate

long-range transcriptional repression through heterochromatin spreading. PLoS Genet *6*, e1000869.

**Grozinger, C.M., and Schreiber, S.L. (2002).** Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol *9*, 3-16.

**Gu, W., and Roeder, R.G. (1997).** Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595-606.

**Guo, D., Hazbun, T.R., Xu, X.J., Ng, S.L., Fields, S., and Kuo, M.H. (2004).** A tethered catalysis, two-hybrid system to identify protein-protein interactions requiring post-translational modifications. Nat Biotechnol *22*, 888-892.

**Haberland, M., Montgomery, R.L., and Olson, E.N. (2009).** The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet *10*, 32-42.

**Hamamoto, S., Nishitsuji, H., Amagasa, T., Kannagi, M., and Masuda, T. (2006).** Identification of a novel human immunodeficiency virus type 1 integrase interactor, Gemin2, that facilitates efficient viral cDNA synthesis in vivo. J Virol *80*, 5670-5677.

**Han, Y., Wind-Rotolo, M., Yang, H.C., Siliciano, J.D., and Siliciano, R.F. (2007).** Experimental approaches to the study of HIV-1 latency. Nat Rev Microbiol *5*, 95-106.

**Hare, S., Di Nunzio, F., Labeja, A., Wang, J., Engelman, A., and Cherepanov, P. (2009).** Structural basis for functional tetramerization of lentiviral integrase. PLoS Pathog *5*, e1000515.

**Hare, S., Gupta, S.S., Valkov, E., Engelman, A., and Cherepanov, P. (2010).** Retroviral intasome assembly and inhibition of DNA strand transfer. Nature *464*, 232-236.

**Harper, A.L., Skinner, L.M., Sudol, M., and Katzman, M. (2001).** Use of patient-derived human immunodeficiency virus type 1 integrases to identify a protein residue that affects target site selection. J Virol *75*, 7756-7762.

**Harris, R.S., Bishop, K.N., Sheehy, A.M., Craig, H.M., Petersen-Mahrt, S.K., Watt, I.N., Neuberger, M.S., and Malim, M.H. (2003).** DNA deamination mediates innate immunity to retroviral infection. Cell *113*, 803-809.

**Hatziioannou, T., Perez-Caballero, D., Yang, A., Cowan, S., and Bieniasz, P.D. (2004).** Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. Proc Natl Acad Sci U S A *101*, 10774-10779.

**He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D.O., and Landau, N.R. (1995).** Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J Virol *69*, 6705-6711.

**Hearps, A.C., and Jans, D.A. (2006).** HIV-1 integrase is capable of targeting DNA to the nucleus via an importin alpha/beta-dependent mechanism. Biochem J *398*, 475-484.

**Heinzinger, N.K., Bukinsky, M.I., Haggerty, S.A., Ragland, A.M., Kewalramani, V., Lee, M.A., Gendelman, H.E., Ratner, L., Stevenson, M., and Emerman, M. (1994).** The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc Natl Acad Sci U S A *91*, 7311-7315.

**Hindmarsh, P., Ridky, T., Reeves, R., Andrake, M., Skalka, A.M., and Leis, J. (1999).** HMG protein family members stimulate human immunodeficiency virus type 1 and avian sarcoma virus concerted DNA integration in vitro. J Virol *73*, 2994-3003.

**Hinnebusch, A.G. (2006).** eIF3: a versatile scaffold for translation initiation complexes. Trends Biochem Sci *31*, 553-562.

**Hock, R., Wilde, F., Scheer, U., and Bustin, M. (1998).** Dynamic relocation of chromosomal protein HMG-17 in the nucleus is dependent on transcriptional activity. EMBO J *17*, 6992-7001.

**Hombrouck, A., De Rijck, J., Hendrix, J., Vandekerckhove, L., Voet, A., De Maeyer, M., Witvrouw, M., Engelborghs, Y., Christ, F., Gijsbers, R.*, et al.* (2007).** Virus evolution reveals an exclusive role for LEDGF/p75 in chromosomal tethering of HIV. PLoS Pathog *3*, e47.

**Howell, B., Larsson, N., Gullberg, M., and Cassimeris, L. (1999).** Dissociation of the tubulin-sequestering and microtubule catastrophe-promoting activities of oncoprotein 18/stathmin. Mol Biol Cell *10*, 105-118.

**Huthoff, H., and Towers, G.J. (2008).** Restriction of retroviral replication by APOBEC3G/F and TRIM5alpha. Trends Microbiol *16*, 612-619.

**Iordanskiy, S., Berro, R., Altieri, M., Kashanchi, F., and Bukrinsky, M. (2006).** Intracytoplasmic maturation of the human immunodeficiency virus type 1 reverse transcription complexes determines their capacity to integrate into chromatin. Retrovirology *3*, 4.

**Ito, A., Kawaguchi, Y., Lai, C.H., Kovacs, J.J., Higashimoto, Y., Appella, E., and Yao, T.P. (2002).** MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. EMBO J *21*, 6236-6245.

**Ito, A., Lai, C.H., Zhao, X., Saito, S., Hamilton, M.H., Appella, E., and Yao, T.P. (2001).** p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. EMBO J *20*, 1331-1340.

**Ivanov, A.V., Peng, H., Yurchenko, V., Yap, K.L., Negorev, D.G., Schultz, D.C., Psulkowski, E., Fredericks, W.J., White, D.E., Maul, G.G.*, et al.* (2007).** PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. Mol Cell *28*, 823-837.

**James, P., Halladay, J., and Craig, E.A. (1996).** Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425-1436.

**Jaskolski, M., Alexandratos, J.N., Bujacz, G., and Wlodawer, A. (2009).** Piecing together the structure of retroviral integrase, an important target in AIDS therapy. FEBS J *276*, 2926-2946.

**Jeanson, L., Subra, F., Vaganay, S., Hervy, M., Marangoni, E., Bourhis, J., and Mouscadet, J.F. (2002).** Effect of Ku80 depletion on the preintegrative steps of HIV-1 replication in human cells. Virology *300*, 100-108.

**Jenkins, T.M., Esposito, D., Engelman, A., and Craigie, R. (1997).** Critical contacts between HIV-1 integrase and viral DNA identified by structure-based analysis and photo-crosslinking. EMBO J *16*, 6849-6859.

**Jones, K.A., and Peterlin, B.M. (1994).** Control of RNA initiation and elongation at the HIV-1 promoter. Annu Rev Biochem *63*, 717-743.

**Jordan, A., Bisgrove, D., and Verdin, E. (2003).** HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J *22*, 1868-1877.

**Jurriaans, S., de Ronde, A., Dekker, J., Goudsmit, J., and Cornelissen, M. (1992).** Analysis of human immunodeficiency virus type 1 LTR-LTR junctions in peripheral blood mononuclear cells of infected individuals. J Gen Virol *73 ( Pt 6)*, 1537-1541.

**Kaiser, S.M., and Emerman, M. (2006).** Uracil DNA glycosylase is dispensable for human immunodeficiency virus type 1 replication and does not contribute to the antiviral effects of the cytidine deaminase Apobec3G. J Virol *80*, 875-882.

**Kajaste-Rudnitski, A., Pultrone, C., Marzetta, F., Ghezzi, S., Coradin, T., and Vicenzi, E. (2010).** Restriction factors of retroviral replication: the example of Tripartite Motif (TRIM) protein 5 alpha and 22. Amino Acids *39*, 1-9.

**Kalpana, G.V., Marmon, S., Wang, W., Crabtree, G.R., and Goff, S.P. (1994).** Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science *266*, 2002-2006.

**Kao, S.Y., Calman, A.F., Luciw, P.A., and Peterlin, B.M. (1987).** Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature *330*, 489-493.

**Katzman, M., and Katz, R.A. (1999).** Substrate recognition by retroviral integrases. Adv Virus Res *52*, 371-395.

**Kiernan, R.E., Vanhulle, C., Schiltz, L., Adam, E., Xiao, H., Maudoux, F., Calomme, C., Burny, A., Nakatani, Y., Jeang, K.T.*, et al.* (1999).** HIV-1 tat transcriptional activity is regulated by acetylation. EMBO J *18*, 6106-6118.

**Kilzer, J.M., Stracker, T., Beitzel, B., Meek, K., Weitzman, M., and Bushman, F.D. (2003).** Roles of host cell factors in circularization of retroviral dna. Virology *314*, 460-467.

**Kim, J.Y., Kim, K.W., Kwon, H.J., Lee, D.W., and Yoo, J.S. (2002). P**robing lysine acetylation with a modification-specific marker ion using high-performance liquid chromatography/electrospray-mass spectrometry with collision-induced dissociation. Analytical chemistry *74*, 5443-5449.

**Kimura, A., Matsubara, K., and Horikoshi, M. (2005).** A decade of histone acetylation: marking eukaryotic chromosomes with specific codes. J Biochem *138*, 647-662.

**Kleiman, L. (2002).** tRNA(Lys3): the primer tRNA for reverse transcription in HIV-1. IUBMB Life *53*, 107-114.

**Konig, R., Zhou, Y., Elleder, D., Diamond, T.L., Bonamy, G.M., Irelan, J.T., Chiang, C.Y., Tu, B.P., De Jesus, P.D., Lilley, C.E.*, et al.* (2008).** Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell *135*, 49-60.

**Kouzarides, T. (2000).** Acetylation: a regulatory modification to rival phosphorylation? EMBO J *19*, 1176-1179.

**Krishnan, L., Matreyek, K.A., Oztop, I., Lee, K., Tipper, C.H., Li, X., Dar, M.J., Kewalramani, V.N., and Engelman, A. (2010).** The requirement for cellular transportin 3 (TNPO3 or TRN-SR2) during infection maps to human immunodeficiency virus type 1 capsid and not integrase. J Virol *84*, 397-406.

**Kutay, U., Bischoff, F.R., Kostka, S., Kraft, R., and Gorlich, D. (1997).** Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell *90*, 1061-1071.

**Lau, A., Kanaar, R., Jackson, S.P., and O'Connor, M.J. (2004).** Suppression of retroviral infection by the RAD52 DNA repair protein. EMBO J *23*, 3421-3429.

**Lau, A., Swinbank, K.M., Ahmed, P.S., Taylor, D.L., Jackson, S.P., Smith, G.C., and O'Connor, M.J. (2005).** Suppression of HIV-1 infection by a small molecule inhibitor of the ATM kinase. Nat Cell Biol *7*, 493-500.

**Le Rouzic, E., and Benichou, S. (2005).** The Vpr protein from HIV-1: distinct roles along the viral life cycle. Retrovirology *2*, 11.

**Lechner, M.S., Begg, G.E., Speicher, D.W., and Rauscher, F.J., 3rd (2000).** Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. Mol Cell Biol *20*, 6449-6465.

**Lee, K., Ambrose, Z., Martin, T.D., Oztop, I., Mulky, A., Julias, J.G., Vandegraaff, N., Baumann, J.G., Wang, R., Yuen, W.*, et al.* (2010).** Flexible use of nuclear import pathways by HIV-1. Cell Host Microbe *7*, 221-233.

**Lee, K.K., and Workman, J.L. (2007).** Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev Mol Cell Biol *8*, 284-295**.**

**Lee, S.P., Xiao, J., Knutson, J.R., Lewis, M.S., and Han, M.K. (1997).** Zn2+ promotes the self-association of human immunodeficiency virus type-1 integrase in vitro. Biochemistry *36*, 173-180.

**Lee, Y.K., Thomas, S.N., Yang, A.J., and Ann, D.K. (2007).** Doxorubicin down-regulates Kruppel-associated box domain-associated protein 1 sumoylation that relieves its transcription repression on p21WAF1/CIP1 in breast cancer MCF-7 cells. J Biol Chem *282*, 1595-1606.

**Lewinski, M.K., and Bushman, F.D. (2005).** Retroviral DNA integration--mechanism and consequences. Adv Genet *55*, 147-181.

**Lewinski, M.K., Yamashita, M., Emerman, M., Ciuffi, A., Marshall, H., Crawford, G., Collins, F., Shinn, P., Leipzig, J., Hannenhalli, S.*, et al.* (2006).** Retroviral DNA integration: viral and cellular determinants of target-site selection. PLoS Pathog *2*, e60.

**Lewis, P., Hensel, M., and Emerman, M. (1992).** Human immunodeficiency virus infection of cells arrested in the cell cycle. EMBO J *11*, 3053-3058.

**Li, L., Olvera, J.M., Yoder, K.E., Mitchell, R.S., Butler, S.L., Lieber, M., Martin, S.L., and Bushman, F.D. (2001).** Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. EMBO J *20*, 3272-3281.

**Li, L., Yoder, K., Hansen, M.S., Olvera, J., Miller, M.D., and Bushman, F.D. (2000).** Retroviral cDNA integration: stimulation by HMG I family proteins. J Virol *74*, 10965-10974.

**Li, M., Mizuuchi, M., Burke, T.R., Jr., and Craigie, R. (2006).** Retroviral DNA integration: reaction pathway and critical intermediates. EMBO J *25*, 1295-1304.

**Li, X., Lee, Y.K., Jeng, J.C., Yen, Y., Schultz, D.C., Shih, H.M., and Ann, D.K. (2007).** Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. J Biol Chem *282*, 36177-36189.

**Li, X., and Sodroski, J. (2008).** The TRIM5alpha B-box 2 domain promotes cooperative binding to the retroviral capsid by mediating higher-order self-association. J Virol *82*, 11495-11502.

**Limon, A., Devroe, E., Lu, R., Ghory, H.Z., Silver, P.A., and Engelman, A. (2002).** Nuclear localization of human immunodeficiency virus type 1 preintegration complexes (PICs): V165A and R166A are pleiotropic integrase mutants primarily defective for integration, not PIC nuclear import. J Virol *76*, 10598-10607.

**Liu, X., Wang, L., Zhao, K., Thompson, P.R., Hwang, Y., Marmorstein, R., and Cole, P.A. (2008).** The structural basis of protein acetylation by the p300/CBP transcriptional coactivator. Nature *451*, 846-850.

**Llano, M., Delgado, S., Vanegas, M., and Poeschla, E.M. (2004a).** Lens epithelium-derived growth factor/p75 prevents proteasomal degradation of HIV-1 integrase. J Biol Chem *279*, 55570-55577.

**Llano, M., Saenz, D.T., Meehan, A., Wongthida, P., Peretz, M., Walker, W.H., Teo, W., and Poeschla, E.M. (2006).** An essential role for LEDGF/p75 in HIV integration. Science *314*, 461-464.

**Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., and Poeschla, E.M. (2004b).** LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. J Virol *78*, 9524-9537.

**Lloyd, A.G., Tateishi, S., Bieniasz, P.D., Muesing, M.A., Yamaizumi, M., and Mulder, L.C. (2006).** Effect of DNA repair protein Rad18 on viral infection. PLoS Pathog *2*, e40.

**Lopez-Verges, S., Camus, G., Blot, G., Beauvoir, R., Benarous, R., and Berlioz-Torrent, C. (2006).** Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions. Proc Natl Acad Sci U S A *103*, 14947-14952.

**Luban, J. (2007).** Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. J Virol *81*, 1054-1061.

**Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000).** Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature *408*, 377-381.

**Luo, K., Wang, T., Liu, B., Tian, C., Xiao, Z., Kappes, J., and Yu, X.F. (2007).** Cytidine deaminases APOBEC3G and APOBEC3F interact with human immunodeficiency virus type 1 integrase and inhibit proviral DNA formation. J Virol *81*, 7238-7248.

**Lutzke, R.A., and Plasterk, R.H. (1998).** Structure-based mutational analysis of the C-terminal DNA-binding domain of human immunodeficiency virus type 1 integrase: critical residues for protein oligomerization and DNA binding. J Virol *72*, 4841-4848.

**Lutzke, R.A., Vink, C., and Plasterk, R.H. (1994).** Characterization of the minimal DNA-binding domain of the HIV integrase protein. Nucleic Acids Res *22*, 4125-4131.

**Madani, N., and Kabat, D. (1998).** An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. J Virol *72*, 10251-10255.

**Maertens, G., Cherepanov, P., Debyser, Z., Engelborghs, Y., and Engelman, A. (2004).** Identification and characterization of a functional nuclear localization signal in the HIV-1 integrase interactor LEDGF/p75. J Biol Chem *279*, 33421-33429.

**Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., and Engelborghs, Y. (2003).** LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. J Biol Chem *278*, 33528-33539.

**Malim, M.H. (2009).** APOBEC proteins and intrinsic resistance to HIV-1 infection. Philos Trans R Soc Lond B Biol Sci *364*, 675-687.

**Manganaro, L., Lusic, M., Gutierrez, M.I., Cereseto, A., Del Sal, G., and Giacca, M. (2010).** Concerted action of cellular JNK and Pin1 restricts HIV-1 genome integration to activated CD4+ T lymphocytes. Nat Med *16*, 329-333.

**Mansky, L.M., and Temin, H.M. (1995).** Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol *69*, 5087-5094.

**Marcello, A. (2006).** Latency: the hidden HIV-1 challenge. Retrovirology *3*, 7.

**Maroun, M., Delelis, O., Coadou, G., Bader, T., Segeral, E., Mbemba, G., Petit, C., Sonigo, P., Rain, J.C., Mouscadet, J.F.*, et al.* (2006).** Inhibition of early steps of HIV-1 replication by SNF5/Ini1. J Biol Chem *281*, 22736-22743.

**Marshall, H.M., Ronen, K., Berry, C., Llano, M., Sutherland, H., Saenz, D., Bickmore, W., Poeschla, E., and Bushman, F.D. (2007).** Role of PSIP1/LEDGF/p75 in lentiviral infectivity and integration targeting. PLoS One *2*, e1340.

**Martinez-Balbas, M.A., Bauer, U.M., Nielsen, S.J., Brehm, A., and Kouzarides, T. (2000).** Regulation of E2F1 activity by acetylation. EMBO J *19*, 662-671.

**Marzio, G., Wagener, C., Gutierrez, M.I., Cartwright, P., Helin, K., and Giacca, M. (2000).** E2F family members are differentially regulated by reversible acetylation. J Biol Chem *275*, 10887-10892.

**Mayer, B.J. (2001).** SH3 domains: complexity in moderation. J Cell Sci *114*, 1253-1263.

**McDonald, D., Vodicka, M.A., Lucero, G., Svitkina, T.M., Borisy, G.G., Emerman, M., and Hope, T.J. (2002).** Visualization of the intracellular behavior of HIV in living cells. J Cell Biol *159*, 441-452.

**Miller, M.D., Farnet, C.M., and Bushman, F.D. (1997).** Human immunodeficiency virus type 1 preintegration complexes: studies of organization and composition. J Virol *71*, 5382-5390.

**Mitchell, R.S., Beitzel, B.F., Schroder, A.R., Shinn, P., Chen, H., Berry, C.C., Ecker, J.R., and Bushman, F.D. (2004).** Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol *2*, E234.

**Miyauchi, K., Kim, Y., Latinovic, O., Morozov, V., and Melikyan, G.B. (2009).** HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. Cell *137*, 433-444.

**Morozov, A., Yung, E., and Kalpana, G.V. (1998).** Structure-function analysis of integrase interactor 1/hSNF5L1 reveals differential properties of two repeat motifs present in the highly conserved region. Proc Natl Acad Sci U S A *95*, 1120-1125.

**Mousnier, A., Kubat, N., Massias-Simon, A., Segeral, E., Rain, J.C., Benarous, R., Emiliani, S., and Dargemont, C. (2007).** von Hippel Lindau binding protein 1-mediated degradation of integrase affects HIV-1 gene expression at a postintegration step. Proc Natl Acad Sci U S A *104*, 13615-13620.

**Mujtaba, S., He, Y., Zeng, L., Farooq, A., Carlson, J.E., Ott, M., Verdin, E., and Zhou, M.M. (2002).** Structural basis of lysine-acetylated HIV-1 Tat recognition by PCAF bromodomain. Mol Cell *9*, 575-586.

**Mujtaba, S., He, Y., Zeng, L., Yan, S., Plotnikova, O., Sachchidanand, Sanchez, R., Zeleznik-Le, N.J., Ronai, Z., and Zhou, M.M. (2004).** Structural mechanism of the bromodomain of the coactivator CBP in p53 transcriptional activation. Mol Cell *13*, 251-263.

**Mujtaba, S., Zeng, L., and Zhou, M.M. (2007).** Structure and acetyl-lysine recognition of the bromodomain. Oncogene *26*, 5521-5527.

**Mulder, L.C., Chakrabarti, L.A., and Muesing, M.A. (2002).** Interaction of HIV-1 integrase with DNA repair protein hRad18. J Biol Chem *277*, 27489-27493**.**

**Murakami, T., and Freed, E.O. (2000).** Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. J Virol *74*, 3548-3554.

**Nakamura, M., Masuda, H., Horii, J., Kuma, K., Yokoyama, N., Ohba, T., Nishitani, H., Miyata, T., Tanaka, M., and Nishimoto, T. (1998).** When overexpressed, a novel centrosomal protein, RanBPM, causes ectopic microtubule nucleation similar to gamma-tubulin. J Cell Biol *143*, 1041-1052.

**Nakayama, E.E., and Shioda, T. (2010).** Anti-retroviral activity of TRIM5 alpha. Rev Med Virol *20*, 77-92.

**Neil, S.J., Eastman, S.W., Jouvenet, N., and Bieniasz, P.D. (2006).** HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. PLoS Pathog *2*, e39.

**Neil, S.J., Sandrin, V., Sundquist, W.I., and Bieniasz, P.D. (2007).** An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein. Cell Host Microbe *2*, 193-203.

**Neil, S.J., Zang, T., and Bieniasz, P.D. (2008).** Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature *451*, 425-430.

**Nielsen, A.L., Ortiz, J.A., You, J., Oulad-Abdelghani, M., Khechumian, R., Gansmuller, A., Chambon, P., and Losson, R. (1999).** Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. EMBO J *18*, 6385-6395.

**Nisole, S., and Saib, A. (2004).** Early steps of retrovirus replicative cycle. Retrovirology *1*, 9.

**Nisole, S., Stoye, J.P., and Saib, A. (2005).** TRIM family proteins: retroviral restriction and antiviral defence. Nat Rev Microbiol *3*, 799-808.

**O'Brien, W.A., Koyanagi, Y., Namazie, A., Zhao, J.Q., Diagne, A., Idler, K., Zack, J.A., and Chen, I.S. (1990).** HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. Nature *348*, 69-73.

**O'Geen, H., Squazzo, S.L., Iyengar, S., Blahnik, K., Rinn, J.L., Chang, H.Y., Green, R., and Farnham, P.J. (2007).** Genome-wide analysis of KAP1 binding suggests autoregulation of KRAB-ZNFs. PLoS Genet *3*, e89.

**Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K.*, et al.* (2004).** Complete sequencing and characterization of 21,243 full-length human cDNAs. Nat Genet *36*, 40-45.

**Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008).** TRIM family proteins and their emerging roles in innate immunity. Nat Rev Immunol *8*, 849-860.

**Panganiban, A.T., and Temin, H.M. (1984).** The retrovirus pol gene encodes a product required for DNA integration: identification of a retrovirus int locus. Proc Natl Acad Sci U S A *81*, 7885-7889.

**Parissi, V., Calmels, C., De Soultrait, V.R., Caumont, A., Fournier, M., Chaignepain, S., and Litvak, S. (2001).** Functional interactions of human immunodeficiency virus type 1 integrase with human and yeast HSP60. J Virol *75*, 11344-11353.

**Peng, G., Greenwell-Wild, T., Nares, S., Jin, W., Lei, K.J., Rangel, Z.G., Munson, P.J., and Wahl, S.M. (2007).** Myeloid differentiation and susceptibility to HIV-1 are linked to APOBEC3 expression. Blood *110*, 393-400.

**Peng, H., Begg, G.E., Schultz, D.C., Friedman, J.R., Jensen, D.E., Speicher, D.W., and Rauscher, F.J., 3rd (2000).** Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. J Mol Biol *295*, 1139-1162.

**Pengue, G., Caputo, A., Rossi, C., Barbanti-Brodano, G., and Lania, L. (1995).** Transcriptional silencing of human immunodeficiency virus type 1 long terminal repeat-driven gene expression by the Kruppel-associated box repressor domain targeted to the transactivating response element. J Virol *69*, 6577-6580.

**Perez-Caballero, D., Hatziioannou, T., Yang, A., Cowan, S., and Bieniasz, P.D. (2005a).** Human tripartite motif 5alpha domains responsible for retrovirus restriction activity and specificity. J Virol *79*, 8969-8978.

**Perez-Caballero, D., Hatziioannou, T., Zhang, F., Cowan, S., and Bieniasz, P.D. (2005b).** Restriction of human immunodeficiency virus type 1 by TRIM-CypA occurs with rapid kinetics and independently of cytoplasmic bodies, ubiquitin, and proteasome activity. J Virol *79*, 15567-15572.

**Peterlin, B.M., and Trono, D. (2003).** Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat Rev Immunol *3*, 97-107.

**Pflum, M.K., Tong, J.K., Lane, W.S., and Schreiber, S.L. (2001).** Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation. J Biol Chem *276*, 47733-47741.

**Pion, M., Granelli-Piperno, A., Mangeat, B., Stalder, R., Correa, R., Steinman, R.M., and Piguet, V. (2006).** APOBEC3G/3F mediates intrinsic resistance of monocyte-derived dendritic cells to HIV-1 infection. J Exp Med *203*, 2887-2893.

**Polesskaya, A., and Harel-Bellan, A. (2001).** Acetylation of MyoD by p300 requires more than its histone acetyltransferase domain. J Biol Chem *276*, 44502-44503.

**Polesskaya, A., Naguibneva, I., Duquet, A., Bengal, E., Robin, P., and Harel-Bellan, A. (2001).** Interaction between acetylated MyoD and the bromodomain of CBP and/or p300. Mol Cell Biol *21*, 5312-5320.

**Pollard, V.W., and Malim, M.H. (1998).** The HIV-1 Rev protein. Annu Rev Microbiol *52*, 491-532.

**Popov, S., Rexach, M., Zybarth, G., Reiling, N., Lee, M.A., Ratner, L., Lane, C.M., Moore, M.S., Blobel, G., and Bukrinsky, M. (1998).** Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. EMBO J *17*, 909-917.

**Popovic, M., Sarngadharan, M.G., Read, E., and Gallo, R.C. (1984).** Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science *224*, 497-500.

**Priet, S., Navarro, J.M., Querat, G., and Sire, J. (2003).** Reversion of the lethal phenotype of an HIV-1 integrase mutant virus by overexpression of the same integrase mutant protein. J Biol Chem *278*, 20724-20730.

**Rachez, C., and Freedman, L.P. (2001).** Mediator complexes and transcription. Curr Opin Cell Biol *13*, 274-280.

**Ragvin, A., Valvatne, H., Erdal, S., Arskog, V., Tufteland, K.R., Breen, K., AM, O.Y., Eberharter, A., Gibson, T.J., Becker, P.B.*, et al.* (2004).** Nucleosome binding by the bromodomain and PHD finger of the transcriptional cofactor p300. J Mol Biol *337*, 773-788.

**Rahman, S., Lu, R., Vandegraaff, N., Cherepanov, P., and Engelman, A. (2007).** Structure-based mutagenesis of the integrase-LEDGF/p75 interface uncouples a strict correlation between in vitro protein binding and HIV-1 fitness. Virology *357*, 79-90.

**Rain, J.C., Cribier, A., Gerard, A., Emiliani, S., and Benarous, R. (2009).** Yeast two-hybrid detection of integrase-host factor interactions. Methods *47*, 291-297.

**Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K.*, et al.* (1985).** Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature *313*, 277-284.

**Reiser, J., Harmison, G., Kluepfel-Stahl, S., Brady, R.O., Karlsson, S., and Schubert, M. (1996).** Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc Natl Acad Sci U S A *93*, 15266-15271.

**Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R.A., and Dynlacht, B.D. (2002).** E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes Dev *16*, 245-256.

**Ren, G., Gao, K., Bushman, F.D., and Yeager, M. (2007).** Single-particle image reconstruction of a tetramer of HIV integrase bound to DNA. J Mol Biol *366*, 286-294.

**Rey, F., BouHamdan, M., Navarro, J.M., Agostini, I., Willetts, K., Bouyac, M., Tamalet, C., Spire, B., Vigne, R., and Sire, J. (1998).** A role for human immunodeficiency virus type 1 Vpr during infection of peripheral blood mononuclear cells. J Gen Virol *79 ( Pt 5)*, 1083-1087.

**Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S.*, et al.* (2001).** The tripartite motif family identifies cell compartments. EMBO J *20*, 2140-2151.

**Rice, P.A., and Baker, T.A. (2001).** Comparative architecture of transposase and integrase complexes. Nat Struct Biol *8*, 302-307.

**Riviere, L., Darlix, J.L., and Cimarelli, A. (2010).** Analysis of the viral elements required in the nuclear import of HIV-1 DNA. J Virol *84*, 729-739.

**Roe, T., Reynolds, T.C., Yu, G., and Brown, P.O. (1993).** Integration of murine leukemia virus DNA depends on mitosis. EMBO J *12*, 2099-2108.

**Rold, C.J., and Aiken, C. (2008).** Proteasomal degradation of TRIM5alpha during retrovirus restriction. PLoS Pathog *4*, e1000074.

**Roth, S.Y., Denu, J.M., and Allis, C.D. (2001).** Histone acetyltransferases. Annu Rev Biochem *70*, 81-120.

**Rowe, H.M., Jakobsson, J., Mesnard, D., Rougemont, J., Reynard, S., Aktas, T., Maillard, P.V., Layard-Liesching, H., Verp, S., Marquis, J.*, et al.* (2010).** KAP1 controls endogenous retroviruses in embryonic stem cells. Nature *463*, 237-240.

**Sabo, A., Lusic, M., Cereseto, A., and Giacca, M. (2008).** Acetylation of conserved lysines in the catalytic core of cyclin-dependent kinase 9 inhibits kinase activity and regulates transcription. Mol Cell Biol *28*, 2201-2212.

**Sadoul, K., Boyault, C., Pabion, M., and Khochbin, S. (2008).** Regulation of protein turnover by acetyltransferases and deacetylases. Biochimie *90*, 306-312.

**Sanchez-Pescador, R., Power, M.D., Barr, P.J., Steimer, K.S., Stempien, M.M., Brown-Shimer, S.L., Gee, W.W., Renard, A., Randolph, A., Levy, J.A.*, et al.* (1985).** Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). Science *227*, 484-492.

**Satou, A., Taira, T., Iguchi-Ariga, S.M., and Ariga, H. (2001).** A novel transrepression pathway of c-Myc. Recruitment of a transcriptional corepressor complex to c-Myc by MM-1, a c-Myc-binding protein. J Biol Chem *276*, 46562-46567.

**Sayah, D.M., Sokolskaja, E., Berthoux, L., and Luban, J. (2004).** Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature *430*, 569-573.

**Schroder, A.R., Shinn, P., Chen, H., Berry, C., Ecker, J.R., and Bushman, F. (2002).** HIV-1 integration in the human genome favors active genes and local hotspots. Cell *110*, 521-529.

**Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., and Rauscher, F.J., 3rd (2002).** SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev *16*, 919-932.

**Schultz, D.C., Friedman, J.R., and Rauscher, F.J., 3rd (2001).** Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes Dev *15*, 428-443.

**Sengupta, N., and Seto, E. (2004).** Regulation of histone deacetylase activities. J Cell Biochem *93*, 57-67.

**Sheehy, A.M., Gaddis, N.C., Choi, J.D., and Malim, M.H. (2002).** Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature *418*, 646-650.

**Shen, H., Cheng, T., Preffer, F.I., Dombkowski, D., Tomasson, M.H., Golan, D.E., Yang, O., Hofmann, W., Sodroski, J.G., Luster, A.D.*, et al.* (1999).** Intrinsic human immunodeficiency virus type 1 resistance of hematopoietic stem cells despite coreceptor expression. J Virol *73*, 728-737.

**Short, K.M., and Cox, T.C. (2006).** Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. J Biol Chem *281*, 8970-8980.

**Shun, M.C., Raghavendra, N.K., Vandegraaff, N., Daigle, J.E., Hughes, S., Kellam, P., Cherepanov, P., and Engelman, A. (2007).** LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. Genes Dev *21*, 1767-1778.

**Shyu, A.B., and Wilkinson, M.F. (2000).** The double lives of shuttling mRNA binding proteins. Cell *102*, 135-138.

**Simon, J.H., Miller, D.L., Fouchier, R.A., Soares, M.A., Peden, K.W., and Malim, M.H. (1998).** The regulation of primate immunodeficiency virus infectivity by Vif is cell species restricted: a role for Vif in determining virus host range and cross-species transmission. EMBO J *17*, 1259-1267.

**Singh, R., Gaiha, G., Werner, L., McKim, K., Mlisana, K., Luban, J., Walker, B.D., Karim, S.S., Brass, A.L., and Ndung'u, T. (2011).** Association of TRIM22 with the type 1 interferon response and viral control during primary HIV-1 infection. J Virol *85*, 208-216.

**Sokolskaja, E., and Luban, J. (2006).** Cyclophilin, TRIM5, and innate immunity to HIV-1. Curr Opin Microbiol *9*, 404-408.

**Solsbacher, J., Maurer, P., Bischoff, F.R., and Schlenstedt, G. (1998).** Cse1p is involved in export of yeast importin alpha from the nucleus. Mol Cell Biol *18*, 6805-6815.

**Sorin, M., Yung, E., Wu, X., and Kalpana, G.V. (2006).** HIV-1 replication in cell lines harboring INI1/hSNF5 mutations. Retrovirology *3*, 56.

**Spange, S., Wagner, T., Heinzel, T., and Kramer, O.H. (2009).** Acetylation of non-histone proteins modulates cellular signalling at multiple levels. Int J Biochem Cell Biol *41*, 185-198.

**Sripathy, S.P., Stevens, J., and Schultz, D.C. (2006).** The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. Mol Cell Biol *26*, 8623-8638.

**Sterner, D.E., and Berger, S.L. (2000).** Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev *64*, 435-459.

**Stevenson, M., Stanwick, T.L., Dempsey, M.P., and Lamonica, C.A. (1990).** HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J *9*, 1551-1560.

**Strahl, B.D., and Allis, C.D. (2000).** The language of covalent histone modifications. Nature *403*, 41-45.

**Strebel, K., Luban, J., and Jeang, K.T. (2009).** Human cellular restriction factors that target HIV-1 replication. BMC Med *7*, 48.

**Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004).** The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature *427*, 848-853.

**Stremlau, M., Perron, M., Lee, M., Li, Y., Song, B., Javanbakht, H., Diaz-Griffero, F., Anderson, D.J., Sundquist, W.I., and Sodroski, J. (2006).** Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor. Proc Natl Acad Sci U S A *103*, 5514-5519.

**Stremlau, M., Perron, M., Welikala, S., and Sodroski, J. (2005).** Species-specific variation in the B30.2(SPRY) domain of TRIM5alpha determines the potency of human immunodeficiency virus restriction. J Virol *79*, 3139-3145.

**Studamire, B., and Goff, S.P. (2008).** Host proteins interacting with the Moloney murine leukemia virus integrase: multiple transcriptional regulators and chromatin binding factors. Retrovirology *5*, 48.

**Suzuki, Y., and Craigie, R. (2007).** The road to chromatin - nuclear entry of retroviruses. Nat Rev Microbiol *5*, 187-196.

**Symington, L.S. (2002).** Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microbiol Mol Biol Rev *66*, 630-670, table of contents.

**Tan, W., Dong, Z., Wilkinson, T.A., Barbas, C.F., 3rd, and Chow, S.A. (2006).** Human immunodeficiency virus type 1 incorporated with fusion proteins consisting of integrase and the designed polydactyl zinc finger protein E2C can bias integration of viral DNA into a predetermined chromosomal region in human cells. J Virol *80*, 1939-1948.

**Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. (2008).** Acetylation is indispensable for p53 activation. Cell *133*, 612-626.

**Terreni, M., Valentini, P., Liverani, V., Gutierrez, M.I., Di Primio, C., Di Fenza, A., Tozzini, V., Allouch, A., Albanese, A., Giacca, M.*, et al.* (2010).** GCN5-dependent acetylation of HIV-1 integrase enhances viral integration. Retrovirology *7*, 18.

**Thys, W., De Houwer, S., Demeulemeester, J., Taltynov, O., Vancraenenbroeck, R., Gerard, M., De Rijck, J., Gijsbers, R., Christ, F., and Debyser, Z. (2011).** Interplay between HIV Entry and Transportin-SR2 Dependency. Retrovirology *8*, 7.

**Tian, C., Xing, G., Xie, P., Lu, K., Nie, J., Wang, J., Li, L., Gao, M., Zhang, L., and He, F. (2009).** KRAB-type zinc-finger protein Apak specifically regulates p53-dependent apoptosis. Nat Cell Biol *11*, 580-591.

**Tissot, C., and Mechti, N. (1995).** Molecular cloning of a new interferon-induced factor that represses human immunodeficiency virus type 1 long terminal repeat expression. J Biol Chem *270*, 14891-14898.

**Topper, M., Luo, Y., Zhadina, M., Mohammed, K., Smith, L., and Muesing, M.A. (2007).** Posttranslational acetylation of the human immunodeficiency virus type 1 integrase carboxyl-terminal domain is dispensable for viral replication. J Virol *81*, 3012-3017.

**Towers, G.J. (2007).** The control of viral infection by tripartite motif proteins and cyclophilin A. Retrovirology *4*, 40.

**Towers, G.J., Hatziioannou, T., Cowan, S., Goff, S.P., Luban, J., and Bieniasz, P.D. (2003).** Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat Med *9*, 1138-1143.

**Trelle, M.B., and Jensen, O.N. (2008).** Utility of immonium ions for assignment of epsilon-N-acetyllysine-containing peptides by tandem mass spectrometry. Analytical chemistry *80*, 3422-3430.

**Tsuruma, R., Ohbayashi, N., Kamitani, S., Ikeda, O., Sato, N., Muromoto, R., Sekine, Y., Oritani, K., and Matsuda, T. (2008).** Physical and functional interactions between STAT3 and KAP1. Oncogene *27*, 3054-3059.

**Turelli, P., Doucas, V., Craig, E., Mangeat, B., Klages, N., Evans, R., Kalpana, G., and Trono, D. (2001).** Cytoplasmic recruitment of INI1 and PML on incoming HIV preintegration complexes: interference with early steps of viral replication. Mol Cell *7*, 1245-1254.

**Turlure, F., Devroe, E., Silver, P.A., and Engelman, A. (2004).** Human cell proteins and human immunodeficiency virus DNA integration. Front Biosci *9*, 3187-3208.

**Turlure, F., Maertens, G., Rahman, S., Cherepanov, P., and Engelman, A. (2006).** A tripartite DNA-binding element, comprised of the nuclear localization signal and two AT-hook motifs, mediates the association of LEDGF/p75 with chromatin in vivo. Nucleic Acids Res *34*, 1653-1665.

**Uchil, P.D., Quinlan, B.D., Chan, W.T., Luna, J.M., and Mothes, W. (2008).** TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. PLoS Pathog *4*, e16.

**Underhill, C., Qutob, M.S., Yee, S.P., and Torchia, J. (2000).** A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. J Biol Chem *275*, 40463-40470.

**Urrutia, R. (2003).** KRAB-containing zinc-finger repressor proteins. Genome Biol *4*, 231.

**Valente, S.T., Gilmartin, G.M., Mott, C., Falkard, B., and Goff, S.P. (2009).** Inhibition of HIV-1 replication by eIF3f. Proc Natl Acad Sci U S A *106*, 4071-4078.

**Van Damme, N., Goff, D., Katsura, C., Jorgenson, R.L., Mitchell, R., Johnson, M.C., Stephens, E.B., and Guatelli, J. (2008).** The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. Cell Host Microbe *3*, 245-252.

**van Gent, D.C., Groeneger, A.A., and Plasterk, R.H. (1992).** Mutational analysis of the integrase protein of human immunodeficiency virus type 2. Proc Natl Acad Sci U S A *89*, 9598-9602.

**van Gent, D.C., Mizuuchi, K., and Gellert, M. (1996).** Similarities between initiation of V(D)J recombination and retroviral integration. Science *271*, 1592-1594.

**van Gent, D.C., Vink, C., Groeneger, A.A., and Plasterk, R.H. (1993).** Complementation between HIV integrase proteins mutated in different domains. EMBO J *12*, 3261-3267.

**Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F., and Debyser, Z. (2006).** Cellular co-factors of HIV-1 integration. Trends Biochem Sci *31*, 98-105.

