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HIV-1 Integration: an Interplay Between HIV-1 Integrase, Cellular and Viral Proteins

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Abstract

To achieve a productive infection, the reverse transcribed cDNA of the human immunodeficiency virus type 1 (HIV-1) has to be inserted in the host cell genome. The main protein required to accomplish this reaction is the virally encoded integrase.* In vitro, *the recombinant integrase is capable of catalyzing the two subsequent reactions of the integration process, namely the 3' processing followed by the strand transfer, without other viral and/or cellular proteins. However, a number of studies indicate that the* in vivo *integration process also involves cellular proteins, assisting the virus to integrate in the cellular genome. These cellular proteins can play a role during different steps of the integration process, including nuclear import, integrase catalysis, integration site selection and DNA gap repair. In this review we summarize the candidate cellular proteins involved in the HIV-1 integration process identified so far and discuss their potential roles during HIV-1 replication. (AIDS Reviews 2005;7:26-43)

Key words

HIV-1 integration. Integrase.

HIV-1 integration

The integration of reverse-transcribed viral cDNA into a host chromosome is an obligatory early step in the HIV-1 life cycle in order to obtain a productive infection. The key protein player in the retroviral integration is the 32 kDa viral integrase (IN), which enters the cell as part of the virion. The viral IN, encoded by the *pol* gene of the virus, is translated as part of a large Gag-Pol polyprotein and is processed into its mature form by the viral protease. The viral IN possesses three structural domains, consisting of a N-terminal domain (residues 1-50) containing a zinc binding motif, a catalytic core domain (50-212) carrying the DD(35)E motif, and a less-conserved C-terminal domain (212-288).

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Retroviral integration occurs in two well-characterized catalytic steps, referred to as 3' processing and strand transfer (Fig. 1). During the 3' processing, IN removes a pGT dinucleotide at each 3' end of the viral long terminal repeats (LTRs), adjacent to a highly conserved CA dinucleotide[1]. This reaction takes place in the cytoplasm within a nucleoprotein complex, referred to as the preintegration complex (PIC)[2]. This nucleoprotein complex contains linear viral DNA and several viral proteins including matrix, reverse transcriptase (RT)[3], IN[2] and nucleocapsid[4]. Cellular proteins have been identified as well in functional PICs, as will be described in more detail in this review. The subsequent strand transfer occurs in the nucleus following the nuclear import of the PIC. In the nucleus, the viral IN mediates a concerted nucleophilic attack by the 3' hydroxyl residues of the viral DNA on phosphodiester bridges located on either side of the major groove in the target DNA[1]. Next, the processed CA-3'-OH viral DNA ends are ligated to the 5'-O-phosphate ends of the target DNA. Since the 3' ends of the target DNA remain unjoined after the strand transfer, the product is a gapped intermediate product in which the 5'-phosphate ends of the viral DNA are not attached to the 3'-OH ends of the host DNA. The integration process is completed by cleavage of the unpaired dinucleotides from the 5' ends of the viral DNA and repair of

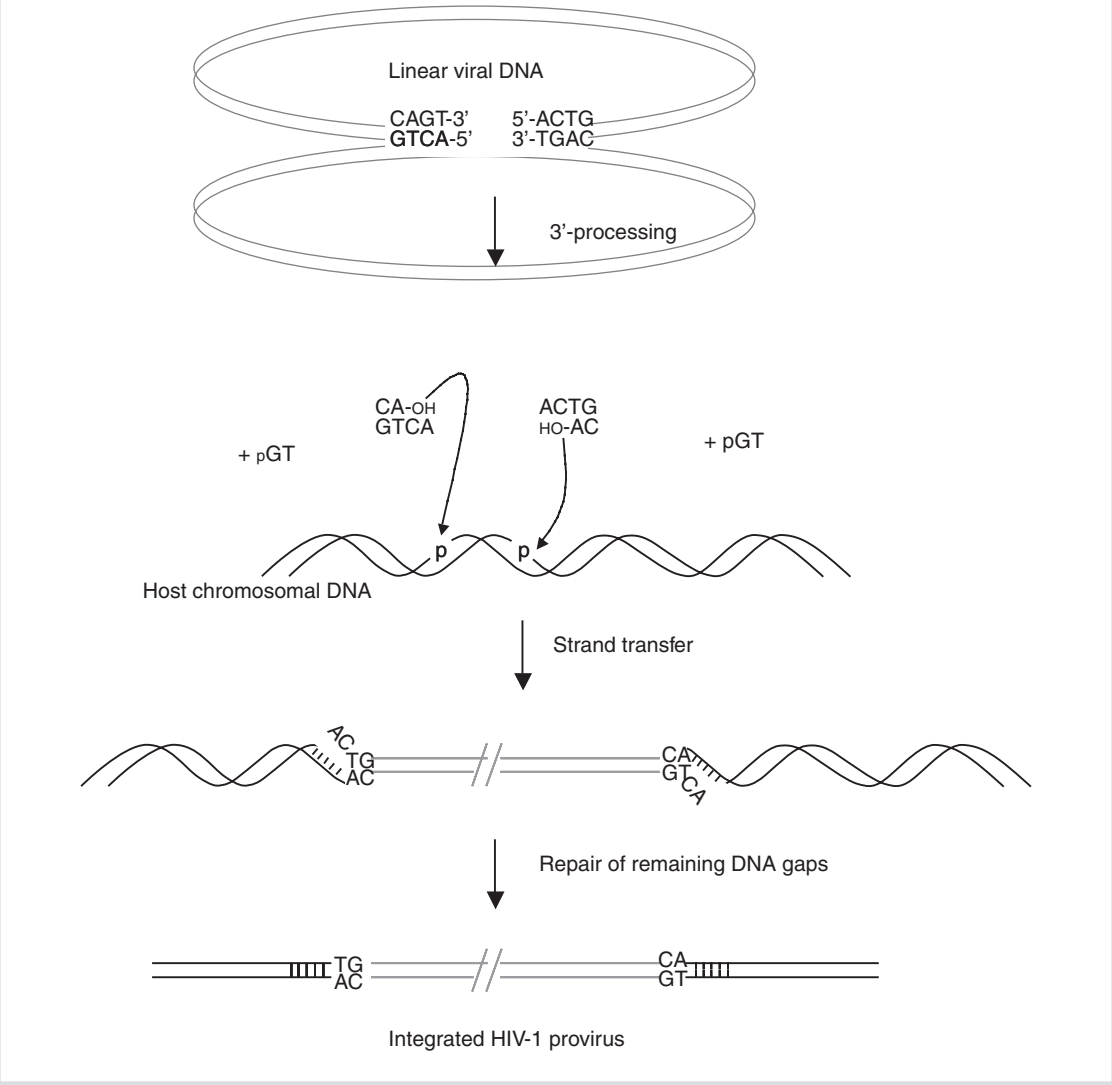

Figure 1. *Outline of the integration reaction* in vivo. *The integration reaction is concerted; both viral DNA ends are inserted into the host chromosomal DNA at the same time. In the case of HIV-1, the distance between the integration sites of both ends is always 5 bp. Repair of the remaining gaps in the chromosomal DNA results in a 5 bp duplication of the host cell genome. The viral ends are probably bridged by cellular host-factors, like BAF or HMGI(Y).*

the single stranded gaps created between the viral and target DNA. This repair is probably accomplished by host-cell DNA repair enzymes[5].

Although purified recombinant IN is necessary and sufficient to perform the basic catalytic activities, 3' processing and strand transfer, in the test tube, a variety of cellular proteins have been implicated as important partners in establishing the integrated provirus in the infected cell. Also, other HIV proteins seem to interact with the IN and/or the integration process. Since retroviral integration is a multistep process, the different cofactors can theoretically play a role during one of the following steps: 1. catalysis; 2. nuclear import of the PIC; 3. target site selection; 4. repair of the DNA gaps. Since several studies have indicated that certain mutations of IN result in pleiotropic effects affecting stages other than integration during HIV-1 replication[6-8], cofactors may also play a role in these effects. In this review we will give an overview of the possible interacting partners of HIV-1 integrase (Table 1 and Fig. 2) reported until now, and their proposed role during HIV-1 replication.

