The impact of nuclear topology on HIV-1 integration

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Una volta, prendere 10 in matematica
in pagella non era cosa da tutti i giorni.
Il Preside volle verificare personalmente
e ti interrogò per ore su tutto il programma,
davanti a tutta la classe; tu non sbagliasti una virgola.
Il giorno dopo il Preside venne a casa tua,
e pregò tuo padre di iscriverti all'Università, a Matematica.
Da dietro una porta socchiusa, e con le lacrime agli occhi,
tu ascoltasti tuo padre costretto a dire di no.
Per tutta la tua vita, lunga e serena,
hai sempre continuato ad amare la lettura e lo studio;
grazie per avermi trasmesso questo amore.

A Ginevra D'Alessio Marini

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INTRODUCTION

1. HIV-1 discovery

Starting in June 1981, several young men affected by severe immunosuppression, opportunistic infections and unusual cancers were admitted as first cases of a new, unknown disease. One year later, more than 400 cases were reported to the Centers for Disease Control and Prevention (CDC) of the United States, and half of them were fatal; the new disease was termed Acquired Immuno Deficiency Syndrome (AIDS).

The infectious nature of this disease was highlighted in 1983, when a new retrovirus, termed LAV (Lymphadenopathy-Associated Virus) was isolated by Dr Luc Montagnier from lymph nodes of one of these patients (Barre-Sinoussi, Chermann et al. 1983). At the same time, Dr. Robert Gallo and Dr Jay Levy isolated a retrovirus from AIDS patients and some still healthy individuals in the risk groups, and termed it HTLV-III (Human T-Cell Leukemia Virus III)(Gallo, Sliski et al. 1983). In 1984, several publications consistently proved that HTLV III was the cause of AIDS (Alter, Eichberg et al. 1984, Gajdusek, Amyx et al. 1984, Gallo, Salahuddin et al. 1984, Popovic, Sarngadharan et al. 1984). In June 1984, Drs. Robert Gallo and Luc Montagnier held a joint press conference to announce that HTLV-III and LAV were almost certainly identical. Later on, the new retrovirus was termed HIV (Human Immunodeficiency Virus). On the same year, almost 7,000 cases were reported to CDC, with more than 2,000 deaths only in United States. This number exponentially increased, so that in August 1988, the number of AIDS patients exceeded 70,000 in the United States only; the CDC predicted that more than 1 million people was infected in that country, without being yet aware of it, and that a new case was

reported every 14 minutes. HIV infection became pandemic and spread worldwide, infecting at least 60 million people and causing more than 25 million deaths (http://www.unaids.org/).

But where exactly did HIV-1 originated? Scientists discovered that HIV-1 and a similar virus termed HIV-2 were the result of the transmission of viruses from primates to humans, that likely occurred in Africa in the 1920s (Sharp and Li 1988, Hahn, Shaw et al. 2000). Indeed simian immunodeficiency viruses (SIVs) were identified in different sub-Saharan primates, suggesting a cross-species infection; however these viruses do not cause disease in their host (Sharp, Bailes et al. 2001). Despite frequent human exposure to SIV-infected monkeys in Africa, only 11 cross-species transmission events have been identified, and only four of them have resulted in a significant human-to-human transmission. Indeed, phylogenetic analysis revealed that the main subtypes of HIV-1 (groups M and O) and HIV-2 (groups A and B) originated from four different infections of SIVs, from chimpanzees and sooty mangabeys respectively (Marx, Apetrei et al. 2004). Recently, it has been defined that such events occurred in Leopoldville (now Kinshasa) in Congo, essentially due to the consumption and manipulation of bushmeat, by which humans are directly exposed to animal blood and secretions. Demographic data proved that pandemic HIV-1 developed in the late ‘50s together with the exponential growth of urban population in West Central Africa and the expansion of fluvial travel and commerce along the Congo river; a subtype of the group M spread to Haiti, then to US and other Western countries (Sharp and Hahn 2011, Faria, Rambaut et al. 2014).

Over the last two decades, HIV has been deeply studied and characterized in unprecedented molecular detail, allowing the development of drugs that, even though unable tot eradicate HIV-1 infection, still can

successful control viral replication in the host and spread. Therefore, in the Western Countries, AIDS has been transformed from a lethal disease into a chronic one, that still represents a heavy burden on the health organizations. Moreover, despite this remarkable success, AIDS remains a devastating and fatal disease in all countries where drugs are not available, including most of the African continent and several Asian countries. The global expenditure for HIV-1 treatment and prevention could reach 35 billion dollars before 2031 (Report from Aids2031 Project, UNAIDS 2010).

2. HIV-1 genome and structure

HIV-1 belongs to the Lentivirus genus, from the Retroviridae family. Lentiviruses (lente-, Latin for "slow") are a group of RNA viruses characterized by a very long incubation period and by a particular tropism for cells of the monocyte/macrophage lineage. They cause chronic persistent infection in different mammalian species. Five main serogroups of lentiviruses have been identified, each corresponding to vertebrate hosts: primates (including HIV-1, HIV-2 and SIVs), felines (FIVs), horses (EIAVs), cattle and sheep/goats (CAEV, VISNA) (Gifford 2012). Not all lentiviruses are pathogenic in their natural hosts, but some of them cause immune system dysfunctions that can be lethal. The virions are composed of a spherical envelope that measures about 100 nm in diameter; the envelope encloses a rod-shaped nucleocapsid that contains both the viral enzyme and some viral enzymes. Once in the host cell, lentiviral RNA genomes are transformed into DNA by the viral reverse transcriptase; the DNA is then incorporated into the cellular genome by the integrase enzyme, thus becoming a provirus capable of replication by exploiting the host transcription machinery.

As other lentiviruses, HIV-1 genome consists of two copies of positive-

stranded RNA (Alizon, Sonigo et al. 1984), containing nine genes, which encode for 19 proteins.

The Gag gene is translated into a p55 polyprotein that, after cleavage, originates p17, p24, p7 and p6. Viral protein p24 forms a capsid that surrounds and encloses the viral genome, which is further protected by nucleocapsd protein p7. The capsid is surrounded by a matrix composed of viral protein p17.

The Pol gene codes for the main viral enzymes, protease (PR), reverse transcriptase (RT), and integrase (IN), which are included in the capsid and are required for the subsequent steps of infection.

The matrix and the capsid are surrounded by an envelope, which is composed by a phospholipid bilayer derived directly from the cellular membrane, and enriched in two viral glycoproteins, gp120 and gp41, which are translated from the Env gene and derive from the cleavage of the gp160 polyprotein. The resulting virion is spherical and with a diameter of 120 nm.

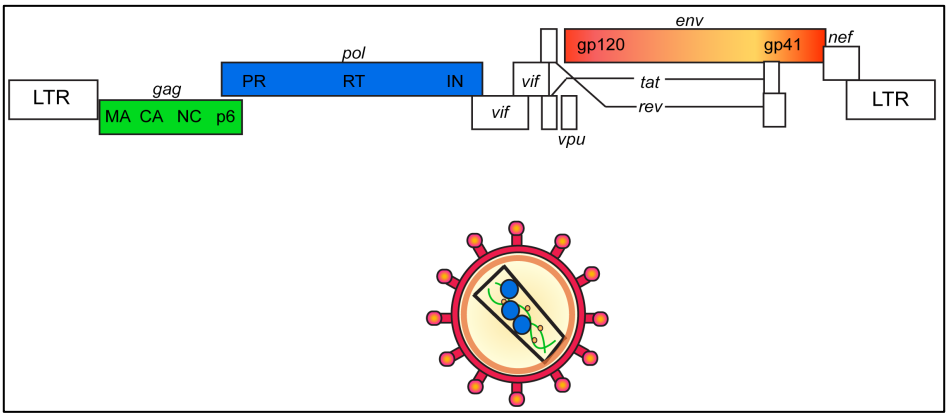


Figure 1. Schematic representation of HIV-1 genome and virion.

The remaining 6 genes (*tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*) are regulatory genes, which code for accessory proteins.

The Tat protein is an essential, strong transcriptional transactivator of the viral promoter, constituted by the Long Terminal Repeats (LTR, see chapter 3 for details) (Berkhout and Jeang 1989), which acts by binding to the TAR RNA element and activating transcription initiation and elongation. It is the first transcription factor interacting with RNA rather than DNA, and it is very similar to prokaryotic anti-termination factors (Ott, Geyer et al. 2011). The human positive transcription elongation factor b (P-TEFb) is an essential human cofactor for Tat transactivation (Mancebo, Lee et al. 1997).

Rev is a protein necessary for HIV-1 expression; it promotes nuclear export and stabilization of viral unspliced mRNA, by binding to Rev-Responsive Element contained in the env region (Chang and Sharp 1990, Pollard and Malim 1998).

Vif plays a role to antagonize the restriction factor APOBEC3G (Sheehy, Gaddis et al. 2003, Stopak, de Noronha et al. 2003), whereas Vpu enhances the release of new virions, by counteracting cellular protein tetherin, able to inhibit this process (Neil, Zang et al. 2008).

Additional proteins auxiliary for a efficient viral propagation are Vpr, that probably plays a role in the nuclear import of preintegration complexes, and Nef, that acts in downregulating both CD4 and MHC molecules from the cell surface and it is essential for efficient viral spread and disease progression in vivo (Malim and Emerman 2008).

3. HIV-1 life cycle: an overview

As other primate lentiviruses, HIV-1 targets cells from the hematopoietic / lymphoid lineage, mainly T helper lymphocytes and macrophages, and at a lesser extent also microglial and dendritic cells; such cells share the feature

of expressing the CD4 receptor and CC-chemokine receptor 5 (CCR5) (Dragic, Litwin et al. 1996) or CXC chemokine receptor 4 (CXCR4) (Feng, Broder et al. 1996). It has been shown that other non hematopoietic, CD4 negative cell types can be infected in vitro (with a very low efficiency) but the significance of such observation in the in vivo pathogenesis is considered to be scarce (Dittmar, McKnight et al. 1997, Clapham and McKnight 2001).

The CD4 receptor interacts with viral envelope protein gp120 (Kwong, Wyatt et al. 1998), and such interaction promotes the binding to the mentioned co-receptors eventually leading to viral entry mostly by direct fusion with the plasma membrane (mediated by gp41)(Chan and Kim 1998), or by endocytosis followed by glycoprotein- and dynamin-dependent fusion with intracellular compartments (Miyauchi, Kim et al. 2009). Such process is extremely conserved among primate lentiviruses.

The binding with one of the coreceptors CCR5 or CXCR4 is crucial for efficient membrane fusion (Bleul, Wu et al. 1997, Dittmar, McKnight et al. 1997, Kozak, Platt et al. 1997). Interestingly, individuals that are homozygous for a mutant CCR5 allele (CCR5D32) were found to remain uninfected even after frequent exposures to HIV (Samson, Libert et al. 1996). This discovery is linked to the “Berlin patient”, remaining nowadays the only individual to have been cured of HIV infection, after having received an allogenic hematopoietic stem cells transplant from a homozygous CCR5D32 (Hutter, Nowak et al. 2009).

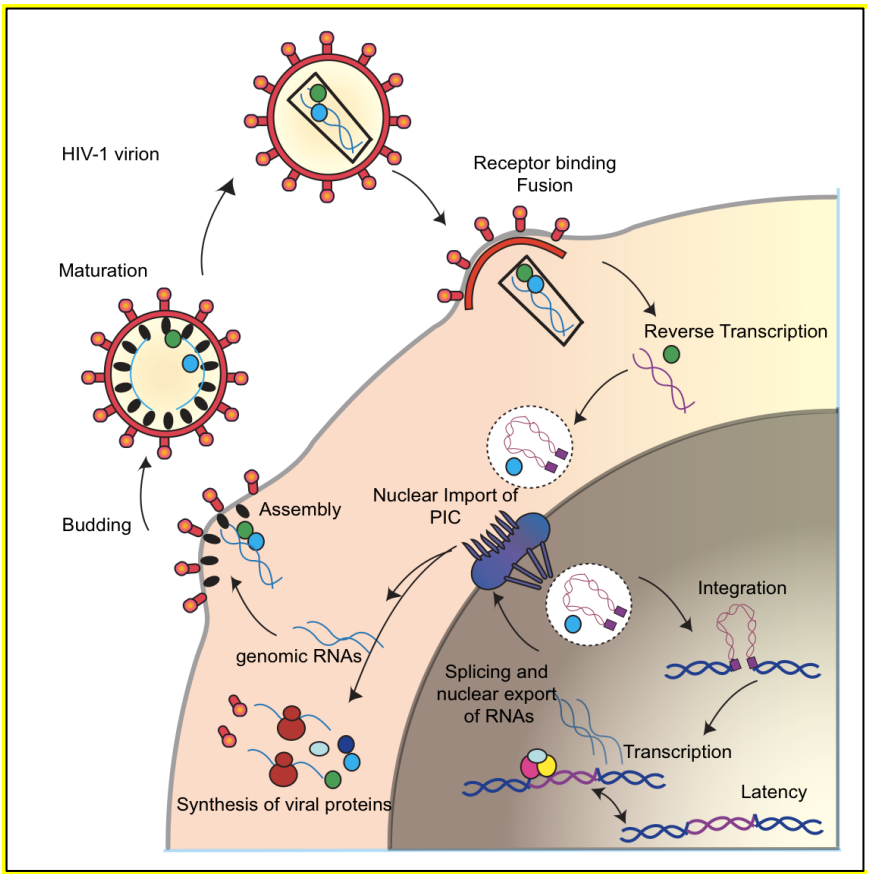


Figure 2. Scheme of the main steps of HIV-1 replication.

Following cell entry, the cooperation of viral proteins (p17 Matrix, Nef and Vif) with host proteins leads to the uncoating of the nucleocapsid, which allows the viral genome to be extruded out from the capsid and released into the cytosol (Dvorin and Malim 2003). Once uncoating is completed, the viral RNA, RT, IN, p17, p7, Vpr and a plethora of cellular cofactors all assemble together into the so-called reverse transcription complex. The reverse transcription starts in the cytoplasm and is completed in the nucleus; the so-called "Long Terminal Repeats" (LTRs) are formed as duplication of the U3, R and U5 sequences at both 5' and 3' ends of the final cDNA

(Gilboa, Mitra et al. 1979) (see paragraph 4.1 for details). However, the exact mechanism leading from viral entry to reverse transcription is still matter of debate; indeed, it has been indeed hypothesized that uncoating might occur simultaneously with the reverse transcription process. Moreover this would occur not in the cytoplasm but rather in the proximity of the nuclear pore complexes (Bichel, Price et al. 2013).

After or during reverse transcription (see paragraph 4.1), both viral (IN, p17, p7, RT and Vpr) and cellular proteins associate with the double-stranded viral DNAs in the so-called Pre-Integration Complex (PIC) (Farnet and Haseltine 1991, Miller, Farnet et al. 1997). The cellular component of the PICs has been deeply studied in the last years through a proteomic approach (Raghavendra, Shkriabai et al. 2010), leading to a long list of cofactors that are included in the PICs even though not always the role for their presence has been determined. Also the molecular mechanism of the PIC entry into the nucleus is still not completely understood; however it has been clearly demonstrated that cellular protein partners are crucial for the nuclear entry of the PIC and viral integration (see paragraph 4.2.1 for major details).

The early steps of viral replication appear to be the main targets for the "restriction factors", namely host proteins exerting an innate anti-viral response.

A recent and comprehensive analysis of such factors is presented in the table http://www.retrovirology.com/content/10/1/106/table/T1 (Abden-Mohsel et al. 2013). The most characterized restriction factors for HIV-1 infection are the TRIM5α, APOBEC3 and SAMHD1.

- TRIM5α blocks HIV-1 infection in simian cells (Stremlau, Owens et al. 2004) at the reverse transcription and nuclear import steps; TRIM5α recognizes motifs within the capsid proteins and interferes with the uncoating process, therefore preventing successful reverse transcription and

transport to the nucleus of the viral genome. Retroviral resistance to TRIM5α can be generated by sequences in the viral capsid protein (Sebastian and Luban 2005, Stremlau, Perron et al. 2006).

- Apolipoprotein B mRNA-editing enzyme 3 family (APOBEC3) can also suppress HIV-1 replication (Sheehy, Gaddis et al. 2002, Sheehy and Erthal 2012). It is a family of cytidine deaminases that hypermutates the viral genomes, impairing viral replication. A deaminase-independent inhibition by APOBEC was also reported (Sasada, Takaori-Kondo et al. 2005, Nguyen, Gummuluru et al. 2007). Vif interacts with APOBEC3G by triggering its degradation via the proteasomal pathway (Donahue, Vetter et al. 2008).
- SAM domain and HD domain-containing protein 1 (SAMHD1) has also been described as an additional restriction factor for HIV-1. SAMHD1 is a phosphohydrolase that can convert nucleotide triphosphate to a nucleoside and triphoshate. In doing so, SAMHD1 depletes the pool of nucleotides available for viral cDNA synthesis by reverse transcriptase, and in this way prevents viral replication (Goldstone, Ennis-Adeniran et al. 2011, Laguette, Sobhian et al. 2011, Ayinde, Casartelli et al. 2012, Baldauf, Pan et al. 2012, Descours, Cribier et al. 2012, Kim, Nguyen et al. 2012). SAMHD1 is also able to restrict other retroviruses, such Murine Leukemia Virus (MLV), SIV, EIAV, FIV, showing a broad antiretroviral activity (Gramberg, Kahle et al. 2013). SAMHD1 is counteracted by the viral protein Vpx, which forms a complex with a E3 ubiquitin ligase, which in turn induces ubiquitination and proteasomal degradation of SAMHD1. Therefore, the dNTP pool is restored and the block to reverse transcription is released (Hrecka, Hao et al. 2011, Ahn, Hao et al. 2012, Hofmann, Logue et al. 2012).

Integration of the proviral genome into host chromosomal DNA is one of the defining features of retroviral replication (Coffin, Hughes et al. 1997).

To access chromatin, some retroviral PICs require nuclear-envelope disassembly during cell division, whereas HIV-1 and other lentiviruses that infect non-dividing cells can directly cross the nuclear envelope to enter the nucleus. In order to integrate their cDNA into the most preferable chromosomal targets, their PICs are transported through the Nuclear Pore Complexes (NPCs) by active translocation (Suzuki and Craigie 2007).

Once inside the nucleus, integration of viral genome into the host DNA is mediated by the IN protein, with the support of several cellular partners and post-translational modifications (see chapter 4.2.1). Although HIV-1 integration is not site-specific, several lines of evidence have indicated that it is not completely random and favors portion of chromatin with particular features. Mainly, HIV-1 prefers to integrate inside actively transcribing genes, possibly to maximize the exploitation of the cellular machinery needed for transcription (see chapter 4.2.2) (Jordan, Defechereux et al. 2001, Schroder, Shinn et al. 2002, Bushman, Lewinski et al. 2005).

After integration into the host genome, the provirus behaves as any human RNA Pol II-transcribed gene: transcription proceeds downstream to the promoter and enhancer elements encoded in the 5' LTR, to the polyadenylation site within the 3' LTR (see chapter 4.3.1). Regulation of HIV-1 gene expression is defined by a complex cross-talk between the local chromatin landscape, host transcription factors, and the viral trans-activating factor Tat (Malim, Fenrick et al. 1989, Fujinaga, Cujec et al. 1998, Marzio, Tyagi et al. 1998, Lusic, Marcello et al. 2003) (see chapter 4.3.2-3). HIV transcription is classically subdivided into an early and a late phase. In the early phase of transcription, in the absence of Rev and Tat regulatory proteins, transcription is triggered by host transcription factors bound to the LTR; the RNA splicing machinery quickly splices mRNA and only Rev, Tat and Nef proteins are produced (Kim, Byrn et al. 1989). In the subsequent step of transcription, Tat boosts initiation and elongation of viral

transcription (Berkhout, Silverman et al. 1989), leading to the production of a single mRNA, which is then processed to generate over thirty different viral transcripts. In the early phase of transcription, short, multiply spliced transcripts (encoding for Tat, Rev and Nef) are produced, while later, singly spliced RNAs and long unspliced RNAs become predominant (for an extensive review, see (Karn and Stoltzfus 2012)). The first short transcripts, which do not contain unspliced exons, exploit the cellular transport of mature cellular mRNAs into the cytoplasm (Cullen 1998); instead, Rev coordinates the export to the cytoplasm of unspliced and partially spliced mRNAs (Malim, Hauber et al. 1989, Pollard and Malim 1998, Greene and Peterlin 2002).

After the production of new viral genomes and the synthesis of several viral proteins, three main steps need to be executed: the assembly of the virion, when essential components for infectivity are packed together; the budding, when the virion acquires its lipid envelope through the fusion with the cellular membrane; and the maturation, when the virion becomes infectious and it is released from the cell (Sundquist and Krausslich 2012). These steps require a multiplicity of cellular pathways and partners, but are coordinated by the viral Gag precursor protein (p55) that guides the entire process. p55 leads this process after moving to the host cell plasma membrane, within membrane microdomains corresponding to the so called lipid rafts, specific membrane sub-domains enriched in cholesterol (Gheysen, Jacobs et al. 1989, Karacostas, Nagashima et al. 1989, Freed 1998, Nguyen and Hildreth 2000, Zheng, Plemenitas et al. 2003). Here, Gag simultaneously triggers membrane binding (through its N-terminal component, MA), virion assembly (through the central domain, CA which will constitute the capsid in the mature virion), and RNA packaging (by the NC domain) (Benjamin, Ganser-Pornillos et al. 2005, Briggs, Riches et al.

2009, Jouvenet, Simon et al. 2009).

At the same time, Env is synthesized as a precursor of about 850 aminoacids and later it is processed as an integral membrane protein. Indeed it undergoes first post-translational processing that includes signal peptide cleavage, folding and trimerization (Haim, Salas et al. 2013). Later it follows the cellular secretory pathway through the endoplasmic reticulum where it gets glycosylated. The resulting gp160 Env precursor is transported to the Golgi where host proteases cleave it into the gp120 (surface) and gp41 (membrane) subunits (Willey, Bonifacino et al. 1988, Stein and Engleman 1990). The trimeric complexes are then inserted into the cell membrane in trimeric complexes (Decroly, Vandenbranden et al. 1994, Bugelski, Maleeff et al. 1995). Gag initially assembles spherical particles, which will bud from the membrane through the interaction with components of the cellular Endosomal Sorting Complex Required for Transport (ESCRT) and the apoptosis-linked-gene 2 interacting protein (Alix) (Martin-Serrano, Zang et al. 2001, Strack, Calistri et al. 2003, Martin-Serrano and Marsh 2007, Martin-Serrano and Neil 2011). After budding, PR is activated and cleaves p55 into its separate components MA, CA, NC and p6 proteins, forming a mature infectious virion (Gottlinger, Sodroski et al. 1989, Peng, Ho et al. 1989, Schatzl, Gelderblom et al. 1991, Kaplan, Zack et al. 1993, Bharat, Davey et al. 2012, Meng, Zhao et al. 2012).

In this context, Tetherin and calcium-modulating cyclophilin Ligand 1 (CAML-1) inhibit viral release; these restriction factors are counteracted by viral Vpu, although its exact mechanism is still under investigation (Neil, Zang et al. 2008, McNatt, Zang et al. 2013).

Interestingly, lipid rafts, where assembly and budding start, are the same compartment in which the myristoylated form of the Nef protein is incorporated, being this a prerequisite for the biological activity of the protein itself (Geyer, Fackler et al. 2001, Zheng, Plemenitas et al. 2003).

Nef promotes the release of more infectious virions by a variety of molecular mechanisms, including: activation of TCR-activated pathways that stimulate HIV transcription (Wang, Kiyokawa et al. 2000, Simmons, Aluvihare et al. 2001, Fenard, Yonemoto et al. 2005); decrease in the expression of CD4 on the cell surface (Lama, Mangasarian et al. 1999, Glushakova, Munch et al. 2001); reduction in the expression of MHC I and thus the visibility of the infected cells to CD8+ cytotoxic T-cells (Collins, Chen et al. 1998); block of apoptosis (Fackler and Baur 2002). The overall result of these activities is to prolong the life of the infected cell to optimize viral replication (Greenway, Holloway et al. 2003).

4. HIV-1 nuclear biology

4.1 Reverse transcription and nuclear import

Following cell-receptor entry, HIV-1 starts reverse transcription of its genome. The viral capsid is able to provide the right environment for this process to occur, but the virus must uncoat its core to enter the nucleus. So far, it is not clear where uncoating takes place: whereas the reverse transcription complex has been visualized in the cytoplasm (McDonald, Vodicka et al. 2002, Hulme, Perez et al. 2011), some studies suggest that uncoating occurs at the NPCs, in parallel with the completion of reverse transcription itself within the capsid (Arhel, Souquere-Besse et al. 2007). This might reduce the exposure of viral RNA/DNA to the cytoplasmic environment and therefore to degradation. Moreover, coupling uncoating with reverse transcription might speed up the entire process, lowering the risk of activating the innate immunity pathways, which are able to sense both the cytosolic viral genomes and viral capsid (Towers, Hatziioannou et al. 2003, Pertel, Hausmann et al. 2011, Schaller, Ocwieja et al. 2011).

Once retro-transcribed, the viral DNA integrates into the host genome; integration is a key feature of retroviruses (Temin 1976), and it ensures expression of viral genes for the subsequent production of new viral particles. In order to integrate, retroviral PICs have to access the nucleus. Some retroviruses, such as MLV, require the nuclear envelope breakdown during mitosis, to get in contact with cellular chromosomes. On the contrary, lentiviruses, including HIV, can enter the nucleus in non-dividing cells, by passing through the NPC (Greene and Peterlin 2002, Bukrinsky 2004). The molecular processes underlining the PIC entry into the nucleus remains elusive. It appears that a redundancy of mechanisms exist, at least in part mediated by the Nuclear Localization Signals (NLS) that have been identified in most of the viral proteins of the PIC (Bouyac-Bertoia, Dvorin et al. 2001).

Importantly, the viral capsid is implicated in the HIV-1 nuclear import. M. Yamashita and M. Emerman have indeed demonstrated that the capsid is crucial for the capacity of HIV-1 to infect non-dividing cells. In particular, substituting the HIV-1 capsid with MLV capsid in the HIV-1 virion, the virus containing the latter is not anymore able to infect non-dividing cells. On the opposite, if the HIV-1 capsid is inserted into a MLV virion, this is able to infect non-dividing cells in a HIV-1-like manner (Yamashita and Emerman 2004). Indeed, the HIV-1 capsid is able to interact with several nucleoporines involved in nuclear import, including Nup153, TNPO3 and Nup358/RANBP2 (Christ, Thys et al. 2008, Lee, Ambrose et al. 2010) (for a comprehensive review, see (Suzuki and Craigie 2007)). Capsid mutants, such as CA N74D, affect its uncoating speed (Yamashita, Perez et al. 2007), and therefore lead to unsuccessful reverse transcription and missed interaction with Nup153, TNPO3 and Nup358. However, such mutants appear to still reach the cellular genome through other, not well-determined import pathways.