**Vandegraaff, N., Devroe, E., Turlure, F., Silver, P.A., and Engelman, A. (2006).** Biochemical and genetic analyses of integrase-interacting proteins lens epithelium-derived growth factor (LEDGF)/p75 and hepatoma-derived growth factor related protein 2 (HRP2) in preintegration complex function and HIV-1 replication. Virology *346*, 415-426.

**Vandegraaff, N., and Engelman, A. (2007).** Molecular mechanisms of HIV integration and therapeutic intervention. Expert Rev Mol Med *9*, 1-19.

**Vandekerckhove, L., Christ, F., Van Maele, B., De Rijck, J., Gijsbers, R., Van den Haute, C., Witvrouw, M., and Debyser, Z. (2006).** Transient and stable knockdown of the integrase cofactor LEDGF/p75 reveals its role in the replication cycle of human immunodeficiency virus. J Virol *80*, 1886-1896.

**Varthakavi, V., Smith, R.M., Bour, S.P., Strebel, K., and Spearman, P. (2003).** Viral protein U counteracts a human host cell restriction that inhibits HIV-1 particle production. Proc Natl Acad Sci U S A *100*, 15154-15159.

**Vink, C., Groenink, M., Elgersma, Y., Fouchier, R.A., Tersmette, M., and Plasterk, R.H. (1990).** Analysis of the junctions between human immunodeficiency virus type 1 proviral DNA and human DNA. J Virol *64*, 5626-5627.

**Violot, S., Hong, S.S., Rakotobe, D., Petit, C., Gay, B., Moreau, K., Billaud, G., Priet, S., Sire, J., Schwartz, O.*, et al.* (2003).** The human polycomb group EED protein interacts with the integrase of human immunodeficiency virus type 1. J Virol *77*, 12507-12522.

**von Schwedler, U., Kornbluth, R.S., and Trono, D. (1994).** The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. Proc Natl Acad Sci U S A *91*, 6992-6996.

**von Schwedler, U., Song, J., Aiken, C., and Trono, D. (1993).** Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. J Virol *67*, 4945-4955.

**Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., and Alizon, M. (1985).** Nucleotide sequence of the AIDS virus, LAV. Cell *40*, 9-17.

**Wang, C., Ivanov, A., Chen, L., Fredericks, W.J., Seto, E., Rauscher, F.J., 3rd, and Chen, J. (2005a).** MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. EMBO J *24*, 3279-3290.

**Wang, C., Rauscher, F.J., 3rd, Cress, W.D., and Chen, J. (2007a).** Regulation of E2F1 function by the nuclear corepressor KAP1. J Biol Chem *282*, 29902-29909.

**Wang, G.P., Ciuffi, A., Leipzig, J., Berry, C.C., and Bushman, F.D. (2007b).** HIV integration site selection: analysis by massively parallel pyrosequencing reveals association with epigenetic modifications. Genome Res *17*, 1186-1194.

**Wang, L., Tang, Y., Cole, P.A., and Marmorstein, R. (2008).** Structure and chemistry of the p300/CBP and Rtt109 histone acetyltransferases: implications for histone acetyltransferase evolution and function. Curr Opin Struct Biol *18*, 741-747.

**Wang, R., Cherukuri, P., and Luo, J. (2005b).** Activation of Stat3 sequence-specific DNA binding and transcription by p300/CREB-binding protein-mediated acetylation. J Biol Chem *280*, 11528-11534.

**Wang, W., Cote, J., Xue, Y., Zhou, S., Khavari, P.A., Biggar, S.R., Muchardt, C., Kalpana, G.V., Goff, S.P., Yaniv, M.*, et al.* (1996).** Purification and biochemical heterogeneity of the mammalian SWI-SNF complex. EMBO J *15*, 5370-5382.

**Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H., and Jones, K.A. (1998).** A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell *92*, 451-462.

**Weichold, F.F., Zella, D., Barabitskaja, O., Maciejewski, J.P., Dunn, D.E., Sloand, E.M., and Young, N.S. (1998).** Neither human immunodeficiency virus-1 (HIV-1) nor HIV-2 infects most-primitive human hematopoietic stem cells as assessed in long-term bone marrow cultures. Blood *91*, 907-915.

**West, K.L. (2004).** HMGN proteins play roles in DNA repair and gene expression in mammalian cells. Biochem Soc Trans *32*, 918-919.

**White, D.E., Negorev, D., Peng, H., Ivanov, A.V., Maul, G.G., and Rauscher, F.J., 3rd (2006).** KAP1, a novel substrate for PIKK family members, colocalizes with numerous damage response factors at DNA lesions. Cancer research *66*, 11594-11599.

**Willetts, K.E., Rey, F., Agostini, I., Navarro, J.M., Baudat, Y., Vigne, R., and Sire, J. (1999).** DNA repair enzyme uracil DNA glycosylase is specifically incorporated into human immunodeficiency virus type 1 viral particles through a Vpr-independent mechanism. J Virol *73*, 1682-1688.

**Wilson, S.J., Webb, B.L., Ylinen, L.M., Verschoor, E., Heeney, J.L., and Towers, G.J. (2008).** Independent evolution of an antiviral TRIMCyp in rhesus macaques. Proc Natl Acad Sci U S A *105*, 3557-3562.

**Wolf, D., Cammas, F., Losson, R., and Goff, S.P. (2008a).** Primer binding site-dependent restriction of murine leukemia virus requires HP1 binding by TRIM28. J Virol *82*, 4675-4679.

**Wolf, D., and Goff, S.P. (2007).** TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell *131*, 46-57.

**Wolf, D., and Goff, S.P. (2009).** Embryonic stem cells use ZFP809 to silence retroviral DNAs. Nature *458*, 1201-1204.

**Wolf, D., Hug, K., and Goff, S.P. (2008b).** TRIM28 mediates primer binding site-targeted silencing of Lys1,2 tRNA-utilizing retroviruses in embryonic cells. Proc Natl Acad Sci U S A *105*, 12521-12526.

**Woodward, C.L., Prakobwanakit, S., Mosessian, S., and Chow, S.A. (2009).** Integrase interacts with nucleoporin NUP153 to mediate the nuclear import of human immunodeficiency virus type 1. J Virol *83*, 6522-6533.

**Wu-Baer, F., Lane, W.S., and Gaynor, R.B. (1996).** Identification of a group of cellular cofactors that stimulate the binding of RNA polymerase II and TRP-185 to human immunodeficiency virus 1 TAR RNA. J Biol Chem *271*, 4201-4208.

**Wu, X., Anderson, J.L., Campbell, E.M., Joseph, A.M., and Hope, T.J. (2006).** Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection. Proc Natl Acad Sci U S A *103*, 7465-7470.

**Wu, X., Li, Y., Crise, B., and Burgess, S.M. (2003).** Transcription start regions in the human genome are favored targets for MLV integration. Science *300*, 1749-1751.

**Yamashita, M., and Emerman, M. (2006).** Retroviral infection of non-dividing cells: old and new perspectives. Virology *344*, 88-93.

**Yang, X.J., and Seto, E. (2007).** HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene *26*, 5310-5318.

**Yap, M.W., Dodding, M.P., and Stoye, J.P. (2006).** Trim-cyclophilin A fusion proteins can restrict human immunodeficiency virus type 1 infection at two distinct phases in the viral life cycle. J Virol *80*, 4061-4067.

**Yap, M.W., Nisole, S., Lynch, C., and Stoye, J.P. (2004).** Trim5alpha protein restricts both HIV-1 and murine leukemia virus. Proc Natl Acad Sci U S A *101*, 10786-10791.

**Yoder, K., Sarasin, A., Kraemer, K., McIlhatton, M., Bushman, F., and Fishel, R. (2006).** The DNA repair genes XPB and XPD defend cells from retroviral infection. Proc Natl Acad Sci U S A *103*, 4622-4627.

**Yuan, Z.L., Guan, Y.J., Chatterjee, D., and Chin, Y.E. (2005).** Stat3 dimerization regulated by reversible acetylation of a single lysine residue. Science *307*, 269-273.

**Yudin, D., and Fainzilber, M. (2009).** Ran on tracks--cytoplasmic roles for a nuclear regulator. J Cell Sci *122*, 587-593.

**Yung, E., Sorin, M., Pal, A., Craig, E., Morozov, A., Delattre, O., Kappes, J., Ott, D., and Kalpana, G.V. (2001).** Inhibition of HIV-1 virion production by a transdominant mutant of integrase interactor 1. Nat Med *7*, 920-926.

**Yung, E., Sorin, M., Wang, E.J., Perumal, S., Ott, D., and Kalpana, G.V. (2004).** Specificity of interaction of INI1/hSNF5 with retroviral integrases and its functional significance. J Virol *78*, 2222-2231.

**Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A., and Chen, I.S. (1990).** HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell *61*, 213-222.

**Zack, J.A., Haislip, A.M., Krogstad, P., and Chen, I.S. (1992).** Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J Virol *66*, 1717-1725.

**Zeng, L., Yap, K.L., Ivanov, A.V., Wang, X., Mujtaba, S., Plotnikova, O., Rauscher, F.J., 3rd, and Zhou, M.M. (2008).** Structural insights into human KAP1 PHD finger-bromodomain and its role in gene silencing. Nat Struct Mol Biol *15*, 626-633.

**Zeng, L., and Zhou, M.M. (2002).** Bromodomain: an acetyl-lysine binding domain. FEBS Lett *513*, 124-128.

**Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000).** HIV-1 genome nuclear import is mediated by a central DNA flap. Cell *101*, 173-185.

**Zhang, J., Attar, E., Cohen, K., Crumpacker, C., and Scadden, D. (2005).** Silencing p21(Waf1/Cip1/Sdi1) expression increases gene transduction efficiency in primitive human hematopoietic cells. Gene Ther *12*, 1444-1452.

**Zhang, J., Scadden, D.T., and Crumpacker, C.S. (2007).** Primitive hematopoietic cells resist HIV-1 infection via p21. J Clin Invest *117*, 473-481.

**Zhang, J.Q., Wang, J.J., Li, W.J., Huang, L., Tian, L., Xue, J.L., Chen, J.Z., and Jia, W. (2009).** Cellular protein TTRAP interacts with HIV-1 integrase to facilitate viral integration. Biochem Biophys Res Commun *387*, 256-260.

**Zhang, Y., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999).** Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev *13*, 1924-1935.

**Zheng, R., Jenkins, T.M., and Craigie, R. (1996).** Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization, and enhances catalytic activity. Proc Natl Acad Sci U S A *93*, 13659-13664.

**Zheng, X.M., Black, D., Chambon, P., and Egly, J.M. (1990).** Sequencing and expression of complementary DNA for the general transcription factor BTF3. Nature *344*, 556-559.

**Zielske, S.P., and Stevenson, M. (2005).** Importin 7 may be dispensable for human immunodeficiency virus type 1 and simian immunodeficiency virus infection of primary macrophages. J Virol *79*, 11541-11546.

**Zielske, S.P., and Stevenson, M. (2006).** Modest but reproducible inhibition of human immunodeficiency virus type 1 infection in macrophages following LEDGFp75 silencing. J Virol *80*, 7275-7280.

**Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D.C., Lukas, J., Bekker-Jensen, S., Bartek, J., and Shiloh, Y. (2006).** Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. Nat Cell Biol *8*, 870-876.

**Publications**

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**Abstract** The viral protein integrase (IN) catalyzes the integration of the HIV-1 cDNA into the host cellular genome. We have recently demonstrated that IN is acetylated by a cellular histone acetyltransferase, p300, which modifies three lysines located in the C-terminus of the viral factor (Cereseto et al. in EMBO J 24:3070–3081, 2005). This modification enhances IN catalytic activity, as demonstrated by in vitro assays. Consistently, mutations introduced in the targeted lysines greatly decrease the efficiency of HIV-1 integration. Acetylation was proven to regulate protein functions by modulating protein–protein interactions. HIV-1 to efficiently complete its replication steps, including the integration reaction, requires interacting with numerous cellular factors. Therefore, we sought to investigate whether acetylation might modulate the interaction between IN and the cellular factors. To this aim we performed a yeast two-hybrid screening that differs from the screenings so far performed (Rain et al. in Methods 47:291–297, 2009; Studamire and Goff in Retrovirology 5:48, 2008) for using as bait IN constitutively acetylated. From this analysis we have identified thirteen cellular factors involved in transcription, chromatin remodeling, nuclear transport, RNA binding, protein synthesis regulation and microtubule organization. To validate these interactions, binding assays were performed showing that acetylation increases the affinity of IN with specific factors. Nevertheless, few two-hybrid hits bind with the same affinity the acetylated and the unmodified IN. These results further underlie the relevance of IN post-translational modification by acetylation in HIV-1 replication cycle.

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**Abbreviations**

| | |
|---|---|
| HIV-1 | Human immunodeficiency virus-1 |
| IN | Integrase |
| PIC | Pre-integration complex |
| GDBD | Gal4 DNA binding domain |
| GAD | Gal4 activation domain |
| HAT | Histone acetyltransferase catalytic domain |
| wt | Wild type |
| mut | Mutated |
| His | Histidine |
| Ade | Adenine |
| Ac-Lys | Acetylated lysines |
| TEV | Tobacco etch virus protease site |
| HA | Hemagglutinin epitope tag |
| WB | Western blot analysis |
| α | Antibodies |
| NLS | Nuclear localization signal |

## Introduction

An essential step in the retroviral life cycle is the integration of the viral DNA into the host cellular genome; a reaction catalyzed by the viral protein integrase (IN). Following virus entry and uncoating in the cytoplasm, the viral double strand cDNA is synthesized through reverse transcription starting from the RNA genome (Coffin et al. 1997). The viral DNA molecules then translocate into the nuclei of infected cells as part of a large nucleoprotein

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complex, the pre-integration complex (PIC), which is formed by IN and other viral/cellular proteins (Suzuki and Craigie 2007). Within PICs both ends of the linear DNA molecule are processed by IN to form recessed 3′ OH termini. At the integration site, IN uses the recessed 3′OH groups to cut opposing strands of chromosomal DNA in a staggered fashion, thus concomitantly connecting the viral DNA 3′ ends to the generated 5′ overhangs. The resultant DNA recombination intermediate harbors single strands discontinuities that must be repaired by the host cellular repair system to complete provirus formation (Vandegraaff and Engelman 2007). Purified HIV-1 IN displays 3′ processing and DNA strand transfer activities that are sufficient to catalyze the cDNA integration reaction in vitro. However, in vivo numerous cellular proteins are required for efficient integration. The host factors regulate IN enzymatic functions by modulating its stability and by mediating nuclear import and perhaps access to specific regions of the chromatin (Goff 2007; Van Maele et al. 2006). We have recently demonstrated that p300, a histone acetyltransferase, binds IN and acetylates three lysines (K264, K266, K273) located in its C-terminus leading to enhanced IN activity. Acetylable lysines are necessary for virus integration and thus for optimal replication, as demonstrated by the inefficient infectivity observed following their mutations into arginine residues (K264, 266, 273R) (Cereseto et al. 2005). Since it has been demonstrated that acetylation modulates the activities of cellular and viral proteins by affecting protein–protein interactions (Sterner and Berger 2000), in this study we investigated whether this protein modification could affect the interaction of IN with cellular factors. To this aim we have employed the tethered catalysis two-hybrid system, a method previously reported to efficiently identify factors binding specifically to acetylated proteins (p53, histones H3 and H4) (Guo et al. 2004). From this screening we identified thirteen new factors binding IN with variable affinities based on the acetylation level of the viral factor.

## Results

### Constitutive acetylation of IN fused to the HAT domain of p300

To produce constitutively acetylated IN, a cDNA cassette was constructed containing a codon-optimized sequence for IN (IN-CO) fused to the acetyltransferase catalytic domain of p300 (IN-HATwt). As control, IN was also fused to a sequence coding for a catalytically inactive HAT containing a D1395Y mutation (IN-HATmut). The IN-HAT fusion proteins produced by these constructs contain at the N-terminus a histidine tag (6xHis) which allows affinity purification of the protein product by means of cobalt based resin columns (see 'Materials and methods'). In addition, the C-terminus of the chimera is in frame with a hemagglutinin (HA) epitope tag used to analyze the fusion protein by immunoblot with anti-HA antibodies. Finally, between the IN and the HAT domains a tobacco etch virus (TEV) proteolytic cleavage site was introduced allowing IN separation from the HAT domain (Fig. 1a).

Both IN-HATwt and IN-HATmut chimera were produced and purified in bacteria (*Escherichia coli*, BL21 strain). The purified products were immunoprecipitated with anti His-tag antibodies and analyzed by Western blot using anti-acetylated lysine (Ac-Lys) antibodies to assess the acetylation status of the recombinant chimera. As shown in Fig. 1b, upper panel, high levels of acetylation were detected with the IN-HATwt chimera at the two quantities tested (1 and 5 μg), while no signal was reported with the same amounts of IN-HATmut chimera. In order to verify appropriate protein loading the same filter was also incubated with antibodies anti-IN (Fig. 1b, lower panel).

Since p300 HAT domain contains 44 lysine residues that are substrate of auto-acetylase catalytic activity, we sought to verify whether the acetylation reported with the IN-HAT chimera (Fig. 1b) included modification of the individual IN domain. To this end IN was separated from the fusion protein through TEV digestion; the cleavage product was purified with nickel affinity chromatography and checked by Western blot analysis using anti-IN antibodies (Fig. 1c, left panel). As shown in Fig. 1c, right panel, IN derived from the IN-HATwt chimera gave a strong acetylation signal with anti-Ac-Lys antibodies, while no acetylation was detected with IN derived from the HATmut. Thus, these results clearly demonstrate that IN is specifically acetylated as a fusion product of HATwt but not of HATmut.

The IN-HAT cDNA cassette was then expressed in yeast cells in frame with the Gal4 DNA-binding domain (GDBD) under the control of a yeast promoter (GDBD-IN-HATwt/mut). The expression of the IN-HATwt/mut chimeras in yeast cells (AH109 strain) showed that the IN-HATwt chimera expressed high levels of acetylated IN, while the IN-HATmut chimera was negative for acetylation (A. Allouch et al., manuscript in preparation).

In conclusion, these results demonstrate that IN expressed as a fusion product with the HAT domain of p300 is highly acetylated. In addition to bacteria, acetylated IN can be expressed in eukaryotic (yeast) cells.

### Two-hybrid screening analysis using the IN-HATwt fusion protein

To identify the cellular factors interacting with acetylated IN, we used the GDBD-IN-HATwt as bait to screen a

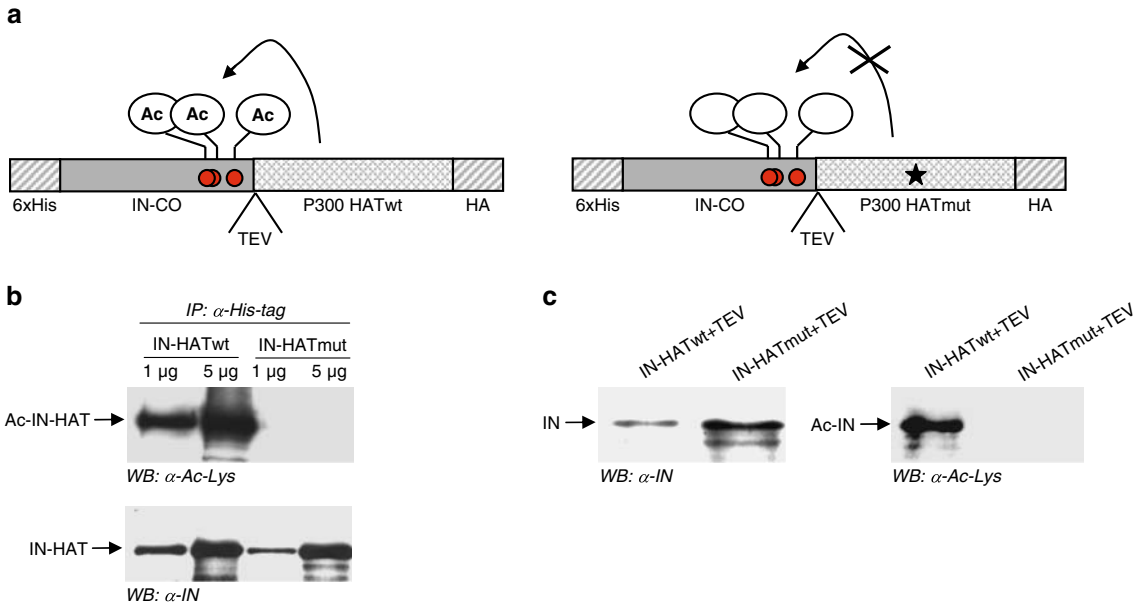


**Fig. 1** Generation of in vitro and in vivo of highly acetylated HIV-1 IN. **a** Schematic representation of the constructs engineered to produce constitutively acetylated IN (IN-HATwt) or control unmodified IN (IN-HATmut). IN codon optimized (*CO*) was fused to HATwt or HATmut (D1395Y). The fusion proteins are tagged at N terminus with 6xHis and at C-terminus with HA. A TEV proteolytic cleavage site is introduced between IN and the HAT domains. **b** Two amounts (1 and 5 µg) of IN-HATwt and IN-HATmut recombinant proteins were immunoprecipitated (*IP*) by anti His-tag antibodies (*α-His*). Immunoprecipitates were analyzed by Western blot (*WB*) using anti-acetylated lysine antibodies (α-Ac-Lys) (*upper panel*) and the same filter was incubated with anti-IN antibodies (*α-IN*) (*lower panel*). **c** 6xHisIN separated from either IN-HATwt or IN-HATmut fusion proteins by TEV digestion was purified by affinity chromatography (see 'Materials and methods') and analyzed by WB using α IN (*left panel*) and αAc-Lys (*right panel*)

human lymphocytes cDNA library fused to the Gal4 activation domain (GAD). The two-hybrid screening was performed in the AH109 yeast strain. This strain contains *Ade* and *His* genes as reporters which allows the selection of positive clones using the selective medium (-Ade and -His). From almost $10.6 \times 10^6$-screened transformants, 754 were positive clones encoding thirteen cellular proteins which are listed in Table 1. According to their proprieties and proposed functions, these cellular factors could be divided in three categories:

1. Transcription regulatory and chromatin remodeling factors: LEDGF/p75, KAP1, BTF3b, THRAP3 and HMGN2.
2. Translation regulatory and RNA binding proteins: eIF3h, eEF1A-1 and hnRNPA2.
3. Nuclear import-export proteins: Exp2 and RanBP9.

In addition to factors grouped in categories, we also identified RPL23, a structural ribosomal subunit, STMN1, a factor involved in microtubule organization and finally CCDC32, a protein with still unknown functions.

These newly identified factors, were subsequently tested by two-hybrid analysis with the IN-HATwt chimera, as well as with each single domain (IN and HATwt), to verify their association properties with the acetylated or unmodified IN and also with the HAT domain contained in the chimera. In addition, the analysis was performed with the GDBD to check the bait specificity. Results shown in Table 2 indicate that all identified factors associate with IN as a separate domain and that the majority do not bind the HAT domain except for THRAP3, RanBP9, eEF1A-1, STMN1 and CCDC32.

In conclusion, thirteen new factors binding to acetylated IN have been identified. These factors positively interact also with IN separated from the HAT domain, suggesting that acetylation modulates but is not absolute requirement for virus cell interaction.

## Binding of two-hybrid factors with acetylated and un-modified IN

To verify the interaction between IN and the cellular factors identified by two-hybrid screening (Table 1), pull down assays with the same factors expressed in human cells have been performed. Experiments were carried out with few selected factors based on their possible involvement in HIV-1 replication: transcription related proteins (BTF3b, THRAP3 and HMGN2) potentially involved in tethering viral integration in transcription units; a nuclear transport factor (Exp2) possibly involved in nuclear–cytoplasmic translocation. In addition, eIF3h, a factor involved in protein synthesis, was tested due to its high frequency of

**Table 1** Cellular factors identified by yeast two-hybrid screening using constitutively acetylated IN (IN-HATwt) as bait

| Protein names (number of isolated clones) | Proposed function | Complete residues: peptides retrieved | GenBank accession number | Reference |
|---|---|---|---|---|
| Lens epithelium-derived growth factor: LEDGF/p75 (6 clones) | Transcription coactivator. Factor interacting with lentiviral INs determining IN association to chromatin. Putative tethering factor for HIV-1 integration | 530: 344–530 | AF063020.1 | Ge et al. (1998), Cherepanov et al. (2003) |
| Krüppel-associated protein 1: KAP1 (3 clones) | Transcription corepressor and DNA damage response factor. Factor inhibiting infectivity of MoMLV in embryonic cells | 835: 304–835 | HSU78773 | Sripathy et al. (2006), Wolf and Goff (2007) |
| Basic transcription factor 3 isoform b: BTF3b (1 clone) | Component of the RNA polymerase II complex required for transcription initiation | 162: 1–162 | NM_001207.4 | Zheng et al. (1990) |
| Thyroid hormone receptor protein 3: THRAP3 (1 clone) | Subunit of the large transcription mediator TRAP complex; positive regulator of RNA polymerase II promoters transcription | 373: 1–361 | BC054046.1 | Rachez and Freedman (2001) |
| High-mobility group nucleosomal binding domain 2: HMGN2 (1 clone) | Component of the HMG non histone chromatin remodeling family of proteins. Inducer of chromatin decondensation and transcription activity | 90: 1–90 | BC014644.1 | West (2004) |
| Ran-binding protein 9: RanBP9 (8 clones) | Ran binding protein involved nuclear transport pathway NLS mediated | 729: 149–729 | BC063849.1 | Gorlich (1998) |
| Exportin 2 (synonyme: CAS): Exp2 (1 clone) | Importin α binding protein. Mediator of importin α nuclear export after NLS cargo release into the nucleus. | 971: 244–971 | BC108309.1 | Kutay et al. (1997), Solsbacher et al. (1998) |
| Eukaryotic translation initiation factor 3 subunit H: eIF3h (726 clones) | Component of the eIF3 complex: promotes translation preinitiation complex formation, mRNA recruitment and scanning for AUG recognition in the ribosomes | 352: 12–352; 172–352 | BC000386.2 | Hinnebusch (2006) |
| Elongation factor 1 alpha 1: eEF1A-1 (1 clone) | Component of the alpha subunit of EF1 complex: promotes protein biosynthesis by delivering aminoacylated tRNA to ribosomes. Binds HIV-1 matrix and nucleocapsid and stimulates HIV-1 transcription. Its yeast homologue binds HIV-1 IN | 46: 268–462 | BC082268.1 | Calado et al. (2002) |
| Heterogenous nuclear ribonucleoprotein A2: hnRNPA2 (1 clone) | RNA binding protein containing two RNA recognition motifs (RRM): involved in mRNA regulation (splicing and trafficking). Regulator of HIV-1 RNA trafficking | 341: 1–180 | NM_002137.2 | Shyu et al. (2000) |
| Stathmin 1: STMN1 (2 clones) | Tubulin binding protein: involved in microtubule depolymerization and signal transduction cascade | 149: 1–149 | BC082228.1 | Cassimeris (2002) |
| Ribosomal protein L23: RPL23 (1 clone) | Structural component of 60S subunit of ribosomes. Activates p53 by inhibiting MDM2 | 140: 18–140 | NM_000978.3 | Berchtold and Berger (1991) |
| Coiled-coil domain containing 32 isoform1: CCDC32 (2 clones) | Unknown functions | 185: 12–185 | BC001673.2 | Ota et al. (2004) |

identification (726 clones) in the two-hybrid screening. These factors were fused to a Flag tag and expressed in HEK293T cells. The derived cell lysates were incubated with either IN-HATwt or IN-HATmut recombinant proteins. Subsequently, the immunocomplexes were recovered with anti-Flag antibodies and analyzed by Western blot with anti-HA and anti-Flag antibodies. As an experimental control for binding specificity the same analysis was performed using HEK293T cells expressing an unrelated control protein, luciferase, fused to the Flag tag (Flag-Luciferase). As shown in Fig. 2a and b higher amounts of IN-HATwt than IN-HATmut were found associated with Flag-Exp2 and Flag-elF3h. Conversely, similar amounts of IN-HATwt and IN-HATmut bound Flag-BTF3b, Flag-THRAP3 and Flag-HMGN2 (Fig. 2c, d, e). Finally, no specific binding was observed with the unrelated control protein, Flag-Luc (Fig. 2f). As shown in lower panels of Fig. 2a–f, incubation of the same filters with anti-Flag antibodies proved that similar amounts of Flag proteins were immunoprecipitated with either IN-HATwt or IN-HATmut. These results suggest that Exp2 and eIF3h bind with higher affinity the acetylated form of IN, whereas BTF3b, THRAP3 and HMGN2 show no preferential binding for either forms of the viral protein. In a separate

**Table 2** Interactions in yeast between GAD prey proteins and GDBD hybrid baits (IN-HATwt, IN and HATwt)

| | IN-HATwt | IN | HATwt | GDBD |
|---|---|---|---|---|
| LEDGF/p75 | + | + | − | − |
| KAP1 | + | + | − | − |
| BTF3b | + | + | − | − |
| THRAP3 | + | + | + | − |
| HMGN2 | + | + | − | − |
| Exp2 | + | + | − | − |
| RanBP9 | + | + | + | − |
| EIF3h | + | + | − | − |
| EEF1A-1 | + | + | + | − |
| HnRNPA2 | + | + | − | − |
| STMN1 | + | + | + | − |
| RPL23 | + | + | − | − |
| CCDC32 | + | + | + | − |

+ Indicates interaction, − indicates no interaction

report we show that another factor, KAP1, also identified by the two-hybrid screening (Table 1), preferentially binds acetylated IN (A. Allouch et al., manuscript in preparation).

Next, the interaction between the two-hybrid hits and the single HAT and IN domains was verified using lysates of HEK293T cells expressing the Flag-tagged factors. As shown in Fig. 3a, upper panel, no binding was observed with the HAT domain, even though high expression of two-hybrid Flag-tagged factors could be detected (lower panel). Thus, these data demonstrate that the interaction between IN and the two-hybrid hits is specific and not mediated by the HAT domain. Moreover, this result suggests that the positive interactions observed by two-hybrid analysis with the HAT domain (Table 2), were likely a result of the synergistic transactivation properties of these factors (THRAP3, RanBP9, eEF1A-1, STMN1 and CCDC32) together with the HAT domain over the yeast promoter.

Finally, the unmodified form of IN (6xHis-IN) was verified by pull down assays with Flag-tagged two-hybrid hits expressed in HEK293T cells. High levels of IN was found associated with BTF3b, HMGN2 and THRAP3 (Fig. 3b), while much lower amounts could be detected in complex with Exp2, eIF3h and KAP1.

Therefore, these data are in agreement with results in Fig. 2, showing that BTF3b, HMGN2 and THRAP3 bind efficiently unmodified IN and do not require IN acetylation. Conversely, the low amounts of Exp2, eIF3h and KAP1

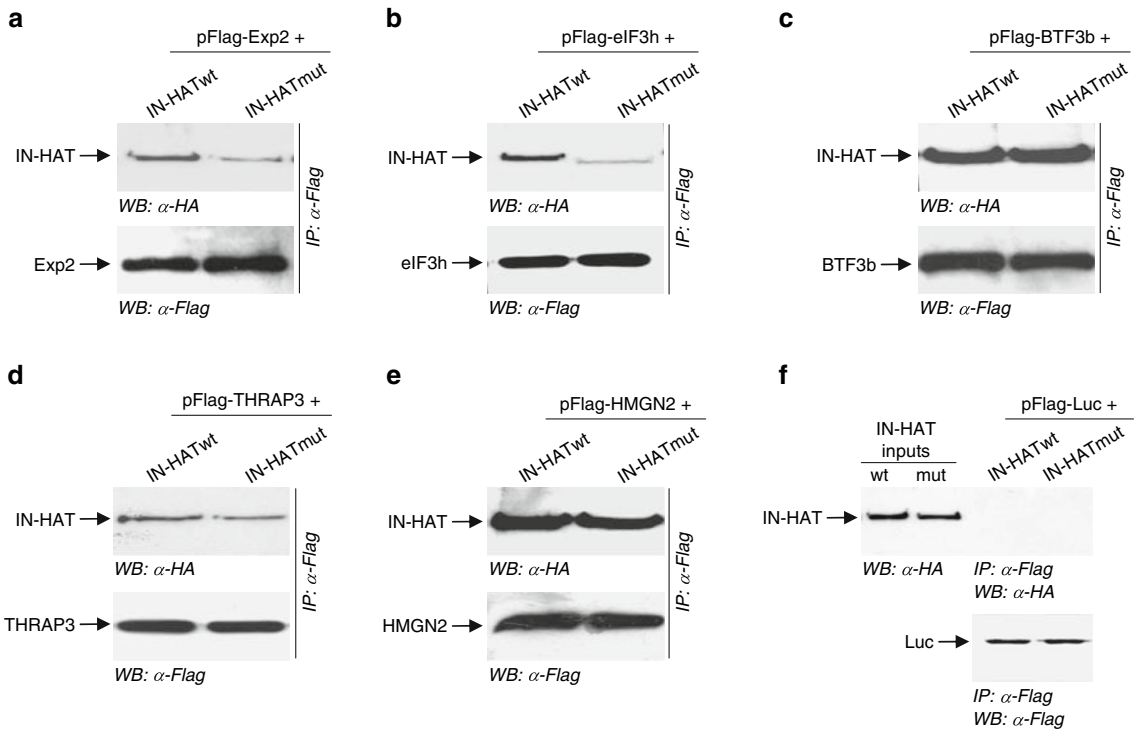


**Fig. 2** Binding analysis between acetylated IN and proteins identified by the two-hybrid screening. **a** Lysates from HEK293T cells expressing Flag-Exp2 (**a**), Flag-eIF3h (**b**), Flag-BTF3b (**c**), Flag-THRAP3 (**d**), Flag-HMGN2 (**e**) and FLAG-Luc (**f**) were incubated with recombinant IN-HATwt or IN-HATmut-HA and immunoprecipitated (*IP*) with monoclonal anti-Flag antibodies. Immunoprecipitates were then analyzed by Western blot (*WB*) with anti-HA antibodies (*α-HA*) (*upper panels*) and with polyclonal anti-Flag antibodies (*α-Flag*) (*lower panels*)

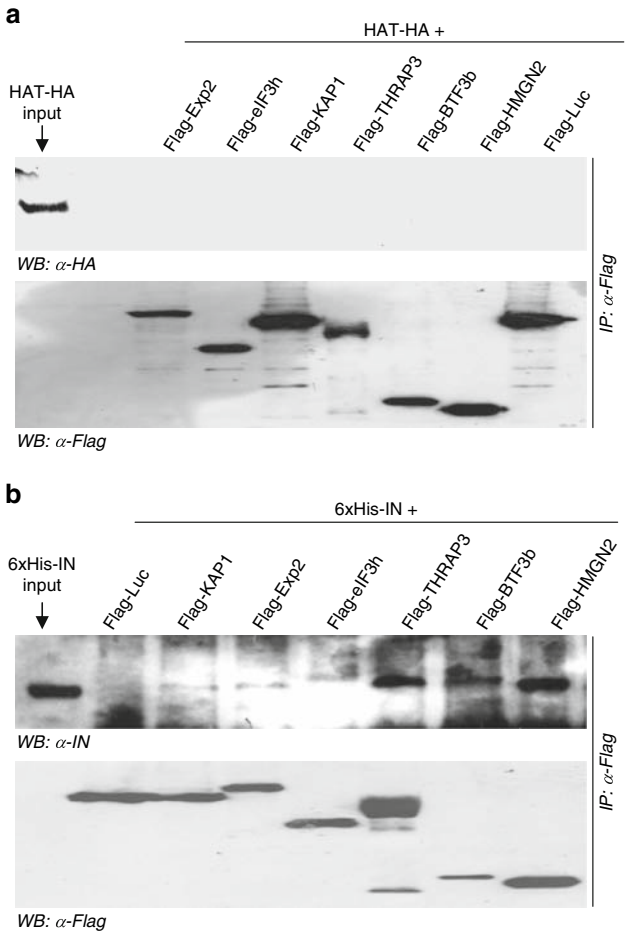


**Fig. 3** Binding between proteins identified by the two-hybrid screening and the HATwt or IN domains. **a** HEK293T cell lysates expressing Flag-eIF3h, Flag-BTF3b, Flag-THRAP3, Flag-HMGN2 or FLAG-Luc were incubated with either HATwt (**a**) or 6xHis-IN (**b**) recombinant proteins and immunoprecipitated (*IP*) with monoclonal anti-Flag antibodies. Immunoprecipitates were then analyzed by Western blot (*WB*) with anti-HA antibodies (*α-HA*) (*upper panels*) and with polyclonal anti-Flag antibodies (*α-Flag*) (*lower panels*)

bound to IN is indicative that IN acetylation enhances binding affinity.

## Discussion

This study exploits the tethered catalysis system (Guo et al. 2004) to produce an HIV-1 viral protein, IN, constitutively acetylated by p300. The construct verified to produce acetylated IN in bacteria, was used to screen by two-hybrid a human lymphocytes cDNA library. From this screening we have identified 13 cellular factors, 12 of which have never been reported to interact with HIV-1 IN. The binding analysis performed by two-hybrid and pull down assays revealed that a basal association of all factors was reported with the unmodified IN while the acetylation of the viral factor variably affect the affinity with the two-hybrid hits.

The newly identified factors interacting with acetylated or un-modified IN showed no obvious simple sequence similarity. Nevertheless, it is plausible that the IN recognizes common elements present in these proteins. In fact, these factors can be grouped in three categories based on their functional properties: (a) transcription regulatory and chromatin remodeling factors; (b) translation regulatory and RNA binding proteins; (c) nuclear import–export proteins.

Interestingly, LEDGF/p75, one of the factors identified in this screening, is one of the most described IN interactor required for efficient HIV-1 integration (Cherepanov et al. 2003; Engelman and Cherepanov 2008). This result validates the system of analysis employed in this study and proves that fusion of IN to the HAT domain does not significantly alter the IN structure. LEDGF/p75 binds the core catalytic domain of HIV-1 IN. Nevertheless, the N-terminal domain of IN was proven to enhance the binding affinity with this factor (Maertens et al. 2003). Therefore, the identification of LEDGF/p75 in our screening might suggest that also the C terminus of IN and its acetylation could affect IN/LEDGF-p75 interaction.

HIV-1 integration preferentially occurs in regions of the chromatin rich in genes transcriptionally active (Bushman et al. 2005; Mitchell et al. 2004; Schroder et al. 2002). Recent studies performed by sequence analysis using the ENCODE annotation (Wang et al. 2007) and by a visualization analysis (Albanese et al. 2008) demonstrated that HIV-1 targets decondensed regions of the chromatin. It has been hypothesized that cellular factors interacting with IN may tether the virus to appropriate sites for integration. Indeed, LEDGF/p75 knockdown and knockout cells show a significant reduction of integration frequency in transcription units (Ciuffi et al. 2005; Shun et al. 2007). Nevertheless, since in the absence of LEDGF/p75 the virus still does not integrate randomly in the genome, additional factors may be required for integration specificity. The screening reported in this study, uncovered factors involved in transcription and chromatin structure regulation, thus good candidate proteins to tether HIV-1 integration. BTF3b and THRAP3, found in the two-hybrid screening, are positive regulators of gene transcription that act by associating with RNA polymerase II (Rachez and Freedman 2001; Zheng et al. 1990). The HMNG2 protein, another two-hybrid hit, is involved in chromatin structure regulation by binding to nucleosomes in a DNA sequence independent manner. This factor induces chromatin decompaction, which in turn facilitates DNA transcription and replication (West 2004). In fact, HMNG2 was found to localize in active transcription chromatin regions (Bustin 2001; Hock et al. 1998). Finally, HMGA1, another HMG family member, was previously reported to stimulate HIV-1 integration by promoting the formation of IN/cDNA complexes (Hindmarsh et al. 1999; Li et al. 2000).

HIV-1 nuclear import occurs through still incompletely understood mechanisms. One of the viral factor hypothesized to be involved in nuclear translocation is the IN protein which contains several putative nuclear localization signals (NLSs) (Bouyac-Bertoia et al. 2001; Gallay et al. 1997; Hearps and Jans 2006). In our study, we identified two proteins that regulate the nuclear importin pathway Exp2 and RanBP9. These factors may trigger the nuclear import of IN by importin $\alpha$ and $\beta$ complex. However, the implications of both importin factors have been explored for HIV-1 infectivity leading to contradictory results. Indeed, recent reports suggested that IN may lack a functional NLS and attributed its karyophilic proprieties to the LEDGF/p75 interacting factor (Devroe et al. 2003; Llano et al. 2004). More recently IN has been demonstrated to interact with a cellular factor, transportin SR2 (TNPO3) which mediates HIV-1 transport into the nucleus (Christ et al. 2008).