Table 1. Overview of the characteristics of some *in vitro* identified potential cellular cofactors of HIV-1 integration

	BAF	HMGA1	DNA-PK	INI1	HSP60	LEDGF/p75
Cellular function	Nuclear assembly/structure, transcriptional regulation?	Transcriptional regulation and chromatin structure	NHEJ of dsDNA breaks, V(D)J recombination	Component of chromatin remodeling SWI/SNF complex	Chaperone protein facilitating folding of newly synthesized proteins	Survival factor, protecting cells against stress-in -duced apoptosis
In vitro effect	Restoring salt-stripped PIC activity	Restoring salt-stripped PIC activity stimulating concerted integration	Not done	Activation of DNA joining of recombinant IN	Stimulation of 3' processing and strand transfer	Pull-down with HIV-1 IN, stimulation of HIV-1 IN strand transfer activity, stimulation of IN-binding to DNA
In vivo effect	Not done	In HMGA1 knock-out chicken cells no effect on retroviral integration was seen	Protecting cells from apoptosis at high MOI infection	Increase in virus production by interacting with IN; cellular redistribution upon infection and colocalization with HIV-1 PIC	ND	Co-immunoprecipitation with HIV-1 IN, nuclear localization and chromosomal tethering of lentiviral IN
IN binding domain	No	No	No	Repeat region 1 (S6 mutant aa183-294)	Binding to IN, but domain ND	C-terminal domain aa 347-429
Presence in HIV-1 virions/PICs	Very low levels/yes	Not done/yes	Not done/Ku is associated with PIC	Yes, with high specificity for HIV-1/ND	Yes/ND	ND/yes
Hypothetical function in viral replication	Compacting viral DNA, promoting formation of intact intasome structure preventing intramolecular and stimulating intermolecular integration	Stabilizing active conformation of IN by modulating DNA structure; role in HIV-1 expression by indirectly recruiting hSWI/SNF complex via ATF-3	Role in circularization, thereby removing pro-apoptotic signal of free dsDNA ends	More evidence for post-integration role; maybe role in nuclear import of PIC and target-site selection	Maybe conversion of viral proteins to active state	Chromosomal tethering and maybe targeting HIV-1 PICs to actively transcribed regions – protecting IN against proteasomal degradation

In vitro versus in vivo

The discovery of new candidate cellular cofactors of integration was originally based on the *in vitro* reconstitution of enzymatic activity of salt-stripped PICs. Purified PICs from infected cells manifest two properties that are not reproduced in assays using recombinant IN and synthetic DNA substrates: a) PICs efficiently insert both viral DNA ends into a target DNA in a concerted way[2,9], whereas reactions with recombinant IN mainly result in the insertion of a single viral DNA end in a single strand of the duplex target DNA[10,11]; b) PICs preferentially integrate the viral DNA intermolecularly into a target DNA, thereby avoiding suicidal intramolecular autointegration[2]. *In vitro* reactions using purified PICs therefore resemble more the *in vivo* situation. High salt treatment of these PICs gives rise to integration-defective complexes, of which the activity can be restored upon addition of host-cell cytoplasmic extracts, suggesting the presence of cellular factors aiding retroviral integration. An alternative method for identifying interacting partners of IN uses the yeast-two-hybrid assay[12]. The potential integration cofactors detected by one of these assays are then analyzed for a possible effect on *in vitro* integration with recombinant IN. Until now, the impact of candidate cofactors on the activity of recombinant IN has often yielded ambiguous results. Moreover, a clear role during *in vivo* integration has not yet been demonstrated for the majority of them. In conclusion, one should keep in mind that *in vitro* results may depend on particular reaction conditions and that the *in vitro* conditions are at best mimicking the *in vivo* situation. Therefore, it is of paramount importance that potential cofactors identified in test tube

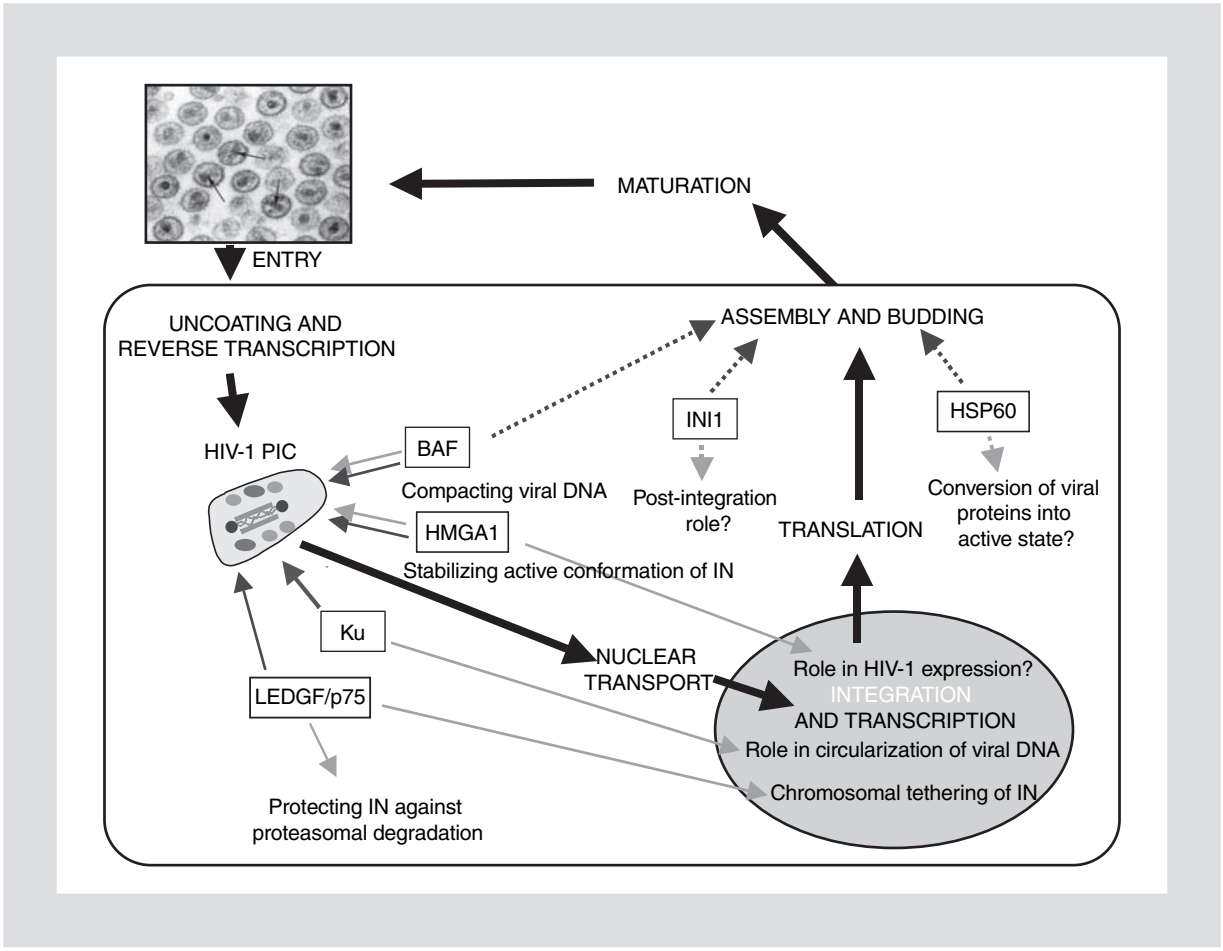

Figure 2. *Potential cellular cofactors of the HIV-1 integration process. After viral entry, uncoating and reverse transcription, the HIV-1 PIC is transported into the nucleus where the viral genome will be integrated. Some cellular proteins that were detected in the PIC are believed to function as cofactors during HIV-1 integration. Potential cellular cofactors are depicted in the figure. Solid dark grey arrows point to their probable localization in the HIV-1 PIC. Dashed dark grey arrows point to their probable presence in the virions. The possible functions of those cellular cofactors during the integration process are given in the picture, and the light grey arrows (solid and dashed) point to the cellular compartment in which the action most probably takes place.*

reactions are carefully evaluated and validated in cell culture experiments.

Host factors: stimulating or restricting HIV-1 replication?

HIV-1 has only a limited genetic make up, although it has to carry out multiple and distinct functions. Consequently, the virus takes advantage of cellular proteins and cellular pathways to complete the different steps in its life cycle. During viral entry, HIV-1 is already dependent on the CD4 cellular receptor and appropriate coreceptor (CXCR4 or CCR5) for internalization in the target cell[13]. During the early phase of HIV-1 infection, including nuclear import and integration, several cellular proteins have been proposed to intervene. Those associated directly or indirectly with HIV-1 IN will be discussed below. Also, Rev, a shuttling protein required for the nuclear export of unspliced and partially spliced viral mRNA, relies upon the nuclear export factor Crm-1 for its proper functioning[14,15], and the transacting protein Tat needs to recruit the cellular protein Cyclin T-1 in order to promote transcriptional activation from the LTR[16]. At the final stage of virus budding, the tumor susceptibility gene 101 (TSG-101) was identified as a cellular cofactor[17]. TSG-101 is a component of the Class E vacuolar protein sorting (Vps) machinery that interacts with a motif in the p6 region of Gag, referred to as the late domain[18].

