Joint PhD Programme in Molecular Biology (JUMBO)

"Post-transcriptional regulation of HIV-1 gene expression by the host factor MATR3"

Phd student: Ambra Sarracino

Laboratory of Molecular Virology

ICGEB (Trieste)

Supervisor: Dr. Alessandro Marcello

Laboratory of Molecular Virology

ICGEB (Trieste)

SUMMARY

ABSTRACT

The post-transcriptional regulation of HIV-1 replication is finely controlled by both viral and host factors. Among the former, Rev is involved in the nuclear export of viral intron-containing mRNAs, a group of transcripts which encodes for viral enzymes and structural proteins, but also constitute the viral genome that will be encapsidated into nascent virions.

To avoid the nuclear retention of these intron-containing transcripts, Rev engages an alternative export route which ultimately involves the CRM-1 export factor; during this process Rev requires the concerted action of several host factors but the details of its interactions are still not fully understood. To dissect this pathway a novel proteomic approach for the immunoprecipitation of the viral RNA was developed in our laboratory : we thus identified the nuclear matrix protein MATR3 as a Rev co-factor which was recruited after mRNA biogenesis during this process (Kula et al., 2011, 2013). We could assess that MATR3 acts in the post-transcriptional steps of viral replication and we could demonstrate its role as a Rev-cofactor during the nuclear export of viral mRNAs.

To establish the functional role of MATR3 during acute viral infection we modulate its levels in Jurkat cells by both knockdown or overexpression. We found that, while MATR3 depletion resulted in the drastic reduction of viral replication, its overexpression leads to enhanced viral particle production. We applied the same approach to primary PBLs and obtained a similar result concluding that MATR3 is a positive regulator of viral replication.

To investigate a possible role for MATR3 in the establishment of viral latency we depleted MATR3 from J-Lat cells, a well-established model of latency. We observed that MATR3 depletion did not impede transcriptional reactivation of the integrated provirus upon TNFα stimulation, but strongly impaired intracellular viral protein production and full viral rescue.

This observation demonstrated that MATR3 depletion affects the post-transcriptional steps of latency reversal suggesting that this factor could play a crucial role during the maintenance of latency.

Since most of the attempts done with Latency Reversal Agents (LRAs), an heterogeneous group of drugs proposed to restore viral transcription, failed to induce full reactivation of the latent provirus (Darcis et al., 2015; Spina et al., 2013) we reasoned that there could be a post-transcriptional block to full viral reactivation

in latently infected cells and we suppose that MATR3 could represent a limiting factor to this process. We confirmed that MATR3 was almost undetectable in resting PBLs but could be promptly upregulated upon cellular activation. MATR3 was not induced by treatment with LRAs, such as SAHA or Romidepsin within PBL from healthy donors and HIV-infected patients.

We propose that the restoration of proper MATR3 levels within latently infected cells could enhance latency reversal in LRAs-treated cells.

INTRODUCTION

THE HIV-1 VIRUS: GENOME, STRUCTURE AND LIFE CYCLE

HIV-1 AND AIDS

The immunodeficiency virus type 1 (HIV-1) is the causative agent of the acquired immunodeficiency syndrome (AIDS), a systemic disease characterized by the dramatic drop in CD4+ T lymphocyte count which ultimately leads to the loss of immune system function.

Although the discovery of HIV-1 virus dated at more than 30 years ago, AIDS is still a mayor public health concern worldwide with a high burden of mortality and morbidity in developing countries (Barré-Sinoussi et al., 1983; Gallo et al., 1983). It has been estimated that around 35million people are HIV-1 infected and 2,3 million of new cases have been diagnosed only in 2012. Although the absolute number of new HIV infections is still high, it represents an historical minimum with a negative trend in morbidity which is remarked also by the huge decrease in death cases during the last decade (ECDC annual epidemiological report 2014).

Success in prevention and treatment of AIDS is due to the development of an efficient antiretroviral therapy based on the combined administration of drugs which target different phases of the viral life cycle. Constant adherence to the therapy results for the patient in the reduction of plasma viremia which is dropped to undetectable levels.

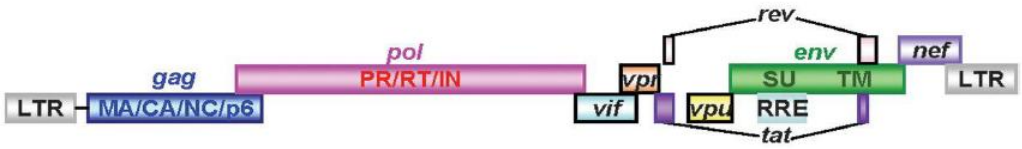
Despite its high efficiency in controlling the progression of the disease, the antiretroviral therapy is a life-long treatment which is not able to eradicate the viral infection. The therapy interruption results in the rebound of viral replication which is due to the existence of a “viral reservoir” in which the virus could remain dormant for an undefined time frame.

The existence of a viral reservoir constitute the major obstacle to HIV-1 eradication since the actual therapy are not able to target those cells which remained unaffected by treatment and invisible by the immune system. Currently, the development of a strategy able to target the viral reservoir represent the major challenge in the fight against AIDS.

HIV-1 GENOME

HIV-1 belongs to the family of Retroviridae (genus lentivirus) and its genome is based on 2 identical molecules of single-stranded RNA (+); each strand consists of about 9kb and carries a 5'CAP and a 3' polyA-tail.

HIV-1 genome contains 3 major structural genes named gag, pol and env which are shared by the other members of the Retroviridae family; moreover it contains 6 non-structural genes (Tat, Rev, Nef, Vpr, Vif, Vpu) encoding for accessory proteins.



HIV-1 genome organization – from Sakuma et al., Biochemical Journal 2012

The viral genome encodes three structural and six non-structural genes flanked by the LTRs. The Gag precursor consists of matrix (MA), capsid protein (CA), nucleocapsid protein (NC) and p6. The Pol gene encodes for reverse-transcriptase (RT), integrase (IN) and protease (PR). The envelope glycoprotein of HIV-1 encodes the polyprotein envelope precursor (gp160), which is eventually cleaved by PR to generate gp120 (SU) and gp41 (TM) domains. The RRE located within the *env* region is also indicated.

The gag gene encodes for a 55 kDa polyprotein then cleaved by the viral protease into the 3 structural proteins composing the virion: the p24 constitute the nucleocapsid, the p17 is the major component of the matrix while the p15, processed in p6 and p7, helps in the proper assembly of the structure.

The pol gene encodes for the viral enzymes: the reverse transcriptase (RT), a DNA-polymerase RNA-dependent which create a double-stranded DNA molecule from the RNA viral template, the integrase (IN) which catalyse the insertion of the viral genome within the host cellular DNA, and the protease (PR) which allows the maturation of both viral polyproteins and budding virions.

The env gene encodes for the two glycoproteins, gp120 and gp41, which form the spikes exposed on the external surface of the virion: these structures are re-

sponsible for the interaction between the virus and the host target cell by mediating the binding to receptors and co-receptors.

The accessory proteins are involved in a variety of functions within the viral life cycle, ranging from the enhancement of transcription to the modulation of the host immune response.

The Tat gene (Trans-Activator of Transcription) is constitute by two separate exons and encodes for a small protein (86-101 amino acids depending on the viral strain) which is crucial to enhance the transcription of the integrated provirus also thanks to its broad interactome (Gautier et al., 2009). The Tat protein binds the cis-acting elements TAR located downstream of the initiation site for transcription and promotes the assembly of the RNA Pol II transcription complex (Brady and Kashanchi, 2005); moreover Tat enhance transcripts elongation by promoting the activity of the p-TEFb factor, but also by recruiting some cellular histone acetyltransferases to remodel the chromatin at the LTR site (Benkirane et al., 1998; Zhu et al., 1997).

The Rev gene produces a 20 kDa protein which shuttles between the nucleus and the cytoplasm to mediate the export of intron-containing viral transcripts; this specific class of viral mRNAs are targeted by Rev because of the presence of the Rev-Responsive Elements (RRE), a sequence located at the 5' within the env gene which is bound by the protein to enhance their export.

The Nef gene (Negative Expression regulatory Factor) encodes for a phosphoprotein involved in the regulation of viral infectivity and in the evasion from the host immune response: after infection of the target cell, Nef, along with the Vpu viral factor, modulate the surface level of the CD4 receptor to avoid both the superinfection by other virions and the cytotoxic activity of CD8+ T lymphocytes. Recently, Nef was also reported to counteract the activity of the host restriction factor SERINC5, a cellular transmembrane protein which, if incorporated into nascent virion, render the particles less infectious. Nef is able to redirect the cellular localization of SERINC5 to the endosomal compartment to avoid its inclusion within the nascent virions (Rosa et al., 2015).

The Vif gene encodes for a small regulatory factor which strongly counteracts the cellular antiviral response during the very early phase of infection: Vif induces the proteasomal degradation of the cellular citidine deaminase APOBEC3G which lethally hypermutates the retroviral genome to inhibit the infection (Chiu et al., 2005).

The Vpr factor plays an important role in the formation of the pre-integration complex and has also been linked to the regulation of the cell cycle and the apoptosis in the infected cells (Planelles and Barker, 2010). Moreover Vpr was shown to directly interact with the transcription factor TFIIB and to be putatively able to bind DNA: thus Vpr can also contribute to induction of viral basal transcription at the LTR (Agostini et al., 1996; Zhang et al., 1998).

STRUCTURE OF THE VIRAL LTR

The viral Long Terminal Repeat (LTR) are non coding sequences of approximately 640bp in length located both at the 5' and 3' end of the provirus; each LTR consists of 2 *Unique* sequences (U3 and U5) and one *Repeat* sequence (R).

The U3 region of the 5'LTR contains the viral promoter with all the functional sequences to allow the initiation of transcription by the cellular RNA polymerase II: enhancer elements surround the TATA box while 2 binding sites for Nf-KB and 3 for Sp-1 ale placed in tandem immediately upstream of the TATA box.

The region immediately upstream the NF-kB binding site, also known as the "modulatory region", is rich in cis-acting binding sites for cellular factors responsible both the repression and the activation of the viral LTR (Jones and Peterlin, 1994; Pereira et al., 2000)

The TAR element is a 26 nucleotide sequence located downstream the initiation of transcription site, within the R region, and forms a highly stable stem-loop structure characterized by stems, pyrimidine-rich bulge and loops; this structure is recognized by Tat and essential for transcriptional transactivation: mutations analysis which disrupt the TAR base pairing were shown to abolish the Tat-driven LTR transcription (Selby et al., 1989).

At the edge of both the LTRs are located the *att* sites, targeted by the viral integrase to insert the provirus into the host genome; the packaging signals (PSI) required for the incorporation of the viral genome into the nascent virion are also located within the LTR.

STRUCTURE OF THE VIRION

The HIV-1 virion is a icosahedral enveloped particle of approximately 145nm.

The external phospholipid envelope is derived by the host cell membrane during the budding process of the nascent virion. On the envelope are exposed the gly-

coprotein spikes composed by heterodimers of gp120 and gp41: these structures mediate the interaction of the virus with the host target cells by recognizing and binding the cellular receptors and co-receptors present at the cellular membrane.

More internally in the structure there is the matrix formed by the myristoylated p17 protein, which allows the correct anchoring of the envelop to the viral capsid.

The most internal part of the virion is the nucleocapsid, a conical structure of p24, which contains both the viral genome associated with the p6 and p7 proteins and the viral enzymes of reverse transcriptase and integrase.

This final structure characterizes the mature virion and is generated by a process of maturation performed by the viral protease, which cleaves the multimeric gag polyprotein to allow the proper assembly of the different layers of matrix and nucleocapsid.

VIRAL LIFE CYCLE

The first contact between the virus and the target cell is established through the interaction of the viral gp120/gp41 heterodimer with the cellular CD4 receptor expressed by the cells of the monocytic-machrophage lineage and by lymphocytes.

The binding with the receptor causes a profound rearrangement in the structure of these proteins and lead to the exposition of those domains responsible for the binding to the cellular co-receptors: the usage of CCR5 as a co-receptor defines the HIV-1 R5-tropic strains, while the preference for the CXCR-4 co-receptor characterizes the HIV-1-X4-tropic strains.

The interaction with the co-receptor causes another structural change in the envelope spikes that induces the exposure of a portion of the peptide which is essential to allow the fusion of the viral and cellular membranes and the release of the viral nucleocapsid into the cellular cytoplasm.

Once in the cytoplasm, the nucleocapsid undergoes a process of disassembly called uncoating, which ends with the release of the viral genome, which is finally available for the subsequent reverse transcription: the viral RT uses the RNA template to generate a double-stranded DNA molecule, which assembles

with the integrase (Farnet and Haseltine, 1991) and likely with p17 and Vpr (Bukrinsky et al., 1993) to form the pre-integration complex (PIC).

The movement of the PIC through the cytoplasm depends on the association with actin (Bukrinskaya et al., 1998) and its import into the nucleus occurs through the nuclear pore complex, a process which seems to be mediated by the Vpr factor (De Rijck and Debyser, 2006).

The integration of viral genome into the host DNA is mediated by the viral integrase with the cooperation of several cellular factors: among them the nuclear protein LEDGF/p75 was found to interact with the integrase and to help its anchoring to the chromatin (Llano et al., 2004), while the DNA binding protein HGMA I seems to induce chromatin remodelling to facilitate the integration (Miller et al., 1997).

Transcription of the viral genes is performed by the RNA polymerase II starting from the viral promoter at the LTR and requires the trans-activator Tat to enhance the assembly of the complex of initiation of transcription and the recruitment of the elongation factors (Kao et al., 1987; Tahirov et al., 2010).

The immature full-length pre-mRNA undergoes a splicing process to generate 3 different classes of transcripts: the unspliced (US; 9kb) the singly-spliced (SS; 4kb) and the multiply-spliced (MS; 2kb) transcripts.

The US and the SS mRNAs encodes mostly for structural proteins and enzymes, but also, in the case of the US transcripts, serves as the viral genome to be encapsidated into new virions; the MS mRNAs are translated into regulatory factors such as Tat and Rev, crucial to allow full viral gene expression by acting both in the transcription and export of viral mRNAs.

The expression of the different viral genes is temporally distinct: regulatory genes (Tat, Rev and Nef) are translated during the early phase of the viral life cycle because of their requirement to allow the late phase genes expression by increasing the efficiency of transcription (Tat) or the mRNAs' nucleo-cytoplasmic export (Rev).

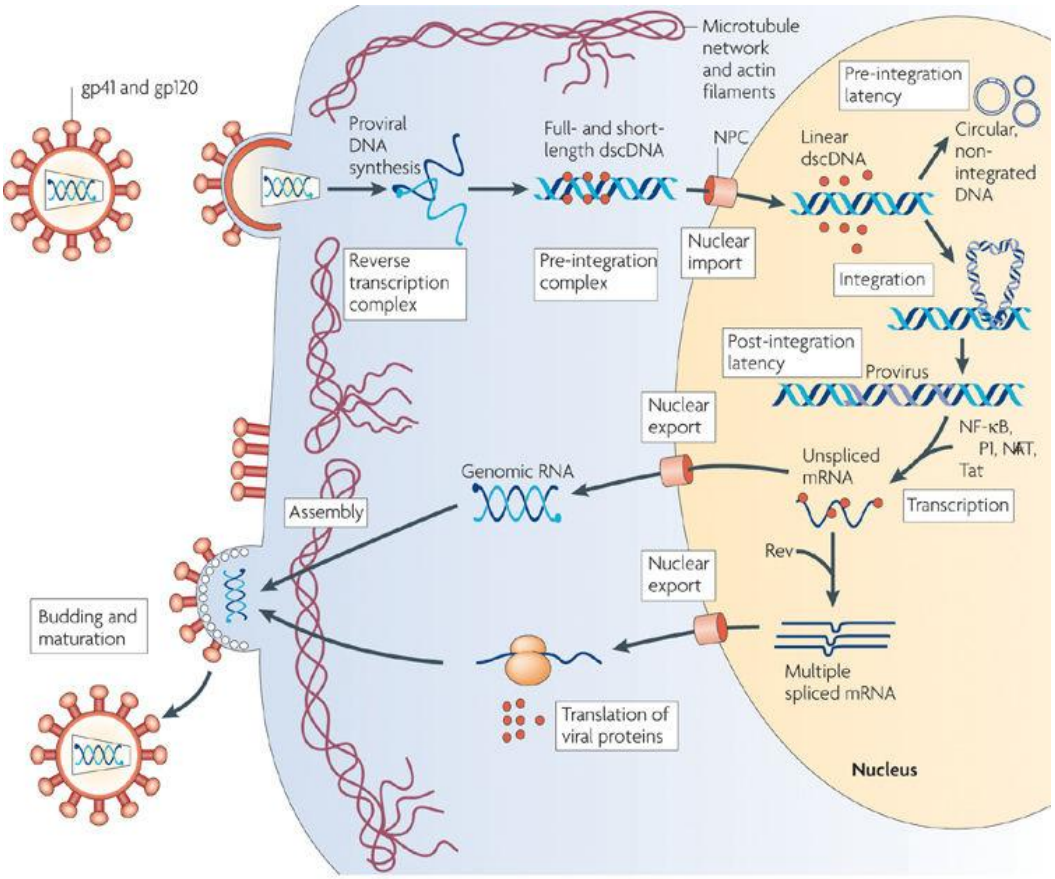
Once produced, the viral transcripts exploit different pathways to be exported to the cytoplasm: MS mRNAs use the same pathway of cellular ones, while the US and the SS transcripts need the presence of Rev to overcome the cellular restriction to the cytoplasmic translocation of intron-containing mRNAs.

The production of viral protein exploits the host cell translation machinery.

A first block to the initiation of translation is given by the presence of several highly structured motifs present at the 5'UTR of both US and MS transcripts: to overcome this block is crucial the RNA helicase DDX3 which binds the 5'UTR and recruits the factor eIF4G and PABP to allow the proper assembly of the pre-initiation complex and to enhance the initiation of translation (Soto-Rifo et al., 2012).

The initiation of translation of US-mRNAs can occur both in a CAP-dependent or IRES-dependent manner (Brasey et al., 2003; Ricci et al., 2008), since two internal ribosomal entry sites are present at the 5'UTR and within the gag coding sequence; the CAP-dependent pathway is preferred during the first 48hours after infection, while the IRES-dependent one is predominant at a later time point or under conditions of translation downregulation (Amorim et al., 2014).

The translation of US mRNAs needs to be finely regulated to guarantee the maintenance of a proper pool of not-translated US transcripts to be enclosed within the nascent virion as genomic DNA. The Rev protein was proposed to play a crucial role in this process in a way tightly related to its concentration: it was suggested that, when low levels of Rev are available, they are mostly bound to the DDX3 host protein which, ultimately, forms a complex with the US transcripts to stimulate their translation (Groom et al., 2009a).



HIV-1 life cycle – from Coiras et al.; Nat. Rev. Microbiol. 2015

After the initial binding to the target cell, the viral genome is relased into the cytoplasm and converted in a molecule of dsDNA, which integrates within the host genome. The viral gene expression relies on the host cellular machinery for viral mRNA transcription and translation. Once translated, the viral proteins and the viral genome start accumulate at the plasma membrane where the nascent virion is assembled and released.

The viral factors Env and Vpu are translated on the rough ER: the glycoprotein Env is inserted co-translationally into the ER membranes, is glycosilated and assembles into trimeric complex, which are then cleaved to separate the gp120 subunit from the transmembrane gp41.

The Env trimeric complex is shuttled to the plasma membrane through the vesicular transport and the protein spikes are inserted within the cellular membrane where the nascent virion is assembled.

The Gag protein and the Gag-Pro polyprotein concentrate at the plasma membrane and it was proposed that a crucial protein level, known as “cooperative threshold”, is required to trigger the activation the assembly process which begin with the interaction between the MA domain of Gag with the plasma membrane (Perez-Caballero et al., 2004; Yadav et al., 2012) and with the viral Env protein.

The Gag and the Gag-Pro proteins alone are able to orchestrate all the events required for the assembly of the nascent virion and particularly the CA domain was proposed to mediate all the processes: indeed it was shown that mature CA has the capacity to assemble into viral particle in vitro (Ehrlich et al., 1992).

The NC domain of Gag is able to bind the PSI sequence within the viral genome and thus is responsible to ensure its encapsidation within the virion (Rein et al., 2011).

The final release of the virion from the plasma membrane is mediated by the Host Endosomal Sorting Complexes Required for Transport machinery (ESCRT); after budding the viral protease cleaves Gag into the several components to allow the formation of the mature structure of the virion with a proper assembled nucleocapsid (Bieniasz, 2009).

EXPORT OF THE VIRAL mRNAs

VIRAL mRNA EXPORT

Upon transcription at the viral LTR, a full length 9kb transcript is generated and processed by the cellular splicing machinery to create 3 different class of mRNA: fully spliced, singly spliced and unspliced.

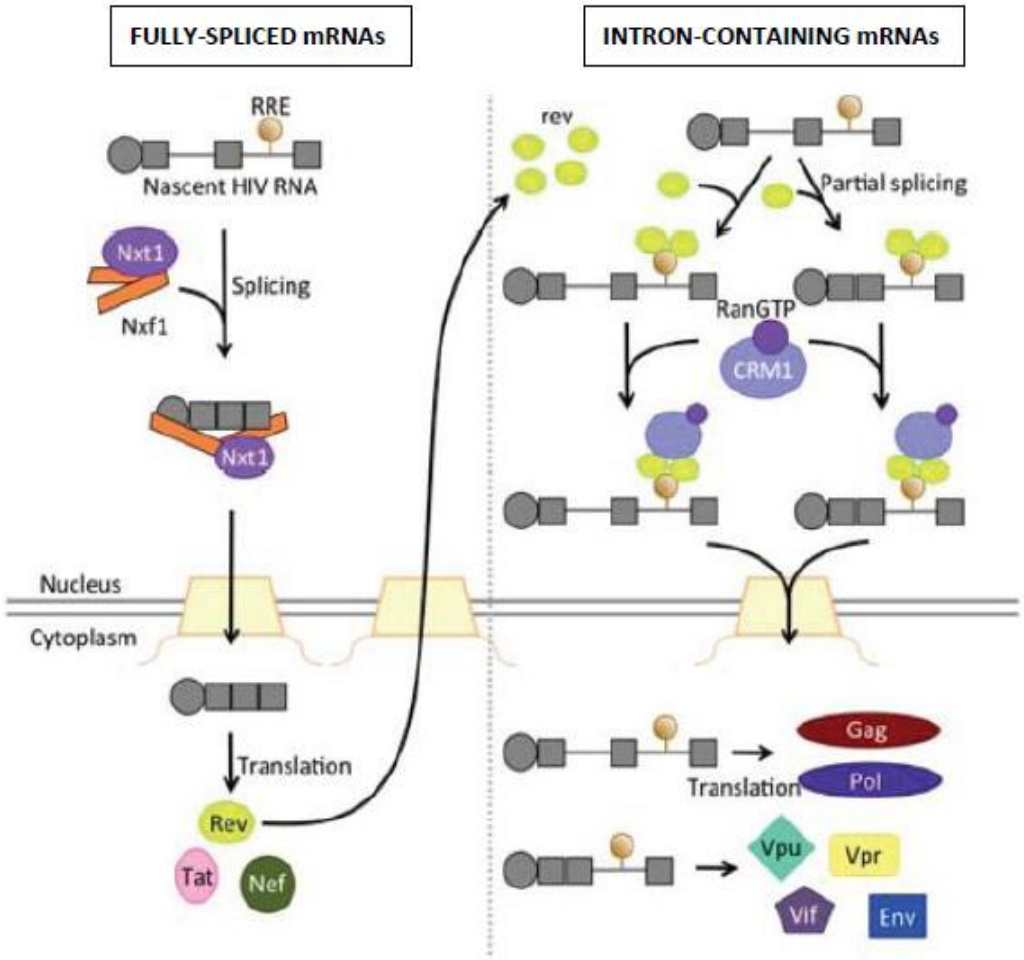
Fully spliced mRNAs encodes for the 3 regulatory protein Tat, Rev and Nef which are also known as “early phase genes” because of their requirement to allow full viral gene expression. Immediately after their processing these group of transcripts are exported to the cytoplasm by the same pathway used by cellular mRNAs, which involves the recruitment of Nxf-1 and of the Trex complex to allow the passage through the Nuclear Pore Complex (NPC).

The presence of introns in the mature form of singly spliced (SS) and unspliced (US) transcripts impede their export through the same pathway exploited by fully spliced mRNAs; these groups of transcripts encoding for structural protein and enzymes are called “late phase genes” and their retention within the nucleus would be an obstacle to full viral replication.

To overcome nuclear retention and degradation of intron-containing transcripts, the Rev protein is essential. The presence of a nuclear localization signal allows Rev to be imported into the nucleus where it targets these mRNAs by binding the Rev-Responsive Element (RRE), a sequence present within the 5’ of the Env gene (Fernandes et al., 2012; Malim et al., 1990). Thus Rev engages these viral mRNAs for an alternative route of nuclear export which ultimately involves the Chromosomal Maintenance 1 (Crm-1) export factor.

Due to its small dimension Rev acts as an adaptor protein in the interaction of the Rev-RNA complex with the cellular factor Crm-1, a karyopherin present in the nuclear export complex.

Crm-1 (also known as Exportin-1) is responsible for the nucleo-cytoplasmic export of cellular proteins and some cellular RNAs such as the ribosomal ones (Okamura et al., 2015); it binds the Ran-GTP in the nucleus and through the hydrolysis of the GTP into GDP is able to catalyze the export of a cargo to the cytoplasm most likely through the interaction with nucleoporins such as Nup98 and Nup214 (Monecke et al., 2009, 2013).



Viral mRNAs export – from Okamura et al.; Genes 2015

HIV-1 spliced transcripts are exported through the canonical cellular export pathway (left panel); intron-containing transcripts are exported through an alternative pathway involving the viral protein Rev and the host export factor Crm-1 (right panel).

To be targeted by Crm-1, the cargo needs to have a specific Nuclear Export Signal (NES) based on a leucine motif (or on other hydrophobic residues): even if the NES present on Rev is unconventional, it still allows the recognition and binding (Güttler et al., 2010; Paraskeva et al., 1999).

Usually Crm-1 binds the cargo in a monomeric form but the EM reconstruction of the complex with Rev-RNA showed unambiguously that, in this specific case,

Crm-1 is present as a dimer and it was proposed that the dimerization functions increases the affinity for Rev's unconventional NES (Booth et al., 2014).

In this view, the unusual dimerization of this factor is essential to the export of Rev-dependent mRNAs and the inability to dimerize would constitute a limit to full viral replication: in line with this idea, comparative sequence analysis of human and murine Crm-1 showed that the 7 amino acids of difference between the two proteins resides in Crm-1's dimer interface and this could provide a possible explanation for the defect in viral Gag protein production in murine cells (Sherer et al., 2011).

The presence of multiple NES on the Rev oligomer would suggests the existence of a panel of equivalent configurations for the complex but the binding possibilities are limited by steric effects due to Crm-1's large size: in fact binding of one Crm-1 subunit to a NES drastically reduces the possible orientations that the second Crm-1 could use to bind another NES (Booth et al., 2014; Daugherty et al., 2010a).

Once the Rev-RNA complex translocates to the cytoplasm, Rev dissociation from the mRNAs occurs by a still unknown mechanism; different modes have been proposed, between the most likely there is the competition with nuclear import machinery for the NLS present in the same motif for RNA binding and the forced ejection of Rev by the ribosome: in any case cellular factors such as helicases are believed to help in complex disassembly (Fankhauser et al., 1991; Hadian et al., 2009; Kutluay et al., 2014; Naji et al., 2012).

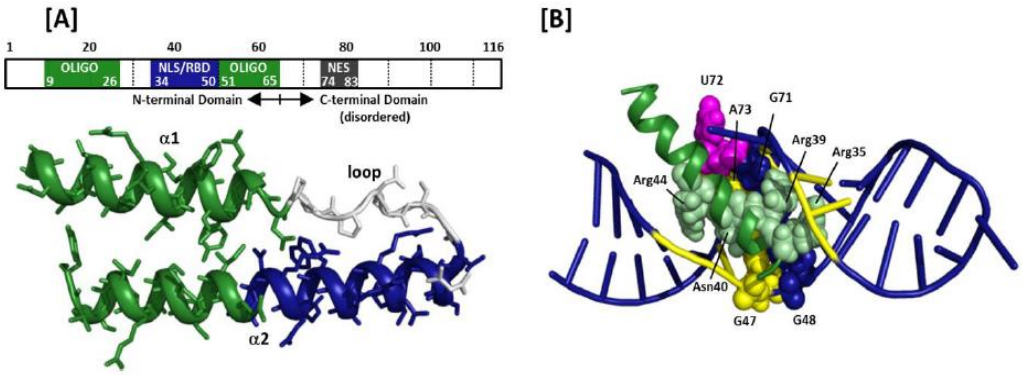
THE REV PROTEIN

The rev gene is transcribed early during the viral life cycle into a completely spliced mRNA and is translated into a small protein with dimensions ranging from 96 to 129 amino acids in patients (in most cases either 116 or 123 amino acids).

It contains a bipartite Oligomerization Domain (OD), an Arginine-Rich Motif (ARM) and a Nuclear Export Sequence (NES).

The ARM domain is an arginine-rich sequence which serves also as a nuclear localization signal (NLS). The ARM is responsible for the initial binding with the RRE on viral transcripts and leads to the recruitment of additional Rev monomers to form the final Rev-RRE complex; the interaction between Rev monomers occur through the OD domain (Zapp et al., 1991). The NES domain, necessary to allow

the Rev shuttling between the nucleus and the cytoplasm, binds the Crm-1 to assure the mRNAs export activity; moreover the NES domain was supposed to be critically important also for other activities such as the degradation of Tat, proposed to be a mechanism involved in the establishment of latency (Lata et al., 2015).