A recent two-hybrid screening performed with IN of another retrovirus, the Moloney Murine Leukemia Virus (MoMLV), identified the murine eIFs2, a subunit part of the translation initiation factor 3 (eIF3) complex (Studamire and Goff 2008). Moreover, in this study it was demonstrated that HIV-1 IN does not interact with eIFs2. The human eIF3f, another component of eIF3 complex, was reported to inhibit HIV-1 replication at post-integration step by interfering with the 3′ end processing of HIV-1 mRNAs (Valente et al. 2009). From our screening we identified eIF3h, another subunit belonging to the eIF3 complex, as a factor interacting with acetylated HIV-1 IN. Thus, since numerous studies report the association of retroviruses with the eIF3, this protein complex presumably plays an important function in the viral replication cycle, even though the detailed molecular mechanism has not yet been unraveled.

One of the two-hybrid hits was eEF1A-1 factor which has been previously reported to be involved in HIV-1 biology. Most interestingly, in agreement with our results, a former study using a yeast expression experimental system suggested that IN interacts with eEF1A-1 (Parissi et al. 2001). Moreover, eEF1A-1 was identified to be involved in HIV-1 replication by binding with the viral gag polyproteins (matrix and nucleocapsid) (Cimarelli and Luban 1999) and also by activating the viral promoter (Wu-Baer et al. 1996). All these reports implicate multi-roles of eEF1A-1 in HIV-1 replication cycle.

STMN1, identified in our two-hybrid screening, regulates microtubule organization by binding tightly tubulin and inducing microtubule destabilization (Cassimeris 2002; Howell et al. 1999). This observation is in line with a previous report showing that HIV-1 IN bind microtubule-associated proteins such as the yeast STU2p, a centrosomal protein, and Dyn2p (dynein light chain protein). It has been hypothesized that IN interaction with these factors may be responsible for IN nuclear import (de Soultrait et al. 2002; Desfarges et al. 2009).

In conclusion, here we report a list of cellular proteins that interact with acetylated HIV-1 IN. These are new potential factors involved in HIV-1 replication by either inhibiting or favoring the virus at specific steps involving IN activity. Further analyses are required to establish their role in HIV-1 biology.

## Materials and methods

### Vectors and constructs

pASK-IN-HATwt and pASK-IN-HATmut to express and purify IN-HATwt and IN-HATmut in bacteria were constructed by cloning IN codon optimized (CO) in frame with the HAT domain of p300 (a.a. 1195–1673) wild-type or mutated (D1395Y) in the pASK-IBA37 plus vector (IBA, Gottingen, Germany) containing at 5′ of the MCS a 6xHis tag. During the cloning procedure a 3′ HA tag and a TEV protease cleavage site between IN and HAT were introduced by PCR. HATwt-HA was cloned in pASK-IBA37 plus by PCR. PINSD-IN encoding for 6xHis-IN was described in (Cereseto et al. 2005). From the pASK-IBA37-IN-HATwt/mut vectors the IN-HATwt/mut, HATwt and IN were PCR amplified and cloned in frame with the GDBD in the pBD-Gal4 vector (Stratagene, La Jolla, CA, USA) for expression in yeast cells. KAP1, BTF3b, THRAP3, HMGN2, Exp2, eIF3h cDNAs were cloned by PCR in pFlag-CMV2 vectors starting from their truncated cDNAs isolated in the two-hybrid screening.

### Yeast two-hybrid screening

A human T-lymphocytes cDNA library fused to Gal4 Activating Domain in a pACT vector (BD biosciences Clontech, Palo Alto, CA, USA) was expressed in AH109 yeast cells and screened with the pGDBD-IN-HATwt (bait) expression vector. Library transformation and screening were performed following manufacturer's instructions (Matchmaker GAL4 two-hybrid system 3). GAD identified prey proteins (Table 1) were co-expressed in AH109 yeast cells with GDBD fused to IN-HATwt, HATwt and IN to check for interactions.

### IN-HATwt and IN-HATmut, IN and HATwt purifications

pASK-IN-HATwt/mut encoding for 6xHis-IN-HATwt/mut-HA, pASK-HATwt encoding for 6xHis-HATwt-HA and pINSD-IN encoding for 6xHis-IN were transformed in

*E. coli* BL21 competent cells (Stratagene, La Jolla, CA, USA). Induction of protein expression was performed using 43 mM anhydrotetracycline hydrochloride (AHT) (for pASK plasmids) or 0.5 mM IPTG (for pINSD-IN) for 4 h at 30°C. Bacteria culture was lysed in binding buffer (1 M NaCl, 20 mM Tris HCl pH 7.9 and 0.5% Triton X-100) containing 1 mM PMSF and protease inhibitor cocktail (Roche, Mannheim, Germany). TALON Metal Affinity Resin (BD Biosciences, Palo Alto, CA, USA) incubated for 2 h at 4°C was used to recover the 6xHis recombinant proteins. Following two washes in binding buffer containing 5 mM imidazole, proteins were eluted using binding buffer containing 200 mM imidazole and dialysed in buffer containing 150 mM NaCl, 50 mM Tris HCl pH 8, 10% glycerol and 0.5 mM EDTA. 6xHis-IN was dialyzed in 1 M NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT and 10% glycerol. For TEV digestion 20 μg of 6xHis-IN-HATwt/mut were incubated with 30 units of AcTEV protease (Invitrogen, Paisley, UK) in 50 mM Tris–HCl pH 8, 0.5 mM EDTA and 1 mM of DTT in 250 μl total volume. To recover His-IN from the digested product the TEV treated samples were adjusted to 1 M NaCl and incubated with Ni-NTA agarose resin (Qiagen, Hilden, Germany) for 2 h at 4°C. Following washes in binding buffer containing 5 mM imidazole, His-IN was eluted using the binding buffer containing 250 mM imidazole and dialysed in 1 M NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT and 10% glycerol. 6xHis-IN was dialysed in 1 M NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT and 10% glycerol.

### Antibodies

Primary antibodies for Western blot analyses were: monoclonal HIV-1 IN antibody (8G4) from NIH AIDS Research and Reference Reagent Program (Germantown, MD), polyclonal anti-acetylated lysines (Cell Signaling Technology, Beverly, MA, USA), polyclonal anti-HA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), polyclonal anti-Flag (Sigma-Aldrich, St Louis, MO, USA) and monoclonal anti-His (Qiagen, Hilden, Germany). Secondary antibodies HRP conjugated anti-mouse or anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, YSA).

### Immunoprecipitations and anti-Flag beads pull down

A total of 1 and 5 μg of either purified and dialyzed 6xHis-IN-HATwt-HA or 6x His-IN-HATmut-HA proteins were incubated with 1 mg of monoclonal anti-His antibody (Qiagen, Hilden, Germany) for 1 h and then 20 μl of Protein G UltraLink resin (Pierce Biotechnology, Rockford, IL, USA) were added for additional 1 h. Immunocomplexes were washed three times in buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 8, 10% glycerol and 0.5 mM EDTA and analyzed by Western blot. For Flag pull down experiments HEK293T expressing Flag proteins (KAP1, BTF3b, THRAP3, HMGN2, Exp2 and eIF3h) were lysed in 50 mM Hepes pH 7.4, 150 mM NaCl and 0.5% NP-40. A total of 250 μg lysate was mixed with 250 ng of recombinant and purified proteins (6xHis-IN-HATwt-HA or 6xHis-IN-HATmutHA, 6xHis-HATwt-HA or 6xHis-IN) together with 5 μM of Lys-CoA (synthesized at the ICGEB Peptide Synthesis Core Faculty, Trieste, Italy). Following 1 h incubation at 4°C, 20 μl of monoclonal anti-Flag M2 antibodies immobilized on agarose beads (Sigma, St Louis, MO, USA) were added and incubated for 1 h. Following washes in lysis buffer samples were analyzed by Western blot using polyclonal anti-HA or monoclonal anti-IN and polyclonal anti-Flag antibodies.

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## References

Albanese A, Arosio D, Terreni M, Cereseto A (2008) HIV-1 pre-integration complexes selectively target decondensed chromatin in the nuclear periphery. PLoS One 3:e2413

Berchtold MW, Berger MC (1991) Isolation and analysis of a human cDNA highly homologous to the yeast gene encoding L17A ribosomal protein. Gene 102:283–288

Bouyac-Bertoia M, Dvorin JD, Fouchier RA, Jenkins Y, Meyer BE, Wu LI, Emerman M, Malim MH (2001) HIV-1 infection requires a functional integrase NLS. Mol Cell 7:1025–1035

Bushman F, Lewinski M, Ciuffi A, Barr S, Leipzig J, Hannenhalli S, Hoffmann C (2005) Genome-wide analysis of retroviral DNA integration. Nat Rev Microbiol 3:848–858

Bustin M (2001) Chromatin unfolding and activation by HMGN(*) chromosomal proteins. Trends Biochem Sci 26:431–437

Calado A, Treichel N, Muller EC, Otto A, Kutay U (2002) Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. EMBO J 21:6216–6224

Cassimeris L (2002) The oncoprotein 18/stathmin family of microtubule destabilizers. Curr Opin Cell Biol 14:18–24

Cereseto A, Manganaro L, Gutierrez MI, Terreni M, Fittipaldi A, Lusic M, Marcello A, Giacca M (2005) Acetylation of HIV-1 integrase by p300 regulates viral integration. EMBO J 24:3070–3081

Cherepanov P, Maertens G, Proost P, Devreese B, Van Beeumen J, Engelborghs Y, De Clercq E, Debyser Z (2003) HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. J Biol Chem 278:372–381

Christ F, Thys W, De Rijck J, Gijsbers R, Albanese A, Arosio D, Emiliani S, Rain JC, Benarous R, Cereseto A et al (2008) Transportin-SR2 imports HIV into the nucleus. Curr Biol 18:1192–1202

Cimarelli A, Luban J (1999) Translation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. J Virol 73:5388–5401

Ciuffi A, Llano M, Poeschla E, Hoffmann C, Leipzig J, Shinn P, Ecker JR, Bushman F (2005) A role for LEDGF/p75 in targeting HIV DNA integration. Nat Med 11:1287–1289

Coffin JM, Hughes SH, Varmus HE (1997) Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

de Soultrait VR, Caumont A, Durrens P, Calmels C, Parissi V, Recordon P, Bon E, Desjobert C, Tarrago-Litvak L, Fournier M (2002) HIV-1 integrase interacts with yeast microtubule-associated proteins. Biochim Biophys Acta 1575:40–48

Desfarges S, Salin B, Calmels C, Andreola ML, Parissi V, Fournier M (2009) HIV-1 integrase trafficking in *S. cerevisiae*: a useful model to dissect the microtubule network involvement of viral protein nuclear import. Yeast 26:39–54

Devroe E, Engelman A, Silver PA (2003) Intracellular transport of human immunodeficiency virus type 1 integrase. J Cell Sci 116:4401–4408

Engelman A, Cherepanov P (2008) The lentiviral integrase binding protein LEDGF/p75 and HIV-1 replication. PLoS Pathog 4:e1000046

Gallay P, Hope T, Chin D, Trono D (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc Natl Acad Sci USA 94:9825–9830

Ge H, Si Y, Roeder RG (1998) Isolation of cDNAs encoding novel transcription coactivators p52 and p75 reveals an alternate regulatory mechanism of transcriptional activation. EMBO J 17:6723–6729

Goff SP (2007) Host factors exploited by retroviruses. Nat Rev Microbiol 5:253–263

Gorlich D (1998) Transport into and out of the cell nucleus. EMBO J 17:2721–2727

Guo D, Hazbun TR, Xu XJ, Ng SL, Fields S, Kuo MH (2004) A tethered catalysis, two-hybrid system to identify protein-protein interactions requiring post-translational modifications. Nat Biotechnol 22:888–892

Hearps AC, Jans DA (2006) HIV-1 integrase is capable of targeting DNA to the nucleus via an importin alpha/beta-dependent mechanism. Biochem J 398:475–484

Hindmarsh P, Ridky T, Reeves R, Andrake M, Skalka AM, Leis J (1999) HMG protein family members stimulate human immunodeficiency virus type 1 and avian sarcoma virus concerted DNA integration in vitro. J Virol 73:2994–3003

Hinnebusch AG (2006) eIF3: a versatile scaffold for translation initiation complexes. Trends Biochem Sci 31:553–562

Hock R, Wilde F, Scheer U, Bustin M (1998) Dynamic relocation of chromosomal protein HMG-17 in the nucleus is dependent on transcriptional activity. EMBO J 17:6992–7001

Howell B, Larsson N, Gullberg M, Cassimeris L (1999) Dissociation of the tubulin-sequestering and microtubule catastrophe-promoting activities of oncoprotein 18/stathmin. Mol Biol Cell 10:105–118

Kutay U, Bischoff FR, Kostka S, Kraft R, Gorlich D (1997) Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell 90:1061–1071

Li L, Yoder K, Hansen MS, Olvera J, Miller MD, Bushman FD (2000) Retroviral cDNA integration: stimulation by HMG I family proteins. J Virol 74:10965–10974

Llano M, Vanegas M, Fregoso O, Saenz D, Chung S, Peretz M, Poeschla EM (2004) LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. J Virol 78:9524–9537

Maertens G, Cherepanov P, Pluymers W, Busschots K, De Clercq E, Debyser Z, Engelborghs Y (2003) LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. J Biol Chem 278:33528–33539

Mitchell RS, Beitzel BF, Schroder AR, Shinn P, Chen H, Berry CC, Ecker JR, Bushman FD (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. PLoS Biol 2:E234

Ota T, Suzuki Y, Nishikawa T, Otsuki T, Sugiyama T, Irie R, Wakamatsu A, Hayashi K, Sato H, Nagai K et al (2004) Complete sequencing and characterization of 21, 243 full-length human cDNAs. Nat Genet 36:40–45

Parissi V, Calmels C, de Soultrait VR, Caumont A, Fournier M, Chaignepain S, Litvak S (2001) Functional interactions of human immunodeficiency virus type 1 integrase with human and yeast HSP60. J Virol 75:11344–11353

Rachez C, Freedman LP (2001) Mediator complexes and transcription. Curr Opin Cell Biol 13:274–280

Rain JC, Cribier A, Gerard A, Emiliani S, Benarous R (2009) Yeast two-hybrid detection of integrase-host factor interactions. Methods 47:291–297

Schroder AR, Shinn P, Chen H, Berry C, Ecker JR, Bushman F (2002) HIV-1 integration in the human genome favors active genes and local hotspots. Cell 110:521–529

Shun MC, Raghavendra NK, Vandegraaff N, Daigle JE, Hughes S, Kellam P, Cherepanov P, Engelman A (2007) LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. Genes Dev 21:1767–1778

Solsbacher J, Maurer P, Bischoff FR, Schlenstedt G (1998) Cse1p is involved in export of yeast importin alpha from the nucleus. Mol Cell Biol 18:6805–6815

Sripathy SP, Stevens J, Schultz DC (2006) The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. Mol Cell Biol 26:8623–8638

Sterner DE, Berger SL (2000) Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 64:435–459

Studamire B, Goff SP (2008) Host proteins interacting with the Moloney murine leukemia virus integrase: multiple transcriptional regulators and chromatin binding factors. Retrovirology 5:48

Suzuki Y, Craigie R (2007) The road to chromatin—nuclear entry of retroviruses. Nat Rev Microbiol 5:187–196

Valente ST, Gilmartin GM, Mott C, Falkard B, Goff SP (2009) Inhibition of HIV-1 replication by eIF3f. Proc Natl Acad Sci USA 106:4071–4078

Van Maele B, Busschots K, Vandekerckhove L, Christ F, Debyser Z (2006) Cellular co-factors of HIV-1 integration. Trends Biochem Sci 31:98–105

Vandegraaff N, Engelman A (2007) Molecular mechanisms of HIV integration and therapeutic intervention. Expert Rev Mol Med 9:1–19

Wang GP, Ciuffi A, Leipzig J, Berry CC, Bushman FD (2007) HIV integration site selection: analysis by massively parallel pyrosequencing reveals association with epigenetic modifications. Genome Res 17:1186–1194

West KL (2004) HMGN proteins play roles in DNA repair and gene expression in mammalian cells. Biochem Soc Trans 32:918–919

Wolf D, Goff SP (2007) TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell 131:46–57

Wu-Baer F, Lane WS, Gaynor RB (1996) Identification of a group of cellular cofactors that stimulate the binding of RNA polymerase II and TRP-185 to human immunodeficiency virus 1 TAR RNA. J Biol Chem 271:4201–4208

Zheng XM, Black D, Chambon P, Egly JM (1990) Sequencing and expression of complementary DNA for the general transcription factor BTF3. Nature 344:556–559

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Poster presentation



# HIV-1 acetylated integrase is targeted by KAP1 (TRIM28) to inhibit viral integration

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Post-translational modifications, such as acetylation, dynamically modulate the chemical and structural properties of proteins generating new protein-protein interfaces. HIV-1 integrase is acetylated by p300 at three specific lysines located in the carboxy terminal domain. In the attempt to understand how acetylation modifies the integration event, we have searched for cellular cofactors that may specifically require acetylation to bind integrase.

To this aim a tethered catalysis system has been exploited to perform a yeast two-hybrid screening. In this assay an integrase constitutively acetylated by fusion with the HAT catalytic domain of p300, was used as a "bait" to screen a human T-cell cDNA library. One of the identified binding factors was KAP1, which showed a higher affinity to integrase following its acetylation. This affinity was confirmed by either pull down assays and *in vivo* co-immunoprecitation in 293T cells. To evaluate the role of KAP1 during HIV-1 life cycle, infections were performed in HeLa and 293T cells transiently and stably silenced for KAP1. Interestingly, the infectivity was 3-10 fold higher than control cells and the analysis of the DNA forms showed a specific enhancement at the level of integration. In a reciprocal experiment overexpression of KAP1 showed a reduction of infectivity by a 50% decrease in integration.

Since integrase activity is positively regulated by acetylation, we then performed experiments to explore whether KAP1 inhibition of viral integration might correlate with modulation of integrase acetylation levels. We demonstrated that KAP1 binding to acetylated integrase indeed induces integrase deacetylation through HDAC1 complex formation. Finally, HDAC1 complex formation is a requirement for KAP1 viral inhibition since no HIV-1 restriction can be observed in cell silenced for HDAC1.

In conclusion, this study reports that KAP1, recently described to restrict M-MLV infectivity in embryonic stem cells at the level of viral transcription, inhibits HIV-1 through a novel mechanism targeting the integration step.

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# GCN5-dependent acetylation of HIV-1 integrase enhances viral integration

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## Abstract

**Background:** An essential event during the replication cycle of HIV-1 is the integration of the reverse transcribed viral DNA into the host cellular genome. Our former report revealed that HIV-1 integrase (IN), the enzyme that catalyzes the integration reaction, is positively regulated by acetylation mediated by the histone acetyltransferase (HAT) p300.

**Results:** In this study we demonstrate that another cellular HAT, GCN5, acetylates IN leading to enhanced 3'-end processing and strand transfer activities. GCN5 participates in the integration step of HIV-1 replication cycle as demonstrated by the reduced infectivity, due to inefficient provirus formation, in GCN5 knockdown cells. Within the C-terminal domain of IN, four lysines (K258, K264, K266, and K273) are targeted by GCN5 acetylation, three of which (K264, K266, and K273) are also modified by p300. Replication analysis of HIV-1 clones carrying substitutions at the IN lysines acetylated by both GCN5 and p300, or exclusively by GCN5, demonstrated that these residues are required for efficient viral integration. In addition, a comparative analysis of the replication efficiencies of the IN triple- and quadruple-mutant viruses revealed that even though the lysines targeted by both GCN5 and p300 are required for efficient virus integration, the residue exclusively modified by GCN5 (K258) does not affect this process.

**Conclusions:** The results presented here further demonstrate the relevance of IN post-translational modification by acetylation, which results from the catalytic activities of multiple HATs during the viral replication cycle. Finally, this study contributes to clarifying the recent debate raised on the role of IN acetylated lysines during HIV-1 infection.

## Background

Integration of reverse transcribed HIV-1 DNA into the cellular genome is catalyzed by the viral IN protein. Even though *in vitro* integration can be solely driven by IN, cellular cofactors are required to complete the reaction *in vivo*. It was recently reported that the cellular HAT p300 interacts with IN and regulates its function through acetylation [1,2]. HATs are enzymes able to transfer acetyl groups from acetyl coenzyme A (acetyl-CoA) to specific lysine residues within the N-terminal tails of nucleosomal histones, leading to chromatin decondensation and transcriptional activation [3,4]. HATs can also acetylate non-histone substrates, such as transcription factors and other nuclear proteins, as well as cytoskeletal components, metabolic enzymes and signalling regulators in the cytoplasm [5]. Acetylation has been reported to regulate the activity of these factors by modulating DNA binding [6-8], protein-protein interactions [9-12], protein stability [13-15], and subcellular localization [16-19]. Growing evidence now indicates that acetylation significantly participates in signaling pathways ultimately regulating viral infectivity [20-26]. Among the viral factors functionally modulated by acetylation is the HIV-1 protein Tat. Tat is acetylated at lysine 28 by PCAF, while residues 50 and 51 are substrates for p300/CBP and GCN5 [27-29]. Acetylation of lysine 28 enhances the ability of Tat to recruit the P-TEFb complex [28], while modification of lysine 50 leads to Tat dissociation from TAR RNA [28,30]. Therefore, even though the final effect of acetylation is an increased transactivation activity on the viral LTR promoter, the modification of each individual lysine differently affects Tat functionality at the molecular level.

We have recently discovered that another HIV-1-encoded protein, IN, is a substrate for p300-mediated

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acetylation. Three lysine residues, located at positions 264, 266, and 273 in the C-terminal domain of IN, were identified as the target sites for modification [1,2]. Acetylation by p300 was shown to increase both IN affinity for DNA and strand transfer activity [1], thus suggesting a potential role for this post-translational modification during viral integration. The importance of IN acetylation for HIV-1 replication was further highlighted by the finding that the mutant virus, in which arginine substitutions were introduced at p300-targeted IN lysines, integrated less efficiently than the wild type [1].

Since proteins modified by acetylation are often substrates for multiple HATs, we sought to investigate whether IN might be acetylated by enzymes other than p300. It has already been reported that MOZ and PCAF (belonging to the MYST and GNAT families of HATs, respectively) are incapable of efficiently acetylating the IN C-terminal domain *in vitro* [2]. Therefore, in this study, another member of the GNAT family, GCN5, was examined. Here we demonstrate that GCN5 binds and acetylates IN both *in vitro* and *in vivo*. GCN5 expression is functionally relevant to HIV-1 infectivity and specifically affects the integration process, likely by modulating the catalytic activity of IN. Interestingly, the four lysines targeted by GCN5 partially overlap with those modified by p300 in the C-terminal domain of IN. A comparative analysis of viral clones mutated at IN lysines acetylated by GCN5 or p300 revealed the same replication defect at the step of integration, thus indicating common roles for the two HATs in regulating IN function.

## Results

### HIV-1 IN is acetylated by GCN5

To examine whether IN is acetylated by GCN5, *in vitro* acetylation assays were performed with recombinant IN and GCN5, both purified as GST fusion proteins. Incubation of the single GST domain with GCN5 in the presence of [$^{14}$C]-acetyl-CoA, and subsequent protein resolution by SDS-PAGE followed by autoradiography, revealed a unique band at the same size as GST-GCN5, corresponding to the auto-acetylation product of the enzyme (Figure 1A, lane 1). Incubation of GST-IN with GST-GCN5 resulted in two major radiolabeled bands, the higher one corresponding to auto-acetylated GST-GCN5 and the lower one to GST-IN (Figure 1A, lane 2), thus demonstrating that GCN5 specifically acetylates IN *in vitro*.

To define which region of IN is acetylated by GCN5, GST-IN fragments with progressive deletions starting from the C-terminus (as schematized in Figure 1C) were used as substrates in *in vitro* acetylation assays, and the corresponding acetylation signals in the autoradiograms were evaluated by densitometric analysis (Figure 1B, left histogram). GST-IN fragment 1-272 was acetylated to a similar extent as full-length IN (Figure 1A, compare lanes 2 and 3, and Figure 1B, left histogram). Acetylation of fragment 1-263 (Figure 1A, lane 4) was reduced by 30% (Figure 1B, left histogram), while a more significant decrease in the signal (ranging from 60% to 70%) was observed using shorter fragments (1-243, 1-234 and 1-212) (Figure 1A, lanes 5-7, and Figure 1B, left histogram). These results indicated that IN is acetylated by GCN5 within the region located between amino acids 244 and 288. As schematically represented in Figure 1C, this region contains five lysines at positions 244, 258, 264, 266, and 273 as possible targets for acetylation. Therefore, in order to exclude that the reduced acetylation of the deleted IN forms resulted from improper protein folding, each of these lysines was replaced with an arginine, an amino acid that cannot be acetylated and conserves a positively charged side chain. The resulting mutants were then tested *in vitro* as substrates for GCN5 activity. In this experiment, IN was tagged with a 6× His epitope in place of GST, in order to obtain better SDS-PAGE resolution between acetylated GCN5 and IN (Figure 1A, lane 8). As reported in the right histogram of Figure 1B, densitometric analysis of radioactivity incorporation highlighted that the mutation of the individual lysines 258, 264, 266, and 273 (Figure 1A, lanes 10-13) caused a reduction in the acetylation level of IN ranging from 40% to 50%, while no significant decrease in the signal was detected upon mutation of lysine 244 (Figure 1A, lane 9). These data suggested that GCN5 acetylates IN at residues 258, 264, 266, and 273. Notably, previous reports demonstrated that another HAT, p300, acetylates lysines 264, 266, and 273 of IN [1,2]. To confirm that GCN5 acetylates lysine 258 in addition to the above-mentioned residues, two mutant forms of IN were assayed for *in vitro* acetylation: one containing mutations at the sites acetylated by both GCN5 and p300 (IN K264,266,273R), and the other carrying these same amino acidic substitutions, with the additional mutation of lysine 258 specifically targeted by GCN5 (IN K258,264,266,273R). The decrease in the radioactive signal detected with IN K264,266,273R was similar to the one obtained with the single-mutated forms (compare lane 14 with lanes 10-13 in Figure 1A, and right histogram of Figure 1B), while the residual acetylation level of IN K258,264,266,273R dropped to 20% with respect to wild type (Figure 1A, lane 15, and Figure 1B, right histogram). These results demonstrated that GCN5 acetylates lysines 264, 266, and 273 of IN, also targeted by p300, and lysine 258 as a specific site of modification.

Next, we investigated whether IN is also acetylated by GCN5 *in vivo*. Codon-optimized Flag-IN [31] was expressed in HEK 293T cells, alone or together with HA-GCN5 wild type or mutated in the catalytic domain

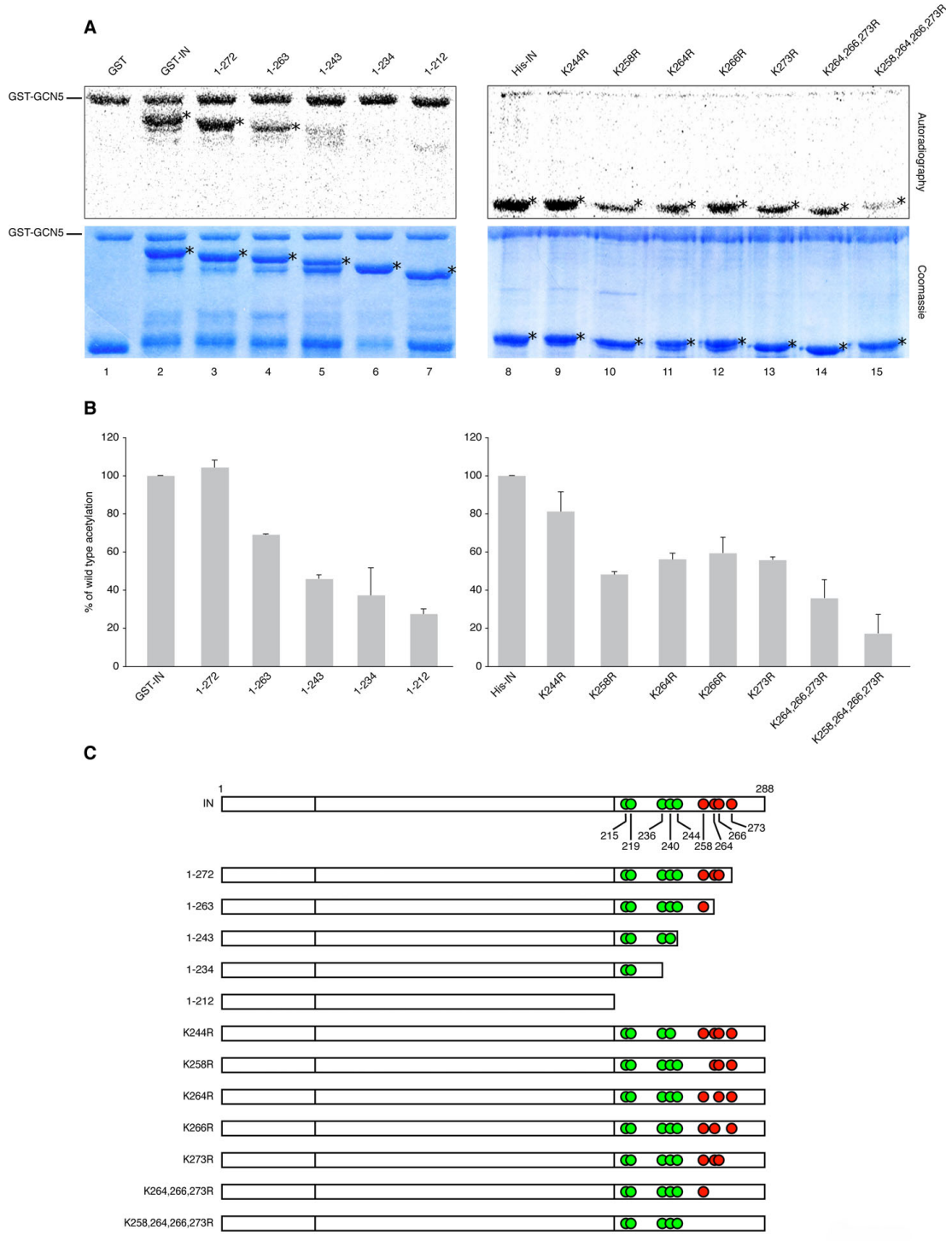


**Figure 1 HIV-1 IN is acetylated by GCN5 *in vitro***. (A) Autoradiography (upper panels) and Coomassie blue staining (lower panels) of *in vitro* acetylation assay with recombinant GST-GCN5 and IN wild type or mutant proteins. Lanes 1-7: GST fusion IN proteins; lanes 8-15: 6× His-tagged IN proteins. In the Coomassie panels, IN proteins used as acetylation substrates are indicated by asterisks; in the autoradiograms, IN proteins found positive for GCN5-mediated acetylation are indicated in the same way. Presented results are representative data from triplicate *in vitro* acetylation assay experiments. (B) Results of densitometric analysis of autoradiograms derived from three independent experiments (means ± standard errors of the means [SEM]) expressed as percent wild type IN acetylation. (C) Schematic representation of IN proteins used for the acetylation assays. The positions of lysines in the C-terminal domain of IN are indicated. Lysines positive for acetylation are shown in red.

(Y260A/F261A) [32]. Immunoprecipitation of IN and subsequent detection by Western blotting with an antibody specific to acetylated lysines revealed the highest acetylation signal in the sample corresponding to IN co-expressed with wild type GCN5 (Figure 2A, upper panel, lane 3). Conversely, expression of IN alone or together with catalytically inactive GCN5 resulted in a lower acetylation signal, likely derived from the activity of endogenous HATs (Figure 2A, upper panel, lanes 2 and 4). In this experiment, the total amounts of immunoprecipitated IN and the expression levels of wild type and mutant GCN5 were verified by Western blot analysis with anti-Flag and anti-HA antibodies, respectively (Figure 2A, middle and lower panels).

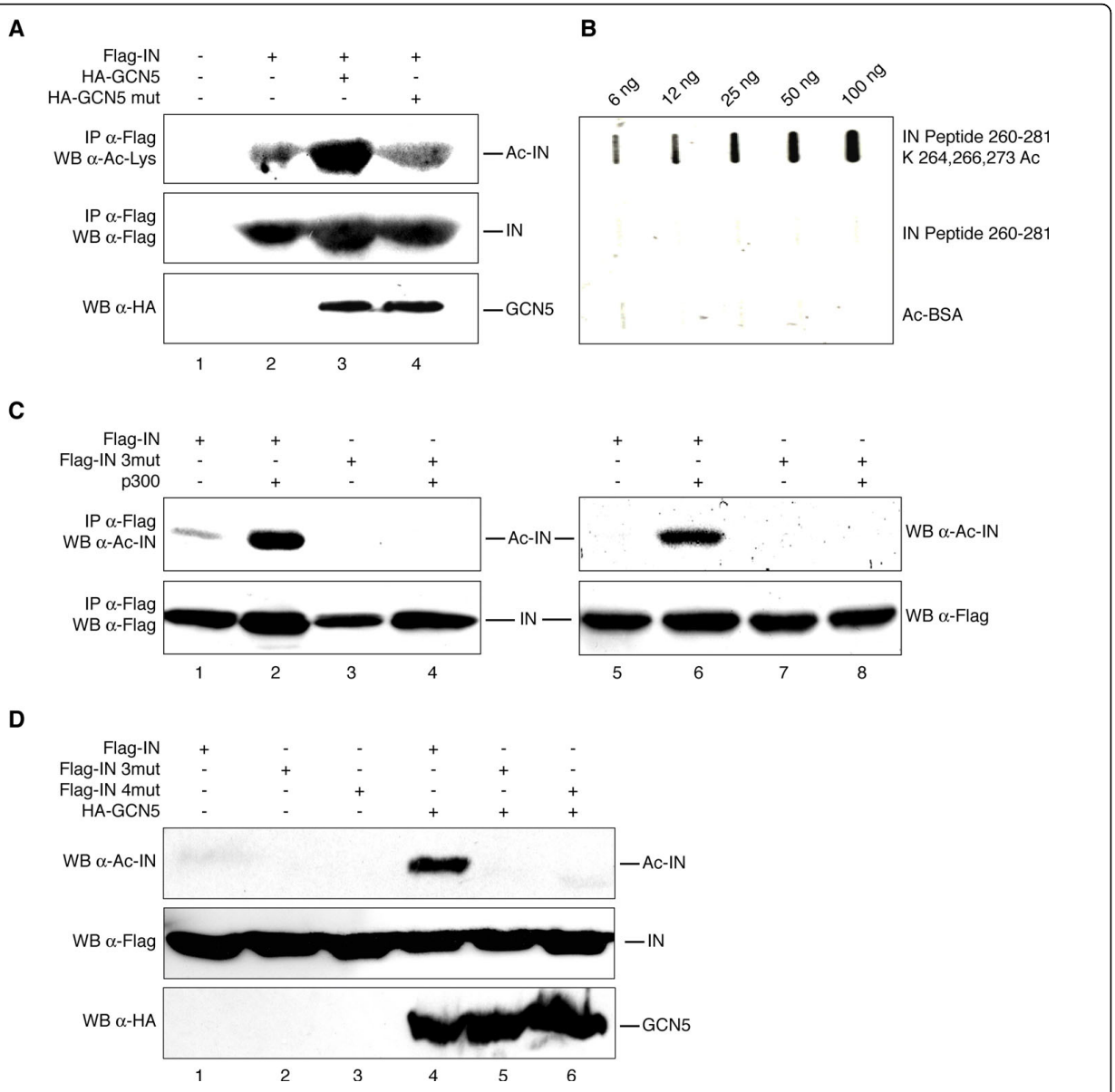


**Figure 2 IN is acetylated by GCN5 *in vivo***. (A) Extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-Flag antibody and analyzed by Western blotting with anti-acetyl-lysine antibody (upper panel) or anti-Flag antibody (middle panel). Lower panel: cell extracts immunoblotted with anti-HA antibody. (B) Acetylated BSA and peptides corresponding to IN amino acids 260-281, either chemically acetylated at lysines 264, 266, and 273, or not acetylated, were blotted onto a nitrocellulose filter and incubated with anti-acetylated IN antibody. (C) Left panels (lanes 1-4): extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-Flag antibody and analyzed by Western blotting with anti-acetylated IN antibody (top panel) or anti-Flag antibody (bottom panel). Right panels (lanes 5-8): extracts from HEK 293T cells transfected with the indicated plasmids analyzed by Western blotting with anti-acetylated-IN antibody (top panel) or anti-Flag antibody (bottom panel). (D) Extracts from HEK 293T cells transfected with the indicated plasmids analyzed by Western blotting with anti-acetylated-IN antibody (upper panel), anti-Flag antibody (middle panel), or anti-HA antibody (lower panel).

### Detection of *in vivo* IN acetylation by a novel anti-acetylated IN antibody

To confirm the *in vitro* observation that IN is a substrate for both GCN5 and p300, an antibody specific to acetylated IN was produced by using an IN-derived peptide for immunization. The IN-derived peptide was chemically acetylated at lysines 264, 266, and 273, which are targeted in common by the two HATs (see the Methods section). As shown in Figure 2B, the purified antibody specifically recognized the acetylated IN peptide in dot blot experiments, while no cross-reactivity was detected with the unmodified peptide or acetylated BSA. This antibody allowed detecting basal levels of IN acetylation by endogenous HATs following immunoprecipitation (Figure 2C, top-left panel, lane 1); additionally, high levels of IN acetylation were detected from cells overexpressing p300 (Figure 2C, top-left panel, lane 2). This result is consistent with our previous study showing that p300 mediates IN acetylation *in vivo* at positions 264, 266, and 273 [1]. Conversely, no signal, expressed either alone or together with p300 (Figure 2C, top-left panel, lanes 3 and 4), was detected with IN K264,266,273R, thus revealing the high specificity of the antibody. In this experiment, the amount of IN (wild type or mutated) immunoprecipitated in each sample was verified by Western blotting with an anti-Flag antibody (Figure 2C, bottom-left panel). The anti-acetylated IN antibody was also used for direct Western blot analysis of cell lysates, producing a strong acetylation signal in the sample corresponding to IN co-expressed with p300 (Figure 2C, top-right panel, lane 6). Therefore, the newly developed antibody showed higher sensitivity than the standard anti-acetyl-lysine antibodies, which require an immunoprecipitation step to reveal IN acetylation. Given the high specificity and sensitivity of the anti-acetylated IN antibody, it was used to confirm the *in vivo* acetylation of IN by GCN5, as well as the mapping of the *in vitro* targeted lysines. As shown in the upper panel of Figure 2D, extracts from cells co-expressing wild type IN and GCN5 revealed a remarkable signal corresponding to IN acetylation (lane 4); while, consistent with the data reported in Figure 2C (top right panel, lane 5), acetylation of the viral enzyme by endogenous HATs was almost undetectable (lane 1). Conversely, no signal with triple- and quadruple-mutant IN, expressed either alone (lanes 2 and 3) or together with GCN5 (lanes 5 and 6) was detected. In this experiment, Western blot analysis of the cell lysates was also performed with anti-Flag and anti-HA antibodies to control the levels of exogenously expressed proteins (Figure 2D, middle and lower panels). Taken together, these data demonstrated that IN is acetylated by GCN5 both *in vitro* and *in vivo*, and the targeted lysines are located in the C-terminal domain at positions 258, 264, 266, and 273.

### IN interacts with GCN5

Since IN is acetylated by GCN5, the interaction between these two factors was investigated. To this aim, HEK 293T cells were transfected with Flag-IN together with HA-GCN5 wild type or mutated in the catalytic domain. After immunoprecipitation with an anti-Flag antibody, both wild type and mutant GCN5 were found to co-precipitate with IN, as demonstrated by Western blot analysis using an anti-HA antibody (Figure 3A, upper panel, lanes 3 and 4). Accordingly, in the reciprocal experiment, where immunoprecipitation was performed with an anti-HA antibody, IN was found to associate with GCN5 (both wild-type and mutant forms) (Figure 3B, upper panel, lanes 3 and 4). In both experiments, the total amounts of immunoprecipitated proteins and the expression levels of IN and GCN5 were verified by Western blotting (Figures 3A and 3B, middle and lower panels).