On the other hand, the cell also tries to defend itself against the invasion of HIV-1. So, besides cellular proteins that aid virus replication, and that will be referred to as cofactors, the cell also harbors proteins counteracting the HIV-1 replication, the so-called “restriction factors”. One of the most studied cellular restriction factors dur-

ing the last two years is the cellular cytidine deaminase (APOBEC3G) identified as a cellular target for the HIV-1 virion infectivity factor (Vif)[19]. Vif protein is required for viral replication in primary CD4+ T-cells and monocytes[20], and it has been demonstrated that in the absence of Vif viral replication is restricted[21]. The proposed mechanism for restriction by APOBEC3G is as follows: in the absence of Vif, APOBEC3G is incorporated into the virions where it subsequently triggers massive deamination of deoxycytidine to deoxyuridine within the retroviral minus (first)-strand cDNA during reverse transcription. This could then provide a trigger for viral destruction by interfering with the plus-strand cDNA synthesis and/or by targeting the viral cDNA for breakdown by the uracil DNA glycosylase-dependent pathway. In the presence of Vif, the amount of APOBEC3G incorporated into the virions is reduced, and experimental results have suggested that Vif somehow promotes degradation of APOBEC3G through an ubiquitination-dependent proteasomal pathway[22]. Except for the block at the level of reverse transcription, additional cellular defense mechanisms appear to target the incoming Gag protein[23]. The species-specific restriction factors, lentivirus susceptibility factor Lv-1 in nonhuman primate cells and restriction factor 1 (Ref-1) in human cells, block for example the infection of specific retroviruses by targeting the incoming viral capsid. Recently, Ref-1 and Lv-1 were shown to be species-specific variants of tripartite motif-5α (TRIM-5α), a factor that confers resistance to HIV-1 infection in rhesus monkeys[24]. TRIM-5α is likely to be an important natural barrier to cross-species retrovirus transmission.

The candidate host proteins involved in the retroviral integration process

Since the integration of HIV-1 cDNA in the host genome, mediated by viral IN, is a critical step in the HIV-1 life cycle, integration has been the focus of many investigations. In the following paragraphs, different cellular proteins will be discussed that are possibly implicated in the retroviral integration step.

The Barrier-to-Autointegration Factor (BAF) prevents suicidal integration in vitro

The absence of intramolecular concerted integration in assays with purified PICs suggested the presence of a cellular factor preventing intramolecular integration. A striking feature of the reactions with PICs, isolated from cells infected with Moloney murine leukemia virus (MoMLV) or HIV-1, is indeed the strong preference to integrate intermolecularly into another target DNA, rather than intramolecularly into their own DNA. Preventing a suicidal autointegration is an important characteristic for a retrovirus, since this would result in the destruction of the viral genome. In 1994, Lee and Craigie provided evidence that the PIC of MoMLV possesses a cellular barrier-to-autointegration factor (BAF) by salt-stripping the purified PICs and restoring the IN activity through addition of cytoplasmic extracts of uninfected cells, but not after adding extracts of MoMLV virions[25]. Later, the involvement of BAF in restoring the salt-inactivated PIC activity of HIV-1 was reported[26]. BAF was originally identified as a single 89-aa polypeptide not matching any other identified protein[27]. Although BAF enhances the intermolecular integration of viral DNA into target DNA in the *in vitro* PIC assay[27], no stimulation of BAF on the activity of recombinant IN by itself was detected[28]. This could be a consequence of a too simplified recombinant IN assay that does not require cellular cofactors. The discovery of BAF was followed by a more detailed analysis of the *in vivo* function of this protein in normal cellular conditions, and by further *in vitro* analysis of the possible mode of action of this protein during integration.

BAF is a highly conserved cellular protein, existing as a dimer in solution[29]. Upon binding to double-stranded unspecific DNA, BAF forms a dodecamer with DNA bound at multiple sites, resulting in a discrete higher-order nucleoprotein complex[30]. Next to binding to double-stranded DNA, several cellular interacting partners have been identified, such as nuclear LEM-domain proteins – the abbreviation "LEM" is derived from the first recognized proteins at the inner nuclear membrane sharing a conserved domain: **L**AP2, **E**merin and **M**AN1[31] – lamin A and transcription activators[32]. The ability of BAF to bridge DNA[30] and the finding that lamina-associated polypeptide (LAP)2β, a nuclear LEM-domain protein associated with the nuclear lamina, interacts with BAF in a two-hybrid screen[33] suggests a possible role in nuclear structure organization. Because RNAi-mediated downregulation of BAF in *C. Elegans* embryos[30] or creation of BAF-null *Drosophila* embryos[34] resulted in lethal phenotypes with aberrant chromosome segregation, mitotic arrest, aberrant nuclear morphology and chromatin clumping, BAF is presumed to play an important role in nuclear assembly. In addition to this structural role, it was recently proposed that BAF could also regulate gene expression in a tissue- or cell-type specific way by interacting with homeodomain transcription factors, as has been shown for CRX (cone-rod homeobox), a homeodomain transcription factor that binds to BAF. The binding of BAF represses the CRX-dependent gene regulation in differentiating retinal cells[35]. Research on the cellular function of BAF is still ongoing, but it is already clear that BAF has various essential functions in the cell. The results of this research will help to clarify the possible role of BAF during retroviral infection and will help evaluate BAF as a potential target for antiviral therapy.

Many studies have been carried out to elucidate the contribution of BAF in the HIV-1 PIC activity. Functional co-immunoprecipitation of endogenous BAF protein with HIV-1 PICs was demonstrated by using antibodies against known viral and cellular PIC components. Additional evidence for the presence of BAF as a cellular component in HIV-1 PICs was obtained by co-immunoprecipitation of IN protein and activity using anti-BAF antibodies[36].

During the analysis of BAF expression in 16 different human tissues, two tissues were identified in which BAF mRNA was undetectable, namely the thymus and peripheral blood leukocytes, both enriched in target cells for HIV-1 infection[37]. This raised the hypothesis of the possible presence of BAF in incoming virions. Indeed, besides being a component of HIV-1 PICs, it was recently proposed that BAF is also present in incoming HIV-1 virions, although at very low levels of approximately zero to three copies per virion[37]. This observation is somehow at odds with previous findings where extracts of MoMLV virions could not reconstitute the salt-stripped PIC activity *in vitro*[27]. Most probably the virion-associated BAF is less crucial than the recruited cytoplasmic BAF in the infected cell. The same group also reported a direct binding of BAF to both p55 Gag and its cleaved product, matrix (MA)[37]. The necessity of MA-BAF interaction for PIC activity is not yet clear, but it cannot be essential under all infectious conditions, since HIV-1 can replicate under certain conditions in the absence of MA[38]. So, BAF might play a role in the assembly and/or activity of HIV-1 PICs through direct binding to MA, as well as to DNA. Nevertheless, it is unlikely that BAF is essential for HIV-1 virion assembly because it is only a minor component that might be even absent from a subset of virions. Why then is BAF incorporated in HIV-1 virions? Two speculations have been made: (1) BAF could be accidentally incorporated due to its affinity for the MA domain of Gag, or (2) it could be incorporated on purpose to promote PIC survival in resting CD4+ T-lymphocytes which lack BAF. Hitherto, most evidence shows that BAF is predominantly recruited from the cytoplasm of infected cells and that it stimulates authentic intermolecular integration, probably by compacting the viral cDNA[27] and promoting the formation of an intact protein-DNA intasome structure, required for correct integration, at the ends of the HIV-1 LTRs[26,39].

More research is required to understand the contribution of BAF during retroviral replication *in vivo* and to answer the following questions: Is BAF essential for retroviral infection? If so, is BAF only acting on the integration step, or is it also involved in other stages? Can BAF be used as an antiviral target? More data from cellular experiments are needed to get a clear view of its normal cellular functions and the possible role of BAF during retroviral infection *in vivo*.

More recently though an interesting finding was reported concerning the association of lamina-associated protein (LAP)-2α, a probable interacting partner of BAF, with the PIC of MoMLV[40]. Whereas LAP-2α only weakly stimulated the intermolecular integration of salt-stripped PICs, it clearly increased the salt resistance of the BAF–DNA complex, implying that LAP-2α may contribute to the efficient acquisition of BAF by the PIC. A more amazing observation was the significant block of the viral replication when LAP-2α was knocked down[40], suggesting a direct or indirect role of LAP-2α, with or without the implication of BAF, during the retroviral replication of MoMLV.

Investigation of the role of a possible LAP-2α-BAF-viral DNA-complex during retroviral replication of MoMLV as well as HIV-1 may be very helpful to analyze the possible *in vivo* contribution of BAF during retroviral replication. Since knocking down BAF expression results in cytotoxicity[30,34], the alternative approach by down-regulating the expression of LAP-2α, as well as other BAF-interacting proteins, followed by analyzing the effect on retroviral replication as well as co-localization studies, can give useful information regarding the possible function of BAF during retroviral infection. Although BAF is directly involved in stimulating the intermolecular integration in the *in vitro* PIC assay, it is most probable that the *in vivo* role, if any, will be indirect.