Cleave and Polyadenylation Factor 6 (CPSF6) is a host restriction factor preventing HIV-1 nuclear entry by targeting the capsid; the N74D capsid mutant is able to evade the interaction with CPSF6 (Lee, Ambrose et al. 2010). Interestingly, a novel HIV-1 restriction factor, interferon-induced Myxovirus Resistance 2 (MX2) protein, was recently described as inhibitor of HIV-1 capsid-dependent nuclear import (Goujon, Moncorge et al. 2013, Kane, Yadav et al. 2013). However MX2 and N74D mutant might be involved into different pathways: indeed the N74D mutant reduced but did not eliminate the sensitivity to overexpression of MX2 (Kane, Yadav et al. 2013).

4.2 Integration

4.2.1 HIV Integrase (IN) and its cofactors

Once inside the nucleus, integration of viral DNA into the cellular genome is mediated by the IN protein, which binds the end of the viral DNA and tethers it to chromatin, where it catalyzes the joining reaction within the host chromosome.

IN is a 32 kDa protein containing three different domains (Engelman 1999, Krishnan, Li et al. 2010, Krishnan and Engelman 2012). The N-terminal domain (NTD, 1-50 residues), important for the IN multimerization and enzymatic activity (Khan, Mack et al. 1991, Schauer and Billich 1992, Zheng, Jenkins et al. 1996, Lee, Xiao et al. 1997), consists of three alpha-helices with coordination of a single Zn^{2+} ion stabilizing the structure (Carayon, Leh et al. 2010). The catalytic core domain (CCD, 51-212 residues) is the most conserved among all retroviral INs. It consists of five stranded alpha-sheets with six surrounding helices; this structure resembles the one of the transposase from the bacteriophage Mu, the Rnase H domain of RT enzyme and the RuvC protein of E. Coli, all belonging to a varied

superfamily of polynucleotidyl transferases (Rice and Mizuuchi 1995). The key catalytic site is the highly conserved D,D(35)E motif, consisting in three conserved acidic amino acids (Asp-64, Asp-116, and Glu-152) with a conserved spacing of 35 amino acids between the second and the third (Engelman and Craigie 1992). The CCD interacts with the target DNA (Heuer and Brown 1997). Mutation of the D,D(35)E motif inhibits IN enzymatic activity (Kulkosky, Jones et al. 1992, Leavitt, Shiue et al. 1993). The C-terminal domain (CTD, 213-288 residues) is needed for proper multimerization, and binds DNA in a non specific manner, possibly representing the first determinant of chromosomal DNA recognition during integration (Engelman, Hickman et al. 1994) (Lu, Ghory et al. 2005).

Immediately after reverse transcription is completed, IN complex is bound to the LTR DNA ends, forming the so called intasome (Wei, Mizuuchi et al. 1997), which will undergo different events before integration is completed (Maertens, Hare et al. 2010, Krishnan and Engelman 2012).

In the first step, the so-called stable synaptic complex (SSC) is transformed into the cleaved donor complex (CDC) by the removal of two nucleotides from each 3' end of viral DNA (3' processing) (Pauza 1990, Vink, van Gent et al. 1991, Vink, Yeheskiely et al. 1991).

In the second step (DNA strand transfer), the CDC is engaged to the target DNA after the attack of the 3' ends to a pair of phosphodiester bonds in the chromatin acceptor, which are separated by 5 nucleotides. At this step, the intasome is called target capture complex (TCC) (Engelman, Mizuuchi et al. 1991). Finally, IN catalyzes the link of the 3' ends to the 5' phosphates of the cleaved host DNA, whereas the 5' ends of the viral genome remain unjoined (strand transfer complex, STC) (Brown, Bowerman et al. 1987, Fujiwara and Mizuuchi 1988, Brown, Bowerman et al. 1989). Afterwards, cellular enzymes with gap repair functions remove two nucleotides on each of the 5' ends of the viral DNA and trigger the ligation of the 5' ends to the

target DNA (Katz, Mack et al. 1992, Yoder and Bushman 2000).

Alternatively, if the viral DNA does not get integrated, it may follow three different fates. The ends of the viral DNA might be joined to form a 2-LTR circle by the non-homologous end joining cellular pathway (NHEJ) (Li, Yoder et al. 2000); the viral genome might result in a single LTR circle through the action of factors of the cellular homologus DNA recombination (Brown, Bowerman et al. 1987, Kilzer, Stracker et al. 2003); viral DNA might auto-integrate into itself producing a rearranged circular structure (Farnet and Haseltine 1991). Notwithstanding that none of these variants produce infectious virus, they seem to be transcriptionally active (Stevenson, Haggerty et al. 1990, Stevenson, Stanwick et al. 1990), leading to selected transcription of *tat* and *nef* genes before integration (Wu and Marsh 2003). The persistence of these forms of circular DNA before integration is related to the phenomenon referred to as pre-integration latency (Coiras, Lopez-Huertas et al. 2009, Sloan and Wainberg 2011).

Even if IN alone is sufficient to promote in vitro integration (Craigie, Fujiwara et al. 1990, Katz, Merkel et al. 1990, Bushman and Craigie 1991, Sinha, Pursley et al. 2002), for efficient integration in the cellular context, the protein requires several host factors, most of which already recruited into the PIC before entering the nucleus (for review see: (Turlure, Devroe et al. 2004, Van Maele, Busschots et al. 2006)).

PICs isolated from HIV-1 infected cells can integrate their endogenous viral cDNA into a target DNA substrate in vivo (Ellison, Abrams et al. 1990, Farnet and Haseltine 1990, Khiytani and Dimmock 2002). Given that the integrating activity of the PICs was disrupted by treating them with high concentration of salts, and that extracts of uninfected cells restored their activity, cellular cofactors had to be involved. The first protein to reconstitute the activity of salt-treated PICs was the high mobility group

protein A1 (HMGA1) (Farnet and Bushman 1997). HMGA proteins are non-histone chromatin-associated proteins that participate in several nuclear processes; in particular, HMGA1 is able to induce local conformational changes in DNA strands, affecting gene expression (for a review see: (Jiang and Pugh 2009)). Inside the PICs, HMGA1 interacts with viral DNA but not with IN and it may help in bridging the viral ends together within the intasome (Farnet and Bushman 1997, Hindmarsh, Ridky et al. 1999, Henderson, Bunce et al. 2000).

Salt treatment of extracted PICs led to the discovery of another cellular cofactor (Lee and Craigie 1994). Indeed, high salt concentrations induced an increase in autointegration events in MLV, by removing Barrier-to-Autointegration Factor (BAF) (Chen and Engelman 1998, Lin and Engelman 2003). This protein, involved in nuclear envelope assembly and organization (Margalit, Brachner et al. 2007), is included in the PICs of both MLV and HIV. As HMGA1, BAF interacts with DNA and not with IN, and it may help compacting the viral DNA and so making it inaccessible for premature integration into itself (Suzuki and Craigie 2002).

Other cellular factors can bind directly IN protein. The first to be discovered was INtegrase Interactor 1 (INI1) that appeared to be able to stimulate integration activity in vitro 10 to 20 folds (Kalpana, Marmon et al. 1994). The importance of this factor, however, was later questioned (Boese, Sommer et al. 2004). More recently, it was reported that defects in IN-INI1 interaction impairs integration in HEK293T and HeLa cells (Mathew, Nguyen et al. 2013). In considering these findings, it appears worth taking into account that INI1 is a component of the Switch/Sucrose Non Fermentable (SWI/SNF) chromatin remodeling complex (for a review see: (Euskirchen, Auerbach et al. 2012)); therefore, rather than acting directly on integrase activity, INI1 might help targeting integration into SWI/SNF-remodeled regions of the genome (Kalpana, Marmon et al. 1994, Lesbats,

Botbol et al. 2011).

One of the most studied cofactors of HIV-1 IN is the product of the *PSIP1* gene, LEDGF/p75 (Maertens, Cherepanov et al. 2003), a transcriptional coactivator implicated in cell differentiation and cellular response to stress (Llano, Morrison et al. 2009). In the context of lentiviral integration, LEDGF/p75 acts by tethering IN to chromatin through its two main domains: an N-terminal PWWP (Pro-Trp-Trp-Pro) chromatin-binding domain and a C-terminal IN-binding domain (Cherepanov, Maertens et al. 2003, Llano, Saenz et al. 2006, Llano, Vanegas et al. 2006, Turlure, Maertens et al. 2006, Busschots, Voet et al. 2007). Depletion of LEDGF/p75 reduces efficiency of HIV-1 integration by 10 fold (Vandekerckhove, Christ et al. 2006), but does not affect IN catalysis (Shun, Raghavendra et al. 2007), suggesting that the LEDGF/p75 function in chromatin engagement is crucial (Busschots, Vercammen et al. 2005, Emiliani, Mousnier et al. 2005) (see paragraph 4.2.2 for details).

Other IN interactors include Polycomb Protein EED (Violot, Hong et al. 2003), HSP60 (Parissi, Calmels et al. 2001), nuclear pore component NUP153 (Woodward, Prakobwanakit et al. 2009), and gap repair protein Rad18 (Mulder, Chakrabarti et al. 2002). A role for emerin in HIV-1 integration was described (Jacque and Stevenson 2006), but it was later disproved by two other studies (Shun, Daigle et al. 2007, Mulky, Cohen et al. 2008).

Moreover, IN is affected by several post-translational modifications. Acetylation by the Histone Acetyl Transferase (HAT) p300 increases its affinity for the DNA template (Cereseto, Manganaro et al. 2005). However, once acetylated, IN can be bound by KRAB-Associated Protein 1 (KAP1), a protein belonging to the TRIM family of antiviral proteins that induces IN deacetylation through the formation of a protein complex that includes

HDAC1 deacetylase; therefore KAP1 may be considered as a restriction factor (Allouch, Di Primio et al. 2011).

Ubiquitination reduces IN stability (Mousnier, Kubat et al. 2007), but is prevented by phosphorylation by c-Jun N-terminal kinase (JNK) and subsequent prolin-isomerization by the Peptidyl-Prolyl cis-trans Isomerase NIMA-interacting 1 protein (Pin1) (Manganaro, Lusic et al. 2010). Finally, IN sumoylation has been also proposed to be necessary in the pre-integration processes (Zamborlini, Coiffic et al. 2011).

4.2.2 Integration site selection

IN requires specific sequences at the end of the viral cDNA, whereas cellular DNA sequences that serve as integration target sites show only weak primary sequence specificity (Stevens and Griffith 1996, Carteau, Hoffmann et al. 1998, Wang, Ciuffi et al. 2007). Yet, integration into the host genome is not random: preferences for particular regions do exist and are different among retroviruses.

Based on a positive correlation between integration frequency and DNase I-hypersensitive sites, early studies of MLV integration suggested that open chromatin might favor this process (Vijaya, Steffen et al. 1986, Rohdewohld, Weiher et al. 1987). The first genome-wide study on the HIV-1 integration target site selection, performed a decade ago using high-throughput DNA sequencing, confirmed that HIV-1 strongly favors integration within active transcription units of the human genome, in gene-rich regions (Schroder, Shinn et al. 2002). The authors also found a group of genes that were upregulated following HIV-1 infection (confirming the results by Corbeil et al. (Corbeil, Sheeter et al. 2001)) and were more often targeted by the integration process. Since then, HIV-1 integration site selection has been studied in many cell types and integration in active transcription units was observed in all cases (Mitchell, Beitzel et al. 2004,

Lewinski, Bisgrove et al. 2005, Barr, Ciuffi et al. 2006, Lewinski, Yamashita et al. 2006, Brady, Agosto et al. 2009). Moreover, HIV integration appears to favor Alu elements (potentially because these repetitive sequences are enriched in gene-rich regions) (Kumar, Mehta et al. 2007), and to avoid the CpG islands present in the gene promoter regions (Mitchell, Beitzel et al. 2004).

Integration of the SIV shows a similar pattern of integration into active genes, suggesting that lentiviruses might have similar target preferences (Crise, Li et al. 2005). In contrast, MLV favors integration at promoter regions (Mitchell, Beitzel et al. 2004), transcription start sites (Wu, Li et al. 2003), DNase-sensitive regions and CpG islands (Lewinski, Yamashita et al. 2006).

To explain target site selection, different models have been proposed. A first model is based on the concept that open chromatin is more accessible to the PIC ("chromatin accessibility model"). This notion is supported by all the information gathered so far about flanking sequences and epigenetic features by genome-wide studies, as HIV-1 integrants are mostly found in active transcription units. However, should chromatin be the sole determinant of target selection, all retroviruses should integrate into the same accessible hotspots, which is not the case. As an example, MLV, but not HIV-1, prefers DNase-sensitive regions, considered as markers for open chromatin. Moreover, in vitro experiments have shown that nucleosome-packed DNA not only does not obstruct integration, but rather naked DNA is disfavored (Pryciak, Sil et al. 1992, Pryciak and Varmus 1992). Therefore, chromatin accessibility cannot be the main determinant of target site selection.

A second model to explain integration site preferences follows the discovery of a cellular protein (LEDGF/p75, the product of the PSIP1 gene) capable of binding both PIC-associated HIV-1 IN (Cherepanov, Maertens et

al. 2003) and chromatin (Maertens, Cherepanov et al. 2003). According to this model, LEDGF/p75 tethers the PIC to the chromatin regions at which it associates (De Rijck, Bartholomeeusen et al. 2010, Ferris, Wu et al. 2010, Gijsbers, Ronen et al. 2010, Gijsbers, Vets et al. 2011).

LEDGF/p75 was originally described as a transcriptional co-activator, p75 (Ge, Si et al. 1998); later, it was proven to be identical to a protein found in a screen for factors involved in lens epithelial cell growth and survival, namely Lens Epithelium-Derived Growth Factor (LEDGF) (Singh, Kimura et al. 2000, Nishizawa, Usukura et al. 2001); hence the double name LEDGF/p75 that is commonly used. LEDGF/p75 is involved in the modulation of stress-related gene expression (Llano, Morrison et al. 2009) and is ubiquitously expressed.

LEDGF/p75 binds tightly lentiviral IN proteins (from HIV, SIV and FIV) (Busschots, Vercammen et al. 2005) through a C-terminal IN-binding domain that interacts with the core domain of IN (Cherepanov, Maertens et al. 2003, Llano, Saenz et al. 2006, Llano, Vanegas et al. 2006, Busschots, Voet et al. 2007). On the other hand, LEDGF/p75 binds DNA through a N-terminal PWWP (Pro-Trp-Trp-Pro) chromatin-binding domain (Turlure, Maertens et al. 2006). A recent study based on the DamID (DNA adenine methyltransferase identification) technology unraveled the genome-wide distribution of LEDGF/p75, showing its association with transcription start sites (TSSs) of active transcription units, Pol2-bound genomic regions and active chromatin markers (H3 and H4 acetylation, H3K4 monomethylation), confirming the correlation between LEDGF/p75 genome-wide distribution and HIV-1 integration (De Rijck, Bartholomeeusen et al. 2010).

LEDGF/p75 depletion from cells does not abolish lentiviral integration completely (Vandekerckhove, Christ et al. 2006), but rather it results in diminished integration in the transcriptional units (Ciuffi, Llano et al. 2005) and a more random HIV integration pattern (Schrijvers, Vets et al.

2012). By combining integration site sequencing with transcriptional profiling of a LEDGF/p75 knock-down cell line, it was found that HIV-1 preferentially targets LEDGF/p75 modulated genes in the control cells, whereas this preference was abolished in the knocked-down cells (Ciuffi, Llano et al. 2005). The importance of LEDGF tethering function is reinforced by a retargeting study, where the PWWP domain was replaced by CBX1 domain, which associates preferentially with pericentric heterochromatin and intergenic regions. The chimeric LEDGF/CBX1 protein was able to redirect lentiviral integration into the CBX1-bound heterochromatic regions (Gijsbers, Ronen et al. 2010).

However, LEDGF/p75 depletion does not abolish completely HIV-1 integration in transcription units, suggesting that probably other factors may be involved in this process. More recently, Hepatoma-derived growth factor related protein (HRP-2) was found as a co-factor of HIV-1 IN in LEDGF/p75-depleted cells. Nevertheless, even if integration frequency into transcription units was reduced in cells in which both LEDGF/p75 and HRP-2 was knocked down, still, the integration distribution was not fully random (Schrijvers, De Rijck et al. 2011, Schrijvers, Vets et al. 2012).

Taking into account that retroviruses differ in their ability to infect dividing or non-dividing cells, a third model to explain integration site selection can also be envisaged, based on the interaction of the PIC with nuclear pore proteins. MLV PICs require the disassembly of the nuclear membrane to enter the nucleus, whereas HIV PICs are able to pass the NPC, therefore not requiring cell division. The involvement of NPC proteins in HIV-1 nuclear import and/or integration has so far been reported for a number of different nucleoporins. For example, Nup62 was recently shown to interact with HIV-1 IN and to contribute to chromatin binding and efficient integration (Ao, Jayappa et al. 2012). Other nucleoporins, such as

Nup85, Nup107, Nup133, and Nup160, which were found in genome-wide screenings for human genes that affect HIV-1 infection, have a less defined role in the viral life cycle (Brass, Dykxhoorn et al. 2008, Zhou, Xu et al. 2008). Two of the nucleoporins also identified in these screenings, Nup153 and Nup358/RanBP2, were explored in the context of both nuclear import and viral integration. Nup153, a protein of the inner nuclear basket, was shown to interact with HIV-1 IN (Woodward, Prakobwanakit et al. 2009) and Vpr (Varadarajan, Mahalingam et al. 2005) proteins. More recently, Nup153 was demonstrated to be responsible for the PIC nuclear import (Di Nunzio, Danckaert et al. 2012) via a viral capsid-dependent mechanism (Matreyek and Engelman 2011). Nup153 depletion from the cells reduced the tendency of HIV-1 to integrate into gene dense regions (Koh, Wu et al. 2013). Despite the ascertained role of nucleoporins in HIV-1 biology, the mechanisms by which these proteins could direct target site selection still remains elusive.

Interestingly, integrity of the capsid and the nuclear import pathway also appears to be important later for integration efficiency and gene targeting. Multiple evidence has been collected to support this conclusion: coumermycin-A1, a drug targeting HIV-1 capsid, impairs integration (Vozzolo, Loh et al. 2010); certain mutations, including N74D, impart different integration patterns into host chromosomes compared to wild type virus (Ocwieja, Brady et al. 2011, Schaller, Ocwieja et al. 2011); small amounts of capsid itself were detected inside the nuclear space (Zhou, Sokolskaja et al. 2011). It has been hypothesized that uncoating might occur inside the nucleus, through the NPC. According this hypothesis, TNPO3 might promote uncoating and displace residual capsid elements by facilitating their export (Zhou, Sokolskaja et al. 2011)

4.2.3 Integration and transcription

The choice of integration sites has strong consequences for basal viral transcription: indeed, this is sensitive to the chromosomal environment and proviruses can be transcriptionally repressed when integrated in heterochromatic regions. Thus, a strong correlation is expected to exist between integration site selection and the propensity of the virus to undergo latency, namely being maintained in a silent and repressed state (Jordan, Defechereux et al. 2001, Jordan, Bisgrove et al. 2003). Latent proviruses did show a slight preference for gene deserts, as opposed to active proviruses that are more likely found into short intergenic regions; since gene deserts are thought to be compacted into heterochromatin, this might contribute to silencing. However, genome-wide analysis of latent integrants showed no striking difference with actively transcribing proviruses, being poorly-expressed proviruses still integrated into highly expressed host genes (Lewinski, Bisgrove et al. 2005, Liu, Dow et al. 2006). In conclusion, based on these data, HIV-1 always integrates into actively transcribing genes; the transcriptional fate of the integrants is then determined successively by the specific chromatin microenvironment and, overall, by the activation state of the infected cell (for more information about transcriptional silencing and latency; see paragraph 4.3.4).

4.3 Transcription.

4.3.1 LTR

After integration into the host genome, the HIV-1 provirus starts transcribing viral mRNA upon activation of its LTR, which acts like a promoter (Rosen, Sodroski et al. 1985). LTRs are generated in their symmetrical configuration during the process of reverse transcription and appear as “repeats” only in the viral cDNA. From a functional point of view,

the LTR can be divided into one of four main regions: the core promoter region, the enhancer region, the modulatory region, the Trans-Activation-Responsive region (TAR).

1. The core promoter region, encompassing the TSS, exerts a positive basal effect on transcription. It contains a TATA box and three tandemly arranged binding sites for the constitutively expressed Sp1 transcription factor (Jones, Kadonaga et al. 1986). Both elements are necessary for basal level of LTR-driven RNA synthesis. As in other eukaryotic cellular promoters, the TATA box is specifically bound by the TBP (TATAA-Binding Protein) subunit of TFIID; mutations of this region result in a marked decrease of both basal transcription and viral replication (Garcia, Harrich et al. 1989). After binding of TFIID on TATA box, TFIIB is recruited, and in turn recruits RNA Pol II to the promoter, definitively establishing the location of the TSS.
2. The enhancer region mediates the transcriptional inducibility of the provirus in response to a variety of stimuli which trigger cellular activation and proliferation (Lusic, Marcello et al. 2003) (Siekevitz, Josephs et al. 1987, Chinnadurai 1991). Located upstream of the core promoter, the enhancer encompasses two partially overlapping binding sites for the inducible transcription factor NF-kB (Nabel and Baltimore 1987) and for STAT5 (Signal Tranducer and Activator of Transcription 5) (Selliah, Zhang et al. 2006), respectively. Noteworthy, also NFAT members can bind the same region of the NF-kB consensus sites, and likely play an important role, particularly in T cells (Kinoshita, Chen et al. 1998). NF-kB has been shown to stimulate both basal and Tat-mediated expression in activated T-cells (Nabel and Baltimore 1987, Siekevitz, Josephs et al. 1987).

3. The modulatory region, formerly called "negative regulatory element" contains several positive and negative regulatory elements, critical for modulating HIV-1 gene expression in response to various stimuli. This region is conserved among isolated HIV strains, and is bound by several cellular proteins such as LEF-1, Ets-1, USF, NFAT, c-Myb and COUP-TF (reviewed in (Pereira, Bentley et al. 2000)). The modulatory region has also been proposed to contain a Negative Regulatory Element (NRE), the deletion of which increases LTR-driven transcription and viral replication (Rosen, Sodroski et al. 1985).
4. The Trans-Activation-Responsive region (TAR) encompasses the 5'-terminal (nucleotides +1 to +59, numbering the TSS as +1) of all viral RNAs. Its importance was defined in concurrence with the findings of Sodroski and colleagues, who showed that LTR-driven expression was dependent on a 86-aminoacids viral product, a transactivating factor they named Tat, a 14 kDa protein conserved in the genomes of all primate lentiviruses (Sodroski, Patarca et al. 1985). To fulfill its functions, Tat binds a hairpin structure present at the 5' end of the nascent viral RNA, named as TAR; this region functions as an RNA sequence rather than as a DNA element. It folds into a highly stable, nuclease-resistant stem-bulge-loop structure which is essential for Tat-mediated LTR transactivation (Berkhout, Silverman et al. 1989) (Berkhout and Jeang 1989, Selby, Bain et al. 1989), as it is suggested by the fact that mutations that destabilise the stem by disrupting base-pairing abolish Tat-stimulated transcription. Furthermore, the TAR element was found to be functional only when placed in the 3' to the HIV-1 promoter and in the correct

orientation and position (Selby, Bain et al. 1989).

4.3.2 Tat

Tat is required for in vivo viral replication (Jeang, Xiao et al. 1999). Unique case among transcriptional activators, Tat functions via RNA rather than DNA promoter elements (Berkhout, Silverman et al. 1989) (Berkhout and Jeang 1989). After binding to TAR, Tat binds specifically to the cellular P-TEFb complex (Marshall and Price 1995, Zhu, Pe'ery et al. 1997, Zhou, Chen et al. 1998) (for comprehensive reviews on P-TEFb see (Bres, Yoh et al. 2008)). P-TEFb contains a cyclin component, cyclin T1, which can form a stable complex with CDK9, Tat and TAR RNA. The formation of the P-TEFb/Tat/TAR ternary complex is essential for recruitement of the processive RNA Pol 2 machinery at the LTR promoter (Bieniasz, Grdina et al. 1998, Zhou, Chen et al. 1998).

At the beginning of the transcription cycle, CDK7-mediated phosphorylation on Serine 5 of RNA Pol II CTD facilitates promoter clearance, but, shortly after initiation, the progression of Pol II is stalled by two negative elongation factors, namely NELF (Negative Elongation Factor) and DSIF (Dichloro-1-b-D-RibofuranosylBenzimidazole riboside (DRB)-Sensitivity-Inducing Factor) (for a review, see: (Ott, Geyer et al. 2011)). To overcome this checkpoint, Tat recruits P-TEFb to the stalled Pol II, forming the stable ternary complex with Tat/TAR/Cyclin T1. P-TEFb phosphorylates serines at the second position (Ser2) within each of the heptapeptide repeats that constitute the C-terminal domain (CTD) of the largest subunit of Pol II. The phosphorylated CTD serves as a platform for the assembly and functioning of different transcription and RNA processing factors. P-TEFb also phosphorylates DSIF and NELF, causing NELF dissociation and the conversion of DSIF into a positive elongation factor, finally allowing the production of full-length transcripts (reviewed in (Barboric and Peterlin 2005)).

It has recently been shown that, in addition to the CycT1/CDK9 core complex, Tat also associates with transcription factors/cofactors ELL2, AFF4, ENL and AF9 that together with some additional proteins form the so-called SEC (Super elongation complex). SEC has been shown to be involved also in activation of basal HIV-1 transcription in the absence of Tat, an event important for the reactivation from latency (He, Liu et al. 2010) (Sobhian, Laguette et al. 2010, Ott, Geyer et al. 2011).

4.3.3 Transcription and chromatin

It is well established that the HIV-1 promoter is structurally remodeled in order to allow efficient transcription (Verdin, Paras et al. 1993, El Kharroubi, Piras et al. 1998). Immediately after integration into the cell genome, and independently from the integration site, the proviral DNA, similar to cellular genes, is organized into a chromatin structure. The chromosomal integration packages the proviral DNA into specifically positioned nucleosomes (Verdin, Paras et al. 1993) (Van Lint, Emiliani et al. 1996).