Rev structural organization and ARM/Stem loop IIB complex- from Rausch and Grice; Viruses 2015

A) Rev organization according to primary sequence and 3D structure; the bipartite oligomerization domain (OLIGO) and the nuclear localization signal/RNA binding domain (NLS/RBD) are shown in green and blue, respectively. The C-terminal domain of Rev, which houses the nuclear export signal (NES) is intrinsically disordered.

B) ARM peptide in complex with stem-loop IIB model RNA. ARM binds in the RNA major groove; the aminoacidic residues and nucleotides involved in direct contact are highlighted.

The Rev protein has a well-established function in the export of intron-containing viral mRNAs and viral vectors, defective for Rev, shows impaired infectivity. The protein level of Rev are finely regulated by a negative feedback loop: a high concentration of Rev increases the rate of export of unspliced transcripts thus reducing the amount of RNAs available for complete splicing and, as a consequence reduces the levels of Rev expression (Felber et al., 1990).

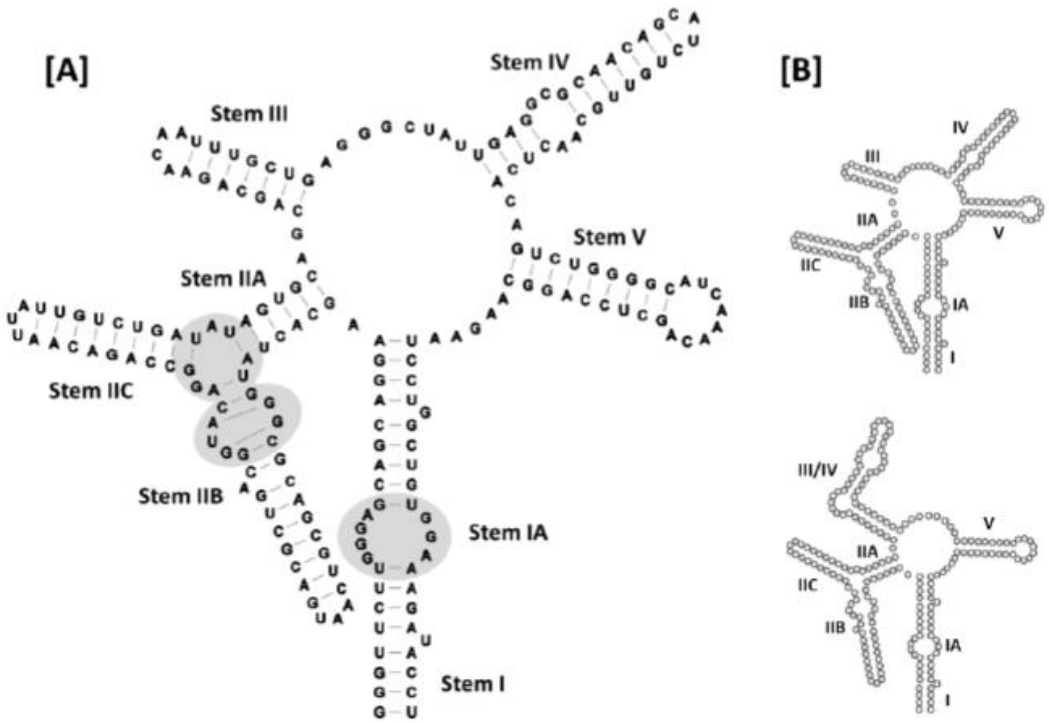
Different research groups reported a discrepancy between the fold increase in cytoplasmic accumulation of unspliced RNAs and in their protein products in

presence and absence of Rev (Emerman et al., 1989; Perales et al., 2005): in presence of Rev, a few-fold increase in the rate of cytoplasmic RNA was associated to a much stronger accumulation of viral protein. These observation suggested an additional role for Rev in the enhancement of RNA translation; this hypothesis was reinforced by the report of a direct interaction between Rev and the polysomes (D'Agostino et al., 1992; Groom et al., 2009b). Moreover, Rev was supposed to have a role also during the assembly of nascent virions by its direct involvement in the packaging of the viral genome (Groom et al., 2009b).

Even if the focus on Rev research was mostly put on its role on export, as frequently observed for retroviral factors, a single protein could fulfil a wide range of tasks and be involved in different events during the viral replication; thus the Rev protein could exert a number of functions which of them are still not fully described.

REV-RRE INTERACTION

The RRE is a highly structured sequence consisting of 242 nucleotides at the 3' of the HIV genome within the Env gene. It exists in two conformational states of 4 or 5 stem loops (since stem III and IV could be fuse as a single stem loop); both conformations are functional for Rev binding although they were shown to be different in the efficiency of mRNA export activity, with the 5 harpin structure displaying the higher export activity (Heaphy et al., 1990; Olsen et al., 1990; Rausch and Le Grice, 2015; Sherpa et al., 2015).



Secondary structures of HIV-RRE - from Rausch and Grice; Viruses 2015

A) RRE 5-stem structure. Established Rev-binding sites at the stem IIB, the stem-loop II junction and the stem IA are indicated in grey ovals.

B) Comparison of the 5 and 4-stem structures. Differences between the two models are limited to the base pairing patterns of nucleotides comprising stem loop III and IV, and stem loop III/IV in the respective structures.

As deduced from mutational studies (Huang et al., 1991; Jayaraman et al.; Malim et al., 1989; Tiley et al., 1992), the RRE carries three major sites for Rev binding: Stem IA, Stem IIB and the Junction between Stem IIABC .

The three-dimensional model of RRE derived from the Small Angle X-ray Scattering (SAXS) shows an “A-shape” folding with Stem IA and Stem IIB close in space, suggesting that a Rev dimer may bind simultaneously to the two sites; taking account of the existence of different RRE conformers the structure depicted may reflect the solution average of multiple structures (Fang et al., 2013).

The Arginine-rich motif (ARM) of Rev is responsible for the interaction with the RRE: the high affinity binding of the ARM to the Stem IIB is an anchor point for the recruitment of six Rev molecules on the RRE sequence till the formation of the complete Rev-RRE ribonucleoprotein complex (RNP) identified as a Rev hexamer on the 242nt RRE (Daugherty et al., 2010b).

The Rev's ARM can use several aminoacidic residues to interact with different binding sites on the mRNA and the interaction mostly rely on RNA structure and ARM orientation than on nucleotide sequence (Jayaraman et al., 2015): the high degree of Rev-RRE complex plasticity may represent the key of the functional robustness to mutations in the Rev sequence.

After the initial binding to the Stem IIB, Rev oligomerization on the RRE was proposed to continue with the formation of a dimer to bind the Junction IIABC; then the binding of additional Rev molecules proceed across the Stem IA till the formation of the Rev hexamer (Bai et al., 2014).

It's still unclear if, to prevent the binding of additional Rev molecules to the viral RNA, the negative feedback mechanism which regulate Rev concentration is implicated, or if cellular factors, such as the hnRNPs, fulfil this task by directly binding the viral RNA (Cochrane et al., 2006; Felber et al., 1990).

The Rev-Rev interaction occurs via the bipartite, hydrophobic OD domain and could lead to at least 3 different orientation of the Rev-Rev complex and this plasticity is useful to recognize the different possible Rev-RRE complex structures (Jayaraman et al.).

CELLULAR FACTORS INTERACTING WITH REV

The activity and localization of Rev are largely dependent on its interaction with a variety of cellular factors: Rev's interactome has been studied both experimentally and computationally to understand the molecular basis of the host-virus interaction and of the cellular restriction to viral infection (Brass et al., 2008; König et al., 2008; Trono and Baltimore, 1990).

Post translational modification of Rev such as phosphorylation and methylation are carried out by CK2 (Meggio et al., 1996) and PRMT6 respectively (Invernizzi et al., 2006) to influence or regulate its activity.

Apart from chemical modification, also some cellular proteins can influence Rev shuttling between distinct cellular compartments: the Importin B binds to Rev's

NLS to induce it's nuclear translocation and factors such as HIC1 (Human I-mfa domain Containing Protein) and NAP1L1 (Nucleosome Assembly Protein 1) have been shown to participate to this process (Cochrane et al., 2009; Gu et al., 2011; Henderson and Percipalle, 1997). In particular the interaction between Rev's NLS and HIC1 have been shown to inhibit Importin B-mediated nuclear import of Rev, but not transportin-mediated one suggesting that the spatial localization of Rev could be regulated in alternative ways (Gu et al., 2011).

To allow the export of viral US-mRNAs Rev takes advantage of the interaction with several host factors: the recruitment of the CRM-1-Ran GTP complex allows the translocation of the Rev-RNP complex through the nuclear pore; other factors involved in this process are the eukaryotic initiation factor-5A (eIF-5A), which induces the interaction between Crm-1 and the nucleoporins (Ruhl et al., 1993), and the helicase DDX3 (or DDX1 in astrocytes) which help the passage of the RNP complex through the pore(Fang et al., 2005; Yedavalli et al., 2004).

Other proteins belonging to the RNA helicases family have been shown to interact with Rev and to affect HIV replication by influencing the mRNA metabolism: among them, DDX3 seems to be implicated in mRNA translation while DDX5 and DDX17 affect both the amount and the export of viral transcripts suggesting a potential involvement in transcription, splicing or export (Naji et al., 2012).

THE REV EXPORT COMPLEX

To better characterize the involvement of host factors in the Rev-mediated nuclear export and to identify novel cellular interactors of the Rev-RNA complex, an approach based on the immunoprecipitation of the MS2-tagged viral RNA (Maiuri et al., 2011) coupled to a mass spectrometry analysis was developed in our laboratory. Through this approach it was possible to identify the PTB-associated Binding Factor (PSF) and Matrin3 (MATR3) as novel components of the Rev-RNA RNP complex (Kula et al., 2011a).

A more detailed analysis of this complex revealed that while MATR3 directly binds to PSF, its interaction with Rev occurrs in a RNA-dependent manner since it was lost upon treatment with the RNAse (Kula et al., 2013a).

Moreover, it was possible to determine that, while PSF and Rev contacted the nascent mRNA at the site of transcription, MATR3 was recruited in a second moment to most probably enhance the Rev-mediated export of viral mRNAs (Kula et al., 2011a; Yedavalli and Jeang, 2011).

PSF binding to the INS sequences of the HIV-RNA was shown to stabilize the viral transcripts (Zolotukhin et al., 2003) and both PSF and MATR3 were previously implicated in the nuclear retention of hyperedited RNAs (Zhang and Carmichael, 2001): the documented involvement of PSF and MATR3 in mRNA metabolism make reasonable to hypothesize that their interaction with Rev is finalized to avoid the nuclear retention of viral transcripts and that they are recruited by Rev to the alternative route of viral mRNA export.

THE NUCLEAR MATRIX PROTEIN MATR3

The nuclear matrix protein MATR3 binds the inner nuclear membrane and, along with the other matrix proteins, forms a fibrogranular network involved in chromatin organization, genome replication and gene expression (Bode et al., 2003).

Concerning its sub-cellular localization, MATR3 is mainly found in the insoluble nucleoplasm with the exception of the nucleolus even if, by biochemical fractionation, it was shown that it's possible to find MATR3 also in the soluble nucleoplasmic fraction and, at lower levels, in the cytoplasm (Coelho et al., 2015; Hibino et al., 2006; Zeitz et al., 2009).

MATR3 is an highly conserved 125 kDa protein which contains a bipartite nuclear localization signal (NLS), two zinc-finger domains (ZF) and two RNA-recognition motifs (RRM); the remaining disordered region of the protein is supposed to mediate protein-protein interactions, as in the case of the PTBP1- RRM Interaction Motif (PRI), a seven amino acid sequence found within this region which is responsible for the interaction with the splicing factor PTB (Coelho et al., 2015; Hibino et al., 2006).

The two ZF domains mediate the interaction with the cellular DNA and the deletion of both of them is required to abolish the DNA binding.

The two RRM domains are responsible for the binding to the target RNA and it has been suggested that the RRM preferentially recognize the AUCUU motif as the optimal RNA target site (Coelho et al., 2015; Hibino et al., 2006).

Several proteins have been reported to interact with MATR3 and, of note, the majority of them are factors involved in RNA binding and metabolism such as PSF, PTB and p54nrb. Also thanks to its broad spectra of interactors, MATR3 was proposed to exert a number of functions ranging from the regulation of mRNA splicing and stability (Coelho et al., 2015; Salton et al., 2011), to the involvement

in viral mRNA export (Kula et al., 2011a, 2013a; Yedavalli and Jeang, 2011) and in the DNA damage response (Salton et al., 2010).

It was reported that Double Strand Breaks (DSBs) induces MATR3 phosphorylation at the Serine208 by the ATM kinase and this modification was proposed as a trigger event to induce the DNA damage response: upon phosphorylation, MATR3 forms a complex with PSF and p54nrb, and their interaction with MATR3 was proposed to be crucial for the correct recruitment and release of the two factors at the damaged site (Salton et al., 2010).

MATR3 was frequently associated to different processes in RNA metabolism.

Transcriptome-wide analysis performed in MATR3 depleted HeLa cells, showed that MATR3 can regulate alternative splicing with or without the cooperation of PTB (Coelho et al., 2015), while MATR3 depletion in U2OS cells was associated to the altered expression of 77 genes. In particular it was shown that upon MATR3 knockdown the levels of these transcripts were reduced and the effect was associated to a reduced RNA stability while a transcriptional effect was excluded (Salton et al., 2011).

Mutations in MATR3 sequence have been associated to the development of a progressive miopathy called Vocal Cord and Pharyingeal weakness with Distal Myopathy (VCPDM) (Senderek et al., 2009) and, more recently, to the familiar form of Amiotrophic Lateral Sclerosis (ALS) (Johnson et al., 2014).

Further investigation on the pathological role of ALS-associated mutations of MATR3 sequence suggested that these mutations altered MATR3 interaction with several components of the cellular Transcription and Export protein complex (TREX) thus leading to altered global mRNA nuclear export (Boehringer et al., 2017).

MATR3 was also identified as a positive regulator of HIV-1 replication as it is required to allow full viral gene expression by enhancing Rev-mediated export of US-mRNAs (Kula et al., 2011a, 2013a; Yedavalli and Jeang, 2011); moreover, it was observed that MATR3 depletion strengthens the antiviral activity of the potent restriction factor ZAP against retroviral infection (Erazo and Goff, 2015).

VIRAL LATENCY

LATENCY: DISCOVERY, DEFINITION AND COMPOSITION OF THE VIRAL RESERVOIR

In the late 1990's different clinical investigation on cohorts of HIV+ patients reported that the administration of the Highly Active Antiretroviral Therapy (HAART) for several years was not sufficient to eradicate the virus: upon treatment discontinuation the plasma viremia recurred in few weeks (Palmer et al., 2008; Zhang et al., 2000).

At the same time it was demonstrated that the virus can establish a latent phase within a small fraction of infected cells: in HAART treated patients it was identified a subset of cells which contained a copy of the integrated provirus (~1 integrated copy of HIV in 1x10^6 CD4+T cells) but didn't express viral particles (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997). These cells carrying a silent provirus were found to be responsible for the rebound of plasma viremia after drug interruption and were defined as the "viral reservoir" established very early during infection. Notably, a fraction of these infected cells contained a defective virus whose expression couldn't be rescued and thus their contribution to the clinically significant pool of the viral reservoir is null.

Latently infected cells are thus defined as the cells which carry at least one copy of integrated HIV DNA; the provirus is silent but inducible and, upon cellular activation, is able to produce and release full viral particles.

Some authors reported that, on the contrary to current opinion, latently infected cells were capable of transcribing low levels of HIV RNA and prevalently unspliced viral transcripts were detected (Lassen et al., 2004; Pasternak et al., 2009). It is unclear if the rate of viral mRNA transcription was sufficient to achieve viral protein production in latent cells; however, the detection of viral transcripts and the absence of full virions suggested the existence of multiple barriers to productive infection within the cells of the viral reservoir.

Several cell types were identified as components of the viral reservoir (Chomont et al., 2009; Palmer et al., 2008); nevertheless the resting central memory CD4+ T lymphocytes were proposed as the most representative ones and are characterized by the lack of activation markers such as CD25 and CD69 (Chun et al., 1995; Han et al., 2007) and by the poor expression of transcription factors like NFAT

and NF-kB, which contribute to the maintenance of the proviral transcriptional silencing (Colin and Van Lint, 2009).

The virus may persist also in macrophages (Igarashi et al., 2001) and in stem or progenitor cells (Carter et al., 2010); due to their short half life dendritic cells and monocytes seems unlikely to represent an important part of the reservoir even if, upon their differentiation into macrophages, they can contribute to the formation of local reservoirs in specific body districts such as the central nervous system (Burdo et al., 2010; Hasegawa et al., 2009).

ORIGIN AND PERSISTENCE OF THE RESERVOIRE

The molecular details which lead to the establishment of a viral reservoir early during the infection is still a matter of debate. Latently infected cells may arise from the infection of activated cells that, after viral integration, return to a resting state, as suggested by the observation that memory T cells are the major component of the viral reservoir (Chun et al., 1995, 1997). However, the infection could also occur directly in resting T cells as observed in vitro (Agosto et al., 2007; Plesa et al., 2007): this event is even more likely to occur in vivo in a cytokine rich environment supported by the presence of dendritic cells and macrophages (Eckstein et al., 2001; Haase, 2005; Swingler et al., 2003).

The prevalent theory about how the reservoir is maintained is that long-living cells, like central memory CD4+ T lymphocytes, persist for the whole life of the patient by homeostatic proliferation induced by cytokines like IL-7 (Chomont et al., 2009; Dahl and Palmer, 2009; Shen and Siliciano, 2008). This theory is supported by the detection over the time of a constant level of latently infected cells as measured by the Infectious Units Per Millions Assay (IUPM) and by the quantification of integrated HIV DNA in patients (Chomont et al., 2009; Finzi et al., 1997; Siliciano et al., 2003; Wong et al., 1997).

However, it was also suggested that different mechanisms of persistence may occur in different subsets of latently infected cells (Chomont et al., 2009).

ASSAYS TO MEASURE THE RESERVOIR

The existence of a viral reservoir constitute the major barrier to viral eradication and thus the measure of its extent can provide an essential parameter to evaluate the success of the therapeutic strategy and to predict the prognosis in patients.

The first assay established to measure the viral reservoir is the Infectious Units Per Millions (IUPM), also called as Viral Outgrowth Assay (VOA), developed by Siliciano and Wong (Finzi et al., 1997; Wong et al., 1997). This method consists of the serial dilution of resting CD4+ T cells isolated from patients under HAART and their activation by PHA in the presence of T lymphoblasts to allow the spreading of infection in such target cells. The count of positive wells for the spreading of infection allows thus to estimate the number of latently infected cells which reverted to productive infection: therefore the major advantage of this method is the detection of the portion of the viral reservoir that can be successfully induced to release infectious virus.

However, in specific groups of patients, like the Elite Suppressors (ES), the size of the viral reservoir is so reduced to be under the limit of detection with the IUPM assay: in this case the measurement of the integrated HIV DNA provides a better alternative to estimate the viral reservoir, even if the presence of defective integrated provirus could lead to its overestimation(Brussel et al., 2005; Chun et al., 1997).

Recently, the group of Chomont (Procopio et al., 2015) developed a novel assay to measure the inducible viral reservoir. The TILDA assay (Tat/Rev Induced Limiting Dilution Assay) is based on the assumption that multiple-spliced HIV RNAs are usually poorly expressed within latent cells and thus its transcriptional induction upon cellular activation can provide the quantification of cells carrying an inducible replication-competent provirus. Thus this assay provides a sensitive detection of that specific subset of cells which carry a non-defective integrated provirus, since it has been observed that many defective HIV genomes have deletions in the Tat/Rev genes and thus their mRNA are unlikely to be detectable (Ho et al., 2013).

MOLECULAR MECHANISMS OF POST-INTEGRATIVE LATENCY

Taking advantage of the previous mentioned assays and of the quantification of the integrated HIV DNA in CD4+ T cells, it was possible to verify that the size of the viral reservoir is not much affected by the administration of the antiretroviral therapy (Chomont et al., 2009; Siliciano et al., 2003) and that each patient seems to maintain a stable number of latently infected cells during all the course of infection.

Thus, the comprehension of the molecular details behind the establishment of latency is required to develop a therapeutic approach targeting all the cells, both

in the acute and latent phase of infection, to achieve a “sterilizing cure” to eradicate the virus.

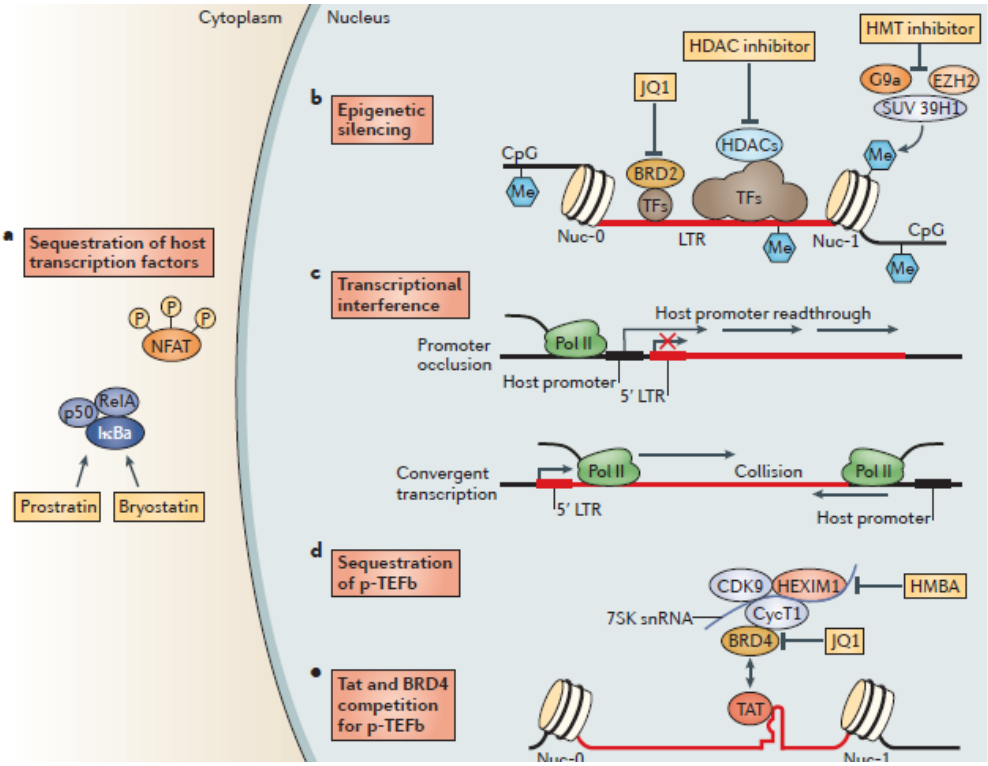
The establishment of latency within infected cells is a complex process depending on multiple mechanisms most of which are still a matter of debate and investigation. During pioneering studies on the topic, a bias was posed in proposing the transcriptional mechanisms as the predominant way to maintain the viral latency.

Nowadays increasing evidence support the idea that, even if the transcriptional silencing of the provirus plays a major role in this process, it could not be sufficient to explain the tightly regulated phenomena of latency, and it was proposed the existence of different post-transcriptional mechanisms contributing to latency to be investigated.

TRANSCRIPTIONAL MECHANISMS IN VIRAL LATENCY

The transcriptional silencing of the provirus is definitely the results of a number of events and condition occurring together within latent cells: the site of integration and the state of chromatin condensation at the viral promoter firstly contribute to render the chromatin inaccessible to the transcription complex.

Moreover, the limited action of the transcription complex, is a crucial block to viral expression and it results as a consequence of both the limited availability and altered activation status of the transcription factors. To impair the transcription al the viral promoter a big contribution is given by the modulation in the levels and in the activity of the viral trans-activator Tat which is absolutely required to enhance both the initiation of transcription and the elongation process. Last but not least, the cellular pathway of RNA interference was also shown to be involved in the maintenance of latency.



Transcriptional mechanisms in viral latency - from Archin et al.; Nat. Rev. Microbiology 2014

HIV-1 transcriptional silencing is maintained by several mechanisms. a) transcription factors are sequestered in the cytoplasm ; b) epigenetic modification at the viral promoter; c) transcriptional interference by RNA PolII readthrough; d/e) inactivation of the p-TEFb complex. Different drugs proposed to revert transcriptional silencing are shown in yellow boxes.

The analysis of the HIV provirus in both in vitro models of latency and in resting CD4+ T cells from HIV+ patients revealed that the preferential sites of integration were regions within actively transcribing genes (Han et al., 2004; Shan et al., 2011), supporting the idea that the establishment of latency is unlikely to be related to integration of the provirus within heterochromatic region.

Nevertheless epigenetic modifications of the chromatin at the local site of integration could influence the availability of the LTR promoter to the cellular transcription factors.

Despite the site of integration, the nucleosome Nuc-0 and Nuc-1 were found to bind the 5'viral LTR respectively upstream of the modulatory region and downstream of the viral promoter thus not only physically impeding the processivity of

the cellular RNA polymerase, but also occupying the binding sites for the transcription factors and the regulatory proteins (Verdin et al., 1993).

A deeper analysis of the chemical modification to Nuc-1 showed markers associated with condensed and silent chromatin, such as the methylation of the histone3 and the lack of proper acetylation (Pearson et al., 2008; Van Lint et al., 1996): such epigenetic modifications are due to the recruitment at the viral promoter of the Histone Methyl-Transferase (HMT) and the Histone Deacetylases (HDAC) by several cellular factors like Sp1, CBF-1 and CTIP2 (Jiang et al., 2007; Marban et al., 2007; Tyagi and Karn, 2007).

The epigenetic modification operated by HMTs and HDACs are important not only to affect the nucleosome assembly. The acetylation of non-histonic substrates is important to regulate the activity of some transcription factors, such as RelA, or cellular factors such as HIC1. HIC1 is a repressor of transcription proposed to be involved in the establishment of latency within microglial cells, the major viral reservoir within the central nervous system, along with CTIP2 and HMGA1; it was shown that its acetylation status, regulated by SIRT1, influences its binding to the viral Tat, thus affecting the Tat-mediated regulation of viral transcription (Le Douce et al., 2016). The activity of Tat in microglial cells was also found to be negatively regulated by CTIP-2 which redirected Tat localization to inactive region of the chromatin (Rohr et al., 2003).

The catalytic activity of HMT is exerted not only on histones but also directly on the CpG islands at the viral 5'LTR, even if it is still unclear whether the methylation status of the DNA represent an important contribution to latency (Blazkova et al., 2012).

The viral LTR contains multiple binding sites to different cellular transcription factors like NF-kB, NFAT, SP1 and the activator protein AP1: the activation status and the availability of such factors appears to be one of the major contributors to the transcriptional block in latency.

The inhibitory homodimeric form of NF-kB, p50/p50, binds the viral LTR to repress transcription, while the active heterodimeric form p50/RelA is sequestered in the cytoplasm by the IkB-α inhibitor; the stimulation of the PKC pathway would result in the release of the active p50/RelA form which leads to the displacement of the p50/p50 complex from the LTR and to the recruitment of HATs to remodel the Nuc-1 to finally induce the initiation of transcription (Zhong et al., 2002).

Once the transcription starts, the RNA Pol II get stalled and needs to be phosphorylated to proceed with the elongation of the transcripts: thus the recruitment of the Positive Transcription Elongation Factor b complex (P-TEFb) is the crucial event to avoid the premature termination of transcritpion.

The P-TEFb complex contains the Cyclin T1 and the cyclin-dependent kinase 9 (CDK9): both of this factors are reported to be expressed at limiting levels in resting CD4+ T cells (Budhiraja et al., 2013). Besides the limited availability of these factors in resting conditions, the P-TEFb complex is sequestered in a large repressed ribonucleoprotein complex containing the 7SK RNA and the proteins HEXIM2, MePCE and LARP7 (Michels et al., 2004): release of the active P-TEFb complex is essential to induce the proper phosphorylation of RNA Pol II by CDK9 and thus to overcome the initial block of the transcriptional machinery (Fujinaga et al., 2004; Ping and Rana, 1999).

POST-TRANSCRIPTIONAL MECHANISMS IN VIRAL LATENCY

The transcriptional silencing of the integrated provirus plays a major role in the maintenance of HIV latency and the removal of these transcriptional blocks is without a doubt fundamental to achieve viral reactivation.

Nevertheless, it was demonstrated that when latently infected cells from HIV+ patients are stimulated with transcriptional inducers to restore viral gene expression, the rescue of full viral particles from these cells was affected, even if a proper level of intracellular viral transcripts was detected (Mohammadi et al., 2014).

This observation demonstrate that overcoming transcriptional limitation is necessary but not sufficient to revert latency: several post-transcriptional blocks exist within latent cells and impede full viral reactivation even upon proper viral transcription. Limiting levels of the host factors involved in mRNA metabolism can affect proper stabilization, splicing and export of the viral mRNAs; moreover the activity of those factors could be altered in resting lymphocytes (Sarracino and Marcello, 2017).

Taking advantage of ultra sensitive methods for intracellular and extracellular viral RNA detection, it was possible to determine that in resting CD4+ T cells from HIV+ patients under HAART, both unspliced and multipli-spliced viral mRNA were present (Lassen et al., 2004). An accurate analysis of the subcellular localization of these mRNAs revealed that they were retained in the nucleus suggesting the

existence of a block in the nuclear export of viral RNAs. Notably, upon cellular activation by TCR stimulation, both US and MS mRNAs exhibited a proper cytoplasmatic distribution within 72 hours (Lassen et al., 2006).

More evidences on the existence of post-transcriptional mechanisms to viral silencing came from the use of in vitro model to reproduce the cellular environment of viral latency.