The High Mobility Group (HMG) chromosomal protein A1 (HMGA1) may affect transcription more than integration

Another host protein has been detected in the same way as BAF, namely by restoring the *in vitro* PIC activity after salt-stripping and addition of an uninfected cell extract. Fractionation of this complementing activity by DNA cellulose yielded HMGA1 (formerly, HMG I[Y]), a nonhistone DNA-binding protein that can modulate transcriptional regulation and chromatin structure[41]. The members of the HMGA family of "high mobility group" proteins possess DNA as well as protein-binding capacities and participate in a wide variety of nuclear processes such as transcription regulation. Importantly, both the transcription of HMGA genes and the biochemical modifications of the HMGA proteins are direct downstream targets of numerous signal transduction pathways, making them exquisitely responsive to various environmental influences[42].

Recombinant HMGA1 was sufficient to restore the integration activity of salt-stripped PICs[41] as well as to generate concerted integration products in an *in vitro* system, employing a small linear blunt-ended donor DNA substrate containing 20 base-pairs representing the LTRs of HIV-1 DNA flanking the *supF* gene, a su-

percoiled plasmid acceptor DNA and recombinant IN[43]. In comparison to BAF, the stimulation of PIC activity by HMGA1 was 500-fold lower[26,41]. Therefore, neither the importance of this protein during integration nor its precise mechanism are known. The knowledge of some common functional features of these HMG proteins may help to understand the role of this potential cofactor of integration. These features include: a) binding to the minor groove of AT-rich double-stranded DNA through DNA binding domains known as A-T hooks; b) recognizing DNA structure rather than sequence; c) preferentially interacting with bent, supercoiled or distorted DNA structures; d) binding to non-B-form DNA structures; e) unwinding, bending and supercoiling of DNA substrates, in the absence of ATP hydrolysis; and f) selectively interacting with other sequence-specific transcription factors as part of gene-transcription regulatory complexes. In mechanistic studies it was shown that HMGA1 proteins must contain multiple DNA-binding domains to stimulate the IN-catalyzed integration *in vitro*, to form a ternary complex with IN[43], to restore HMGA1-depleted PIC activity, and to increase intermolecular ligation of LTR ends *in vitro*[44]. Next, DNA ligase-mediated ring closure assays have demonstrated that HMGA1 is capable of bending short, rigid pieces of DNA into closed circles[43], suggesting that HMGA1 might bring the ends of the donor DNA into close proximity by bending the donor DNA and thereby facilitating concerted integration. In other studies it was demonstrated that HMGA1 protein is capable of unwinding DNA substrates *in vitro*[45,46]. Additionally, Scottoline, et al.[47] showed that the 3'-processing activity, mediated by HIV-1 IN, requires base-pair disruption at the terminus of the viral DNA. Taken together, these findings may point to the involvement of the DNA-binding activity of HMGA1, bringing the LTR ends into close proximity, and facilitating the binding of IN proteins to the viral ends by unwinding the LTR termini. By doing so, HMGA1 could stabilize IN in an active conformation. A similar mechanism has been put forward for the architectural DNA-binding protein, integration host factor (IHF), in phage lambda integration. Since HMGA1 proteins are also able to interact with other proteins, coprecipitation experiments were performed to demonstrate a possible interaction between HMGA1 and HIV-1 IN, but no stable interaction could be detected[43].

In addition to these *in vitro* data, one might wonder if there is also evidence for a role of HMGA1 as a likely cofactor for integration *in vivo*. So far the most compelling evidences are: a) the association of HMGA1 with MoMLV PICs from infected cells[44]; b) the co-fractionation of HMGA1 with HIV-1 PICs[41]; and c) the depletion of reconstitution activity from HIV-1 PICs by anti-HMGA1 antibody[41]. On the other hand, a more recent report surprisingly showed that chicken cells lacking HMGA1 were not deficient in cell growth or in retroviral integration, suggesting that HMGA1 protein is most probably not required for integration[48]. A more detailed analysis during retroviral replication, for example by partially knocking down HMGA1 in human cells, should answer the question of whether or not HMGA1 plays a role during the retroviral life cycle.

Given the abilities of HMGA1 to recognize and alter the structure of DNA regions, and to interact specifically with a large number of proteins, mostly transcription factors[42], Henderson, et al.[49] raised the possibility that HMGA1 may play an important role during HIV-1 transcription, besides the described role in the *in vitro* integration activity. They analyzed the interaction of HMGA1 with the HIV-1 5' LTR and were able to localize multiple high-affinity HMGA1 binding sites using DNase-I footprinting. Most of these sites overlap with important transcription factor binding sites, among which is an important site for activator protein-1 (AP-1) binding located downstream of the transcriptional start site at the boundary of nuc-1[50]. This AP1-3 site appears to be important for transcriptional activation in response to a broad range of external stimuli[51,52]. Whereas the presence of HMGA1 can inhibit the binding of one transcription factor to the AP1-3 site, such as Fos-Jun, it can also enhance the binding of another factor, such as the inducible AP-1 factor, ATF-3[49]. These results argue for a fundamental role of HMGA1 in HIV-1 expression by determining the nature of transcription factor-promoter interactions, possibly by chromatin remodeling. Recently, the possible role of HMGA1 during HIV-1 transcription was strengthened by the finding that ATF-3 seemed responsible for targeting the human SWI/SNF (hSWI/SNF) chromatin remodeling complex to the HIV-1 promoter, and that this recruitment of hSWI/SNF required HMGA1 proteins[53], since HMGA1 enhances the binding of ATF-3 to the AP1-3 site.

Although HMGA1 was discovered as a stimulating cofactor for restoring the integration activity of salt-stripped PICs *in vitro*, it now looks like HMGA1 might contribute to a greater extent to HIV-1 transcription rather than to integration *in vivo*.

DNA-dependent protein kinase (DNA-PK) is a component of the NHEJ pathway and protects the infected cell against apoptosis

Since the DNA strand transfer step of retroviral integration results in a gapped intermediate containing unpaired 5' ends adjacent to five-base gaps, completion of integration requires repair of these gaps and joining of the 5' viral ends to the host DNA. Although one unconfirmed report suggested that IN would pro-

vide DNA polymerase activity to fill in the gaps[54], it is generally accepted that cellular DNA repair enzymes do the job. In mammalian cells, the nonhomologous end-joining (NHEJ) pathway represents the major mechanism for repairing double-stranded DNA (dsDNA) breaks[55]. This NHEJ is mediated by DNA-dependent protein kinase (DNA-PK), which is activated by dsDNA ends[56] and is composed of a 450-kDa catalytic subunit, DNA-PK_{CS}, and the dsDNA-binding Ku70/86 heterodimer. DNA-PK is also involved in V(D)J recombination, and mice bearing a truncated mutation of DNA-PK_{CS} suffer a severe combined immunodeficiency (SCID) due to defects in double-strand break repair and V(D)J rearrangement, leading to a lack of mature B and T lymphocytes[57,58]. These features of the NHEJ pathway and, in particular, DNA-PK made it conceivable to hypothesize a role in DNA gap repair during the late steps of retroviral integration.

The first experiments using retroviral vectors and NHEJ-deficient cells led to some controversial results. In one study, Daniel, et al.[59] showed a substantial reduction in retroviral DNA integration, measured indirectly by reporter-gene activity and after selecting stably transduced clones, in DNA-PK-deficient murine SCID cells after retrovirus transduction at a multiplicity of infection (MOI) of 0.2 to 1. Moreover, it was demonstrated that retroviral transduction of DNA-PK-deficient cells, as well as cells deficient in other components of the DNA-PK pathway (Ku and XRCC4), induced a high degree of apoptosis compared to wild-type cells, but only when integration-competent vectors were used. Similar results were obtained for two other components of the NHEJ pathway, namely XRCC4 and DNA ligase IV[60]. Based on these observations, the authors proposed that the retroviral integration intermediate might be detected as DNA damage by the host cell, and that the completion of the integration would need the NHEJ-mediated repair pathway to avoid the proapoptotic signal mediated by the DNA gaps.

Almost one year later, our group questioned the requirement of DNA-PK for lentivirus integration, although we also observed increased apoptosis after lentiviral transduction in DNA-PK_{CS} and Ku-deficient cells, but only at high MOI (> 1 transducing unit (TU) per cell)[61]. When transducing with low vector titers (< 1 TU/cell), transduction was even more efficient in the NHEJ-deficient cells as compared to NHEJ-competent cells, and no evidence of apoptosis was detected. Strikingly, transduction of SCID mouse brain with lentiviral vectors was as efficient as in control mice. Taking into account these different observations, DNA-PK is probably not required for gap repair during retroviral integration, although it could play a protective role during high titer infections, preventing apoptosis of the host cell.