To map the region of IN mediating the interaction with GCN5, pull-down assays were carried out between GST-GCN5 immobilized on glutathione-Sepharose beads and IN deletion mutants labeled with [$^{35}$S]-Met by *in vitro* translation. As shown in Figure 3C, the affinities of IN fragments 1-272 and 1-263 to GST-GCN5 (13% binding efficiency) were similar to that of full-length IN (16% binding efficiency). Conversely, the GCN5/IN interaction significantly decreased using fragments containing further deletions towards the N-terminus (1-243 and 1-234). These results indicated that the C-terminal region of IN located between amino acids 244 and 288 is involved in binding to GCN5.

### Acetylation by GCN5 increases IN catalytic activity *in vitro*

To explore the effect of GCN5-mediated acetylation on the catalytic activity of IN, constitutively acetylated recombinant IN was produced by exploiting the "tethered catalysis" approach [33,34]. This method allows the production of a constitutively acetylated protein by tethering the factor of interest to the catalytic domain of a specific HAT enzyme. Based on this approach, as schematized in Figure 4A, a chimeric construct was generated where 6× His-tagged IN was fused at its C-terminal end with the HAT domain of GCN5 (amino acids 6-300). To obtain a control that cannot be acetylated, the same chimera was constructed using the inactive mutant of GCN5 Y260A/F261A. In addition, a sequence coding for Tobacco Etch Virus (TEV) protease recognition site was inserted between IN and GCN5 coding sequences to allow for the separation of the two domains. The fusion proteins expressed from the two chimeric constructs were purified, digested with TEV protease, and the acetylation levels of the resulting IN proteins analyzed by Western blotting with an anti-

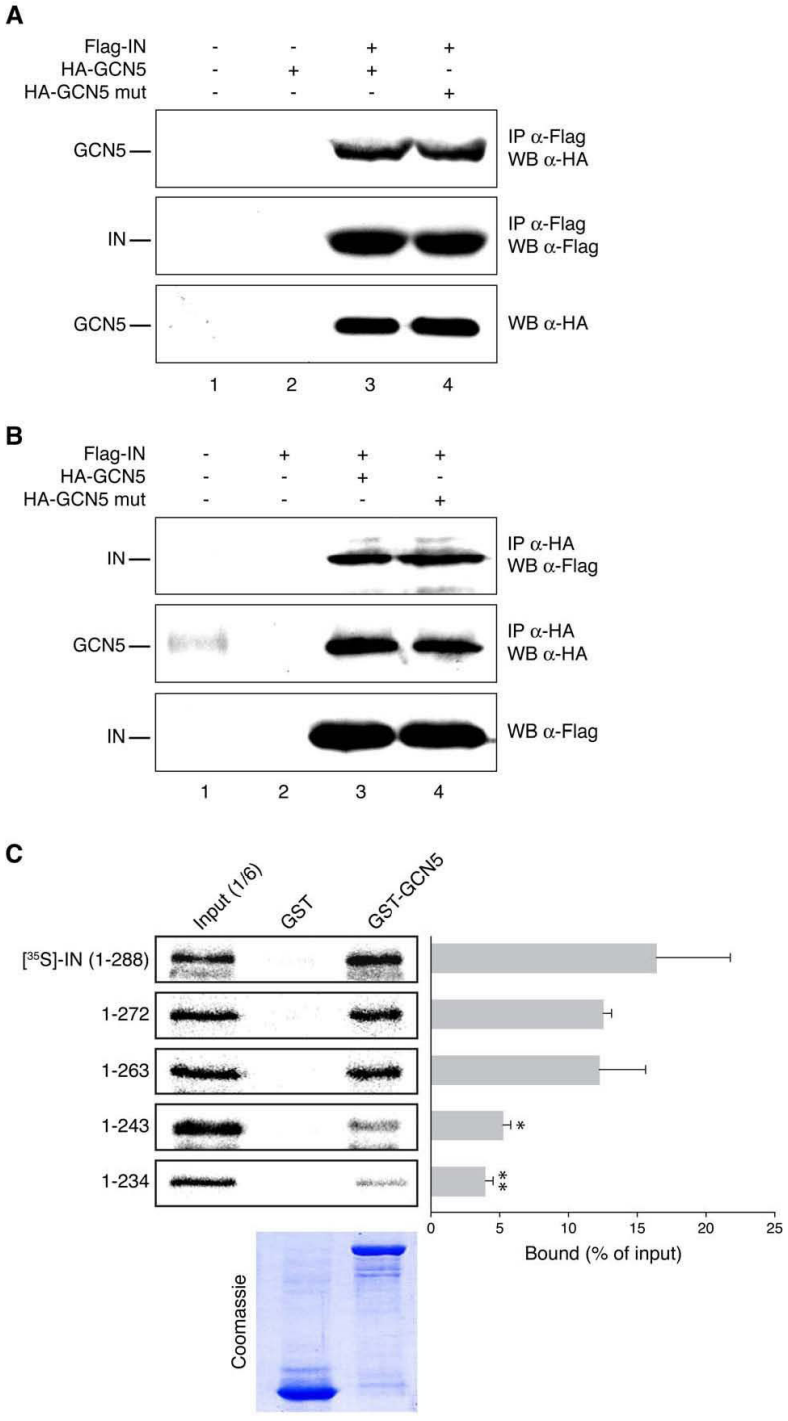


**Figure 3 IN interacts with GCN5 both *in vitro* and *in vivo***. (A) Extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-Flag antibody and analyzed by Western blotting with anti-HA antibody (upper panel) or anti-Flag antibody (middle panel). Lower panel: extracts immunoblotted with anti-HA antibody. (B) Extracts from HEK 293T cells transfected with the indicated plasmids were immunoprecipitated using anti-HA antibody and analyzed by Western blotting with anti-Flag antibody (upper panel) or anti-HA antibody (middle panel). Lower panel: extracts immunoblotted with anti-Flag antibody. (C) Autoradiography and Coomassie Blue staining of *in vitro* binding assays with GST-GCN5 and $^{35}$S-IN or the indicated $^{35}$S-IN fragments. The histogram represents the results of three independent experiments (means ± SEM), where the amounts of bound proteins are expressed as percentages of the corresponding radiolabeled inputs. Statistical significance of the binding percentages was calculated by using the Student's two-sided *t* test. Asterisks directly above bars indicate differences in binding efficiency to GST-GCN5 between IN deleted forms and full-length IN. **, $P < 0,01$; *, $P < 0,05$. Conversely, where asterisks are not present, values obtained did not significantly differ ($P > 0,05$) from those obtained with control, non-silenced cells.

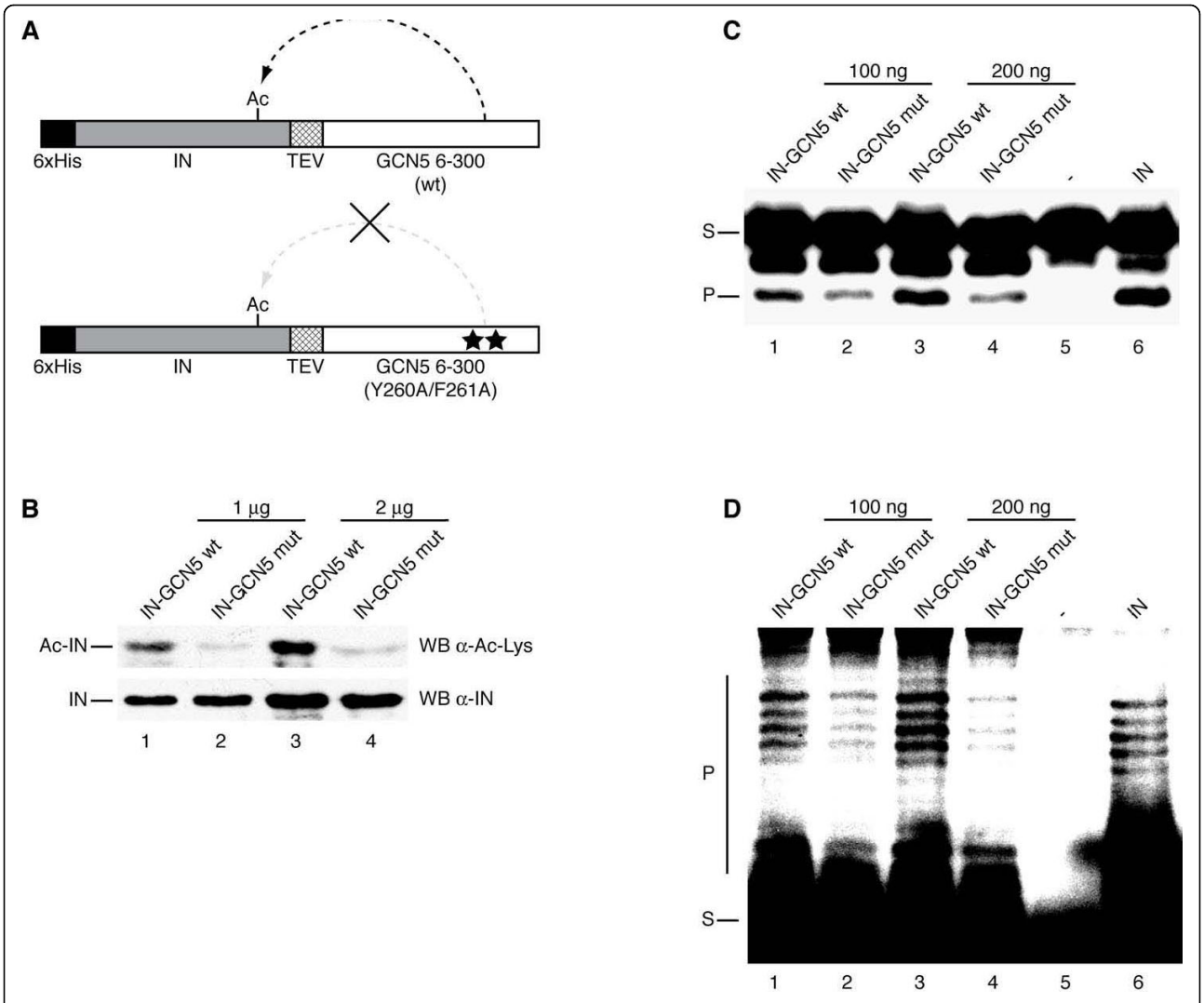


**Figure 4 GCN5-mediated acetylation increases the catalytic activity of IN**. (A) Schematic representation of IN-GCN5 tethered catalysis constructs. Full-length IN, tagged with a N-terminal 6× His epitope, is fused in frame with TEV proteolytic site and cloned upstream of the 6-300 amino acid region of wild type GCN5 (IN-HAT wt) or its catalytically inactive allele (IN-HAT mut). (B) 1 µg and 2 µg of IN derived from IN-HAT wt (lanes 1 and 3, respectively), or 1 µg and 2 µg of IN derived from IN-HAT mut (lanes 2 and 4, respectively) were analyzed by Western blotting with anti-acetyl-lysine antibody (top panel) or anti-IN antibody (bottom panel). (C) 3' -end processing activity of IN derived from IN-HAT wt (lane 1: 100 ng; lane 3: 200 ng) or IN-HAT mut (lane 2: 100 ng; lane 4: 200 ng). Lane 5: DNA substrate; lane 6: DNA substrate with 40 ng of 6× His-tagged IN. (D) Strand transfer activity of IN derived from IN-HAT wt (lane 1: 100 ng; lane 3: 200 ng) or IN-HAT mut (lane 2: 100 ng; lane 4: 200 ng). Lane 5: DNA substrate; lane 6: DNA substrate with 40 ng of 6× His-tagged IN. In (C) and (D), the DNA substrate (S) and the catalytic products (P) are indicated.

acetyl-lysine antibody. IN derived from the wild type GCN5 fusion scored positive for acetylation, while no significant signal was detected with IN derived from the GCN5 mutant chimera (Figure 4B, top panel, compare lanes 1 and 3 with lanes 2 and 4). In this experiment, the levels of loaded proteins were verified by incubating the same membrane with an antibody directed against IN (Figure 4B, bottom panel).

Constitutively acetylated recombinant IN and the non-acetylated control were tested *in vitro* for 3'-end processing and strand transfer activities. In the 3'-end processing reaction, recombinant IN was incubated with a [$^{32}$P]-labeled DNA substrate (S) and the excision of 2 nucleotides evaluated by measuring the radioactive signal of the shorter product (P). In Figure 4C the comparative analysis by densitometry of the bands corresponding to the 3'-end processed template, indicated that acetylated IN (100 ng in lane 1 and 200 ng in lanes 3) was two- to three-fold more active than non-acetylated controls (lanes 2 and 4 respectively). In the strand transfer assay, a [$^{32}$P]-labeled oligonucleotide was used as a substrate (S) and IN activity was evaluated by

measuring the radioactive signal derived from the ladder of higher molecular weight products (P). Constitutively acetylated IN, at two different doses (100 ng and 200 ng), was more active than non-acetylated IN (Figure 4D, compare lanes 1 and 3 with lanes 2 and 4). This was consistent with the 3'-end processing results. Finally, densitometric analysis of the autoradiograms indicated that the two amounts of acetylated IN were five- to ten-fold more active than the corresponding non-acetylated controls.

Taken together, these results demonstrated that GCN5-mediated acetylation enhances the catalytic activity of IN *in vitro*.

#### HIV-1 infectivity is reduced in GCN5 knockdown cells

In order to assess the physiological relevance of the IN/GCN5 interaction during HIV-1 replication cycle, viral infectivity upon GCN5 depletion in target cells was monitored. Transient knockdown of GCN5 expression was obtained in HeLa cells using a specific short interfering RNA (siRNA), while stably silenced HEK 293T cell clones were selected after transduction with a lentiviral vector (pGIPZ from Open Biosystems, Inc.) encoding a short hairpin RNA (shRNA) targeting GCN5 (GCN5 shRNAmir). As a control for the transient knockdown experiments, HeLa cells were transfected with a non-targeting siRNA (unrelated to any human genomic sequence), while stable silencing experiments were checked by using two HEK 293T polyclonal cell lines, one expressing a mismatched, non-targeting GCN5 shRNAmir (GCN5 shRNAmir mut) and the other carrying an empty pGIPZ vector. As shown in the top panels of Figure 5A, siRNA- and shRNAmir-mediated knockdown reduced GCN5 expression to a similar extent. Silenced cells were then infected with an *env*-deleted, VSV-G pseudotyped NL4.3 virus expressing the luciferase reporter gene (indicated hereafter as NL4.3-Luc), and luciferase activity was measured 48 hours after infection. As shown in Figure 5B, a two- to three-fold reduction in luciferase activity was detected in both transiently and stably silenced cells, thus indicating that knockdown of GCN5 expression in target cells reduces HIV-1 infectivity. To determine which step of viral replication was affected by GCN5 depletion, cells were collected at various time points after infection, and measurements of the different HIV-1 DNA species were performed by real time quantitative PCR (RT-Q-PCR). Total HIV-1 DNA was quantified with the use of primers annealing to the luciferase reporter gene, in order to avoid cross-reaction with the integrated pGIPZ lentiviral vectors present in stably transduced cell lines. As shown in Figure 5C, no significant alterations in total HIV-1 DNA levels were detected in cells either transiently or stably silenced, thus indicating that reverse transcription was not affected by the reduction of GCN5 expression. SiRNA-treated cells were analyzed 48 hours post-infection by Alu-LTR nested PCR to detect integrated HIV-1 DNA, while stable knockdown cell clones were processed two weeks after infection using primers specific to the luciferase gene. This was necessary in order to dilute non-integrated HIV-1 DNA and avoid cross-reaction with the integrated pGIPZ lentiviral vectors. Proviral DNA was about two-fold less in all GCN5 knockdown cells, either treated with siRNA or transduced with shRNAmir-encoding lentiviral vectors (Figure 5D). Finally, a two-fold increase in the amount of two-LTR circles was detected in both stably and transiently silenced cells (Figure 5E). Since the increase in two-LTR circles often correlates with a defect at the step of integration [35], these data are collectively consistent with reduced integration efficiency in GCN5 knockdown cells.

#### Mutations at IN acetylation sites cause a defect in HIV-1 replication at the integration step

Since the IN lysines acetylated by GCN5 partially overlap with those targeted by p300, a comparative analysis was performed to evaluate the role of these residues during the HIV-1 replication cycle. To this aim, single-round infections were performed, using *env*-deleted NL4.3-Luc viruses expressing either IN K264,266,273R (NL4.3-Luc-3mut), or IN K258,264,266,273R (NL4.3-Luc-4mut). Luciferase activity was measured 48 hours after infection, revealing an average five-fold reduction in infectivity for both mutant viruses as compared to wild type (Figure 6A). To determine which step of viral replication was affected by the lysine-to-arginine substitutions, DNA was extracted from cells at several time points after infection and the different HIV-1 DNA species were measured by RT-Q-PCR. Infection with NL4.3-Luc-3mut and 4mut, as well as with wild type virus, resulted in similar levels of total HIV-1 DNA at 24 hours post-infection (Figure 6B), indicating that reverse transcription was not affected by the amino acidic substitutions. Integrated HIV-1 DNA was quantified at 48 hours post-infection by Alu-LTR nested PCR, showing a five-fold reduction in the number of proviruses for both mutant clones with respect to wild type (Figure 6C). These data indicated decreased integration efficiency upon mutation of IN lysines targeted by acetylation. Consistently, a three-fold increase in the amount of two-LTR circles was detected at 24 hours post-infection with both NL4.3-Luc-3mut and 4mut (Figure 6D), confirming a specific defect at the step of integration and no alterations during viral nuclear import.

To investigate the role of IN acetylated lysines during HIV-1 replication in a T-cell line, two NL4.3-derived clones were generated, expressing either the triple- or

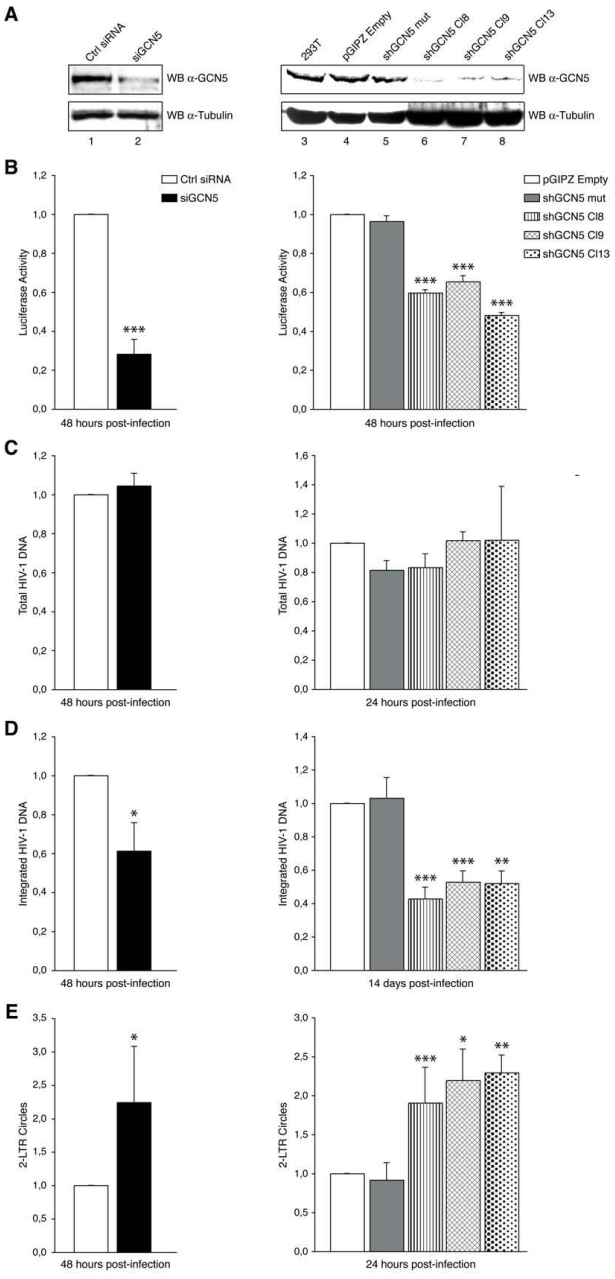


**Figure 5**

**Figure 5 GCN5 depletion in infected cells reduces HIV-1 integration**. (A) Left panels: extracts from siRNA-treated Hela cells analyzed by Western blotting with anti-GCN5 antibody (top) or anti-α-tubulin antibody (bottom). Lane 1: cells transfected with non-targeting siRNA (Ctrl siRNA); lane 2: cells transfected with GCN5-targeting siRNA (siGCN5). Right panels: extracts from stable GCN5 knockdown HEK 293T cell clones or control cells immunoblotted with anti-GCN5 antibody (top panel) or anti-α-tubulin antibody (bottom panel). Lane 3: untransduced HEK 293T cells; lane 4: HEK 293T cells carrying empty pGIPZ vector; lane 5: HEK 293T cells expressing mutant, non-targeting GCN5 shRNAmir; lanes 6-8: HEK 293T clones (Cl8, Cl9 and Cl13) expressing GCN5 shRNAmir. (B) siRNA-treated Hela cells (left histogram) or HEK 293T cells stably transduced with pGIPZ lentiviral vectors (right histogram) were infected with NL4.3-Luc and analyzed for luciferase activity 48 hours after infection. The histograms represent percentages of luciferase activity relative to control, non-silenced cells. Means ± SEM from three independent experiments are reported. (C-E) Total DNA extracted from siRNA-treated HeLa cells (left histograms) or HEK 293T cells stably transduced with pGIPZ lentiviral vectors (right histograms) was analyzed by RT-Q-PCR for total HIV-1 DNA (C), integrated HIV-1 DNA (D), and two-LTR circles (E). In (C-E), results are presented as percentages relative to control, non-silenced cells. Reported values are means ± SEM from three independent experiments. Statistical significance values shown in (B-E) were calculated by using the Student's two-sided *t* test. Asterisks directly above bars indicate differences between knockdown and control, non-silenced cells. ***, $P < 0,001$; **, $P < 0,01$; *, $P < 0,05$. Conversely, where asterisks are not present, values obtained did not significantly differ ($P > 0,05$) from those obtained with control, non-silenced cells.

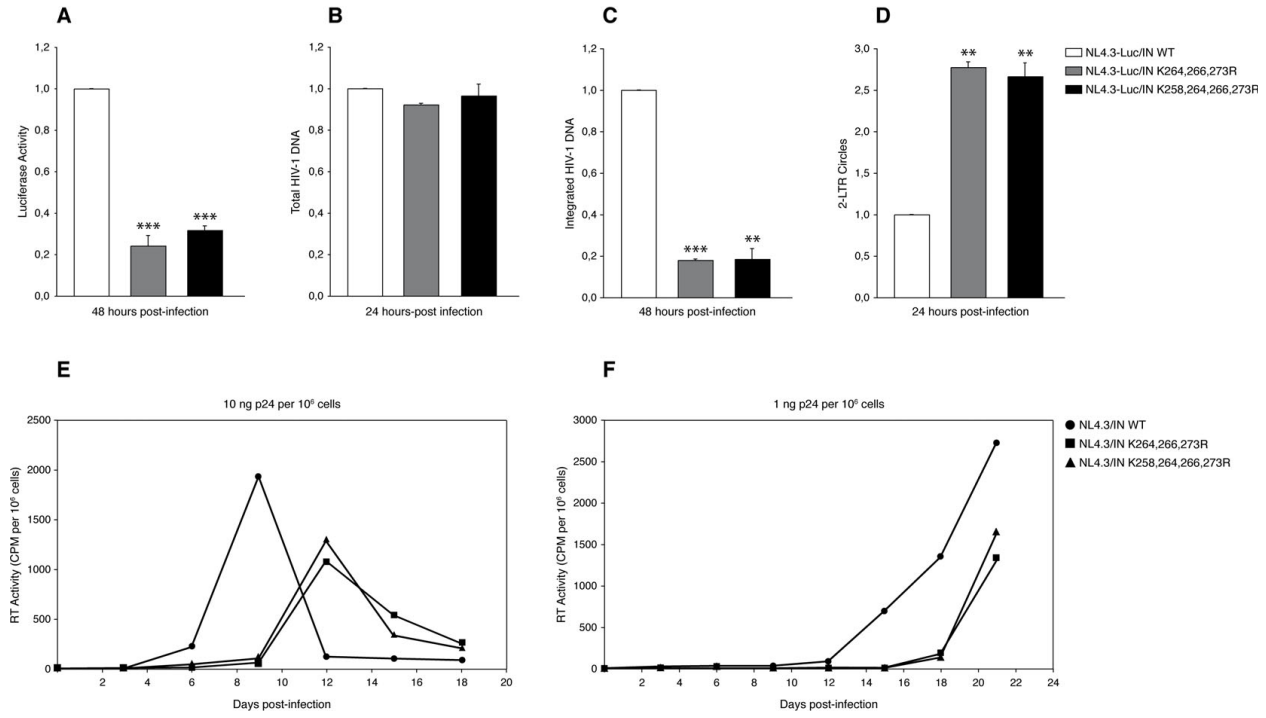


**Figure 6 Mutations at IN acetylation sites cause a replication defect at the step of integration**. (A) HEK 293T cells infected with NL4.3-Luc/IN WT, NL4.3-Luc/IN K264,266,273R, or NL4.3-Luc/IN K258,264,266,273R were analyzed for luciferase activity 48 hours after infection. (B-D) Total DNA extracted from HEK 293T cells infected with the same viral clones as in (A) was analyzed by RT-Q-PCR for total HIV-1 DNA at 24 hours after infection (B), integrated HIV-1 DNA at 48 hours after infection (C) and two-LTR circles at 24 hours after infection (D). In (A-D), results are presented as percentages relative to cells infected with NL4.3-Luc/IN WT virus. Reported values are means ± SEM from three independent experiments. Statistical significance values shown in (A-D) were calculated by using the Student's two-sided *t* test. Asterisks directly above bars indicate differences between cells infected with mutant viruses and cells infected with wild type virus. ***, $P < 0,001$; **, $P < 0,01$. Conversely, where asterisks are not present, values obtained did not significantly differ ($P > 0,05$) from those obtained with cells infected with wild type virus. (E) RT activity detected in the culture supernatants of CEM cells at different time points after infection with NL4.3/IN WT, NL4.3/IN K264,266,273R, or NL4.3/IN K258,264,266,273R. (F) Infections performed as in (E), using 10-fold lower viral loads.

quadruple-mutant IN (NL4.3-3mut and NL4.3-4mut, respectively). One million CEM T-cells were infected with the resulting viruses using two different amounts of p24 antigen (10 ng or 1 ng). Viral replication was followed by measuring HIV-1 reverse transcriptase (RT) activity in the culture supernatants every three days over a period of 21 days. As shown in Figure 6E, cells infected with the higher viral load (10 ng of p24) of wild type virus showed a peak of HIV-1 replication around day 9 post-infection. Conversely, infections with the same amounts of NL4.3-3mut and -4mut resulted in delayed peaks at day 12. Notably, at the infectivity peak, the RT amounts produced by both mutant HIV-1 clones were approximately half of that obtained with wild type virus. By using the lower viral load (1 ng of p24), the replication curve of wild type virus started to raise quite steeply around day 12 post infection, while for both mutant clones the curves started to appear at day 15. Detectable RT production was observed for both mutant viruses at day 18, thus with 6 days of delay compared to the kinetics of the wild type virus (Figure 6F). In conclusion, mutations introduced in the virus at IN acetylation sites targeted by both GCN5 and p300 (K264, K266, and K273), or additional mutation at lysine 258 specifically acetylated by GCN5 *in vitro*, determined similar decreases in viral integration and infectivity.

## Discussion

The results presented in this study reveal that GCN5 is a novel HAT which interacts with IN. GCN5 binding to the C-terminal domain of IN leads to the acetylation of IN at lysines 258, 264, 266 and 273, located within the same region required for the two proteins to interact. We have recently demonstrated that the carboxy terminus of IN is a substrate for another cellular HAT, p300, which acetylates IN lysines at positions 264, 266, and 273 [1], a finding that was also later confirmed by Topper and coworkers [2]. Therefore, based on previous and present studies, three IN lysines (K264, K266, and K273) are acetylated by both HATs, while lysine 258 appears to be specifically targeted by GCN5. Our mapping of the HAT-interacting regions of IN based on

*in vitro* binding assays is consistent with a recent report which presented two models of full-length IN complexed with GCN5 and p300 [36]. Both models predict that the IN C-terminal tail located between amino acids 271 and 288, due to its high flexibility, could easily adapt to the binding pocket of GCN5, as well as to that of p300 (Figure 7). Interestingly, lysine 273 is included in this unstructured region and is therefore expected to be the residue most prone to acetylation. In fact, since lysines 264 and 266 are located in close proximity to a sandwich of two three-stranded antiparallel β-sheets, their binding and acetylation would require a more complex unfolding of this stable secondary structure. Based on this model, we may hypothesize that IN lysine 273 is the first residue contacted and acetylated by the HAT enzyme, whether GCN5 or p300. This event might in turn induce a conformational change in the C-terminal portion of IN, which could facilitate the modification of the other two lysines. This hypothesis is also compatible with the data reported by Topper and coworkers, demonstrating a hierarchy of reactivity between the three residues modified by p300, with lysine 273 as the key site targeted for acetylation [2].

A comparative analysis, aimed at establishing the roles of the two HATs during the HIV-1 replication cycle, revealed that the mutant viruses expressing either IN K264,266,273R or IN K258,264,266,273R exhibited the same replication deficiency, specifically affecting the step of integration. These results indicated that acetylation of IN C-terminal lysines 264, 266, and 273 is required for maximal HIV-1 integration efficiency, while acetylation of lysine 258, although observed *in vitro*, does not appear to play any significant role during infection.

Proteins modified by acetylation, including viral factors, are often targeted by multiple HATs in a redundant manner. For instance, HIV-1 Tat is acetylated at lysines 50 and 51 by p300/CBP and GCN5, leading in both cases to an increased transactivation activity of the modified protein on the viral LTR promoter [27-30]. The action of two different HATs on common sites of the same substrate may be ascribed to the importance of acetylation for the functionality of the target protein. However, in the case of IN, the reduced viral integration capacity detected in GCN5 knockdown cells indicated that endogenous p300 is not able to fully compensate for the lack of GCN5 so as to completely restore HIV-1 infectivity.

The role of IN acetylation at lysines 264, 266 and 273 during the HIV-1 replication cycle has been the subject of a recent debate. Our former study showed that the replication level of a HIV-$1_{BRU}$ clone expressing a triple-mutant Flag-tagged IN (Flag-IN K264,266,273R) was severely impaired, and that the replication deficiency was specifically due to a block at the integration step [1]. In subsequent reports, the untagged triple-mutant virus showed either no replication defect [2], or a five-fold infectivity decrease in single-round infections [37]. Moreover, by using a genetic assay where integration was evaluated through the number of cell clones containing proviruses, one report [2] detailed almost half decreased integration efficiency, while the other [37] indicated a 14-fold lower residual integration rate. In

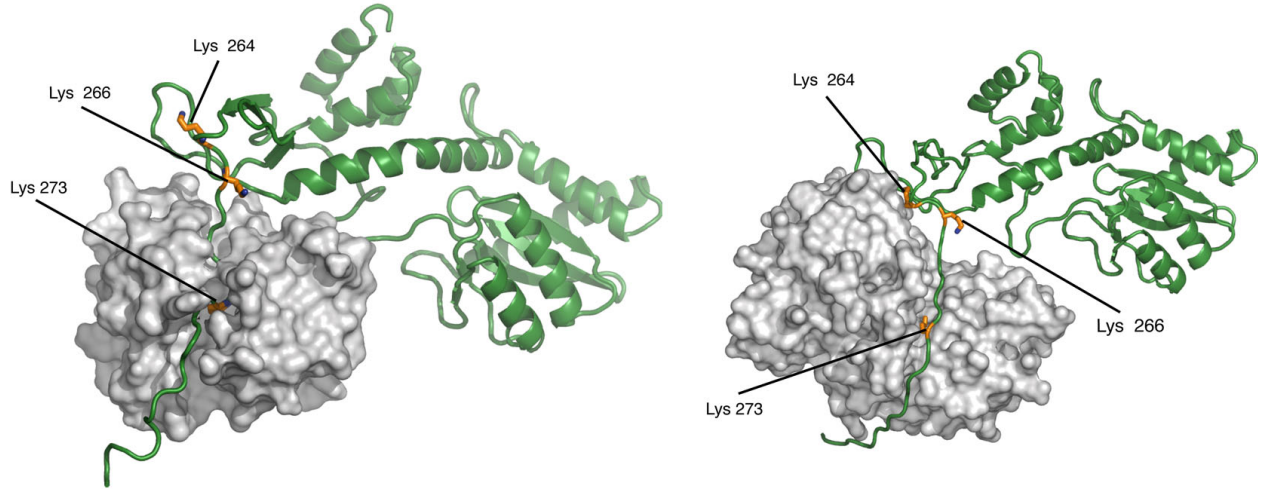


**Figure 7 Three-dimensional models of IN complexes with GCN5 and p300**. (A) Three-dimensional model of the IN/GCN5 complex. IN is represented in green and GCN5 in light grey. (B) Three-dimensional model of the IN/p300 complex. IN is represented in green and p300 in light grey. In (A) and (B), the three lysine residues in the C-terminal domain of IN that are acetylated by both GCN5 and p300 (Lys 264, Lys 266, and Lys 273) are shown in yellow. GCN5 and p300 are rendered as surfaces, while IN as a cartoon to highlight the C-terminal unfolded portion which inserts in the binding pockets of the two HATs.

the present study, we performed single- and multiple-round infections with HIV-1 clones encoding IN either mutated at the positions targeted by both GCN5 and p300 (IN K264,266,273R), or carrying an additional lysine-to-arginine substitution at the site specifically modified by GCN5 (IN K258,264,266,273R). In multiple-round replication experiments, both mutant clones showed reduced virus production and delays in the peaks of infectivity with respect to wild type. The discrepancy of these findings with the data reported by Topper *et al.* [2] might be due to the different time-courses of analyses: although working in the same experimental conditions (10 ng of p24 antigen per $1\times 10^6$ CEM cells), the detection of RT activity in the culture supernatants over a period of 21 days allowed us to monitor the peak of HIV-1 replication, while Topper and coworkers terminated the replication curve before the highest point of viral infectivity was reached (at 12 days post infection).

Moreover, consistent with Apolonia *et al.* [37], we detected a five-fold infectivity decrease in single-round replication assays performed with IN triple- and quadruple-mutant viruses. The five-fold infectivity decrease paralleled a five-fold reduction in the number of proviruses, as measured by RT-Q-PCR. Taken together, the results presented in all the different reports suggest that acetylation of IN C-terminal lysines 264, 266, and 273 represents a mechanism which, by finely regulating the integration process, contributes to determine the efficiency of HIV-1 replication.

Identification of lysines 258, 264, 266, and 273 as the targets of GCN5 activity on IN does not exclude that additional residues might be acetylated, as indicated by the residual acetylation level of the quadruple-mutant IN (Figure 1A, lane 15). Finally, IN could also be subject to different post-translational modifications, such as methylation, sumoylation, or ubiquitination [38-41], which might open up new mechanisms of modulation of IN function.

## Conclusions

This study demonstrates that, in addition to the formerly reported p300, another HAT, GCN5, acetylates the C-terminal domain of IN. Similar to p300, GCN5-mediated acetylation is required for efficient viral integration, thus reinforcing the role of this post-translational modification for HIV-1 replication.

## Methods

### Plasmids

Construction of pGEX-IN has already been described [1]. pcDNA3-HA-IN was obtained by subcloning IN sequence from pGEX-IN plasmid into pcDNA3-HA vector. pGEX-IN and pcDNA3-HA-IN deletion mutants were produced by PCR amplification of IN with primers specific to the deleted versions. pASK-IBA37-IN was constructed by subcloning IN sequence from pGEX-IN plasmid into pASK-IBA37 vector (IBA GmbH, Göttingen, DE). pFlag-IN codon optimized (c.o.) was kindly provided by A. Engelman. pASK-IBA37-IN point mutants and pFlag-IN c.o. K264,266,273R or K258,264,266,273R were obtained by PCR-based site-directed mutagenesis starting from the corresponding plasmids encoding wild type IN.

pGEX-GCN5 was a kind gift of M. Benkirane. pGEX-GCN5 deletion mutants were produced by PCR amplification of GCN5 with primers specific to the truncated forms. pcDNA3-HA-GCN5 was constructed by subcloning GCN5 sequence from pGEX-GCN5 plasmid into pcDNA3-HA vector. pcDNA3-HA-GCN5 (Y260A/F261A) [32] was obtained by PCR-based site-directed mutagenesis starting from the plasmid encoding wild type GCN5.

For production of IN-GCN5 tethered catalysis constructs, the sequence coding for the 6-300 amino acid region of GCN5 was amplified by PCR from pcDNA3-HA-GCN5 or pcDNA3-HA-GCN5 (Y260A/F261A) and cloned into a pASK-IBA37 vector in frame with c.o. IN. The sequence encoding TEV protease recognition site was inserted by PCR between IN and GCN5 cDNAs.

pGIPZ and pGIPZGCN5 lentiviral vectors were purchased from Open Biosystems (Huntsville, AL). The sequence of GCN5 shRNAmir inserted into the pGIPZGCN5 vector is as follows: 5'-CCCATTCATT CCCTGGCATTAATAGTGAAGCCACAG ATGTATT AATGCCAGGGAATGAATGGT-3'. For production of the pGIPZGCN5 mut vector, four point mutations were introduced in the shRNAmir cassette of pGIPZGCN5, obtaining the following sequence: 5'-CCCATTCA**AA GG**CTGGCA TTAATAGTGAAGCCACAGATGTATT AATGCCAG**CCTT**TGAATGGT-3', where mutated nucleotides are underlined.

The NL4.3-Luc *env*-deleted virus expressing the luciferase reporter gene was produced from the pNL4.3.Luc. R-E- molecular clone obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. IN sequence was subcloned from the molecular clone pHXB2 for construction of pNL4.3.Luc.R-E-/IN WT and pNL4.3/IN WT plasmids. The IN mutations in pNL4.3.Luc.R-E-/IN K264,266,273R, pNL4.3.Luc.R-E-/IN K258,264,266,273R and in pNL4.3/IN K264,266,273R, pNL4.3/IN K258,264,266,273R were introduced by PCR-based site-directed mutagenesis using either pNL4.3.Luc. R-E-/IN WT or pNL4.3/IN WT as template.

The envelope plasmid pMDG and the packaging plasmid pCMVΔR8.91 were kindly provided by Z. Debyser.

### *In vitro* acetylation assay

HAT assays were performed as previously described [1], with minor modifications. Briefly, GST or 6× His-tag fusion proteins were incubated with GST-GCN5 and [$^{14}$C]-acetyl-CoA in HAT buffer (50 mM Tris-HCl, pH 8.0, 5% glycerol, 0.1 M EDTA, 50 mM KCl and 2 mM sodium butyrate) in a final volume of 30 μl for 45 min at 30°C. Acetylated proteins were visualized by phosphoimaging (Cyclone) after separation by SDS-PAGE.

### In vitro binding assay

[$^{35}$S]-labeled IN proteins used for *in vitro* binding assays were produced from the corresponding pcDNA3-HA plasmids by using the TNT Reticulocyte Lysate System (Promega Corp., Madison, WI). Analysis of *in vitro* binding between GST fusion proteins and [$^{35}$S]-IN or [$^{35}$S]-IN fragments was performed as previously described [1]. Briefly, GST fusion proteins (1 μg) immobilized on agarose beads, after pre-treatment in a solution containing DNase I 0.25 U/μl and RNase H 0.25 U/μl, were incubated with 600 c.p.m. of *in vitro* translated [$^{35}$S]-proteins in a solution containing 0.2 mg/ml ethidium bromide. Following extensive washes, the reaction mixtures were resolved by SDS-PAGE and radiolabeled proteins visualized by phosphoimaging (Cyclone).

### Recombinant proteins production and proteolytic processing

GST fusion proteins were expressed and purified from *Escherichia Coli* BL21 as already described [1].

N-terminal 6× His-tagged IN proteins were expressed in *Escherichia Coli* BL21 and purified by metal ion affinity chromatography (BD TALON Metal Affinity Resin, BD Biosciences, Palo Alto, CA) according to a previously reported protocol [42]. Proteolytic processing of IN-GCN5 chimeras was performed by incubating 20 μg of fusion protein with 30 U of TEV protease (AcTEV Protease, Invitrogen, Inc., Carlsbad, CA) in a buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1 M NaCl, 1 mM DTT and 10% glycerol, overnight at 4°C. 6× His-tagged IN was then recovered from the reaction mixture by adsorption on BD TALON Resin.