In an in vitro model of latency based on the direct infection of resting PBLs, abundant levels of unspliced viral transcripts were detected, while the spliced forms were less represented demonstrating an inefficient splicing process in such conditions (Pace et al., 2012). The concomitant analysis of intracellular viral proteins revealed that the level of Gag was much lower than expected based on its high mRNA concentration suggesting an additional block in the nuclear export of the unspliced mRNAs.

These observations suggested that, in latently infected cells, both viral mRNA biogenesis and their nuclear export are affected thus leading to defects in viral protein production.

In accordance with the hypothesis that defective mRNA processing could contribute to post-integrative latency, it was reported that several factors involved in mRNA metabolism are limiting in CD4+ T cells under resting conditions and that their expression is increased only upon cellular activation (Mohammadi et al., 2014); some of these factors were previously found to interact with the viral RNA and in some cases it was shown that their overexpression can revert latency both in cellular models and in primary lymphocytes from patients.

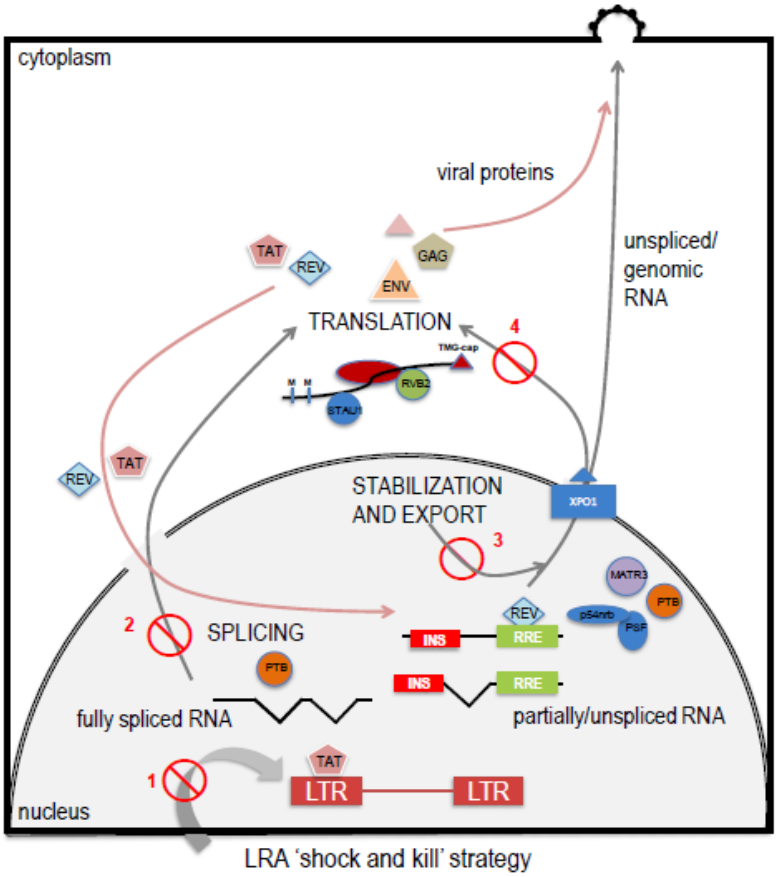
The Polypyrimidine Tract Binding Protein (PTB) is a cellular factor involved in the post-transcriptional regulation of gene expression by different processes, like mRNA splicing (Kosinski et al., 2003; Singh et al., 2004); PTB was found to associate with the viral mRNA (Black et al., 1996) and to be barely expressed in cells of the viral reservoir. Notably it was demonstrated that PTB overexpression in resting latently infected CD4+ T cells was able to restore full virus production (Lassen et al., 2006).

The PTB-associated Splicing Factor (PSF) plays several roles in cellular mRNA metabolism (Emili et al., 2002; Shav-Tal and Zipori, 2002) and was proposed to enhance viral RNA stability by preventing their degradation (Kula et al., 2013b), a role shared with the heterogenous ribonuclear protein A1 (hnRNP). In lympho-

cytes, the activity of PSF and hnRNP A1 is finely regulated by phosphorilation via the MAP-kinase signal-integrating kinases (Mnks). The phosporylation of PSF occurs differently in resting and activated CD4+ T cells and this influence the interaction of PSF with other host factors leading to a modulation in its activity (Buxadé et al., 2008)

In particular, in resting lymphocytes, the protein kinase GSK3 phosphorylates PSF which, in turn, strongly associates with TRAP150 and BTF, two host factors implicated in mRNA processing, stability and, along with hnRNP A1, in splicing regulation. It was demonstrated that TRAP150 binds PSF on its RNA-recognition motifs (RRM), an interaction which abrogate PSF binding to target mRNAs (Heyd and Lynch, 2010; Yarosh et al., 2015).

PSF was found to be a part of the Rev-RNA ribonucleoproteic complex along with the nuclear matrix protein MATR3, a factor linked to the Rev-mediated export (Kula et al., 2011b). Both MATR3 and PSF were found to be limiting under resting conditions and to be promptly upregulated upon cellular activation suggesting that their limiting levels could affect proper mRNA stabilization and export during latency.



Post-Transcriptional blocks to latency reversal – modified from Sarracino and Marcello, Curr. Pharm. Des. 2017

HIV-1 transcriptional silencing is maintained by different mechanisms.

1) epigenetic and molecular blocks inhibit HIV-1 expression; transcriptional activation is obtained by LRAs;

2) efficient splicing and export of fully-spliced transcripts is require to allow the production of regulatory proteins;

3) limiting levels of cellular Rev co-factors results in nuclear retention of viral intron-containing mRNAs;

4) translation of properly chemically modified viral transcripts is inhibited in latent cells by several mechanisms involving mRNA degradation and their sequestration in cellular granules.

The involvement of miRNA-mediated silencing in the process of latency was demonstrated by showing the physical association between Ago2 and HIV mRNAs and their co-localization within P-bodies (Chable-Bessia et al., 2009). Remarkably silencing of several RNAi effectors such as DGCR8 or Drosha, lead to reactivation of latent provirus from PBMC of HIV+ patients under cART. More-

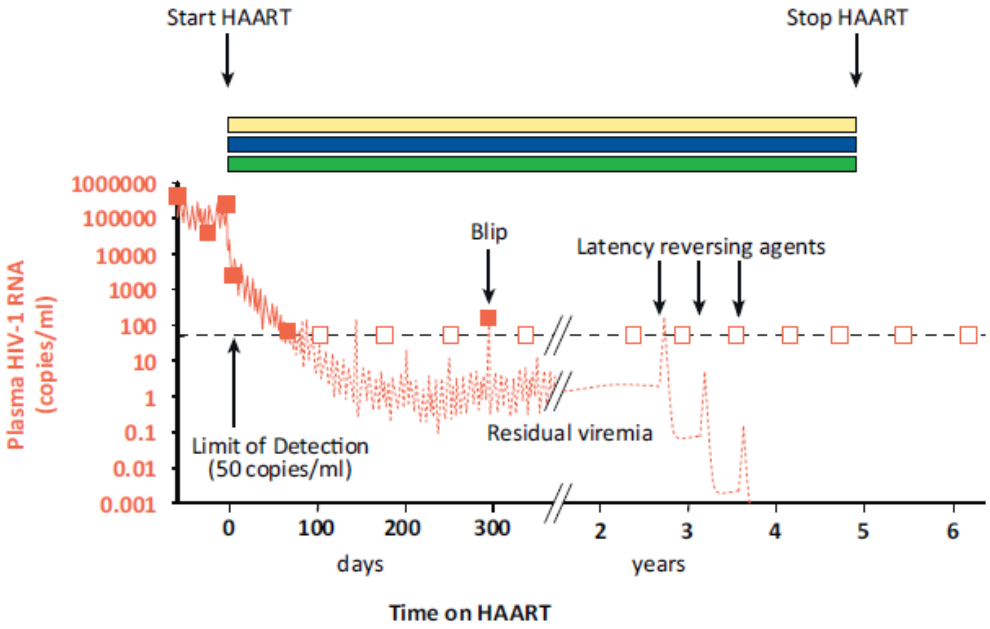
over, the 3'UTR of HIV mRNAs contains several putative binding sites for cellular miRNAs such as miR-125b, miR-150, miR-223, miR-382 and miR-28 (Huang et al., 2007); all of them were found to be more abundant in resting than in activated CD4+ T cells and to specifically restrict viral replication.

TOWARD A STRATEGY TO CLEAR THE VIRAL RESERVOIR

Since it was observed that long-lasting treatment with the antiretroviral drugs was not sufficient to eradicate the virus, a first attempt to clear the viral reservoir was done with intensified drug regimens; unfortunately it was found that the use of higher drug dosage didn't affect the size of the reservoir (Gandhi et al., 2010).

Currently, different strategies have been suggested to specifically target the viral reservoir.

Among them, the "shock and kill" therapy proposes that, the reactivation of the silent provirus from latently infected cells, could be achieved by pharmacological treatment with transcriptional inducers collectively named as Latency Reversal Agents (LRAs). Viral reactivation will consequently lead to the clearance of the infected cells by cytotoxic T lymphocytes whose activity could be also stimulated by the concomitant administration of cytokines.



Use of LRAs to achieve a sterilizing cure – from Durand, Blankson and Siliciano; Trends Immunol. 2012

Patients on a three-drug HAART therapy regimen (yellow, blue and green boxes) have undetectable levels of viremia, even if occasional "blips" can occur. Ideally, the administration of LRAs should induce a transient increase in viremia followed by a decrease in the size of the latent reservoir.

To this task, a panel of drugs has been tested alone or in combination to restore proper viral transcription: chromatin remodelling agents, NF-kB inducers and P-TEFb stimulators are the best characterized compounds.

Among chromatin remodelling agents, we find inhibitors of the histone deacetylase (HDACi) and inhibitors of the DNA methyltransferase (DNMTi); the latter ones are less exploited since the role of DNA methylation in the establishment of latency is still controversial (Blazkova et al., 2012), even if some studies (Bernhard et al., 2011; Bouchat et al., 2012; Imai et al., 2010) showed that inhibitors of the methylases G9a and SUV39H1 can revert HIV latency by inhibiting the methylation of Nuc-1.

The panel of HDACi is an heterogeneus group of drugs including both natural and synthetic compounds which showed different specificity for the different classes of HDACs: this group of drugs is well characterized thanks to their use in clinic for

their anti-cancer properties and for the treatment of neurodegenerative disorders.

Among the most potent HDACi there are Valproic Acid (VPA), suberoylanilide hydroxamic acid (SAHA), Romidepsin and Panabinostat; their effect is not limited to histones but also non-histonic proteins can represent a substrate for their activity. In the case of the NF-kB subunit p65, the deacetylation by HDAC3 induces its binding to the IkB-α inhibitor thus leading to its sequestration into an inactive complex which results in transcriptional silencing; the restoration of proper acetylation on p65 leads its release from the inhibitory complex to allow the formation of the active NF-kB heterodimer and the iniziation of transcription.

Thanks to its broad spectra of actions directed to different events in the transcriptional process, the group of HDACi constitute a powerful class of drugs for latency reversal. The potency of HDACi to viral reactivation have been widely tested in several latency models and in resting CD4+ T cells from HIV+ patients (Quivy et al., 2002; Reuse et al., 2009; Søgaard et al., 2015); unfortunately the results are frequently controversial since it was not possible to achieve a complete latency reversal in all the condition tested.

The activation of transcription factors NF-kB and AP-1 is achieved by stimulation of the protein kinase C (PKC) pathway and thus PKC agonists, such as prostratin and bryostatin-1, have been tested alone or in combination with HDACi as latency reversal agents in different model of latency (Mehla et al., 2010; Pérez et al., 2010; Reuse et al., 2009).

To enhance the activity of the elongation factor P-TEFb different approaches have been proposed. The stimulation of the P13/Akt pathway induces the release of P-TEFb from the inhibitory complex formed with HEXIM and the 7 SK RNA while the inhibition of the bromodomain protein 4 (BRD4), which bind directly to P-TEFb on the same binding site of Tat, would allow the rescue of proper Tat activity on the enhancement of transcripts elongation.

The hexamethylene bisacetammide (HMBA) is an activator of P13/Akt pathway and was tested both in latency models and in CD4+ T cells from HIV+ patients for its ability to revert latency; unfortunately all those tests gave disappointing results (Choudhary et al., 2008; Yang et al., 2009).

JQ1 is a small molecule which acts as a selective inhibitor of BRD4; it was tested in different latent cell models but gave inconsistent results: in the latency model

developed by the group of Siliciano, JQ1 proved to be effective but these result was not confirmed in other cellular models (Boehm et al., 2013).

Despite the promising approach of the “shock and kill” strategy to target the viral reservoir by acting on proviral transcription, the results accumulated during the past years of experimentation are disappointing.

The restoration of viral transcription seems to be not sufficient to revert the latent state suggesting that several steps of the viral replication are not successfully induced by the currently available LRAs thus leading to the failure of these treatments. The development of drugs to overcome the post-transcriptional blocks to latency reversal would provide a powerful tool which, used in combination to the well-known LRAs, could provided a more incisive strategy to target the viral reservoir.

CURRENT MODELS FOR THE STUDY OF LATENCY

Investigation on the molecular mechanisms behind viral silencing and the necessity to test therapeutic strategies to target the viral reservoir, require a proper in vitro model of latency; the small number of latently infected cells in patients and their lack of a proper distinguishable markers makes the attempt to derive such cells directly from patients almost not feasible.

Moreover the use of immortalized cell lines, even if useful and practical, pone several issuses such as the clonal nature of the integration site, the altered cellular environment related to the immortalizing mutations and the cycling nature due to an imbalanced growth control.

The development of in vitro models of latency based on primary cells appear to be the best way to study the characteristics of the viral reservoir in a more physiological environment and different laboratories have explored the possibility to reproduce latent infection by taking advantage of different techniques.

The model developed in Lewin’s laboratory (Cameron et al., 2010; Saleh et al., 2007) exploits the use of several chemokines to induce rearrangement in the cytoskeleton of resting CD4+ cells without causing activation: such structural changes allow the establishment of proper infection with a wild-type NL-4.3 HIV strain.

The model proposed by Siliciano (Yang et al., 2009) is articulated in two different steps to isolate latently infected CD4+ T cells: during the first step, cells are TCR

stimulated and transduced to express Bcl-2 to overcome apoptosis. After a brief culture in IL-2 these active cells are infected with a replication-defective NL 4.3 GFP reporter virus and left in culture for at least 3 weeks to allow the establishment of latency: the cytofluorimeter is then used to sort only the GFP-negative cells showing a resting memory phenotype.

The Spina model (Spina et al., 1995, 1997) is based on the observations that latent infection can be established in resting CD4+ T cells upon exposure of a mixed population of primary CD4+ T lymphocytes, including both dividing and quiescent cells, to wild type HIV. Basically co-colture of dividing infected cells is used to obtain latent infection in non-replicating bystander lymphocytes.

Direct infection of resting primary CD4+ T cells is exploited also in the model that the Greene's group (Lassen et al., 2012) derived from the modification of O'Doherty model (Swiggard et al., 2005); infection of quiescent cells is achieved by increasing the efficiency of virus delivery (a replication competent NL 4.3 containing luciferase reporter gene in place of Nef) by the use of spinoculation.

Taking account of the different subsets of cellular populations exploited and the variety of techniques and cytokines or chemokines implied in the presented systems, it seems to be unlikely to obtain a uniform result from experiments performed in these different models.

AIM OF THE THESIS

The identification of MATR3 as a component of the Rev-RNA nuclear complex prompted an investigation of its role in the course of HIV-1 infection. My thesis work is therefore finalized to assess the functional role of MATR3 during viral replication, both during the acute infection and upon establishment of the latent phase. To this aim, I investigated the effect of MATR3 modulation in Jurkat cells and primary PBLs in the context of infection with a replication-competent HIV-1. I either depleted or overexpressed MATR3 within HIV-infected lymphocytes and assessed the impact on viral replication in terms of viral mRNA metablosm, viral protein production and full virion release.

To study the putative role of MATR3 in the establishment of HIV-1 latency I used the J-Lat cell model. I evaluated the effect of MATR3 depletion on latency reversal when the transcription of the silent provirus is restored by TNFα stimulation: I analysed both the effect on transcriptional reactivation and full viral particle rescue from latent cells.

To better characterize the actual cellular context of latency I used primary PBLs from healthy donors and from HIV-infected patients and analysed the levels of MATR3 during treatment with clinically-tested Latency Reversal Agents.

Finally I tried to establish a proper model of viral latency, based on either primary or immortalized lymphocytes, to test the effect that MATR3 overexpression exert on latency reversal when is combined to treatment with Latency Reversal Agents.

In conclusion I found that MATR3 is a positive regulator of HIV-1 replication during acute infection in Jurkat cells and primary PBLs: I could assess that MATR3 acts as a Rev co-factor to enhance the export of intron-containing viral transcripts and I could exclude its involvement in the process of viral transcription.

I assessed that MATR3 depletion affects latency reversal in J-Lat cells in a post-transcriptional way and that MATR3 levels are limiting in primary resting PBLs from healthy donors and HIV-1 infected patients. Moreover I confirmed that the low basal expression of MATR3 is not induced by treatment with LRAs while cellular activation by PHA or TCR stimulation promptly increase MATR3 levels. These last observations suggest that MATR3 could be one of the limiting factors

to latency reversal and that its inadequate expression within cells of the viral reservoir could affect their capacity to restore full viral particles production upon treatment with LRAs.

PUBLICATIONS FROM THIS WORK

1) The Relevance of Post-Transcriptional Mechanisms in HIV Latency Reversal. *Sarracino A, Marcello A.* 2017. Curr Pharm Des 23:4103-4111.

2) Post-transcriptional regulation of HIV-1 gene expressionduring replication and reactivation from latency by nuclear matrix protein MATR3. *Sarracino A, Gharu L. et al.* SUBMITTED FOR PUBLICATION.

RESULTS

MATR3 depletion affects acute HIV-1 viral replication in human lymphocytes

The Rev-mediated nuclear export of viral intron-cointaining mRNAs requires the concerted action of several host-factors exploited by the virus to assure the efficient completion of its replication cycle. The cellular component of the nuclear matrix MATR3 was previously identified in our laboratory to be involved in this process and to specifically interact with Rev.

When I joined the laboratory of Molecular Virology the post-doc Lavina Gharu was working on this project and thus the initial stages of my work are closely related to hers: figure 1, 2 and 3 are essentially her contribution and I worked along with her to repeat some of those data.

To better investigate the role of MATR3 in the context of HIV-1 infection we screened 5 different shRNA targeting MATR3 mRNA (either in the ORF or in the 3'UTR) to stably deplete this factor from target cells, either immortalized or primary T-cell lines.

Each shRNA was expressed from a lentiviral vector pseudotyped with the VSV-envelope protein (VSV-G) as described in Materials and Methods; a non-targeting scramble shRNA was chosen as a control (shCTRL).

Jurkat cells were transduced and kept under puromicin selection for 3-4 days; cells were then lysed in Laemmli Buffer and the level of MATR3 was analyse by immunoblot.

Two shRNA (sh905 and sh906) showed the strongest efficacy in inducing an efficient depletion of MATR3 (Figure 1A).

In order to assess the persistence of the depletion in Jurkat cells the levels of MATR3 were measured at different time points following selection. As shown in Figure 1B, specific and long-lasting suppression of MATR3 could be observed up to two weeks : sh905 and sh906 were thus selected for the subsequent studies throughout this work. To exclude any cytoxic effect related to MATR3 depletion we checked cell viability of Jurkat cells transduced with sh905, sh906 and shCTRL lentivectors by tripan blue exclusion test: we could thus assess that MATR3 depletion didn't affect cell viability (Figure 1C).

FIG. 1

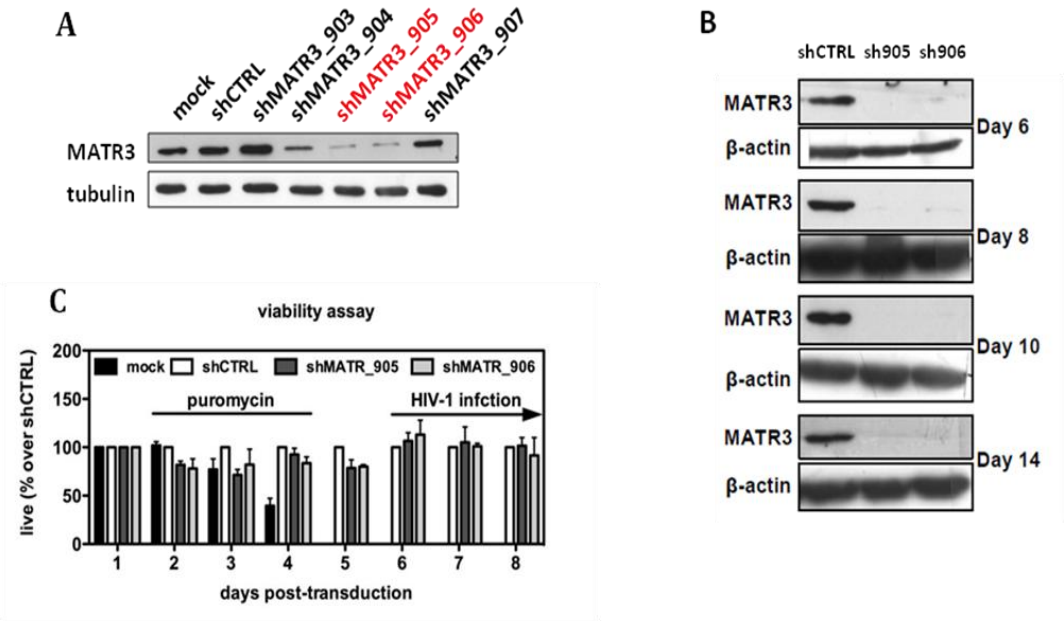


Fig.1- screening of shRNA targeting MATR3

A) HeLa cells were transduced with different lentivectors targeting MATR3 (shMATR) or with a control (shCTRL); 4 days after puromicin selection cells were harvested and analysed by immunoblot. Tubulin is the loading control. B) Jurkat cells were transduced with lentivectors targeting MATR3 (sh905; sh906) or with a control (shCTRL); MATR3 depletion was monitored for two weeks (B). Actin is the loading control. C) The Tripan Blue exclusion test of cell viability showed no cytotoxic effect upon MATR3 depletion.

To investigate the role of MATR3 in the context of acute HIV-1 infection, Jurkat cells were transduced with the above mentioned sh905 , sh906 or shCTRL lentiviruses and kept under puromicin selection for 4 days. MATR3 depleted Jurkat cells were then infected with the full-lenght HIV-1 NL4.3 and the overall effect on viral replication was assessed by viral particle quantification in the culture media through p24 ELISA.

MATR3 depleted cells exhibited a strong impairment in viral replication since no increase in extracellular viral particle was observed up to 14 days post infection (Figure 2A).

To confirm the essential role of MATR3 in HIV-1 replication in a more physiological cellular model we recapitulated this experiment in primary PBLs. Again upon MATR3 depletion a striking impairment in HIV-1 replication was observed for both sh905 and sh906 compared to shCTRL as shown in Figure 2B.

These data confirm the role of MATR3 as a positive regulator of HIV-1 replication during acute infection.

FIG. 2

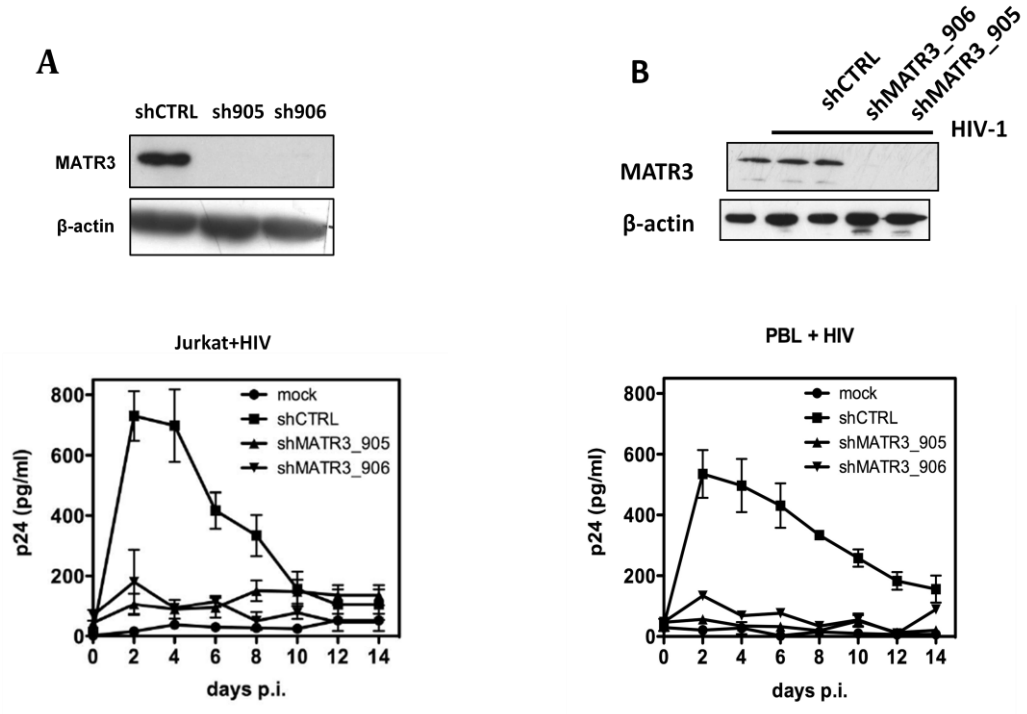


Fig.2-MATR3 depletion affects viral repliaction in Jurkat cells and in PHA-activated primary PBLs.

A) MATR3 depleted Jurkat cells were infected with the full lenght HIV-1 NL4.3. Viral replication was monitored for two weeks by p24 ELISA on culture supernatants.
B) PBLs isolated from healthy donors were activated with PHA for 72 hours and transduced with lentivectors to deplete MATR3. MATR3 depleted PBLs were infected with the replication competent HIV-1 NL4.3 and viral replication was monitored by p24 ELISA on culture supernatants.

MATR3 acts post-transcriptionally

Previous work in our laboratory identified MATR3 as a co-factor in Rev-mediated nuclear export of intron-containing HIV-1 RNAs. In order to confirm if this was the case also in acute HIV-1 infection we infected MATR3- depleted Jurkat cells with a VSV-G pseudotyped HIV-1 vector NL4.3-R-E-luc containing the firefly luciferase reporter gene within the Nef region which is expressed as the product of a fully spliced transcript.

Since we could not observe differences in luciferase expression upon MATR3 depletion we could exclude a direct role of MATR3 in viral transcription (Figure 3A); moreover the lack of effect on luciferase expression also indicate that MATR3 depletion was not affecting the metabolism of fully spliced viral mRNAs.

To investigate a possible effect of MATR3 depletion on the nuclear export of intron-containing viral mRNAs nuclear and cytoplasmic fraction of infected cells were separated (Figure 3D) to quantify the relative amount of US-mRNAs by qPCR in the two compartment.

The amount of US transcripts was significantly reduced within the cytoplasm and slightly increased in the nucleus (Figure 3B): a trend compatible with a defective export of this specific class of mRNAs was also confirmed by the immunoblot for the intracellular viral Gag protein, fully dependant on the export of unspliced viral mRNAs, which was reduced upon MATR3 depletion (Figure 3C).

These experiments confirmed that MATR3 regulates nuclear export and expression of intron-containing HIV-1 mRNAs also in the context of acute viral infection.

FIG.3

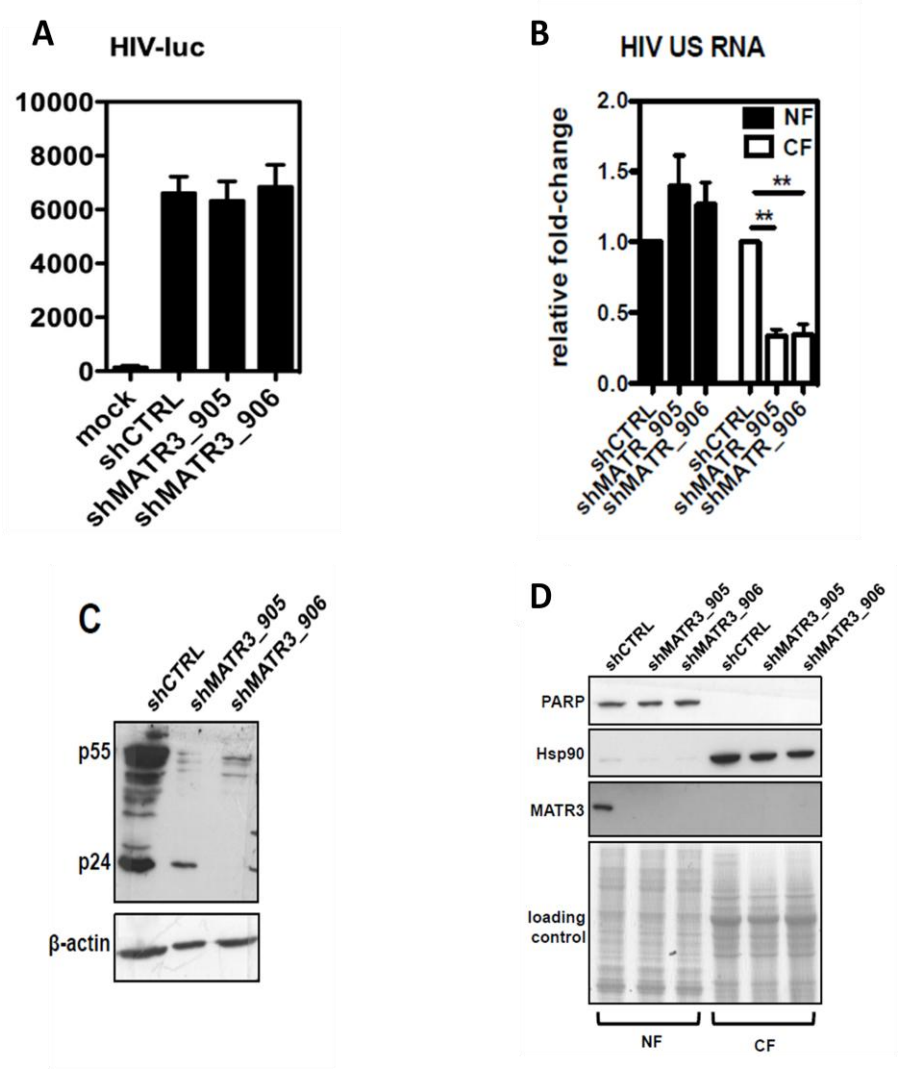


Fig 3- MATR3 acts post-transcriptionally. MATR3 depleted Jurkat cells were infected with the viral vector pNL-4.3-R-E-luc; luciferase expression was measured 48hours post infection and normalized to total protein level measured by Bradford Assay (A). Results from three indipendent experiments are shown as mean values + SD. Unspliced RNA levels were quantified by real time PCR in the nuclear and cytoplasmic fraction of MATR3 depleted Jurkat cells (B). Purity of nucleo-cytoplasmic fraction (D) is assessd by immunoblot for the cytoplasmic marker Hsp90 and the nuclear marker PARP. As expected MATR3 is detected only in the nuclear fraction of CTRL Jurkat cells. RNA levels are normalized to GAPDH. Results from three indipendent experiments are shown as mean values + SD. MATR3 depleted Jurkat cells were infected with the replication competent HIV-1 NL4.3 and 48 hours post- infection cells were harvested and the levels of intracellular Gag analysed by immunoblotting (C). B-actin is the loading control.