This controversy and the notion that NHEJ pathway proteins normally only repair double-strand breaks, but not single-stranded gaps[62] as present in the integration intermediate, gave rise to a new proposal for the function of the NHEJ pathway during the early phase of retroviral infection. The dsDNA ends produced during reverse transcription may serve as a proapoptotic signal during retroviral infection. Therefore, it was investigated whether the NHEJ pathway could be implicated in the removal of this proapoptotic signal by DNA circularization. Indeed, when cells lacking the NHEJ pathway components (Ku80, XRCC4 or ligase IV) were transduced with HIV-based vectors, no 2-LTR circles could be detected[63]. A similar decrease in 2-LTR circle formation was reported after HIV-1 infection in Ku-80-depleted human cells[64]. DNA-PK_{CS}, on the contrary, seemed dispensable for the DNA end joining that forms 2-LTR circles[65]. The 2-LTR circular form of the viral cDNA is produced by ligation of the LTR sequences at the viral cDNA ends, and is believed to represent an unproductive pathway leading to loss of the viral genome[66,67]. The very systematic study by the Bushman group[63] resulted in a set of data that support a protective role of the NHEJ pathway during the early steps of retroviral infection. It was found that: a) Ku associates with the HIV-1 PICs without being necessary for the integration activity; b) the NHEJ system is responsible for the formation of 2-LTR circles, thereby preventing the induction of apoptosis; c) infection of NHEJ-deficient cells leads to apoptosis in a MOI-dependent way, paralleling both previous studies[59,61]; and d) the unintegrated linear cDNA provides the proapoptotic signal, since a block in integration, but not a block in reverse transcription, induced apoptosis in the infected NHEJ-deficient cells.

Ku by itself has been proposed to negatively regulate HIV-1 transcription in experiments that employed either integrated HIV-1 provirus or circular plasmid DNA, devoid of free dsDNA ends[68]. Although this cannot be excluded, the exact contribution still awaits more convincing evidence and independent confirmation.

In conclusion, these observations highlight a protective role for the NHEJ pathway – comprising Ku, DNA-PK, XRCC4 and DNA ligase IV[69] – by directly or indirectly suppressing apoptosis in infected cells by removing the proapoptotic signal of unintegrated viral cDNA. Whether Ku is also involved in the integration process, as originally postulated, cannot be ruled out completely, but awaits independent confirmation.

Other cellular proteins play a possible role in DNA repair during HIV-1 integration

For the following proteins that are candidates for a role in DNA repair during the late stage of integration, only weak evidence is available.

1. Poly(ADP-ribose) polymerase-1 (PARP-1) is a predominantly nuclear enzyme and is activated by either single- or double-stranded DNA breaks to attach ADP-ribose groups to nuclear proteins, including itself[70]. It has been implicated in cellular processes that require DNA cleavage and rejoining reactions, such as DNA replication, recombination and repair. In one report, HIV-1 infection of cells derived from PARP-1$^{-/-}$ mice was aborted at the viral integration step[71]. These findings are at odds with an earlier report of our group showing a lack of inhibition of lentivirus vector-mediated transduction and HIV-1 replication by 3-methoxybenzamide, a known PARP inhibitor[61]. Recently, some experiments pointed toward a putative role of PARP-1 during HIV-1 transcription, through the regulation of the expression of histone acetyltransferase (HAT)[72,73].

2. ATR (ATM and Rad3-related) kinase belongs to a family of large phosphatidylinositol-3-kinase-related protein kinases, to which also DNA-PK belongs[74]. It is also implicated in the cellular response to DNA damage and is more specifically involved in the regulation of cell-cycle checkpoints[75]. A reduction in the number of cells, stably transduced by HIV-1- or ASV-based retroviral vectors, was observed when those cells were treated with caffeine, an inhibitor of cellular DNA repair, through its presumed effects on ATR kinase[76]. However, a lack of specificity for the effect of caffeine on ATR casts doubts on a potential role of ATR during retroviral integration. In fact, another group demonstrated the activation of the ATR-mediated DNA damage response by Vpr, finally resulting in G_2 cell-cycle arrest[77].

3. RAD52 and RAD18 are both DNA repair proteins. RAD52 is involved in the homologous recombination (HR) pathway[78], whereas RAD18 participates in DNA post-replication/translesion repair[79]. In cells, RAD18 forms a stable heterodimer with RAD6, an E2 ubiquitin-conjugating enzyme, after binding to single-stranded DNA breaks[80,81]. RAD18 was shown to associate with HIV-1 IN and to co-localize in a subset of co-transfected cells in the same sub-nuclear structures[82]. A possible hypothesis, based on studies of bacteriophage Mu transposition, proposes that the formed RAD6-RAD18 heterodimer participates in DNA repair by destabilizing PIC proteins, thereby facilitating access for the cellular DNA repair proteins to the gapped DNA[82]. Although it cannot be excluded that RAD18 is involved in the integration process by interacting with, and thereby maybe regulating, the stability of IN, more functional studies are needed to provide more relevant proof.

On the other hand, cellular overexpression of RAD52 seemed to suppress retroviral infection accompanied by a decrease in 2-LTR circle formation, but without affecting the cell viability[83]. Given the proposed role for Ku and the NHEJ pathway in the formation of 2-LTR circles during HIV-1 replication[63,64], Lau, et al.[83] reported that RAD52 competed with and effectively displaced Ku from the ends of HIV-1 cDNA, suggesting that RAD52 could interfere with Ku-directed NHEJ repair activity. A possible model has been proposed according to which RAD52 may prevent the association of other proteins that bind to the retroviral cDNA, such as Ku[83], and that are needed to form the active PIC. Since the suppressive effect of RAD52 needs overexpression of the protein in cells, it is not really certain that this cellular protein plays a relevant role during retroviral infection at physiologic concentration, unless the expression level of RAD52 increases during HIV-1 infection.

As a final conclusion, one could postulate that different DNA repair proteins may be involved in a redundant manner and in a cell-specific way during the early stage that comprises the circularization of viral cDNA and/or the final gap repair following strand transfer.

Integrase interactor 1 (INI1) was the first integrase-binding partner identified

Using the *in vitro* yeast-two-hybrid system, a human gene product, called INI1, was identified that tightly interacted with HIV-1 IN *in vitro* and activated its DNA-joining activity[12]. The sequence of this gene suggested that it was the human homolog of yeast SNF5, a transcriptional activator and component of the chromatin remodeling SWI/SNF complex[84]. This multiprotein complex activates transcription by remodeling chromatin, using the energy of ATP hydrolysis to facilitate access of transcription factors to regulatory DNA sequences[85]. A similar complex has been isolated from mammalian cells and INI1 was shown to be part of this mammalian SWI/SNF complex[86]. Since INI1 directly binds to HIV-1 IN and is involved in chromatin remodeling, the question arose whether INI1 played a role during HIV-1 infection. If so, at what stage of the replication cycle does it intervene? Is the interaction with HIV-1 IN specific? Different studies provided some answers.

INI1 is a 385-amino-acid protein with three highly conserved regions, including two direct imperfect repeats, repeat 1 (Rpt1) and repeat 2 (Rpt2), a C-terminal coiled-coil domain and a homology region 3 (HR3)[87]. Deletion analysis of INI1 distinguished the Rpt1 region of INI1 as necessary and sufficient to interact with HIV-1 IN. The same analysis also detected a nonspecific DNA binding activity upstream of Rpt1, not required for IN binding[87]. Besides binding to integrase, INI1 seems to interact with a variety of cellular and viral proteins[88-91], suggesting the presence of domains important for protein-protein interaction. Furthermore, a masked nuclear export sequence (NES) was identified at the beginning

of Rpt2[92]. Possibly, this NES might be unmasked during HIV-1 infection by a still-undefined mechanism.

The minimal IN-interaction domain of INI1 (aa183-294), named S6, acted as a specific trans-dominant inhibitor of the late steps of HIV-1 replication upon cellular overexpression[93]. The presence of the S6 fragment in producer cells consistently reduced the virus release by a factor of 10,000 to 100,000, without affecting the intracellular p24 levels. On the other hand, mutations in the S6 fragment that disrupt the IN interaction, abrogated the inhibitory effect on virus production. Furthermore, Yung, et al.[93] demonstrated that for S6 inhibition, IN had to be part of Gag-Pol, since trans-complementation of IN during particle production abolished the inhibitory effect of S6. Importantly, the cellular localization of S6 differed from that of INI1. Instead of being nuclear, the S6 protein was cytoplasmic. This ectopic expression could at least in part explain the effect on the post-integrative steps that also occur in the cytoplasm. The experiments with the trans-dominant mutant of INI1 were helpful to understand the mechanism underlying the trans-dominant inhibition, but did not clarify the role of full-length INI1 during retroviral replication. The addition of full-length INI1 in the MON cell line, genetically null for INI1, resulted in a clear increase in the production of infectious virus particles. In addition, INI1 has been found as a host protein in the HIV-1 virions. Although INI1 is rather abundant in most cell lines, the interaction of INI1 with IN, the negative effect of S6 on virus release, and the presence of INI1 in the virions, are all very specific for HIV-1[94].