The 5' LTR, independent from the integration site, is incorporated into two distinct nucleosomes, nuc-0 and nuc-1, precisely positioned with respect to cis-acting regulatory elements, and separated by a nuclease-hypersensitivity region (Verdin, Paras et al. 1993). This arrangement undergoes remodeling, when transcription from HIV-LTR is activated (Verdin, Paras et al. 1993, Marcello, Lusic et al. 2004). Interestingly, several genome-wide studies of nucleosome positioning have revealed that most RNA Pol II-transcribed genes carry a similar chromatin conformation, with nucleosomes precisely positioned at promoters that are remodeled during transcription activation by covalent epigenetic modifications on both DNA and histones (for a review see (Schones, Cui et al. 2008)).

Moreover, Tat establishes interactions with a vast number of cellular

partners that eventually stimulate transcription after rearrangement of the chromatin structure (Lusic, Marcello et al. 2003) (Marcello, Lusic et al. 2004). Indeed, after HIV-1 integration into chromatin, several transcription factors can recruit HDACs to the proviral LTR under basal conditions; once recruited, HDACs are able to deacetylate histone proteins locally, and lead to transcriptional silencing (Margolis, Somasundaran et al. 1994, Jiang, Espeseth et al. 2007, Marban, Suzanne et al. 2007). To counteract this mechanism, the viral Tat protein is able to induce the remodeling of the nucleosome arrangement downstream of the transcription-initiation site by recruiting to the LTR the transcriptional coactivator p300 and the closely related CREB-binding protein (CBP), (Marzio, Tyagi et al. 1998), P300/CBP-associated factor P/CAF (Benkirane, Chun et al. 1998, Lusic, Marcello et al. 2003), GCN5 (Lusic, Marcello et al. 2003), all three having histone acetyltransferase activity. Consequently, acetylation of both histones H3 and H4 occurs at discrete nucleosomal regions before the onset of viral mRNA transcription, relieving chromatin repression (Lusic, Marcello et al. 2003). HDAC inhibitor treatment is sufficient to activate silent, latent HIV (Van Lint, Emiliani et al. 1996, Savarino, Mai et al. 2009).

4.3.4 Transcriptional silencing: latency

Most of HIV-1 integrants are productive, and viral replication is completed within days. However, a small population of resting memory CD4+ cells can enter a transcriptionally inactive state, or “latent state”, as long as these cells remain quiescent (Perelson, Essunger et al. 1997). Latent reservoirs have an extremely long half-life and cannot be cleared even after several decades of antiviral therapy (Siliciano, Kajdas et al. 2003). Indeed, upon cessation of highly active antiretroviral therapy (HAART), viral replication rapidly resumes (Chomont, El-Far et al. 2009). Therefore, latency represents the main obstacle for HIV-1 purge, and understanding the mechanisms controlling silencing and reactivation of the provirus may have

strong implications for the study of HIV-1 pathogenesis.

HIV-1 latency was both documented in HIV-1 infected patients (Chun, Finzi et al. 1995, Finzi, Hermankova et al. 1997) and reproduced in T cell cultures. The latent reservoirs are established within days after acute HIV infection, and continuously repopulated during active viral replication; approximately 1 out of $1x10^6$ CD4+ T cells harbors a replication-competent but transcriptionally silent provirus (Chun, Carruth et al. 1997, Chun, Engel et al. 1998). Latency can occur both pre- and post-integration. Pre-integration latency is mainly due to incomplete reverse transcription (Zack et al. 1990); restriction factor APOBEC3G is able to inhibit both reverse transcription and then integration by modifying the linear cDNA substrate. (Bishop, Verma et al. 2008, Sloan and Wainberg 2011). Unintegrated latent genomes are labile and decay within 2 days, therefore it can be excluded that pre-integration latency is originating the long-term persistence of latently infected cell populations (Pierson, Zhou et al. 2002). A more recent study, however, has also identified a preintegration, inducible reservoir in HIV+ patients (Petitjean, Al Tabaa et al. 2007).

On the other hand, post-integration latency has been clearly detected in HAART- treated HIV+ patients; post-integration latency may be established in T cells infected during the decline of a T cell activating event (Chun, Carruth et al. 1997). In these memory CD4+ T cells, the provirus, which is successfully integrated into the host genome, is silenced but its transcription can be reactivated by a variety of stimuli, such as antigens, cytokines, mitogens or chemicals like phorphol esters (Chomont, El-Far et al. 2009).

As previously mentioned, remodeling of chromatin conformation is an important determinant of regulation of HIV gene expression (Hakre, Chavez et al. 2011). Histones on nuc-0 and nuc-1 in the HIV LTR are constitutively deacetylated in cellular models of latency, and HDAC inhibitors are able to

reactivate silent proviruses (Van Lint, Emiliani et al. 1996). As already mentioned in paragraph 4.3.3, HDACs can be recruited to the viral promoter through different transcriptional repressors such as Ying Yang 1 (YY-1)(Margolis, Somasundaran et al. 1994), late SV40 factor (LSF) (Coull, Romerio et al. 2000), the p50 subunit of NF-kB complex (Williams, Chen et al. 2006), the c-promoter binding factor CBF-1 (Tyagi and Karn 2007), the COUP-TF interacting protein CTIP2 (Marban, Suzanne et al. 2007). Also, transcription factors are able to recruit HDACs such as AP-4 (Imai and Okamoto 2006), c-Myc and Sp1 (Jiang, Espeseth et al. 2007). Deacetylated nucleosomes create a repressive environment that discourages binding of the transcriptional machinery to HIV-1 LTR.

This information highlights the importance of HDACs in establishing and maintaining HIV latency, and has prompted the start of clinical trials entailing the use of the HDAC inhibitor valproic acid in order to stimulate the latent reservoir and thus eradicate the remaining infected cells with normal therapeutics – with the so-called "shock and kill therapy" (Demonte, Quivy et al. 2004, Lehrman, Hogue et al. 2005, Routy 2005). However, adding valproic acid to HAART-treated patient did not ablate the latent HIV reservoir in patients (Savarino, Mai et al. 2009, Routy, Angel et al. 2012, Routy, Tremblay et al. 2012).

Another class I HDAC inhibitor, vorinostat (also known as suberanilohydroxamic acid and abbreviated as SAHA) was shown to induce virus production in vitro from resting CD4+ T-cells of HIV+ patients on ART with levels of plasma HIV RNA below the detection limit (Archin, Espeseth et al. 2009, Edelstein, Micheva-Viteva et al. 2009). This was reproduced in patients, where vorinostat was able to disrupt latency and HIV RNA expression was increased of about 5-fold upon vorinostat treatment (Archin, Liberty et al. 2012, Archin, Vaidya et al. 2012). Later, Rasmussen and colleagues performed a comparative analysis of the effect of various

HDACs inhibitors, each of which displaying a different degree in the stimulation of HIV-1 expression in latently infected cell lines. According their findings, the HDAC inhibitor panobinostat was the most potent, even in a very low concentration range, followed by givinostat, belinostat, vorinostat and valproic acid (Rasmussen, Schmeltz Sogaard et al. 2013). Entinostat is another HDAC inhibitor that is nowadays matter of investigation (Wightman, Lu et al. 2013).

Histone methylation also regulates post-integration latency. The histone methyltransferase (HMT) Suv39H1 is attracted to the viral promoter via CTIP-2 (Marban, Suzanne et al. 2007) and is able to add tri-methylation on histone H3 lysine 9 on silent HIV proviruses; this event recruits heterochromatin protein 1 HP1 thereby initiating or maintaining the formation of heterochromatin (du Chene, Basyuk et al. 2007). Similarly, another HMT, G9a, is able to deposit repressive di-methylation at histone H3 lysine 9, inhibiting basal and induced HIV-1 gene expression (Imai, Togami et al. 2010). These findings have suggested the possible use of HMT inhibitors in anti-HIV therapy, maybe in combination with HDAC inhibitors (Bernhard, Barreto et al. 2011, Bouchat, Gatot et al. 2012). Recently a third HMT, G9a-like protein GLP, was found to have a role similar to G9a (Ding, Qu et al. 2013).

Our group also provided evidence for an important role of Promyelocytic Leukemia Protein nuclear bodies (PML NBs) in the regulation of HIV-1 latency through the control of G9a and H3K9me2 deposition (Lusic, Marini et al. 2013). Indeed, we were able to show that silent HIV-1 provirus resides in close proximity to PML NBs; the presence of PML protein, able to interact with G9a, ensures the deposition of the repressive H3K9me2 along the viral genome. Upon activation, PML NBs are displaced from HIV-1 proviruses via an actin-dependent mechanism that set the virus free from

transcriptional repression. Arsenic, a drug triggering selectively PML degradation, is able to strongly transactivate the viral promoter, also in a primary model of latency (Lusic, Marini et al. 2013, Ott and Verdin 2013).

Tri-methylation of histone H3 lysine 27 and H2A ubiquitylation via Polycomb group protein may also play a crucial role in epigenetic silencing accounting for HIV-1 latency. Indeed, the knockdown of HMT enhancer of Zeste 2 (EZH2), a key component of Polycomb repressive complex 2 (PRC2), induced strong reactivation of the proviruses in a T cell model of latency (Freed 1998).

Finally, DNA methylation on HIV LTR may also be involved in post-integration latency. DNA methylation is a common repressive epigenetic mark that preferentially is deposited on CpG-rich sequences. The HIV promoter is methylated in latently-infected cells (Bednarik, Cook et al. 1990, Schulze-Forster, Gotz et al. 1990, Chavez, Kauder et al. 2011) and this correlates with its transcriptional repression: induction of viral gene expression results in demethylation of the LTR (Ishida, Hamano et al. 2006), whereas methylated CpG islands recruit the methyl-CpG-binding protein MBD2, that in turn can recruit HDACs (Kauder, Bosque et al. 2009). It has been shown that inhibition of cytosine methylation abrogates HDACs recruitment, contributing to viral silencing (Kauder, Bosque et al. 2009). However, according another study, such treatment provokes the opposite effect and it reactivates viral transcription (Pion, Jordan et al. 2003). Therefore, it has been suggested that the HIV-1 DNA methylation may be not uniform over all the integration events, but rather it might be affected by the local context depending on each integration site; according to such hypothesis, considering a population of cells with different integrants (each of them with a different methylation state) the global effect will result to be limited (Pion, Jordan et al. 2003).

The situation appears to be contradictory also in vivo. A study showed that

HIV DNA methylation is a rare event in HIV+ aviremic patients, suggesting that the effective role of methylation in latency is limited (Blazkova, Murray et al. 2012); another study performed in the same year, showed enrichment of DNA methylation in long-term non-progressor patients, suggesting that this modification may contribute to LTR silencing instead (Palacios, Perez-Pinar et al. 2012). Further work is clearly needed to better define this issue.

All together, the findings collected so far underline the importance of chromatin modeling and epigenetic regulation in the establishment and control of viral latency, and highlights the importance of these studies for the future development of viral eradication strategies.

5. Nuclear organization

As already mentioned, a key step in HIV-1 life cycle is its proper integration into the cellular genome, after entering the nucleus; chromatin structure is important both for integration site selection and, later, for the regulation of gene expression. However, the nucleus is not a homogenous organelle, but is extremely organized in subdomains and compartments. Thus, the actual position in the nuclear space of protein factors and DNA domains also needs to be taken into account when considering molecular regulation of HIV integration and expression (Figure 3).

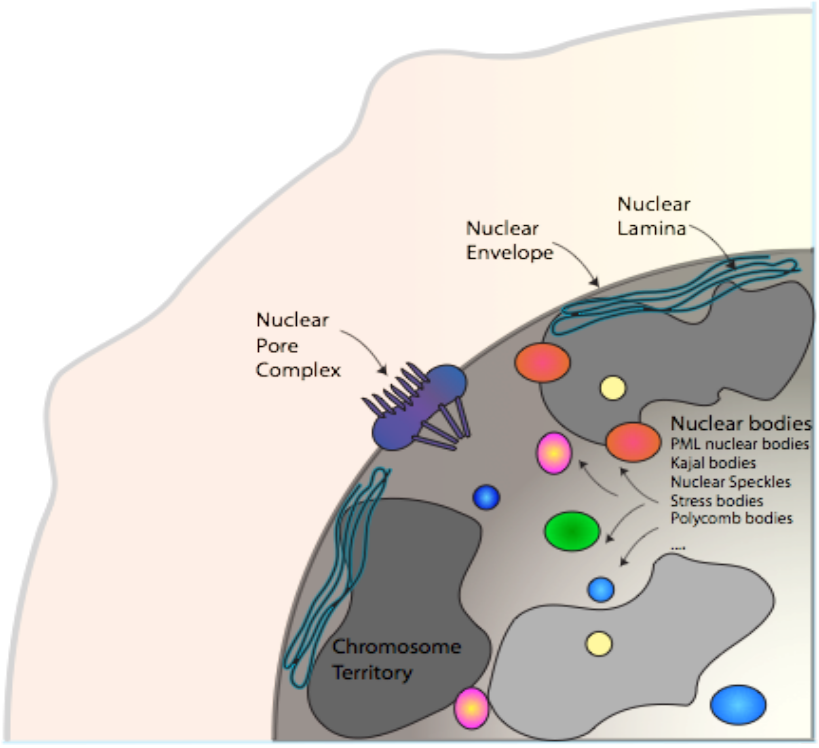


Figure 3. Nuclear organization.

The three-dimensional organization of the genome in the nucleus started to be revealed after the improvement of a few technologies, such as Fluorescent in situ Hybridization (FISH), Chromatin Immuno-Precipitation (ChIP) and its derivatives (ChIP on chip, ChIP-seq), Chromosome Conformation Capture (3C) and, more recently, the Dam-ID technique.

5.1 The concept of chromosome territory

The idea that each chromosome may occupy a specific territory in the nucleus is very ancient, dating back at the beginning of the 20th century when Theodor Boveri first hypothesized that, during interphase, chromosomes keep a peculiar positioning in the nuclear space; actually,

Boveri was the first to introduce the term of chromosome territory (CT) (Boveri 1909). It was only in the '70s that Thomas Cremer confirmed experimentally the existence of CTs, by using in situ hybridization techniques that later allowed detecting specific portions of the genome by laser confocal microscopy (Eils, Dietzel et al. 1996, Croft, Bridger et al. 1999) (Cremer, Cremer et al. 1982).

Specific parts of chromatin can be arranged in a non-random fashion, either in terms of radial distances or of neighborhood and proximity to other nuclear structures. Entire CTs were confirmed to keep specific positions of the nuclear space, and these positions were also found to differ strongly between different cell types, adding a new layer of complexity (Mayer, Brero et al. 2005). Whether radial-positioning matters is still under debate, with various studies arguing in favor and other against this possibility. The supporting and denying evidence is summarized in Figure 4.

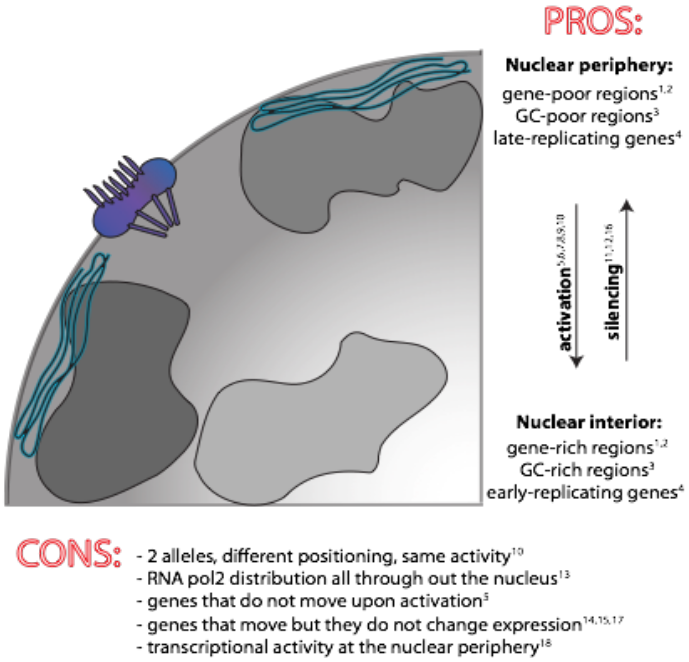


Figure 4. Summary of the main findings about the relationship between radial gene positioning and transcription.

Gene-dense, GC-rich regions, together with early replicating genes are often found in the nuclear interior, whereas the distribution of gene-poor and GC-poor regions, together with late replicating genes, positively correlate with nuclear periphery (Ferreira, Paolella et al. 1997, Croft, Bridger et al. 1999, Boyle, Gilchrist et al. 2001, Gilbert 2001). Moreover, a set of genes involved in the differentiation of various murine cell types move from periphery to the interior upon activation (Kosak, Skok et al. 2002, Hewitt, High et al. 2004, Williams, Azuara et al. 2006) (Ragoczy, Bender et al. 2006, Takizawa, Gudla et al. 2008), whereas movement of genes from the interior to the periphery correlates with silencing in yeast (Akhtar and Gasser 2007, Brown and Silver 2007).

However, in disagreement with the possibility that gene location is relevant, two alleles of the same gene might have different position but show the same transcriptional activity; RNA polymerase 2 does not show any accumulation at the interior but it is distributed throughout the nucleus (Wansink, Schul et al. 1993); many genes have been shown to move without change in expression, or to not move upon activation (Hewitt, High et al. 2004, Zink, Amaral et al. 2004, Meaburn and Misteli 2008).

Tethering experiments were performed in order to solve the problem, but they, in turn, also generated opposite results: some studies found that reporter genes are repressed when tethered to the periphery via emerin (Reddy, Zullo et al. 2008), other shows transcriptional activity even when genes were tethered to Lamin B (Kumaran and Spector 2008), still others showed different behaviors for different genes tethered to LAP2 (Finlan, Sproul et al. 2008).

All together, it is clear that different sets of genes show different behavior, probably due to the way the expression of these genes is regulated during development and differentiation. Moreover, probably the physical association with specific factors at the nuclear periphery is more important

than radial positioning itself (Takizawa, Meaburn et al. 2008). Indeed, nuclear periphery appears as a heterogenous compartment, playing opposite roles in the regulation of gene expression (for reviews, see: (Spector 2003, Cremer, Cremer et al. 2006, Fraser and Bickmore 2007, Sexton, Schober et al. 2007, Finlan and Bickmore 2008)).

5.2 Complexity at the edge of nucleus: the nuclear lamina

The nuclear envelope consists of a double lipid membrane with interspersed nuclear pore complexes (NPCs), acting as selective channels for nuclear import and export. In metazoan cells, the nucleoplasmic side of the nuclear envelope is covered by the nuclear lamina (NL), described already in the first studies employing nuclear electron microscopy (Fawcett 1966). NL consists of a dense network of filamentous proteins named lamins, which act in the first place as physical support for keeping the nuclear shape and structure (Gerace, Blum et al. 1978, Gerace and Blobel 1982, Gerace, Comeau et al. 1984). Lamins are divided into three groups (A, B, C) according to their molecular weight (Aebi, Cohn et al. 1986, Peter, Kitten et al. 1989, Vorburger, Lehner et al. 1989). The two major lamins in vertebrates, lamin A and C, derive from alternative splicing after transcription of the *LMNA* gene, whereas *LMNB1* and *LMNB2* genes encode B-type lamins (Lin and Worman 1993, Lin and Worman 1995, Machiels, Zorenc et al. 1996). Lamin expression is differentially regulated throughout development, where *LMNA* products seem to play a role in postnatal development, whereas *LMNB* genes are involved in embryogenesis (for a comprehensive review, see (Burke and Stewart 2013)).

Lamin proteins self-assemble first into polar dimer through “head-to-tail” interaction, and eventually into high order, not polarized structures (Heitlinger, Peter et al. 1991, Strelkov, Schumacher et al. 2004). Different

types of lamins form separate networks that only rarely overlap; moreover A- and B-type lamins have different disassembly and assembly properties during mitosis (Ottaviano and Gerace 1985, Aebi, Cohn et al. 1986, Glass and Gerace 1990).

Interestingly, at least a portion of lamins is nucleoplasmic in the interphase, and most likely they are not only the intermediate blocks for NL assembly, but also may play an active role in DNA replication and transcription. Whereas nucleoplasmic B-type lamins are static, A-type ones are highly mobile, even reinforcing the idea that they may play different functions (Shimi, Pfleghaar et al. 2008).

Beside their crucial role in mechanical stability of the nuclear shape, lamins are involved in nuclear membrane disruption and assembly during mitosis and in mitotic spindle formation; moreover lamins are involved in DNA replication by direct binding to factors at replication foci and, interestingly, also in DNA repair and telomere regulation: indeed *LMNA* mutation is linked to progeria, a premature aging syndrome (Cao and Hegele 2003, Mounkes, Kozlov et al. 2003).

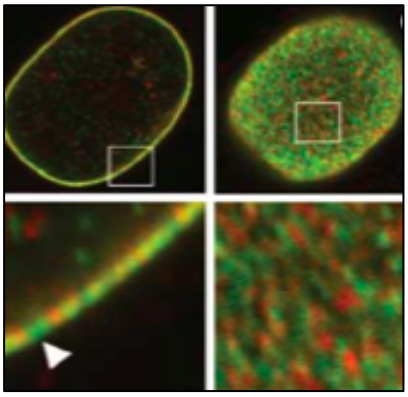


Figure 5. Networks of Lamin A/C (green) and B1 (red) observed by confocal immunofluorescence localization (adapted from Shim et al., 2008).

Finally, lamins have recently been described to also regulate transcription (Brown and Silver 2007). They can interact with transcription factors modulating their function either directly or via lamin-binding proteins such as emerin and LAP2β (Nili, Cojocaru et al. 2001, Shaklai, Amariglio et al. 2007). Gene-poor chromatin and inactive genes are often found in contact with the NL, suggesting that this region might represent a silencing environment that directly is in contact with part of the genome, both in yeast and in metazoans (Belmont, Zhai et al. 1993, Shaklai, Amariglio et al. 2007, Shevelyov and Nurminsky 2012). In fact, in some cases, tethering of genes to the nuclear periphery is associated with their silencing (Kumaran and Spector 2008, Reddy, Zullo et al. 2008).

Given that Chromatin Immunoprecipitation – Sequencing technique has never been efficient for Lamin B1 proteins (van Steensel and Dekker 2010), it was possible to detect which regions of the genome are associated with Lamin B1 only thanks to the development of DamID technique (van Steensel and Henikoff 2000; Vogel et al. 2007). Regions associated with Lamin B1 have been mapped in different cell types, first of all in Drosophila (Pickersgill, Kalverda et al. 2006) and later in human fibroblasts (Guelen, Pagie et al. 2008). Here, more than 1300 Lamin Associated Domains (LADs), 0.1-1 Mb in size, are distributed along chromosomes with a specific pattern, are mostly heterochromatic and contain repressed genes and gene-poor regions. The borders of LADs are characterized by the presence of the insulator protein CTCF and CpG islands. Interaction of chromatin with lamin has strong effect on the epigenetic modificiation of histones: LADs are enriched in silencing marks such as bi- and trimethylated H3K9 and trimethylated H3K27, whereas are devoid of H3K4met2 and Pol2 (Guelen, Pagie et al. 2008).

Lamin DamID approach was also applied to embryonic stem cells (ESCs), multipotent neural precursor cells and terminally differentiated astrocytes, in order to verify whether LADs might be different at different developmental stages. The different cell types were shown to have a similar but not identical nuclear architecture; changes in interaction with NL involve single transcription units and cluster of genes, without altering the flanking regions. Detachment from the NL renders silent genes more prone to transcription initiation, suggesting that NL may favor repression of specific subset of genes during development (Peric-Hupkes and van Steensel 2010).

The establishment of the LADs appears to occur during late anaphase, in mitosis. Lamin-Associating-Sequences (LASs), defined by the presence of GAGA motifs, direct the interaction of the surrounding regions with the NL. HDAC3 participates in the regulation of this process, via histone deacetylation that may facilitate the binding of other heterochromatic proteins (Zullo, Demarco et al. 2012). Consistently, it has been shown that forced hyperacetylation by trichostatin A (TSA) treatment reduces binding to NL and relocation from the nuclear periphery, suggesting that chromatin marks may represent the cause, rather than the consequence, of genome-NL interactions (Pickersgill, Kalverda et al. 2006). However, it seems that NL-associated HDAC3 may also contribute to maintain the silenced state of peripheral chromatin (Somech, Shaklai et al. 2005). Methylation of H3K9 was also found to be important for the NL anchoring of genes in C. elegans (Towbin, Gonzalez-Aguilera et al. 2012). A more detailed analysis carried out in single cells of human origin, showed that LADs contact NL in an intermittent way; each contact leads to transcriptional repression and deposition of H3K9 methyl mark. G9a, a H3K9 methyltransferase, appears to be one of the main regulators of LAD-NL associations (Kind, Pagie et al. 2013).

5.3 Complexity at the edge of nucleus: nuclear pores

The only means of access to the nucleoplasm during interphase, is through the NPCs, the largest protein complexes in eukaryotic cells (about 60 MDa in yeast and 90-120 MDa in humans). Mammalian nuclei contain approximately 3000-5000 NPCs per nucleus that are essential for trafficking between nucleoplasm and cytoplasm. Indeed, NPCs form selective channels allowing the diffusion of small molecules and regulating the transport of macromolecules with high specificity (Capelson and Hetzer 2009). NPCs were first observed almost 60 years ago, by pioneering work using transmission electron microscopy (Callan and Tomlin 1950, Gall 1954), which showed a structure with 8-fold rotational symmetry in the plane of the NE. Later, by scanning electron microscopy, the complex structure of NPCs was revealed: a membrane-embedded scaffold built around a central transport channel, a cytoplasmic ring, a nuclear ring and eight filaments attached to each ring; the filaments are attached to a distal ring to form the so-called nuclear basket (Beck, Forster et al. 2004, Alber, Dokudovskaya et al. 2007, Beck, Lucic et al. 2007, Frenkiel-Krispin, Maco et al. 2010, Maimon, Elad et al. 2012).

The NPC is composed of multiple copies of about 30 different proteins, called nucleoporines (Nups), (Cronshaw, Krutchinsky et al. 2002), that can be divided into several classes:

- transmembrane Nups, anchoring NPCs to the NE;
- core Nups, constituting the channel;
- FG-repeats containing Nups, providing for selective transport;
- peripheral Nups, initiating import/export of macromolecules.

The scaffold components are considered the most stable cellular structure (Daigle, Beaudouin et al. 2001, D'Angelo, Raices et al. 2009, Savas, Toyama et al. 2012, Toyama 2013), whereas many peripheral Nups (such as Nup153

and Nup98) are highly dynamic and shuttle between NPC and nucleoplasm (Rabut, Doye et al. 2004).