### Immunoprecipitation and Western blotting

For immunoprecipitation, cell pellets were lysed 36 hours after transfection in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycolic acid) containing 10 mM sodium butyrate (Sigma, Inc.) and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics). Anti-Flag M2 affinity resin or rat monoclonal anti-HA antibody were incubated overnight at 4°C with the cell lysates (2 mg for coimmunoprecipitation or 4 mg for in vivo acetylation experiments). The HA-immune complexes were precipitated by incubation with Ultra-Link Immobilized Protein G (Pierce Biotechnology, Inc., Rockford, IL). The precipitated complexes were then extensively washed and analyzed by Western blotting using the appropriate antibodies.

### Antibodies

The following primary antibodies were used: rabbit anti-acetylated-lysine (Cell Signaling Technology, Inc., Danvers, MA); mouse anti-Flag M2 (Sigma, Inc., St Louis, MO), either free or bound to agarose beads; rat anti-HA Clone 3F10 (Roche Diagnostics, Indianapolis, IN); mouse anti-IN 8G4, obtained from the AIDS Research and Reference Reagent Program; rabbit anti-GCN5 H-75 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mouse anti-α-tubulin Clone B-5-1-2 (Sigma, Inc.).

For the production of a polyclonal, anti-acetylated IN antibody, three rabbits were immunized with a peptide corresponding to amino acids 261-280 of the IN sequence, chemically acetylated at lysines 264, 266 and 273, after conjugation with Maleimide Activated mcKLH (Pierce Biotechnology, Inc.). The IgG fraction was obtained from collected sera with the use of ImmunoPure (A) IgG Purification Kit (Pierce Biotechnology, Inc.). The purified samples were then passed over a column conjugated with the unmodified IN peptide to remove the antibody cross-reacting with non-acetylated IN.

Secondary horseradish peroxidase (HRP)-conjugated antibodies against mouse or rabbit Igs were purchased by Santa Cruz Biotechnology, Inc. For Western blot analysis with anti-acetylated-lysine antibody, Biotin-SP-conjugated AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and ECL Streptavidin-HRP conjugate (Amersham Biosciences Corp., Piscataway, NJ) were employed.

### Cell cultures and virus production

HeLa and HEK 293T cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. HEK 293T cells stably transduced with pGIPZ vectors were grown with the addition of puromycin 2 μg/ml. CEM cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

To produce *env*-deleted, VSV-G pseudotyped NL4.3-Luc viruses, 6× $10^6$ HEK 293T cells were transfected with 20 μg of pNL4.3.Luc.R-E- (wild-type or mutated) and 5 μg of the envelope plasmid pMDG using the PEI reagent (Sigma, Inc.). The cell culture supernatant was collected 48 h after transfection and filtered through a 0.45 μM pore size filter.

NL4.3 replication competent viruses were prepared as described for NL4.3-Luc viral clones, using 25 μg of pNL4.3 plasmid (wild-type or mutated) for transfections.

For the generation of viral vector stocks, HEK 293T cells were transfected with 10 μg of the packaging plasmid pCMVΔR8.91, 5 μg of pMDG and 20 μg of the gene transfer plasmid (pGIPZ, pGIPZGCN5, or pGIPZGCN5 mut), following the protocol used for virus production. The cell culture supernatant was collected twice, at 48 h and 72 h after transfection, filtered through a 0.45 μM pore size filter and concentrated by ultracentrifugation at 110000 × g for 2 h at 4°C.

Both viruses and viral vectors were titered by quantification of p24 antigen in cell culture supernatants with an enzyme-linked immunoabsorbent assay (Innogenetics, Gent, Belgium).

#### Transient and stable knockdown of GCN5 expression

GCN5-targeting siRNA (Dharmacon Research, Boulder, CO) had the following plus-strand sequence: 5'-AACCAUGGAGCUGGUCAAUGAAA-3'. As a non-silencing control, Dharmacon ON-TARGETplus siCONTROL Non-Targeting Pool was employed.

HeLa cells, seeded in 6-well plates ($1.5 \times 10^6$ cells/well), were transfected twice at a 24 h interval with 150 nM siRNA using Gene Silencer reagent as recommended by the manufacturer (Gene Therapy Systems, Inc., San Diego, CA). Cells trypsinized after 20 h were either plated for infections, or lysed for Western blot analysis.

For production of stably silenced cell lines, HEK 293T cells, seeded in 24-well plates ($5 \times 10^4$ cells/well), were transduced with shRNAmir-encoding pGIPZ lentiviral vectors and grown in medium containing 2 μg/ml puromycin.

#### Infectivity and IN activity assays

For single-round replication assays, siRNA-treated HeLa cells ($2.5 \times 10^6$/well) or HEK 293T cells ($5 \times 10^6$/well) were seeded in 6-well plates and incubated for 3 h, in a total volume of 500 μl, with 50 or 100 ng p24 antigen of NL4.3-Luc virus (wild type or mutated), respectively. Cells were collected 48 h after infection for measurement of luciferase activity (Luciferase Assay System, Promega Corp.).

Viral stocks used in infections for measurement of HIV-1 DNA species by RT-Q-PCR were pre-treated for 1 h at 37°C with 160 U/ml Turbo DNase (Ambion, Inc., Austin, TX).

For multiple-round infections, $1 \times 10^6$ CEM cells were incubated with 1 ng or 10 ng p24 antigen of NL4.3 virus (wild-type or mutated) in a total volume of 500 μl for 3 h. Every 3 days, supernatants were collected and viral titers determined by a $^{32}$P-based RT assay performed by standard procedures.

To evaluate IN catalytic activity *in vitro*, 3'-end processing and strand transfer reactions were performed with recombinant IN proteins as previously described [1].

#### Real-time quantitative PCR analysis

Total DNA was extracted from HEK 293T cells with the DNeasy Tissue Kit (QIAGEN, Valencia, CA) at different time points after infection. Amplification reactions were performed with the Light Cycler 480 instrument (Roche Diagnostics). Quantification of total HIV-1 DNA was performed with a pair of primers and a fluorogenic hybridization probe annealing to the luciferase reporter gene of NL4.3-Luc viral clone. The sequences of the primers and the probe are as follows: forward primer, LucFw, 5'-GAAGAGATACGCCCTGGTTCC-3'; reverse primer, LucRev, 5'-TGTGATTTGTATTCAGCCCATATCG-3'; and probe, LucProbe, 5'-FAM-TTCATAGCTTCTGCCAACCGAACGGACA-3' - BlackBerry Quencher. Reaction mixtures contained 500 ng of total genomic DNA, 1× Light Cycler 480 Probe Master (Roche Diagnostics), 300 nM each forward and reverse primers and 200 nM probe in a total volume of 20 μl. After an initial denaturation step (95°C for 10 min), the cycling profile was 40 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Quantifications of proviral DNA at 48 h post infection (Alu-LTR nested PCR) and of two-LTR circles were performed according to previously described protocols [43]. For detection of integrated HIV-1 DNA in HEK 293T cells transduced with pGIPZ vectors, cells were maintained in culture for two weeks and proviruses were quantified using LucFw, LucRev primers and LucProbe.

As an internal standard for normalizing the amount of cellular genomic DNA, the level of human β-globin DNA was determined in each sample using primers and fluorogenic hybridization probe that were previously described [44]. The amplification conditions included a hot start at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and extension at 60°C for 1 min.

#### Statistical analysis

Paired comparisons were carried out using two-tailed Student's *t*-tests, assuming equal variance between samples to determine differences at the 5% level; all data points (including outliers) were included in the analysis for significance.

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**Authors' contributions**
MT designed and performed the experiments, analyzed the data, wrote the manuscript; PV performed the experiments and analyzed the data; VL designed the experiments and analyzed the data; MIG performed the experiments and analyzed the data; CDP performed the experiments and helped in the design of the study; ADF performed the computational analysis; VT performed the computational analysis; AAII performed the experiments and analyzed the data; AAIb performed the experiments and analyzed the data; MG designed the research and analyzed the data; AC designed the research, analyzed the data and wrote the manuscript

**Competing interests**
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**References**

1. Cereseto A, Manganaro L, Gutierrez MI, Terreni M, Fittipaldi A, Lusic M, Marcello A, Giacca M: **Acetylation of HIV-1 integrase by p300 regulates viral integration.** *Embo J* 2005, **24**:3070-3081.
2. Topper M, Luo Y, Zhadina M, Mohammed K, Smith L, Muesing MA: **Posttranslational acetylation of the human immunodeficiency virus type 1 integrase carboxyl-terminal domain is dispensable for viral replication.** *J Virol* 2007, **81**:3012-3017.
3. Carrozza MJ, Utley RT, Workman JL, Cote J: **The diverse functions of histone acetyltransferase complexes.** *Trends Genet* 2003, **19**:321-329.
4. Roth SY, Denu JM, Allis CD: **Histone acetyltransferases.** *Annu Rev Biochem* 2001, **70**:81-120.
5. Yang XJ, Seto E: **HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention.** *Oncogene* 2007, **26**:5310-5318.
6. Gu W, Roeder RG: **Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain.** *Cell* 1997, **90**:595-606.
7. Marzio G, Wagener C, Gutierrez MI, Cartwright P, Helin K, Giacca M: **E2F family members are differentially regulated by reversible acetylation.** *J Biol Chem* 2000, **275**:10887-10892.
8. Yao YL, Yang WM, Seto E: **Regulation of transcription factor YY1 by acetylation and deacetylation.** *Mol Cell Biol* 2001, **21**:5979-5991.
9. Bannister AJ, Miska EA, Gorlich D, Kouzarides T: **Acetylation of importin-alpha nuclear import factors by CBP/p300.** *Curr Biol* 2000, **10**:467-470.
10. Cohen HY, Lavu S, Bitterman KJ, Hekking B, Imahiyerobo TA, Miller C, Frye R, Ploegh H, Kessler BM, Sinclair DA: **Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis.** *Mol Cell* 2004, **13**:627-638.
11. Kovacs JJ, Murphy PJ, Gaillard S, Zhao X, Wu JT, Nicchitta CV, Yoshida M, Toft DO, Pratt WB, Yao TP: **HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor.** *Mol Cell* 2005, **18**:601-607.
12. Yuan ZL, Guan YJ, Chatterjee D, Chin YE: **Stat3 dimerization regulated by reversible acetylation of a single lysine residue.** *Science* 2005, **307**:269-273.
13. Bernassola F, Salomoni P, Oberst A, Di Como CJ, Pagano M, Melino G, Pandolfi PP: **Ubiquitin-dependent degradation of p73 is inhibited by PML.** *J Exp Med* 2004, **199**:1545-1557.
14. Jeong JW, Bae MK, Ahn MY, Kim SH, Sohn TK, Bae MH, Yoo MA, Song EJ, Lee KJ, Kim KW: **Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation.** *Cell* 2002, **111**:709-720.
15. Li M, Luo J, Brooks CL, Gu W: **Acetylation of p53 inhibits its ubiquitination by Mdm2.** *J Biol Chem* 2002, **277**:50607-50611.
16. Blander G, Zalle N, Daniely Y, Taplick J, Gray MD, Oren M: **DNA damage-induced translocation of the Werner helicase is regulated by acetylation.** *J Biol Chem* 2002, **277**:50934-50940.
17. Kawaguchi Y, Ito A, Appella E, Yao TP: **Charge modification at multiple C-terminal lysine residues regulates p53 oligomerization and its nucleus-cytoplasm trafficking.** *J Biol Chem* 2006, **281**:1394-1400.
18. Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, Gu W, Accili D: **FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction.** *Cell Metab* 2005, **2**:153-163.
19. Thevenet L, Mejean C, Moniot B, Bonneaud N, Galeotti N, Aldrian-Herrada G, Poulat F, Berta P, Benkirane M, Boizet-Bonhoure B: **Regulation of human SRY subcellular distribution by its acetylation/deacetylation.** *Embo J* 2004, **23**:3336-3345.
20. Alfonso P, Quetglas JI, Escribano JM, Alonso C: **Protein pE120R of African swine fever virus is post-translationally acetylated as revealed by post-source decay MALDI mass spectrometry.** *Virus Genes* 2007, **35**:81-85.
21. Madison DL, Yaciuk P, Kwok RP, Lundblad JR: **Acetylation of the adenovirus-transforming protein E1A determines nuclear localization by disrupting association with importin-alpha.** *J Biol Chem* 2002, **277**:38755-38763.
22. Mu JJ, Tsay YG, Juan LJ, Fu TF, Huang WH, Chen DS, Chen PJ: **The small delta antigen of hepatitis delta virus is an acetylated protein and acetylation of lysine 72 may influence its cellular localization and viral RNA synthesis.** *Virology* 2004, **319**:60-70.
23. Poulin DL, Kung AL, DeCaprio JA: **p53 targets simian virus 40 large T antigen for acetylation by CBP.** *J Virol* 2004, **78**:8245-8253.
24. Shimazu T, Komatsu Y, Nakayama KI, Fukazawa H, Horinouchi S, Yoshida M: **Regulation of SV40 large T-antigen stability by reversible acetylation.** *Oncogene* 2006, **25**:7391-7400.
25. Xie AY, Bermudez VP, Folk WR: **Stimulation of DNA replication from the polyomavirus origin by PCAF and GCN5 acetyltransferases: acetylation of large T antigen.** *Mol Cell Biol* 2002, **22**:7907-7918.
26. Zhang Q, Yao H, Vo N, Goodman RH: **Acetylation of adenovirus E1A regulates binding of the transcriptional corepressor CtBP.** *Proc Natl Acad Sci USA* 2000, **97**:14323-14328.
27. Col E, Caron C, Seigneurin-Berny D, Gracia J, Favier A, Khochbin S: **The histone acetyltransferase, hGCN5, interacts with and acetylates the HIV transactivator, Tat.** *J Biol Chem* 2001, **276**:28179-28184.
28. Kiernan RE, Vanhulle C, Schiltz L, Adam E, Xiao H, Maudoux F, Calomme C, Burny A, Nakatani Y, Jeang KT, Benkirane M, Van Lint C: **HIV-1 tat transcriptional activity is regulated by acetylation.** *Embo J* 1999, **18**:6106-6118.
29. Ott M, Schnolzer M, Garnica J, Fischle W, Emiliani S, Rackwitz HR, Verdin E: **Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity.** *Curr Biol* 1999, **9**:1489-1492.
30. Deng L, de la Fuente C, Fu P, Wang L, Donnelly R, Wade JD, Lambert P, Li H, Lee CG, Kashanchi F: **Acetylation of HIV-1 Tat by CBP/P300 increases transcription of integrated HIV-1 genome and enhances binding to core histones.** *Virology* 2000, **277**:278-295.
31. Limon A, Devroe E, Lu R, Ghory HZ, Silver PA, Engelman A: **Nuclear localization of human immunodeficiency virus type 1 preintegration complexes (PICs): V165A and R166A are pleiotropic integrase mutants primarily defective for integration, not PIC nuclear import.** *J Virol* 2002, **76**:10598-10607.
32. Paulson M, Press C, Smith E, Tanese N, Levy DE: **IFN-Stimulated transcription through a TBP-free acetyltransferase complex escapes viral shutoff.** *Nat Cell Biol* 2002, **4**:140-147.
33. Allouch A, Cereseto A: **Identification of cellular factors binding to acetylated HIV-1 integrase.** *Amino Acids* 2009.
34. Guo D, Hazbun TR, Xu XJ, Ng SL, Fields S, Kuo MH: **A tethered catalysis, two-hybrid system to identify protein-protein interactions requiring post-translational modifications.** *Nat Biotechnol* 2004, **22**:888-892.
35. Engelman A: **In vivo analysis of retroviral integrase structure and function.** *Adv Virus Res* 1999, **52**:411-426.
36. Di Fenza A, Rocchia W, Tozzini V: **Complexes of HIV-1 integrase with HAT proteins: Multiscale models, dynamics, and hypotheses on allosteric sites of inhibition.** *Proteins* 2009, **76(4)**:946-58.
37. Apolonia L, Waddington SN, Fernandes C, Ward NJ, Bouma G, Blundell MP, Thrasher AJ, Collins MK, Philpott NJ: **Stable gene transfer to muscle using non-integrating lentiviral vectors.** *Mol Ther* 2007, **15**:1947-1954.
38. Mulder LC, Muesing MA: **Degradation of HIV-1 integrase by the N-end rule pathway.** *J Biol Chem* 2000, **275**:29749-29753.

39. Tasaki T, Mulder LC, Iwamatsu A, Lee MJ, Davydov IV, Varshavsky A, Muesing M, Kwon YT: **A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons.** *Mol Cell Biol* 2005, **25**:7120-7136.
40. Llano M, Delgado S, Vanegas M, Poeschla EM: **Lens epithelium-derived growth factor/p75 prevents proteasomal degradation of HIV-1 integrase.** *J Biol Chem* 2004, **279**:55570-55577.
41. Devroe E, Engelman A, Silver PA: **Intracellular transport of human immunodeficiency virus type 1 integrase.** *J Cell Sci* 2003, **116**:4401-4408.
42. Bushman FD, Engelman A, Palmer I, Wingfield P, Craigie R: **Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding.** *Proc Natl Acad Sci USA* 1993, **90**:3428-3432.
43. Brussel A, Sonigo P: **Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus.** *J Virol* 2003, **77**:10119-10124.
44. Tan W, Dong Z, Wilkinson TA, Barbas CF, Chow SA: **Human immunodeficiency virus type 1 incorporated with fusion proteins consisting of integrase and the designed polydactyl zinc finger protein E2C can bias integration of viral DNA into a predetermined chromosomal region in human cells.** *J Virol* 2006, **80**:1939-1948.

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# KAP1 INHIBITS HIV-1 INTEGRATION

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**SUMMARY**

**HIV-1 integrase (IN), the enzyme that catalyzes the integration of the viral cDNA into the host cellular genome is positively regulated by acetylation induced by the cellular histone acetyl transferase (HAT) p300 (Cereseto et al., 2005). Acetylation increases IN affinity to DNA and promotes IN enzymatic activity (Cereseto et al., 2005). To investigate the relevance of IN acetylation in the context of HIV-1 infection, we searched for cellular partners selectively binding acetylated IN *in vivo*. By two-hybrid screening using constitutively acetylated IN as bait, we identified KAP1, a protein belonging to the TRIM family of antiviral proteins as a novel IN interactor that regulates HIV-1 infection. We found that KAP1 binds acetylated IN and induces its deacetylation through the formation of a protein complex including the deacetylase HDAC1. Modulation of intracellular KAP1 levels in different cell types including T-cells, the primary HIV-1 target, revealed that KAP1 curtails viral infectivity by selectively affecting HIV-1 integration. The KAP1-dependent viral inhibition was found to require HDAC1 activity since cells deficient for this deacetylase were insensitive to KAP1-induced resistance. This study reveals that KAP1 is a novel cellular factor restricting HIV-1 infection and underscores the relevance of IN acetylation as a crucial step in the viral infectious cycle.**

## INTRODUCTION

A key step in retroviral life cycle is the integration of the double-stranded viral cDNA into the host cellular genome; a reaction catalyzed by the viral integrase (IN) protein (Vandegraaff and Engelman, 2007).

The double–stranded DNA molecule is reverse transcribed from the viral RNA genome soon after virion entry into the cytoplasm. The DNA ends of the neo synthesized viral genome are processed by IN, producing recessed 3' OH termini at each end of the linear molecule (Vandegraaff and Engelman, 2007). The recessed 3' ends are then used in a nucleophylic attack on the host DNA to covalently join the viral and the host genomes (Vandegraaff and Engelman, 2007). The terminal cleavage and the strand transfer steps can be modeled *in vitro* with purified IN (Vandegraaff and Engelman, 2007). Nevertheless, in cells integration occurs within a large nucleoprotein complex termed the pre-integration complex (PIC), which is formed by the viral cDNA, viral proteins and numerous cellular factors playing important regulatory roles (Suzuki and Craigie, 2007; Van Maele et al., 2006; Vandegraaff and Engelman, 2007). One of the cellular factors exploited by HIV-1 during the integration process is the histone acetyl transferase (HAT) p300, which specifically acetylates the carboxy terminus of IN (Cereseto et al., 2005). We have previously reported that acetylation of IN lysines 264, 266 and 273 increases catalytic activity of this enzyme and that their mutation (K264,266,273R) significantly impairs viral replication (Cereseto et al., 2005; Terreni et al., 2010). Since post-translational modifications, like acetylation, dynamically change the chemical and structural properties of proteins generating new protein-protein interfaces, we wanted to search for possible cellular partners selectively interacting with acetylated IN. Although, most IN interacting factors were identified through the yeast two-hybrid approach (Van Maele et al., 2006) conventional two-hybrid screening, however, does not allow identification of factors that bind specifically to acetylated baits. We therefore developed a tethered catalysis two-hybrid system where constitutively acetylated IN is used as bait. The tethered catalysis system exploits

the *cis* enzymatic activity of a HAT domain, which determines constitutive acetylation of the substrate to which it is fused. From this screening, KAP1 (also known as TRIM28 or Tif-1 beta) has emerged as a new factor that interact specifically with acetylated IN. KAP1 was initially identified as a transcriptional corepressor that is recruited to its target genes by interacting with the Kruppel-associated box-domain-containing (KRAB) zinc-finger DNA proteins (Friedman et al., 1996; Peng et al., 2000). KAP1 mediates gene silencing (Ivanov et al., 2007; Sripathy et al., 2006) by recruiting on target promoters the NuRD histone deacetylase (HDAC) complex (Schultz et al., 2001), the histone methyltransferase SETDB1 (Schultz et al., 2002) and the heterochromatin-associated protein HP-1 (Lechner et al., 2000). KAP1 belongs to the tripartite motif (TRIM) protein family which display antiviral properties, targeting retroviruses in particular (Nisole et al., 2005). Importantly, KAP1 has been recently reported to restrict a gamma-retrovirus, M-MLV, in embryonic cells by silencing the proviral transcription (Wolf and Goff, 2007). Mounting evidence now suggest that KAP1 in addition to its documented transcriptional corepressor activity is involved more broadly in various nuclear functions (e.g. DNA damage response (White et al., 2006; Ziv et al., 2006).

KAP1 acts by forming protein complexes containing HDACs leading to the reversible acetylation of non histone proteins such as p53 and E2F1 (Tian et al., 2009; Tsuruma et al., 2008; Wang et al., 2005; Wang et al., 2007). As acetylation is a dynamic regulatory mechanism that involves the deacetylation activity of the HDAC protein family, in this study accordingly we analyzed the dynamic mechanisms regulating the acetylation levels of IN through KAP1. In particular, we examined whether that KAP1 mediated inhibition of viral integration may represent a novel cell defense mechanism from viral infection.

## RESULTS

### Two hybrid screening with acetylated IN

To identify cellular factors binding specifically to acetylated IN we have performed a tethered catalysis two-hybrid screening where IN used as bait is constitutively acetylated. To this aim a chimera where IN is fused to the HAT domain of p300 (IN-HATw) (Figure 1A, left panel) was engineered. To allow the separation of IN from the HAT domain, a proteolytic cleavage site for TEV was introduced between IN and HAT. As control the same chimera was constructed containing a catalytically inactive HAT domain (IN-HATm) (Figure 1A, right panel).

The IN-HAT chimeras were purified from bacteria and verified for acetylation by Western blot using antibodies specific for acetylated IN. Figure 1B, left panel, shows a high molecular size band corresponding to the full length IN-HATw chimera while no signal was detected in the IN-HATm control. The TEV digested products of both chimeras were also analyzed using antibodies against acetylated IN. IN-HATw showed a band at the same size of IN while no signal could be detected from the cleavage of IN-HATm (Figure 1B, right panel), thus indicating that IN is indeed acetylated within the IN-HATw chimera.

In addition, specific acetylation of lysines 264, 266 and 273 formerly identified by site directed mutagenesis (Cereseto et al., 2005), was verified by mass spectrometry analysis of the IN-HATw chimera (Figure S1). To perform the two hybrid screen, IN-HATw and IN-HATm were then cloned in a yeast expression vector (PBD-Gal4) in frame with GAL4 DNA binding domain (GDBD) at the N-terminus and a HA-tag at the C-terminus. Following transformation of yeast cells the level of IN expression and acetylation was checked by immunoblot analysis showing specific acetylation only in the GDBD-IN-HATw sample (Figure 1C, upper panel).

The vector expressing the GDBD-IN-HATw chimera was then co-transformed in yeast cells (AH109) with a human lymphocytes cDNA library cloned in a yeast expression vector (pACT) fused to GAl4 Activation Domain (GAD). The AH109 reporter strain carries ADE2, HIS3, lacZ and MEL1 genes. From the two hybrid selective medium (-Ade,-His) we obtained 754 positive clones (Ade+, His+) encoding for thirteen factors. Three clones encoded for the same domain (a.a 304-835) of the KAP1 gene (HSU78773). KAP1 has drawn our attention because it was recently reported to restrict M-MLV in embryonic stem cells (Wolf and Goff, 2007), and so potentially involved in HIV-1 biology.

To test the specificity of interaction between KAP1 and acetylated IN, a comparative analysis was performed by co-transforming GAD-KAP1 in yeast with the following GDBD/HA fusion proteins: IN-HATw, IN-HATm, HATw, HATm or Gal4-BD alone. These two-hybrid analyses showed that KAP1 interacts more efficiently with the acetylated IN (IN-HATw) than the unmodified IN (IN-HATm and IN), and no interaction was detected between KAP1 and the individual single domains (HATw, HATm and Gal4-BD) (Figure 1D). These results indicate that KAP1-IN interaction is highly favored by IN acetylation catalyzed by p300.

**Interaction between acetylated IN and KAP1**

To validate the interaction between the acetylated IN and KAP1 the binding of the two proteins was further analyzed in a mammalian system. KAP1 fused to a Flag-tag was expressed in HEK293T cells and the lysate incubated with the IN-HATw and IN-HATm recombinant proteins. Flag immunoprecipitates were then analyzed by Western blot. As shown in Figure 2A, upper-left panel, higher amounts of IN-HATw bind KAP1 as compared to IN-HATm. The same experiment was performed using IN isolated from the HATw or HATm domains following proteolytic cleavage of the recombinant chimera with the TEV protease. In Figure 2A (upper-right panel) a doublet at the same molecular size of IN was clearly visible with anti-Flag-KAP1 immunocomplexes incubated with IN derived from the

IN-HATw chimera, while no bands were detected using IN-HATm. The doublet observed in the IN-HATw immunoprecipitation is probably due to degradation of the IN protein during purification procedures since similar doublets are also observed in Figure 1B. These data clearly demonstrate that KAP1 binds preferentially the acetylated IN than the non-acetylated protein.

To further analyze the IN/KAP1 interaction *in vivo*, Flag-tagged IN was expressed in HEK293T cells and the amount of complexed endogenous KAP1 was evaluated following anti-Flag immunoprecipitation. As shown in Figure 2B, using anti-KAP1 antibodies endogenous KAP1 was detected on IN immunoprecipitates, while no bands were found in samples expressing the control Flag. To verify the role of IN acetylated lysines located in the carboxy-terminus, the same immunoprecipitation experiment was performed using a plasmid expressing Flag-IN mutated in lysines targeted for acetylation (264, 266 and 273) (Cereseto et al., 2005). Lower amounts of endogenous KAP1 bound mutated IN as compared to wild-type IN (Figure 2B). This observation was further verified using untagged INw and INm (Figure S2A).

To further prove the role of acetylation in IN binding with KAP1 both IN-HATw and IN-HATm chimeras were incubated with in vitro $^{35}S$ labeled KAP1. As shown in Figure 2C, IN-HATw binds KAP1 with higher affinity than IN-HATm. Since acetylated IN lysines enhanced the association with KAP1, we then specifically analyzed the role of the IN carboxy domain, which contains the acetylable lysines (264, 266 and 273). To this aim, pull-down experiments were performed by incubating recombinant IN domains fused to glutathione-S-transferase (GST) with in vitro $^{35}S$ labeled KAP1. KAP1 was found to bind the GST-C-terminus IN at the same extent (~10%of the input) of the GST- full length IN, while no interaction was observed between KAP1 and either the N-terminus or the catalytic domain (Figure 2D).

In conclusion, IN and KAP1 interact both *in vivo* and *in vitro* and IN/KAP1 complex formation, which is favored by IN acetylation, occurs through the C-terminal domain of IN.

**KAP1 inhibits HIV-1 integration**

To evaluate the role of KAP1 during HIV-1 infection, this factor was knocked down in HeLa cells by either transient siRNA treatment or stable shRNA (Figure 3A). Western blot analysis revealed that the expression of KAP1 was lowered in cells treated with a pool of siRNA directed against KAP1, as compared to cells untreated or transfected with control siRNA (Figure 3A, left panel). Similarly, HeLa cell clones stably expressing KAP1 shRNA (sh-1-KAP1) through a lentiviral vector (GIP-Z) showed reduced expression of KAP1 as compared to cell clones stably expressing mismatched shRNA (Figure 3A, right panel). Single round infectivity experiments with a VSV-G-pseudotyped HIV-1 clone carrying the luciferase gene as reporter (NL4.3-Luc), showed that cells in which KAP1 was knocked down were substantially more sensitive to infection (Figure S3A).

Since KAP1 was reported to possess transcriptional co-repressor activity and to limit M-MLV infectivity by blocking viral transcription, we first tested its possible involvement in viral events other than those regulated by IN. For this purpose, experiments were carried out using the HIV-1 IN (D64E) mutant, which is integration-defective, yet does express luciferase from its circular unintegrated forms. These infections showed no variation of luciferase activity in KAP1 knockdown cells, indicating that KAP1 does not affect replication steps unrelated to IN activity, including viral transcription (Figure S4A). This conclusion is further sustained by the lack of the regulation of transcriptional activity reported from a HIV-1 clone transiently transfected in KAP1 knockdown HeLa cells, as well as from a stably integrated HIV-1 based viral vector carried in Jurkat T-cells (J-lat A1) (Figures S4B and S4C).

These results strongly suggest that KAP1 regulates viral infectivity during the early events of viral replication, before transcription. We therefore sought to quantify, by real-time quantitative PCR (qPCR), the different DNA viral forms characterizing the HIV-1 replication cycle (late reverse transcription products, unintegrated 2-LTRs circles and integrated DNA) in KAP1 knockdown cells

infected with HIV-1. No significant variations ($P > 0.05$) were observed in cDNA synthesis (late reverse transcripts) (Figure 3B), implying that KAP1 has no observable effect on reverse transcription. In contrast, quantification of the 2-LTR circles showed that, these un-integrated forms were significantly less abundant in KAP1 knockdown cells (Figure 3C). 2-LTR circles are molecules of circularized retroviral cDNA that fail to integrate. These viral DNA forms directly correlate with cDNA nuclear translocation, while are inversely correlated with the efficiency of integration. Finally, consistent with the luciferase activity results (Figure S3A) a 8- and 2-fold ($P < 0.05$) increases were observed in integrated viral DNA using, respectively, siRNA or shRNA, against KAP1 (Figure 3D).

Additionally, to verify that the observation so far reported are not cell type dependent the same experiments were also reproduced in a different cell line (HEK293T) (Figures S4A and S5).

To verify also that inhibition of viral integration is determined by specific downregulation of KAP1, the integrated viral DNA was measured in KAP1 knockdown cells where the KAP1 expression was re-established by using a KAP1 cDNA resistant to shRNA (sh3'KAP1). In these cells viral integration returned to baseline demonstrating specific activity of the KAP1 shRNA (Figure 3E). Taken together, the increase in the levels of integrated viral DNA and the decrease in 2-LTR circles in the KAP1 knockdown cells, support the conclusion that KAP1 negatively regulates viral integration.

Since KAP1 has been shown to restrict infectivity of M-MLV in embryonic stem cells (ECSs) by inhibiting viral transcription, we tested whether M-MLV might also be affected by KAP1 at the integration step. To discern the integration from the transcription step, these experiments were performed by using a M-MLV vector carrying a CMV driven luciferase gene. As shown in Figure S6, M-MLV infectivity is not affected in KAP1 HeLa knocked down cells correlating with no specific effect at the integration step.

To further verify the activity of KAP1 on viral integration, experiments were performed by infecting cells over-expressing KAP1. Consistently with KAP1

knockdown experiments, high levels of KAP1 (Figure 3F, left panel) inhibit HIV-1 infection as measured by luciferase activity (Figure S3B). Notably, decreased infectivity correlates with significantly reduced levels of integrated viral DNA ($P < 0.05$), while reverse transcripts products are not significantly altered (Figure 3F, middle and right panels).

In conclusion these results demonstrate that KAP1 decreases viral infectivity by specifically downregulating the efficiency of the integration event.

**KAP1 inhibits HIV-1 integration via IN acetylation-dependent mechanism in lymphocytic cells**

To specifically test the role of IN acetylation in KAP1-dependent viral inhibition, we exploited a viral clone (pNL4.3-Luc-3mut) carrying IN mutated in the lysines targeted for acetylation (K264, 266, 273R). As previously shown this virus was less infectious than the parental NL4.3-Luc (Figures 4A and Figure S3C). However, as observed above (Figure 3D), while the wild-type virus showed increased proviral DNA formation in KAP1 knockdown HeLa cells, the integration of the mutated virus was not significantly altered ($P > 0.05$) in cells expressing low levels of KAP1 (Figures 4A and Figure S3C). Therefore these data further demonstrate that IN acetylation is necessary for KAP1 mediated inhibition of viral integration.

To test the role of KAP1 in cells naturally targeted by HIV-1, similar experiments were performed in primary blood lymphocytes (PBLs) purified from 4 healthy donors and in a T-cell line (CEMss). PBLs knocked down for KAP1 by electroporated siRNAs (Figure 4B, left panel) showed almost 3 fold ($P < 0.05$) increased viral integration following infection with NL4.3-Luc, however, viral integration remained unaltered in KAP1 knockdown cells infected with NL4.3-Luc-3mut (Figure 4B). Similar results were observed in CEMss cells knocked down by either a cocktail of 5 KAP1 shRNA (sh-5-KAP1) or by a single shRNA contained in the cocktail (sh-3'KAP1) (Figure 4C). The specificity of KAP1 mediated viral

inhibition was further proven by the lack of enhancement of viral integration observed in KAP1 knockdown CEMss cells (sh-3'KAP1) expressing a non-targetable KAP1 cDNA (Figure 4C, right panel, fourth column). In these experiments the levels of total viral DNA were checked showing no significant variations (data not shown).

In conclusion these data strongly support the role of IN acetylation in KAP1 viral inhibition in different cell types.

**KAP1 interaction decreases IN acetylation through HDAC1**

Results so far reported in this study demonstrate that KAP1 inhibition of viral infectivity is mediated by the interaction of the cellular protein with acetylated IN. Therefore, in the attempt to delineate the molecular mechanism of this phenomena, the IN acetylation was analyzed relative to KAP1 expression. To this aim IN was expressed in HEK293T cells together with KAP1 and the acetylation levels evaluated using antibodies against acetylated IN. The over-expression of KAP1 significantly decreased the acetylation of IN as compared to IN expressed in the absence of exogenous KAP1 (Figure 5A, upper panel). The level of IN acetylation was then analyzed in HeLa cell clones stably knocked down for KAP1 (Figure 3A). Transfection of Flag-IN in KAP1 silenced HeLa cells revealed that the levels of IN acetylation were substantially increased (Figure 5B, upper panel). Since HDACs are responsible for protein deacetylation, we verified the activity of these enzymes on IN. We observed that HDAC activity is associated with IN (Figure S7A) and trichostatin A (TSA), a potent inhibitor of these enzymes, increases the acetylation levels of IN (Figure S7B). Moreover, HDAC1 purified from HEK293T cells but not the catalytically inactive form (H141A) (Hassig et al., 1998) lowered the acetylation levels of recombinant IN, thus demonstrating that HDAC1 specifically regulates the acetylation of IN (Figure S7C).

Interestingly, KAP1 has been reported to form complexes with HDACs determining the deacetylation of its binding partners (Tian et al., 2009; Tsuruma

et al., 2008; Wang et al., 2005; Wang et al., 2007). We thus sought to investigate whether IN deacetylation induced by KAP1 would be mediated by complex formation with HDACs.

Co-immunoprecipitation experiments of Flag-HDAC1 and Flag-HDAC3 co-expressed with HA-IN showed higher amounts of IN associated with HDAC1 than with HDAC3. In addition, the same membrane blotted with anti-KAP1 antibodies revealed endogenous KAP1 together with the HDAC1 complex and not with HDAC3 (Figure 5C second panel from top). These data thus suggest that IN forms a complex with HDAC1 and KAP1. To explore the hypothesis that the association of IN with HDAC1 is mediated by KAP1, the binding of HDAC1 with IN was checked in HEK293T cells over-expressing KAP1. Immunoprecipitation of HDAC1 revealed much higher associated IN in cells over-expressing KAP1 as compared to control cells (Figure 5D and FigureS2B). These results strongly suggest that KAP1 plays a role in IN/HDAC1 complex formation. To further prove this hypothesis, KAP1 knockdown cells were transfected with both HA-IN and Flag-HDAC1, and HDAC1 immunoprecipitates were verified for the amounts of associated IN. When KAP1 expression was downregulated the levels of IN complexed with HDAC1 were strikingly lower than control cells expressing mismatched KAP1 shRNA (Figure 5E, upper panel).

In conclusion these data clearly demonstrate that KAP1 induces IN deacetylation by favoring HDAC1 binding to IN.

**KAP1 inhibits HIV-1 integration through HDAC1**

Results here reported indicate that KAP1 lowers IN acetylation through HDAC1 tethering. Since our previous reports demonstrate that acetylation of IN positively regulates viral integration (Cereseto et al., 2005; Terreni et al., 2010), we assumed that HDAC1 deacetylase activity might downregulate HIV-1 integration. To verify this hypothesis we analyzed viral integration in cells treated with HDACs inhibitors. Infection of cells treated with TSA or with a specific inhibitor of HDAC1, MS-275 (Nishioka et al., 2008), determined an increase of

viral integration (As shown in Figure 6A), while total viral DNA remained unaltered (data not shown). To better pinpoint the role of HDAC1 in viral integration, infections were then performed in HDAC1 knockdown cells (Figure 6B, left panel). We observed that in conditions of low HDAC1 expression the integration (Alu-LTR) was increased of almost four fold, while the un-integrated 2-LTR circles were decreased of two-fold (Figure 6B, middle and right panels). To verify that HDAC1 affects specifically the integration reaction the total viral DNA was also quantified showing no major alteration in cells silenced for HDAC1 (data not shown).

Since results so far reported strongly suggest that KAP1 inhibition of viral integration might occur through HDAC1 deacetylase activity, we sought to analyze the interplay between these factors during HIV-1 integration. To this aim infections were performed in cells where the expression of both HDAC1 and KAP1 was simultaneously modulated by silencing (HDAC1) and over-expression (KAP1) (Figure 6C, left panel). In agreement with results reported in Figure 3F, integration of viral cDNA was substantially reduced in cells over-expressing KAP1 (Figure 6C, right panel), while the amounts of the late reverse transcripts remained unaltered (data not shown). In cells that were silenced for HDAC1, however, over-expression of KAP1 did not show any viral inhibitory effect (Figure 6C, right panel). These experiments were also performed in HDAC3 knockdown cells, where no significant modulation of viral integration was observed (Figure S8), consistently with the weak association of this enzyme with IN and the absence of KAP1 in the IN-HDAC3 complex (Figure 5C). Therefore, these data clearly demonstrate that KAP1 integration-specific inhibitory effects occurs through HDAC1 activity. The role of HDAC1 in KAP1 inhibitory effects was further sustained by the results showing that the KAP1 N-terminal domain (1-381) involved in viral inhibition correlate with HDAC1 binding properties, while the inactive KAP1 C-terminal domain (617-835) was not able to bind HDAC1 (Figure S9).

Collectively, these data clearly show that KAP1 inhibits viral integration by recruiting HDAC1 to IN leading to deacetylation of the viral protein.

## DISCUSSION

Acetylation of HIV-1 IN enhances viral integration (Cereseto et al., 2005; Terreni et al., 2010). The role of this modification during the viral life cycle is proven by the decreased infectivity and provirus formation following inhibition of IN acetylation (Apolonia et al., 2007; Cereseto et al., 2005; Terreni et al., 2010; Topper et al., 2007).