MATR3 overexpression enhances HIV-1 replication in human lymphocytes

Next we wished to investigate the effect of overexpressing MATR3 in acute HIV-1 infection. To this end a flag-tagged version of MATR3 was cloned into the pWPI_BLR lentiviral vector as described in materials and methods.

Transduction of Jurkat cells with the lentiviral vector to express the flag-MATR3 (fMATR) resulted in higher MATR3 RNA and protein levels (Figure 4A; 4B) compare to cells transduced with a control lentiviral vector (CTRL).

FIG. 4

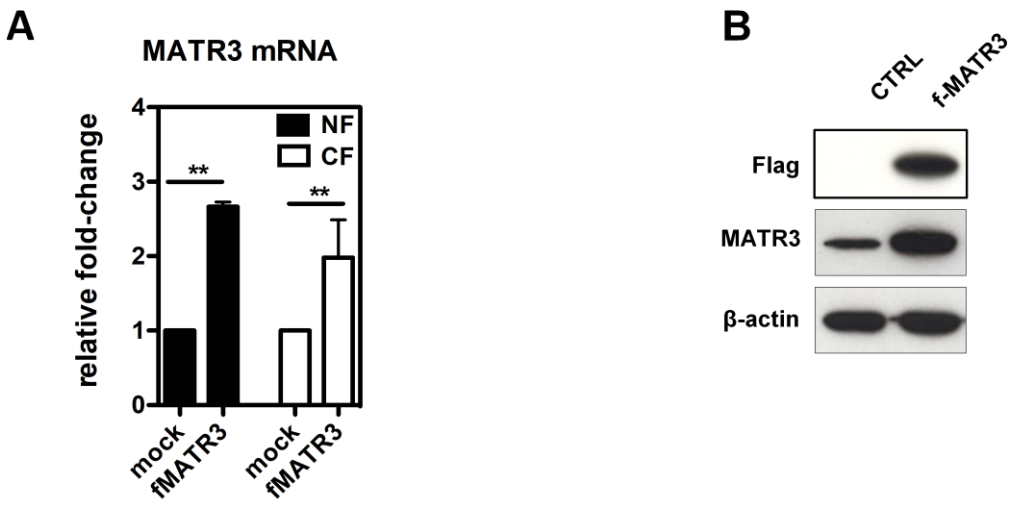


FIG.4- Efficient MATR3 overexpression in Jurkat cells. Transduction of Jurkat cells with a lentivector to deliver a flag-tagged MATR3 (fMATR) resulted in higher MATR3 mRNA (A) and protein levels (B) compared to cells trated with the control vector (CTRL). RNA levels were normalized to GAPDH; B-actin is the loading control in the immunoblot. Results from two indipendent experiments expressed as mean values + SD.

Infection of these cells with HIV-1 NL4.3 resulted in increased intracellular Gag (Figure 5A); nucleo-cytoplasmic fractionation of infected Jurkat cells overexpressing MATR3 revealed a major decrease of US-HIV RNA in the nucleus while MS HIV RNA were not affected (Figure 5B; 5C). Decrease of US RNA in the nucleus is compatible with an enhanced export of these transcripts mediated by MATR3 through the Rev pathway while the steady-state invariant US RNA level in the cytoplasm could be explained by the increased production of viral particle (Figure 5A).

However we couldn't exclude a MATR3-dependent effect on stability of HIV RNA mediated by the zinc-finger antiviral protein (ZAP)- complex as proposed by Erazo and Goff (Erazo and Goff, 2015).

FIG. 5

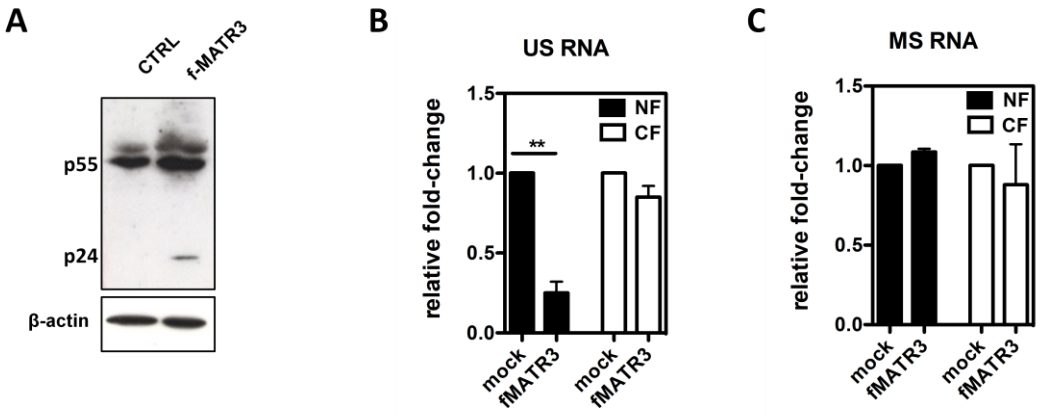


FIG.5- MATR3 overexpression enhances the export of US mRNAs. Jurkat cells overexpressing MATR were infected with the replication competent HIV-1 NL 4.3; 48 hours post infection the cells were harvested and subjected to nuclear-cytoplasmic fractionation. MATR3 overexpression (fMATR) resulted in increased intracellular Gag levels; B-actin is the loading control (A). Quantitave analysis of viral US (B) and MS (C) mRNAs in nuclear and cytoplasmic fraction was performed by real time PCR; RNA levels are normalized to GAPDH. Results from two indipendent experiment expressed as mean values+SD.

To finally confirm that MATR3 is a positive regulator of HIV-1 replication primary PBMCs activated for 4 days with PHA/IL2 were transduced to overexpress

MATR3 and than infected with HIV-1 NL4.3.Viral production was monitored in the culture media with a p24 ELISA and showed that increased levels of MATR3 resulted in higher amount of viral particles in the supernatant (Figure 6).

FIG.6

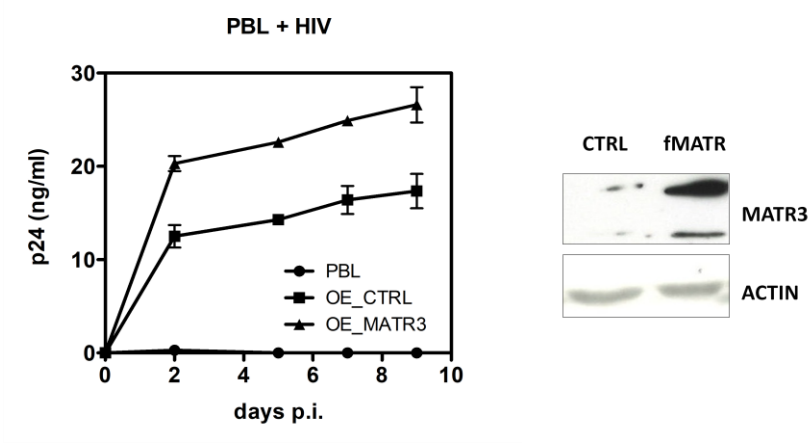


FIG.6- MATR3 overexpression enhances viral replication in primary PBLs

PHA-activated primary PBLs were transduced with a lentivector to overexpress MATR3 and infected with the replication competent HIV-1 NL 4.3.Viral replication was monitored by p24 ELISA in culture supernatant. Results from two indipendent experiments expressed as mean values+SD.

These data complement the depletion data described above and confirm the role of MATR3 as a post-transcriptional regulator of HIV-1 RNA export and ultimately viral replication.

However, a major hurdle to HIV-1 eradication is the persistence of viral reservoir that carry a silent, but transcriptionally competent, integrated copy of the viral genome.

Since few study have explored post-transcriptional blocks to HIV-1 reactivation we addressed this topic further.

MATR3 depletion affects latency reversal in J-lat cells

To address the question if MATR3 can play any role in the reactivation of HIV-1 latency we took advantage of a well known model for the study of latency, J-lat cells, which contain an integrated but silent provirus encoding for GFP as a reporter gene for viral transcription, expressed from an HIV MS mRNA.

Cells were transduced with shMATR3 lentiviral vectors to deplete MATR3 (Figure 7A) and then stimulated with TNFα to induce HIV transcription from the latent provirus. As shown in Figure 7B, cell-associated Gag protein levels were markedly reduced upon MATR3 depletion with sh905 and sh906. Consistently, p24 levels in the medium were also reduced (Figure 7C), while HIV-1 transcription was not affected by MATR3 depletion (Figure 7D).

These experiments show that MATR3 removal results in a reduced ability of TNFα to reactivate HIV-1 from latency. One intriguing hypothesis is that certain host factors, which are essential for HIV-1 gene expression, might be limiting and/or their activity might be perturbed, in latently infected cells.

FIG.7

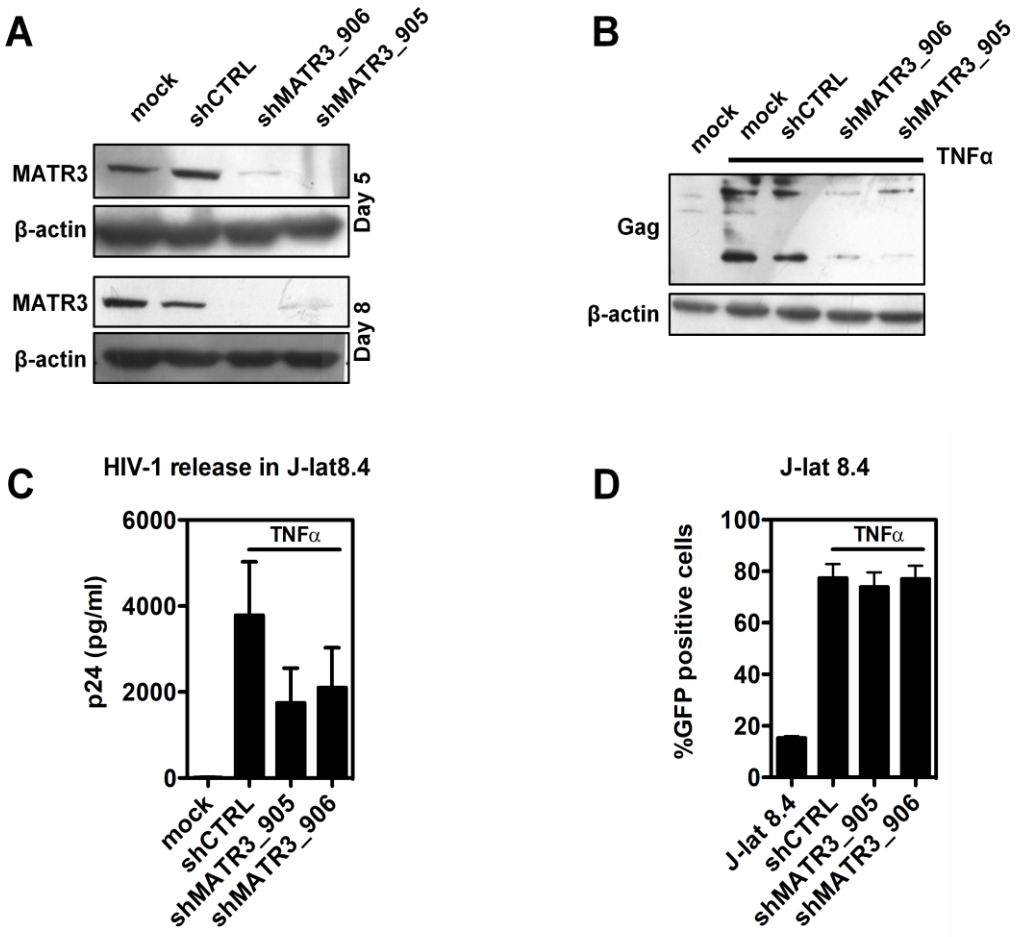


FIG.7- MATR3 depletion affects the post-transcriptional steps of viral reactivation in J-lat cells

J-lat 8.4 cells were transduced with a lentivector to knockdown MATR3 (shMATR) leading to an efficient and stable MATR3 depletion (A). MATR3 depleted J-Lat 8.4 were stimulated with TNFα for 48 hours and viral reactivation was assessed in terms of intracellular Gag (B), viral particle in the supernatant by p24 ELISA (C) and GFP expression by FACS analysis (D). B-actin is the loading control in the immunoblots. Results from three indipendent experiments presented as mean+SD.

MATR3 is limiting in primary resting PBMCs

To better characterize the cellular context of the viral reservoir we analysed the expression levels of several host factors in quiescent primary PBMCs and PBLs.

Primary PBMCs and PBLs purified from healthy donors were cultured in IL2 alone or activated with PHA for different time points (24, 48, 72 hrs or 6 days) and their protein extract was analysed with an immunoblot. We checked for factors involved in mRNA synthesis and metabolism at different stages, from transcription (Cyclin T1) to RNA stabilization and export (MATR3, PSF, PTB).

We observed that these factors were barely expressed in quiescent PBLs and readily induced by PHA activation (Figure 8A). The same was observed for purified primary CD4+ T cells (Figure 8B) that represents the best-characterized reservoir of latent HIV-1.

Hence, levels of MATR3 and other post-transcriptional factors required for full reactivation of HIV-1 are low in quiescent lymphocytes, possibly contributing to HIV-1 latency.

FIG.8

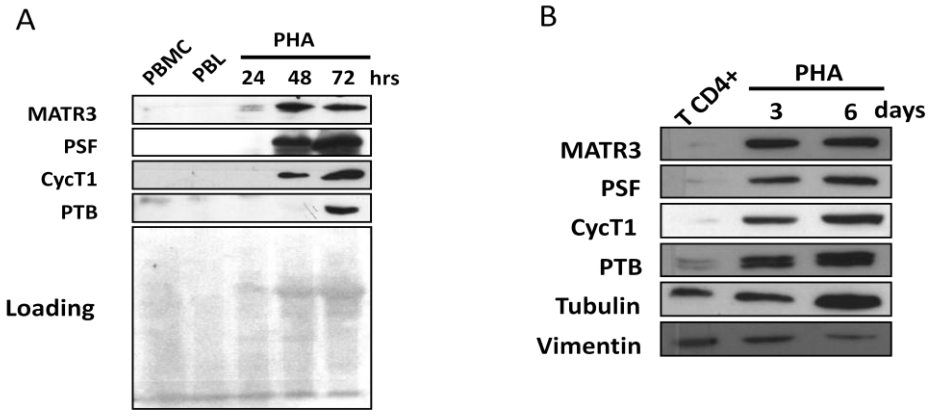


FIG.8- Expression of MATR3 in primary lymphocytes

Primary PBMC, PBLs (A) and CD4+T cells (B) were isolated from healthy donors as described in Materials and Methods.The levels of transcriptional and post-transcriptional factrors (MATR3, PSF, CyclinT1, PTB) were analysed by immunoblot prior and post-PHA activation.Ponceau staining (A) or Vimentin (B) were used as loading control.

MATR3 is not modulated by LRAs

We focused our analysis on the cellular context of resting primary PBMCs which are the cell types mainly composing the viral reservoir: we sought to investigate deeper the actual behaviour of MATR3 and its modulation upon pharmacological treatments with the newly proposed latency reversing agents (LRAs).

To this aim we selected several drugs from the panel of LRAs and tested the effect these drugs exert on endogenous MATR3 levels within primary resting PBMCs.

The drugs selected for the screening are transcriptional inducer acting through different mechanism and in particular we choose the PTEN inhibitor Disulfiramin, the two histone deacetilase inhibitor SAHA and Romidepsin and the BET-inhibitor JQ1 in combination to the PKC agonist Ingenol B; we used PHA as a positive control for cellular activation.

Romidepsin is a relatively newly discovered HDAC inhibitor whose potent action in latency reversal is arousing increasing interest (Søgaard et al., 2015) while SAHA has a well-established use in clinic. SAHA was approved in 2006 for the treatment of cutaneous T cell lymphoma (CTCL) and is currently tested in several clinical trials for other types of cancer. Moreover, in preclinical investigation, SAHA demonstrated to be a powerful activator of latent HIV-1 transcription in different cellular model of latency (Archin et al., 2009).

The combination of JQ1 and Ingenol B is an intriguing approach which associate the nuclear translocation of NF-kB through the PKC pathway induced by Ingenol B to the release of P-TEFb induced by JQ1 (Darcis et al., 2015).

To set the appropriate concentration to use for each drug we refer to the plasma concentration (when available), the tolerated doses and the peak of efficacy as reported from literature.

In particular we used the following drug concentration:

-Disulfiramin 0,5uM

-SAHA 0,5uM

-Romidepsin 0,0175uM

-JQ1 (0,5uM) in combination to Ingenol B (10nM)

PBMCs isolated from healthy donors were cultured in IL2 alone or stimulated with one or a combination of drugs for 3 and 6 days; MATR3 levels were then analysed at the protein level by immunoblotting.

The relative amount of MATR3 after each treatment is shown as fold change to unstimulated PBMCs and represent the average obtained from three healthy donors (Figure 9).

FIG. 9

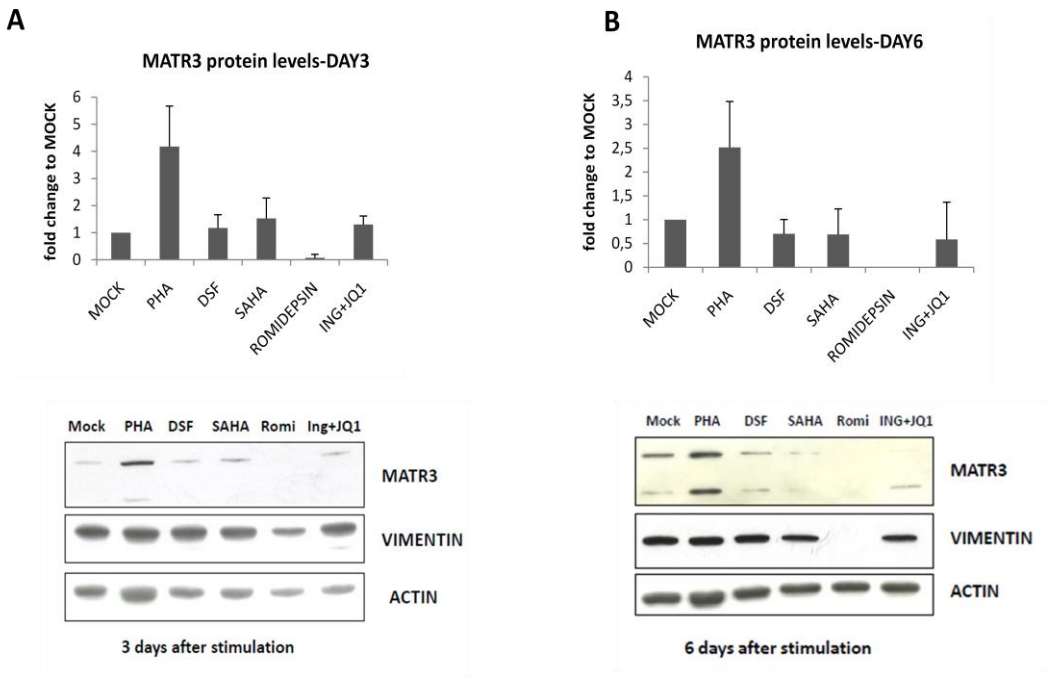


FIG.9- MATR3 is not modulated upon treatment with LRAs in PBLs from healthy donors

Primary PBLs were isolated from healthy donors as described in Materials and Methods and stimulated with different LRAs or PHA-activated; after 3 (A) and 6 days (B) of treatment cells were harvested and MATR3 levels analysed by immunoblot. Vimentin is the loading control. Western Blot quantifications of MATR3 relative levels are normalized to Vimentin by ImageJ and are obtained from immunoblot analysis on 3 different donors; MATR3 protein level is expressed as fold change to MOCK and reported as mean values+SD.

None of the tested drug was able to induce MATR3 protein level either after 3 or 6 days of treatment, while PHA consistently increased MATR3 protein levels.

Among the tested drugs we focused our attention on SAHA because of its well-established use in clinic: we added an extra donor to the measurement and we confirmed again that MATR3 protein level remained low and not induced after 3 and 6 days of SAHA treatment in resting PBMCs from healthy donors (Figure 10).

FIG. 10

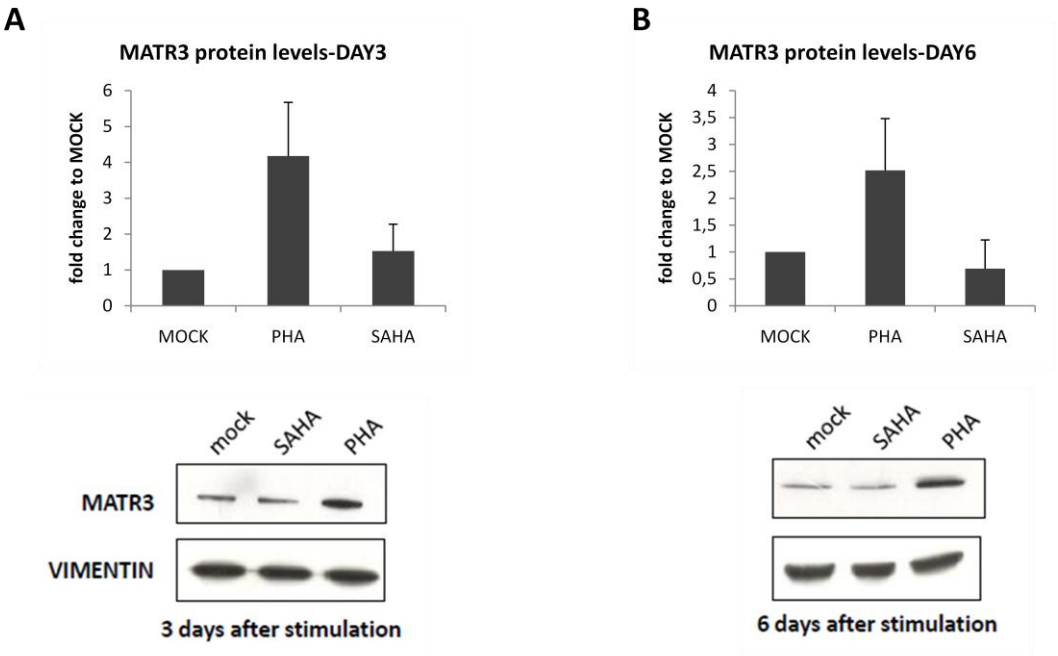


FIG.10- MATR3 is not modulated upon treatment with SAHA in PBLs from healthy donors

Primary PBLs isolated from healthy donors were stimulated with 0,5uM of SAHA or PHA-activated for 3 (A) and 6 days (B); the cells were then harvested and MATR3 levels analysed by immunoblot.Vimentin is the loading control. Western Blot quantification are obtained from immunoblot analysis on 4 different donors; MATR3 relative levels are normalized to MOCK and expressed as mean values+SD.

To exclude a possible fluctuation of MATR3 protein levels during SAHA treatment at shorter time points we evaluated the effect of SAHA stimulation at 18 and 24

hrs. As shown in Figure 11, no modulation of MATR3 occurs upon stimulation with SAHA.

FIG.11

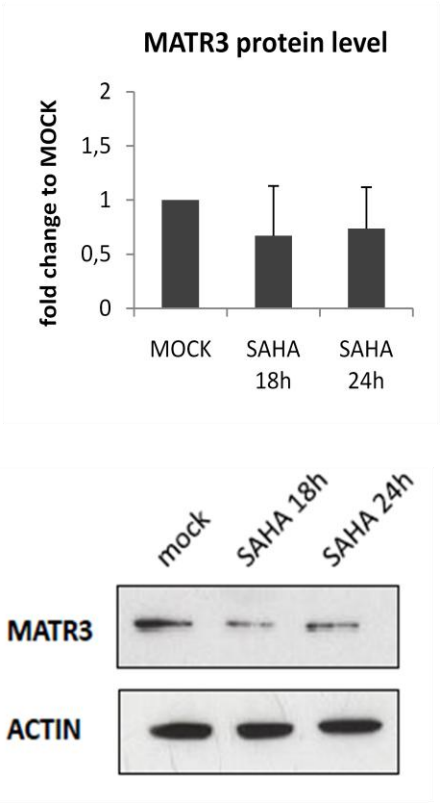


FIG.11- MATR3 is not modulated upon short treatment with SAHA in PBLs from healthy donors

Primary PBLs isolated from healthy donors were stimulated with 0,5uM of SAHA or PHA-activated for 18 and 24 hours; the cells were then harvested and MATR3 levels analysed by immunoblot. Actin is the loading control. Western Blot quantification are obtained from immunoblot analysis on 2 different donors; MATR3 relative levels are normalized to MOCK and expressed as mean values+SD.

The inability of LRAs to increase MATR3 levels within resting PBMCs from healthy donors lead us to extend the observation to the actual context of viral reservoirs ex-vivo by testing the same drugs in PBMCs isolated from aviremic HIV+ patients under cART.

The experiments on samples from HIV+ patients were done in collaboration with Dr.Ania Kula and Dr.Carine Van Lint at the University of Bruxelles (ULB) because they have access to a well established cohort of HIV+ patients from the St-Pierre Hospital; qPCR data analysis were performed in collaboration with Alexander Pasternak at University of Amsterdam (AMC).

PBMCs isolated from 3 HIV+ patients were treated for 3 and 6 days with disulfiramin, SAHA, romidepsin or Ingenol B + JQ1 while anti-CD3/CD28 antibodies were used as a positive control for cellular activation; the induction of MATR3 was measured at the RNA level by qPCR.

The results obtained in cells from HIV+ patients recapitulate the same scenario observed from healthy donors in which none of the tested drugs was increasing MATR3 levels after both 3 and 6 days of treatment (Figure 12A). We focused our analysis on SAHA and included 4 extra patients achieving a similar result (Figure 12B).

FIG. 12

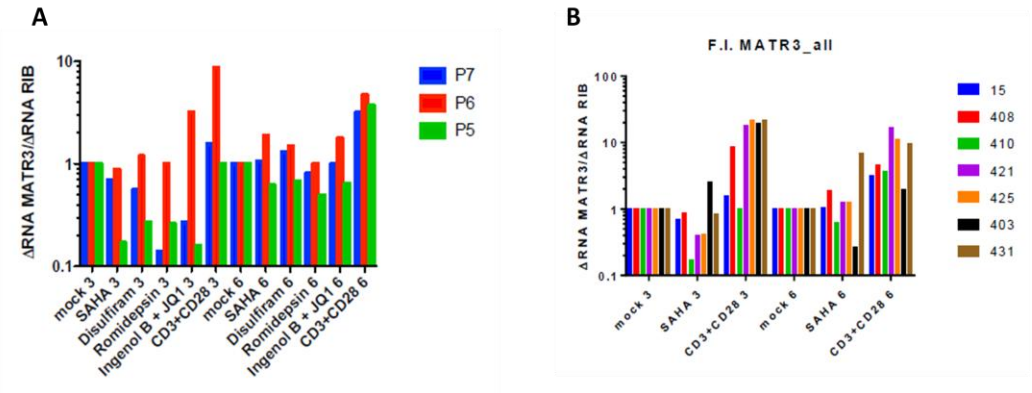


FIG.12- MATR3 is not modulated upon treatment with LRAs in PBLs from HIV-infected patients

Primary PBLs were isolated from aviremic HIV+ patients as described in Materials and Methods and stimulated with different LRAs (A), with SAHA (B) or CD3/CD28-activated for 3 and 6 days; MATR3 RNA levels were analysed by quantitative real timePCR and normalized to total cellular RNA. MATR3 levels are normalized to MOCK; the number of patients included in the analysis is 3 for all the LRAs (A) and 7 for SAHA (B).
A) Data sets were analysed using the Student's t-test with significance at $p<0.05$. SAHA3 vs mock3 p=0.1917; Disulfiram3 vs mock3 p=0.351; Romidepsin3 vs mock3 p=0.1909; IngB+JQ1-3 vs mock3 p=0.8525; CD3+CD28-3 vs mock3 0.3819; SAHA6 vs mock6 p=0.6606; Disulfiram6 vs mock6 p=5874; Romidepsin6 vs mock6 p=0.2681; IngB+JQ1-6 vs mock6 p=0.7147 ; CD3+CD28-6 vs mock6 p=0.0237.
B) Data sets were analyzed using a paired, nonparametric Wilcoxon test. $p<0.05$ was considered statistically significant. SAHA3 vs mock3 p=0.2969; CD3+CD28-3 vs mock3 p=0.0313; SAHA6 vs mock6 p=0.4688; CD3+CD28-6 vs mock6 p=0.0156.