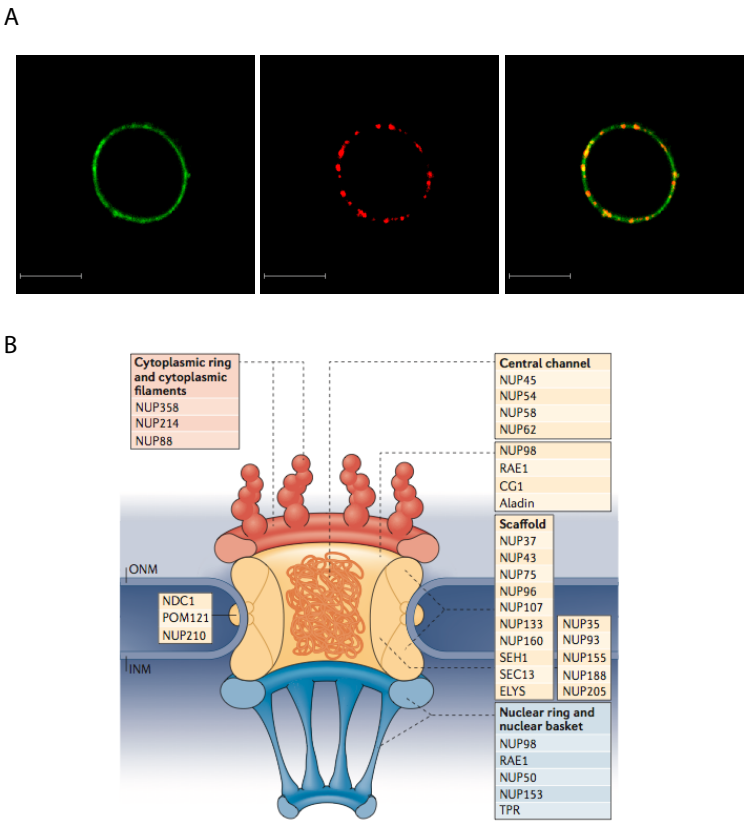


Figure 6. The nuclear pore complex. **A)** Immunofluoresce of T-lymphocytes nucleus with antibody for FG-rich nucleoporines (mAb414, stained in red) and antibody for Lamin-B1 (green). Bar: 5 µm. **B)** Schematic representation of the human nuclear pore complex (adapted from Raices et al., 2012)

The main function of NPC is the control of nucleocytoplasmic transport (Wente and Rout 2010). Ions and small molecules are able to passively diffuse through the NPC, whereas molecules larger than 40 kDa need to be actively transported by various protein factors (Feldherr and Akin

1997, Keminer and Peters 1999); the biggest cargoes transportable are 39 nm in diameter (Pante and Kann 2002). Translocation requires at least a transport signal that is encoded in the protein sequence (nuclear localization sequences or nuclear export sequences), and a shuttling receptor able to recognize that signal (Pemberton and Paschal 2005). These factors are needed to either bring cargoes to the NPC (directly or through adaptors) or facilitate the translocation across the channel. FG-repeats of the Nups constituting the inner channel form a dense meshwork that excludes macromolecules, representing therefore a "virtual gate"; however nuclear carriers often can bind FG-repeats containing Nups and can diffuse through the NPC, overcoming the barrier (Lim, Huang et al. 2006, Lim, Fahrenkrog et al. 2007). The directionality of transport through the NPC is driven by the formation of a Ran-GTP gradient that affects the abilities of nuclear carriers to interact with their substrate and changes their abilities to bind or release their cargoes (for a review see (Wente and Rout 2010)).

Besides this crucial role, NPC has been suggested to have several transport-independent functions, and in particular, it has been connected to gene regulation (Strambio-De-Castillia, Niepel et al. 2010). First hints about a connection between chromatin and NPC were obtained already in the '70s, when NPCs were described to be surrounded by decondensed chromatin regions (Engelhardt and Pusa 1972). Later, high-resolution images of mammalian nuclei confirmed a non-random association of decondensed chromatin with NPCs, suggesting a direct relationship between NPCs and active genes (Taddei, Hediger et al. 2004, Akhtar and Gasser 2007). This was further supported by the finding that Tpr, a nucleoporin of the inner basket (Cordes, Reidenbach et al. 1997), is needed to maintain a heterochromatin-free zone under the NPC (HEZ, Heterochromatin Exclusion Zones). Therefore, Tpr could indirectly affect the expression of genes

proximal to NPCs, by acting as a boundary and preventing the spread of heterochromatin in these regions (Krull, Dorries et al. 2010). A similar boundary activity was observed for Nup2, the yeast homologue of human Nup50 (Ishii, Arib et al. 2002). According to the so-called gene-gating hypothesis, proposed in the mid '80s, genome-NPC interaction would represent an advantageous coregulation of the two endpoints of nuclear gene expression, that is the production of mRNA and its eventual exit into the cytoplasm (Blobel 1985). Up to now, this idea was confirmed only in yeast, where a specific and functional interaction between nucleoporins and gene promoter (Nup-PI) was detected (Schmid, Arib et al. 2006). Interestingly, a genome-wide screening performed in Drosophila identified a portion of the genome (about 25%) that is bound to the orthologue of Tpr, Megator, and its binding partner Nup153 (Hase and Cordes 2003); these regions are decorated by chromatin marks typical for active transcription (Vaquerizas, Suyama et al. 2010). Silencing of Nup153 caused a decrease in transcription of genes within these NPC-associated regions, strengthening the idea of a functional role for the NPC-genome connection (Vaquerizas, Suyama et al. 2010).

In yeast, a subset of Nups plays a more direct role in transcriptional activation. *GAL* genes, necessary to metabolize galactose as an alternative carbon source, are usually dispersed randomly inside the nucleus when cells are grown in glucose. Upon galactose administration, all these genes are shifted to the nuclear periphery and associate with NPC components. Such repositioning has been proposed to promote efficient transcriptional activation (Casolari, Brown et al. 2004, Dieppois, Iglesias et al. 2006, Green, Jiang et al. 2012). Similar behavior was observed for other genes such as *INO1* (Brickner and Walter 2004) and *HXK1* (Taddei, Van Houwe et al. 2006). The recruitment to NPC involves specific DNA zip codes that target genes to the nuclear periphery (Ahmed, Brickner et al. 2010, Light, Brickner

et al. 2010, Brickner, Ahmed et al. 2012). Moreover, the movement has been shown to depend on the SAGA-TREX2 complex and THO-TREX complex (Cabal, Genovesio et al. 2006, Luthra, Kerr et al. 2007), which appear to be responsible for coupling of transcription and mRNA export to the cytoplasm (see (Rondon, Jimeno et al. 2010, Garcia-Oliver, Garcia-Molinero et al. 2012) for specific reviews).

Interestingly, in yeast, the strong transactivation caused by repositioning of genes to NPC might occur also through a local establishment of a DNA loop, an event considered as a booster for transcription. Gene loops are established by juxtaposition of promoter and terminator region, and HPC appears to be involved in this process (Laine, Singh et al. 2009, Hampsey, Singh et al. 2011). Especially interesting is the context of transcriptional memory, i.e. a gene "remembering" its previous transcriptionally active state (Tan-Wong, Wijayatilake et al. 2009). In yeast, short term repression (i.e., due to glucose administration) results in a retained gene loop at the NPC of *GAL* genes, through the nucleoporin Mlp1 (homologue of human Tpr); transcription factors are retained in the loop scaffold, whereas Pol2 leaves the template. Upon reinduction, Pol2 is able to load onto the promoter and begin faster transcription due to the retention of transcription factors on the gene loop, speeding up the production of mRNA. On the other hand, a longer term repression results in the abolishment of loop structure of *GAL* genes, and their dissociation from the NPC, with concomitant loss of associated transcription factors; therefore transcription will require de novo formation of the transcription complex, with slower kinetics of reinduction (Tan-Wong, Wijayatilake et al. 2009).

Recently, Nups have been shown to also be associated with mammalian genomes: for instance, Nup93 exhibits specific interaction with the genome, which was shown to be dependent on histone modification

(Brown, Kennedy et al. 2008). Another example is represented by Nup153 and Tpr (homolog of yeast Mlp1), which are necessary for the hypertranscription of the male X-chromosome in Drosophyla (Mendjan, Taipale et al. 2006). Moreover, in Drosophila cells, a pool of nucleoplasmics Nups have been shown to be associated with the genome and to be able to affect gene expression. In particular, these nucleoplasmic Nups were shown to specifically bind genes involved in differentiation and development, which undergo transcription induction, whereas, in contrast, peripheral Nups seem to rather bind either constitutively expressed or inactive genes (Capelson, Liang et al. 2010, Kalverda and Fornerod 2010, Kalverda, Pickersgill et al. 2010, Liang, Franks et al. 2013). This function of nucleoplasmic Nups may also have evolved in order to let Nups act on the genome throughout the nucleus, limiting the movement of the genes. Interestingly, no zip code has been described yet for the binding of mammalian genes to nucleoporines.

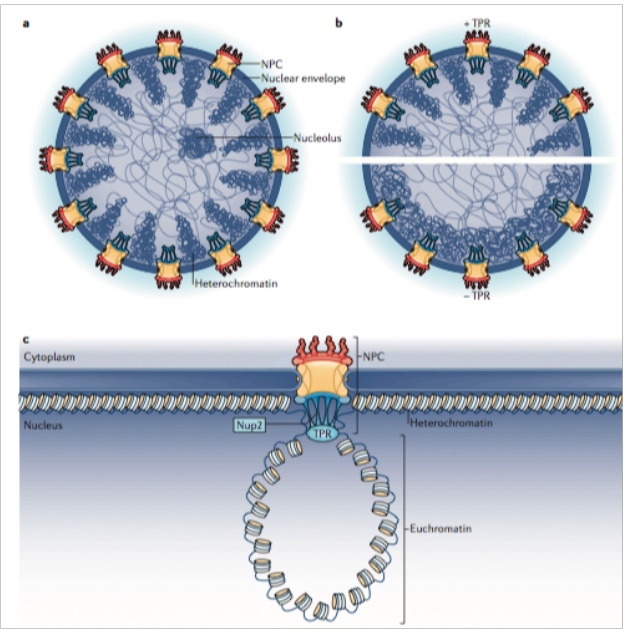


Figure 7. Tpr role in nuclear organization (adapted from Raices et al., 2012)

SYNOPSIS

One of the main events in the HIV-1 viral cycle is the integration into the host genome, mainly in CD4+ T-cells. Once integrated, HIV-1 exploits cellular mechanisms for fine-tuning of its gene expression. HIV-1 integrates preferentially in active genes and so far hundreds of integration sites have been sequenced in infections obtained in vitro or in samples derived from infected patients. Among these integration sites, some are targeted more often than others, representing Recurrent Integration Genes (RIGs); chromatin structure and nuclear topology may represent a determinant factor for target-site selection during the integration process. However, the encounter of the viral cDNA with the complex architecture of the mammalian nucleus has not been investigated so far in the context of integration site selection.

We have explored the role of nuclear topology in integration site selection and we show that HIV-1 preferentially integrates inside the active genes that are localized in the nuclear periphery. By exploiting 3D Immuno DNA FISH in activated human primary CD4+ cells we found that the most significant integration sites localize preferentially in the outer shells of the nucleus. Consistently, we also visualized the newly integrated provirus at the nuclear periphery in primary CD4+ T-cells, whereas the virus avoids the inner part of the nucleus. HIV-1 also avoids the Lamin Associated Domains at the nuclear periphery, as revealed by bioinformatics analysis.

In order to understand whether HIV-1 positioning at the nuclear periphery depends on the integration of the virus into the cellular genome, we applied 3D Immuno DNA FISH (Fluorescence In Situ Hybridization) on cells in which integration was blocked. We observed unintegrated virus dispersed

inside the nuclear space, confirming that HIV-1 positioning is integration-dependent and that integration takes place at the nuclear periphery.

Furthermore, by chromatin immunoprecipitation on primary infected CD4+ T-cells, we found that active HIV-1 genomic sequences bound by RNA Polymerase II are also bound by nucleoporines of the inner basket of the Nuclear Pore Complex (NPC). In particular, we found that Nup153, Nup98 and Nup62, already identified in several studies as important factors involved in integration process, bind the viral promoter directly, suggesting a direct connection between integrase action and the tethering of the virus to the proximity of the nuclear pores.

We also report that the nucleoporin Tpr is a novel interactor binding the HIV-1 genome. Interestingly, its association with the genome was only observed in a transcriptionally active population of proviruses, whereas it was lost in the case of silencing. We also verified that, when Tpr was silenced, latent proviruses strongly reduced their ability to transcribe, suggesting a functional role for HIV-1 association with NPC.

Taken together, our results demonstrate that due to nuclear topology, HIV-1 integration preferentially occurs into peripheral but active genes connected with the NPC, and that this proximity is exploited by the virus during its replication.

RESULTS

1. HIV-1 integration in $CD4^+$ T cells occurs in the nuclear periphery.

We wondered whether HIV-1 might localize preferentially in one nuclear compartment to the detriment of others. To explore this possibility we examined the nuclear position of the HIV-1 provirus in primary human $CD4^+$ T cells by 3D- FISH combined with immunostaining. $CD4^+$ T cells were extracted from fresh human blood of healthy blood donors (Manganaro, Lusic et al. 2010) and activated in vitro by stimulation with anti-CD3/CD28 antibodies. At 48 hours after stimulation, cells were infected with 1 μg p24 /1x10^6 cells of HIV-1 obtained from the Env- molecular clone HIV-1 $_{NL4-3/E-R-}$, which performs a single-round infection once pseudotyped with VSV-G and expresses a luciferase reporter gene in lieu of Nef (Connor, Chen et al. 1995). Four days after infection, most HIV-1 DNA was integrated into the host cell genome, as confirmed by real-time Alu-PCR amplification (Figure 8A). We confirmed that the integrated provirus was transcriptionally active by real time PCR analysis of viral mRNA (Perkins, Lusic et al. 2008) (Figure 8B).

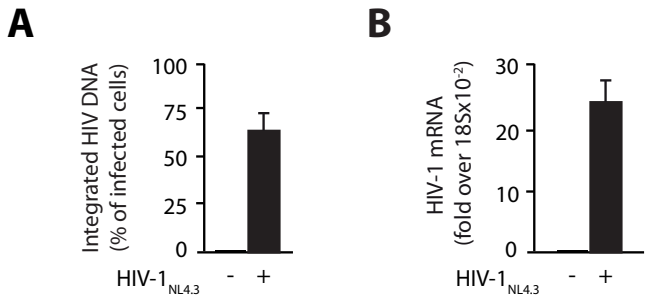


Figure 8. A) Quantification of integrated HIV-1 DNA by Real Time Alu PCR in activated CD4+ T cells, 4 days after infection with HIV-$1_{NL4.3}$. Values are means and s.e.m. of three experiments after normalization for the amount of total genomic DNA.

B) Quantification of HIV-1 mRNA by Real Time PCR in activated CD4+ T cells, 4 days after infection with HIV-$1_{NL4.3}$. Values are means and s.e.m. of three experiments after normalization for the amount of host 18S RNA.

After proper fixation, the nuclear envelope was stained using mAb414-antibody, which recognizes a family of nucleoporins characterized by the presence of phenylalanine-glycine (FG)-repeats; a specific probe recognizing HIV-1 was prepared. After hybridization and probe detection, FISH analysis was carried out as described in detail in the Material and Methods section. 3D-stacks of slides of fixed cells were captured by confocal microscopy and the HIV-1 signal-to-envelope distance and nuclear diameter were determined. Given that each cell might have a slightly different size, measurements were normalized over nuclear radius (r, defined as half of the middle of the mAb414-cy3 ring). Distances were binned in three concentric shells of equal surface area; it is assumed that each zone contains equal amount of genome. Zone 1 has a width of 0 (i.e. the nuclear envelope) to 0.19 x r; zone 2 corresponds to 0.19 x r to 0.43 x r; and zone corresponds 3 0.43 x r to 1 (i.e. the nuclear center) (Hediger, Neumann et al.

2002, Nagai, Dubrana et al. 2008, Schober, Ferreira et al. 2009), as shown in the scheme in Figure 9.

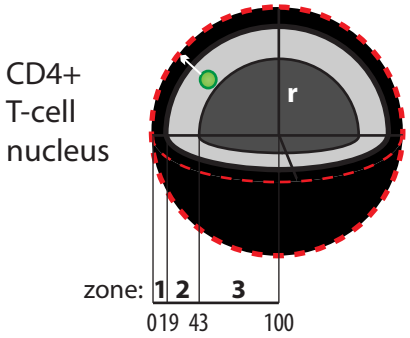


Figure 9. Scheme of the subdivision of nucleus into three concentric shells of equal surface (zone 1, zone 2, zone 3) (Hediger, Neumann et al. 2002, Nagai, Dubrana et al. 2008, Schober, Ferreira et al. 2009); after normalization over nuclear radius, FISH measurements were binned into these classes.

In the infected cells, 1-2 proviruses could be visualized per cell, as shown in the representative images displayed in Figure 10A; 160 proviruses were analyzed, collected in three independent experiments. The results obtained indicated that the vast majority of HIV-1 integrations were in zone 1, corresponding to the outer shell of the nucleus (75.2% of integration events were within 1 µm under the nuclear envelope); less than 5% of the integrations were in zone 3, corresponding to the inner portion of the nucleus (Figure 10B). We wanted to confirm this observation by infecting $CD4^+$ T cells with a virus carrying the $HIV\text{-}1_{Bru}$ wild type envelope. By analyzing 42 proviral integrations in three independent experiments, we observed that more than 90% of proviruses resided in zones 1 or 2 (Figures 10C and D).

Next, we used 3D Immuno-DNA FISH to estimate the number and position

of the proviruses harbored by individual $CD4^+$ T cells from two HIV-infected patients. Peripheral blood mononuclear cells (PBMCs) were obtained before the two patients initiated HAART, when their $CD4^+$ levels dropped below 300 cells/µl. We analyzed approximately 30 HIV-1-infected, $CD4^+$ T cells for each patient and found an average of 1.7 integrations per cell. Proviruses were found within 1 mm under the nuclear envelope (zone 1; ~50% of cases on average) or in zone 2 (~30% of cases), while more rarely in the central region of the nucleus (~20% of cases; representative images in Figure 10E and 10G and distributions in Figure 10F and 10H).

For both ex vivo and in vivo infected $CD4^+$ T cells, the distribution of proviral integration sites was significantly different from that expected from random integration according to chromatin distribution in the nucleus ($P<0.001$).

The selective localization of integrated HIV-1 DNA in the periphery of the nucleus suggested that nuclear topology is an important determinant of HIV-1 integration site selection.

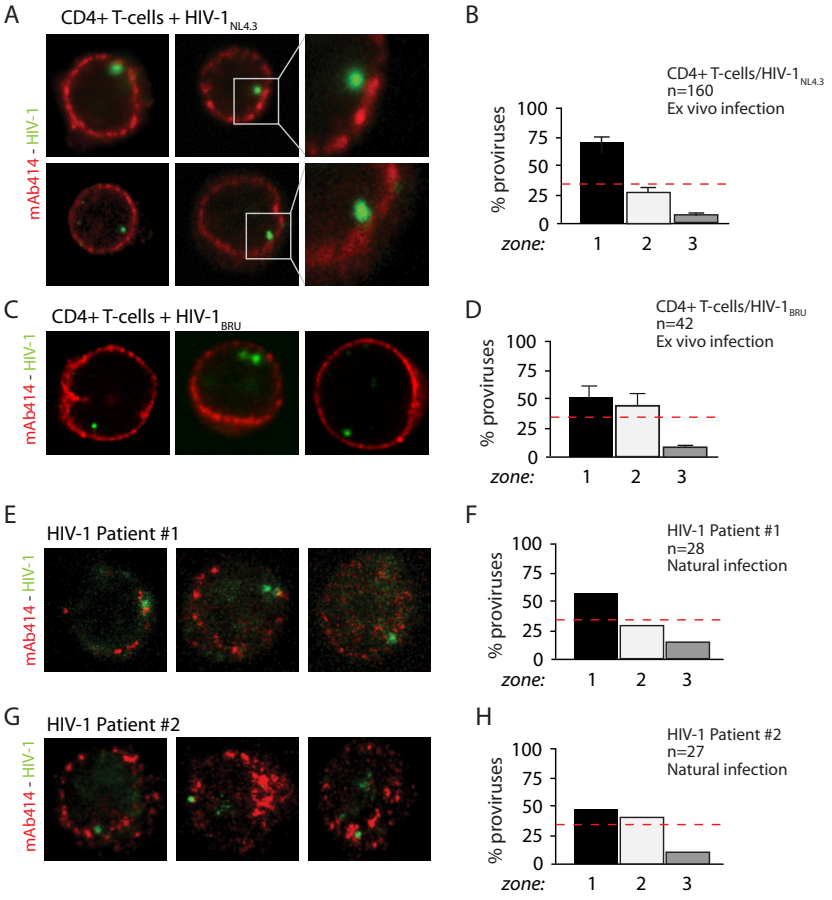


Figure 10. A-B) 3D Immuno-DNA FISH of HIV-$1_{NL4.3}$ (green) in activated CD4+ T cells stained for NPC (red), 4 days after infection; distribution of distances of proviruses from the nuclear envelope, normalized over nuclear radius (n = 160 proviruses measured in 3 independent experiments) and divided into the three concentric zones of equal surface. Evenly distributed random proviruses would be enriched equally in the 3 zones (33% each, indicated by a red dashed line).

C-D) 3D Immuno-DNA FISH of HIV-1_{BRU} (green) in activated CD4+ T cells stained for NPC (red), 4 days after infection, and distribution of distances of proviruses from the nuclear envelope, normalized over nuclear radius and divided into the three zones.

E-H) 3D Immuno-DNA FISH of HIV-1 (green) in CD4+T cells stained for NPC (red), collected from two different HIV+ patients, and relative distributions.

We checked the distribution of the proviruses also in other cell types, specifically in primary macrophages and in the monocytic cell line U937. Again, we found that HIV-1 localized in the external part of the nucleus also

in these cells (Figure 11). Therefore we concluded that the localization of the provirus is not cell-type dependent.

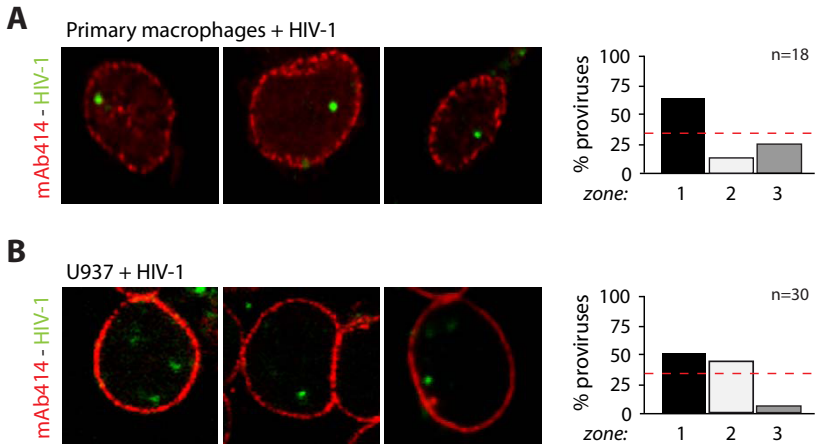


Figure 11. 3D Immuno-DNA FISH of HIV-1 proviruses (green) in primary macrophages **(A)** and monocytic U937 cell line **(B)** stained for NPC (red), 4 days after infection; distribution of distances of proviruses from the nuclear envelope, normalized over nuclear radius and divided into the three concentric zones of equal surface.

n = number of measured proviruses.

We decided to verify whether the peripheral localization is a feature of lentiviruses or rather a general propensity of retroviruses. Therefore we performed FISH on Jurkat T cells infected with an empty HIV-1 based lentiviral vector (Figure 12A) and an empty MLV gamma-based retroviral vector (Figure 12C). Whereas the lentiviral vector localized in the nuclear periphery, the majority of FISH signals derived from retroviral vector were observed in the second and third zones of the nucleus. We also tested a promoter-less lentiviral vectors, unable of transcribing; the vector behaved as a regular lentiviral vector with an active promoter (Figure 12B).

Therefore we concluded that transcriptional activity of lentiviral vector prior to integration does not seem to influence integration site selection.

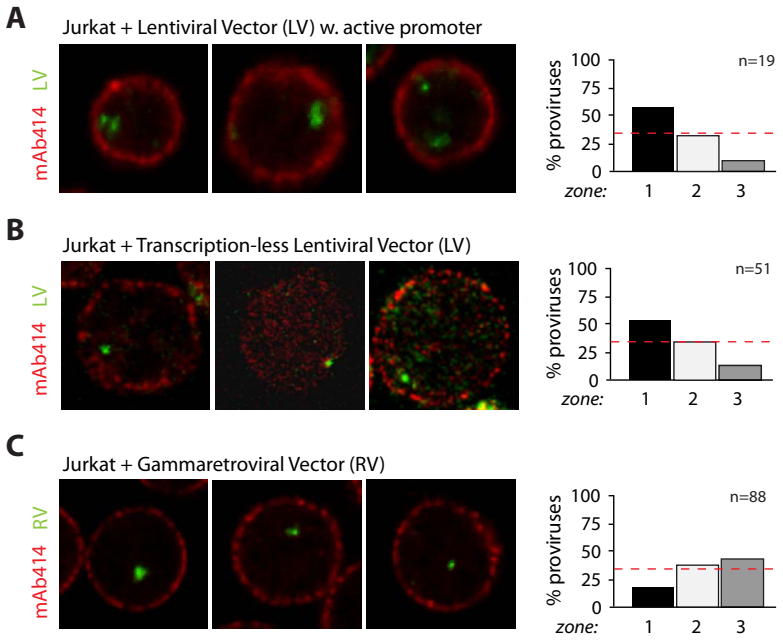


Figure 12. 3D Immuno-DNA FISH signals (green) in Jurkat T cells stained for NPC (red), 4 days after infection with lentiviral vector with active promoter **(A)**, transcription-less lentiviral vector **(B)** and gamma-retroviral vector **(C)**; distribution of distances of proviruses from the nuclear envelope, normalized over nuclear radius and divided into the three concentric zones of equal surface.

n = number of measured proviruses.

2. HIV-1 localization at the nuclear periphery depends on efficient integration.

Visualization of HIV-1 DNA by FISH, which is based on a probe

corresponding to the proviral DNA, is able to detect both integrated and episomal DNA. Thus, we wanted to assess the localization of HIV-1 DNA in conditions when integration is blocked. In order to address this issue, we impaired the integration process by using two different approaches:

1) targeting the viral integrase function directly;
2) targeting integration indirectly, through silencing of IN cellular cofactors.