Post-translational modifications, such as acetylation, regulate protein functions by modifying protein-protein interactions. Since HIV-1 IN is functionally regulated by the association with cellular co-factors, we hypothesized that acetylation might modulate the association of IN with known or still unidentified cellular factors. To this aim we have carried out a modified two-hybrid screening that differ from all screening so far performed for using as "bait" an IN constitutively acetylated through its fusion with the HAT domain of p300 (Allouch and Cereseto, 2009). This screening revealed that KAP1 interacts with acetylated IN and with a lower affinity to the un-modified form. Investigation on the role of KAP1 during viral replication cycle showed that KAP1 downregulation enhances viral infectivity due to specific increase in viral integration. This observation has been further confirmed by reciprocal experiments showing that KAP1 over-expression reduces provirus formation. Cellular antiretroviral defense blocks viral infectivity at specific time points during the viral replication cycle. In addition to the well defined restriction factors, a growing numbers of host cellular genes have been described to reduce susceptibility to retrovirus infection (Boulanger et al., 2005; Ganesh et al., 2003; Naghavi et al., 2005; Naghavi et al., 2007; Turelli et al., 2001). Among the factors so far identified only one, $p21^{Cip1}$, has been described to decrease infectivity by interfering with viral integration (Zhang et al., 2005; Zhang et al., 2007). Since $p21^{Cip1}$ is a cell cycle checkpoint protein playing a role in DNA damage response, it has been speculated that DNA damage pathways activated by the virus are responsible for $p21^{Cip1}$ antiviral activity; however, the molecular mechanism underlying viral integration inhibition

has not been delineated (Zhang et al., 2007). Here we demonstrate that KAP1 inhibits viral infectivity by specifically interfering with viral integration. By binding to the acetylated IN, KAP1 induces HDAC1 complex formation leading to IN deacetylation and reduced integration efficiency. We have proven that HDAC1 is essential for KAP1 mediated viral inhibition since over-expression of KAP1 does not reduce integration in cells silenced for HDAC1. Conversely, HDAC3 which is not part of the IN/KAP1 complex, does not affect the inhibitory activity of KAP1. The HDAC1-KAP1 interplay in HIV-1 inhibition is further sustained by the demonstration that the efficacy of the truncated KAP1 domains on HIV-1 replication correlates with their binding properties with HDAC1 (see Supplementary Figure S9 for further discussion). Interestingly, recently it has been reported that HDAC1 affects HIV-1 reverse transcription (Sorin et al., 2009). However, our data show a specific involvement of this enzyme during the integration event and not during reverse transcription. These apparent discrepancy could be explained by the fact that Sorin et al. (2009) analyze HDAC1 interacting with nascent viral particles, while in this study HDAC1 is analyzed in post-entry steps.

Emerging new evidence indicate that KAP1 is involved in acetylation/deacetylation activities unrelated to transcriptional control. In fact, KAP1 has recently been shown to inactivate proteins involved in DNA damage response (p53 and E2F1) by deacetylating these proteins through HDACs tethering (Tian et al., 2009; Wang et al., 2005; Wang et al., 2007). Notably, it has been suggested that unintegrated HIV-1 cDNA is substrate for double-DNA strand break repair factors, which in turn activate DNA damage response (Daniel et al., 1999; Jeanson et al., 2002; Kilzer et al., 2003; Li et al., 2001; Lloyd et al., 2006). Moreover, ATM that predominantly detects DNA double strand breaks, has been demonstrated to be activated following HIV-1 infection and its inhibition through a small molecule (KU-55933) drastically decreases cellular susceptibility to viral infection (Lau et al., 2005; Perfettini et al., 2008). Since KAP1 is ubiquitously expressed showing no major restriction of HIV-1 infectivity,

we propose a model where ATM activated by unintegrated HIV-1 phosphorylates KAP1 leading to disassembly of the IN/HDAC1 complex. This would ultimately reverse HDAC1 deacetylase activity associated to IN, thus allowing the reconstitution of IN acetylation and restored levels of integration efficiency. Very similar results have been reported to regulate p53 activity through an acetylation/deacteylation cycle regulated by KAP1 (Tian et al., 2009). In fact, this study shows that in unstressed cells p53 is inactivated by deacetylation through KAP1 mediated HDAC1 binding. In response to DNA damage ATM rapidly activates p53 through phosphorylation of KAP1 leading to p53/HDAC1 complex disruption and p53 acetylation. Further studies are required to verify a similar model for IN regulation.

KAP1 has also been described to restrict M-MLV in embryonic carcinoma and embryonic stem cells. However, in that case viral inhibition occurs at the level of transcription specifically in embryonic stem cells (ECSs), while here we show that integration of the M-MLV cDNA is not affected by KAP1. In ESCs infected with M-MLV, KAP1 is part of a large protein complex that binds the proviral primer binding site (PBS) leading to transcriptional repression (Wolf and Goff, 2007, 2009; Wolf et al., 2008). KAP1 mediated inhibition of viral transcription appears to be shared among retroviruses containing PBS sequences complementary to $tRNA^{Pro}$ such as M-MLV and HTLV-1, or $tRNA^{Lys-1,2}$ such as visna, spuma and Mason-Pfeizer monkey virus (Wolf and Goff, 2009; Wolf et al., 2008). Conversely, the HIV-1 PBS does not contain either $tRNA^{Pro}$ or $tRNA^{Lys-1,2}$ complementary sequences, and is thus not susceptible to KAP-1 mediated transcriptional repression as also confirmed by our results. Interestingly, the KAP1 inhibitory activity on transcription is responsible for controlling endogenous retroelements during early embryonic development (Rowe et al., 2010), suggesting a potential role of KAP1 in HIV-1 post-integration latency. However, we observed that KAP1 is expressed at the same levels in cells harboring silent or activated provirus as well as in the main cell types involved in HIV-1 latency (Figure S10). Hence, these data together with the absence of KAP1 in

transcriptional repression suggested by other reports (Wolf and Goff, 2009; Wolf et al., 2008), disfavor a hypothetical role of KAP1 in post-integration latency.

The different molecular mechanism of KAP1 restriction might parallel the dual activities of this factor at the cellular level: transcriptional as a corepressor and protein activity regulator through acetylation.

In summary, our study demonstrates that KAP1 inhibits HIV-1 integration through a novel cellular defense pathway targeting acetylated IN.

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## Vectors and constructs

pASK-IN-HATw/m to produce recombinant IN-HATw/m were constructed by cloning IN codon optimized (CO) in frame with the HAT domain of p300 (a.a. 1195-1673) wild-type or mutated (D1395Y) in the pASK-IBA37 plus vector (IBA, Gottingen, Germany) containing at 5' of the MCS a 6xHis tag. A 3'HA tag and a Tobacco Etch Virus (TEV) protease cleavage site were introduced by PCR. From the pASK-IBA37-IN-HATw/m vectors the IN-HATw/m, HATw/m and IN were cloned in the pBD-Gal4 vector (Stratagene, La Jolla, CA). Full-length KAP1 cDNA was purchased from Open Biosystem (Huntsville, AL) and cloned by PCR in pcDNA3.0, pFlag-CMV2 vectors and in pAIP lentiviral vector (kindly provided by J. Luban, Geneve University, Switzerland) in frame with HA (pAIP-HA-KAP1). pFlag-IN CO was kindly provided by A. Engelman (Dana-Farber Cancer Institute, Boston, MA) and K264,266,273R mutations were introduced by PCR. Expression vectors of GST-IN full length and GST-IN truncated domains (N-terminus, Core and C-terminus) have been previously described (Cereseto et al., 2005). pFlag-HDAC1 and pFlag-HDAC3 have been previously described (Sabo et al., 2008).
pNL4.3.Luc.R-E- was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). PNL4.3-Luc-3mut (K264,266,273R) has been previously described (Terreni et al., 2010).

## Yeast two hybrid screen

A human T-lymphocytes cDNA library fused to Gal4 Activating Domain in a pACT vector (BD biosciences Clontech, Palo Alto, CA) was expressed in AH109 yeast cells and screened with the pGBD-IN-HATw (bait) expression vector. Library transformation and screening were performed following manufacturer's instructions (Matchmaker GAL4 two hybrid system 3). GAD-KAP1 was co-expressed in AH109 yeast cells with GDBD fused to IN-HATw/m, HATw/m and IN to check for interactions.

## IN-HATw and IN-HATm purification and TEV digestion

pASK-IN-HATw/m encoding for 6xHis-IN-HATw/m were transformed in *E. coli* Arctic Express RIL competent cells (Stratagene) and induction of protein expression was performed using 43 mM anhydrotetracycline hydrochloride (AHT) for 24 hours at 13 °C. Bacteria culture was lysed in binding buffer (1M NaCl, 20 mM Tris HCl pH 7.9 and 0.5% Triton X-100). 6xHis-IN-HATw/m were purified using TALON Metal Affinity Resin (BD Biosciences). For TEV digestion 20 µg of 6xHis-IN-HATw/m were incubated with 30 units of AcTEV protease (Invitrogen, Paisley, UK) in 120 mM NaCl, 50 mM Tris-HCl pH 8, 0.5 mM EDTA and 1 mM of DTT in 250 µl total volume. 6xHis-IN TEV digested products were adjusted to 1 M NaCl and recovered using Ni-NTA agarose resin (Qiagen, Hilden, Germany).

## Virus productions and infections

NL4.3-Luc and NL4.3-Luc-3mut viruses pseudotyped with the Vesicular Stomatitis Virus-G (VSV-G) envelope were produced by transfecting $5x10^6$ HEK293T cells with 20 µg pNL4.3.Luc.R-E- or pNL4.3-Luc-3mut and 5 µg p-MDG-VSV-G using 150 nM polyethylenimine (PEI) reagent (Sigma, St. Louis, MO). Viral supernatants were collected 48 hours post transfection, quantified for their HIV-1 p24 antigen content using Innotest HIV Antigen mAb kit (INNOGENETICS N.V., Gent, Belgium) and DNAse I treated before infection (40 U/ml) (TURBO DNAse-Applied Biosystems, Foster City, CA).

Infections of transiently knocked down cells were performed for 2 hours using: 100 ng p24 for $6x10^5$ HeLa cells 48 hours after siRNA transfection; 250 ng p24 for $5x10^5$ CEMss cells 60 hours post-transduction with KAP1 shRNAs lentiviral vectors; 500 ng p24 for $2x10^5$ PBLs 24 hours after siRNA transfection. For back-complementation experiments (HeLa or CEMss cells) were infected 60 hours post LKO.1-sh-3'KAP1 and AIP-HA-KAP1 (1-835) transductions using 250 ng p24 NL4.3-Luc (VSV-G) HIV-1 virus for $5x10^5$ cells. Infections of stable knockdown HeLa cell clones ($3x10^5$) were performed using 300-1200 ng p24. Infections of

HEK293T cells transfected 24 hours in advance with pcDNA3-KAP1 (6.25 μg for $1.2x10^6$ cells with PEI) were performed using 125 ng p24 for $2,5x10^5$ cells.

**Transient/stable knockdowns and back-complementation**

Transient knockdowns mediated by siRNAs and controls experiments were performed by using smart pool siRNAs pre-designed by Dharmacon Inc. (Chicago, IL). The KAP1 pool contains the following four siRNAs: 1) 5'-GACCAAACCUGUGCUUAUG-3'; 2) 5'-GAUGAUCCCUACUCAAGUG-3'; 3) 5'-GCGAUCUGGUUAUGUGCAA-3'; 4) 5'- AGAAUUAUUUCAUGCGUGA-3'. The HDAC1 pool contains the following four siRNAs: 1) 5'-CUAAUGAGCUUCCAUACAA-3'; 2) 5'-GAAAGUCUGUUACUACUAC-3'; 3) 5'-GGACAUCGCUGUGAAUUGG-3'; 4) 5'-CCGGUCAUGUCCAAAGUAA-3'. $1x10^5$ HeLa cells were treated with 200 nM KAP1 siRNAs or 150 nM HDAC1 siRNAs and equivalent amounts of control siRNAs (ON-TARGET plus Non-targeting Pool) by using the Gene Silencer siRNA transfection reagent (Gene Therapy Systems,Inc., San Diego, CA). PBLs were isolated from Ficoll-purified peripheral blood monocytes using a high density hyper-osmotic Percoll density gradient (Sigma) as previously described (Repnik et al., 2003). Following activation with PHA-P (2 μg/ml) (Sigma) and 5 days culture in IL-2 (25 U/ml) (Roche, Mannheim, Germany), $5x10^6$ cells were electroporated using the nucleofector II Amaxa biosystems instrument (Lonza, Switzerland) with 600 nM KAP1 siRNAs or control siRNAs in 100 μl T-cell Nucleofector solution (Lonza).

CEMss cells were transiently knocked down by transducing a pool of 5 LKO.1 lentiviral vectors each expressing a shRNAmir against the KAP1 gene (sh-5-KAP1) (400 ng p24 each vector for $1x10^5$ cells): 1) 5'-CCTGGCTCTGTTCTCTGTCCT-3' (sh-3'-KAP1); 2) 5'-GAGAATTATTTCATGCGTGAT-3'; 3) 5'-GAGGACTACAACCTTATTGTT-3'; 4) 5'-CTGAGACCAAACCTGTGCTTA-3'; 5) 5'-GACCACCAGTACCAGTTCTTA-3'. For back-complementation experiments $1x10^5$ HeLa or CEMss cells were knocked down by transducing 3 μg p24 of LKO.1-sh-3'KAP1 targeting the 3' untranslated region (3'UTR) of KAP1 gene and KAP1 was

expressed using 2 μg p24 AIP-HA-KAP1.

Stable KAP1 knockdown cells were obtained by expressing a shRNAmir (sh-1-KAP1: 5'-CCACTGAGGACTACAACCTTA-3') (Open Biosystem) through a lentiviral vector system (GIPZ-sh-1-KAP1). As control a mismatch oligonucletide (sh-MM: 5'-CCACTGAGCTGAACAACCTTA-3') containing four mutations in sh-1-KAP1 sequence was expressed through the GIPZ lentiviral vector (GIPZ-sh-MM).

Lentiviral vectors containing shRNAs or KAP1 cDNA were obtained by collecting supernatants from HEK293T cells 48 hours post transfection with 20 μg transfer gene, 10 μg lentiviral packaging vector (p8.91) and 5 μg pMDG-VSV-G for pseudotyping.

**Real time quantitative PCR (qPCR)**

Integrated HIV-1 copies by Alu-LTR and 2 LTRs circles were analyzed by qPCR as previously described (Brussel and Sonigo, 2003). Total viral DNA was analyzed as previously described (Butler et al., 2001). Total and integrated HIV-1 DNA quantifications in cells carrying GIPZ, LKO.1 or AIP lentiviral DNAs was performed by using primers/probes specific for the luciferase gene in NL4.3-Luc 24 hours or 15 days post infection respectively to avoid cross reactivity between viral LTR (Terreni et al., 2010). MLV late reverse transcripts and integrated MLV DNA at 24 hours and 15 days post infection respectively were quantified using primers/probes specific to the luciferase gene that recognize LNC-CMV-eGFPT2A-fLuc MLV viral vector (Terreni et al., 2010). A kinetic PCR assay for human beta-globin DNA was carried out as endogenous control as previously described (Tan et al., 2006). The fold increase/decrease ratios of the real time data were calculated using the mathematical model for the relative quantification in the real time-PCR (Pfaffl, 2001) where the HIV-1 gene is the "target gene" and β–globin gene is the "reference gene".

**Immunoprecipitations and Flag-KAP1 pull downs**

GBD-IN-HATw/m expression and immunoprecipitations in yeast cells were performed as previously described (Guo et al., 2004) with minor modifications. For Flag immunoprecipitation experiments HEK293T or HeLa cells expressing Flag tagged proteins were lysed in NEHN buffer (300 mM NaCl, 50 mM Hepes pH 7.5, 0.5% NP-40, 20% glycerol and 1 mM EDTA). 500 µg-2000 µg lysates were incubated with 30 µl anti-Flag M2 agarose beads (Sigma) followed by Western blot analysis. KAP1 binding to Flag-INw/m was performed using transfected cells treated with 1 µM TSA for 8 hours.

For Flag pull down experiments HEK293T expressing Flag-KAP1 were lysed in 50 mM Hepes pH 7.4, 150 mM NaCl and 0.5% NP-40. Lysates (250 µg) containing a HAT inhibitor (Lys-CoA) were incubated with 250 ng of either 6xHis-IN-HATw/m or 6xHis-IN TEV digested products and pulled down by anti-Flag M2 agarose beads (Sigma) followed by Western blot analysis.

**Antibodies**

Primary antibodies for Western blot analyses were: monoclonal HIV-1 IN antibody (8G4) from NIH AIDS Research and Reference Reagent Program, polyclonal anti-KAP1 (Bethyl laboratories, Inc., Montgomery, TX), polyclonal anti-HA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-HDAC1 (Upstate, Temecula, CA). Anti-Flag (polyclonal and monoclonal-Clone M2), anti-Tubulin (CloneB-5-1-2) were all purchased from Sigma. Antibodies anti-acetylated IN have been previously described (Terreni et al., 2010).

***In vitro* binding assays**

*In vitro* translated $^{35}$S-KAP1 was produced using TNT T7 Reticulocyte Lysate System (Promega) and pcDNA3-KAP-1 as template. GST-IN full length and GST-IN truncated domains (N-terminus, Core and C-terminus) preparations and *in vitro* binding have been previously described (Cereseto et al., 2005). *In vitro* binding between 6xHis-IN-HATw or 6xHis-IN-HATm and $^{35}$S-KAP1 was performed

by incubating 1000 ng of either recombinant purified proteins with 1000 cpm of $^{35}$S-KAP-1, 20 µg BSA and 15 µl of Ni-NTA agarose (settled beads volume) in 1000 µl pull down buffer (150 mM NaCl, 2 mM MgCl2, 25 mM imidazole, 0.1 % NP-40, 25 mM imidazole and 50 mM Tris-HCl pH 7.4) for 5 hours at 4 °C. Following three washes in pull down buffer, the reaction mixture was separated by SDS-PAGE gel analyzed by phosphoimaging (Cyclone).

**Statistical analysis.**

Statistical significance of the results obtained in the HIV-1 infectivity assays was assessed by the two-tailed Student's t-test assuming equal variance between samples to determine differences at the 5% level of significance.

## References


Allouch, A., and Cereseto, A. (2009). Identification of cellular factors binding to acetylated HIV-1 integrase. Amino Acids.

Apolonia, L., Waddington, S.N., Fernandes, C., Ward, N.J., Bouma, G., Blundell, M.P., Thrasher, A.J., Collins, M.K., and Philpott, N.J. (2007). Stable gene transfer to muscle using non-integrating lentiviral vectors. Mol Ther *15*, 1947-1954.

Boulanger, M.C., Liang, C., Russell, R.S., Lin, R., Bedford, M.T., Wainberg, M.A., and Richard, S. (2005). Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression. J Virol *79*, 124-131.

Brussel, A., and Sonigo, P. (2003). Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J Virol *77*, 10119-10124.

Butler, S.L., Hansen, M.S., and Bushman, F.D. (2001). A quantitative assay for HIV DNA integration in vivo. Nat Med *7*, 631-634.

Cereseto, A., Manganaro, L., Gutierrez, M.I., Terreni, M., Fittipaldi, A., Lusic, M., Marcello, A., and Giacca, M. (2005). Acetylation of HIV-1 integrase by p300 regulates viral integration. Embo J *24*, 3070-3081.

Daniel, R., Katz, R.A., and Skalka, A.M. (1999). A role for DNA-PK in retroviral DNA integration. Science *284*, 644-647.

Friedman, J.R., Fredericks, W.J., Jensen, D.E., Speicher, D.W., Huang, X.P., Neilson, E.G., and Rauscher, F.J., 3rd (1996). KAP-1, a novel corepressor for the highly conserved KRAB repression domain. Genes Dev *10*, 2067-2078.

Ganesh, L., Burstein, E., Guha-Niyogi, A., Louder, M.K., Mascola, J.R., Klomp, L.W., Wijmenga, C., Duckett, C.S., and Nabel, G.J. (2003). The gene product Murr1 restricts HIV-1 replication in resting CD4+ lymphocytes. Nature *426*, 853-857.

Guo, D., Hazbun, T.R., Xu, X.J., Ng, S.L., Fields, S., and Kuo, M.H. (2004). A tethered catalysis, two-hybrid system to identify protein-protein interactions requiring post-translational modifications. Nat Biotechnol *22*, 888-892.

Hassig, C.A., Tong, J.K., Fleischer, T.C., Owa, T., Grable, P.G., Ayer, D.E., and Schreiber, S.L. (1998). A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. Proc Natl Acad Sci U S A *95*, 3519-3524.

Ivanov, A.V., Peng, H., Yurchenko, V., Yap, K.L., Negorev, D.G., Schultz, D.C., Psulkowski, E., Fredericks, W.J., White, D.E., Maul, G.G.*, et al.* (2007). PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. Mol Cell *28*, 823-837.

Jeanson, L., Subra, F., Vaganay, S., Hervy, M., Marangoni, E., Bourhis, J., and Mouscadet, J.F. (2002). Effect of Ku80 depletion on the preintegrative steps of HIV-1 replication in human cells. Virology *300*, 100-108.

Kilzer, J.M., Stracker, T., Beitzel, B., Meek, K., Weitzman, M., and Bushman, F.D. (2003). Roles of host cell factors in circularization of retroviral dna. Virology *314*, 460-467.

Lau, A., Swinbank, K.M., Ahmed, P.S., Taylor, D.L., Jackson, S.P., Smith, G.C., and O'Connor, M.J. (2005). Suppression of HIV-1 infection by a small molecule inhibitor of the ATM kinase. Nat Cell Biol *7*, 493-500.

Lechner, M.S., Begg, G.E., Speicher, D.W., and Rauscher, F.J., 3rd (2000). Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. Mol Cell Biol *20*, 6449-6465.

Li, L., Olvera, J.M., Yoder, K.E., Mitchell, R.S., Butler, S.L., Lieber, M., Martin, S.L., and Bushman, F.D. (2001). Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. EMBO J *20*, 3272-3281.

Lloyd, A.G., Tateishi, S., Bieniasz, P.D., Muesing, M.A., Yamaizumi, M., and Mulder, L.C. (2006). Effect of DNA repair protein Rad18 on viral infection. PLoS Pathog *2*, e40.

Naghavi, M.H., Hatziioannou, T., Gao, G., and Goff, S.P. (2005). Overexpression of fasciculation and elongation protein zeta-1 (FEZ1) induces a post-entry block to retroviruses in cultured cells. Genes Dev *19*, 1105-1115.

Naghavi, M.H., Valente, S., Hatziioannou, T., de Los Santos, K., Wen, Y., Mott, C., Gundersen, G.G., and Goff, S.P. (2007). Moesin regulates stable microtubule formation and limits retroviral infection in cultured cells. EMBO J *26*, 41-52.

Nishioka, C., Ikezoe, T., Yang, J., Takeuchi, S., Koeffler, H.P., and Yokoyama, A. (2008). MS-275, a novel histone deacetylase inhibitor with selectivity against HDAC1, induces degradation of FLT3 via inhibition of chaperone function of heat shock protein 90 in AML cells. Leuk Res *32*, 1382-1392.

Nisole, S., Stoye, J.P., and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. Nat Rev Microbiol *3*, 799-808.

Peng, H., Begg, G.E., Schultz, D.C., Friedman, J.R., Jensen, D.E., Speicher, D.W., and Rauscher, F.J., 3rd (2000). Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. J Mol Biol *295*, 1139-1162.

Perfettini, J.L., Nardacci, R., Bourouba, M., Subra, F., Gros, L., Seror, C., Manic, G., Rosselli, F., Amendola, A., Masdehors, P.*, et al.* (2008). Critical involvement of the ATM-dependent DNA damage response in the apoptotic demise of HIV-1-elicited syncytia. PLoS One *3*, e2458.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res *29*, e45.

Repnik, U., Knezevic, M., and Jeras, M. (2003). Simple and cost-effective isolation of monocytes from buffy coats. J Immunol Methods *278*, 283-292.

Rowe, H.M., Jakobsson, J., Mesnard, D., Rougemont, J., Reynard, S., Aktas, T., Maillard, P.V., Layard-Liesching, H., Verp, S., Marquis, J.*, et al.* (2010). KAP1 controls endogenous retroviruses in embryonic stem cells. Nature *463*, 237-240.

Sabo, A., Lusic, M., Cereseto, A., and Giacca, M. (2008). Acetylation of conserved lysines in the catalytic core of cyclin-dependent kinase 9 inhibits kinase activity and regulates transcription. Mol Cell Biol *28*, 2201-2212.

Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., and Rauscher, F.J., 3rd (2002). SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev *16*, 919-932.

Schultz, D.C., Friedman, J.R., and Rauscher, F.J., 3rd (2001). Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes Dev *15*, 428-443.

Sorin, M., Cano, J., Das, S., Mathew, S., Wu, X., Davies, K.P., Shi, X., Cheng, S.W., Ott, D., and Kalpana, G.V. (2009). Recruitment of a SAP18-HDAC1 complex into HIV-1 virions and its requirement for viral replication. PLoS Pathog *5*, e1000463.

Sripathy, S.P., Stevens, J., and Schultz, D.C. (2006). The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. Mol Cell Biol *26*, 8623-8638.

Suzuki, Y., and Craigie, R. (2007). The road to chromatin - nuclear entry of retroviruses. Nat Rev Microbiol *5*, 187-196.

Tan, W., Dong, Z., Wilkinson, T.A., Barbas, C.F., 3rd, and Chow, S.A. (2006). Human immunodeficiency virus type 1 incorporated with fusion proteins consisting of integrase and the designed polydactyl zinc finger protein E2C can bias integration of viral DNA into a predetermined chromosomal region in human cells. J Virol *80*, 1939-1948.

Terreni, M., Valentini, P., Liverani, V., Gutierrez, M.I., Di Primio, C., Di Fenza, A., Tozzini, V., Allouch, A., Albanese, A., Giacca, M.*, et al.* (2010). GCN5-dependent acetylation of HIV-1 integrase enhances viral integration. Retrovirology *7*, 18.

Tian, C., Xing, G., Xie, P., Lu, K., Nie, J., Wang, J., Li, L., Gao, M., Zhang, L., and He, F. (2009). KRAB-type zinc-finger protein Apak specifically regulates p53-dependent apoptosis. Nat Cell Biol *11*, 580-591.

Topper, M., Luo, Y., Zhadina, M., Mohammed, K., Smith, L., and Muesing, M.A. (2007). Posttranslational acetylation of the human immunodeficiency virus type 1 integrase carboxyl-terminal domain is dispensable for viral replication. J Virol *81*, 3012-3017.

Tsuruma, R., Ohbayashi, N., Kamitani, S., Ikeda, O., Sato, N., Muromoto, R., Sekine, Y., Oritani, K., and Matsuda, T. (2008). Physical and functional interactions between STAT3 and KAP1. Oncogene *27*, 3054-3059.

Turelli, P., Doucas, V., Craig, E., Mangeat, B., Klages, N., Evans, R., Kalpana, G., and Trono, D. (2001). Cytoplasmic recruitment of INI1 and PML on incoming HIV preintegration complexes: interference with early steps of viral replication. Mol Cell *7*, 1245-1254.

Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F., and Debyser, Z. (2006). Cellular co-factors of HIV-1 integration. Trends Biochem Sci *31*, 98-105.

Vandegraaff, N., and Engelman, A. (2007). Molecular mechanisms of HIV integration and therapeutic intervention. Expert Rev Mol Med *9*, 1-19.

Wang, C., Ivanov, A., Chen, L., Fredericks, W.J., Seto, E., Rauscher, F.J., 3rd, and Chen, J. (2005). MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. Embo J *24*, 3279-3290.

Wang, C., Rauscher, F.J., 3rd, Cress, W.D., and Chen, J. (2007). Regulation of E2F1 function by the nuclear corepressor KAP1. J Biol Chem *282*, 29902-29909.

White, D.E., Negorev, D., Peng, H., Ivanov, A.V., Maul, G.G., and Rauscher, F.J., 3rd (2006). KAP1, a novel substrate for PIKK family members, colocalizes with numerous damage response factors at DNA lesions. Cancer Res *66*, 11594-11599.

Wolf, D., and Goff, S.P. (2007). TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell *131*, 46-57.

Wolf, D., and Goff, S.P. (2009). Embryonic stem cells use ZFP809 to silence retroviral DNAs. Nature *458*, 1201-1204.

Wolf, D., Hug, K., and Goff, S.P. (2008). TRIM28 mediates primer binding site-targeted silencing of Lys1,2 tRNA-utilizing retroviruses in embryonic cells. Proc Natl Acad Sci U S A *105*, 12521-12526.

Zhang, J., Attar, E., Cohen, K., Crumpacker, C., and Scadden, D. (2005). Silencing p21(Waf1/Cip1/Sdi1) expression increases gene transduction efficiency in primitive human hematopoietic cells. Gene Ther *12*, 1444-1452.

Zhang, J., Scadden, D.T., and Crumpacker, C.S. (2007). Primitive hematopoietic cells resist HIV-1 infection via p21. J Clin Invest *117*, 473-481.

Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D.C., Lukas, J., Bekker-Jensen, S., Bartek, J., and Shiloh, Y. (2006). Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. Nat Cell Biol *8*, 870-876.

## Figures

**Figure 1. Identification of KAP1 in yeast two hybrid screen using constitutively acetylated integrase as a bait**

(A) Schematic representation of IN-HATw (left) and IN-HATm (right) chimeras. IN codon optimized is fused to the p300 HAT domain either wild-type (HATw) or mutated (HATm). Between the IN and the HAT domains a TEV protease cleavage site is inserted to allow separation of the two domains.

(B) IN-HATw and IN-HATm fusion recombinant proteins undigested (-TEV) or TEV digested (+TEV) were immunoblotted with anti-acetylated IN specific antibodies ($\alpha$AcIN) (left panel). The immunoblot was re-probed with $\alpha$IN antibodies for total IN (right panel).

(C) The IN-HATw and IN-HATm chimeras expressed in yeast cells (AH109) were immunoprecipitated with $\alpha$HA antibodies and immunoblotted with $\alpha$AcIN antibodies to detect acetylated IN. Total IN was detected with $\alpha$IN antibodies.

(D) Interaction efficiencies of the IN-HATw and IN-HATm chimeras and single domains (HAT, IN and GDBD) with KAP1 in yeast two-hybrid assay.

**Figure 2. KAP1 preferentially binds acetylated IN than the unmodified form *in vivo* and *in vitro***

(A) HEK293T cell lysates expressing Flag-KAP1 were incubated with the IN-HATw or IN-HATm chimeras (left panel) and with IN isolated from the IN-HATw (IN-Ac+) or from IN-HATm (IN-Ac-) by TEV digestion (right panel). The $\alpha$Flag immunocomplexes were blotted with $\alpha$HA antibodies to detect bound IN-HAT chimeras and re-probed with $\alpha$Flag antibodies to check for KAP1 expression.

(B) HEK293T cells expressing either Flag-IN wild type (Flag-INw) or mutated at acetylable lysines (K264, 266, 273R) (Flag-INm) were immunoprecipitated with αFlag antibodies and blotted using α-KAP1 antibodies.
(C) *In vitro* binding between $^{35}$S-KAP1 and IN-HATw or IN-HATm recombinant proteins. The graph expresses the amounts of KAP1 bound protein as percentages of the binding reaction: $^{35}$S-KAP1 + IN-HATw.
(D) *In vitro* binding between $^{35}$S-KAP1 and recombinant full length IN fused to GST (GST-IN) or each single recombinant IN domain fused to GST: N-terminus (GST-IN-Nt), catalytic (GST-IN-Cat) and C-terminus (GST-Ct). The graph expresses the amounts of KAP1 bound protein as percentages of input ($^{35}$S-KAP1). In (C) and (D) the upper panels show the gels exposed to Phosphoimaging (Cyclone) and the lower panels the Coomassie staining of the SDS-PAGE gels.

**Figure 3. KAP1 inhibits HIV-1 integration**

(A) Left panel: expression of KAP1 in HeLa cells transiently knocked down with smart pool siRNAs (si-KAP1) and in control HeLa cells treated with a pool of non targeting siRNAs (si-CTR). Right panel: expression of KAP1 in HeLa cell clones (H14a, H18 and H19) stably knocked down with shRNA expressed by a lentiviral vector (sh-1-KAP1) and in control HeLa cells expressing a mismatched sh-1-KAP1 (shMM).
(B-D) Transient and stable KAP1 knockdown HeLa cells were infected with NL4.3-Luc HIV-1 virus and analyzed by qPCR for late reverse transcripts 24 hours post infection (hpi) (B), 2-LTR DNA circles 24 hpi (C) and integrated HIV-1 DNA 48 hpi (siRNA treated cells) or 15 days post-infection (dpi) (sh-1-KAP1) (D).
(E) Integrated NL4.3-Luc HIV-1 DNA following infection (15 dpi) of HeLa cells KAP1 knockdown (sh-3'KAP1), back-complemented (sh-3'KAP1 + HA-KAP1) or control treated (GIPZ). Expression of KAP1 was verified by immunoblot.
(F) Expression of KAP1 in HEK293T cells transfected with KAP1 (pcDNA-KAP1), empty vector (pcDNA) or un-transfected (UT) (left panel). Over-expressing KAP1

cells (pcDNA-KAP1) and control cells (pcDNA) were infected with NL4.3-Luc HIV-1 virus and analyzed by qPCR for integrated HIV-1 DNA (Alu-LTR) (middle panel) and late reverse transcripts (right panel) at 48 hpi.
All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments). qPCR absolute values are reported in Table S1.

**Figure 4. KAP1 inhibition of HIV-1 integration depends on integrase lysines (K264, K266 and K273) targeted for acetylation by p300 and occurs in HIV-1 natural target cells**

(A) KAP1 knockdown (H14a and H18) and control (shMM) HeLa cell clones were infected with NL4.3-Luc or NL4.3-Luc-3mut and analyzed by qPCR for integrated HIV-1 DNA (15 dpi).
(B) Primary T-cells (PBLs) transiently knocked down for KAP1 (si-KAP1) and control treated (si-CTR) were infected with NL4.3-Luc or NL4.3-Luc-3mut HIV-1 and analyzed for integrated HIV-1 DNA (Alu-LTR, 48 hpi). Protein expression was verified by immunoblot.
(C) Integrated NL4.3-Luc HIV-1 or NL4.3-Luc-3mut HIV-1 DNA following infection (15 dpi) of CEMss cells KAP1 knockdown (sh-5-KAP1) and control treated (GIPZ) and integrated NL4.3-Luc HIV-1following infection (15 dpi) of CEMss KAP1 knockdown (sh-3'KAP1) and back-complemented (sh3'KAP1 + HA-KAP1). Protein expression was verified by immunoblot.
All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments).
qPCR absolute values are reported in Table S1.

**Figure 5. KAP1 decreases HIV-1 IN acetylation by inducing IN/HDAC1 complex formation**

(A) Flag-IN expressed in HEK293T with or without exogenous KAP1 (pcDNA-KAP1), immunoprecipitated with αFlag antibodies and blotted with anti-acetylated IN specific antibodies (αAcIN) to determine IN acetylation levels. Expression of KAP1 and total IN was determined by immunoblot using αKAP1 and αIN antibodies respectively.

(B) Flag-IN expressed in either HeLa cell clones stably knocked down for KAP1 by sh-1-KAP1 (H14a, H18 and H19) or in control cells (sh-MM) was immunoprecipitated with αFlag antibodies and blotted with αAcIN antibodies. Total IN was detected by re-probing with αFlag antibodies.

(C) HA-IN was co-expressed with either Flag-HDAC1 or Flag-HDAC3 in HEK293T cells. αFlag immunoprecipitates were blotted with αHA (IN), αKAP1 and αFlag (HDAC1 and HDAC3) antibodies. Total IN was detected by αHA immunoblot.

(D) HA-IN and Flag-HDAC1 were co-expressed in HEK293T cells with or without exogenous KAP1 (pcDNA-KAP1). αFlag (HDAC1) immunoprecipitates were blotted with αHA (IN) antibodies. The expression levels of KAP1, HDAC1 and IN were controlled by immunoblot using the indicated antibodies.

(E) HA-IN and Flag-HDAC1 were co-expressed in KAP1 stably knocked down cell clones (H14a, H18 and H19) and in control cells (sh-MM). αFlag (HDAC1) immunoprecipitates were blotted with αHA (IN) antibodies. Expression levels of IN and HDAC1 were controlled by immunoblot using the indicated antibodies.

**Figure 6. KAP1 inhibition of HIV-1 integration is mediated by HDAC1**

(A) HeLa cells treated with 600 nM MS-275 or with 800 nM TSA were infected with NL4.3-Luc HIV-1 and analyzed by qPCR for integrated HIV-1 DNA (Alu-LTR) at 24 hpi.

(B) Expression of HDAC1 in HeLa cells transiently knocked down with smart pool siRNA (si-HDAC1) and in control HeLa cells treated with a pool of non-targeting siRNA (si-CTR) (left panel). HeLa cells transiently knocked down for HDAC1 were infected with NL4.3-Luc HIV-1 and analyzed by qPCR for integrated HIV-1 DNA

(Alu-LTR) at 48 hpi (middle panel) and 2-LTR DNA circles at 24 hpi (right panel).C) Expression of KAP1 and HDAC1 in HEK293T cells transfected with pcDNA-KAP1 and transiently knocked down with si-HDAC1 (left panel). HEK293T cells over-expressing KAP1 (pcDNA-KAP1) were co-treated or not with si-HDAC1, subsequently infected with NL4.3Luc HIV-1 and analyzed by qPCR for integrated HIV-1 DNA (Alu-LTR) at 48 hpi (right panel).

All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments).

qPCR absolute values are reported in Table S1.