Along with the effect exerted on MATR3 level we also checked for the efficiency of SAHA in latency reversal within the same samples: both cellular-associated and extracellular viral RNAs were quantified by qPCR. The measurement of intracellular MS viral transcripts indicate the ability of SAHA to reactivate the transcription from the silent provirus while the presence of extracellular viral RNAs would indicate a complete rescue of full viral particles.

As shown in Figure 13A, cell-associated viral RNAs increased after 6 days of SAHA treatment at levels comparable to the one obtained with the positive control indicating that SAHA is a strong inducer of transcription at the LTR site. However removal of transcriptional blocks after SAHA treatment is not followed by full viral particle rescue (Figure 13B) which suggest the presence of additional blocks in

one or more post-transcriptional steps which impede the completion of the viral life cycle.

These observation reveal that transcriptional activation is necessary but not sufficient to revert latency from cells within the viral reservoir. Since MATR3 have been found to be limiting during SAHA treatment we hypothesized a role for factors involved in mRNA export and stabilization in the maintenance of latency.

FIG. 13

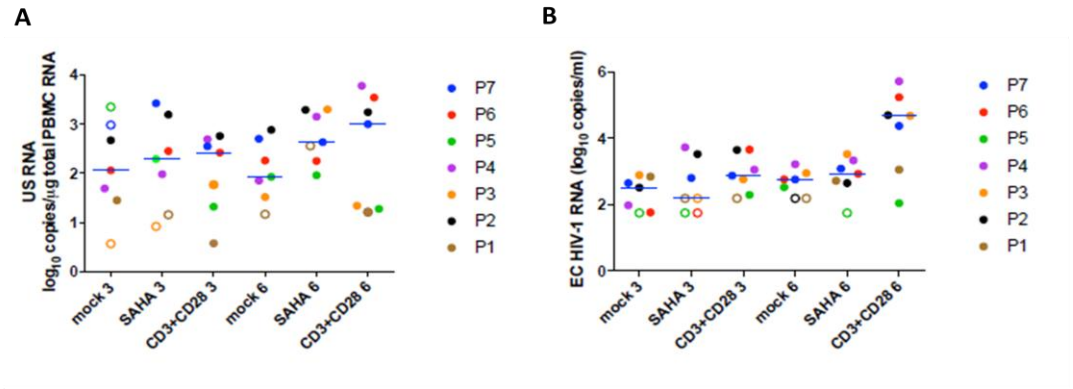


FIG.13- Treatment with SAHA efficinetly induces HIV-1 transcription

A) Cell-associated unspliced HIV-1 RNA (CA-US HIV-1 RNA) extracted from *ex vivo* cultures of CD8-depleted PBLs from 7 aviremic HIV+ patients was quantified by RT-qPCR and expressed as HIV RNA copy numbers/µg of total cellular RNA. Open symbols indicate undetectable samples and report an estimated value calculated as 50% of a detection limit per sample. The detection limit depended on the amounts of cellular RNA and therefore differed between samples. The medians are represented. Data sets were analysed using a paired, nonparametric Wilcoxon test. $p<0.05$ was considered statistically significant. SAHA3 vs mock3 p=0.1875; CD3+CD28-3 vs mock3 p=0.3125; SAHA6 vs mock6 p=0.2188; CD3+CD28-6 vs mock6 p=0.2969. B) Extra-cellular genomic viral RNA (EC HIV-1 RNA) was quantified using RT-qPCR and reported as HIV RNA copy numbers/milliliter of plasma. Symbols and statistics as reported for Figure 13A. SAHA3 vs mock3 p=0.6875; CD3+CD28-3 vs mock3 p=0.1563; SAHA6 vs mock6 p=0.2969; CD3+CD28-6 vs mock6 p=0.0313.

J-Lat 8.4 are responsive to SAHA and Romidepsin treatment

To explore if MATR3 is a limiting factor to latency reversal we opted for its over-expression in J-Lat cells treated with LRAs to induce proviral transcription.

In our hypothesis MATR3 overexpression can help full viral particle rescue from transcriptionally activated J-lat cells by compensating the inefficient induction of post-transcriptional steps after LRAs treatment.

To test our hypothesis the J-Lat model can be useful thanks to the immortalized cell type, easy to manipulate, and to the presence of a reporter gene within an already silent but inducible provirus; nevertheless a major limitation of this model is posed by the high levels of MATR3 present within J-Lat cells.

J-Lat 8.4 cells were stimulated for 48 hours with the following LRAs at the indicated concentrations:

-SAHA (0,5uM- 1uM- 1,5uM)

-disulfiramin (5uM)

-Ingenol B (0,4uM)

-JQ1 (0,5uM)

-Romidepsin (0,006uM - 0,0175uM - 0,026uM)

The reported concentrations were selected referring to literature; TNFa (30ng/ml) served as a positive control.

Responsiveness to drug treatment was evaluated in terms of both GFP expression by FACS analysis and US-HIV mRNA quantification by qPCR.

As shown in Figure 14A, J-lat cells strongly respond to Romidepsin treatment at both concentration tested while transcriptional induction occurred with SAHA only at the higher concentration used; a weak transcriptional induction is also observed in response to Ingenol B.

Results obtained from FACS analysis (Figure 14B) were confusing since not significant increase in the percentage of GFP+ cells was observed even with the drugs which showed increased US mRNAs transcription from the qPCR analysis, with the exception of Romidepsin (0,0175uM).

FIG. 14

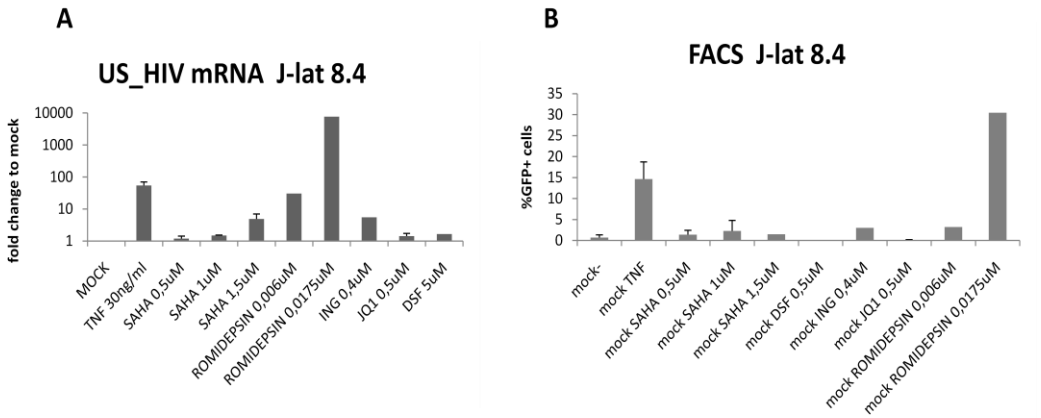


FIG.14- SAHA and Romidepsin rescue viral transcription in J-Lat8.4

J-Lat 8.4 cells were stimulated for 48 hours with different LRAs as indicated in Materials and Methods. Viral transcription was assessed by real time PCR quantification of the US RNAs (A) or by cytoflorimetric analysis of the GFP+ cells (B). US RNAs are normalized to GAPDH and expressed as fold change to MOCK.

MATR3 overexpression doesn't help full viral particle rescue in combination to LRAs in J-lat 8.4

J-lat 8.4 cells were transduced with lentiviruses to overexpress (OVER) or not (CTRL) MATR3, kept under blasticidin selection for 2 weeks and then stimulated with the above mentioned LRAs for 48 hours.

Full viral particle rescue was evaluated by p24 ELISA quantification in the cell culture media.

No significant difference was observed after transcriptional induction of the provirus in cells treated to overexpress MATR3 compare to the control in terms of full viral particle rescue (Figure 15).

FIG. 15

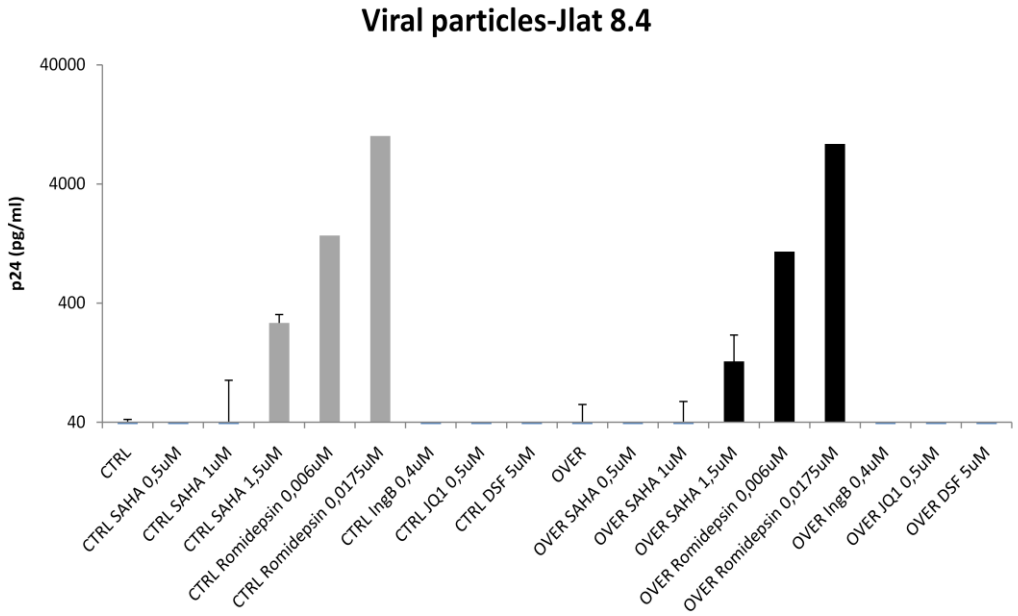


FIG.15- MATR3 overexpression doesn't enhance viral rescue in LRAs-treated J-Lat 8.4

J-Lat 8.4 cells culture transduced with a lentivector to overexpress MATR3 (OVER) or with a control lentivector (CTRL) were grown under blasticidin selection. and stimulated for 48 hours with different LRAs as indicated in Materials and Methods. Full viral particle rescue was assessed by p24 ELISA quantification on culture supernatants.

MATR3 overexpression is not occurring efficiently in J-lat 8.4

Since we couldn't detect any positive effect to latency reversal from the combination of MATR3 overexpression to LRAs treatment, we hypothesized that the high basal level of MATR3 within J-Lat 8.4 could make less appreciable any affect on viral replication related to an additional increase in MATR3 protein level.

We checked the effective amount of MATR3 within OVER or CTRL J-lat 8.4 and surprisingly find out that even upon efficient expression of the exogenous flag-tagged version of MATR3 after lentiviral transduction, the overall amount of this factor was not increased both at protein and RNA level (Figure 16).

We concluded that J-lat 8.4 finely controlled the levels of MATR3 when an additional amount of the factor is provided exogenously in a way to minimize the variation in its total amount.

FIG. 16

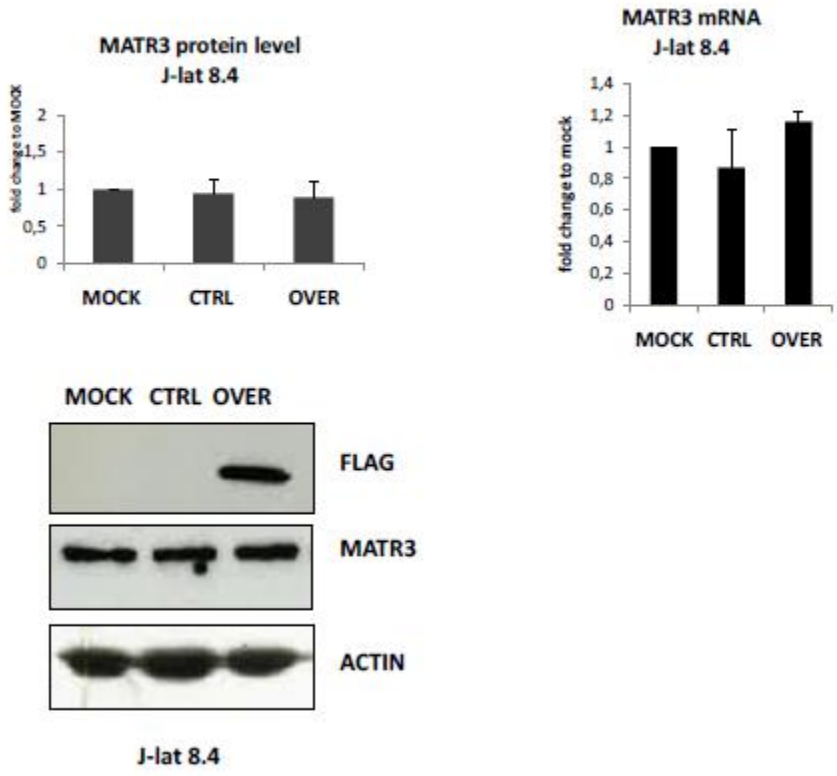


FIG.16- MATR3 overexpression is not efficiently achieved in J-Lat 8.4

J-Lat 8.4 cells culture transduced with a lentivector to overexpress MATR3 (OVER) or with a control lentivector (CTRL) were grown under blasticidin selection. Whole cell lisates were analysed by immunoblot to assess the expression of the exogenous flag-tagged MATR3 (FLAG) and the total levels of MATR3; B-actin is the loading control. Western Blot quantification is obtained by two different experiments and relative levels of MATR3 are expressed as fold change to MOCK. The level of MATR RNA measured by real time-qPCR are normalized to GAPDH and expressed as fold change to MOCK. Results from two different experiments.

J-Lat 6.3 are responsive to SAHA treatment

The limitation faced with the attempt to overexpress MATR3 within J-Lat 8.4 lead us to change the clone of J-Lat used and repeat our test in J-Lat 6.3.

In this case we focused on a single drug, SAHA, which was tested at three concentration as before(0,5uM- 1uM- 1,5uM); 48 hours post-stimulation transcriptional induction of the provirus was assessed checking both GFP expression by FACS analysis and US-HIV mRNA transcription by qPCR. J-Lat 6.3 were found to be responsive to SAHA treatment at the two higher concentration tested (Figure 17).

FIG. 17

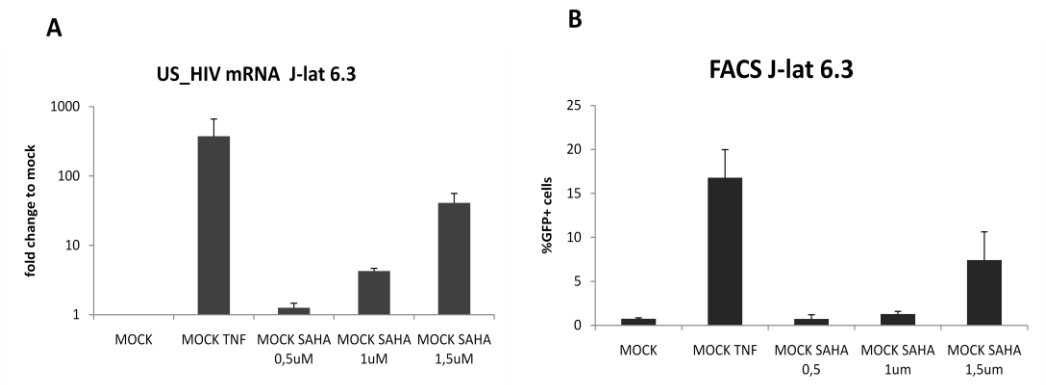


FIG.17- SAHA rescues viral transcription in J-Lat6.3

J-Lat 6.3 cells were stimulated for 48 hours with different concentration of SAHA. Viral transcription was assessed by real time PCR quantification of the US RNAs (A) or by cytoflorimetric analysis of the GFP+ cells (B). US RNAs are normalized to GAPDH and expressed as fold change to MOCK.

MATR3 overexpression in not occurring efficiently in J-Lat 6.3

J-Lat 6.3 cells were transduced with a lentivirus to deliver the flag-tagged MATR3 (OVER) or with a control lentivirus (CTRL) and kept under blasticidin selection for 2 weeks; the effective amount of MATR3 within the cells was checked by both immunoblotting and qPCR.

As observed for J-Lat 8.4 even upon efficient expression of the exogenous flag-MATR3 the overall amount of the factor was not increased (Figure 18).

FIG. 18

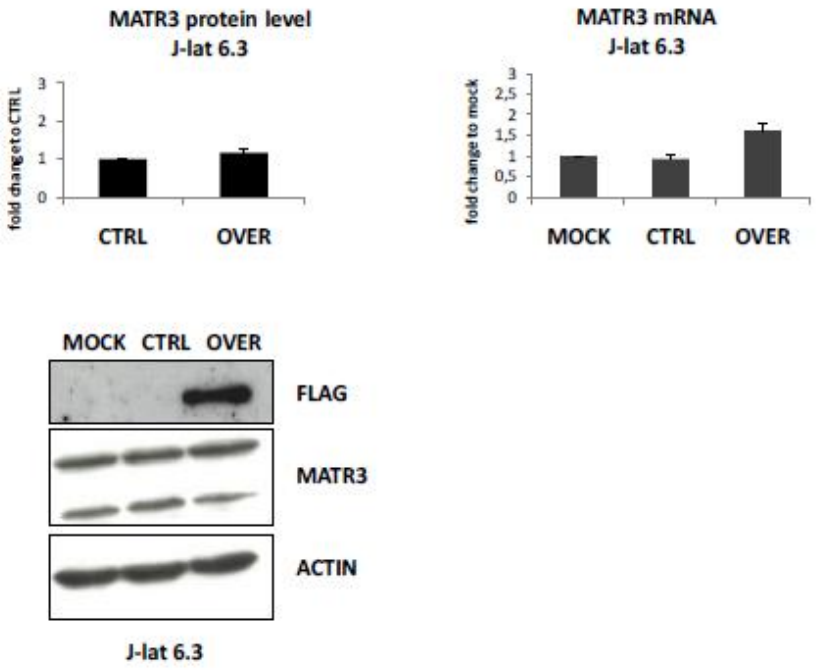


FIG.18- MATR3 overexpression is not efficiently achieved in J-Lat 6.3

J-Lat 6.3 cell culture transduced with a lentivector to overexpress MATR3 (OVER) or with a control lentivector (CTRL) were grown under blasticidin selection. Whole cell lisates were analysed by immunoblot to assess the expression of the exogenous flag-tagged MATR3 (FLAG) and the total levels of MATR3; B-actin is the loading control. Western Blot quantification is obtained by two different experiments and relative levels of MATR3 are expressed as fold change to CTRL. The level of MATR RNA measured by real time-qPCR are normalized to GAPDH and expressed as fold change to MOCK. Results from two different experiments.

Development of a model of latency based on resting primary PBLs

As suspected J-Lat cell line turned out to be an unsuitable model to verify our hypothesis about the combinatorial effect of MATR3 overexpression and LRAs. The problem seems related to the already high levels of MATR3 in these cells that cannot be up-regulated further by overexpression. Indeed an eventual positive effect on latency reversal related to an increased amount of MATR3 could be masked under condition in which such factor is not limiting.

With the aim to verify our hypothesis in a proper and more physiological cellular model we started to work on the set up of a model of latency based on the direct infection of resting primary PBLs.

The task to isolate latently infected cells directly from the blood of HIV+ patients is not feasible to our purpose because of the extremely low number of infected cells carrying an intact and inducible silent provirus: it has been estimated that out of 1 million of CD4+ T cells isolated from the blood of an HIV+ patient only 10-100 cells contain a replication-competent provirus (Eriksson et al., 2013; Ho et al., 2013)

To overcome this limitation several model of latency based on the ex-vivo infection of primary PBLs have been proposed by different laboratory during the past years.

Given the refractory of quiescent PBLs to HIV-1 infection, the majority of these models require a step of cellular activation prior to infection to render the process more efficient: the infected cells should be then left in culture for a reasonable time to allow a complete reversion to a resting state.

To make an estimation of the time required by activated PBLs to revert to a resting phenotype we activated primary PBLs with PHA (3ug/ml) and IL-2 (20U/ml) for 4 days; cells were then washed to remove the activation stimuli and cultured in IL2 alone. The activation status of CD4+ T cells subset was monitored by checking the expression of the activation markers CD69 and HLA-DR by FACS analysis.

As shown in Figure 19, PHA addition resulted in almost 40% of cells expressing one or both the activation markers. Unfortunately once activated these cells hardly come back to a complete resting phenotype: a big percentage of them (~ 25% in our analysis) still express activation markers after 14 days in culture. Longer culture in absence of any activation stimulus only resulted in cell death.

FIG.19

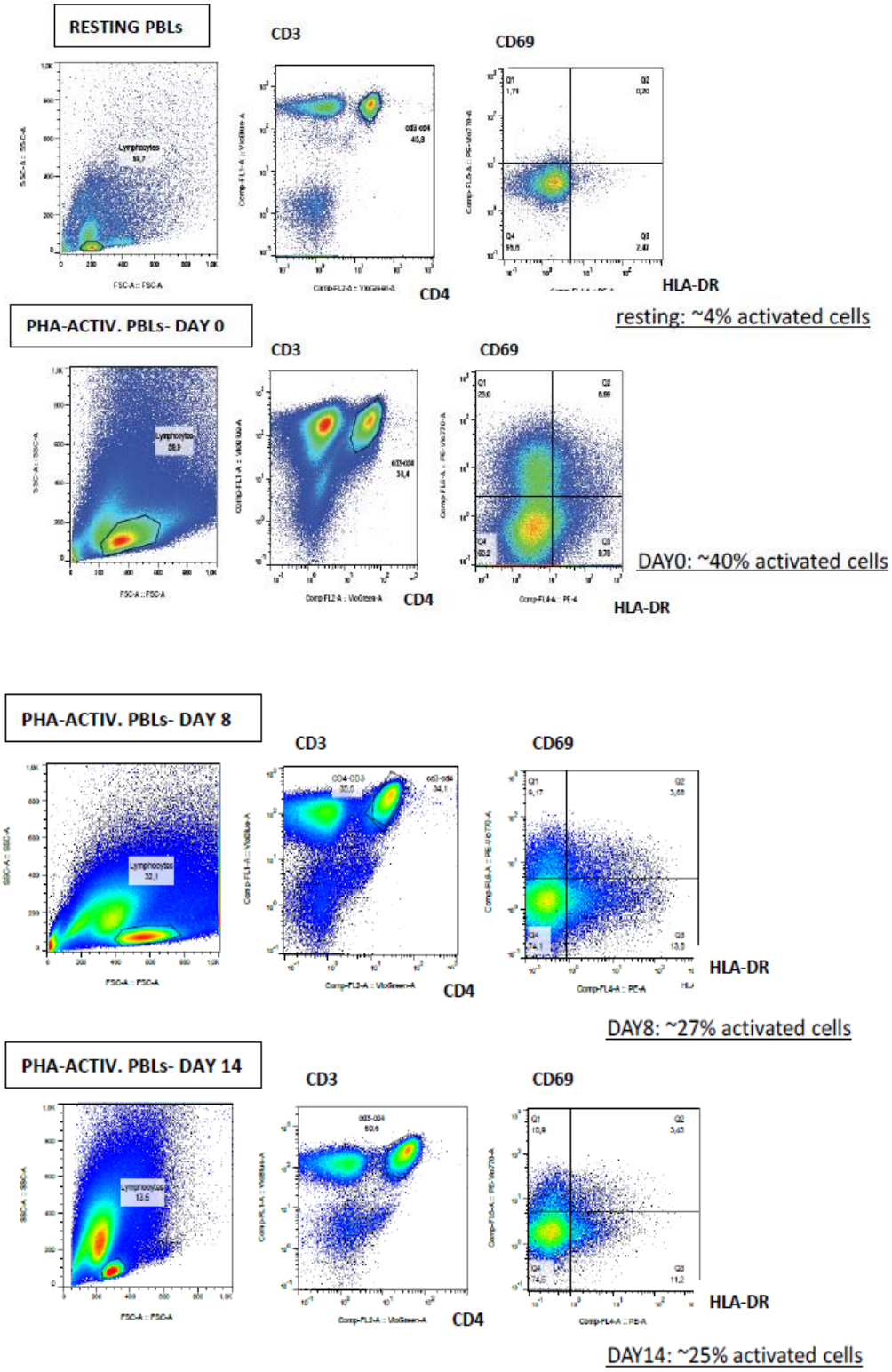

RESTING PBLs
CD3
CD69
CD4
HLA-DR
resting: ~4% activated cells
PHA-ACTIV. PBLs- DAY 0
CD3
CD69
CD4
HLA-DR
DAY0: ~40% activated cells
PHA-ACTIV. PBLs- DAY 8
CD3
CD69
CD4
HLA-DR
DAY8: ~27% activated cells
PHA-ACTIV. PBLs- DAY 14
CD3
CD69
CD4
HLA-DR
DAY14: ~25% activated cells

FIG.19- The majority of PHA-activated PBLs doesn't revert to resting state

Resting primary PBLs isolated from healthy donors were activated with PHA-IL2 as described before; after 4 days PHA was removed and the cells cultured in IL2 alone. Cytofluorimetric analysis was done at days: 0 (after PHA removal), 8, 14. Cells were stained with BV421-antiCD3, BV510-antiCD4, PE-Cy7-antiCD69, PE-antiHLA-DR,.

The approach based on pre-activated primary PBLs have the obvious advantage to facilitate the manipulation of these cells by increasing the efficiency of both infection and lentiviral transduction; nevertheless a major limitation to our purpose is represented by the fact that cellular activation induces MATR3 levels (Figure 8; Mohammadi et al., 2014) thus rendering this factor not more limiting in such a cellular context.

This consideration along with the limitation to have a real quiescent environment lead us to abandon the model based on pre-activated PBMCs and focus more on the direct infection of resting primary PBLs.

Two main features would render this model on resting PBLs ideal: the limiting levels of MATR3, which we know to be unaffected even by LRAs stimulation, and a more physiological and reliable context of latency.

In a setting of latently infected resting PBLs we are going to combine transcriptional reactivation induced by SAHA treatment for 72 hrs to post-transcriptional stimulation achieved by MATR3 overexpression through lentiviral transduction; this would provide us the proof of concept that MATR3 is not only a positive regulator of viral replication but is also a limiting factor in reactivation from latency in cells forming the viral reservoir.

The set up of such a model would consist of three main step: MATR3 overexpression, HIV-1 infection and SAHA stimulation to reactivate the provirus.

SAMHD1 depletion doesn't affect the resting state of primary PBLs

Resting PBLs are refractory to HIV-1 infection given the presence of restriction factors which counteract the process. Among them SAMHD1 proved to block the very first steps of viral replication by depleting the pool of dNTPs available within the cytoplasm of the host cell to allow the retrotranscription of the viral genome.

In several reports it was shown how the depletion of SAMHD1 can alleviate the restriction to HIV-1 infection from resting PBLs: the retroviral factor Vpx was found to specifically degrade SAMHD1 through the proteasomal pathway.

To deliver Vpx within resting PBLs SIVmac-based Viral Like Particles containing Vpx (Vpx-VLPs) are available.

VSVG-pseudotyped Vpx-VLPs were used to treat resting PBLs for 24 and 48 hours; SAMHD1 depletion was then assessed by immunoblotting and revealed an efficient removal of the restriction factor within 48 hours (Figure 20A). We asked if the depletion of SAMHD1 could somehow affect MATR3 protein levels and confirmed by immunoblotting that this was not the case (Figure 20B).

FIG. 20

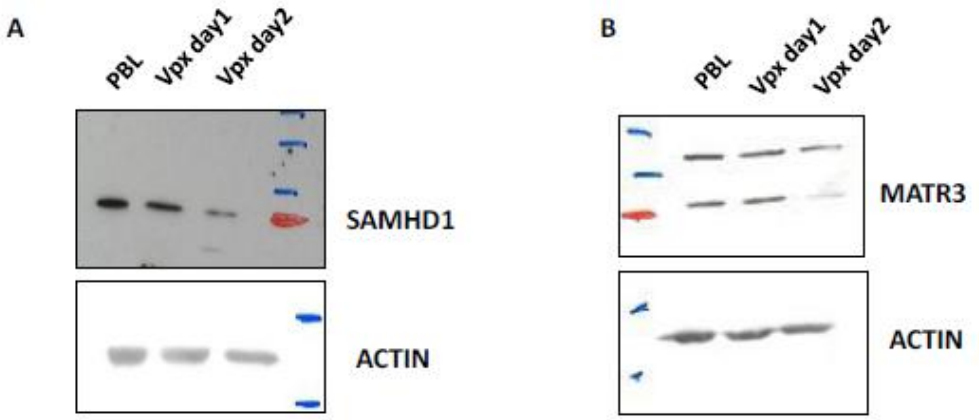


FIG.20- SAMHD1 depletion doesn't affect MATR3 expression

Resting primary PBLs isolated from healthy donors were treated with VSV-G pseudotyped Vpx-VLPs for 24 and 48 hours. Whole cell lysates were blotted for SAMHD1 (A) and MATR3 (B); B-actin is the loading control.

Next we wanted to assess if treatment with Vpx-VLPs can alter the activation status of resting PBLs: 2x10^6 resting PBLs isolated from healthy donors were treated (PBL-Vpx) with 12ng of Vpx-VLPs or left untreated (PBL); 6 days post

treatment the resting status of the cells was checked by FACS through the staining for the activation markers CD69 and HLA-DR.