To affect directly IN function, we used different strategies. On the one hand, we took advantage of two HIV-1 clones harboring single-point mutations in the integrase catalytic domain (class I IN mutations; IN(D64E) and IN(D116N)), which are unable to integrate into the host cell genome (Lu, Limon et al. 2005, Negri, Michelini et al. 2007). On the other hand, we inhibited integration of the HIV-1$NL_{4\text{-}3/E\text{-}R\text{-}}$ viral clone using raltegravir, a prototype of the first generation of clinically approved IN inhibitors (Hicks and Gulick 2009). Viral DNA in $CD4^+$ T cells was visualized by FISH at 4 days after infection with the two defective clones, or infected in the presence of raltegravir. Signal localization was determined with respect to the nuclear envelope (stained with an antibody against the NPC or lamin B1). Representative immuno-DNA FISH images are shown in Figure 13A. In all cases, only 10-20% of viruses were found in zone 1, as compared to 70% observed with the HIV-1$NL_{4\text{-}3/E\text{-}R\text{-}}$ viral clone in the absence of raltegravir. In the vast majority of the infected cells, most of the FISH signals were detected in zones 2 and 3 of the nucleus, hence distant from the nuclear envelope. As revealed by Alu-PCR assessment, the viral genome detected by FISH most likely did not correspond to integrated DNA (Figure 13B) but was highly enriched in not integrated, 2-LTR circles, as expected (Figure 13C).

Thus, HIV-1 positioning in the periphery of the nucleus requires functional

IN, whereas not integrated viral cDNA appears to be positioned randomly in the inner zones of the nucleus.

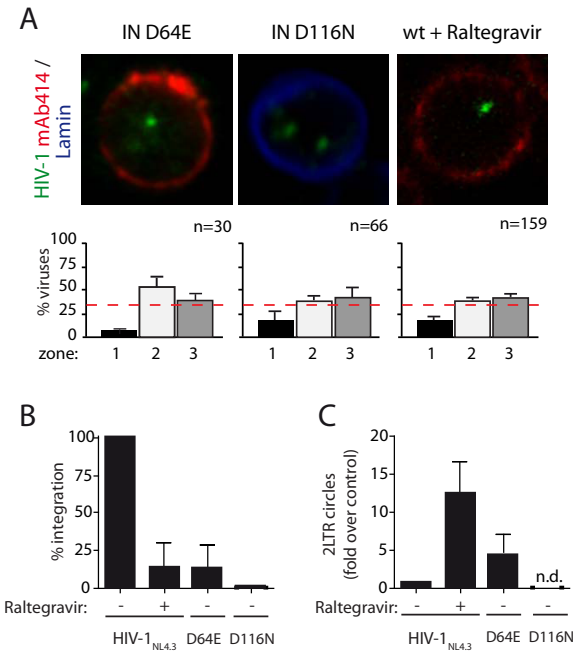


Figure 13. A) 3D Immuno-DNA FISH of HIV-1 (green) in activated CD4+ T cells stained for NPC (red) or lamin B1 (blue), 4 days after infection with mutant viruses IN(D64E) or IN(D116N), or cells infected with HIV-$1_{NL4.3}$ upon raltegravir treatment; on the right, distribution of distances normalized and divided as described above in the 3 zones for the three experimental conditions. **B)** Real time Alu PCR and C) 2 LTR quantification in activated CD4+ T cells 4 days after infection with mutant viruses IN(D64E) or IN(D116N), or cells infected with HIV-$1_{NL4.3}$ upon raltegravir treatment. Values are mean and s.e.m. of three experiments after normalization over control HIV-$1_{NL4.3}$ infection. n.d.: not determined. n = number of measured signals.

In parallel, we reduced the efficiency of viral integration by downregulating two cellular cofactors known to be involved in this process, namely a chromatin tethering factor LEDGF/p75 (Shun, Raghavendra et al. 2007) and

the inner nuclear basket protein Nup153 (Matreyek and Engelman 2011, Koh, Wu et al. 2012, Koh, Wu et al. 2013). Upon knock down of the two proteins by RNAi in Jurkat T cells, controlled by immunoblot (Figure 14A) and following infection with the HIV-$1_{NL4-3/E-R-}$ viral clone, we confirmed reduction in integration by Alu PCR (Figure 14B). By FISH, we observed a clear repositioning of viral DNA from the nuclear periphery (zone 1) to the inner portion of the nucleus (Figure 14C), confirming what we observed with the IN-defective viruses.

In the case of Nup153, the effect of the knock down was specific, since it was rescued, in U2OS cells, by the expression of a Nup153 cDNA devoid of the transcript 3'UTR and thus resistant to the siRNA used (Figure 15). We also performed the same experiment in Jurkat T cells (scheme of the experiment in Figure 16A); even if transfection efficiency was lower compared to U2OS cells, we could observe a rescue in the Nup153 level (Figure 16B) and HIV-1 integration level as well (Figure 16C). After silencing of endogenous Nup153, we stained Nup153-GFP transfected cells with anti-GFP to distinguish them in the population, and performed FISH for HIV using a red-fluorescent secondary antibody. As shown in Figure 16D, HIV signals detected in these cells are localized again in the nuclear periphery. Collectively, these results show that HIV-1 positioning in the periphery of the nucleus depends on efficient integration process, whereas episomal viral cDNA can reach inner zones of the nucleus.

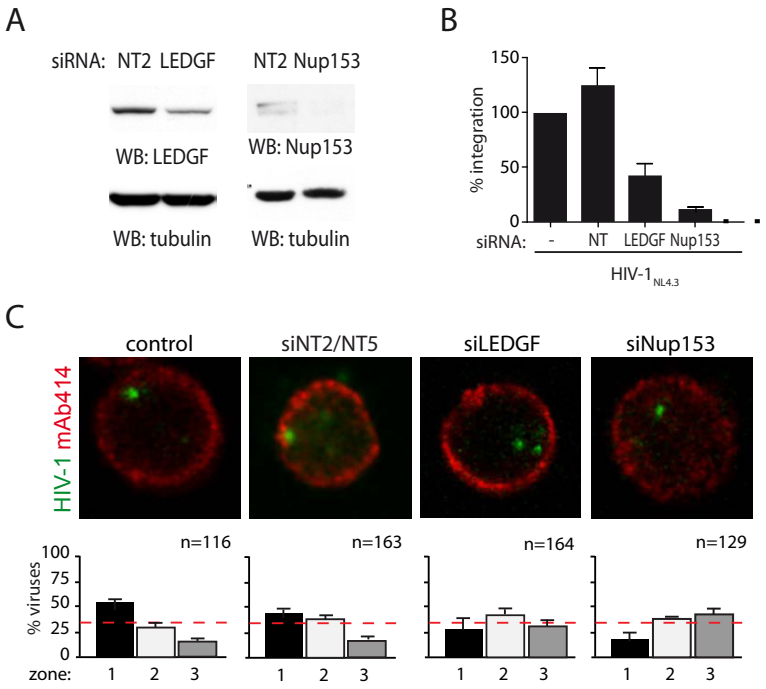


Figure 14. A) Western blot showing protein levels for LEDGF/p75 and Nup153 at the moment of infection, 36 hours after siRNA transfection. **B)** Real time Alu PCR in Jurkat cells infected with HIV-$1_{NL4.3}$ and previously transfected with a not targeting siRNA (NT) or siRNAs targeting LEDGF/p75, or siRNA targeting Nup153 as indicated. Samples were normalized over control infection.

C) 3D Immuno-DNA FISH of HIV-1 DNA (green) in Jurkat cells stained for NPC (red), 4 days after infection with HIV-$1_{NL4.3}$, in different conditions: no transfection (control), transfections with not targeting siRNA (siNT2/NT5), LEDGF siRNA (siLEDGF), or Nup153 siRNA (siNUP153). The graphs show the average results from 3 independent experiments. n = number of measured signals.

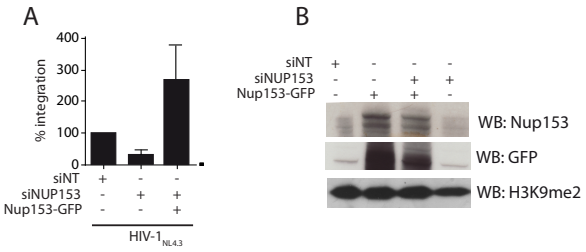


Figure 15. U2OS transfection with Nup153 plasmid. **A)** Alu PCR and **B)** western blot analysis of U2OS cells 48 hours after transfection with non-targeting siRNA (siNT), siRNA for Nup153 (siNUP153) and cells transfected with both siRNA for Nup153 and NUP153-GFP expression plasmid.

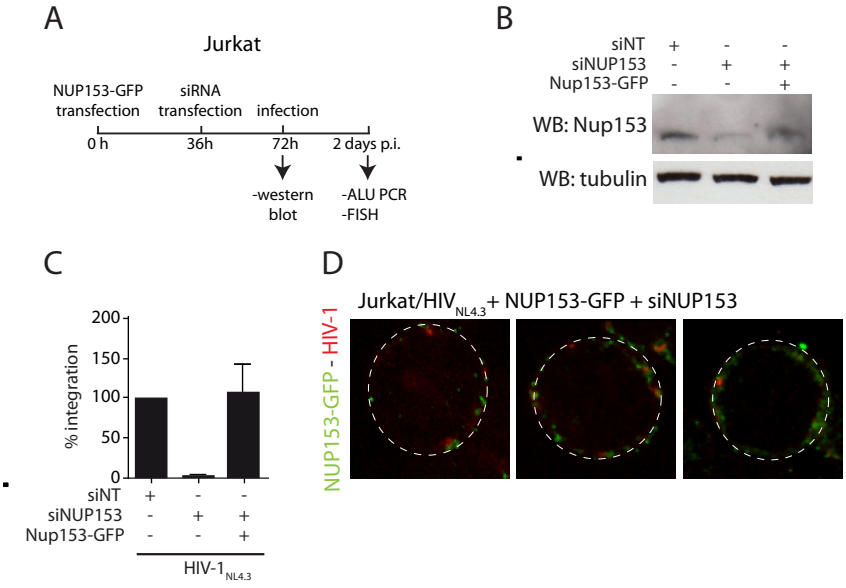


Figure 16. Jurkat transfection with Nup153 plasmid. **A)** Scheme of the experiment performed in Jurkat cells. EGFP-Nup153* contains the coding region for Nup153 tagged with EGFP, but is devoid of the 3'-UTR of the mRNA, which is the target of the anti-Nup153 siRNA#4. **B)** Western blot showing Nup153 protein level at the moment of infection. siNT: not targeting siRNA. **C)** Real time Alu-PCR in Jurkat cells 2 days after infection with HIV-$1_{NL4.3}$. Values are mean and s.e.m of three experiments after normalization over Jurkat transfected with a control, non targeting siRNA (siNT). **D)** Representative images of 3D Immuno-DNA FISH of HIV-1 DNA (red) in Jurkat cells transfected first with the EGFP-Nup153* expression plasmid and then with the siRNA#4, targeting endogenous Nup153. Distribution of HIV-1 FISH signals according to the three concentric zones in cells expressing EGFP.

3. HIV-1 Recurrent Integration Genes (RIGs) localize in the outer shell of the nucleus.

We next hypothesized that either the virus is able to integrate all over the nucleus and is repositioned to the nuclear periphery afterwards, or the integration takes place at the nuclear periphery; in the latter case, integration sites should be already positioned at the nuclear periphery before infection. We therefore analyzed the spatial position of some of the loci targeted by HIV-1 in primary, activated CD4+ T cells in the absence of HIV-1 infection. To select the most relevant loci, we cross-compared the lists of sequenced targeted sites derived from six different studies, as summarized in Table 1. These lists included two studies performed by in vitro infection of primary $CD4^+$ T cells from normal individuals (one from Brady et al. (Brady, Agosto et al. 2009) and the other generated by Mavilio and collaborators (F. Mavilio et al., unpublished data); one study from an in vitro infected $CD4^+$ T cell line (SupT1; (Schroder, Shinn et al. 2002)); and three lists obtained from HIV-1 infected individuals (Han, Lassen et al. 2004, Liu, Dow et al. 2006, Ikeda, Shibata et al. 2007). These lists comprise the vast majority of published HIV-1 integration sites into the genome in activated $CD4^+$ T cells (Table 1). Altogether, these lists contain 1136 HIV-1 integration sites into different, unique genes.

We started noticing that some of these hot sites were present in different lists. Therefore, by using a script written in R, we cross-compared the 6 lists in order to find overlaps. Interestingly, out of these 1136 genes, 126 were found to recur in 2 lists, 24 in 3 lists and 6 in at least 4 lists. These 156 genes with recurrent HIV-1 integration are hereafter named HIV Recurrent Integration Genes (RIGs) and are enlisted in the Attachment 1. The probability of detecting this number of recurrent genes by chance was highly

unlikely ($P<1x10^{-9}$) as estimated by computer simulation that randomly selected genes from a total of 25000 different genes and counted the number of genes selected more than once (Figure 17). According to this distribution around 20 genes in average can be selected by chance, with a maximum of 50; instead, 156 is very far from this distribution and very unusual.

List	Source	Nr. Published Sequences	Nr. Unique Intragenic Sites	Reference
Brady et al.	Primary, activated CD4+ T cells, in vitro infection	524	265	(Brady et al., 2009)
Mavilio et al.	Primary, activated CD4+ T cells, in vitro infection	638	329	Unpublished
Schroeder et al.	Sup T1, in vitro infection	642	294	(Schroder et al., 2002)
Liu et al.	PBMCs and tissues from HIV patients	42	32	(Liu et al., 2006)
Ikeda et al.	CD4+ T cells from HIV patients	463	158	(Ikeda et al., 2007)
Han et al.	CD4+ T cells from HIV patients	74	58	(Han et al., 2004)
TOTAL	1136			
Nr. Genes in 4 lists	6			
Nr. Genes in 3 lists	24			
Nr. Genes in 2 lists	126			
TOTAL NR. RECURRENT GENES	156			

Table 1. HIV-1 integration sites considered in this work. Out of the indicated numbers of sequences identified by the 6 considered studies, 1136 were within individual genes; of these, 156 recurred in 2 or more studies.

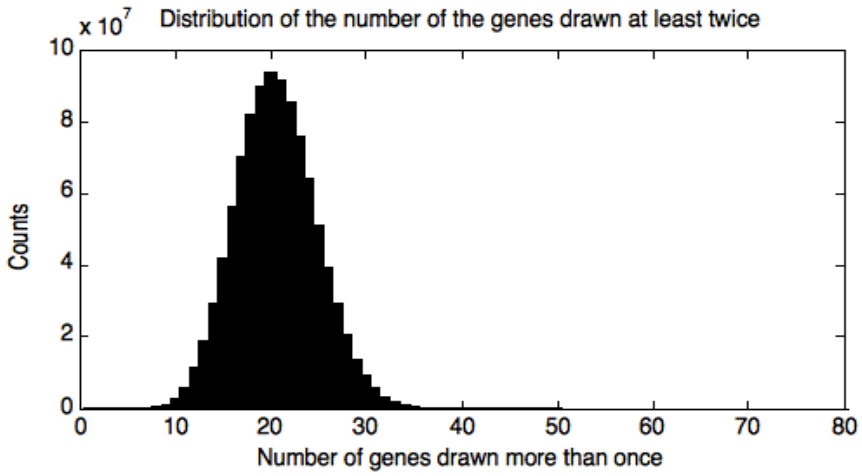


Figure 17. Distribution of the number of the genes drawn at least twice.

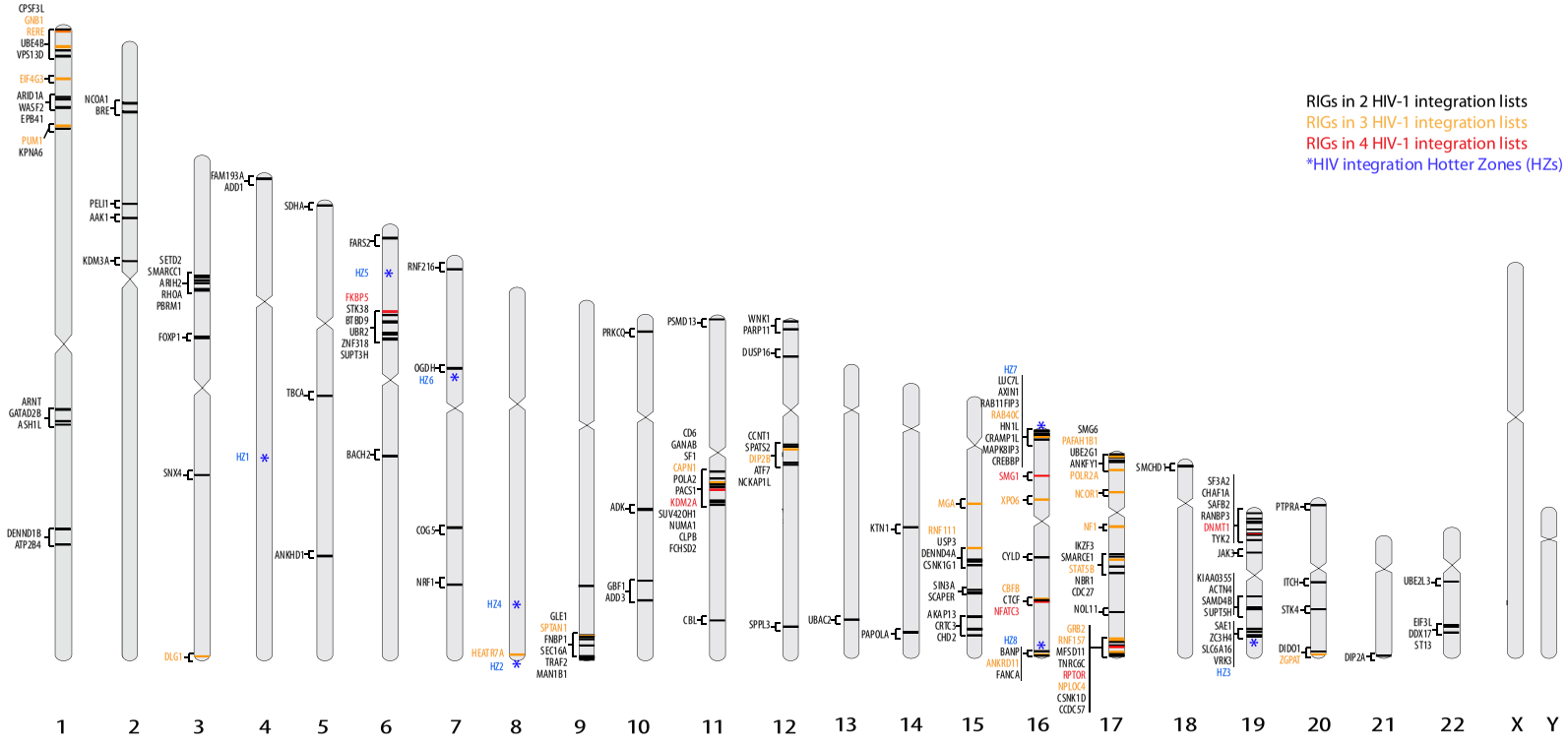
Interestingly, when we expanded our list to other ~12000 integration sites (Sherrill-Mix, Lewinski et al. 2013), 5221 of which were unique genes, we observed that RIGs were again highly targeted and represented more often than the others. Whereas other (non –RIG) genes were targeted 2.2 times in average, our 156 RIGs were selected with an average of 8.96 times (P value < 0.001). This value is significantly higher that an equally sized group of genes randomly selected from the integration sites list.

In addition we have analyzed data from recently published work by (Wagner, McLaughlin et al. 2014) (Maldarelli, Wu et al. 2014), in which a high number of integrants derived from HIV-1 patients were sequenced. More than half of the RIGs (83 out of 156) were confirmed to be found in a group of patients reported by Maldarelli et al. (P value < 0.001) (Maldarelli, Wu et al. 2014), whereas a lower, yet still significant number of RIGs (32/156) were found in the study performed by Wagner (Wagner, McLaughlin et al. 2014) (P value < 0.001). This analysis with in vivo

integrants further confirms that RIGs are a bona fide hottest spots of integration.

We decided to plot RIGs onto the human chromosome map using the Idiograph webtool (Kin and Ono 2007). On the same map, we also indicated the location of the "hotter zones" (HZs) defined by Bushman and collaborators, where integration density was found to be remarkably high ((Wang, Ciuffi et al. 2007); list of sites at: http://www.bushmanlab.org/tutorials/ucsc).

Interestingly, the chromosomal localization of RIGs was not randomly distributed along the chromosomes, but appeared to cluster into specific regions (Figure 18). In particular, multiple RIGs were present in chromosomes 1, 6, 11, 15, 16, 17 and 19, and the terminal parts of chromosome 9. In 5 out of 8 cases, these RIGs also were in proximity to the HZs. Even if clustering of these genes partially overlaps with the presence of gene-dense regions (therefore it is more likely to find genes in those regions), we wondered whether the spatial position of those portions of chromosomes might be localized in a particular compartment inside the nuclear space.


RIGs in 2 HIV-1 integration lists
RIGs in 3 HIV-1 integration lists
RIGs in 4 HIV-1 integration lists
*HIV integration Hotter Zones (HZs)
1
2
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6
7
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20
21
22
X
Y

Figure 18 (previous page). Human chromosome map showing the localization of 156 HIV RIGs. Genes found in 4, 3 and 2 HIV-1 integration lists are highlighted in red, orange and black respectively. Hotter genomic regions (HZs), favored for HIV-1 integration as described in (Wang, Ciuffi et al. 2007) are highlighted in blue and depicted by a star.

To assess the physical localization of RIGs and HZs inside the cell nucleus, we applied 3D Immuno-DNA FISH on primary $CD4^+$ T cells from healthy donors upon in vitro activation by CD3/CD28 stimulation. Twenty-one different bacterial artificial chromosomes (BACs) were used as FISH probes; 6 BACs corresponded to those RIGs that were present in 4 lists; 4 BACs corresponded to those genes that were present in 3 lists, and which were found to be relevant HIV-1 integration sites in natural infections (samples derived from HIV infected individuals); other 4 BACs were from the HZs; finally, 7 BACs mapped to control genes that are not targeted by HIV-1. Each BAC clone ID used for this study can be found in the Attachment 1. For each BAC used the surrounding genomic region was analyzed; in the proximity of the regions covered by BACs within 1 Mb there were other 44 RIGs or integrants meaning that these selected regions were indeed the hot zones for integration. Moreover, 10 Mb surrounding the selected BACs contained additional 125 integration sites.

The radial position of each allele visualized by BAC hybridization was measured along the axis between the center of the nucleus and the nuclear edge; as in Figure 9, the nucleus was subdivided into three concentric shells of equal surface area. Representative FISH images and the corresponding distribution graphs are shown in Figure 19A for the 14 BACs corresponding to the analyzed RIGs and HZs. For most of the probes, there was a clear gradient in the allele localization, which decreased from the nuclear envelope towards the center of the nucleus; hybridization signals were rarely observed in the inner portion of the nucleus (zone 3).

We then asked what was the spatial distribution of the genes that were not

reported to be target of HIV integration neither in the six analyzed HIV-1 integration lists in CD4+ T cells nor inside the HZs. We selected 7 genes expressed in CD4+ T cells (*CD28*, *CD4*, *GAPDH*, *HEATR6*, *PACS2*, *KDM2B*, *ACTN1*; http://biogps.org (Su, Wiltshire et al. 2004) (Wu, Orozco et al. 2009)). According to the published transcriptomic datasets, no statistical difference in the expression levels was observed between HIV RIGs and control genes, whereas both groups were significantly more expressed than random genes ($P<0.005$). We measured the radial distances for a total of 522 alleles (the number of alleles for each gene/loci is indicated in Figure 19B, along with representative images).

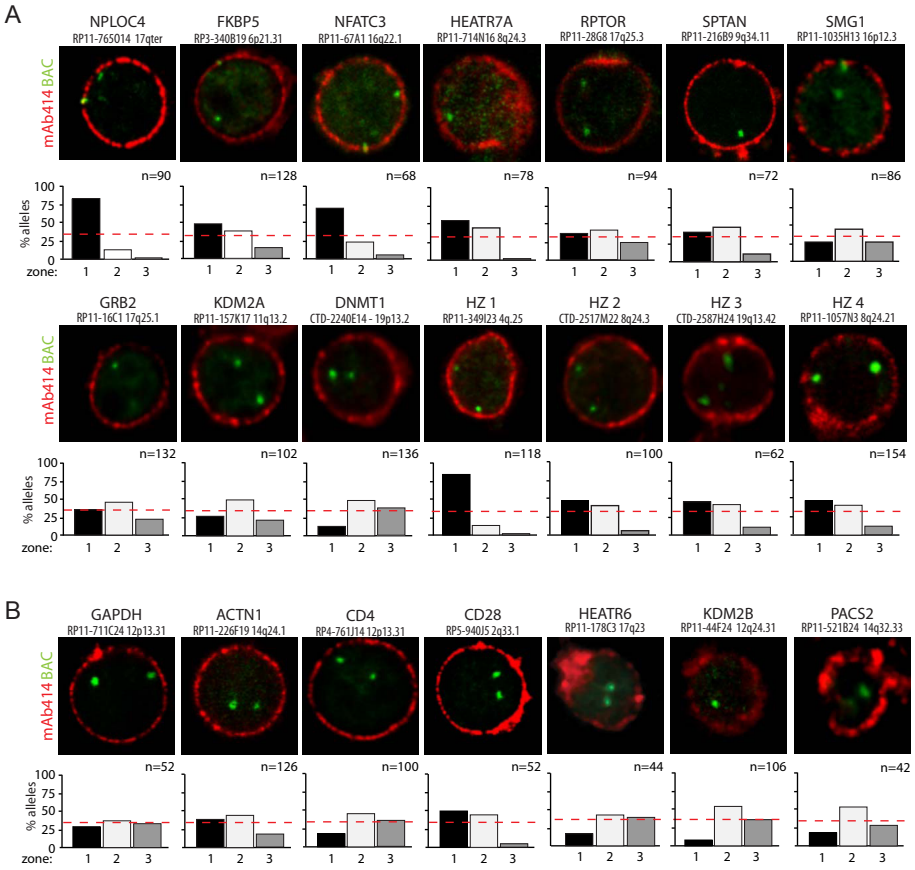


Figure 19. Localization of HIV RIGs at the nuclear periphery. **A)** 3D Immuno-DNA FISH of 10 HIV RIGs and 4 HZs in activated CD4+ T cells (green: BAC probe-DIG-FITC-labeled; red: NPC staining by mAb414). Distances of alleles from the nuclear envelope were normalized and divided into the three concentric zones (shown in Fig. 1B) for each of the following RIGs and HZs: NPLOC4 (n=90), FKBP5 (n=128), NFATC3 (n=68), HEATR7A (n=78), RPTOR (n=94), SPTAN (n=72), SMG1 (n=80), GRB2 (n=132), KDM2A (n=102), DNMT1 (n=136), HZ1 (n=118), HZ2 (n=100), HZ3 (n=62), HZ44 (n=154). Evenly distributed random genes would be enriched equally in the 3 zones (33% each, indicated by a red dashed line).