**Figure 1**

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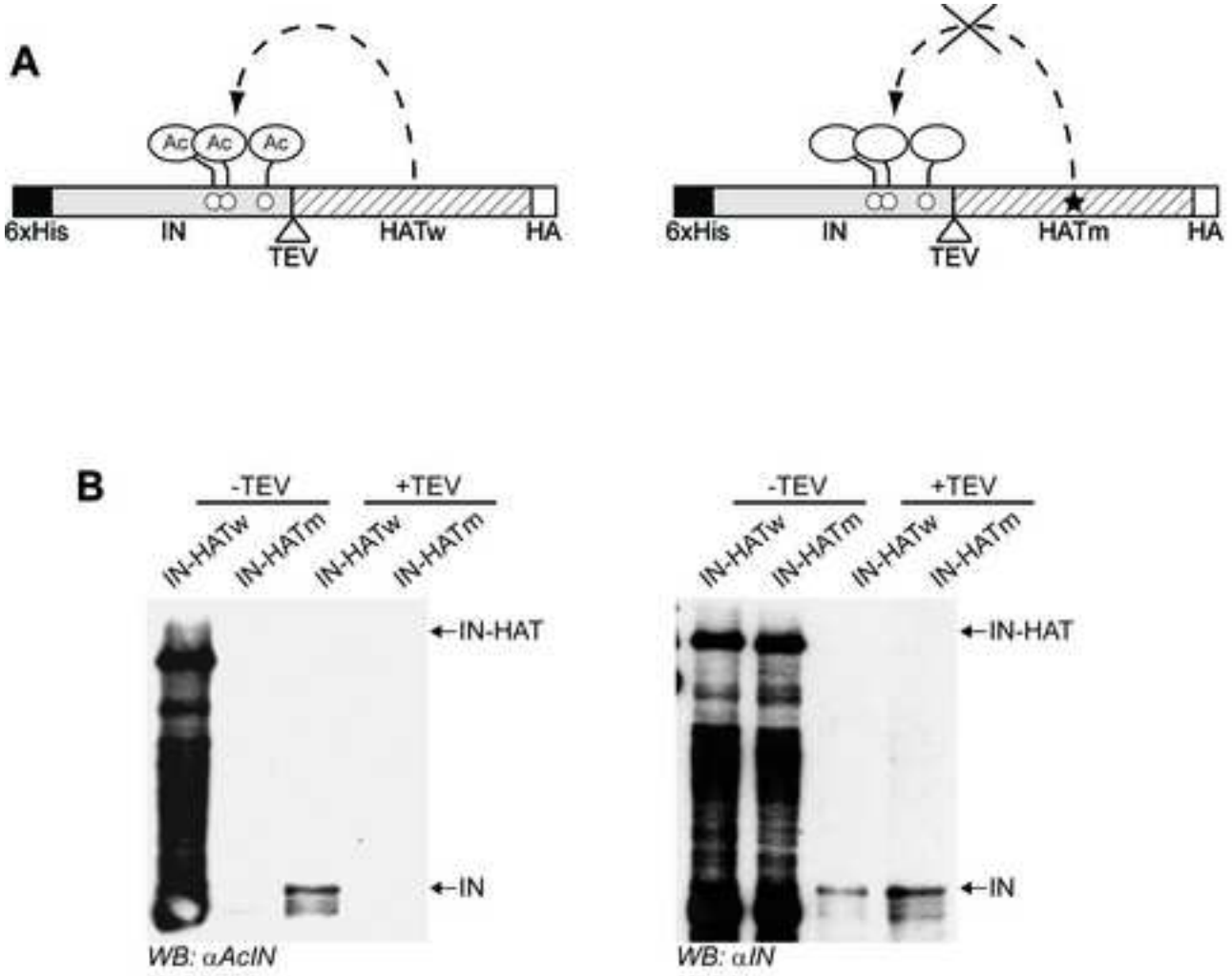


**Figure 1**

**Figure 2**

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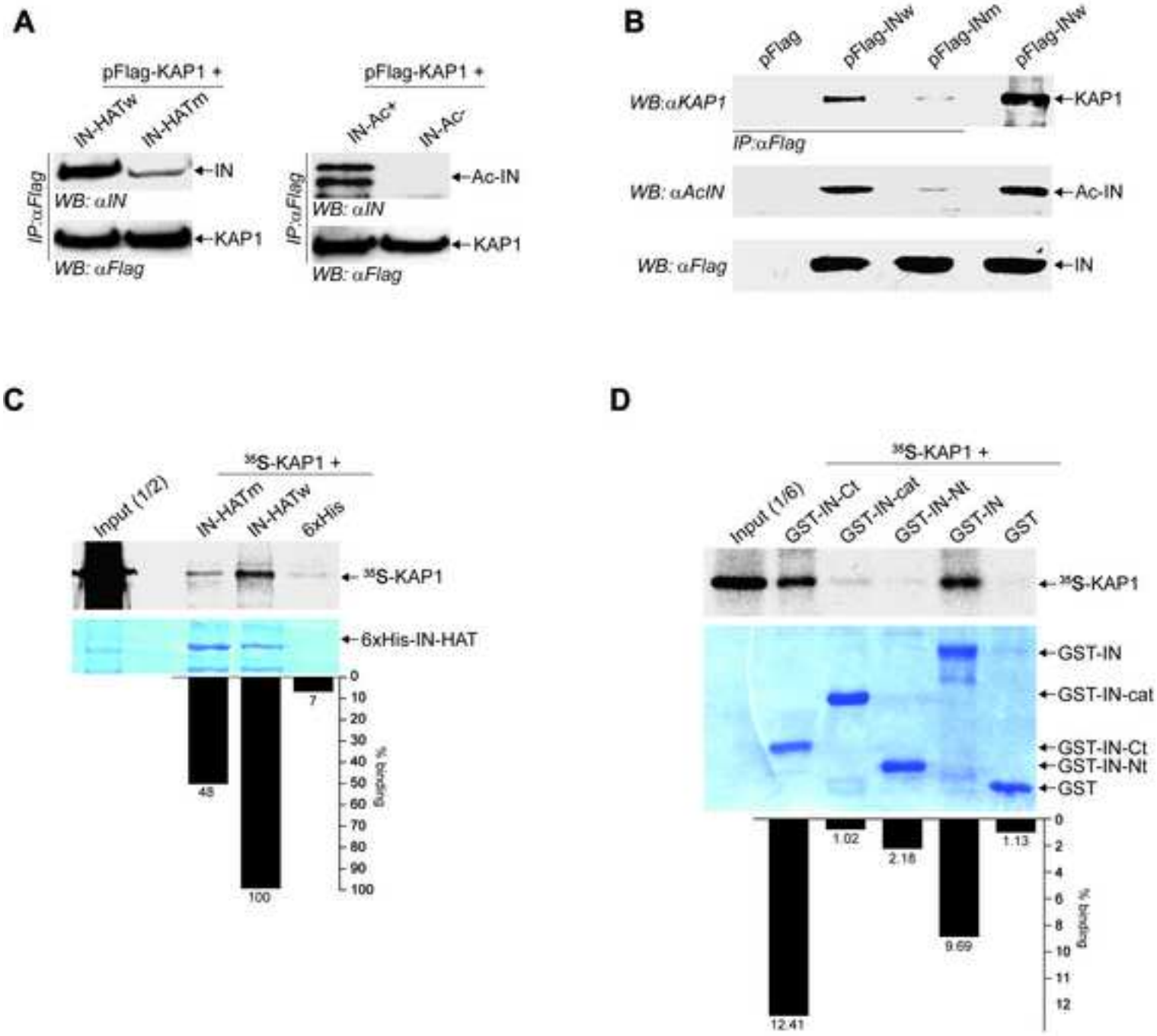


Figure 2

Figure 3

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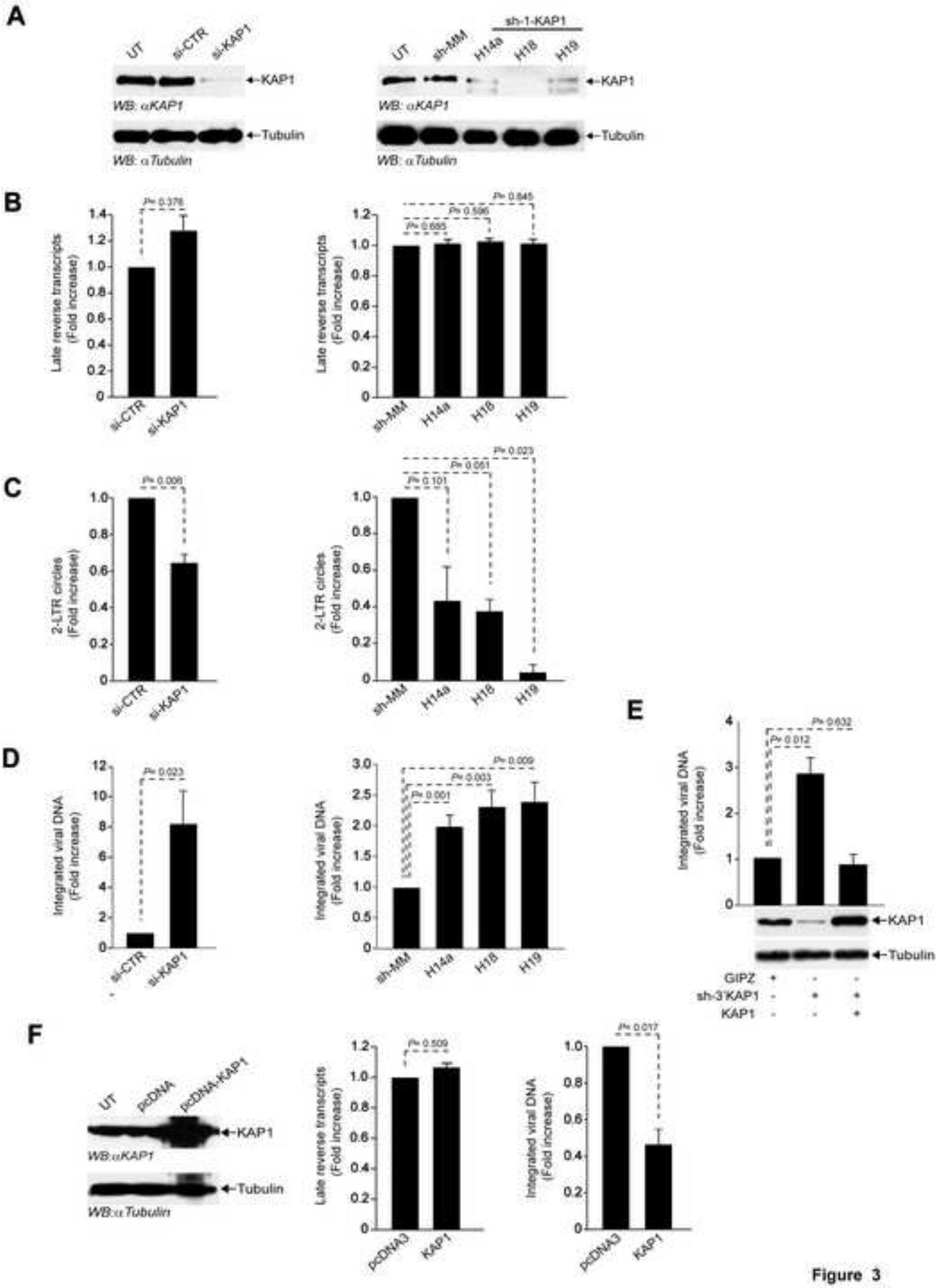


Figure 3

Figure 4

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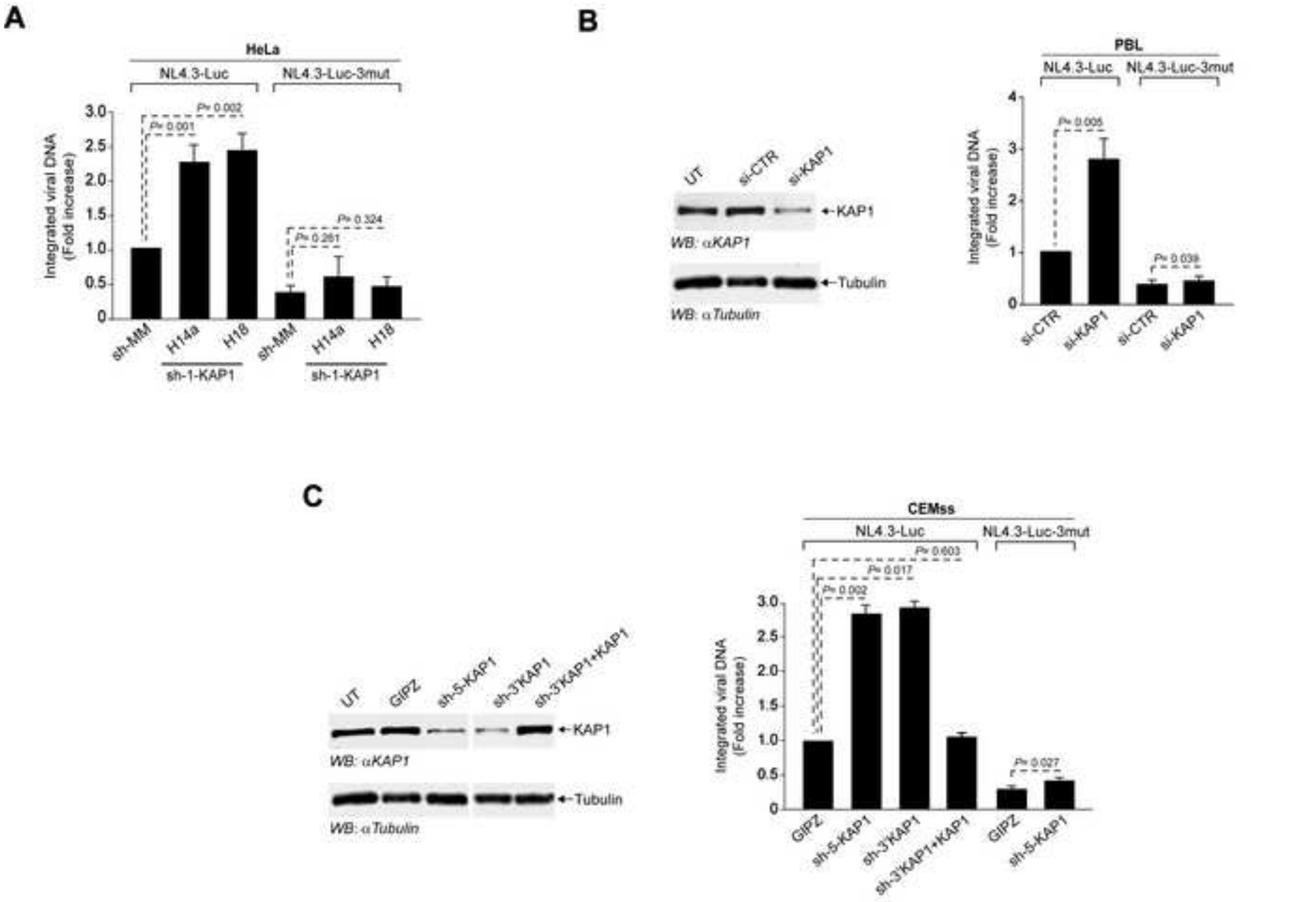


Figure 4

Figure 5

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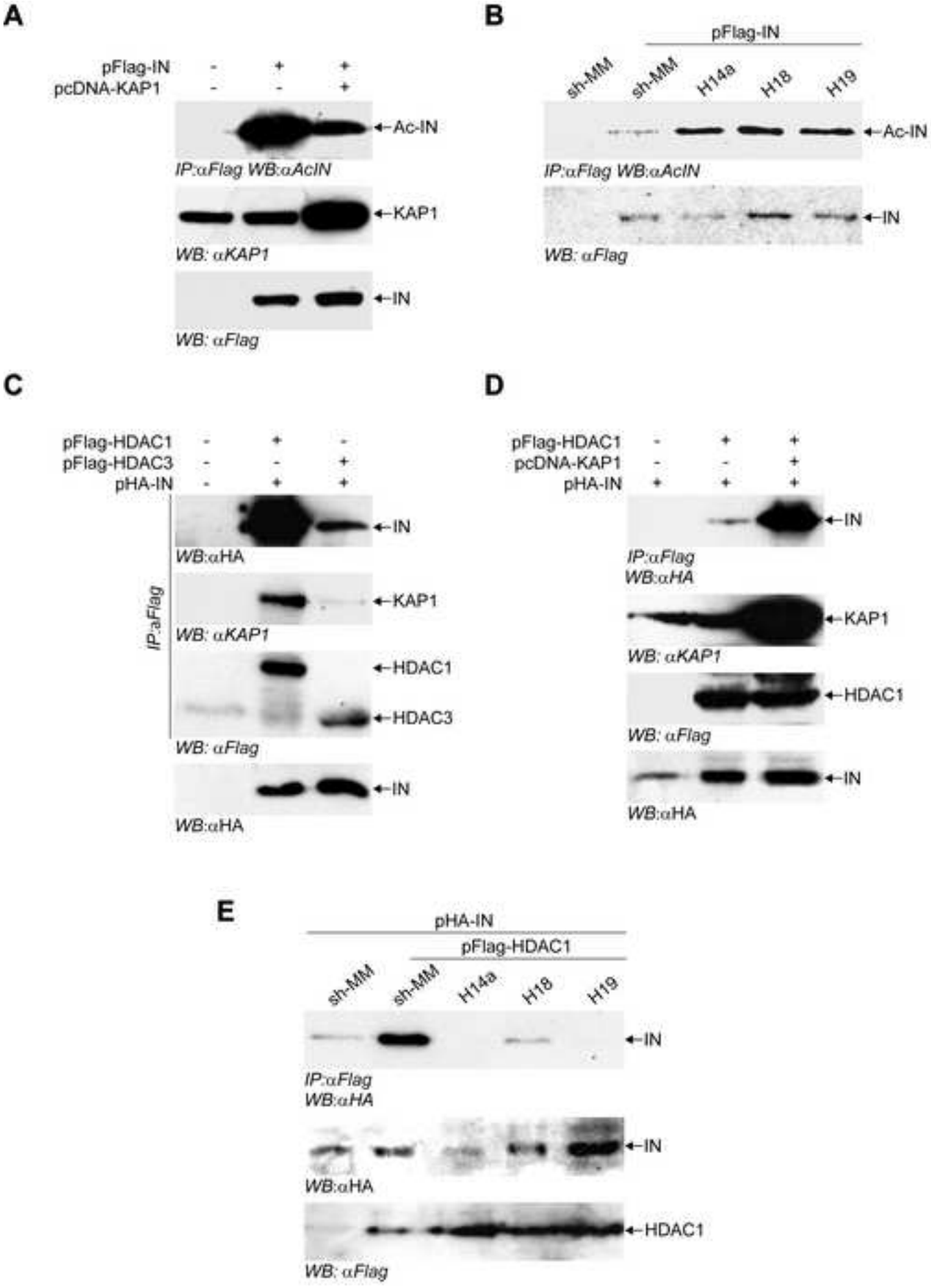
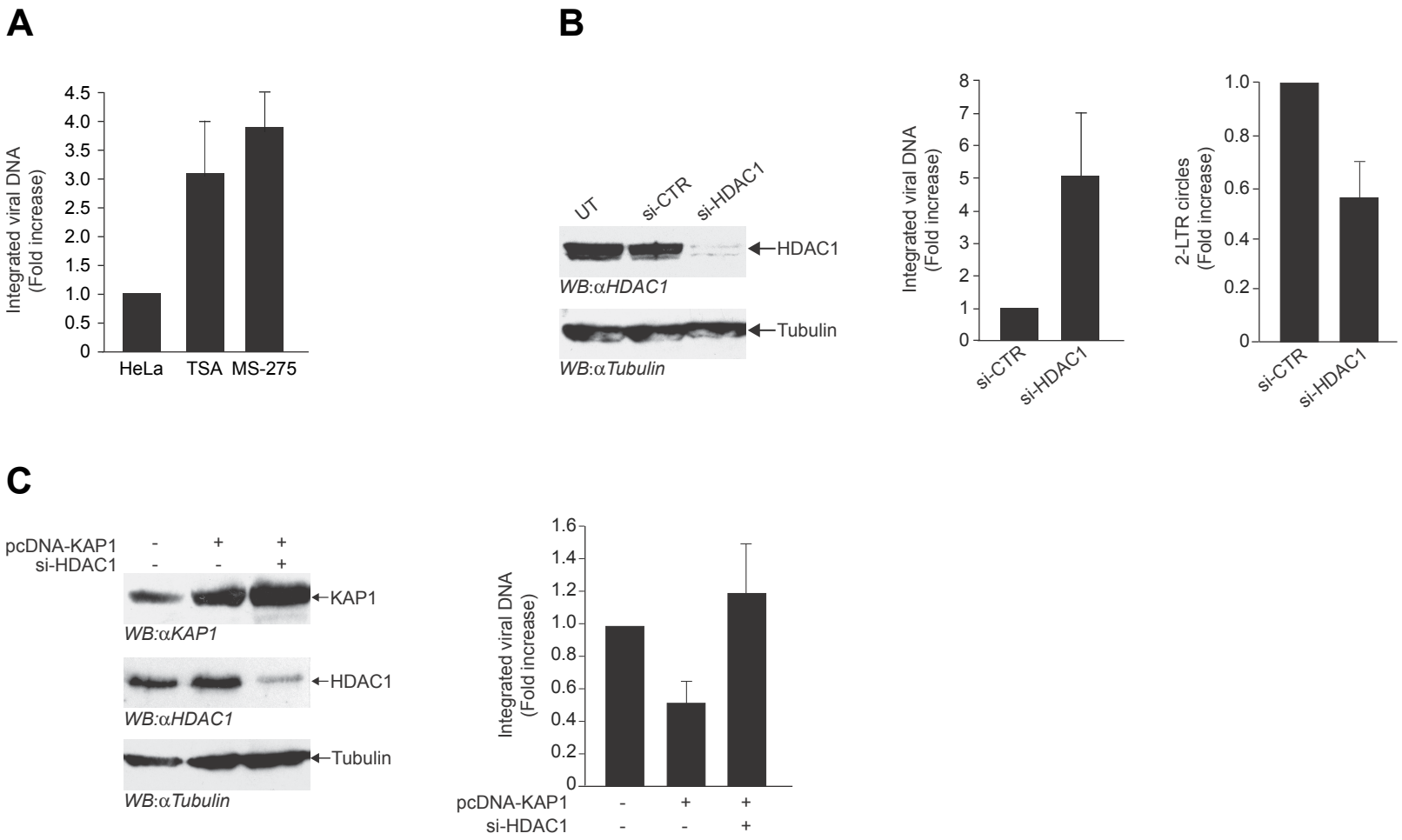



Figure 5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# Supplemental Information

## I- Supplemental Data

### *Figure S1 related to Figure 1*

### Identification of Ac-K264, Ac-K266 and Ac-K273 by MS-MS (MALDI-TOF/TOF) in the IN-HATw fusion recombinant protein

The identification of acetylated lysines in IN-HATw recombinant protein was performed by mass-spectrometry analysis of IN-HATw recombinant protein. The acetylation of the IN lysines was supported by an adequate ladder of y (and b) ion family and by the presence of known marker ions for acetylated lysine: the acetylated lysine immonium ion at m/z 143.1 and the ion at m/z 126.1, which is a fragment ion induced by the loss of NH3 from the ions at m/z 143.11-2 (Kim et al., 2002; Trelle and Jensen, 2008).

(A) Mass spectrum of AcK264 and AcK266 lysines. Fragmentation for bi-acetylated ionAcK264AAcK266IIR.

(B) Mass spectrum of AcK273. Fragmentation for acetylated ion DYGAcK273QMAGDDCamCVASR. CamC stands for carbamidomethylated cysteine.

In (A) and (B) AcK stands for acetylated lysines. Mapped y ion family sequence in red. Mapped b ion family sequence in green. Lysine acetylation marker ions marked in yellow.

### *Figure S2 related to Figures 2 and 5*

### Associations of untagged integrases with KAP1 and HDAC1

To prove that the HA or Flag tag fused to IN does not affect the association with either KAP1 or HDAC1 co-immunoprecipitations were performed using untagged IN.

(A) Endogenous KAP1 binds preferentially acetylated IN (untagged). HEK293T cells expressing either IN wild type untagged (INw) or mutated at lysine acetylation sites (K264, 266, 273R) (INm) were immunoprecipitated with αKAP1 antibodies and blotted using α-IN antibodies.

(B) KAP1 increases the association of HDAC1 with IN (untagged). IN and Flag-HDAC1 were co-expressed in HEK293T cells with or without exogenous KAP1 (pcDNA-KAP1). αFlag (HDAC1) immunoprecipitates were blotted with α-IN antibodies. The expression levels of KAP1, HDAC1 and IN were controlled by immunoblot using the indicated antibodies.

## *Figure S3 related to Figures 3 and 4*

**KAP1 affects viral infectivity through a mechanism involving IN acetylable lysines (K264, K266, K273)**

The KAP1 effect on HIV-1 infectivity was evaluated by measuring the luciferase activity produced by an HIV-1 vector expressing the luciferase gene and containing an IN wt (NL4.3-Luc) or mutated in the three acetylable lysines (NL4.3-Luc-3mut)

(A) KAP1 knockdown increases HIV-1 infectivity. Transient and stable KAP1 knockdown HeLa cells (Figure 3A) were infected with NL4.3-Luc HIV-1 virus and analyzed for luciferase activity (Luc Activity) 48 hpi.

(B) KAP1 over-expression decreases HIV-1 infectivity. HEK293T cells over-expressing KAP1 (pcDNA-KAP1) and control cells (pcDNA) (Figure 3F) were infected with NL4.3-Luc HIV-1 virus and analyzed for luciferase activities (Luc Activity) 48 hpi.

(C) KAP1 does not affect the infectivity of NL4.3-Luc-3mut carrying IN mutated at acetylable lysines (K264,266,273R). KAP1 knockdown (H14a and H18) and control (shMM) HeLa cell clones were infected with NL4.3-Luc or NL4.3-Luc-3mut and analyzed for luciferase activity 48 hpi.

All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments).

***Figure S4 related to Figure 3***

**KAP1 does not regulate the HIV-1 LTR promoter**

To verify whether KAP1 might affect HIV-1 transcription three different approaches were employed:

(A) Left panel: stable KAP1 knockdown HeLa cell clones (H14a, H18 and H19) and control cells expressing a mismatched KAP1 shRNA (sh-MM) were infected with D64E HIV-1 virus. Luciferase activity was evaluated at 48 hpi (Luc Activity).

Right panel: the same experiment was performed using HEK293T cells (T-pool) and cell clones (T14 and T15) KAP1 stably knocked down and control cells expressing a mismatched KAP1-shRNA (shMM).

Graphs are represented in fold increase of infectivity of KAP1 knockdown cells with respect to control cells (error bars represent standard deviations from at least two independent experiments).

(B) HeLa cells KAP1 stably knocked down (H14a, H18 and H19) and control cells (sh-MM) were transfected with pNL4.3.Luc.R-E- HIV-1 together with pCMV-*Renella* luciferase to normalize for transfection efficiency. Results are represented as a ratio of firefly luciferase

(pNL4.3.Luc.R-E-) to *Renella* luciferase (pCMV-Renella).

(C) KAP1 knockdown does not affect transcription of latent or activated integrated HIV-1 in J-lat A1 cells (Jordan et al., 2003). Percentage of GFP positive J-lat A1 cells measured by FACS, non activated or TPA-activated following KAP1 knockdown (sh-5-KAP1). Expression of KAP1 in non activated or TPA-activated J-lat A1 cells following KAP1 knockdown (sh-5-KAP1) and in control cells (AIP) was verified by immunoblot (error bars represent standard deviations from at least two independent experiments).

## *Figure S5 related to Figure 3*

**KAP1 knockdown increases HIV-1 integration in HEK293T cells**

To prove that KAP1 inhibition of HIV-1 integration is not cellular specific, KAP1 was knocked down in HEK293T cells and viral steps analyzed by luc activity or by qPCR for different HIV-1 DNA products.

(A) Expression of KAP1 in HEK293T cells (T-pool) and cell clones (T14 and T15) stably knocked down with KAP1 shRNA expressed by a lentiviral vector (GIPZ-sh-1-KAP1) and in control HEK293T cells expressing a four mutations mismatched KAP1-shRNA (GIPZ-sh-MM).

(B-E) HEK293T cells stably silenced for KAP1 were infected with NL4.3-Luc HIV-1 virus and analyzed for luciferase activity (Luc Activity) at 48 hours post-infection (hpi) (B) and by qPCR for late reverse transcripts at 24 hpi (C), 2-LTR circles at 24 hpi (D) and integrated HIV-1 DNA at 15 dpi (E).

All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments).

qPCR absolute values are reported in Table S1.

## *Figure S6 related to Figure 3*

**Human KAP1 does not affect M-MLV integration in HeLa cells**

(A) Expression of KAP1 in HeLa cells knocked down with smart-pool siRNAs (siKAP1) and control HeLa cells treated with a pool of non targeting siRNAs (siCTR).

(B-D) HeLa KAP1 knockdown cells were infected with HIV-1 or with MLV viruses and analyzed for luciferase activity (B), integrated viral DNA by qPCR at 15 dpi (C) and late reverse transcripts at 24 hpi (D).

All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments).

***Figure S7 related to Figure 5***

**HIV-1 integrase is deacetylated by HDAC1**

(A) HDACs are associated with IN *in vivo*. HEK293T cell lysates expressing Flag-IN were immunoprecipitated using anti Flag antibodies (αFlag). The HDAC activities of the αFlag immunoprecipitates in the presence or not of the TSA were detected using an *in vitro* fluorescence-based assay.

(B) IN acetylation is regulated by the cellular HDACs. HEK293T cells expressing pCMV-IN were treated or not with TSA and analyzed by Western Blot for IN acetylation and for total IN using anti acetylated IN (α–Ac-IN) and anti IN (α–IN) antibodies respectively.

(C) IN is directly deacetylated by HDAC1. Left panel: $^{14}C$ labeled *in vitro* acetylated GST-IN (Ac-IN) was incubated with the purified Flag-HDAC1 in the presence or not of TSA (4μM), with Flag-HDAC1 catalytically inactive (H141A) or with the control eluates. The upper panel shows the gel exposed to Phosphoimaging (Cyclone) and the lower panel the Coomassie staining of the SDS-PAGE gel. Right panel: the purified Flag-HDAC1 and Flag-HDAC1 (H141A), used for the *in vitro* IN deacetylation assays, were analyzed by Western Blot using the anti Flag (αFlag) and anti KAP1 (αKAP1) antibodies.

***Figure S8 related to Figure 6***

**HDAC3 is dispensable in KAP1 mediated inhibition of HIV-1 integration**

(A) Expressions of KAP1 and HDAC3 in HEK293T cells over-expressing KAP1 and knocked down or not for HDAC3 using a pool of HDAC3 siRNAs (si-HDAC3).

(B-C) HEK293T cells were infected with NL4.3-Luc HIV-1 virus at 24 hours post transfection with pcDNA3-KAP1 and si-HDAC3. The integrated provirus (Alu-LTR) (B) and late reverse transcripts (C) were analyzed by qPCR 48 hpi.

All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments). qPCR absolute values are reported in Table S1.

***Figure S9 related to Figure 6***

**Identification of the KAP1 domain determining KAP1 mediated inhibition of HIV-1 integration**

**Results**

In order to determine which region of KAP1 is required for HIV-1 inhibition, different fragments of the cellular protein (schematized in Figure S9A) were tested in viral integration. As shown in Figure S9B, integration of HIV-1 was inhibited at the same extent as full length by the 1-381 and 1-616 fragments, while the 617-835 fragment showed no decreased integration. The reverse transcription remained unaltered (data not shown). To further verify the role of each KAP1 fragments in HIV-1 infectivity reciprocal experiments were performed in KAP1 knockdown CEMss cells expressing each single domain resistant to KAP1 shRNA (Figure S9C, left panel). Expression of 1-381, 1-616 and of a KAP1 mutant (W664A) defective in Mi2$\alpha$ binding restored viral integration to baseline at the same extent as full length KAP1, while the 617-835 fragment could not revert KAP1 knockdown integration phenotype (Figure S9C, right panel). The amounts of late reverse transcripts remained unaltered (data not shown).

Since our results indicate that KAP1 activity on HIV-1 integration occurs through HDAC1, we then evaluated the binding capacity of each KAP1 fragment with HDAC1. As shown in Figure S9D, left panel, similar amounts of fragment 1-616 and full length KAP1 co precipitated with Flag-HDAC1. Due to different migration conditions, a separate gel reported in the right panel of Figure S9D, shows that fragment 1-381 is associated with HDAC1, while the 617-835 fragment is not. Therefore, the associations of HDAC1 with the 1-381 and 1-616 correlate with

the capacity of these fragments to inhibit viral integration and to reverse the KAP1 knockdown phenotype. Since the RBCC domain corresponding to 1-381 fragment has been shown to trimerize (Peng et al., 2000), its binding with HDAC1, here reported, may result from the oligomerization with endogenous KAP1. Therefore, to verify this interaction MEFs KAP1 knock-out cells (Wiznerowicz et al., 2007) were employed showing a positive binding between HDAC1 and the 1-381 fragment (Figure S9E), as observed in HEK293T cells.

It has been reported that the KAP1 region involved in transcriptional silencing mediated by the NuRD-HDAC1 correspond to the PHD-Bromo domain localized in the C-terminal region (Schultz et al., 2001). Therefore, our data suggest that the molecular mechanisms leading to the recruitment of HDAC1 by KAP1 in the integration inhibition (N-terminal) do not correspond to the mechanisms involved in the transcription repression (C-terminal).

**Figure S9**

(A) Schematic representation of HA-tagged KAP1 FL (1-835) and deletion mutants (1-381, 1-616 and 617-835).

(B) Integrated NL4.3-Luc HIV-1 DNA (Alu-LTR) 48 hpi of HEK293T cells expressing KAP1 full length (1-835) or KAP1 deletion mutants (1-381, 1-616 and 617-835). Protein expression was verified by immunoblot.

(C) Integrated NL4.3-Luc HIV-1 DNA 15 dpi in CEMss cells knocked down for KAP1 (sh3'KAP1) and back-complemented with KAP1 full length (1-835), KAP1 deletion mutants (1-381, 1-616 and 617-835) or a mutant KAP1 (W664A) defective in Mi2$\alpha$ binding. Protein expression was verified by immunoblot.

(D) Lysates from HEK293T cells co-expressing Flag-HDAC1 with HA-KAP1 full length (1-835) or HA-KAP1 (1-616) (left panel) and lysates from HEK293T cell co-expressing Flag-HDAC1 with HA-KAP1 full length (1-835), HA-KAP1 (1-381), or HA-KAP1 (617-835) (right panel) were immunoprecipitated with $\alpha$-Flag

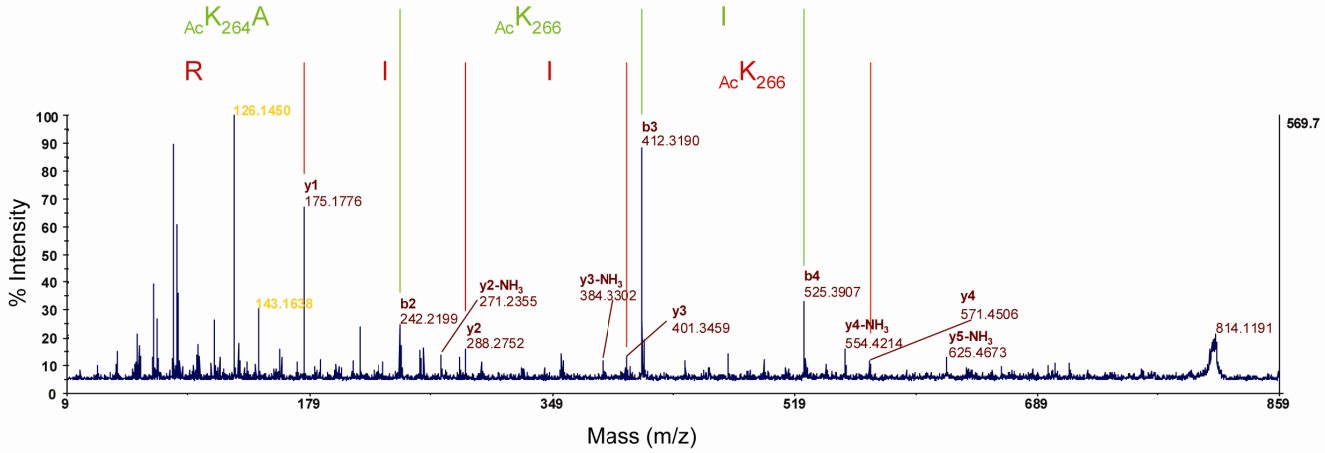
antibodies and blotted with α-HA antibodies. Expression levels of KAP1 full length or deletion mutants and HDAC1 were verified by using the indicated antibodies.
(E) Lysates from MEFs KAP1 KO cells co-expressing Flag-HDAC1 with HA-KAP1 (1-381) were immunoprecipitated with α-Flag antibodies and blotted with α-KAP1 antibodies. Expression levels of KAP1 (1-381) and HDAC1 were verified using the indicated antibodies.
All graphs are represented in fold increase with respect to control cells (error bars represent standard deviations from at least two independent experiments).
qPCR absolute values are reported in Table S1.

***Figure S10 related to Discussion section***

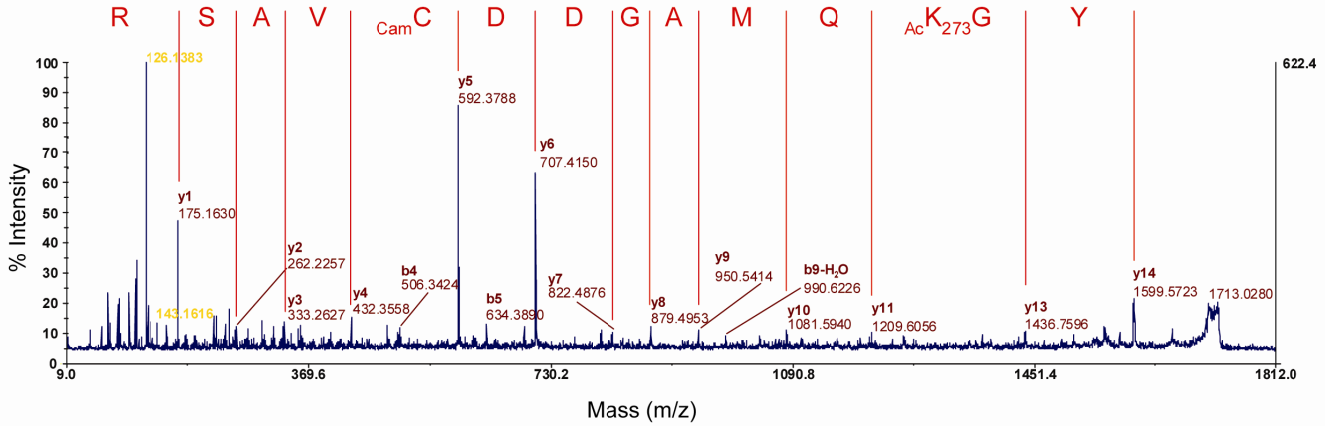
**KAP1 and HDAC1 expressions in Resting or Activated PBLs and in Naive (CD45RA+) or Memory (CD45RO+) CD4+ T cells**

Equal number of Resting or Activated PBLs (2 x $10^6$) were analyzed by western blot for KAP1 and HDAC1 expressions using the indicated antibodies (αKAP1 and αHDAC1). Equal amounts of proteins (60 μg) from CD45RA+ Naive or CD45RO+ Memory CD4+ T cells were analyzed by Western blot for KAP1 and HDAC1 expressions using the indicated antibodies (αKAP1 and αHDAC1).