As shown in Figure 21, CD4+T lymphocytes don't increase the expression of early and late activation marker after Vpx-VLPs treatment.

FIG. 21

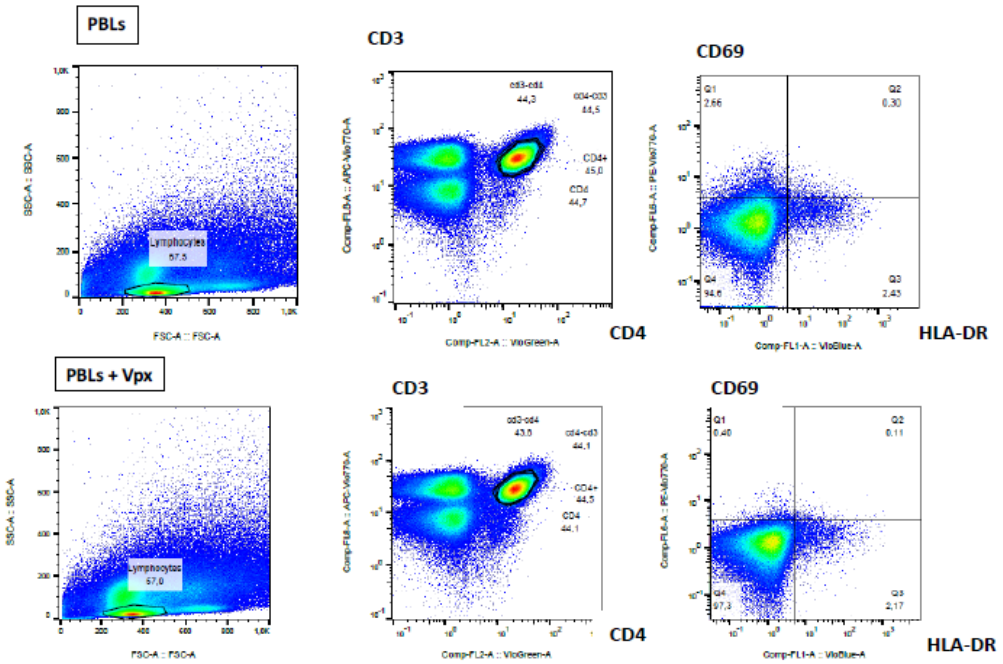


FIG.21- SAMHD1 depletion doesn't activate resting PBLs

Resting primary PBLs isolated from healthy donors were treated with VSV-G pseudotyped Vpx-VLPs. Cytofluorimetric analysis was done 6 days post treatment; cells were stained with APC-Cy7-antiCD3, BV510-antiCD4, PE-Cy7-antiCD69, BV421-antiHLA-DR.

MATR3 overexpression is not achieved along with HIV-1 infection

SAMHD1 depleted cells were infected with 450ng of a full length clone of HIV-1 containing the GFP reporter gene under the CMV promoter (kindly provided by Dr.Monsef Benkirane). The presence of the GFP gene under the control of CMV allow the constitutive expression of the reporter even under silencing condition at the LTR viral promoter thus allowing the identification of all the infected cells.

8 hours post infection PBLs were transduced with 600ng of lentivirus to express the flag-MATR3 and cultured in IL-2 containing media.

5 days post infection/transduction cells were analysed by FACS to assess the efficiency of infection (p24 staining and GFP) and the efficiency of lentiviral transduction and flag-MATR3 expression (flag staining).

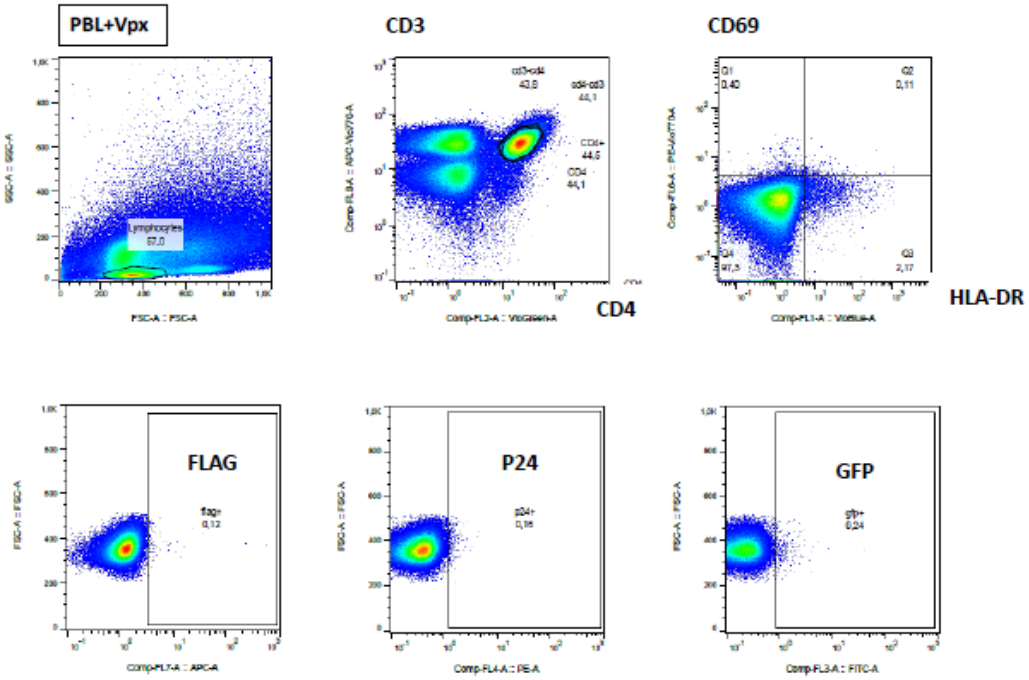
The markers for HIV-1 infection gave contrasting results: an high percentage of p24 positive cells was detected while the GFP expression was not observed (Figure 22).

Limitation in the detection of GFP signal were frequently encountered even in other experiments with the same virus which probably made the p24 staining more reliable to assess the efficiency of infection, which remained anyway still uncertain to be precisely defined.

Unfortunately, flag-MATR3 was not detected after lentiviral transduction as showed by the anti-flag staining (Figure 22).

Several attempts were conducted to optimize the step of lentiviral transduction by changing both the concentration of lentivirus used and the timing of transduction but all of them gave disappointing results: the concomitant events of HIV-1 infection and MATR3 overexpression didn’t occur efficiently in resting primary PBLs during our tests.

Fig. 22



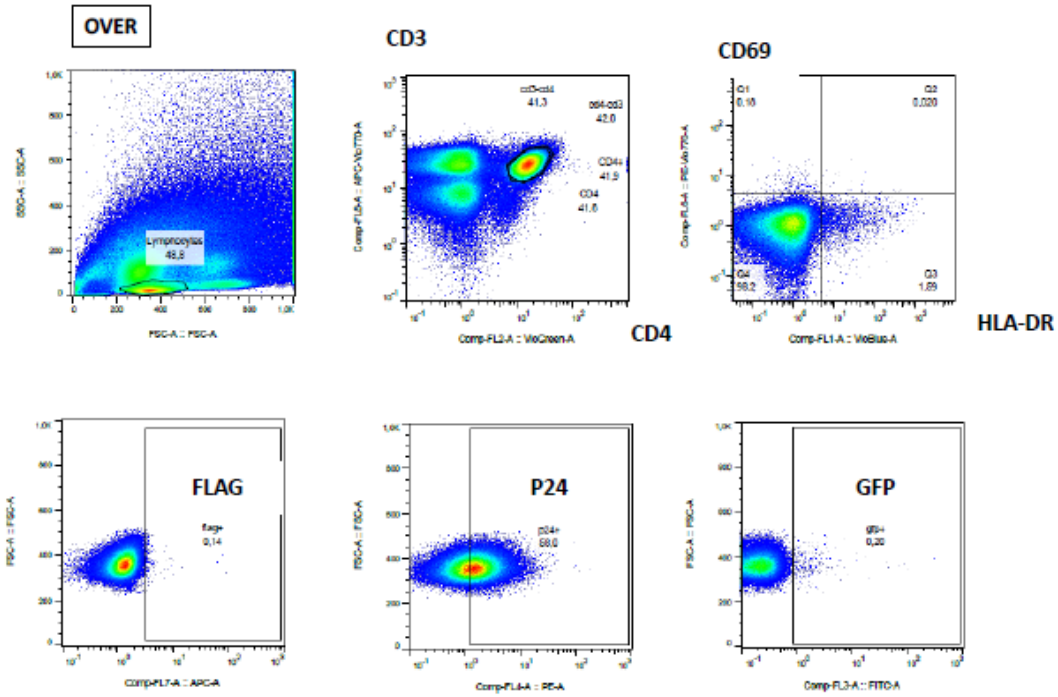


FIG.22- MATR3 overexpression is not achieved along with HIV-1 infection Resting primary PBLs isolated from healthy donors were treated with VSV-G pseudotyped Vpx-VLPs as before. 48 hours after treatment , SAMHD1-depleted PBLs were transduced with the lentivector to express flag-MATR3 (OVER) and infected with a replication competent HIV-1 containing the GFP as a reporter gene. Cytofluorimetric analysis was done 5 days post treatment; cells were stained with APC-Cy7-antiCD3, BV510-antiCD4, PE-Cy7-antiCD69, BV421-antiHLA-DR, APC-antiFLAG, RD1-p24.

DISCUSSION

The HIV-1 viral life cycle is a complex process which relies on the host cellular machinery. Proviral transcription and RNA metabolism occur with the concerted action of cellular pathways and viral factors, co-evolved to fully exploit the host environment to guarantee its replication.

To avoid the translation of defective mRNAs which would result in the production of possibly deleterious proteins, the cellular "quality control" pathway of nonsense-mediated decay along with a finely regulated export pathway, assure that only properly processed mRNAs are exported to the cytoplasm. Thus the presence of viral intron-containing mRNAs at their mature stage could represent a major obstacle for the virus to the completion of the first steps of viral gene expression: unspliced transcripts should be retained in the nucleus and targeted for degradation or sequestration. To overcome this obstacle HIV-1 has evolved the viral factor Rev which is involved in the specific pathway of intron-containing mRNA export. To this aim Rev engages an alternative route for viral mRNA export which ultimately involves the CRM-1 transport receptor, used by the eukaryotic cells to allow cytoplasmic translocation of protein cargos and specific type of RNAs.

The exact pathway which goes from Rev multimerization on viral mRNA to CRM1 recruitment has not been fully elucidated and some steps still remain elusive; thus a better understanding of the Rev interaction with the host proteins during the export process would help to identify the crucial host factors involved and characterize better the details of this process. With this aim it was exploited in our laboratory an approach based on the immunoprecipitation of viral RNA coupled to a mass-spectrometry analysis which allow the identification of the host protein MATR3 as an element of the Rev/RNA complex (Kula et al., 2011).

MATR3 is a component of the nuclear matrix, a complex structure which organize both spatially and functionally the nucleus: for this reason MATR3 can be involved in a plethora of role ranging from structural definition of discrete compartments to functional involvement in genome expression (Bode et al., 2003; Coelho et al., 2015; Salton et al., 2010, 2011).

The literature analysis on MATR3 revealed that this factor has been related to several processes in mRNA metabolism: its involvement in defective mRNA retention was found to be exerted in concerted action with PSF and p54nrb (Zhang

and Carmichael, 2001) while its action on alternative splicing regulation was associated to PTB (Coelho et al., 2015), thus suggesting that MATR3 can be recruited to different complexes and the factors engaged help defining its action.

In the context of host-virus interaction, MATR3 was reported to have a role in retroviral infection. Both for Moloney Murine Leukemia Virus (MoMu-LV) and HIV-1 it was found that MATR3 is a negative regulator of ZAP-mediated retroviral restriction since its depletion resulted in a strengthened antiviral activity (Erazo and Goff, 2015). Specifically for HIV-1, it was previously reported by us and other groups, that MATR3 positively affect viral replication (Kula et al., 2013; Yedavalli and Jeang, 2011): thus my thesis work aims to a better definition of the involvement of MATR3 in the context of HIV-1 infection both in the acute and the latent phase.

To point out a functional role for MATR3 during HIV-1 acute infection, we knocked-down this factor in Jurkat cells: a first striking observation we made is that MATR3 depletion in HIV-1 infected Jurkat cells results in a dramatic drop of viral replication. Notably we could recapitulate the same phenotype also in MATR3 depleted primary PBLs in the context of acute infection confirming that MATR3 is an essential factor to HIV-1 replication.

Our preliminary investigations suggested that MATR3 is recruited after transcription and interacts with Rev in an RNA-dependent manner. Thus to better characterize the effective role of MATR3 in the viral life cycle we further investigated the outcome of MATR3 depletion on HIV-1 transcription and mRNA metabolism.

By infecting Jurkat cells with an HIV-1 vector containing the luciferase gene as a reporter for transcription we could confirm that MATR3 depletion didn't affect proviral transcription during acute infection meanwhile we observed an intriguing effect on viral mRNA distribution. Intron-containing viral transcripts accumulated in the nucleus of infected Jurkat cells deprived of MATR3 and consequently intracellular viral protein production was impaired; on the other hand fully spliced viral transcripts were not affected and no imbalance in their nucleo-cytoplasmic distribution was observed. We therefore suggested that the efficiency of Rev-mediated export was compromised by MATR3 depletion thus affecting post-transcriptional steps of viral replication.

To verify our hypothesis on the role of MATR3 as a Rev co-factor during HIV-1 acute infection we sought to test if an increase in MATR3 protein levels could boost mRNA export and viral replication.

Exogenous delivery of a flag-tagged MATR3 into infected lymphocytes resulted in the effective increase in the total amount of MATR3 and, as expected, this caused an effect specifically on US-mRNA metabolism, while MS mRNAs were not affected. In particular, intron-containing mRNAs showed a reduced nuclear level, without a concurrent increase in their cytoplasmic localization: this observation can suggest either an enhanced export or a reduced stability of this mRNAs.

It was previously reported by Erazo and Goff (Erazo and Goff, 2015) that changes in the levels of MATR3 can affect HIV-1 mRNAs stability; in particular they noticed that MATR3 knockdown strengthen the ZAP-mediated degradation of both MS and US viral transcripts thus leading to a dramatic drop in viral replication.

In our case we observed that the reduction of viral mRNAs concentration occurred upon MATR3 overexpression and only in the nucleus; moreover the effect we reported was specific for US mRNAs while MS ones were unaffected.

Nevertheless, to rule out a possible effect of MATR3 overexpression on viral mRNAs stability we checked the level of intracellular viral proteins and the viral particle production. The increase in intracellular Gag and in viral particle production associated to MATR3 overexpression excluded any effect on mRNA stability and confirmed that MATR3 is a positive regulator of HIV-1 replication which acts as a Rev-cofactor to mediate the nuclear export of US viral mRNAs.

The discovery of new cellular factors involved in HIV-1 replication contributes to broaden the knowledge of the mechanisms behind viral infection. From the first contact with the target cell to the release of new virions every step of the viral life cycle is carried out with the concerted action of different host factors and thus any perturbation in their optimal function could greatly affect the progression of viral replication.

It’s easy to understand how the establishment of post-integrative latency within infected cells requires a peculiar cellular environment in which the transcriptional and post-transcriptional steps of viral replication are somehow defective. It is known that resting PBLs which mainly compose the HIV-1 viral reservoir exhibit a drastically altered cellular context compared to the one found in replicating

PBLs: thus the better comprehension of the phenomena of latency can't be separated from a deeper understanding of the cellular context where it is established.

Extensive studies have been conducted to reveal the transcriptional blocks present in latently infected cells. The epigenetic modifications to chromatin at the integration site are known to play an important role in maintaining a silent provirus: both increased methylation and inadequate acetylation contribute to render the LTR sites inaccessible to the transcription complex (Jiang et al., 2007; Marban et al., 2007; Pearson et al., 2008; Tyagi and Karn, 2007; Van Lint et al., 1996).

Moreover, the majority of the cellular factors involved in transcription are expressed at limiting levels or kept in an inactive state thus rendering the process inefficient: the suboptimal nuclear translocation of NF-kB and the sequestration of the elongation factor P-TEFb into an inactive complex play a crucial role in the transcriptional silencing of the provirus (Budhiraja et al., 2013; Fujinaga et al., 2004; Michels et al., 2004; Ping and Rana, 1999; Zhong et al., 2002).

The detailed characterization of the transcriptional blocks behind proviral silencing is the starting point of the so-called "shock and kill" strategy, one of the most intriguing therapeutic approach proposed to clear the viral reservoir. The "shock and kill" strategy proposes that the clearance of the viral reservoir can be achieved by reverting the latent state of the virus through its transcriptional induction: the return into active replication would render the infected cells visible to the host immune system leading to their targeting and removal. To achieve this goal several drugs, collectively called the Latency Reversal Agents (LRAs), have been proposed. The transcriptional induction is achieved by different mechanisms, from the addition or removal of selective epigenetic histone modification to the direct stimulation of the activity of the factors involved in the transcriptional pathway. The panel of LRAs includes Histone Methyltansferase Inhibitors (HMTIs), Histone Deacetylase Inhibitors (HDACIs), NF-kB activators and P-TEFb inducers; in vitro investigations with single or combination of these drugs reveal that most of them were able to induce in-vitro transcription at the LTR site.

Despite the efficient removal of transcriptional blocks an effective rescue of the full virus from latent cells is still lacking with most of the drug tested suggesting that other limitations impede the completion of the viral life cycle (Darcis et al., 2015, 2016; Van Lint et al., 2013).

The contribution of post-transcriptional steps to the maintenance of latency has been poorly investigated during the past years even though some crucial discoveries started to unveil the possible role played by factors involved in mRNA metabolism to the phenomena (Sarracino and Marcello, 2017).

Several primary cell models have been used to investigate post-transcriptional latency: in the CD4+ T cell model used by Pace et al. (Pace et al., 2012) it was found that reduced level of MS mRNAs were associated to the nuclear retention of US ones, suggesting how inefficient splicing and export of viral mRNAs can contribute to latency. In the model of Saleh et al. (Saleh et al., 2011) in which resting primary CD4+ T cells were treated with CCL19 prior to infection was found that the levels of US mRNAs were reduced possibly due to a nuclear retention or a cytoplasmic degradation of MS ones.

Importantly, in the work of Lassen et al. (Lassen et al., 2006) the ectopic expression of the polypyrimidine-tract binding protein (PTB) in resting CD4+ T cells from HIV+ patients was able to rescue the proper export of both fully and partially-spliced viral transcripts underlining the role that limiting levels of host factors involved in mRNA metabolism can play in the maintenance of latency.

The transcriptomic analysis of resting PBLs revealed that most of the factors involved in mRNA splicing, stabilization and export are poorly expressed unless an activation stimulus is provided thus suggesting how the maintenance of a quiescent state rely also on the limitation in mRNA processing (Mohammadi et al., 2014).

We found interesting that factors like PSF, PTB and MATR3 were shown to be poorly expressed in resting cells: to assess the actual expression of those factors in quiescent PBLs and CD4+ T cells we checked their protein level prior and post-activation with PHA and we confirmed their barely detectable basal expression, which is induced only after activation.

To better characterize the modulation of MATR3 level in the cellular context found in latency and upon exposure to drugs for AIDS treatment we sought to test if drugs selected from the panel of LRAs have any effect on the induction of MATR3 expression. To this aim we stimulated primary PBLs from healthy donors with the PTEN inhibitor Disulfiramin, the two histone deacetilase inhibitor SAHA and Romidepsin or the BET-inhibitor JQ1 in combination to the PKC agonist Ingenol B and observed that none of the tested drugs or combination was able to increase the low levels of MATR after 3 and 6 days of stimulation.

To extend this observation to the actual context of latent HIV-1 infection we repeated this test in PBLs isolated from aviremic HIV+ patients under antiretroviral therapy (ART). We could confirm that HIV-1 infected primary resting PBLs treated with LRAs still express limiting amount of MATR3 and that only cellular activation, in this case by TCR stimulation by anti-CD3/anti-CD28 beads, is able to raise MATR3 protein levels, indicating that the mode of induction is essential.

The assessment that MATR3, which we previously identified as a crucial factor for HIV-1 replication, is poorly expressed within cells composing the viral reservoir lead us to wonder about a possible involvement of this host factor in the maintenance of latency. Limiting level of MATR3 and other factors involved in mRNA processing and export could be responsible for the failure of the attempts to efficiently rescue the full virus from latent cells under condition in which the LRAs render the virus transcriptionally active.

To begin our investigation on the possible role of MATR3 in the context of viral latency we sought to verify if it's depletion in the well-established latency model of J-Lat has any affect on viral reactivation. We transduced J-Lat 8.4 cells with a lentivector coding for a shRNA targeting MATR3 and confirmed an efficient depletion of the factor. Upon administration of TNFα, we could confirm by checking GFP expression, that transcriptional activation of the integrated provirus occurred efficiently and that MATR3 removal was not affecting this process. Interestingly we could observe that both intracellular Gag and virion quantification in the culture media were decreased in MATR3- depleted J-lat cells: the defect on full viral particle production in condition of efficient viral transcription clearly indicated that MATR3 depletion affected the post-transcriptional steps of latency reversal.

The results obtained from J-lat cells supported our hypothesis about the necessity of post-transcriptional factors such as MATR3 to guarantee that, the transcriptional input to viral reactivation provided by TNFα or other drugs, lead to a complete replication cycle.

In this view, a revised approach to the "shock and kill" strategy would include drugs capable of inducing those processes involved in post-transcriptional RNA processing to remove any block to full viral reactivation.

In our previous tests with LRAs we could observe that MATR3 levels remained limiting after treatment with all the drugs. Among those drugs we focus on SAHA because of its potency as a transcriptional inducer and of its well-established

clinical use and we extended our observation to investigate the effect that SAHA alone has on the latency reversal.

To this aim we stimulated PBLs from HIV+ patients with 0,5uM of SAHA, a dosage within the window of the plasmatic concentration usually found in treated patients. The latent virus was responsive to SAHA and its transcription was efficiently restored as was stated by the appearance of intracellular viral transcripts which range levels comparable to the one obtained by cellular activation. Interestingly, the transcriptional induction rescued after stimulation with SAHA was not associated to full virion production, contrary to the appearance of complete viral particles observed after cellular activation by TCR stimulation.

Synergistic combination of LRAs have been tested with the aim to achieve a stronger effect on latency reversal (Darcis et al., 2015). We suggest that a new intriguing approach would be to combine stimuli acting at different stages: a potent transcriptional inducer to revert transcriptional silencing to a stimulus acting post-transcriptionally to assure that nascent viral RNAs are efficiently processed. We believe it would be interesting to test if the restoration of proper MATR3 levels within latently infected cells could help overcome the limitation to full virion production faced upon SAHA administration.

We started working on a suitable cellular model of latency to test our hypothesis: ideally we would need a cellular setting in which MATR3 levels are limiting in a way to be able to obtain a major increase in its total amount by delivering an exogenous version of the factor with the help of a lentiviral tool.

Clearly the best cellular model to our purpose would be the one provided by resting infected primary PBLs because of the almost intact and physiological latency environment; moreover we could previously assess that MATR3 levels are limiting under such conditions and not affected by SAHA administration.

Several protocols are available to set up a latency model based on primary PBLs: however the majority of them require a previous step of cellular activation to increase the efficiency of HIV-1 infection. Infected cells should be then cultured for a reasonable time in absence of any activation stimulus to allow a complete reversion to a quiescent phenotype (Pace et al., 2011; Spina et al., 2013).

To estimate the time in culture needed by PHA-activated PBLs to come back to the resting state we monitored their activation status after PHA removal by checking the expression of early and late activation markers over a time frame of

three weeks. We observed that once activated, primary PBLs harshly come back to the resting state and a substantial part of them still express activation markers after two weeks from PHA removal; an additional week of culture only resulted in cell death.

Another major disadvantage to the use of a model based on pre-activated PBLs to our purpose, is that cellular activation increases MATR3 levels thus rendering this factor not more limiting in this cellular context. In the transcriptomic analysis provided by the group of Talenti (Mohammadi et al., 2014) in which cellular gene expression is analysed in latently infected cells from a well-described latency model based on CD4+ T cells, it was found that the levels of different factors, and between them MATR3, remained sub-limiting after cellular activation, even after the long cellular culture used to allow the establishment of latency.

Considering the limitation in restoring a proper quiescent state and the modulation in MATR3 levels occurring after cellular activation, we abandoned the idea to use a model based on pre-activated PBLs for our purpose.

An alternative approach to set up a latency model in primary PBLs propose the direct infection of resting cells: two major advantages of this model consist in the preservation of the actual quiescent cellular context in which the viral latency is established and in the maintenance of limiting levels of MATR3.

Nevertheless several restrictions counteract HIV-1 viral infection of resting PBLs in vitro.

A major block to HIV-1 infection is constituted by the presence of the host factor SAMHD1 which acts as a potent restriction factor to viral infection. SAMHD1 is a cellular nuclease which exhibit a phospohydrolase activity by removing a phosphate group from deoxynucleoside triphosphate (dNTPs). Its action results in the reduction of the dNTPs pool, which, in turns affect the efficiency of reverse transcription during retroviral infection; the antiviral effect of SAMHD1 is more pronounced in resting cells because of the already exiguous pool of dNTPs (Descours et al., 2012; Goldstone et al., 2011).

It was shown that SAMHD1 depletion enhances HIV-1 infection: thus its removal from resting PBLs could provide a useful strategy to render these cells more permissive to viral infection in vitro. Recently it was discovered that the retroviral factor Vpx enhances SAMHD1 degradation through the proteasomal pathway;

notably this factor is not expressed by HIV-1 but only by other retroviruses such as HIV-2 and SIV (Hofmann et al., 2012; Laguette et al., 2011).

To deliver the Vpx factor within resting PBLs a viral tool based on SIVmac backbone have been developed: these SIVmac-derived Viral Like Particles containing Vpx (Vpx-VLPs) can be pseudotyped with the VSV envelope protein (VSV-G) to increase the efficiency of delivery (Geng et al., 2014; Nègre et al., 2000).

We decided to take advantage of this strategy to render resting PBLs more permissive to viral infection, to infect these cells with HIV-1 to establish a model of latency and in this model to combine the overexpression of MATR3 to the treatment with LRAs to finally test if the two approach can act in synergy to revert viral latency.

We treated resting PBLs with VSV-G pseudotyped Vpx-VLPs and we could assess an efficient degradation of SAMHD1 occurring in 48 hours; to verify if the treatment with Vpx-VLPs could somehow affect MATR3 expression we simultaneously checked the levels of MATR3 protein and assure that there was no effect on its expression. To exclude that the treatment with Vpx-VLPs alter the quiescent state of the cells we analysed the expression of early (CD69) and late (HLA-DR) activation markers before and after treatment; we confirmed that incubation with viral like particles and Vpx delivery didn't activate the resting PBLs.

Resting PBLs, treated with Vpx-VLPs to be more permissive to viral infection, were infected with a replication competent HIV-1 containing the additional GFP gene as a reporter for viral infection: the GFP gene is cloned under the CMV promoter in a way to allow its expression even under condition where the transcription at the LTR is silenced.

To decide the concentration of lentivirus to use to deliver the flag-MATR3 within resting PBLs, we conducted some preliminary test by checking the efficiency of FLAG-MATR3 expression using increasing amount of lentivector. The concentration selected was the lower one at which we started to detect the FLAG-MATR3 expression since we observed a marked cellular toxicity upon lentiviral transduction. The transduction with the lentivector to deliver the flag-tagged MATR3 was performed the same day of the infection with the aim to reduce the time passing after the treatment with Vpx-VLPs to avoid the recovery of SAMHD1 restriction activity.

The efficiency of infection was evaluated by cytofluorimetric analysis checking for the expression of GFP and intracellular p24. The first thing we noticed is that the percentage of GFP positive cells doesn't correspond to the percentage of p24 positive ones which suggest that one of the two markers is not reliable to estimate the rate of infected cells. Problems in the detection of GFP signal were frequently encountered in different experiments using the same virus: thus the p24 staining was probably more reliable to assess the efficiency of infection. Nevertheless the percentage of p24 positive cells is so high that likely could be overestimated due to a residual contamination with the input virus used for the infection: thus the exact percentage of infected cells remained still uncertain.

The efficiency of lentiviral transduction was assessed by cytofluorimetric analysis of the intracellular FLAG: unfortunately no FLAG expression was detected concomitantly to HIV-1 infection under the tested condition. Attempts to increase the time frame occurring between the viral infection and the lentiviral transduction to 24 hours didn't help achieving a better efficacy of the two process simultaneously.

In conclusion we faced technical issues, especially related to the expression of the exogenous FLAG-MATR3, which don't allow us to set up a proper model of latency based on the direct infection of resting PBLs to verify the actual contribution of MATR3 to the reactivation of the latent provirus.

Since we had to put aside the idea to use primary cells for our latency model we evaluated the possibility to utilize the classic latency model of J-Lat. Although the J-Lat cell line can represent a very useful tool for the study of latency thanks to the immortalized cell type, easy to manipulate, and to the presence of a reporter gene within an already silent and inducible provirus, we had some hesitancy to the employment of the J-Lat model to our task.

A major doubt arises from the consideration that J-Lat cells are not limiting in the levels of MATR3: thus it is necessary to keep in mind that the high basal level of the factor could make less appreciable any affect on viral replication related to an additional increase in MATR3 protein level. The general approach would remain unchanged: transcriptional induction provided by LRAs stimulation would be combined to MATR3 overexpression obtained by lentiviral transduction to deliver its FLAG-tagged version.

It is known that J-Lat 8.4 are responsive to treatment with TNF-α, a cytokine able to efficiently rescue the expression of the integrated provirus: a preliminary

screening with different LRAs was thus necessary to assess if J-Lat 8.4 are responsive to any other of the tested drugs. We stimulated J-Lat 8.4 cells with SAHA, Romidepsin, Disulfiramin, Ingenol and JQ1 at different concentration; the transcriptional induction of the virus was assessed by checking both the GFP expression and the transcription of the unspliced viral mRNAs: we found that both Romidepsin and SAHA are able to rescue the transcription of the latent virus.