B) 3D Immuno-DNA FISH and measurements in activated CD4+T cells of each of the following control genes: GAPDH (n=52), ACTN1 (n=126), CD4 (n=100), CD28 (n=52), HEATR6 (n=44), KDM2B (n=106), PACS2 (n=42).

Figure 20 summarizes the above-described findings. Out of the 1420 RIG alleles that were overall analyzed, 44% mapped in zone 1, 41.5% in zone 2 and only 14.5 % in zone 3. Taking into account that the average nuclear

diameter of $CD4^+$ T cells is ~7 µm, this analysis demonstrates that 63% of RIGs and HZs alleles were concentrated within ~1 µm below the nuclear membrane (as indicated in Figure 20 with a dashed line). The global distribution of control genes was instead significantly different: overall, 25.6 % of alleles were found present in zone 1, 47.6 % in zone 2, and 26.8 % in zone 3. The percentage of control gene alleles found within 1 µm from the nuclear envelope was 45.5% (P<0.01).

These results show that genes that are recurrently found as integration sites for HIV-1, are localized preferentially at the nuclear periphery; this observation is compatible with the previous finding that HIV is localized very close to the nuclear envelope.

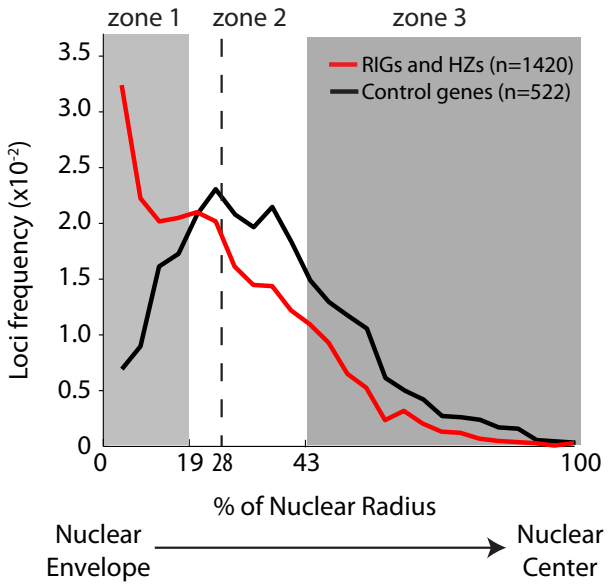


Figure 20. Distribution of the relative distances of all measured alleles (HIV RIGs and HZs: n=1420, control genes: n=522) from the nuclear envelope. Gray areas in the background mark the three zones. The dashed line indicates the approximate distance of 1µm from the nuclear edge of a CD4+ T cell nucleus.

Of interest, available information indicate that, despite most of the HIV-1

targets are common in different cell types of blood cells, there are however subtle differences for a few genes. For example, in contrast to $CD4^+$ T lymphocytes, HIV-1 almost never targets the *IKZF3* locus in $CD34^+$ hematopoietic progenitor cells (Cattoglio, Facchini et al. 2007, Cattoglio, Pellin et al. 2010) ($P<1x10^{-12}$), whereas the *TAP2* gene from the chromosome 6 MHC class II locus is never targeted in $CD4^+$ cells while it is highly targeted in $CD34^+$ cells ($P<1x10^{-13}$). To test whether this remarkable difference reflects a different topographic localization of the two loci in the two cell types, we decided to apply 3D immuno-DNA FISH to primary, cord blood $CD34^+$ cells and peripheral blood, activated $CD4^+$ T cells. Strikingly, we observed that the *IKZF3* locus was preferentially localized in zones 1 and 2 in $CD4^+$ cells (>80% of alleles), whereas it was almost absent from zone 1 in $CD34^+$ cells (<6% alleles; $P<0.001$). Conversely, the *TAP2* locus was absent from zone 1 in $CD4^+$ cells (<8% of alleles), whereas it was distributed between zones 1 and 2 of the nucleus in $CD34^+$ cells (>90% of alleles; $P<0.001$ Figure 21).

Together, these data clearly indicate that the cellular genes that are more often targeted by HIV-1, prior to HIV-1 infection, are preferentially located in the outer shell of the nucleus, further underlining the importance of a specific nuclear topology in HIV-1 integration site selection.

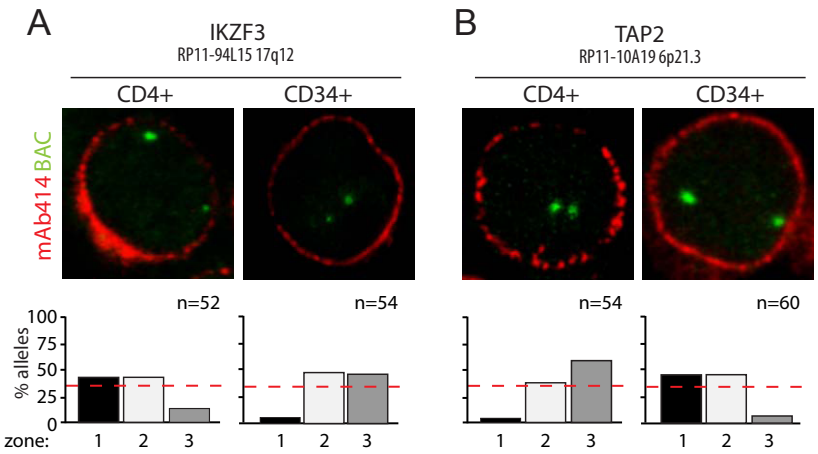


Figure 21. Representative images of 3D Immuno-DNA FISH of IKZF3 (A) and TAP2 (B) genes (green) in $CD4^+$ T cells and $CD34^+$ hematopoietic stem cells, stained for NPC (red), and the corresponding distributions of distances of alleles (IKZF3 n=54, TAP2 n=60) from the nuclear envelope, normalized and divided into the three analyzed concentric zones.

4. RIGs are transcriptionally active genes at the nuclear periphery.

It is well accepted that HIV-1 integrates into actively transcribing genes, thus suggesting that RIGs also pertain to the class of actively transcribed genes. However, nuclear periphery is mostly an heterochromatic compartment, where most of genes are in a repressed, silenced state. Therefore, we wanted to assess whether the selected RIGs were actually transcriptionally active genes.

We took advantage of the available information, obtained by ChIP-seq experiments, on the association of specific genomic sequences with RNA Pol2 and selected chromatin marks in $CD4^+$ T cells, for active (H3K36me3, H3K4me3, H4K16Ac, and H4K20me1) and repressed transcription

(H3K9me2, H3K9me3, H3K27me3) (Barski, Cuddapah et al. 2007, Wang, Zang et al. 2009). We first assessed the RNA Pol2 distribution over the transcription start site (TSS) region of our RIGs and cold genes, and compared this distribution with those found in the one thousand most expressed (active) and one thousand least expressed (silent) genes in $CD4^+$ T cells, using data from the GNF SymAtlas (Su, Wiltshire et al. 2004), as described in (Barski, Cuddapah et al. 2007). We found that the RNA Pol2 associated with RIGs with a pattern superimposable to that of active genes, peaking at the TSSs. In contrast, no RNA Pol2 was detected on the cold genes, where HIV-1 never integrates (Figure 22A). In an analogous manner, the distribution of K9-acetylated histone 3 (H3K9ac) was identical in RIGs and active genes, showing two peaks neighboring the TSS (Figure 22B). Other markers of chromatin correlating with active transcription, such as H3K36me3, H3K4me3, H4K16Ac, and H4K20me1, were also found enriched on HIV RIGs similar to their distribution on genes active in $CD4^+$ T cells (Figure 22C, 22F and 22G respectively). Of potential interest, a distribution superimposable between active genes and RIGs was also observed for the H3K4me2 histone mark, which was shown to be enriched at the LAD borders together with RNA Pol2 (Figure 22E; (Guelen, Pagie et al. 2008)). On the other hand, markers of facultative (H3K9me2) and constitutive (H3K9me3 and H3K27me3) chromatin were found enriched both on cold genes (where HIV-1 never integrates) and on silent genes in $CD4^+$ T cells, but not on RIGs (Figure 22H, 22I and 22J respectively).

In summary, HIV RIGs are genes that despite being localized preferentially at the nuclear periphery match the typical profile of transcriptionally active genes, being decorated by histone marks of open chromatin.

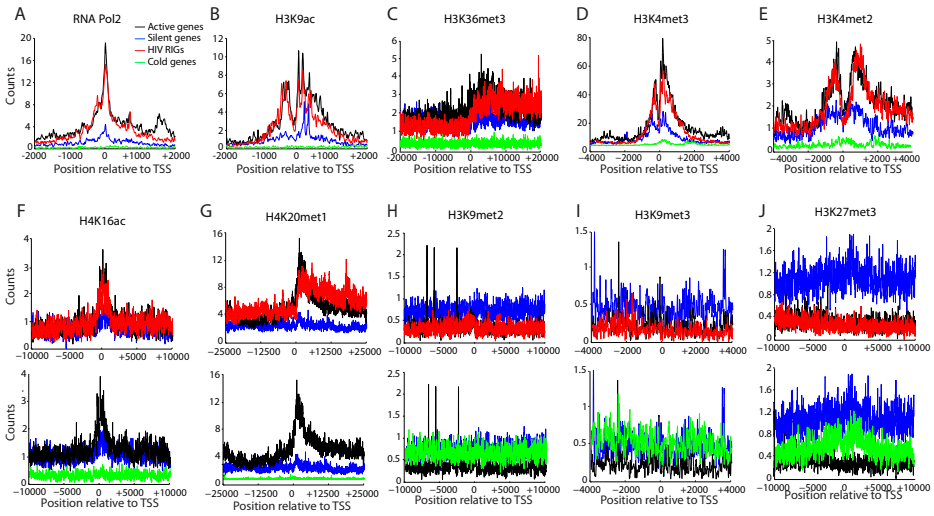


Figure 22. ChIP-seq profiles for HIV RIGs, cold genes and controls. A-J) Profiles of Pol2, histone methylation and acetylation indicated above each panel, around the TSS for HIV RIGs (red) and cold genes (green), compared to highly active (black) and silent (black) genes in activated CD4+ T cells. HIV RIGs show profiles similar to those of active genes, whereas the cold genes profiles match the ones of silent genes. Data are from Barski et al., 2007 and Wang et al., 2009.

5. HIV RIGs are excluded from the nuclear Lamin Associated Domains (LADs).

Our FISH analysis showed that both HIV-1 proviral DNA in infected cells and recurrent HIV-1 integration genes, in the absence of infection, preferentially reside in the nuclear periphery. Since HIV-1 preferentially integrates into transcriptionally active genes, we wondered what was the relationship between RIGs and nuclear LADs, which, on the contrary, are known to contain approximately 4000 transcriptionally inactive genes and to be enriched in heterochromatin (Guelen, Pagie et al. 2008, van Steensel and

Dekker 2010).

By a script written in R, we first compared the list of genes know to be present within LADs (Guelen, Pagie et al. 2008) with the list of HIV RIGs, as well as with each individual integration list used in this study. We found that more than 90% of HIV-1 RIGs laid outside the LADs, in contrast to what could be expected from a random gene distribution ($P<0.001$ considering all RIGs together; Figure 23A). When considering each individual HIV-1 integration site list independently, there was a highly statistically significant difference from random integration in all cases except one. The same trend was observed for five out if six individual integration lists. Finally, we analyzed a group of 61 genes, which we termed "cold genes"; these genes are never targeted by HIV-1 (A. Recchia and F. Mavilio, unpublished data) and are transcriptionally inactive in $CD4^+$ T cells (according to the published transcriptomic datasets). We found that 80% of these genes resided inside the LADs ($P<0.001$). As shown in representative immuno-FISH images for three of these genes (CNTN4, GPC5 and PTPRD), the localization of >90% of alleles was confirmed to be in zone 1, close to the nuclear envelope (Figure 23B).

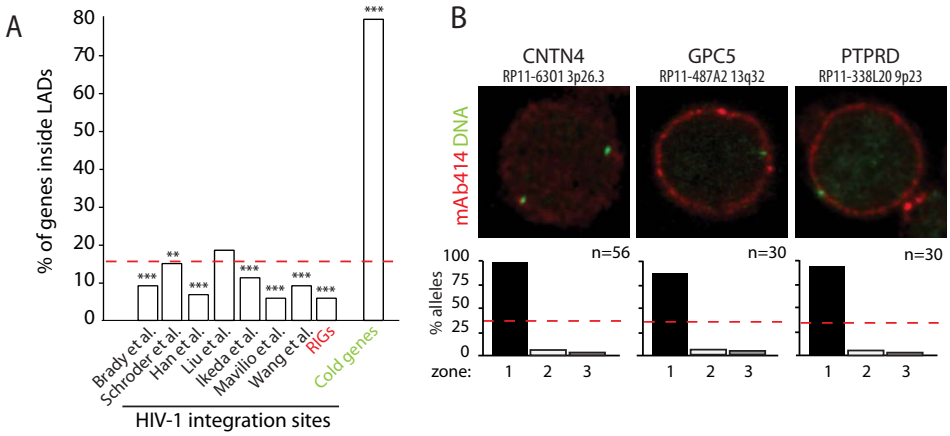


Figure 23. A) Different lists of integration loci, included HIV RIGs, were crossed with list of all the genes present inside LADs. Most HIV integration loci are significantly depleted in LADs, compared to a random distribution (indicated by a red dotted line), with the following P-values: Brady et al. P=1.27 x10^{-8}, Schroeder et al. P=0.008, Han et al. P=0.001, Liu et al. P=0.36, Ikeda et al. P=0.0007, Mavilio et al. P=1.32 x 10^{-15}, Wang et al. HZs P=0.0003, HIV RIGs P=2.09 x 10^{-10}. 80% of genes that are never targeted by HIV-1 (cold genes) are significantly enriched inside LADs (P= 3.25 x10^{-19}). Star code for statistical significance: *** P-value <0.001, ** P -value <0.01, * P -value <0.05.

B) 3D Immuno-DNA FISH in activated $CD4^+$ T cells of three cold genes predicted to be inside LADs by bioinformatic analysis (green: BAC probe-DIG-FITC-labeled; red: NPC staining). Distances of alleles from the nuclear envelope were normalized and divided into the three concentric zones; CNTN4 (n=56), GPC5 (n=30), PTPRD (n=30).

Finally, thanks to a collaboration with the Bioinformatics Group in ICGEB, a program in MatLab was developed: this program aligned all 1,344 known LADs by their left or right border, as described by Guelen and collaborators (Guelen, Pagie et al. 2008), and calculated the localization of RIGs with respect to these combined borders. We found that the vast majority of RIGs (87.2%) were located outside the LADs in contrast to a random distribution of genes (68.2%) (P<0.001), taking into account the lower gene density within LADs (Figure 24A).

Interestingly, aligning the 1344 LADs border we observed that RIGs are distributed in the so-called inter-LAD domains with a preferential distribution in the upstream of the LAD borders, (Guelen, Pagie et al. 2008); after the LAD start, there was a strong drop of their distribution inside of LADs (Figure 24B). These results clearly indicate that RIGs are located in the outer shell of nucleus but are excluded from the LADs.

Next we wanted to validate the usage of LAD coordinates (derived from analysis performed in human fibroblasts) on genes important in lymphoblastoid cells. Fibroblasts and lymphocytes are assumed to have a very similar topology and therefore certain factors are supposed to have similar positioning in their genomes. As a proof of principle, we chose the

positioning of CTCF insulator factor, shown to have peculiar peaks of positioning on the LAD borders and to be a delimiter of LADs in fibroblasts (Guelen, Pagie et al. 2008). We plotted CTCF ChIP-seq data obtained from primary CD4+ T-cells (Barski, Cuddapah et al. 2007) on LAD coordinates derived from fibroblasts, and were able to observe the same distribution (Figure 24C), confirming that LADs are indeed very similar between fibroblasts and lymphocytes, thus further strengthening our results.

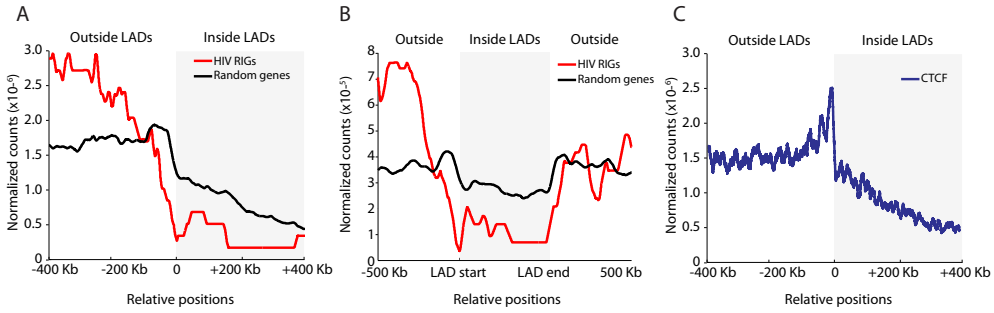


Figure 24. LAD bioinformatic analysis. **A-B)** Profile of the distribution of HIV RIGs (red) and of a random set of genes (black) around aligned LAD border regions. The gray area with positive genomic coordinates indicates the regions inside LADs; the white area with negative coordinates is outside LADs. **C)** CTCF distribution on the LAD border.

Next, we wanted to understand whether altering the contacts between LADs and the nuclear lamina might affect the position of HIV-1 integration inside the nucleus. For this purpose we took advantage of the notion that the H3K9me2 histone mark is crucial for anchoring genes to the nuclear envelope and that its reduction, obtained by inhibiting the G9a methyltransferase, causes a massive detachment of LADs from the nuclear envelope (Kind, Pagie et al. 2013). We treated primary $CD4^+$ T cells with Bix01294, a drug targeting G9a (Kubicek, O'Sullivan et al. 2007), for 8 hours prior to HIV-1 infection, followed by FISH analysis of HIV-1

integration sites (Figure 25A for a scheme of the experiment). Upon G9a inhibition, we detected a strong decrease in H3K9me2 levels (Figure 25B), which was paralleled by HIV-1 integration in more central zones of the nucleus (36.5 and 28% of viruses in zones 2 and 3 respectively; $P<0.01$ compared to wild type infection; Figure 25C and 25D). Of potential interest, central localization of the provirus was also concomitant with a >2.5-fold increase in HIV-1 integration levels (Figure 25E), possibly due to an overall reduction in the heterochromatic regions of the nucleus.

This result shows that perturbation of the nuclear topology and chromatin affects the choice of HIV-1 integration site.

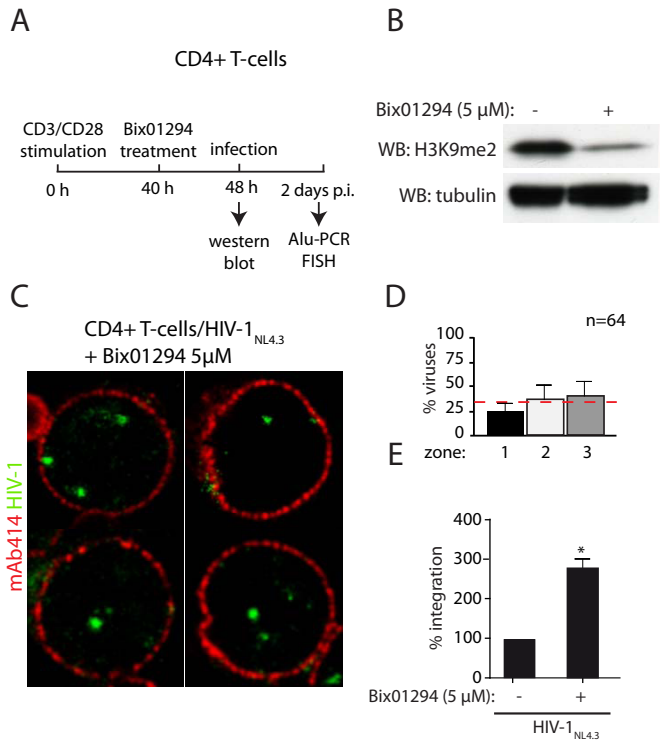


Figure 25. Bix01294 experiment. **A)** Scheme of the experiment for Bix01294 treatment of

CD4+ T cells prior infection. **B)** Western blot of H3K9me2 in control versus Bix01294-pretreated cells. **C)** 3D Immuno-DNA FISH of HIV-1 (green) in Bix01294-pretreated $CD4^+$ T cells stained for NPC (red), 2 days after infection with $HIV\text{-}1_{NL4.3}$.

D) Distribution of distances normalized and divided in the 3 zones. Random distribution is indicated by a red dotted line. **E)** Real time Alu PCR in control versus Bix01294-pretreated cells, 2 days after infection with $HIV\text{-}1_{NL4.3}$ .Values are mean and s.e.m. of three experiments after normalization over control $HIV\text{-}1_{NL4.3}$ infection.

6. HIV-1 genome associates with the Nuclear Pore Complex (NPC).

Since the NPC proteins are known to be involved in gene regulation and to interact with chromatin (Capelson, Liang et al. 2010, Kalverda and Fornerod 2010, Kalverda, Pickersgill et al. 2010, Vaquerizas, Suyama et al. 2010, Liang, Franks et al. 2013), we wondered whether the HIV-1 DNA might directly associate with the nucleoporins after integration into the cellular genome. To assess this possibility, we performed ChIP assays on the HIV-1 provirus in primary $CD4^+$ T cells by using sets of primers spanning the LTR promoter, the region immediately downstream the TSS corresponding to the position of nucleosome-1 and the proviral sequence corresponding to the gag gene 5' end (primers sets PPR1, NUC1A and U1A respectively; Figure 26A). We also designed, based on our FISH results, specific primers for positive and negative control regions, namely the HIV RIG NPLOC4, the cold gene PTPRD, localized in the nuclear periphery, outside and inside the LADs, respectively. As additional controls, we also analyzed two genomic sequences both localized on chromosome 19 in the lamin B2 DNA replication origin, one mapping at the origin itself (B48) and the second approximately 5 kb downstream of it (B13) (Giacca, Zentilin et al. 1994). Control immunoprecipitation with murine IgGs (Figure 26B) scored negative with all primers, whereas Mcm2, a protein of the Pre-Replication Complex, readily bound only the B48 origin of DNA replication while was almost undetectable at all the other viral and genomic regions (Figure 26C).

Four days after primary $CD4^+$ T cell infection, antibodies against USF-1 and p65/RelA efficiently immunoprecipitated the viral promoter region, consistent with the active transcription state of the provirus at this time point (Figure 8B). At the same time, RNA Pol2 was mainly associated with the provirus coding sequence, confirming our previous findings (Perkins, Lusic et al. 2008, Della Chiara, Crotti et al. 2011) (Figure 26D-F). Interestingly, binding to the viral genome was observed using the anti-nucleoporin mAb414 antibody (Figure 26G). Enrichment after mAb414 ChIP was also observed for the NPLOC4 gene, but not for the LAD gene PTPRD.

To further characterize the interaction of the NPC proteins with the HIV-1 provirus, we performed ChIP with antibodies specific for Nup153, Nup98, Nup62 and Tpr. With the exception of Nup62, which is present in the central nuclear pore channel, these nucleoporins are localized in the pore nuclear basket; all four proteins are known to participate in nucleoplasmic gene binding and regulation (Capelson and Hetzer 2009). We found that all four investigated nucleoporins bound the HIV-1 proviral DNA (Figure 26H-K).

These data show that HIV-1 integrates in the peripheral zone of the nucleus of $CD4^+$ T cells, in proximity to the NPC, and that NPC proteins associate with the integrated viral DNA sequence, either directly or indirectly.

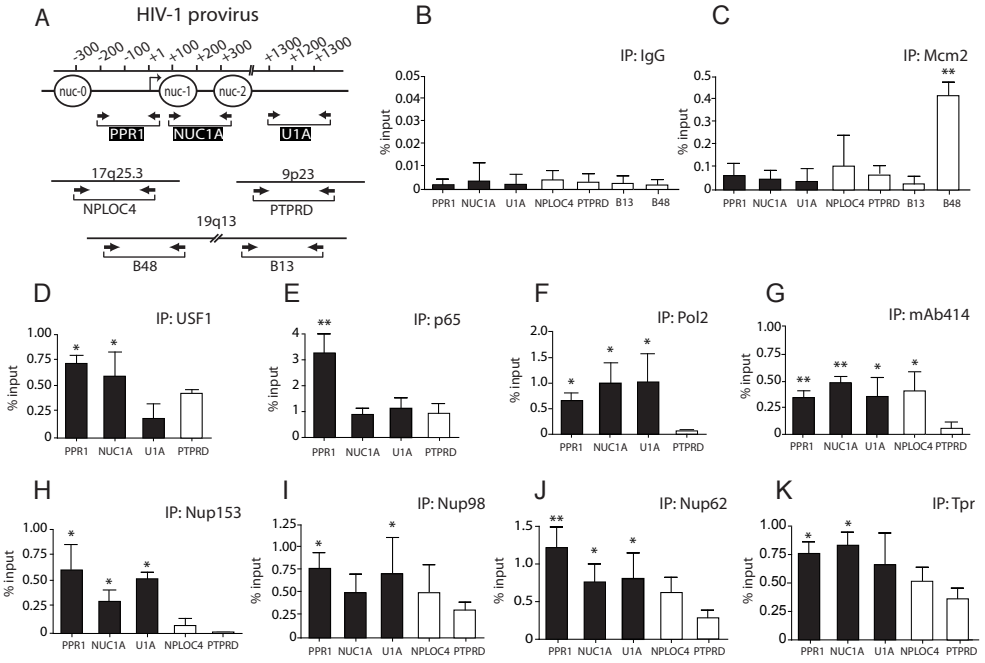


Figure 26. Association of HIV-1 provirus with nucleoporins. **A)** Positions of primers used for amplification of the HIV-1 provirus; the position of the LTR, including the transcription start site (+1) is indicated, along with the known nucleosomal arrangement at the 5' genome region. NPLOC4 was used as positive control gene (HIV RIGs, enriched in zone 1, outside LADs), whereas PTPRD was used as negative control gene (never targeted by HIV, enriched in zone 1, inside LADs). **B-K)** ChIP in CD4+ T cells, 4 days after HIV-1 infection. For each analyzed region, the amount of immunoprecipitated chromatin using the indicated antibodies was normalized according to the input amount of chromatin. The graphs show the mean and s.e.m. from at least three independent experiments. Star code for statistical significance: *** P-value <0.001, ** P -value <0.01,* P -value <0.05.