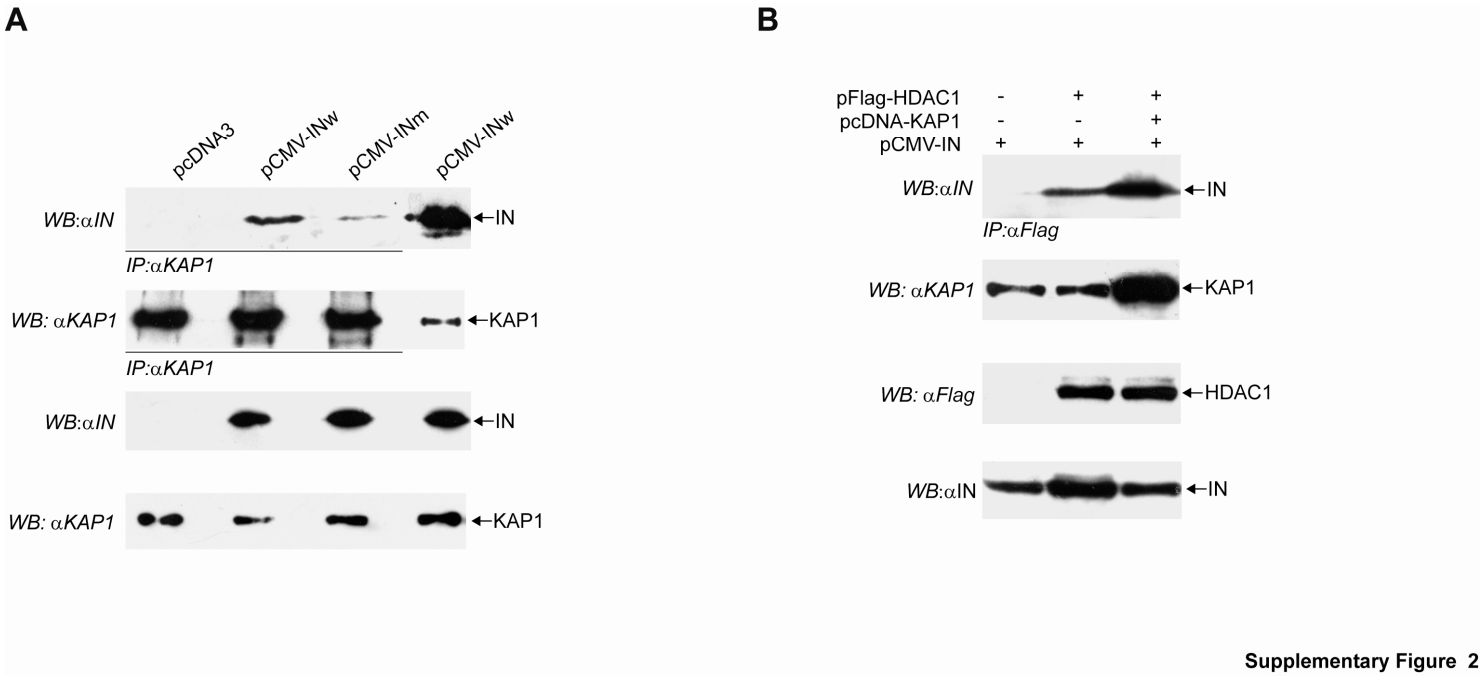
**A**



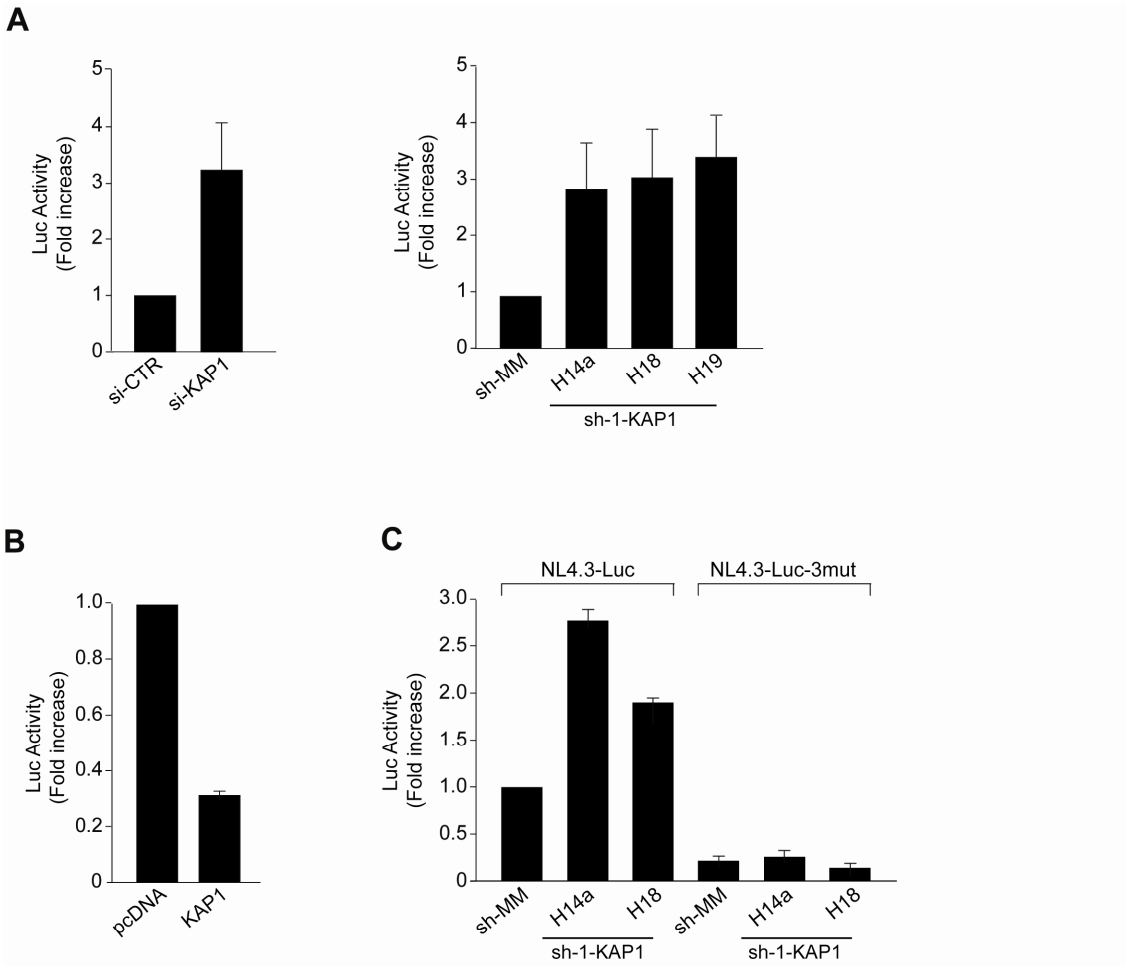

**B**



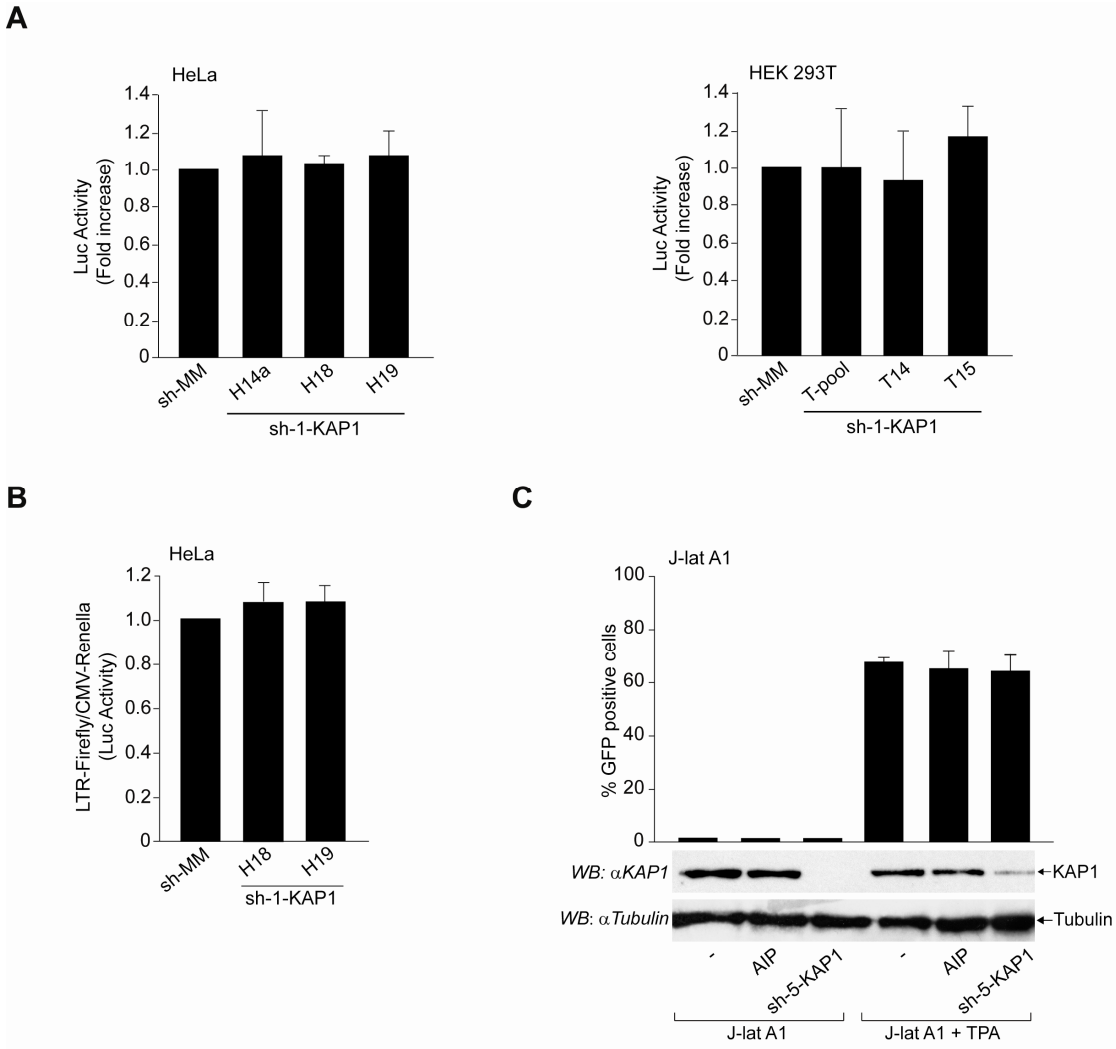

**Supplementary Figure 1**



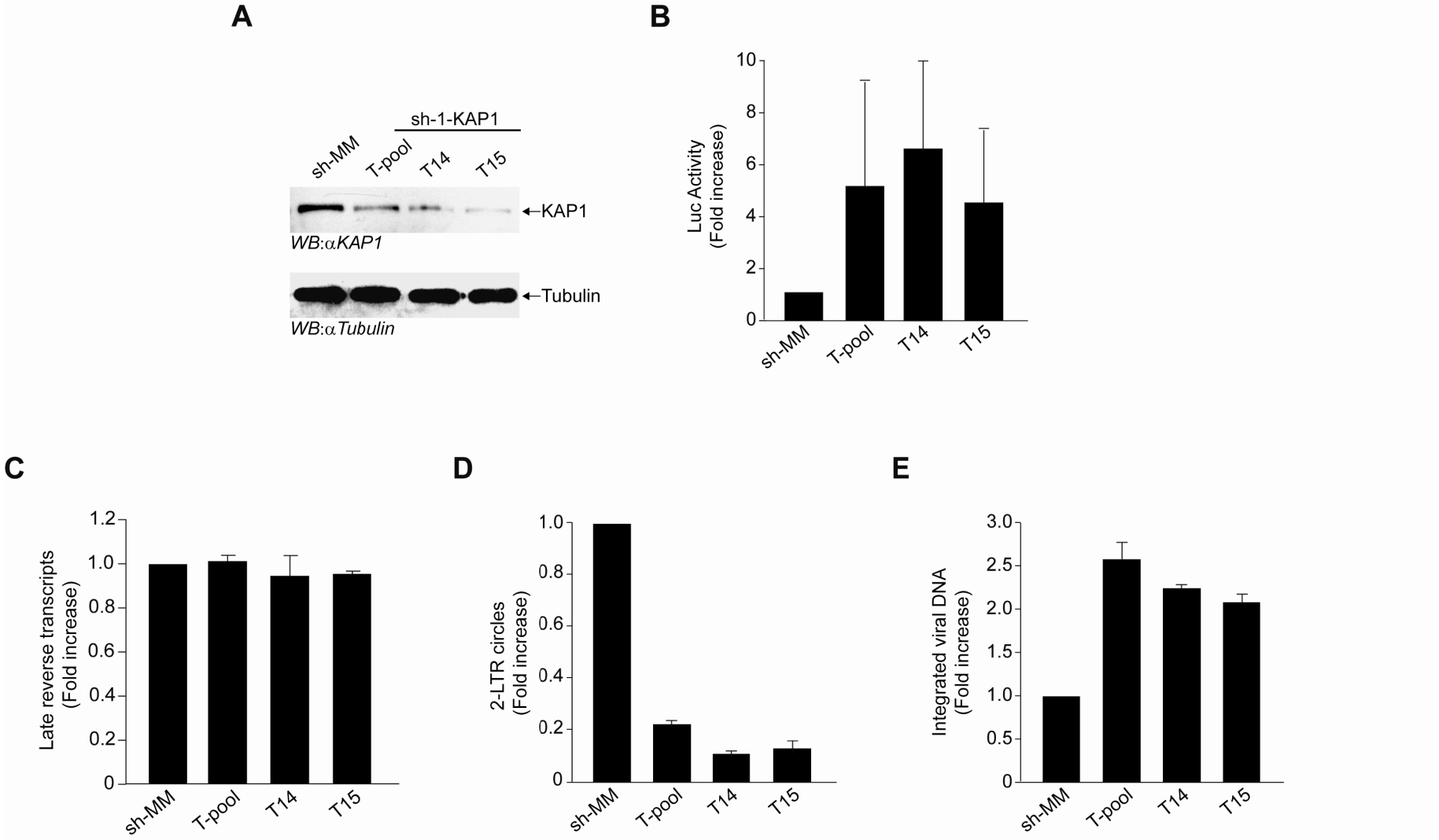

**Supplementary Figure 2**



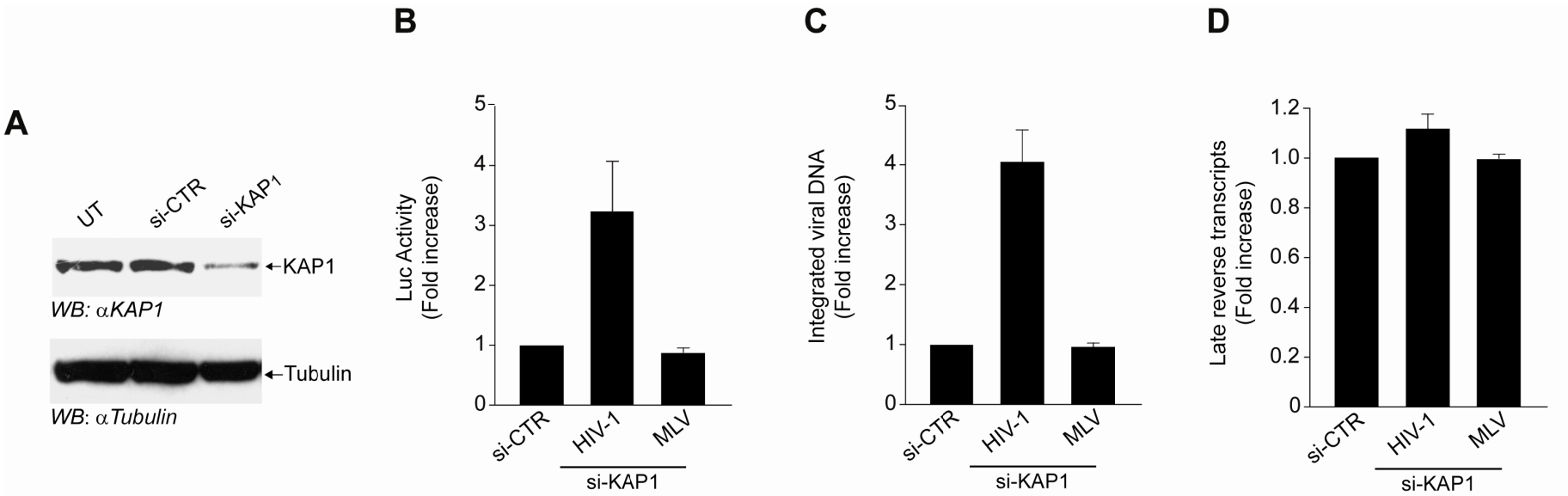

**Supplementary Figure 3**



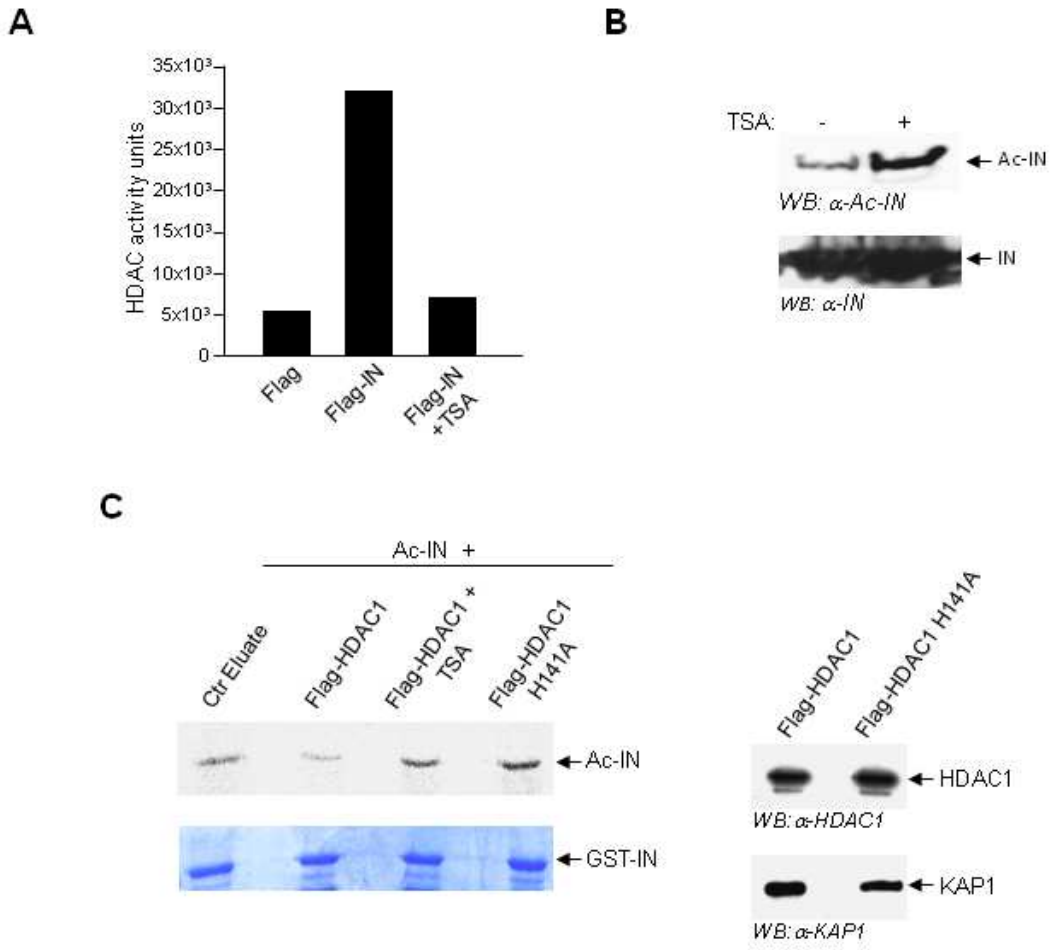

**Supplementary Figure 4**



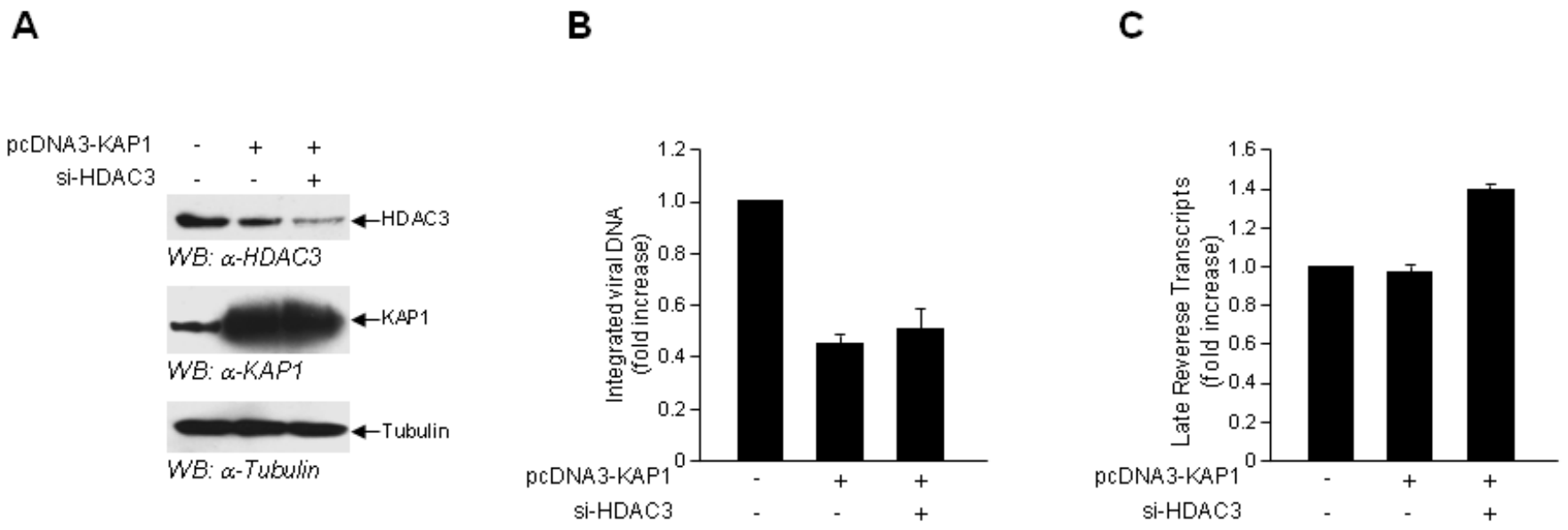

Supplementary Figure 5



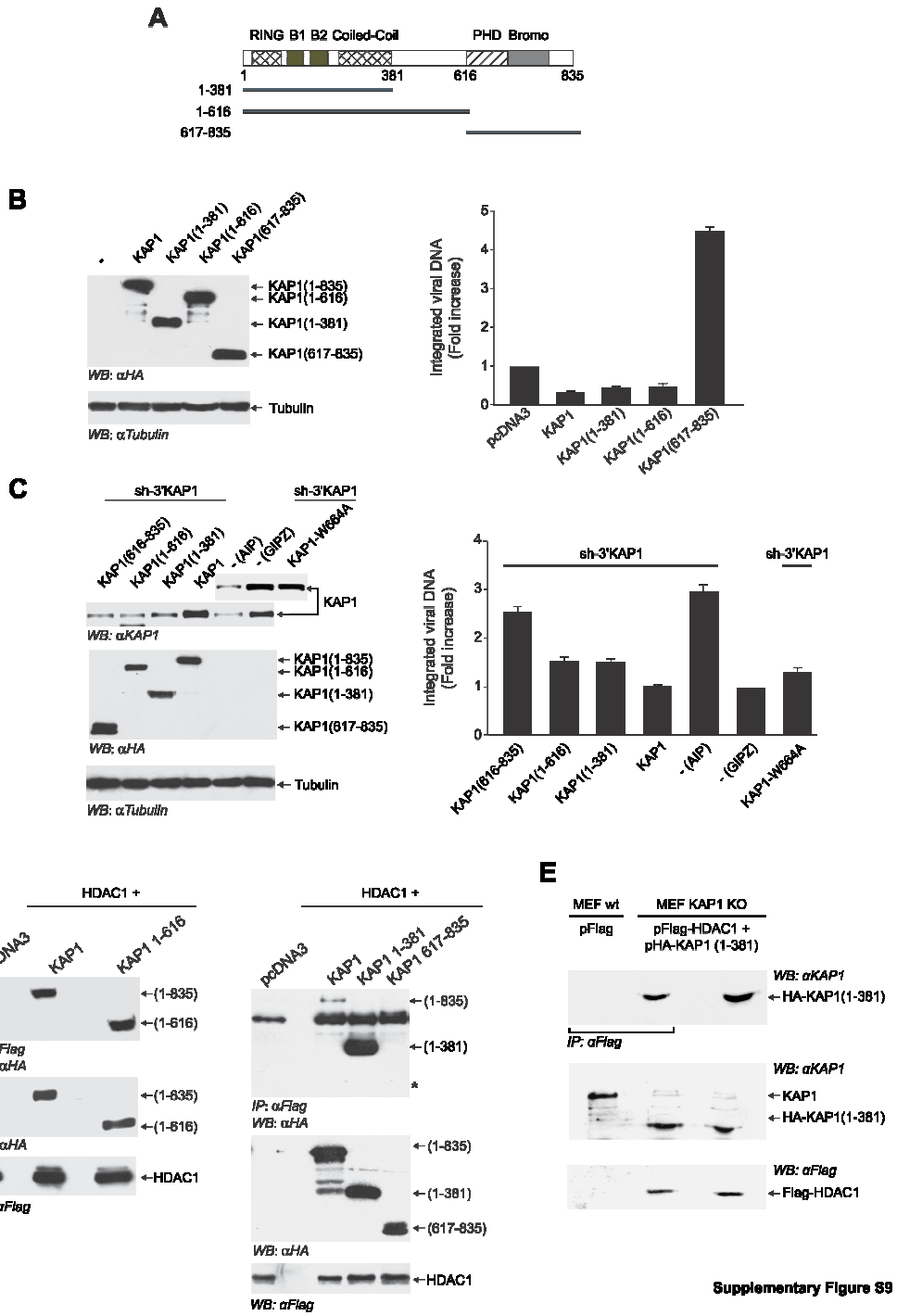

**Supplementary Figure 6**



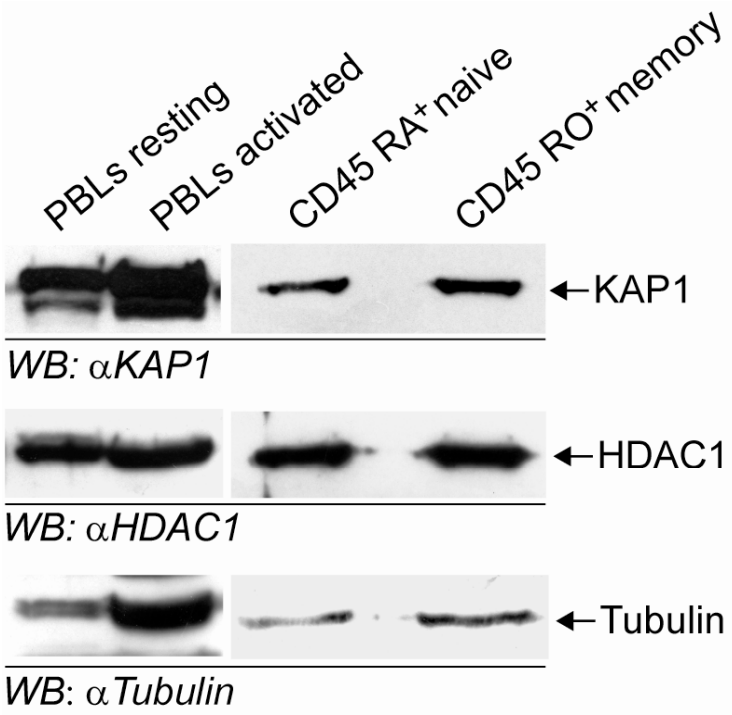

**Supplementary Figure 7**




**Supplementary Figure 8**




**Supplementary Figure S9**




**Supplementary Figure 10**

## II- Supplemental Experimental Procedures

### Vectors and constructs

Untagged INw and INm were cloned by PCR in pCDNA3.0 vector starting from their respective pFlag IN CO constructs. pD64E was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). The pFlag-HDAC1 (H141A) and the pAIP-HA-KAP1 (W644A) were produced by PCR starting from the original vectors. KAP1 deletion mutants (1-381, 1-616 and 617-835) were cloned by PCR in pAIP lentiviral vector in frame with HA and in pcDNA3HA vector.

MLV transfer gene pLNC-CMV-eGFPT2A-fLuc was a kind of gift from Z. Debyser (KU Leuven University, Belgium).

### Identification of constitutively acetylated IN acetylation sites by mass spectrometry

Recombinant purified IN-HATw (250ng) was separated by SDS-PAGE and stained by Coomassie blue. The corresponding band was excised and treated with trypsin as previously described (Shevchenko et al., 1996) with minor modifications. Peptides were analyzed by mass spectrometry (MS) and tandem mass spectrometry (MSMS) performed with a 4800 MALDI TOF/TOF Analyzer mass spectrometer from Applied Biosystems. Searching extracted unambiguously identified proteins and manually curated monoisotopic peak lists against the SwissProt database (version 57.0), UniProtKB database (version 15.0) or NCBI database using the software program Mascot Server 2.1.04 (Matrix Science).

### Co-immunoprecipitations

Untagged INw and INm co-immunoprecipitations were performed using the same conditions described in EXPERIMENTAL PROCEDURES. Untagged INw and INm were detected with a mouse monoclonal IN antibody IN2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

## Cells

Naïve (CD45RA+) and Memory (CD45RO+) CD4+ T cells were isolated starting from PBLs that were purified as described in EXPERIMENTAL PROCEDURES. CD4+ T cells were isolated from PBLs by negative selection with CD4+ T cells isolation kit (Miltenyi Biotec). Naïve (CD45RA+) and Memory (CD45RO+) T cells were isolated from the CD4+ population using respectively CD45RA and CD45RO microbeads (Miltenyi Biotec).

The Mouse embryonic fibroblasts KAP1 Knockout cells (MEFs KAP1 KO) derived from homozygous KAP1 floxed mice were previously described (Wiznerowicz et al., 2007) and are kind of gifts from Didier Trono (School of Life Sciences, EPFL, Lausanne, Switzerland).

## HIV-1 LTR transcription assay in KAP-1 knockdown HeLa and J-lat A1 Cells

HeLa KAP1 knockdown cells were transfected with pNL4.3.Luc.R-E- and CMV-*Renella*. Luciferase activity was measured 48 hours post transfection using the DualGlo Luciferase assay Kit (Promega). J-lat A1 cells (Jordan et al., 2003) (1 x $10^5$) untreated or TPA treated (10 nM TPA, for 24 hours) were transduced with 2 μg p24 of sh-5-KAP1 described in EXPERIMENTAL PROCEDURES or AIP lentiviral vector as control. Western blot analysis and percentage of GFP positive cells (analyzed by FACS) were performed 60 hours post LKO.1-sh-5-KAP1 transduction.

## Deacetylase Assays

Quantification of HDAC activity associated with IN was performed on Flag and Flag-IN immunoprecipitates obtained from 2 mg of HEK293T transfected cells by using the HDAC Fluorometric Activity Assay kit (Upstate).

*In vitro* HDAC1 activity was performed by using Flag-HDAC1 and Flag-HDAC1 H141A purified from HEK293T transfected cells by using the FLAG M Purification Kit (Sigma). Elutions were performed with Flag peptides in 100 mM KCl, 0.2% NP-40 buffer. Purified Flag-HDAC1 and Flag-HDAC1 H141A (1μg) were

incubated with GST-IN (1μg) *in vitro* acetylated by the HAT p300 in the presence of the $^{14}$C-Acetyl CoA as previously described (Cereseto et al., 2005) in HDAC buffer (10 mM Tris HCl pH 7, 100 mM NaCl, 5 mM $MgCl_2$ and 5 % glycerol) at 30°C for 2 hours. The reactions were resolved on SDS-PAGE and analyzed by coomassie blue staining and phosphoimaging (Cyclone).

**Transient knockdowns and KAP1 back-complementation in CEMss cells**

$1x10^5$ CEMss cells were knocked down by transducing 3 μg p24 of LKO.1-sh-3'KAP1 targeting the 3' untranslated region (3'UTR) of KAP1 gene and KAP1 was expressed using 2 μg p24 AIP-HA-KAP1 or AIP-HA-KAP1 deletion mutants (1-381, 1-616 and 617-835) and 4 μg AIP-HA-KAP1 (W664A).

At 60 hours post transductions $5x10^5$ CEMss cells were infected with 250 ng p24 NL4.3-Luc (VSV-G) HIV-1 virus.

The HDAC3 siRNA pool (smart pool siRNAs pre-designed by Dharmacon Inc. Chicago, IL) contains: 1) GGAAUGCGUUGAAUAUGUC; 2) GCAUUGAUGACCAGAGUUA; 3) AAAGCGAUGUGGAGAUUUA; 4) GGAAAGCGAUGUGGAGAUU.

$3x10^5$ HEK293T cells were transfected with pcDNA3-KAP1 (1,5 μg with PEI) and then treated with 100 nM siHDAC3 using Gene silencer. At 24 hours post siRNAs transfection, infections were performed using 125 ng p24 for $2,5x10^5$ cells.

**HIV-1 and MLV infectivity assays**

Transient and stable knockdown HeLa cells and transfected HEK293T cells were infected as described in EXPERIMENTAL PROCEDURES. HEK293T KAP-1 knockdown and control cells ($2,5x10^5$) were infected using 25 ng p24 of either NL4.3-Luc or D64E HIV-1 pseudotyped with VSV-G for 2 hours in 1 ml total volume. Infectivity was measured by luciferase activity quantification using the Luciferase Assay System Kit (Promega) and normalized to total protein concentrations. Real time quantitative PCR (qPCR) to analyze late reverse transcripts, 2-LTR and integrated viral DNA were performed as described in EXPERIMENTAL PROCEDURES.

MLV was produced as previously described (Christ et al., 2008). Real time quantitative PCR (qPCR) to analyze MLV late reverse transcripts at 24 hpi and integrated MLV DNA at 15 dpi were performed by using primers/probes specific to the luciferase gene (see EXPERIMENTAL PROCEDURES) in LNC-CMV-eGFPT2A-fLuc MLV.

## III- Supplemental References


Cereseto, A., Manganaro, L., Gutierrez, M.I., Terreni, M., Fittipaldi, A., Lusic, M., Marcello, A., and Giacca, M. (2005). Acetylation of HIV-1 integrase by p300 regulates viral integration. The EMBO journal *24*, 3070-3081.

Christ, F., Thys, W., De Rijck, J., Gijsbers, R., Albanese, A., Arosio, D., Emiliani, S., Rain, J.C., Benarous, R., Cereseto, A.*, et al.* (2008). Transportin-SR2 imports HIV into the nucleus. Curr Biol *18*, 1192-1202.

Jordan, A., Bisgrove, D., and Verdin, E. (2003). HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J *22*, 1868-1877.

Kim, J.Y., Kim, K.W., Kwon, H.J., Lee, D.W., and Yoo, J.S. (2002). Probing lysine acetylation with a modification-specific marker ion using high-performance liquid chromatography/electrospray-mass spectrometry with collision-induced dissociation. Anal Chem *74*, 5443-5449.

Peng, H., Begg, G.E., Schultz, D.C., Friedman, J.R., Jensen, D.E., Speicher, D.W., and Rauscher, F.J., 3rd (2000). Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. J Mol Biol *295*, 1139-1162.

Schultz, D.C., Friedman, J.R., and Rauscher, F.J., 3rd (2001). Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes & development *15*, 428-443.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem *68*, 850-858.

Trelle, M.B., and Jensen, O.N. (2008). Utility of immonium ions for assignment of epsilon-N-acetyllysine-containing peptides by tandem mass spectrometry. Anal Chem *80*, 3422-3430.

Wiznerowicz, M., Jakobsson, J., Szulc, J., Liao, S., Quazzola, A., Beermann, F., Aebischer, P., and Trono, D. (2007). The Kruppel-associated box repressor domain can trigger de novo promoter methylation during mouse early embryogenesis. J Biol Chem *282*, 34535-34541.

**Table S1**

| Figure 3 | Medium of at least two independent experiments | Standard deviations |
|---|---|---|
| | **Late Reverse transcripts** | |
| **Figure 3B left panel** | **(Copies/$10^6$ Cells)** | |
| si-CTR | 66545 | 19771,4525 |
| si-KAP1 | 84251 | 23672,3885 |
| **Figure 3B right panel** | | |
| sh-MM | 848911,2835 | 58864,6731 |
| H14a | 866446,8395 | 79054,6096 |
| H18 | 869849,1565 | 67167,2621 |
| H19 | 856845,65 | 72831,6591 |
| | **2 LTR circles (Copies/$10^6$** | |
| **Figure 3C left panel** | **Cells)** | |
| si-CTR | 17179,48333 | 4444,56191 |
| si-KAP1 | 11157,319 | 3402,39641 |
| **Figure 3C right panel** | | |
| sh-MM | 5700,5365 | 1228,24377 |
| H14a | 2647,268 | 1939,27146 |
| H18 | 2182,86 | 840,311557 |
| H19 | 291,5285 | 339,465702 |
| | **Integrated viral DNA** | |
| **Figure 3D left panel** | **(Copies/$10^6$ Cells)** | |
| si-CTR | 6566,727333 | 1517,65767 |
| si-KAP1 | 54482,978 | 23085,5902 |
| **Figure 3D right panel** | | |
| sh-MM | 144075,135 | 21948,5591 |
| H14a | 292730,065 | 70364,3867 |
| H18 | 341198,195 | 90057,6783 |
| H19 | 345544,035 | 6155,06047 |
| **Figure 3E** | | |
| GIPZ | 8347,371333 | 1895,8304 |
| sh3'KAP1 | 22874,59667 | 6370,68095 |
| sh3'KAP1+KAP1 | 7634,748333 | 4230,43603 |
| | **Late Reverse transcripts** | |
| **Figure 3F middle panel** | **(Copies/$10^6$ Cells)** | |
| pcDNA3 | 15178,5 | 2734,38192 |
| KAP1 | 15551,5 | 2783,8794 |
| | **Integrated viral DNA** | |
| **Figure 3F right panel** | **(Copies/$10^6$ Cells)** | |
| pcDNA3 | 14185,695 | 52,8420898 |
| KAP1 | 8178,749 | 1136,05331 |

| Figure 4 | Medium of at least two independent experiments | Standard deviations |
|---|---|---|
| | **Integrated viral DNA (Copies/$10^6$ Cells)** | |
| **Figure 4A** | | |
| sh-MM + NL4.3-Luc | 11117,03 | 1326,815164 |
| H14a + NL4.3-Luc | 22626,46 | 6450,850312 |
| H18 + NL4.3-Luc | 23438,525 | 6900,902565 |
| sh-MM + NL4.3-Luc-3mut | 5683,688 | 559,4657137 |
| H14a + NL4.3-Luc-3mut | 8888,4635 | 4836,011464 |
| H18 + NL4.3-Luc-3mut | 5716,7585 | 1014,276088 |
| **Figure 4B** | | |
| si-CTR+ NL4.3-Luc | 8485,9715 | 2444,27821 |
| si-KAP1+ NL4.3-Luc | 19285,1125 | 3171,217047 |
| si-CTR+ NL4.3-Luc-3mut | 3082,93725 | 1174,354288 |
| si-KAP1+ NL4.3-Luc-3mut | 3844,06075 | 918,6291123 |
| **Figure 4C** | | |
| GIPZ+ NL4.3-Luc | 24436,83333 | 3951,675208 |
| sh-5-KAP1+ NL4.3-Luc | 59869,26667 | 8683,191391 |
| GIPZ+ NL4.3-Luc-3mut | 10410,80233 | 1313,384708 |
| sh-5-KAP1+ NL4.3-Luc-3mut | 13603,49333 | 4390,79871 |
| | | |
| GIPZ + NL4.3-Luc | 83256,94 | 14241,73868 |
| sh-3'KAP1 + NL4.3-Luc | 179578,4 | 16623,939 |
| sh-3'KAP1+KAP1+ NL4.3-Luc | 80487,515 | 891,8808542 |

| Figure 6 | Medium of at least two independent experiments | Standard deviations |
|---|---|---|
| | **Integrated viral DNA (Copies/$10^6$ Cells)** | |
| **Figure 6A** | | |
| HeLa | 5219,5775 | 74,7447223 |
| TSA | 16391,605 | 7498,51386 |
| | | |
| HeLa | 2988,42 | 777,548759 |
| MS-275 | 10968,36 | 1059,81164 |
| **Figure 6B middle panel** | | |
| si-CTR | 3980,107 | 2303,46847 |
| si-HDAC1 | 30642,86767 | 26038,3323 |
| | **2 LTR circles (Copies/$10^6$ Cells)** | |
| **Figure 6B right panel** | | |
| si-CTR | 5028,779 | 3005,92574 |
| si-HDAC1 | 2855,263333 | 1932,31539 |
| | **Integrated viral DNA (Copies/$10^6$ Cells)** | |
| **Figure 6C** | | |
| pcDNA + si-CTR | 52342,5 | 2701,85501 |
| pcDNA-KAP1 + si-CTR | 26960,5 | 5103,18964 |

| | | |
|---|---|---|
| pcDNA-KAP1 + si-HDAC1 | 61738,5 | 12516,4971 |
| **Figure S5** | **Medium of at least two independent experiments** | **Standard deviations** |
| **Figure S5C** | **Late Reverse transcripts (Copies/$10^6$ Cells)** | |
| sh-MM | 93095,0435 | 17288,70918 |
| T-pool | 95006,9675 | 22280,02321 |
| T14 | 90671,765 | 11187,06992 |
| T15 | 85352,2355 | 14099,71558 |
| **Figure S5D** | **2 LTR circles (Copies/$10^6$ Cells)** | |
| sh-MM | 5678,0645 | 752,7752726 |
| T-pool | 1406,474 | 200,6542771 |
| T14 | 715,7585 | 148,6571799 |
| T15 | 850,107 | 148,1883681 |
| **Figure S5E** | **Integrated viral DNA (Copies/$10^6$ Cells)** | |
| sh-MM | 16241,163 | 1157,413593 |
| T-pool | 40368,72635 | 796,2677844 |
| T14 | 34849,34929 | 2482,251163 |
| T15 | 32509,842 | 1193,3926 |
| **Figure S8** | **Medium of at least two independent experiments** | **Standard deviations** |
| **Figure S8B** | **Late Reverse transcripts (Copies/$10^6$ Cells)** | |
| pcDNA + si-CTR | 148355,5 | 12533,4677 |
| pcDNA-KAP1 + si-CTR | 144806,5 | 14338,0042 |
| pcDNA-KAP1 + si-HDAC3 | 209178 | 13614,63396 |
| **Figure S8C** | **Integrated viral DNA (Copies/$10^6$ Cells)** | |
| pcDNA + si-CTR | 126551,5 | 15096,02267 |
| pcDNA-KAP1 + si-CTR | 57042,5 | 692,2575388 |
| pcDNA-KAP1 + si-HDAC3 | 59263,5 | 14455,38393 |
| **Figure S9** | **Medium of at least two independent experiments** | **Standard deviations** |
| **Figure S9B** | **Integrated viral DNA (Copies/$10^6$ Cells)** | |
| pcDNA3 | 31100,5 | 9654,12888 |
| KAP1 | 9533 | 3799,99184 |
| KAP1 (1-381) | 14079 | 5026,115 |
| KAP1 (1-616) | 14975 | 7141,77849 |
| KAP1 (617-835) | 137849 | 39928,9057 |

**Figure S9C**

| | | |
|---|---|---|
| GIPZ | 84142,93 | 10103,071 |
| sh-3'KAP1 + AIP | 242767 | 12700,7692 |
| sh-3'KAP1 + KAP1 | 81536,565 | 4394,35045 |
| sh-3'KAP1 + KAP1 (1-381) | 126929,9 | 2944,53406 |
| sh3'KAP1+ KAP1 (1-616) | 128783,6 | 6792,1849 |
| sh-3'KAP1 + KAP1 (617-835) | 207383,55 | 2327,01771 |
| | | |
| GIPZ | 48295,04 | 5443,68984 |
| sh-3'KAP1 + AIP | 119105,7 | 4738,88823 |
| sh-3'KAP1 + KAP1 | 49497,28 | 2681,60347 |
| sh-3'KAP1 + KAP1 (W664A) | 63646,4 | 940,833857 |

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