Next we combined the treatment with the above mentioned drugs to the overexpression of MATR3: J-Lat 8.4 cells transduced with a lentivector to express the FLAG-MATR3 were stimulated for 48 hours with the different drugs and then we evaluated the effect on full viral particle rescue by looking at the viral titre in the culture media. Unfortunately we couldn't detect any positive effect to latency reversal from the combination of MATR3 overexpression to LRAs treatment.

We then hypothesized that, as supposed, the high basal level of MATR3 within J-Lat 8.4 could mask any effect on viral replication related to an additional increase in MATR3 protein level.

We checked the effective amount of MATR3 within these cells and surprisingly find out that even upon efficient expression of the exogenous flag-tagged version of MATR3 upon lentiviral transduction, the overall amount of the factor was not increased. This observation was quite unexpected and we suppose that the overall amount of MATR3 is finely regulated within J-Lat 8.4 cells in a way to minimize drastic increase in its expression.

To verify if this limitation in efficiently increasing MATR3 levels was specific for J-Lat 8.4, we tried to recapitulate the same approach in a different J-Lat clonal cell line, the J-Lat 6.3.

First of all we verified that this cell line was suitable to our purpose by checking the effect of the previously screened LRAs on viral transcription: we could confirm that J-Lat 6.3 cells responded to treatment with SAHA and Romidepsin by inducing high levels of viral mRNA transcription. Once confirmed their responsiveness to LRAs, we transduced J-Lat 6.3 cells with a lentivector to express the FLAG-MATR3 and checked the effective expression of the exogenous factor. We observed again that, even upon efficient delivery and expression of the exogenous MATR3, the total amount of the protein was unaffected and no significant increase in its concentration was reported.

Unfortunately we couldn’t succeed in the attempt to overexpress MATR3 within J-Lat cells since it seems that the overall amount of this factor are finely regulated to avoid drastic variation in its expression.

In conclusion we couldn’t find a suitable model of latency, either based on primary or immortalized cells, in which establish the proper condition to verify if the limiting levels of MATR3 are responsible for the post-transcriptional blocks which maintain the virus in its latent state.

CONCLUSION AND REMARKS

HIV-1 full viral gene expression is achieved with the concerted action of host and viral factors. We identified the host protein MATR3, a component of the nuclear matrix, as a Rev-cofactor recruited to engage the alternative nuclear export pathway through CRM1 to allow the cytoplasmic translocation of viral intron-containing mRNAs.

In this work we defined a functional role for MATR3 in the context of acute infection with a replication competent HIV-1; moreover we provide some evidences about the likely contribution of MATR3 to the maintenance of a latent state within the viral reservoir.

We modulate the level of MATR3 both in T cell line and primary PBLs and we proved that MATR3 knockdown results in the nuclear retention of unspliced viral transcripts thus dramatically affecting viral replication. On the contrary, if MATR3 levels are increased, the positive input to viral mRNA export enhances not only viral protein production but finally leads to a remarkable raise in viral replication.

We observed that MATR3, like other factors involved in mRNA metabolism, is poorly expressed in resting PBLs suggesting that the cellular environment in which viral latency is established could rely on limiting levels of these factors to maintain the virus in a latent state. Moreover we showed that the drugs currently proposed to revert the transcriptional silencing of the virus don't alter the expression of MATR3 which remain limiting throughout the treatment which lead to removal of transcriptional blocks.

Specifically for SAHA, one of the best characterized drug in the panel of the tested ones, we showed that the re-establishment of efficient viral transcription doesn't lead to the production of complete viral particles revealing that removal of transcriptional blocks is necessary but not sufficient to allow the latent virus to come back to active replication.

Intriguingly this observation uncover an aspect of viral latency, the existence of post-transcriptional blocks, which remain mostly elusive and would need further exploration to shed some light on what are the critical steps to be targeted to allow the virus to leave the latent state.

We demonstrated in J-Lat cells, a classic model of latency, that MATR3 depletion impede the full viral rescue under condition of optimal viral transcription and we

propose that, due to a block in nuclear mRNA export caused by MATR3 limiting levels, the viral cycle get stuck in the production of structural proteins to assemble the nascent virion.

In this work we lack a direct evidence that the restoration of adequate levels of MATR3 within latently infected cells can provide the “post-transcriptional boost” to the initial SAHA’s “transcriptional kick” to revert viral latency.

Despite the technical difficulties we encounter to inspect this last scenario, we believe that at this point is essential a further exploration of the post-transcriptional blocks which render the “shock and kill” approach still unsuccessful.

A better comprehension of the pathways which are obstructed during the establishment of the latent state and the discovery of the crucial factors indispensable to revert such condition could significantly improve the development of a clinical strategy to clear the viral reservoir leading finally to a “sterilizing cure” to effective eradicate the HIV-1 viral infection.

MATERIALS AND METHODS

CELLS

Human embryonic kidney 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (penicillin/streptomycin).

Jurkat and J-Lat cells obtained from NIH Research and Reference Reagent Program were cultured in Rosewell Park Memorial Istitute medium (RPMI) supplemented with 10% FBS and antibiotics.

Peripheral Blood Mononuclear Cells (PBMCs) and Peripheral Blood Lymphocytes (PBLs) were purified from healthy donors' buffy coat obtained from "Ospedale Maggiore of Trieste".

PBMCs were purified through Ficoll gradient (Ficoll-Hystopaque Lonza) and cultured in RPMI supplemented with 10% heat inactivated FBS and antibiotics. After an overnight culture non-adherent PBLs were collected and grown in the same media containing only IL2 (20U/ml) to maintain the resting state or IL2 (20U/ml) and PHA (5ug/ml) to activate them.

LENTIVIRAL VECTORS

pLKO.1 lentiviral vectors expressing shRNAs targeting MATR3 or a scramble CTRL were obtain from Open Biosystems (TRCN0000074903-904-905-906-907 or shCTRL).

The pWPI-flagMATR3 lentiviral vector was produced by cloning the flag-MATR3 cDNA into the pWPI plasmid (kindly provided by Gualtiero Alvisi, University of Padova).

Briefly, the flag-MATR3 sequence was amplified by PCR from the pCMV-flagMATR3 plasmid adding the restriction sites for AscI and MluI at the 5' and 3' site of the gene respectively.

The obtained sequence was inserted into the pWPI_BLR transfer vector digested with Asc I and Mlu I and the plasmid was amplified in XL10 gold bacteria. Ampicillin resistant bacterial colonies were screened for the presence of the pWPI-

flagMATR plasmid whose identity was verified by both enzimatic digestion and sequencing (Figure 23).

The VSV-G pseudotyped lentivirus to overexpress MATR3 was tested on Jurkat cells and the efficient expression of the exogenous protein was assessed by an immunoblotting for FLAG (Figure 24).

Fig.23 - cloning strategy

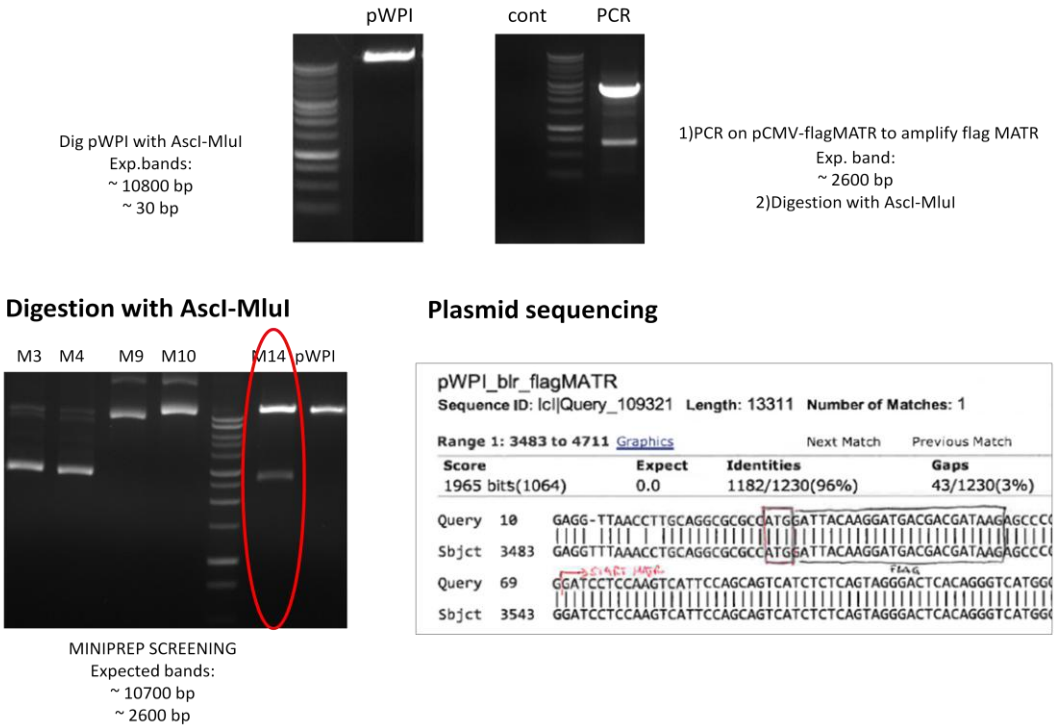


FIG.23- cloning strategy to obtain the pWPI-flagMATR3 lentiviral vector. The flag-MATR sequence containing the restriction sites for AscI-MluI was amplified from the pCMV-flagMATR plasmid and cloned into the pWPI vector digested AscI-MluI. Mini prep screening was performed by enzimatic digestion with AscI-MluI and the positive clone was checked by sequencing.

FIG. 24

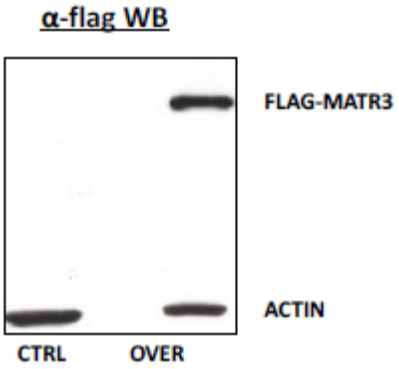


FIG.24- Validation of the flag-MATR lentiviral vector. Jurkat cells were transduced with the VSV-G pseudotyped lentivirus to express the flag-MATR3 (OVER) or with a control lentivector (CTRL) and selected by Blasticidin resistance. Whole cell lysates were blotted for FLAG; B-actin is the loading control.

TRANSFECTION AND TRANSDUCTION

Lentiviral particles to knockdown MATR3 were produced by calcium-phosphate transfection of 293T cells with the transfer vector (pLKO-shRNA; pLKO-scramble), the packaging plasmid (psPAX2) and the VSV-G coding plasmid (pMD2G), (all from Addgene).

Lentiviral particles to express the flag-tagged MATR3 were produced by co-transfecting the transfer vector (pWPI; pWPI-flagMATR), the packaging plasmid (psPAX2) and the VSV-G coding plasmid (pMD2G).

Lentivirus-containing supernatant was collected 48 hours post-transfection, centrifuged to remove cell debris, filtered through 0,45nm filter and stock at –80°C.

When needed lentiviral-containing supernatant was concentrated 100 folds by ultracentrifugation at 27000 rpm and quantified by p24 ELISA (Retrotek, Zeptometrix).

To deplete or overexpress MATR3 1x10^6 cells were incubated with the lentivirus in 6-well plate for 16 hours; cells were then washed with PBS to

remove unbound lentiviral particles and 24 hours post-transduction cells were put under puromicin (1ug/ml) or blasticidin (10ug/ml) selection.

HIV-1 VIRUS PRODUCTION

The replication competent HIV-1 virus was produced by calcium-phosphate transfection of 293T cells with the full-length HIV-1 clone pNL4.3.

48 hours post-transfection virus-containing supernatant was collected, clarified by centrifugation, filtered through 0,45nm filter and stock at -80°C. Viral titre was quantified by p24 ELISA (Innogenetics).

A replication competent HIV-1 virus containing the GFP reporter gene in frame with Nef and under the control of CMV promoter was kindly provided by Dr. Monsef Benkirane.

ANTIBODIES

For immunoblotting the following antibodies have been used: MATR3 (A300-590A, Bethyl laboratories, dilution 1:5000), HIV-1 p55 and p24 (HIV-1 p24 sc-65462, Santa Cruz, dilution 1:250), FLAG (Sigma, dilution 1:5000), B-Actin-HRP (Sigma, 1:10000), Vimentin (Cell Signalling, dilution 1:1000), PARP (Enzo Life), Hsp90 (Enzo Life), PSF (Sigma P2860, 1:1000), Cyclin T1 (C-20-SC-8128, 1:200), PTB (rabbit polyclonal produced in house, 1:1000).

For cytofluorimetrics analysis the following antibodies have been used: APC-flag (Biolegend, 637307, dilution 1:50), RD1-p24 (Beckman KC57-RD1, dilution 1:40), APC-Cy7-CD3 (Biolegend, dilution 1:25), BV510-CD4 (Biolegend, dilution 1:25), BV421-HLA-DR (Biolegend, dilution 1:25), PE-Cy7-CD69 (Biolegend, dilution 1:25).

DRUGS AND REAGENTS

The following drugs have been used: SAHA (SML0061 Sigma), disulfiram (PHR1690 Sigma),

romidepsin (S3020, Selleckchem), JQ1 (2091-1 BioVision).

IngenolB was kindly donated by Luiz F. Pianowski, Kyolab/Amazônia Fitomedicamentos, Valinhos, Sao Paulo, Brazil.

Other reagents include: Histopaque – 1077 (10771, Sigma), Polybrene (Sigma-H9268), Phytohemagglutinin (PHA L1668-5MG, Sigma), Interleukin-2 (H7041,

Sigma), TNF-α (T0157, Sigma), Puromycin (ant-pr-1 InvivoGen), Blasticidin (ant-bl-1 InvivoGen).

LUCIFERASE ASSAY

Jurkat cells were infected with 1µg/ml of HIV-1 pNL4.3R-E-luc pseudotyped with VSV-G envelope for 4 hours. The infected cells were washed twice and further incubated at 37°C for 48 hours.

48 hour post infection the cells were harvested and lysed in passive lysis buffer (Promega) and the levels of luciferase activity were measured by the Single-Luciferase-Reporter assay (Promega) as directed by manufacturers. For normalization, total protein concentration in each extract was determined with a Bio-Rad protein assay kit.

NUCLEAR AND CYTOPLASMIC FRACTIONS

To separate nuclear and cytoplasmic fractions cells were washed in cold PBS and lysate in Buffer A (10mM Tris HCl, 10mM NaCl, 3mM MgCl, 10% glycerol, 0,1% NP40, 0,5mM DTT, protease inhibitor).

Lysed cells were then spin 2300 rpm and the cytoplasmic fraction was collected as supernatant; the pellet was washed twice in Buffer A and finally resuspended in Buffer B (Buffer A supplemented with 0,5% deoxycholate) to collect the nuclear fraction .

Purity of nuclear and cytoplasmic fractions was assessed by immunoblotting for PARP and Hsp90.

RNA EXTRACTION AND qRT-PCR

RNA extraction was done using UPzol (biotech rabbit) according to manufacturer's protocol; residual DNA was removed by DNAse I digestion (Invitrogen) and 500ng of RNA were used to synthesize cDNA using the MMLV reverse transcriptase (Invitrogen).

An equal amount of cDNA from all samples was used to run quantitative real time PCR (qRT-PCR) with SYBR Green dye (KAPA SYBER FAST qPCR Bio-Rad iCycle, KAPA Biosystem); GAPDH served as an housekeeping gene to quantify viral and cellular mRNA abundance.

The primers sequences used in the qRT-PCR reaction are the following:

MATR3 FW: 5’-TCT TGG GGG ACC AGC AGT TGG A-3’

MATR3 REV: 5’-GCT AGT TTC CAC TCT GCC TTT CTG C-3’

GAPDH FW: 5’-CAT GAG AAG TAT GAC AAC AGC-3’

GAPDH REV: 5’-AGT CCT TCC ACG ATA CCA AAG-3’

US_HIV FW: 5’-CTG AAG CGC GCA CGG CAA-3’

US_HIV REV: 5’-GAC GCT CTC GCA CCC ATC TC-3’

MS_HIV FW: 5’-TCT ATC AAA GCA ACC CAC CT-3’

MS_HIV REV: 5’-CGT CCC AGA TAA GTG CTA AG-3’

The primer set for US_HIV amplifies the 5’UTR-Gag region while the one for MS_HIV amplifies the Tat-Rev exon.

HIV-1 REPLICATION KINETICS

Jurkat cells or PHA-activated primary PBLs were infected with 40-70 ng of replication competent HIV-1 for 3 hours (or overnight) at 37°C; cells were then washed twice to remove unbound viral particle and cultured in RPMI 10% FBS (supplemented with 20U/ml of IL2 for PBLs).

Every two days supernatant from infected cultures was collected and viral particle production was quantified by p24 ELISA (Retrotek or Innogenetics).

LRAs TREATMENT ON PBL FROM HEALTHY DONOR

Resting primary PBLs were isolated from healthy donors’ peripheral blood and cultured in RPMI containing 10% heat inactivated FBS and IL2 (20U/ml).

6x10^6 cells were left untreated or stimulated for 3 and 6 days with the following drugs at the indicated concentration:

- Disulfiramin 0,5uM

- SAHA 0,5uM

- Romidepsin 0,0175uM

- JQ1 (0,5uM) in combination to Ingenol B (10nM)

PHA (5ug/ml) was used as a positive control for cellular activation.

3 and 6 days post treatment the cells were lysed in Laemmli buffer to collect the protein extract.

LRAs TREATMENT ON J-LAT

2x10^6 J-Lat 8.4 or J-Lat 6.3 cells were left untreated or stimulated with the following drugs at the reported concentrations:

-SAHA (0,5uM- 1uM- 1,5uM)

-disulfiramin (5uM)

-Ingenol B (0,4uM)

-JQ1 (0,5uM)

-Romidepsin (0,006uM - 0,0175uM - 0,026uM)

-TNFa (30ng/ml)

48 hours post treatment culture supernatant was collected and cells were lysed in UPzol, in Laemmli buffer or analysed by FACS.

The induction of transcription at the LTR viral promoter was evaluated by both GFP expression at the cytofluorimeter and RT-qPCR for the US-HIV mRNA normalized to GAPDH.

Viral particles in the supernatant were quantified by p24 ELISA.

FACS ANALYSIS

J-Lat cells were fixed in 4% PFA for 10 minutes, washed twice with PBS and analysed by BD FacsCalibur to check GFP expression.

FACS analysis on primary resting PBLs were done by MACSQuant Analyzer.

Samples preparation was done as following: cells were fixed in 4% PFA for 10 minutes, washed twice in PBS and permeabilized in Permeabilization Buffer (0,5% triton-X, 20mM Hepes pH 7.4, 50mM NaCl, 3mM MgCl, 300mM sucrose) to allow intracellular staining with anti-FLAG and anti-p24 antibodies.

After intracellular staining cells were washed in PBS 0,1% tween and stained for surface markers (CD3, CD4, CD69, HLA-DR).

HIV+ PATIENTS

Seven HIV-1-infected individuals were selected at the St-Pierre Hospital (Brussels, Belgium) on the basis of the following criteria: all volunteers were treated with cART for at least 1 year, had an undetectable plasma HIV-1 RNA level (20 copies/ml) for at least 1 year and had a level of CD4+ T lymphocytes higher than 300 cells/mm3 of blood. Characteristics (age, CD4+ T cell count, CD4+ nadir, antiviral regimens) of each patient are presented in Table 1.

Ethical approval was granted by the Human Subject Ethics Committees of the Saint-Pierre Hospital (Brussels, Belgium). All individuals enrolled in the study provided written informed consent for donating blood.

Table 1

patients	Total HIV-1 DNA copies/10^6 cells	Age	CD4+ T cell count	nadir	Last treatments
P1	94	41	433	433	STB
P2	1329	46	807	331	KVX RTV ATV
P3	184	57	359	10	3TC RTV DRV RLT ETV MVC
P4	317	47	16	16	KVX RTV DRV
P5	553	56	789	321	TRU ATV
P6	1627	58	X	25	TRU RTV DRV
P7	1442	59	1083	92	RTV DRV ETV MVC

Abbreviations: STB,elvitegravir/cobicistat/emtricitabine/tenofovir; KVX, Kivexa; RTV, ritonavir; ATV, atazanavir; 3TC, lamivudine; DRV, darunavir; RLT raltegravir, ETV, etravirine; MVC, maraviroc; TRU, truvada, ETV, etravirine.

QUANTIFICATION OF CELL-FREE HIV RNA IN CULTURE MEDIA OF PATIENTS CELLS

CD8+ depleted PBMCs were isolated from blood of HIV+ patients.

6x10^6 cells were cultured in LGM-3 Growth Medium (Lonza) and treated with the following drugs at the indicated concentrations:

- Disulfiramin 0,5uM

- SAHA 0,5uM

- Romidepsin 0,0175uM

- JQ1 (0,5uM) in combination to Ingenol B (10nM)

Anit-CD3-anti-CD28 antibodies were used as a positive control for cellular activation.

Three and six days after treatment, culture supernatants from patient cell cultures were collected for RNA extraction using QIAamp Viral RNA Mini kit (Qiagen). HIV-1 RNA levels were quantified using the Generic HIV Charge Viral kit (Biocentric) according to the manufacturer's instructions (detection limits of 110 HIV-1 RNA copies/ml or 300 HIV-1 RNA copies/ml depending on tested supernatant volumes).

QUANTIFICATION OF CELL-ASSOCIATED HIV RNA FROM PATIENTS SAMPLES

Total RNA was isolated from patient's CD8+- depleted PBMCs using the Boom isolation method (22).

The RNA was treated with DNase (DNA-free kit; Ambion) and reverse transcribed using random primers and SuperScript III reverse transcriptase (all from Invitrogen). Cell-associated HIV RNA was quantified using a qPCR assay specific for the HIV *gag* region (23). The amounts of HIV-1 RNA were

normalized to total cellular inputs, which were quantified in separate qPCR assays, using the detection kit for 18S ribosomal RNA (Applied Biosystems, Foster City, CA), and were expressed as the number of copies per microgram of total RNA.

STATISTICS

Typically three independent experiments in triplicate repeats were conducted for each condition examined. Average values are shown with standard deviation and p-values, measured with a Student's t-test. Only significant values are indicated by the asterisks above the graphs ($p<0.01$ = ** highly significant; $p<0.05$ = * significant). Data from patients were analysed using paired, non-parametric Wilcoxon tests ($p<0.05$ = significant). All tests were two-sided.

BIBLIOGRAPHY

Agostini, I., Navarro, J.M., Rey, F., Bouhamdan, M., Spire, B., Vigne, R., and Sire, J. (1996). The human immunodeficiency virus type 1 Vpr transactivator: cooperation with promoter-bound activator domains and binding to TFIIB. J. Mol. Biol. *261*, 599–606.

Agosto, L.M., Yu, J.J., Dai, J., Kaletsky, R., Monie, D., and O'Doherty, U. (2007). HIV-1 integrates into resting CD4+ T cells even at low inoculums as demonstrated with an improved assay for HIV-1 integration. Virology *368*, 60–72.

Amorim, R., Costa, S.M., Cavaleiro, N.P., da Silva, E.E., and da Costa, L.J. (2014). HIV-1 Transcripts Use IRES-Initiation under Conditions Where Cap-Dependent Translation Is Restricted by Poliovirus 2A Protease. PLoS ONE *9*.

Archin, N.M., Keedy, K.S., Espeseth, A., Dang, H., Hazuda, D.J., and Margolis, D.M. (2009). Expression of latent human immunodeficiency type 1 is induced by novel and selective histone deacetylase inhibitors. AIDS Lond. Engl. *23*, 1799–1806.

Bai, Y., Tambe, A., Zhou, K., and Doudna, J.A. (2014). RNA-guided assembly of Rev-RRE nuclear export complexes. eLife *3*, e03656.

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

Benkirane, M., Chun, R.F., Xiao, H., Ogryzko, V.V., Howard, B.H., Nakatani, Y., and Jeang, K.T. (1998). Activation of integrated provirus requires histone acetyltransferase. p300 and P/CAF are coactivators for HIV-1 Tat. J. Biol. Chem. *273*, 24898–24905.

Bernhard, W., Barreto, K., Saunders, A., Dahabieh, M.S., Johnson, P., and Sadowski, I. (2011). The Suv39H1 methyltransferase inhibitor chaetocin causes induction of integrated HIV-1 without producing a T cell response. FEBS Lett. *585*, 3549–3554.

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

Black, A.C., Luo, J., Chun, S., Bakker, A., Fraser, J.K., and Rosenblatt, J.D. (1996). Specific binding of polypyrimidine tract binding protein and hnRNP A1 to HIV-1 CRS elements. Virus Genes *12*, 275–285.

Blazkova, J., Murray, D., Justement, J.S., Funk, E.K., Nelson, A.K., Moir, S., Chun, T.-W., and Fauci, A.S. (2012). Paucity of HIV DNA methylation in latently infected, resting CD4+ T cells from infected individuals receiving antiretroviral therapy. J. Virol. *86*, 5390–5392.

Bode, J., Goetze, S., Heng, H., Krawetz, S.A., and Benham, C. (2003). From DNA structure to gene expression: mediators of nuclear compartmentalization and dynamics. Chromosome Res. Int. J. Mol. Supramol. Evol. Asp. Chromosome Biol. *11*, 435–445.

Boehm, D., Calvanese, V., Dar, R.D., Xing, S., Schroeder, S., Martins, L., Aull, K., Li, P.-C., Planelles, V., Bradner, J.E., et al. (2013). BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism. Cell Cycle *12*, 452–462.

Boehringer, A., Garcia-Mansfield, K., Singh, G., Bakkar, N., Pirrotte, P., and Bowser, R. (2017). ALS Associated Mutations in Matrin 3 Alter Protein-Protein Interactions and Impede mRNA Nuclear Export. Sci. Rep. *7*, 14529.

Booth, D.S., Cheng, Y., and Frankel, A.D. (2014). The export receptor Crm1 forms a dimer to promote nuclear export of HIV RNA. eLife *3*, e04121.

Bouchat, S., Gatot, J.-S., Kabeya, K., Cardona, C., Colin, L., Herbein, G., De Wit, S., Clumeck, N., Lambotte, O., Rouzioux, C., et al. (2012). Histone methyltransferase inhibitors induce HIV-1 recovery in resting CD4(+) T cells from HIV-1-infected HAART-treated patients. AIDS Lond. Engl. *26*, 1473–1482.

Brady, J., and Kashanchi, F. (2005). Tat gets the “green” light on transcription initiation. Retrovirology *2*, 69.

Brasey, A., Lopez-Lastra, M., Ohlmann, T., Beerens, N., Berkhout, B., Darlix, J.-L., and Sonenberg, N. (2003). The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G2/M phase of the cell cycle. J. Virol. *77*, 3939–3949.

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

Brussel, A., Delelis, O., and Sonigo, P. (2005). Alu-LTR real-time nested PCR assay for quantifying integrated HIV-1 DNA. Methods Mol. Biol. Clifton NJ *304*, 139–154.

Budhiraja, S., Famiglietti, M., Bosque, A., Planelles, V., and Rice, A.P. (2013). Cyclin T1 and CDK9 T-loop phosphorylation are downregulated during establishment of HIV-1 latency in primary resting memory CD4+ T cells. J. Virol. *87*, 1211–1220.

Bukrinskaya, A., Brichacek, B., Mann, A., and Stevenson, M. (1998). Establishment of a Functional Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcription Complex Involves the Cytoskeleton. J. Exp. Med. *188*, 2113–2125.

Bukrinsky, M.I., Sharova, N., McDonald, T.L., Pushkarskaya, T., Tarpley, W.G., and Stevenson, M. (1993). Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. U. S. A. *90*, 6125–6129.

Burdo, T.H., Soulas, C., Orzechowski, K., Button, J., Krishnan, A., Sugimoto, C., Alvarez, X., Kuroda, M.J., and Williams, K.C. (2010). Increased monocyte turnover from bone marrow correlates with severity of SIV encephalitis and CD163 levels in plasma. PLoS Pathog. *6*, e1000842.

Buxadé, M., Morrice, N., Krebs, D.L., and Proud, C.G. (2008). The PSF.p54nrb complex is a novel Mnk substrate that binds the mRNA for tumor necrosis factor alpha. J. Biol. Chem. *283*, 57–65.

Cameron, P.U., Saleh, S., Sallmann, G., Solomon, A., Wightman, F., Evans, V.A., Boucher, G., Haddad, E.K., Sekaly, R.-P., Harman, A.N., et al. (2010). Establishment of HIV-1 latency in resting CD4+ T cells depends on chemokine-induced changes in the actin cytoskeleton. Proc. Natl. Acad. Sci. U. S. A. *107*, 16934–16939.

Carter, C.C., Onafuwa-Nuga, A., McNamara, L.A., Riddell, J., Bixby, D., Savona, M.R., and Collins, K.L. (2010). HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs. Nat. Med. *16*, 446–451.

Chable-Bessia, C., Meziane, O., Latreille, D., Triboulet, R., Zamborlini, A., Wagschal, A., Jacquet, J.-M., Reynes, J., Levy, Y., Saib, A., et al. (2009). Suppression of HIV-1 replication by microRNA effectors. Retrovirology *6*, 26.

Chiu, Y.-L., Soros, V.B., Kreisberg, J.F., Stopak, K., Yonemoto, W., and Greene, W.C. (2005). Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. Nature *435*, 108–114.