Interestingly, when ChIP was performed on IN-defective D64E virus (see FISH images in Fig. 13A), mAb414 and Nup153 antibodies could hardly be detected bound to the unintegrated viral sequences, whereas RNA Pol 2 bound the integrated and unintegrated viral sequences to similar extent (Figure 27).

We also performed an initial attempt at analyzing cellular DNA sequences immunoprecipitated with the mAb414 in primary CD4+ T cells (ChIP-seq), to obtain a database of sequences that are associated with NPC in these cells; the same analysis was also performed in Bix01294 treated cells. We used the same program derived from Barski et al., used for the ChIP-seq data in

Figure 22, to plot the mAb414-bound sequences around the TSS of RIGs, compared to a group of equal size consisting of randomly selected genes.

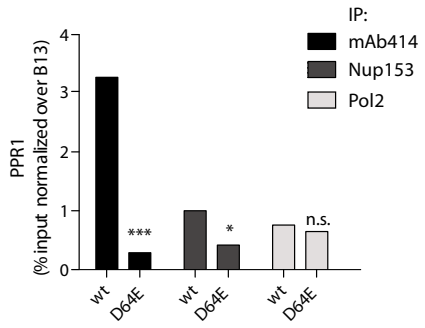


Figure 27. ChIP in CD4+ T cells, 4 days after infection with HIV-1 wildtype or IN-D64E mutant. For PPR1 region, the amount of immunoprecipitated chromatin using the indicated antibodies was normalized according to the input amount of chromatin, and then over control gene B13. Star code for statistical significance: *** P-value <0.001, ** P -value <0.01, * P -value <0.05.

We found that RIGs were enriched on the TSS compared to the random genes (Figure 28A). After Bix01294 treatment, both RIGs and random genes lost this enrichment compared to untreated cells (Figure 28B), suggesting that the drug effect on the nuclear organization is strong and leads to gross changes not only at the level of nuclear lamina, as previously published (Kind, Pagie et al. 2013) but also at the NPCs. These preliminary data need confirmation and validation.

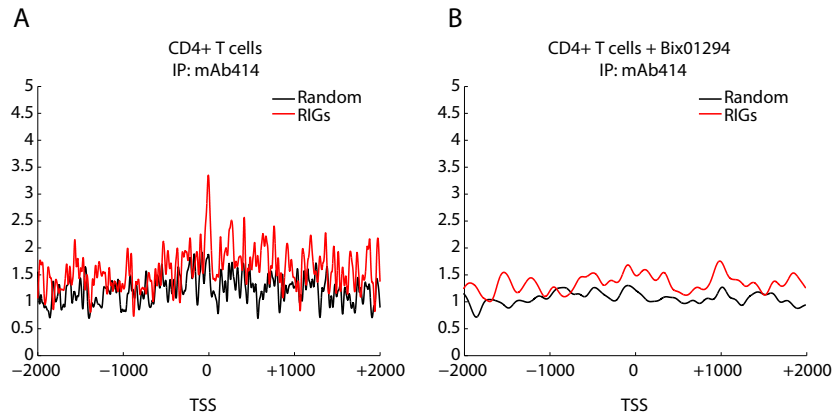


Figure 28. ChIP-seq in control vs Bix01294 pretreated CD4+ T cells.

Profiles of mAb414 binding around the TSS for HIV RIGs (red) and an equal number of randomly selected genes (black) in CD4+ T cells, treated without **(A)** or with **(B)** Bix01294 prior to infection. HIV RIGs show a small enrichment over the random genes.

7. Role of Tpr in HIV-1 positioning.

Given that we found Tpr as a novel interactor with HIV-1 genome, we wondered whether it might play a role in HIV-1 integration process. Therefore we silenced Tpr in Jurkat cells before infecting them with $HIV_{NL4.3}$ (scheme of the experiment in Figure 29A).

We assessed the knockdown of Tpr by western blot after 36 h (Figure 29B), and interestingly, we did not observe any significant change in HIV-1 integration level, as detected by Alu-PCR (Figure 29C). This is consistent with the conclusion that Tpr has no functional effect on the integration process itself, unlike in the case of Nup153 knockdown when integration efficacy was strongly impaired (see Figure 14).

However, FISH analysis showed that HIV-1 is integrated in the nuclear center in the absence of Tpr (Figure 29D-E), implying that Tpr has a indirect

or direct role in HIV-1 positioning at the nuclear envelope. Tpr has important functions in the organization of chromatin boundaries, keeping heterochromatin-exclusion zones under the nuclear pores. Therefore we hypothesize that once the nuclear organization under the pore is disrupted in Tpr knockdown, HIV-1 is unable to integrate in the nuclear periphery any longer, given that heterochromatin is spread all over the zone 1 of the nucleus and creates an unfavorable environment for the integration process to occur. This finding therefore strengthens the conclusion that nuclear organization is important for HIV-1 integration site selection.

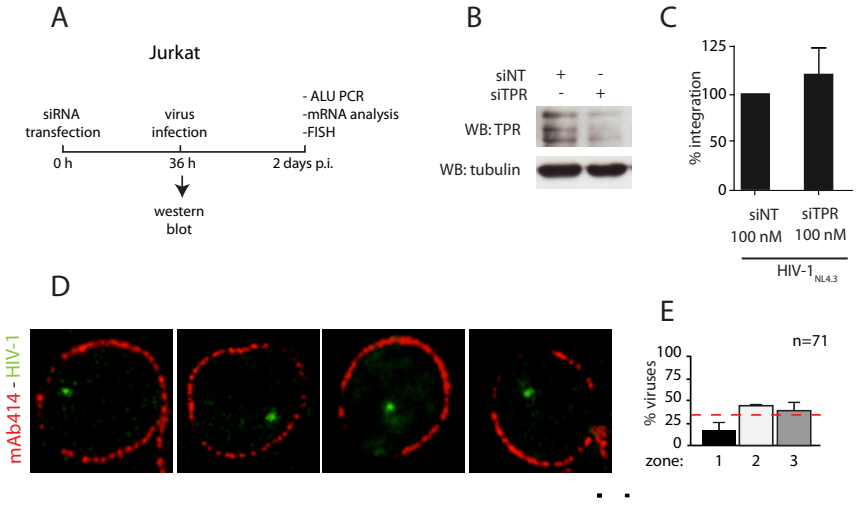


Figure 29. Tpr silencing effect on integration and positioning. **A)** Scheme of the experiment.

B) Western blot showing protein levels for LEDGF/p75 , Nup153 and Tpr at the moment of infection, 36 hours after transfection with the respective siRNAs. **C)** Real time Alu PCR in Jurkat cells infected with HIV-$1_{NL4.3}$ and previously transfected with a not targeting siRNA (NT) or siRNAs targeting Tpr as indicated. Samples were normalized over control infection. **D-E)** 3D Immuno-DNA FISH of HIV-1 DNA (green) in Jurkat cells stained for NPC (red), 4 days after infection with HIV-$1_{NL4.3}$, in Tpr knockdown cells. The graph shows the average results from 3 independent experiments.

8. Transcriptionally active proviruses associate with the nuclear pore.

Next we wanted to understand what the consequences of proviral integration in the periphery of the nucleus and in the neighborhood of the NPC might be in the context of the virus life cycle. One of the hallmarks of HIV-1 is its capacity to finely tune expression of its genes with the activation state of the host cells and to undergo latency in the absence of stimulation. First we took advantage of a Jurkat T-cell derivative, the J-Lat clone 15.4 (Jordan, Bisgrove et al. 2003), which harbors a silent provirus that can be reactivated by stimulation with the phorbol ester TPA (Figure 30A). Immuno DNA-FISH on control and TPA-reactivated J-Lat15.4 cells showed that HIV retained its peripheral localization in both latent and reactivated cells (89% vs. 86% of proviruses in zones 1 and 2, respectively; Figure 30B). Similar results were also obtained in a primary model for HIV-1 latency, according to which $CD4^+$ T-cells were infected and then kept in culture for 2 weeks to allow the virus to enter latency, followed by reactivation using anti-CD3/CD28 antibodies (Bosque and Planelles 2009, Bosque and Planelles 2010, Lusic, Marini et al. 2013). Along this protocol, HIV-1 transcription paralleled cell activation (Figure 30C), however most of the provirus still appeared at the nuclear periphery in both latent and activated cells (Figure 30D).

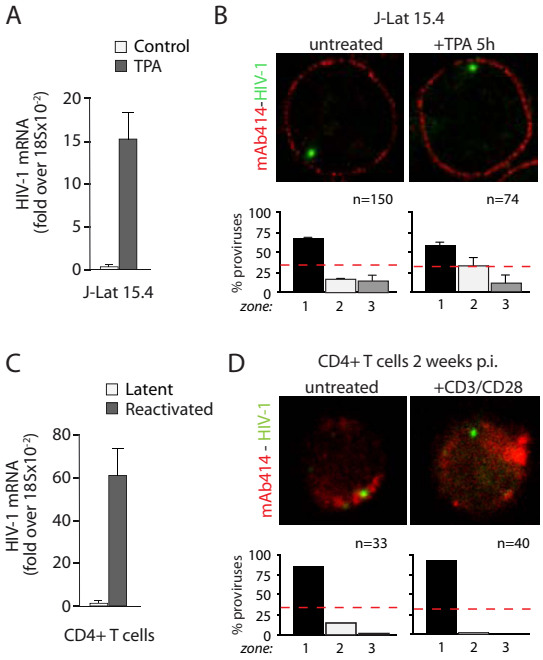


Figure 30. Localization of HIV-1 proviruses in silent versus reactivated cells. **A)** RT-PCR measurement of HIV-1 mRNA levels in mock- or TPA-treated J-Lat clone 15.4 normalized over the 18S house-keeping gene. Control vs TPA: P<0.001. **B)** 3D Immuno-DNA FISH of HIV-1 DNA (green) in J-Lat 15.4 cells stained for NPC (red) before and after TPA reactivation**. C)** Quantification of HIV-1 mRNA by Real Time PCR in latent and reactivated $CD4^+$ T cells, 14 days after infection with $HIV\text{-}1_{NL4.3}$. Values are means and s.e.m. of three experiments after normalization for the amount of host 18S RNA**. D)** 3D Immuno-DNA FISH of HIV-1 DNA (green) in latent or CD3/CD28 reactivated primary $CD4^+$ stained for NPC (red).

Despite microscopic evidence of provirus persistence at the nuclear periphery, ChIP experiments using antibodies against the NPC revealed substantial modification in the association of the provirus with the nuclear pore. While the viral genome was not associated with the pore in latent conditions, binding became apparent for the nucleosome-1 and gag gene 5'-end regions after TPA induction using both the anti-nucleoporin mAb414 antibody and an antibody specific for Tpr (Figure 31A and 31B

respectively).

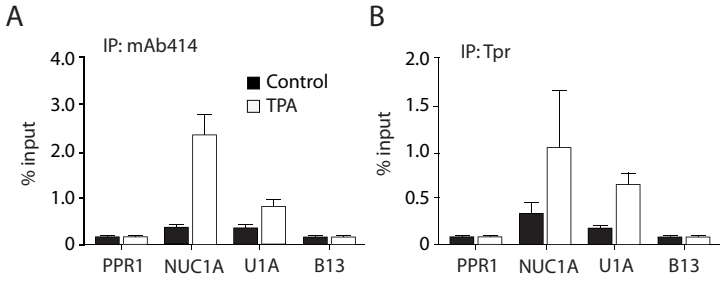


Figure 31. Chromatin ImmunoPrecipitation in 15.4 J-Lat. ChIP in control and TPA stimulated J-Lat 15.4 cells using anti-mAb414 (A) and –Tpr (B) antibodies. Analysis performed as in Figure 24B-K.

These results prompted us to investigate whether there might be a direct involvement of nucleoporins in the regulation of HIV-1 transcription. We transiently silenced Tpr in unstimulated J-Lat cells and, 36 hours later, activated viral expression by TPA (scheme of experiment in Figure 32A). We confirmed the efficient silencing by western blot (Figure 32B), and observed a marked decrease of LTR-driven gene expression in the Tpr-knock down cells (Figure 32C). Consistently with these results, in HeLa cells transfection of two different quantities of an anti-Tpr siRNA reduced Tpr protein levels (Figure 33B; scheme of experiment in Figure 33A) and resulted in a dose dependent decrease in the activity of the LTR promoter (Figure 33C), whereas the integration levels were constant (Figure 33D).

These results reveal that the close association of the provirus with the nuclear pore has a role in the regulation of HIV-1 gene expression. In particular, Tpr, which is involved in the maintenance of a heterochromatin-free zone at the nuclear periphery and in the HIV-1 integration at the nuclear envelope (Figure 29), appears also to directly participate in the regulation of HIV-1 transcriptional activation.

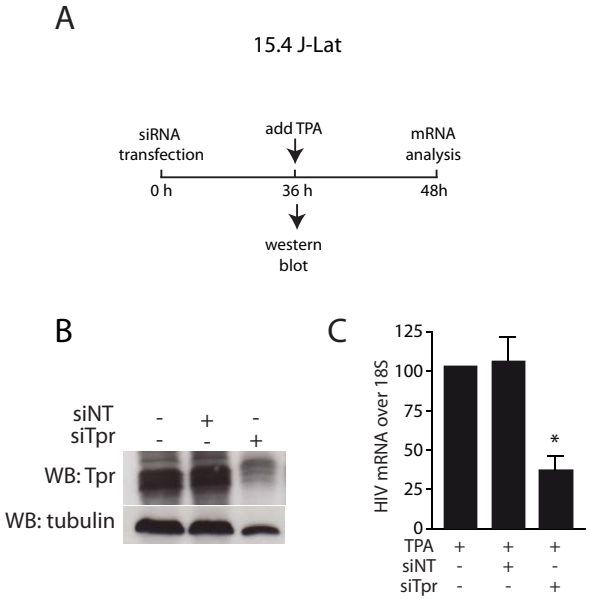


Figure 32. Silencing of Tpr in 15.4 J-Lat **A)** Scheme of the experiment performed in 15.4 J-Lat cells. **B)** Immuno-blot for Tpr, 36 hours after transfection with siNT or siTpr. Anti-tubulin used as a loading control. **C)** J-Lat 15.4 transfected with siNT or siTpr were induced with TPA, and the levels of viral mRNA were compared to non transfected TPA treated cells. Normalization was over 18S gene. TPA vs. siTpr+TPA: $P<0.005$.

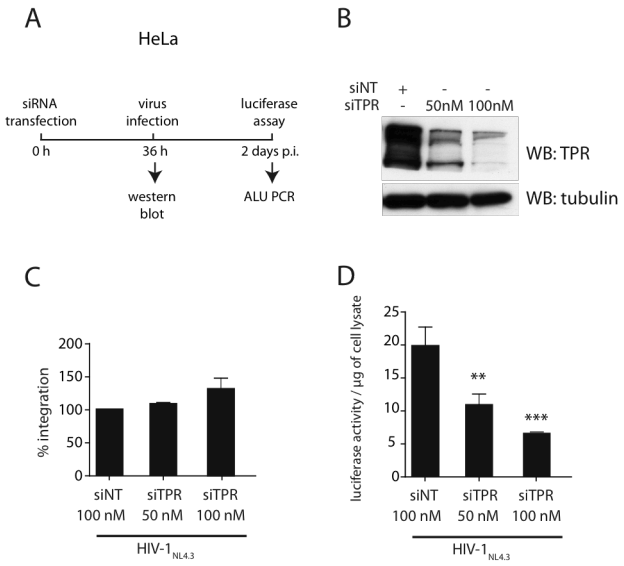


Figure 33. Silencing of Tpr in Hela cells.**A)** Scheme of the experiment performed in HeLa cells. **B)** Western blot showing Tpr protein level at the moment of infection, after treatment with a a not targeting siRNA (siNT) or siRNA targeting Tpr at two different doses. Values are mean and s.e.m of three experiments after normalization over HeLa transfected with a control non targeting siRNA. **C)** Real time Alu PCR in HeLa infected with HIV-$1_{NL4.3}$ and previously transfected with a not targeting siRNA (siNT) or siRNA targeting Tpr at two different doses. Values are mean and s.e.m of three experiments after normalization over HeLa transfected with a control non targeting siRNA. **D)** Luciferase activity assay in HeLa infected with HIV-$1_{NL4.3}$ and previously transfected with a not targeting siRNA (siNT) or siRNA targeting Tpr at two different doses. Values are mean and s.e.m of three experiments. Star code for statistical significance: *** P-value <0.001, ** P-value <0.01, * P-value<0.05.

DISCUSSION

Over the last ten years, there has been growing interest in understanding the spatial organization of the genome inside the nucleus and the role of this architecture in gene function (Cavalli and Misteli 2013, Misteli 2013, Misteli and Warren 2013). It is now well accepted that gene positioning in the nucleus is not random, with transcriptionally active genomic regions aggregating together in the so-called transcriptional factories located in decondensed chromatin, whereas transcriptionally repressed regions are often found at the nuclear periphery.

The results presented in this thesis add a three-dimensional view to the process of HIV-1 integration and show that nuclear architecture plays an essential role in integration site selection. We report that the virus takes advantage of the nuclear topology to integrate into the chromatin of active genes that are located in the periphery of the cell nucleus in correspondence of the nuclear pore. Highly targeted integration genes are distributed in a topologically non-random manner, enriched in open chromatin marks and excluded from the LADs. Several proteins of the NPC bind the provirus, almost exclusively present in the periphery of the nucleus, within 1 μm from the nuclear edge. Taken together, these findings indicate that the PIC, once transported through the NPC channel, preferentially targets those regions of open chromatin that are proximal or even attached to the nuclear pore, while excluding the more internal regions in the nucleus as well as the regions that are equally at the nuclear periphery but associated with the nuclear lamins.

1. Identification and localization of recurrent integration genes.

One of the main finding of this PhD project is the observation that the proviral HIV-1 DNA accumulates in the nuclear periphery in in vitro and in vivo infections. We were the first to visualize HIV-1 DNA in cells derived from patients, and confirmed the observation that HIV-1 can integrate 1 or 2 times in one cells, but rarely more.

We hypothesized two scenarios: either integration takes place at the nuclear envelope, or it takes place wherever in the nuclear space and subsequently the integrated provirus is retargeted to the nuclear periphery. Therefore we aimed to identify the localization of the host genes that are often sites of integration; to select the most informative ones, we decided to perform a meta-analysis of multiple studies in which sequencing of HIV-1 integration sites was performed. We were able to identify a limited group of genomic regions, defined as Recurrent Integration Genes (RIGs).

To date, this is the first study where such kind of comparison was performed, and we believe that the identification of recurrent genes in different lists, derived from different samples and analyzed in different laboratories, might significantly contribute a better understanding the target-site selection. We found that such genes, once plotted onto the chromosome map, are often grouped in small clusters; this might imply that a wider chromosomal region, rather than a specific gene, is targeted by HIV-1 integrase. These clusters were less visible in other studies (such as in (Wang, Ciuffi et al. 2007)), probably because of two main reasons:

1) extremely high number of sequenced integration sites, that despite providing enormous information about the characteristics and features of such sites, does not allow to identify true hot spots, unless

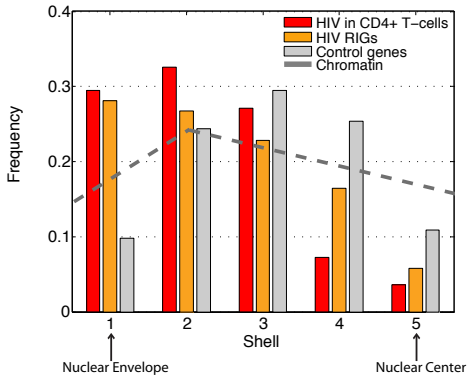
bioinformatics is coupled with topological information;

2) high doses of viral titer used in in vitro studies, that might distort the preferences of targeting and increase the noise of the output.

FISH analysis of selected RIGs showed that these genes are distributed preferentially at the nuclear periphery. We analyzed 14 of RIGs and hot zones, and 7 neutral regions as controls. As previously described by Hediger et al. (Hediger, Neumann et al. 2002), measurements of allele-to-membrane were normalized over the nuclear radius, and then distributed into three concentric shells of equal surface. It has been shown that there is a correlation between the estimated gene density of each chromosome and its average position within the nucleus (Croft, Bridger et al. 1999, Boyle, Gilchrist et al. 2001), meaning that genes are not equally distributed in the nuclear space; rather, it has been estimated that gene rich regions are preferentially localized in the outer shell of the nucleus. The subdivision of nuclear space into three or more concentric shells of equal surface areas has been suggested as a way to avoid bias and normalize radial measurements, in order to assess significant enrichment in one shell or another (Hediger, Neumann et al. 2002). When all alleles were plotted together we could observe a significant difference in the distribution, with a strong preference of RIGs toward the nuclear periphery, as shown in Figure 20. Measurements of FISHed RIGs were also analyzed by another approach, shown below in Figure 34: the nucleus was divided into 5 concentric shells of equal surface; distribution of cellular genome/chromatin, represented as dashed grey line, was calculated considering the total number of genes for each chromosome and the frequency of each chromosome in each shell, as in (Boyle, Gilchrist et al. 2001). According to this study, the spatial distribution of chromosomes in five concentric shells from the outer to inner zone are 17.5%, 24%,

21.9%, 19.4%, and 16.9%. The plot shows that HIV-1 (red bars) and RIGs (orange bars) are more frequently observed in the first, outer shell, with respect to control genes (black bars) and to the total chromatin inside the cell (grey lines). The lower table summarizes the percentage obtained for each zone, for each dataset.

A



B

	zone 1	zone 2	zone 3	zone 4	zone 5
chromatin	17.5 %	24 %	21.9 %	19.4 %	17 %
control genes	9.7 %	24.3 %	29.5 %	25.3 %	10.9 %
HIV RIGs	28 %	26.7 %	22.8 %	16.4 %	5.7 %
HIV-1	29.5 %	32.5%	27.1 %	7.2%	3.6%

Figure 34. Distribution of gene positioning into 5 shells of equal surface. **A)** Distribution of chromatin, control genes, RIGs and HIV-1 proviruses in nuclear space, after subdivision in 5 shells . **B)** Percentage obtained for each zone, for each dataset.

It has to be taken into account that zone 1 of the nuclear space is constituted of the LADs and peripheral heterochromatin attached to it. Therefore, to exclude that RIGs might fall inside LADs, we wrote a program that was able to show the distribution of RIGs around the LAD border, and found that these genomic regions are specifically characterized by being excluded from the repressed chromatin corresponding to the LADs. This finding is

consistent with the characteristics of LADs enriched in repressive histone marks and gene-deserts. Moreover, these calculations strongly reinforce the idea that HIV-1 integration preferentially occurs in the remaining regions in zone 1 outside LADs (corresponding to 6.8% of total genes); these remaining regions are presumably the ones associated with the pore and probably the same containing RIGs.

Subsequent bioinformatics analysis was performed with the aim to better characterize RIGs and verify their transcriptional state. We exploited the data present in the literature (Barski, Cuddapah et al. 2007, Wang, Zang et al. 2009) to describe the TSSs of RIGs, As a reference, we selected two sets of genes, as described in those papers: a set of actively transcribing genes in CD4+ lymphocytes, and a set of repressed genes. In addition, we used a set of genes, never targeted by integration, that we termed cold genes. By such analysis, we showed that RIGs had features of active genes in CD4+ T cells, and were significantly different from repressed genes. On the contrary, cold genes showed an opposite profile, as expected.

Here, by using a combined FISH and bioinformatics analysis, we describe RIGs as a set of genes that are active and are located at the nuclear periphery, and are the preferred target of HIV-1 integration. Positioning of a gene at the nuclear periphery, in the proximity of the NPC, appears to be an essential requisite for being targeted by HIV-1 integration. Our data show that the IKZF3 locus, which is often targeted by HIV-1 in $CD4^+$T cells but not in $CD34^+$ hematopoietic progenitor cells, has a peripheral localization in the former but central in the latter cell type. Conversely, the TAP2 gene in the MHC class II locus, which is peripheral in $CD34^+$ cells, has numerous integrations in these cells but not in $CD4^+$ T lymphocytes, where it is located centrally. Thus, cell-dependent gene positioning inside the nuclear space is

an essential determinant of HIV-1 integration preferences.

In in vitro infected CD4+ T cells, 4 days after infection, HIV and at least one of RIGs (NPLOC4 on chromosome 17qter) are specifically associated with the nuclear periphery regions defined by the presence of the nuclear pore. Indeed, the zones of the nuclear periphery underlying the NPCs have been shown to be rich in euchromatin, and proposed to facilitate the coupling of mRNA production and its export into the cytoplasm (Blobel 1985). So far, the anchoring of active genes to the NPC has been confirmed by several studies performed both in yeast (Schmid, Arib et al. 2006, Taddei, Van Houwe et al. 2006, Tan-Wong, Wijayatilake et al. 2009, Ahmed, Brickner et al. 2010) and Drosophila (Capelson, Liang et al. 2010, Kalverda and Fornerod 2010, Kalverda, Pickersgill et al. 2010, Vaquerizas, Suyama et al. 2010).

We hypothesized that RIGs might be included in the part of genome bound to NPC. To verify this possibility, we performed a first attempt to perform ChIP-seq experiment in primary CD4+ T cells using the mAb414 antibody, to test whether RIGs might actually be bound by the NPC proteins. To date, no similar studies have been performed and no data are available on which genes are bound or connected to the NPC in human lymphocytes, or where in the gene NPC might bind (TSS, promoters, enhancer, gene body etc). We observed binding of the NPC proteins all over the TSS of RIGs, but these are preliminary data still to be validated.

2. HIV-1 integration at the nuclear periphery: possible molecular reasons.

An interesting question arising from this study relates to the understanding of why HIV-1 specifically integrates into the NPC-associated genomic

regions. We hypothesize three different possibilities. First, the HIV-1 PIC might recognize specific chromatin marks at these regions. While we cannot exclude this possibility, all the chromatin marks we have analyzed were common between RIGs and other actively transcribed genes inside the nucleus, including H3K4me2, which, together with RNA Pol2, was reported to define the NPC-LAD transitions (Guelen, Pagie et al. 2008). The only exception found among the analyzed histone marks, was H4K20me1, a histone mark involved in DNA damage response and DNA replication, and that seems to act as a boundary of active chromatin; H4K20me1 was more enriched in the RIGs rather than the active genes (Chai, Nagarajan et al. 2013). In this context, it is nevertheless important to observe that a specific determinant that clearly disfavors integration is the presence of the H3K9me2 mark. This is a common mark in the LADs, where normally HIV-1 never integrates. In addition, perturbation of the G9a methyltransferase function, the major determinant of the H3K9me2 modification, results in integration of the virus also in the inner portion of the nucleus.