The potential contribution of INI1 during HIV-1 infection has been investigated in combination with another nuclear protein, PML[95]. The promyelocytic leukemia (PML) protein is one of the main components of a nuclear substructure known as POD (promyelocytic oncogenic domain). Both POD and PML have been associated with acute promyelocytic leukemia[96,97]. The precise cellular function of PODs is unknown, but it has been suggested that they could be implicated in transcriptional regulation and could somehow be associated with a number of viruses. Early during HIV-1 infection, a dramatic subcellular exportin-mediated redistribution of INI1 and PML was observed[95]. The significant cytoplasmic re-localization of nuclear INI1 was detected as soon as 30 minutes after HIV-1 infection; four hours post-infection, INI1 was again mainly localized in the nucleus, together with PML. This cytoplasmic recruitment required viral entry, but was independent from the HIV-1 envelope, and was observed for VSV-G-pseudotyped HIV- as well as MLV-derived vectors. Thus, this cellular response on incoming viral particles did not seem to be HIV-1 specific, in contrast to the HIV-1 specificity of INI1 described by Yung, et al.[94]. By visualization of the incoming viral particles, co-localization of the HIV-1 PICs with INI1 was shown suggesting an association with this nuclear protein on the way to the nucleus. Whereas INI1 may have a positive influence on HIV-1 replication, PML was proposed to act merely as an antiviral response, since inhibiting PML by arsenic trioxide efficiently increased the cell's susceptibility to HIV-1 infection, without disturbing the cytoplasmic re-localization of INI1[95]. However, these findings were questioned later by the observation that the stimulatory effect of arsenic trioxide was not dependent on the presence of PML[98].

Taking all these observations into account, it is at present unclear whether INI1 is really required for HIV-1 replication. And although INI1 originally was found to stimulate IN activity *in vitro*, no strong evidence has been provided for a possible role during the HIV-1 integration process *in vivo*. Evidence for a possible role of INI1 during the post-integration steps of HIV-1 replication is stronger. INI1 probably interacts with IN within the context of Gag-Pol. Interaction with the S6 fragment results in a trans-dominant inhibition of HIV-1 replication. Given the observed cellular re-localization of INI1 during HIV-1 infection, a role for the virus-associated and/or the cellular INI1 during the early steps of HIV-1 infection, such as PIC nuclear import and/or integration process, remains possible as well[95]. Although INI1, as a component of the SWI/SNF complex, has been hypothesized to play a role in integration site selection or HIV-1 transcription, no experimental evidence has been produced to support this idea.

Human and yeast heat shock protein 60 (HSP60)

HSP60 is an ubiquitous chaperone playing an essential role in cells by binding newly synthesized proteins and facilitating their folding[99]. Using IN-affinity chromatography of yeast cell extracts, HSP60 was identified to interact with HIV-1 IN[100]. Recombinant human HSP60 (hHSP60) and HIV-1 IN were shown to interact *in vitro*; the catalytic core of IN was characterized as determinant for the interaction. Moreover, addition of hHSP60 stimulated the *in vitro* 3' processing and strand-transfer activities of IN, and protected IN from thermal denaturation[100]. Since hHSP60 has been shown to co-purify with HIV-1[101] and to associate with several viruses, such as Hepatitis B virus (HBV)[102], the association between HIV-1 IN and hHSP60 might be biologically relevant during infection. More convincing *in vivo* data are needed to prove the model according to which the chaperone hHSP60 would interact with viral proteins to promote their conversion into an active state.

Human Polycomb group embryonic ectoderm development (EED) protein

Human EED belongs to the family of the widely conserved *Polycomb* group of genes and is involved in

chromatin remodeling, more particularly in the maintenance of the silent state of chromatin[103]. A previous study identified an interaction between the viral MA protein and human EED in a yeast-two-hybrid assay[104]. Since MA is also a component of the HIV-1 PIC[3], Violot, et al.[105] explored the possibility of a protein-protein interaction between EED and IN and the existence of a ternary complex of EED, MA and IN. IN and EED were shown to interact *in vitro* as well as in yeast, but such analysis has not been carried out in human cells so far. In HIV-1 infected cells, though, co-localization of EED, IN and MA was observed at early time points after infection by means of immunoelectron microscopy. An apparent positive effect of EED on the *in vitro* integration reaction was also reported[105]. More recently, the interaction of EED with the HIV-1 Nef protein was also reported[106]. The multiple interactions observed with EED question the specificity and biological relevance of the observations.

Lens Epithelium-Derived Growth Factor (LEDGF)/p75 tethers HIV-1 integrase to the chromosomes

Recently, a new binding partner of HIV-1 IN has been identified in our laboratory following a study of the HIV-1 IN complexes present in nuclei of human cells that stably overexpress the viral IN from a synthetic gene[107]. Cherepanov, et al. demonstrated that the complexes isolated were associated with a cellular protein with an apparent molecular mass of 76 kDa. This novel IN interactor proved to be identical to lens epithelium derived growth factor (LEDGF/p75). So far, LEDGF/p75 is the first HIV-1 IN-interacting protein reported to form a distinct complex with IN in human cells.

Prior to our description of the link with HIV integrase, LEDGF/p75 had been independently identified by at least three groups. The p75 protein was first identified as a 75 kDa protein that co-purified with the transcriptional co-activator PC4, suggesting a role in transcriptional regulation[108]. Independently, a cDNA clone coding for a protein identical to p75 has been isolated from a lens epithelial cell (LEC) library with antibody from a cataract patient[109]. This protein was then named "lens epithelium derived growth factor" and it was proposed that addition of the protein to the culture medium of LECs, cos7 cells, skin fibroblasts, and keratinocytes stimulated their growth and prolonged cell survival. Based on sequence similarity, LEDGF/p75 is a member of the hepatoma-derived growth factor (HDGF) family[110]. Although GFP-LEDGF was found to be secreted in culture medium and internalized into cells, these observations could not be reproduced by other groups[111]. Furthermore, later studies characterized LEDGF/p75 as a survival factor[112]. LEDGF/p75 is also emerging as a common nuclear autoantigen in a variety of inflammatory conditions, including atopic dermatitis, asthma and interstitial cystitis[113]. Originally the nuclear autoantigen was designated dense fine speckles 70 antigen (DFS70), based on the staining of dense fine speckles in the nucleus by the autoantibodies and the detection of a ~70 kDa protein by these antibodies[114]. Analysis of protein sequence databases revealed that DFS70, the transcriptional co-activator p75 and LEDGF/p75 are identical to each other. The cellular protein is now generally referred to as LEDGF/p75, although the protein is probably not a growth factor and neither specific to lens epithelium.

LEDGF/p75 contains 530 amino acids and several functional domains: (1) in the N-terminal part of LEDGF/p75 a PWWP domain of approximately 80 residues is present that functions as a protein-protein interaction domain[115] and/or DNA-binding domain[116]; (2) a functional nuclear localization signal (NLS), GRKRKAEKQ (amino acids: 148-156), was recently reported[117]; (3) in accord with its ability to interact with HIV-1 IN, an evolutionary conserved integrase-binding domain (IBD) of approximately 80 amino acids (amino acids 347-429) was recently mapped to the C-terminus[118]; and (4) computer predictions indicated four potential DNA-binding domains of which one helix-turn-helix (HTH) and a basic leucine zipper are located at the N-terminus and two HTHs at the C-terminus[119]. LEDGF/p75 is predominantly localized in the nucleus where it is intimately associated with the chromosomes[120]. The *LEDGF* gene also encodes a smaller splice variant, p52, which shares a region of 325 residues with LEDGF/p75 at the N-terminus, but contains eight additional amino acids[108]. In contrast to LEDGF/p75, p52 has a stronger and more general transcriptional co-activator activity[108] and, interestingly, fails to interact with HIV-1 IN *in vitro* as well as in living cells[121].