Second, the HIV-1 PIC might be directed to the RIGs by its association with specific nuclear proteins. Silencing of LEDGF/p75, Nup153 or Tpr determined the relocation of viral DNA into inner portion of the nucleus. In the case of the first two proteins, which are well established IN cofactors (Cherepanov, Maertens et al. 2003, Shun, Raghavendra et al. 2007, Di Nunzio, Danckaert et al. 2012, Koh, Wu et al. 2013), the extent of integration itself was markedly diminished, which suggests that the effect of these knock-downs on HIV-1 integration might be the consequence of impaired IN activity. Indeed, inhibition of IN activity via raltegravir, or through the use of IN mutated viruses, lead to similar relocalization of viral DNA into the zones 2 and 3. Therefore, our results on one hand indicate that the HIV-1 PIC has an intrinsic capability of spreading all over the nuclear

space; interestingly, according a previous study, PIC was observed in the nuclear periphery and not freely diffused in the nuclear space (Albanese, Arosio et al. 2008), therefore the presence of LEDGF/p75 and Nup153 is needed for the tethering PIC to the nuclear envelope. On the other hand, the HIV-1 PIC needs to contain a functional IN in order to be tethered at the nuclear periphery. We concluded that the integration process itself needs to be efficient and functionally accessorized by both viral and host factors. As previously described, the silencing of Tpr also provoked the localization of HIV-1 in the inner nuclear space, but, in this case, the integration rate was not affected; therefore, FISH signals might correspond to integrated provirus in the nuclear center. Interestingly, the absence of Tpr has been shown to expand the heterochromatic regions in proximity of the nuclear pore (Krull, Dorries et al. 2010, Raices and D'Angelo 2012), thus restricting the localization of the actively transcribed genes to the central zone of the nucleus. Therefore, we speculate that, in the absence of Tpr, the HIV-1 pre-integration complex might find no possibility for integration in the nuclear periphery.

A third argument that supports the choice of RIGs as sites of HIV-1 integration is the short half-life of viral IN, and thus the need, for the PIC, to achieve rapid integration into genomic DNA upon entry into the nucleus. Our previous work indeed showed that efficient integration of HIV-1 viral DNA in primary $CD4^+$ T cells requires post-translational modification of HIV-1 IN by JNK and Pin1 (Manganaro, Lusic et al. 2010), which significantly increase IN stability. Despite these modifications, however, the functional half-life of integration competent cDNA remains relatively short (Bukrinsky, Stanwick et al. 1991, Chun, Carruth et al. 1997), which might explain the inability of the PIC to reach inner portions of the nucleus. This explanation is also consistent with the observation that pharmacological

inhibition of IN, or infection with HIV-1 mutants carrying an enzymatically defective IN, results in the presence of not integrated viral DNA which is not restricted to the nuclear periphery, as one would expect should viral DNA be constrained in this region by specific interactions. Overall, we suggest a model of target-site selection where all these three possibilities play a role and are strictly connected with the principles of nuclear architecture and genome topology (Figure 35).

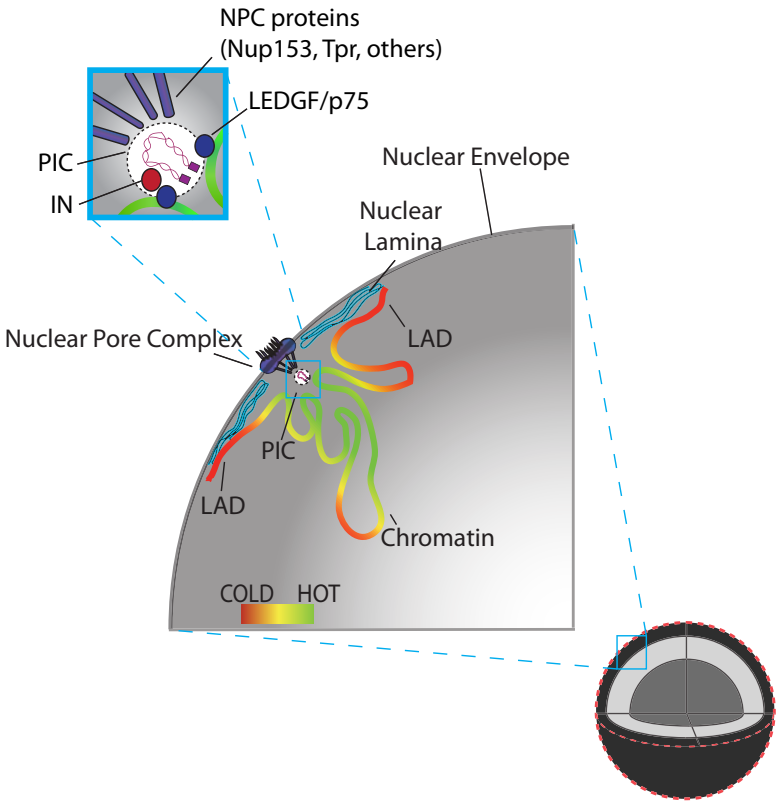


Figure 35. Proposed model of HIV-1 integration site selection. After its entry through the NP, the viral cDNA might integrate in the closest active chromatin (green zones), avoiding both LADs and the inner part of the nucleus (red zones), by unfavorable chromatin accessibility and nuclear topology, respectively. The peripheral localization depends on the efficiency of integration process itself, since functional integrase an cellular cofactors are important for proper localization. This suggests that integration may occur as early as possible after nuclear entry.

The localization of HIV-1 proviral DNA in close association with the nuclear pore is consistent with a number of observations, obtained over the last few years, that several NPC components play a role in HIV-1 infection. Nup62 was shown to interact with HIV-1 IN and contribute to chromatin binding and efficient integration (Ao, Jayappa et al. 2012). Other nucleoporins, such as Nup85, Nup107, Nup133, and Nup160, Nup153 and Nup358/RanBP2 were found in genome-wide screenings for human genes that affect HIV-1 infection (Brass, Dykxhoorn et al. 2008, Konig, Zhou et al. 2008, Zhou, Xu et al. 2008). In particular, Nup153, a protein of the inner nuclear basket, was reported to interact with HIV-1 IN (Woodward, Prakobwanakit et al. 2009) and Vpr (Varadarajan, Mahalingam et al. 2005) and participate in PIC nuclear import (Di Nunzio, Danckaert et al. 2012) via interaction with the viral capsid (Matreyek and Engelman 2011). Nup153 depletion from the cells reduced the tendency of HIV-1 to integrate into gene-dense regions (Koh, Wu et al. 2013). Of interest, in Drosophila, both Nup153 (Vaquerizas, Suyama et al. 2010) and Nup98 (Capelson, Liang et al. 2010, Kalverda, Pickersgill et al. 2010, Liang, Franks et al. 2013) bind active chromatin, and might thus participate in the tethering of the HIV-1 PIC with active chromatin regions. Our work now show that the same nucleoporins are associated with both the NPLOC4 RIG in the absence of viral infection and with actively transcribed HIV-1 DNA after integration, further supporting the conclusion that the provirus remains anchored to the nuclear pore.

3. Nuclear pores and transcription.

The role of the Tpr nucleoporin in the HIV-1 life cycle deserves more comments. Our results underscore a possible role of Tpr protein not only in

regulating HIV-1 integration choice as a component of the nuclear pore, but also in participating in the subsequent step of LTR-controlled gene expression. In this respect, it is worth noting that the yeast homologue of Tpr, the Mlp2 protein, is also present in the inner basket region and mediates association of the NPC with the gene loop structures that are characteristic of actively transcribed genes in S. cerevisiae in the context of the process so-called transcriptional memory (Tan-Wong, Wijayatilake et al. 2009). Genes the regulation of which depends on the nutrient availability are recruited to the nuclear pore and maintained in a looped state that will favor their re-activation, whenever the same nutrient will be presented in the environment.

Interestingly, previous work performed in our lab in collaboration with the N. Proudfoot laboratory has indeed shown that the HIV-1 provirus also forms a gene loop, which depends on ongoing transcription (Perkins, Lusic et al. 2008). Thus, it might be hypothesized that integration of the HIV-1 DNA in close correspondence with the nuclear pore not only reflects the molecular events occurring upon nuclear entry of the PIC, but is also a requirement for productive HIV-1 gene expression. This hypothesis is supported by previous observation that described viral RNA production at the nuclear periphery (Dieudonne, Maiuri et al. 2009).

In the last months, we collected some interesting preliminary results about nuclear topology and HIV-1 latency. We have tackled this issue by FISH analysis for the HIV-1 localization in a primary model of latency and we observed HIV-1 localized at the periphery both in the silenced and in the reactivated state. This phenomenon is reproduced in the 15.4 J-Lat clone, a cell line harboring a single copy of HIV-1-GFP that is silent but still able to be reactivated upon TPA/PHA stimulation. However by ChIP we could observe that, in silent cells, HIV-1 is not bound to nucleoporins, whereas

after reactivation we observed an increase in the binding levels. This implies that the micro-environment changes from a not-NPC compartment (peripheral heterochromatin? LADs?) to a NPC-associated compartment, whereas the macro-environment (i.e. the nuclear periphery) does not change. Given that the integration sites of latent cells are not substantially different from the ones actively transcribing (Lewinski, Bisgrove et al. 2005), it is tempting to hypothesize that the positioning of HIV in repressed periphery is a consequence and not a cause of latency establishment: active genes where HIV-1 is integrated (at the nuclear periphery) might be only lately incorporated in the repressed portion of peripheral chromatin. In particular, PML nuclear bodies, that we proved to be involved in the repression of latent viruses (Lusic, Marini et al. 2013), might exert this function by impeding HIV-1 genome to contact with NPC compartments or even reorganizing the entire chromosomal environment. Indeed, by ChIP, we described an opposite behavior of PML compared to nucleoporines, being the former enriched in the silent state and released in the activated one. We also performed some preliminary experiments showing that the distance to PML bodies is inversely correlated to the distance to the nuclear membrane (Figure 36A), whereas J-Lat with PML knock-down show signals almost exclusively attached to the nuclear envelope (Figure 36B). At 16 hours after infection, tis trend is more evident as shown in Figure 36C: we observed that when HIV-1 is in the inner part of the nucleus PML is very often bound to it; on the contrary the distance increases when HIV-1 is more close to the envelope. These data need further extensive investigation in order to validate a possible connection between PML bodies and the positioning of HIV-1 at the nuclear membrane.

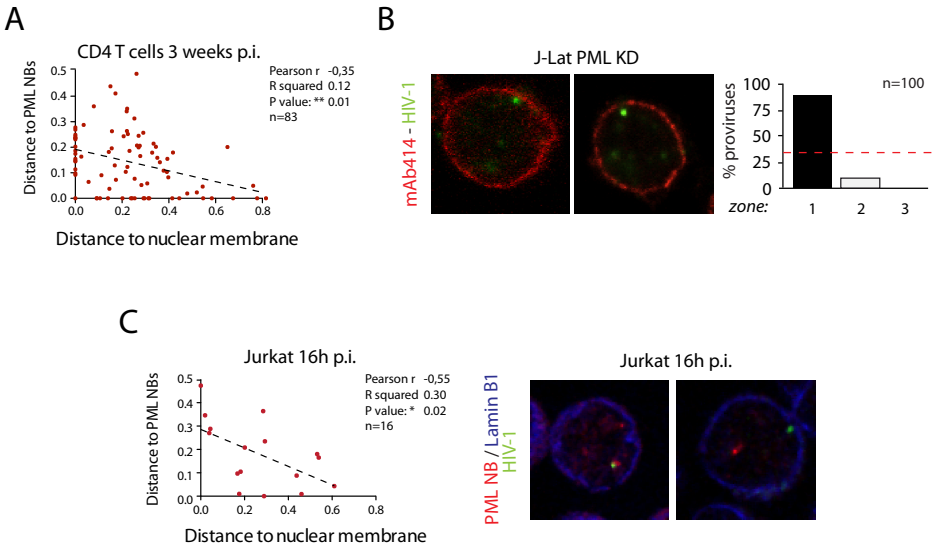


Figure 36. A) Correlation between the distance to PML NBs and to nuclear envelope in a FISH experiment performed in CD4+ T cells 3 weeks after HIV-1 infection. **B)** 3D Immuno-DNA FISH of HIV-1 DNA (green) in latent J-Lat after PML knockdown. **C)** Correlation between the distance to PML NBs and to nuclear envelope in FISH experiment performed in Jurkat T cells 16 hours after HIV-1 infection with two corresponding images.

Taken together, the data collected so far support a model where latency might be considered as a phenomenon of long transcriptional memory: latent viruses might reside at the nuclear periphery, forced by PML nuclear bodies, that will enrich them of marks, typical of LADs, such as H3K9me2 through G9a recruitment, (Lusic, Marini et al. 2013). Nuclear periphery might be considered a perfect compartment for a parasite that will need first to hide, and then to be in a position to replicate as quickly as possible. Later, when stimulation is given, viral DNA might undergo tethering to the NPC compartment and, we speculate, gene looping; in such way, transcription might be fast coupled with nuclear export of viral RNA. This might be the matter of further investigation.

MATERIALS AND METHODS

Virus production

For the production of viral stocks of HIV-$1_{NL4.3}$, we used a plasmid obtained from the Env- molecular clone pNL4-3/E^-R^-, a kind gift from Dr Nathaniel Landau. This viral clone harbors a frameshift mutation introduced near the 5' end of env gene (Connor, Chen et al. 1995) and performs a single-round infection once pseudotyped with Vesicular Stomatitis Virus –G (VSV-G); this renders the virus incapable of spreading (and causing massive cell death).

We produced viral clone HIV-1_{BRU} as previously described (Manganaro, Lusic et al. 2010). The Integrase (IN) defective packaging plasmid pcHelp/IN-, kind gift from Dr Andrea Cara, contains a D116N mutation in the IN genome, preventing the function of the IN protein (Lu, Limon et al. 2005) pD64E mutant plasmid, which is similarly integration-defective, was obtained from the NIH AIDS Research and Reference Reagent Program (Negri, Michelini et al. 2007).

Lentiviral vector pLV-THM was obtained from Addgene, whereas pCLL-18GFP was a kind gift of dr. Alessandra Recchia (University of Modena). Gammaretroviral vector was a kind gift of prof. Greg Towers (UCL, London).

Infectious viral stocks were generated by transfecting viral DNA in HEK 293T cells and collecting supernatants after 48h. Viral production was quantified in the supernatants for HIV-1 p24 antigen content using Innotest HIV antigen mAB kit (INNOGENETICS N.V. Gent, Belgium).

Primary cell isolation, culture, and infection

Primary human CD4+ T cells were isolated by Ficoll gradient separation, followed by purification with CD4 MicroBeads (Miltenyi Biotec). Cells were activated with a cocktail of beads containing $4.5x10^5$ beads coated with antiCD3 and antiCD28 antibodies (Dynabeads Human T-Activator CD3/CD28 Dynal/Invitrogen), and plated in complete medium with IL-2 (30 U/ml, Sigma-Aldrich) for 4 days at 37^0C.

$1x10^6$ activated CD4+ T cells were infected with 0.5-0.75 µg/ml of p24 of virus for 4-5 hours at 37^0C. After infection, the cells were kept in culture at $1x10^6$cells/ml in complete RPMI 1640 medium supplemented with IL-2 and CD3/CD28 beads.

For raltegravir treatment, 10 µM raltegravir (obtained from NIH AIDS Research and Reference Reagent Program) was added together with the virus during the infection, and it was later supplemented in the medium.

For generating primary model of latency, naïve CD4+ T cells were isolated, cultured and infected as described in Lusic et al., 2013.

Primary human CD34+ cells were isolated, cultured and infected as described in Repnik et al., 2003.

Cell culture and transfection

Jurkat lymphoblastoid cell line, Jurkat J-Lat 15.4 clone and U937 monocytic cell line were kept in culture in complete RPMI 1640 medium. Transfection of Jurkat with p-EGFP-Nup153 expression plasmid (Daigle et al., 2001) obtained from Euroscarf was performed using Fugene HD (Promega), according manufacturer's instructions.

$3x10^6$ cells were transfected with siRNA smart pools targeting LEDGF/p75, or Nup153 proteins or with a non-targeting (NT) siRNA as a negative control (Dharmacon, Thermo Scientific). Transfection was performed with Amaxa Nucleofection Device II (Amaxa), using the Amaxa nucleofection Kit V according to the manufacturer's instructions.

For western blot analysis, cells were harvested and homogenized in a lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate supplemented with protease inhibitors (Roche) for 10 min at 4 °C and sonicated (Bioruptor) for 5 minutes. Equal amounts of total cellular proteins (30 μg), as measured with Bradford reagent (Biorad), were resolved by 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (GE Healthcare), and then probed with primary antibody (anti-Nup153 (Santa Cruz, sc-101544), anti-LEDGF/p75 (BD Biosciences, #611714), anti-GFP (LifeTechnologies, A6455, followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (GE Healthcare).

Once the protein silencing was assessed, cells were infected with 0.5-0.75 μg/ml of p24 of viral clone NL4-3/E^-R^- as previously described. 24 and 48 hours after infection samples were collected for further analysis.

Integration assay (Alu PCR)

Infected cells were tested for integration of HIV-1 by isolating genomic DNA from 10^6 cells with DNeasy Tissue Kit (Qiagen). 100 ng of genomic DNA was subjected to quantitative Alu-LTR PCR for integrated provirus or for 2 LTR circles as previously described (Manganaro et al., 2010).

qRT-PCR

For the quantification of HIV transcript levels, RNA was purified from the cells with the Nucleospin RNA II purification kit (Macherey-Nagel), and the mRNA levels were quantified by TaqMan qRT-PCR by using

HIV-1 or IL2 primers and probe (Lusic et al., 2013), and housekeeping gene 18S and GAPDH as a controls.

Luciferase activity assay
Cells were harvested 48 hours post infection, and luciferase activity was measured with Luciferase assay kit (Promega). Viral expression was expressed normalized over μg of total cell extracts.

Cell preparation for 3D Immuno-DNA FISH
Three-dimensional FISH combined with immunostaining was performed according to protocols in Solovei et al.m 2010. Culture or primary cells were resuspended at 3x 10^6 /ml in 5% Foetal Bovine Serum (FBS) in PBS and cell suspension was allowed to attach to the glass cover slips, previously coated with Poly-L-Lysine. Cells were fixed in 4% paraformaldehyde in 0.3X hypotonic PBS for 10 min, permeabilized with PBS/0.5%Triton X-100 for 10 min and left in PBS/20%glycerol for 1hr. Cells were than blocked in PBS/5% Fetal Horse Serum (FHS) for 45 min and primary antibody (anti-NPC mAb414, Covance; anti-LaminB, Abcam ab16048; anti-GFP, LifeTechnologies A6455) was added for an overnight incubation at $+4^0$C in a humid chamber. The following day, cells were washed 5 times in PBS-T (PBS with 0.05% Tween) and the secondary antibody (Jackson Laboratories) was used for 45 minutes at room temperature (1/1000 dilution). After 5 washings in PBS-T, cells were additionally crosslinked with EGS (Ethylen Glycol-bis(succinic acid N-hydroxysuccinimide ester) (Sigma E-3257) for 10', washed and permeabilized again in PBS-T/0.5% Triton X-100. After washing the cells in PBS/0.05% Triton X-100, cells were rinsed and incubated in 0.1N HCl (freshly prepared) for 10min. Cells were left in PBS/20% glycerol for at least 45min, and were then subjected to 5 cycles of freeze and thaw in liquid nitrogen and PBS/20% glycerol. Additional washings in PBS/0.05% Triton –X100 preceded an overnight incubation in 50% formamide/2xSSC (hybridization buffer). The following day, cells were treated with RNAse A (100 μg/ml in 2XSSC) in a humid chamber at 37^0C for 1hr, were rinsed again in 50% formamide/2xSSC for at least 1 hour (or over-night) and were then subjected to hybridization with the appropriate probe.

Probe for hybridization in 3D Immuno-DNA FISH:
For visualization of loci of interest, specific BAC clones (selected from Chori sites and purchased from Invitrogen) were isolated according manufacturer's instructions. The listing of the BACs with their IDs and the genes they contain is provided in **Attachment 1**. 3 μg of BAC DNA

was labeled with digoxigenin by Dig-Nick Translation (Roche) at 15^0C.
For visualization of HIV-1, lentiviral vectors or gamma-retroviral vectors inside primary CD4+ T cells or Jurkat, 3 µg of the relative plasmid was labeled by Nick Translation in the presence of 16-dUTP Biotin nucleotides at 15^0C for 3hr.
In both cases, probes were checked on agarose gel and then they were cleared by using Illustra Microspin G-25 column (GE Healthcare) and precipitated in the presence of Cot-1 DNA (Roche) and DNA from herring sperm (Sigma). Finally, after ethanol precipitation the probes were resuspended in 10 µl formamide, incubated at 37^0C for 15-20min and 10µl of 20%Dextran in 4XSSC was added for a final volume of 20µl.

Hybridization set up and development
1-10 µl probe was loaded on glass cover slips with the cells, and the cells were sealed on glass cover slides with rubber cement and heat denatured on heat block at 75^0C for 4min. Hybridization was carried out for 48 hours at 37^0C in a humid chamber. 3 washings in 2XSSC (10 min each) were followed with 3 washings in 0.5X SSC at 56^0C.
FISH development for Dig-labeled BACs was performed by using FITC-labeled anti-Digoxigenin antibody (Roche), whereas biotin-labeled HIV-1 probes were detected by TSA Plus system from Perkin Elmer, that allows significant amplification of the signal, by using an anti biotin antibody (SA-HRP) and a secondary antibody with a fluorescent dye (usually FITC for HIV).

Microscopy
3D-stacks of slides of fixed cells were captured on a Zeiss LSM 510 META confocal microscope (Carl Zeiss Microimaging) with a x63 NA 1.4 Plan-Apochromat oil objective. The pinhole of the microscope was adjusted to obtain an optical slice of < 1.0 µm for any wavelength acquired.
Distances observed between the FISH signals and the nuclear envelope were measured by LSM 510 Image Examiner Software (Zeiss) and Volocity (Perkin Elmer); measurements were normalized over nuclear radius (defined as half of the middle of the mAb414-TRITC ring), and then binned in three classes of equal surface area as previously described (Hediger et al., 2002).

Chromatin immunoprecipitation (ChIP)
$30x10^6$ CD4+ T cells were washed 2 times in PBS prior to crosslinking with 11% Formaldehyde for 10 minutes at room temperature, followed by

termination of the reaction with 125mM glycine on ice. Cell pellet was washed 2 times with PBS and was lysed in 0.5% NP-40 buffer (10mM Tris-Cl pH7.4, 10mM NaCl, 3mM $MgCl_2$, 1mM PMSF and Protease Inhibitors). Obtained nuclei were washed once in the same buffer without NP-40. Lysis of the nuclei was performed using the same buffer containing 4% of NP-40 at 37^0C for 15 min, upon which Micrococcal Nuclease was added (120 units of the enzyme), and the reaction was stopped with 3mM EGTA. DNA was additionally sheared by sonication to an average size of DNA fragments below 500 bps. Extracts were precleared by 2 rounds of incubation with IgGs and agarose beads, followed by centrifugation at 3500 rpm for 5-10 minutes in centrifuge. 400 µl of the lysate was then incubated with 4 µg of the indicated antibody overnight at 4^0C, followed by a 4 hr incubation with MagnaChIP Protein A/G Magnetic Beads (Millipore). Beads were then washed thoroughly with RIPA150, with LiCl – containing buffer and with TE, RNAse treated for at least 30 minutes at 37^0C, and Proteinase K treated for at least 2 hours at 56^0C. Decrosslinking of protein–DNA complexes was performed by an overnight incubation at 65^0C. DNA was then extracted by phenol – chloroform extraction followed by ethanol precipitation and was quantified by real time PCR. The following antibodies were used ChIP: mAb414 (Covance, MMS-120R), anti-Pol2 (Santa Cruz, sc-9001), anti-USF1 (Santa Cruz, sc-229), anti-NF-kB p65 subunit (Santa Cruz, sc-109X), anti-Nup153 (Abcam, ab-93310), anti-Nup98 (Cell Signaling, #2598), anti-Nup62 (BD Biosciences, #610497), anti-Tpr (Santa Cruz, sc-67116), anti-Mcm2 (Abcam), mouse IgG (Santa Cruz, sc-2025).

Bioinformatic analysis and statistics

Five lists of HIV-1 integration sites were collected from published works; a still unpublished lists of integration sites in CD4+ T cells was gently provided from Dr Fulvio Mavilio.

156 genes, called HIV-1 Recurrent Integration Genes (HIV RIGs) genes, were found in more than one lists and their genomic position were plotted on the chromosome map using Idiographica webtool (http://www.ncrna.org/idiographica).

Genomic coordinates of 8 selected hotter zones, where integration density was found higher than expected, were found on Bushman Lab websites and reported in **Attachment 1** (http://www.bushmanlab.org/tutorials/ucsc).

The calculation of the probability of finding 156 genes present in more than one list by chance was carried out via computer simulation. A program was written to randomly draw, from 25000 genes, 265, 329, 294, 32, 158 and 58 genes respectively, and to count the genes drawn more than once. The simulation was repeated 10^9 times. Calculations were

performed using Matlab 2011R.
Expression of HIV RIGs and control genes was derived from published transcriptomic data in CD4+ T cells, using biogps.org, and was compared to random sets of genes; Mann-Whitney-Wilcoxon test assessed that HIV RIGs and control genes are more transcribed than random sets of genes (P-value<0.005).
LAD coordinates were obtained from whereas genes inside LAD were derived from BioMart Ensembl and called LAD genes. Then, the P-value of the common genes in the LAD genes, HIV RIGs, the 6 integration lists and cold genes were calculated by pairwise comparison of each combination, followed by analysis of significance using statistics with hypergeometric distributions. The profile of aligned LAD border regions was carried out in the same was as in Guelen et al., 2008. Chi-squared test was applied to compare the distribution across the LAD border of HIV RIGs with the one of 3000 random genes that were generated without replacement using RSA-tool (http://rsat.ulb.ac.be/random-genes_form.cgi)
ChIP-seq profile analyses were carried out the same way as in Barski et al., 2007. 1000 most expressed and 1000 less expressed genes were obtained as in and called Active and Silent genes, respectively. The TSS coordinates of the genes were obtained with UCSC Table Browser.
Comparison between groups for expression data was performed using the non-parametric Mann-Whitney-Wilcoxon Rank Sum Test; comparison of gene distributions was analyzed using the chi-squared test. For the FISH, ChIP and Real Time PCR results, the reported values are means and s.e.m., calculated from at least 3 independent samples. For statistical comparison of three or more groups, one-way ANOVA followed by Turkeys post-hoc test was used. A value of $P<0.05$ was considered significant.

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