As already mentioned, LEDGF/p75 acts as a survival factor in the cell; it is involved in promoting mammalian cell growth and protecting the cell against stress-induced cell death. The pro-survival role of LEDGF/p75 is exerted via transcriptional activation of stress-related/anti-apoptotic proteins, such as HSP27, αB-crystallin, HSP90, and antioxidant protein 2 (AOP2)[119]. It has been demonstrated that the cultivation of LECs under heat or oxidative stress resulted in elevated levels of LEDGF mRNA and protein, associated with a higher level of stress-related proteins[112]. In respect to these results, LEDGF/p75 has been shown to be a DNA-binding protein with affinity for heat shock and stress-related DNA elements[122]. LEDGF/p75 is a transcriptional co-activator *in vitro* and interacts with PC4, the VP16 activation domain and general transcription factors[108]. Additionally, LEDGF/p75 is also implicated in some autoimmune disorders; in prostate cancer, the protein acts as a nuclear autoantigen generating an autoan-

tibody response[123,124]. It was demonstrated that LEDGF/p75 could be cleaved by caspases-3 and -7, the main effector caspases in apoptosis, resulting in two cleavage fragments of 65 and 58 kDa. Cellular overexpression of these cleaved LEDGF/p75 fragments clearly abrogated the pro-survival role of LEDGF/p75 [125]. Therefore, LEDGF/p75 probably plays a key role in the balance between cell survival and cell death. Precise mechanisms still need more experimental data.

Given the notion that LEDGF/p75 acts as a survival factor and a transcriptional co-activator in the cell, our original observation[107] that the protein stably interacts with one of the key players of retroviral integration, the IN, stirred the field considerably. These results were the impetus to embark on a journey to investigate the possible role of LEDGF/p75 during HIV-1 replication.

In our laboratory, Cherepanov identified LEDGF/p75 as a cellular partner in HIV-1 IN complexes isolated from the nuclei of 293 T-cells stably expressing the viral IN[107]. The precise stoichiometry of the IN-LEDGF complex has not been elucidated, but the simplest model, based on an estimated molecular mass of 400 kDa for the complex isolated, suggests a symmetrical complex containing a pair of IN tetramers and two subunits of LEDGF. HIV-1 IN is known to localize predominantly in the nucleus upon overexpression[126]. More remarkably, the distribution of nuclear IN perfectly matches with that of LEDGF/p75 [107,121]. The IN-LEDGF/p75 direct interaction was confirmed by an *in vitro* pull-down assay using the recombinant proteins. Moreover, the addition of recombinant LEDGF/p75 to an *in vitro* mini-HIV-based IN assay clearly enhanced the strand transfer activity of the recombinant HIV-1 IN[107], suggesting a contribution of LEDGF/p75 during the HIV-1 integration process. Noteworthy, LEDGF/p75 was previously reported as upregulated in HIV-infected cells[127].

To investigate in more detail the precise role of the interaction between LEDGF/p75 and HIV-1 IN, the recently developed RNA interference technology was employed to study the effect of partial depletion of endogenous LEDGF/p75 on the cellular localization of HIV-1 IN and on the outcome of HIV-1 replication. Knocking-down endogenous LEDGF/p75 completely abolished the nuclear localization of HIV-1 IN as well as its association with chromosomes in cells transiently transfected with the IN fused to EGFP[121]. Co-localization studies of different HIV-1 EGFP-IN deletion mutants in the absence or presence of over-expressed LEDGF/p75 revealed that both the N-terminal zinc-binding domain and the core domain of HIV-1 IN are involved in the interaction with LEDGF/p75. In fact, the core domain proved to be the main determinant for the interaction, since over-expression of LEDGF/p75 was able to restore nuclear/chromosomal localization of the IN core domain but not of the N-terminus. In accord, Llano, et al.[128] reported the requirement of LEDGF/p75 for the nuclear localization of IN derived from HIV-1 and feline immunodeficiency virus (FIV). Not surprisingly, the knock-down of endogenous LEDGF/p75 by small interfering RNA (siRNA) did not affect the cytoplasmic distribution of the non-karyophilic integrase of MoMLV.

Whether the IN/LEDGF interaction is specific for lentiviruses or a general characteristic for all retroviral INs has been addressed in our laboratory in much detail using *in vitro* assays and recombinant INs from different lentiviruses and retroviruses[129]. The interaction is clearly specific for lentiviruses. In the same manuscript we describe the interesting observation, based on fluorescence correlation spectroscopy (FCS) experiments, that LEDGF/p75 stimulates the binding of HIV-1 IN to DNA 40-fold. This *in vitro* result may reflect the molecular mechanism of LEDGF/p75 *in vivo*, acting as a tethering factor for HIV-1 IN to the chromosomes. This function may also explain the apparent nuclear accumulation of HIV-1 IN and association to mitotic chromosomes, which are both abolished by knock-down of LEDGF/p75.

Subsequent experiments addressed whether LEDGF/p75 is necessary during HIV-1 replication and/or affect nuclear import of the HIV-1 PIC. In single-round infections with HIV-1 and FIV-derived vectors in stable LEDGF/p75-deficient cells, identical kinetics of 2-LTR circle formation were seen as in control cells, questioning a direct effect of LEDGF/p75 on the nuclear import of HIV-1 and FIV vector PICs[128]. Likewise, no difference in reporter gene expression was detected after transduction with these vectors, not even after passaging, ruling out an effect of LEDGF/p75 on the integration of the vector genome. Replication of HIV-1 virus in LEDGF/p75-deficient Jurkat cells was not significantly reduced compared to the same cell line that was back-complemented with a siRNA-resistant LEDGF expression clone as monitored by p24 analysis. These latter results are at odds with findings from our group in collaboration with the group of Benarous[130]. Using yeast-two-hybrid analysis Benarous, et al. identified LEDGF/p75 independently as a binding partner of HIV-1 IN[131]. The interaction mapped to the C-terminal domain of LEDGF/p75. Together, we have shown that transient and stable siRNA-mediated knockdown of LEDGF/p75 reduces HIV replication significantly. HIV-1 replication is rescued upon back-complementation. Intriguingly, and in accord with the Poeschla group, no inhibition of HIV-1 vector transduction is seen pointing to a possible difference in the integration process. In a second approach, mutations in IN that abolish the interaction with p75 were identified using yeast-two-hybrid analysis. Although the Q168A recombinant IN displays normal IN activity *in vitro*, viruses containing INQ168A are defective for replication due to a specific block at the integration step. Nuclear import is not hampered. Both

RNAi- and mutant-based experiments thus point to an important role of LEDGF/p75 in HIV replication.

Does LEDGF/p75 play a role in nuclear import? The dramatic effect of siRNA-based knockdown of LEDGF/p75 on the nuclear localization of HIV-1 IN initially suggested a possible role in nuclear import[121]. In fact, in collaboration with the Engelman group, we later showed LEDGF to contain a classical NLS[117]. Llano, et al.[128] claimed that LEDGF/p75 is present in the PIC, although this observation awaits more convincing data and independent confirmation. However, the Q168A virus we constructed yielded normal levels of 2-LTR circles. Moreover, in direct nuclear import assays we have shown that recombinant HIV-1 IN is still actively imported in the nucleus in the absence of LEDGF/p75[130]. In contrast, mutants of HIV-1 IN defective for interaction with LEDGF/p75 failed to associate with the mitotic chromosomes. Finally, addition of a proteasome inhibitor to cells defective for LEDGF/p75 restores IN accumulation in the nucleus; nuclear LEDGF/p75 does apparently protect IN from proteolytic degradation in the nucleus.

A recent report confirmed our earlier data[121] that the presence of LEDGF/p75 increases the stability of HIV-1 IN in the cell. Knockdown of LEDGF/p75 resulted in an increase in ubiquitinated HIV-1 IN that could be rescued by restoration of LEDGF/p75 levels[132]. This protection of HIV-1 integrase from the proteasome by LEDGF/p75 did not require chromatin tethering, or nuclear localization of the IN-LEDGF complex. The only prerequisite for protection from proteasomal degradation was the interaction between LEDGF/p75 and HIV-1 IN. The most plausible hypothesis at this time is that LEDGF/p75 plays a role in the protection of IN against proteasomal degradation and in the tethering of IN to the chromosomal DNA by increasing the affinity of IN for DNA and/or the targeting of the lentiviral PIC to actively transcribed regions, in accord with the preferential integration of HIV-1 into transcriptionally active regions[127]. The lack of interaction between LEDGF/p75 and MoMLV IN, may be consistent with the fact that the oncoretrovirus MoMLV favors integration in promoter regions[133]. A change in integration-site selection during HIV-1 replication in LEDGF/p75-deficient cells may be associated with an apparent defect in integration, dependent on the assay used to detect proviral DNA, and may be associated with a reduction in viral replication, although this may depend on cell type and activation state.

Many questions still need to be addressed: (a) Does LEDGF/p75 also promote the enzymatic activity of HIV-1 IN *in vivo*? Or is the *in vitro* effect related to the increased affinity of HIV-1 IN for DNA? (b) Does LEDGF/p75 play a role as a survival factor during HIV-1 infection, regulating a balance between cell survival and apoptosis? (c) Does the increase in affinity for DNA also relate to the binding of HIV-1 IN to viral DNA in the cytoplasm? (d) Why is HIV replication not inhibited in the Jurkat LEDGF-deficient cell line and why are HIV vectors not inhibited at all?

At this stage it is not completely clear whether LEDGF/p75 is absolutely required during HIV-1 infection and further detailed study will be necessary to get a better insight into why exactly LEDGF/p75 associates with free lentiviral IN.

Viral proteins that possibly play a role in HIV-1 integration

During the early steps of the HIV-1 replication cycle, a transition of the reverse transcription complex (RTC) to the PIC occurs. The PIC carries out the final integration of the viral cDNA in the host genome. Since this PIC contains additional to IN several other viral proteins, such as nucleocapsid protein and reverse transcriptase (RT), these may be involved in the integration process.

Nucleocapsid protein assists integration by coating the DNA

The nucleocapsid protein (NCP) is a small, highly basic protein characterized by two zinc finger domains[134] and generated by proteolytic processing of the Gag polyprotein during virus maturation[135]. It has been well documented that the protein is necessary for virion formation and recognition, packaging, and stabilization of the viral RNA genome. Mutations in the zinc finger domains of the NCP clearly reduced viral infectivity[136]. Besides contributions to virus formation and RNA encapsidation, NCP has been proposed to have a role in events occurring early after viral entry. Initially, the function of NCP was mainly linked to the RTC where it has been shown to be active in stimulating RNA dimerization, in annealing the primer tRNA to the primer binding site (PBS) and to assist the initiation of cDNA synthesis[137]. The major mechanism of action of NCP is probably through coating of the viral RNA genome. The finding that NCP could also interact with viral DNA, in addition to RNA, raised the possibility that NCP may play a role *in vivo* during provirus synthesis and/or integration[4]. NCP would then act as a nucleic acid chaperone, combining helix-destabilizing and strand-annealing properties[138]. *In vitro,* a direct interaction between RT and NCP was observed as well as an enhancement of full-length cDNA synthesis by increasing RT processivity[139]. Moreover, it has been reported that both RT and NCP are required to efficiently complete the final steps in the synthesis of the central DNA flap *in vitro*, caused by the cPPT-CTS region[140]. Since NCP apparently promotes viral DNA synthesis by its nucleic acid chaperone capacity, it was interesting to analyze whether the coating of the viral genome by

NCP could also influence the integration step of HIV-1. Several groups demonstrated the stimulatory effect of NCP on the strand transfer and coupled integration reactions by recombinant HIV-1 IN *in vitro* [28,141-143]. The extent of stimulation though was dependent on the reaction conditions, but it was relevant that NCP could specifically promote the Mg^{2+}-dependent DNA integration at low enzyme concentration, since Mg^{2+} is the likely metal cofactor *in vivo*[141]. Purified NCP was also able to stimulate the activity of salt-stripped PICs[41]. A potential role for NCP during HIV-1 infection *in vivo* was studied by infecting cells with mutant viruses, followed by the analysis of the quantity and composition of the viral DNA generated during HIV-1 replication[144]. This study revealed that the reduced infectivity of viruses mutated in the NCP zinc finger domains resulted from defective reverse transcription and integration as a result of decreased protection of the full-length viral DNA.

A possible model for the contribution of NCP during viral DNA synthesis and subsequent integration is primarily based on the coating of the viral genome by NCP that increases its stability. The helix-destabilizing and strand-annealing properties could promote binding of the tRNA primer to the PBS, initiating the reverse transcription, and could promote the 5'-3' jumps occurring during reverse transcription. Once the double-stranded viral DNA is synthesized, the binding of NCP to the DNA protects it from nuclease digestion and could facilitate IN recognition of the DNA ends by leaving the terminal LTR ends unbound. A possible impact of NCP during the RTC-to-PIC transition is conceivable. Although maybe not essential for integration, NCP likely assists the integration process in the infected cell.

Reverse transcriptase, a physical and a functional binding partner

RT is a heterodimer of p66 and p51 subunits, catalyzing the reverse transcription from the viral RNA to double-stranded viral cDNA in a cytoplasmic nucleoprotein complex, named the reverse transcription complex (RTC)[145]. Since integration is the following step in the replication cycle, carried out by the viral IN residing in the PIC, and given the observation that some IN mutations inhibit viral replication by blocking viral DNA synthesis[7,8,146], RT and IN were proposed to interact with each other to coordinate their functions within the multifunctional HIV-1 replication complexes.

This direct interaction has been clearly demonstrated *in vitro* by several groups[147-149]. The interaction is not mediated by nucleic acid bridging, as shown in a pull-down assay with RT and IN after pretreatment with micrococcal nuclease[146]. Deletion analysis of RT to map the IN-binding domain revealed two separate domains: the fingers-palm domain and the C-terminal half of the connection sub-domain. The RT-interacting domain of IN was mapped to the C-terminal domain, since it was the only domain, in contrast to the N-terminal zinc-binding domain and the catalytic-core domain, still able to bind to heterodimeric RT[149]. Somehow contradictory to this observation, viruses harboring the C130S mutation in IN, located in the core domain, were replication defective due to a complete absence of reverse transcripts. Furthermore, purified C130S-containing IN failed to interact with RT *in vitro*, although this mutant was still enzymatically active[148]. This suggests that the C130S mutation could disrupt the protein recognition interface of the C-terminal domain of IN and consequently abolish its ability to interact with RT. However, additional experiments are required to conclude unequivocally that the defect caused by the C130S mutation in IN is direct and specific for initiating reverse transcription.

The two key viral enzymes physically interact *in vitro* and the interaction appears to be directly or indirectly functionally relevant *in vivo*, but the question remains whether RT-IN interaction affects the enzymatic activity of the enzymes. Although some controversial data have been reported, most probably due to different reaction conditions, we can nevertheless conclude from *in vitro* data that: (1) RT inhibits the 3' processing activity of IN at RT:IN ratios exceeding 1:1 *in vitro*[147,149,150]; (2) the addition of RT stimulates the strand-transfer activity of IN *in vitro*[149]; (3) IN does not affect the RNA-dependent DNA polymerase activity nor processivity of RT[149]; (4) the DNA-dependent DNA polymerase activity of RT is inhibited by IN, possibly by competition for nonspecific DNA binding[147].

Whether these *in vitro* findings are biologically relevant remains unanswered, but one might expect that correct composition, proper folding and specific interactions of the different components in the RTC and PIC, including IN and RT, are important for a productive HIV-1 infection. Further studies should be undertaken to increase our understanding of the dynamics of RT-IN interactions during the early steps of the viral replication *in vivo*.

Conclusions and perspectives: validated cofactors as new antiviral targets

Although the viral IN can perform 3' processing and strand-transfer activities *in vitro* in the absence of additional viral and cellular proteins, the *in vivo* integration process is far more complex. An ever-expanding arsenal of cellular proteins has been proposed to be implicated in this retroviral integration step, either by directly interacting with the IN protein, by binding to

the viral cDNA and/or repairing the DNA gaps of the integration intermediate. Most of these proteins have been identified and/or analyzed using *in vitro* assays, which were also used to validate them as IN cofactors. In cell culture experiments, though, it has proven much more difficult to demonstrate unambiguously that a potential cellular protein is necessary for retroviral integration. For some, the cellular experiments even pointed to another role during HIV-1 replication, distinct from integration (e.g. HMGA1). Validation of these different candidate IN cofactors in cell culture remains a formidable task in this research area for the next couple of years. This effort has to be seen in the general effort in cell biology and functional proteomics to understand (real-time) protein-protein interactions in the living cell. In so doing, it will be important to analyze cell- and virus-strain specificity for the proposed cofactors, given the reported controversies in this field (e.g. for LEDGF/p75). Knockdown experiments using RNA interference technology should be performed very carefully using the appropriate controls[151]. State-of-the-art knockdown experiments will be very informative regarding the cellular role of the cofactor and the requirement of the protein for a productive HIV-1 infection. During this validation procedure, one should keep in mind the likely redundancy in the use of cellular proteins promoting HIV-1 replication. Furthermore, a good candidate IN cofactor should be analyzed in primary cells targeted by HIV-1, not only in cell lines.

Each independently confirmed and validated cellular cofactor for HIV-1 integration could constitute a promising new therapeutic target. Even if a given cofactor only augments HIV replication 10-fold, its specific inhibition may result in a significant reduction in viral replication. Toxicity associated with inhibition of the cellular protein may be anticipated by the knockdown data and limited by identification of compounds specifically interfering with the protein-protein interaction. Moreover, targeting a cellular protein instead of a viral protein would probably minimize the risk of antiviral resistance.

When considering that the HIV-1 virus employs a very complex network of interactions to achieve its final goal (i.e. to integrate in the host genome and produce new viral particles) an analogy arises to describe the task of scientists trying to unravel these interactions: interact and integrate.

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