Chomont, N., El-Far, M., Ancuta, P., Trautmann, L., Procopio, F.A., Yassine-Diab, B., Boucher, G., Boulassel, M.-R., Ghattas, G., Brenchley, J.M., et al. (2009). HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat. Med. *15*, 893–900.

Choudhary, S.K., Archin, N.M., and Margolis, D.M. (2008). Hexamethylbisacetamide and disruption of human immunodeficiency virus type 1 latency in CD4(+) T cells. J. Infect. Dis. *197*, 1162–1170.

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

Chun, T.W., Carruth, L., Finzi, D., Shen, X., DiGiuseppe, J.A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T.C., et al. (1997). Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature *387*, 183–188.

Cochrane, A., Murley, L.L., Gao, M., Wong, R., Clayton, K., Brufatto, N., Canadien, V., Mamelak, D., Chen, T., Richards, D., et al. (2009). Stable complex formation between HIV Rev and the nucleosome assembly protein, NAP1, affects Rev function. Virology *388*, 103–111.

Cochrane, A.W., McNally, M.T., and Mouland, A.J. (2006). The retrovirus RNA trafficking granule: from birth to maturity. Retrovirology *3*, 18.

Coelho, M.B., Attig, J., Bellora, N., König, J., Hallegger, M., Kayikci, M., Eyras, E., Ule, J., and Smith, C.W. (2015). Nuclear matrix protein Matrin3 regulates alternative splicing and forms overlapping regulatory networks with PTB. EMBO J. *34*, 653–668.

Colin, L., and Van Lint, C. (2009). Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies. Retrovirology *6*, 111.

D'Agostino, D.M., Felber, B.K., Harrison, J.E., and Pavlakis, G.N. (1992). The Rev protein of human immunodeficiency virus type 1 promotes polysomal association and translation of gag/pol and vpu/env mRNAs. Mol. Cell. Biol. *12*, 1375–1386.

Dahl, V., and Palmer, S. (2009). Establishment of drug-resistant HIV-1 in latent reservoirs. J. Infect. Dis. *199*, 1258–1260.

Darcis, G., Kula, A., Bouchat, S., Fujinaga, K., Corazza, F., Ait-Ammar, A., Delacourt, N., Melard, A., Kabeya, K., Vanhulle, C., et al. (2015). An In-Depth Comparison of Latency-Reversing Agent Combinations in Various In Vitro and Ex Vivo HIV-1 Latency Models Identified Bryostatin-1+JQ1 and Ingenol-B+JQ1 to Potently Reactivate Viral Gene Expression. PLoS Pathog. *11*, e1005063.

Daugherty, M.D., Liu, B., and Frankel, A.D. (2010a). Structural basis for cooperative RNA binding and export complex assembly by HIV Rev. Nat. Struct. Mol. Biol. *17*, 1337–1342.

Daugherty, M.D., Booth, D.S., Jayaraman, B., Cheng, Y., and Frankel, A.D. (2010b). HIV Rev response element (RRE) directs assembly of the Rev homooligomer into discrete asymmetric complexes. Proc. Natl. Acad. Sci. U. S. A. *107*, 12481–12486.

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

Eckstein, D.A., Penn, M.L., Korin, Y.D., Scripture-Adams, D.D., Zack, J.A., Kreisberg, J.F., Roederer, M., Sherman, M.P., Chin, P.S., and Goldsmith, M.A. (2001). HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues. Immunity *15*, 671–682.

Ehrlich, L.S., Agresta, B.E., and Carter, C.A. (1992). Assembly of recombinant human immunodeficiency virus type 1 capsid protein in vitro. J. Virol. *66*, 4874–4883.

Emerman, M., Vazeux, R., and Peden, K. (1989). The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization. Cell *57*, 1155–1165.

Emili, A., Shales, M., McCracken, S., Xie, W., Tucker, P.W., Kobayashi, R., Blencowe, B.J., and Ingles, C.J. (2002). Splicing and transcription-associated proteins PSF and p54nrb/nonO bind to the RNA polymerase II CTD. RNA N. Y. N *8*, 1102–1111.

Erazo, A., and Goff, S.P. (2015). Nuclear matrix protein Matrin 3 is a regulator of ZAP-mediated retroviral restriction. Retrovirology *12*, 57.

Eriksson, S., Graf, E.H., Dahl, V., Strain, M.C., Yukl, S.A., Lysenko, E.S., Bosch, R.J., Lai, J., Chioma, S., Emad, F., et al. (2013). Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. PLoS Pathog. *9*, e1003174.

Fang, J., Acheampong, E., Dave, R., Wang, F., Mukhtar, M., and Pomerantz, R.J. (2005). The RNA helicase DDX1 is involved in restricted HIV-1 Rev function in human astrocytes. Virology *336*, 299–307.

Fang, X., Wang, J., O'Carroll, I.P., Mitchell, M., Zuo, X., Wang, Y., Yu, P., Liu, Y., Rausch, J.W., Dyba, M.A., et al. (2013). An unusual topological structure of the HIV-1 Rev response element. Cell *155*, 594–605.

Fankhauser, C., Izaurralde, E., Adachi, Y., Wingfield, P., and Laemmli, U.K. (1991). Specific complex of human immunodeficiency virus type 1 rev and nucleolar B23 proteins: dissociation by the Rev response element. Mol. Cell. Biol. *11*, 2567–2575.

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

Felber, B.K., Drysdale, C.M., and Pavlakis, G.N. (1990). Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. J. Virol. *64*, 3734–3741.

Fernandes, J., Jayaraman, B., and Frankel, A. (2012). The HIV-1 Rev response element: an RNA scaffold that directs the cooperative assembly of a homo-oligomeric ribonucleoprotein complex. RNA Biol. *9*, 6–11.

Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, R.E., Quinn, T.C., Chadwick, K., Margolick, J., Brookmeyer, R., et al. (1997). Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science *278*, 1295–1300.

Fujinaga, K., Irwin, D., Huang, Y., Taube, R., Kurosu, T., and Peterlin, B.M. (2004). Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element. Mol. Cell. Biol. *24*, 787–795.

Gallo, R.C., Sarin, P.S., Gelmann, E.P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V.S., Mann, D., Sidhu, G.D., Stahl, R.E., Zolla-Pazner, S., et al. (1983). Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science *220*, 865–867.

Gandhi, R.T., Zheng, L., Bosch, R.J., Chan, E.S., Margolis, D.M., Read, S., Kallungal, B., Palmer, S., Medvik, K., Lederman, M.M., et al. (2010). The effect of raltegravir intensification on low-level residual viremia in HIV-infected patients on antiretroviral therapy: a randomized controlled trial. PLoS Med. *7*.

Gautier, V.W., Gu, L., O'Donoghue, N., Pennington, S., Sheehy, N., and Hall, W.W. (2009). In vitro nuclear interactome of the HIV-1 Tat protein. Retrovirology *6*, 47.

Groom, H.C.T., Anderson, E.C., Dangerfield, J.A., and Lever, A.M.L. (2009a). Rev regulates translation of human immunodeficiency virus type 1 RNAs. J. Gen. Virol. *90*, 1141–1147.

Groom, H.C.T., Anderson, E.C., and Lever, A.M.L. (2009b). Rev: beyond nuclear export. J. Gen. Virol. *90*, 1303–1318.

Gu, L., Tsuji, T., Jarboui, M.A., Yeo, G.P., Sheehy, N., Hall, W.W., and Gautier, V.W. (2011). Intermolecular masking of the HIV-1 Rev NLS by the cellular protein HIC: novel insights into the regulation of Rev nuclear import. Retrovirology *8*, 17.

Güttler, T., Madl, T., Neumann, P., Deichsel, D., Corsini, L., Monecke, T., Ficner, R., Sattler, M., and Görlich, D. (2010). NES consensus redefined by structures of PKI-type and Rev-type nuclear export signals bound to CRM1. Nat. Struct. Mol. Biol. *17*, 1367–1376.

Haase, A.T. (2005). Perils at mucosal front lines for HIV and SIV and their hosts. Nat. Rev. Immunol. *5*, 783–792.

Hadian, K., Vincendeau, M., Mäusbacher, N., Nagel, D., Hauck, S.M., Ueffing, M., Loyter, A., Werner, T., Wolff, H., and Brack-Werner, R. (2009). Identification of a heterogeneous nuclear ribonucleoprotein-recognition region in the HIV Rev protein. J. Biol. Chem. *284*, 33384–33391.

Han, Y., Lassen, K., Monie, D., Sedaghat, A.R., Shimoji, S., Liu, X., Pierson, T.C., Margolick, J.B., Siliciano, R.F., and Siliciano, J.D. (2004). Resting CD4+ T cells from human immunodeficiency virus type 1 (HIV-1)-infected individuals carry integrated HIV-1 genomes within actively transcribed host genes. J. Virol. *78*, 6122–6133.

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

Hasegawa, A., Liu, H., Ling, B., Borda, J.T., Alvarez, X., Sugimoto, C., Vinet-Oliphant, H., Kim, W.-K., Williams, K.C., Ribeiro, R.M., et al. (2009). The level of monocyte turnover predicts disease progression in the macaque model of AIDS. Blood *114*, 2917–2925.

Heaphy, S., Dingwall, C., Ernberg, I., Gait, M.J., Green, S.M., Karn, J., Lowe, A.D., Singh, M., and Skinner, M.A. (1990). HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. Cell *60*, 685–693.

Henderson, B.R., and Percipalle, P. (1997). Interactions between HIV Rev and nuclear import and export factors: the Rev nuclear localisation signal mediates specific binding to human importin-beta. J. Mol. Biol. *274*, 693–707.

Heyd, F., and Lynch, K.W. (2010). Phosphorylation-dependent regulation of PSF by GSK3 controls CD45 alternative splicing. Mol. Cell *40*, 126–137.

Hibino, Y., Usui, T., Morita, Y., Hirose, N., Okazaki, M., Sugano, N., and Hiraga, K. (2006). Molecular properties and intracellular localization of rat liver nuclear scaffold protein P130. Biochim. Biophys. Acta *1759*, 195–207.

Ho, Y.-C., Shan, L., Hosmane, N.N., Wang, J., Laskey, S.B., Rosenbloom, D.I.S., Lai, J., Blankson, J.N., Siliciano, J.D., and Siliciano, R.F. (2013). Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. Cell *155*, 540–551.

Huang, J., Wang, F., Argyris, E., Chen, K., Liang, Z., Tian, H., Huang, W., Squires, K., Verlinghieri, G., and Zhang, H. (2007). Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. Nat. Med. *13*, 1241–1247.

Huang, X.J., Hope, T.J., Bond, B.L., McDonald, D., Grahl, K., and Parslow, T.G. (1991). Minimal Rev-response element for type 1 human immunodeficiency virus. J. Virol. *65*, 2131–2134.

Igarashi, T., Brown, C.R., Endo, Y., Buckler-White, A., Plishka, R., Bischofberger, N., Hirsch, V., and Martin, M.A. (2001). Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): Implications for HIV-1 infections of humans. Proc. Natl. Acad. Sci. U. S. A. *98*, 658–663.

Imai, K., Togami, H., and Okamoto, T. (2010). Involvement of Histone H3 Lysine 9 (H3K9) Methyltransferase G9a in the Maintenance of HIV-1 Latency and Its Reactivation by BIX01294. J. Biol. Chem. *285*, 16538–16545.

Invernizzi, C.F., Xie, B., Richard, S., and Wainberg, M.A. (2006). PRMT6 diminishes HIV-1 Rev binding to and export of viral RNA. Retrovirology *3*, 93.

Jayaraman, B., Mavor, D., Gross, J.D., and Frankel, A.D. (2015). Thermodynamics of Rev-RNA interactions in HIV-1 Rev-RRE assembly. Biochemistry (Mosc.) *54*, 6545–6554.

Jayaraman, B., Crosby, D.C., Homer, C., Ribeiro, I., Mavor, D., and Frankel, A.D. RNA-directed remodeling of the HIV-1 protein Rev orchestrates assembly of the Rev–Rev response element complex. eLife *3*.

Jiang, G., Espeseth, A., Hazuda, D.J., and Margolis, D.M. (2007). c-Myc and Sp1 Contribute to Proviral Latency by Recruiting Histone Deacetylase 1 to the Human Immunodeficiency Virus Type 1 Promoter. J. Virol. *81*, 10914–10923.

Johnson, J.O., Pioro, E.P., Boehringer, A., Chia, R., Feit, H., Renton, A.E., Pliner, H.A., Abramzon, Y., Marangi, G., Winborn, B.J., et al. (2014). Mutations in the Matrin 3 gene cause familial amyotrophic lateral sclerosis. Nat. Neurosci. *17*, 664–666.

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

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

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

Kosinski, P.A., Laughlin, J., Singh, K., and Covey, L.R. (2003). A complex containing polypyrimidine tract-binding protein is involved in regulating the stability of CD40 ligand (CD154) mRNA. J. Immunol. Baltim. Md 1950 *170*, 979–988.

Kula, A., Guerra, J., Knezevich, A., Kleva, D., Myers, M.P., and Marcello, A. (2011a). Characterization of the HIV-1 RNA associated proteome identifies Matrin 3 as a nuclear cofactor of Rev function. Retrovirology *8*, 60.

Kula, A., Guerra, J., Knezevich, A., Kleva, D., Myers, M.P., and Marcello, A. (2011b). Characterization of the HIV-1 RNA associated proteome identifies Matrin 3 as a nuclear cofactor of Rev function. Retrovirology *8*, 60.

Kula, A., Gharu, L., and Marcello, A. (2013a). HIV-1 pre-mRNA commitment to Rev mediated export through PSF and Matrin 3. Virology *435*, 329–340.

Kula, A., Gharu, L., and Marcello, A. (2013b). HIV-1 pre-mRNA commitment to Rev mediated export through PSF and Matrin 3. Virology *435*, 329–340.

Kutluay, S.B., Zang, T., Blanco-Melo, D., Powell, C., Jannain, D., Errando, M., and Bieniasz, P.D. (2014). Global changes in the RNA binding specificity of HIV-1 Gag regulate virion genesis. Cell *159*, 1096–1109.

Lassen, K.G., Bailey, J.R., and Siliciano, R.F. (2004). Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4+ T cells in vivo. J. Virol. *78*, 9105–9114.

Lassen, K.G., Ramyar, K.X., Bailey, J.R., Zhou, Y., and Siliciano, R.F. (2006). Nuclear retention of multiply spliced HIV-1 RNA in resting CD4+ T cells. PLoS Pathog. *2*, e68.

Lassen, K.G., Hebbeler, A.M., Bhattacharyya, D., Lobritz, M.A., and Greene, W.C. (2012). A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs. PloS One *7*, e30176.

Lata, S., Ali, A., Sood, V., Raja, R., and Banerjea, A.C. (2015). HIV-1 Rev downregulates Tat expression and viral replication via modulation of NAD(P)H:quinine oxidoreductase 1 (NQO1). Nat. Commun. *6*, 7244.

Le Douce, V., Forouzanfar, F., Eilebrecht, S., Van Driessche, B., Ait-Ammar, A., Verdikt, R., Kurashige, Y., Marban, C., Gautier, V., Candolfi, E., et al. (2016). HIC1 controls cellular- and HIV-1- gene transcription via interactions with CTIP2 and HMGA1. Sci. Rep. *6*.

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

Maiuri, P., Knezevich, A., Bertrand, E., and Marcello, A. (2011). Real-time imaging of the HIV-1 transcription cycle in single living cells. Methods San Diego Calif *53*, 62–67.

Malim, M.H., Hauber, J., Le, S.Y., Maizel, J.V., and Cullen, B.R. (1989). The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature *338*, 254–257.

Malim, M.H., Tiley, L.S., McCarn, D.F., Rusche, J.R., Hauber, J., and Cullen, B.R. (1990). HIV-1 structural gene expression requires binding of the Rev trans-activator to its RNA target sequence. Cell *60*, 675–683.

Marban, C., Suzanne, S., Dequiedt, F., de Walque, S., Redel, L., Van Lint, C., Aunis, D., and Rohr, O. (2007). Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. EMBO J. *26*, 412–423.

Meggio, F., D'Agostino, D.M., Ciminale, V., Chieco-Bianchi, L., and Pinna, L.A. (1996). Phosphorylation of HIV-1 Rev protein: implication of protein kinase CK2 and pro-directed kinases. Biochem. Biophys. Res. Commun. *226*, 547–554.

Mehla, R., Bivalkar-Mehla, S., Zhang, R., Handy, I., Albrecht, H., Giri, S., Nagarkatti, P., Nagarkatti, M., and Chauhan, A. (2010). Bryostatin modulates

latent HIV-1 infection via PKC and AMPK signaling but inhibits acute infection in a receptor independent manner. PloS One *5*, e11160.

Michels, A.A., Fraldi, A., Li, Q., Adamson, T.E., Bonnet, F., Nguyen, V.T., Sedore, S.C., Price, J.P., Price, D.H., Lania, L., et al. (2004). Binding of the 7SK snRNA turns the HEXIM1 protein into a P-TEFb (CDK9/cyclin T) inhibitor. EMBO J. *23*, 2608–2619.

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

Mohammadi, P., di Iulio, J., Muñoz, M., Martinez, R., Bartha, I., Cavassini, M., Thorball, C., Fellay, J., Beerenwinkel, N., Ciuffi, A., et al. (2014). Dynamics of HIV latency and reactivation in a primary CD4+ T cell model. PLoS Pathog. *10*, e1004156.

Monecke, T., Güttler, T., Neumann, P., Dickmanns, A., Görlich, D., and Ficner, R. (2009). Crystal structure of the nuclear export receptor CRM1 in complex with Snurportin1 and RanGTP. Science *324*, 1087–1091.

Monecke, T., Haselbach, D., Voß, B., Russek, A., Neumann, P., Thomson, E., Hurt, E., Zachariae, U., Stark, H., Grubmüller, H., et al. (2013). Structural basis for cooperativity of CRM1 export complex formation. Proc. Natl. Acad. Sci. U. S. A. *110*, 960–965.

Naji, S., Ambrus, G., Cimermančič, P., Reyes, J.R., Johnson, J.R., Filbrandt, R., Huber, M.D., Vesely, P., Krogan, N.J., Yates, J.R., et al. (2012). Host cell interactome of HIV-1 Rev includes RNA helicases involved in multiple facets of virus production. Mol. Cell. Proteomics MCP *11*, M111.015313.

Okamura, M., Inose, H., and Masuda, S. (2015). RNA Export through the NPC in Eukaryotes. Genes *6*, 124–149.

Olsen, H.S., Nelbock, P., Cochrane, A.W., and Rosen, C.A. (1990). Secondary structure is the major determinant for interaction of HIV rev protein with RNA. Science *247*, 845–848.

Pace, M.J., Graf, E.H., Agosto, L.M., Mexas, A.M., Male, F., Brady, T., Bushman, F.D., and O'Doherty, U. (2012). Directly infected resting CD4+T cells can produce

HIV Gag without spreading infection in a model of HIV latency. PLoS Pathog. *8*, e1002818.

Palmer, S., Maldarelli, F., Wiegand, A., Bernstein, B., Hanna, G.J., Brun, S.C., Kempf, D.J., Mellors, J.W., Coffin, J.M., and King, M.S. (2008). Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy. Proc. Natl. Acad. Sci. U. S. A. *105*, 3879–3884.

Paraskeva, E., Izaurralde, E., Bischoff, F.R., Huber, J., Kutay, U., Hartmann, E., Lührmann, R., and Görlich, D. (1999). CRM1-mediated Recycling of Snurportin 1 to the Cytoplasm. J. Cell Biol. *145*, 255–264.

Pasternak, A.O., Jurriaans, S., Bakker, M., Prins, J.M., Berkhout, B., and Lukashov, V.V. (2009). Cellular levels of HIV unspliced RNA from patients on combination antiretroviral therapy with undetectable plasma viremia predict the therapy outcome. PloS One *4*, e8490.

Pearson, R., Kim, Y.K., Hokello, J., Lassen, K., Friedman, J., Tyagi, M., and Karn, J. (2008). Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. J. Virol. *82*, 12291–12303.

Perales, C., Carrasco, L., and González, M.E. (2005). Regulation of HIV-1 env mRNA translation by Rev protein. Biochim. Biophys. Acta *1743*, 169–175.

Pereira, L.A., Bentley, K., Peeters, A., Churchill, M.J., and Deacon, N.J. (2000). A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Res. *28*, 663–668.

Pérez, M., de Vinuesa, A.G., Sanchez-Duffhues, G., Marquez, N., Bellido, M.L., Muñoz-Fernandez, M.A., Moreno, S., Castor, T.P., Calzado, M.A., and Muñoz, E. (2010). Bryostatin-1 synergizes with histone deacetylase inhibitors to reactivate HIV-1 from latency. Curr. HIV Res. *8*, 418–429.

Perez-Caballero, D., Hatziioannou, T., Martin-Serrano, J., and Bieniasz, P.D. (2004). Human immunodeficiency virus type 1 matrix inhibits and confers cooperativity on gag precursor-membrane interactions. J. Virol. *78*, 9560–9563.

Ping, Y.H., and Rana, T.M. (1999). Tat-associated kinase (P-TEFb): a component of transcription preinitiation and elongation complexes. J. Biol. Chem. *274*, 7399–7404.

Planelles, V., and Barker, E. (2010). Roles of Vpr and Vpx in modulating the virus-host cell relationship. Mol. Aspects Med. *31*, 398–406.

Plesa, G., Dai, J., Baytop, C., Riley, J.L., June, C.H., and O'Doherty, U. (2007). Addition of deoxynucleosides enhances human immunodeficiency virus type 1 integration and 2LTR formation in resting CD4+ T cells. J. Virol. *81*, 13938–13942.

Procopio, F.A., Fromentin, R., Kulpa, D.A., Brehm, J.H., Bebin, A.-G., Strain, M.C., Richman, D.D., O'Doherty, U., Palmer, S., Hecht, F.M., et al. (2015). A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-infected Individuals. EBioMedicine *2*, 874–883.

Quivy, V., Adam, E., Collette, Y., Demonte, D., Chariot, A., Vanhulle, C., Berkhout, B., Castellano, R., de Launoit, Y., Burny, A., et al. (2002). Synergistic activation of human immunodeficiency virus type 1 promoter activity by NF-kappaB and inhibitors of deacetylases: potential perspectives for the development of therapeutic strategies. J. Virol. *76*, 11091–11103.

Rausch, J.W., and Le Grice, S.F.J. (2015). HIV Rev Assembly on the Rev Response Element (RRE): A Structural Perspective. Viruses *7*, 3053–3075.

Rein, A., Datta, S.A.K., Jones, C.P., and Musier-Forsyth, K. (2011). DIVERSE INTERACTIONS OF RETROVIRAL GAG PROTEINS WITH RNAs. Trends Biochem. Sci. *36*, 373–380.

Reuse, S., Calao, M., Kabeya, K., Guiguen, A., Gatot, J.-S., Quivy, V., Vanhulle, C., Lamine, A., Vaira, D., Demonte, D., et al. (2009). Synergistic Activation of HIV-1 Expression by Deacetylase Inhibitors and Prostratin: Implications for Treatment of Latent Infection. PLoS ONE *4*.

Ricci, E.P., Soto Rifo, R., Herbreteau, C.H., Decimo, D., and Ohlmann, T. (2008). Lentiviral RNAs can use different mechanisms for translation initiation. Biochem. Soc. Trans. *36*, 690–693.

Rohr, O., Lecestre, D., Chasserot-Golaz, S., Marban, C., Avram, D., Aunis, D., Leid, M., and Schaeffer, E. (2003). Recruitment of Tat to Heterochromatin Protein HP1 via Interaction with CTIP2 Inhibits Human Immunodeficiency Virus Type 1 Replication in Microglial Cells. J. Virol. *77*, 5415–5427.

Rosa, A., Chande, A., Ziglio, S., De Sanctis, V., Bertorelli, R., Goh, S.L., McCauley, S.M., Nowosielska, A., Antonarakis, S.E., Luban, J., et al. (2015). HIV-1 Nef

promotes infection by excluding SERINC5 from virion incorporation. Nature *526*, 212–217.

Ruhl, M., Himmelspach, M., Bahr, G.M., Hammerschmid, F., Jaksche, H., Wolff, B., Aschauer, H., Farrington, G.K., Probst, H., and Bevec, D. (1993). Eukaryotic initiation factor 5A is a cellular target of the human immunodeficiency virus type 1 Rev activation domain mediating trans-activation. J. Cell Biol. *123*, 1309–1320.

Saleh, S., Solomon, A., Wightman, F., Xhilaga, M., Cameron, P.U., and Lewin, S.R. (2007). CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: a novel model of HIV-1 latency. Blood *110*, 4161–4164.

Salton, M., Lerenthal, Y., Wang, S.-Y., Chen, D.J., and Shiloh, Y. (2010). Involvement of Matrin 3 and SFPQ/NONO in the DNA damage response. Cell Cycle Georget. Tex *9*, 1568–1576.

Salton, M., Elkon, R., Borodina, T., Davydov, A., Yaspo, M.-L., Halperin, E., and Shiloh, Y. (2011). Matrin 3 Binds and Stabilizes mRNA. PLoS ONE *6*.

Sarracino, A., and Marcello, A. (2017). The Relevance of Post-Transcriptional Mechanisms in HIV Latency Reversal. Curr. Pharm. Des. *23*, 4103–4111.

Selby, M.J., Bain, E.S., Luciw, P.A., and Peterlin, B.M. (1989). Structure, sequence, and position of the stem-loop in tar determine transcriptional elongation by tat through the HIV-1 long terminal repeat. Genes Dev. *3*, 547–558.

Senderek, J., Garvey, S.M., Krieger, M., Guergueltcheva, V., Urtizberea, A., Roos, A., Elbracht, M., Stendel, C., Tournev, I., Mihailova, V., et al. (2009). Autosomal-Dominant Distal Myopathy Associated with a Recurrent Missense Mutation in the Gene Encoding the Nuclear Matrix Protein, Matrin 3. Am. J. Hum. Genet. *84*, 511–518.

Shan, L., Yang, H.-C., Rabi, S.A., Bravo, H.C., Shroff, N.S., Irizarry, R.A., Zhang, H., Margolick, J.B., Siliciano, J.D., and Siliciano, R.F. (2011). Influence of host gene transcription level and orientation on HIV-1 latency in a primary-cell model. J. Virol. *85*, 5384–5393.

Shav-Tal, Y., and Zipori, D. (2002). PSF and p54(nrb)/NonO--multi-functional nuclear proteins. FEBS Lett. *531*, 109–114.

Shen, L., and Siliciano, R.F. (2008). Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. J. Allergy Clin. Immunol. *122*, 22–28.

Sherer, N.M., Swanson, C.M., Hué, S., Roberts, R.G., Bergeron, J.R.C., and Malim, M.H. (2011). Evolution of a species-specific determinant within human CRM1 that regulates the post-transcriptional phases of HIV-1 replication. PLoS Pathog. *7*, e1002395.

Sherpa, C., Rausch, J.W., Le Grice, S.F.J., Hammarskjold, M.-L., and Rekosh, D. (2015). The HIV-1 Rev response element (RRE) adopts alternative conformations that promote different rates of virus replication. Nucleic Acids Res. *43*, 4676–4686.

Siliciano, J.D., Kajdas, J., Finzi, D., Quinn, T.C., Chadwick, K., Margolick, J.B., Kovacs, C., Gange, S.J., and Siliciano, R.F. (2003). Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nat. Med. *9*, 727–728.

Singh, K., Laughlin, J., Kosinski, P.A., and Covey, L.R. (2004). Nucleolin is a second component of the CD154 mRNA stability complex that regulates mRNA turnover in activated T cells. J. Immunol. Baltim. Md 1950 *173*, 976–985.

Søgaard, O.S., Graversen, M.E., Leth, S., Olesen, R., Brinkmann, C.R., Nissen, S.K., Kjaer, A.S., Schleimann, M.H., Denton, P.W., Hey-Cunningham, W.J., et al. (2015). The Depsipeptide Romidepsin Reverses HIV-1 Latency In Vivo. PLoS Pathog. *11*, e1005142.

Soto-Rifo, R., Rubilar, P.S., Limousin, T., de Breyne, S., Décimo, D., and Ohlmann, T. (2012). DEAD-box protein DDX3 associates with eIF4F to promote translation of selected mRNAs. EMBO J. *31*, 3745–3756.

Spina, C.A., Guatelli, J.C., and Richman, D.D. (1995). Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. J. Virol. *69*, 2977–2988.

Spina, C.A., Prince, H.E., and Richman, D.D. (1997). Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro. J. Clin. Invest. *99*, 1774–1785.

Swiggard, W.J., Baytop, C., Yu, J.J., Dai, J., Li, C., Schretzenmair, R., Theodosopoulos, T., and O'Doherty, U. (2005). Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. J. Virol. *79*, 14179–14188.

Swingler, S., Brichacek, B., Jacque, J.-M., Ulich, C., Zhou, J., and Stevenson, M. (2003). HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection. Nature *424*, 213–219.

Tahirov, T.H., Babayeva, N.D., Varzavand, K., Cooper, J.J., Sedore, S.C., and Price, D.H. (2010). Crystal structure of HIV-1 Tat complexed with human P-TEFb. Nature *465*, 747–751.

Tiley, L.S., Malim, M.H., Tewary, H.K., Stockley, P.G., and Cullen, B.R. (1992). Identification of a high-affinity RNA-binding site for the human immunodeficiency virus type 1 Rev protein. Proc. Natl. Acad. Sci. U. S. A. *89*, 758–762.

Trono, D., and Baltimore, D. (1990). A human cell factor is essential for HIV-1 Rev action. EMBO J. *9*, 4155–4160.

Tyagi, M., and Karn, J. (2007). CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. EMBO J. *26*, 4985–4995.

Van Lint, C., Emiliani, S., Ott, M., and Verdin, E. (1996). Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. EMBO J. *15*, 1112–1120.

Verdin, E., Paras, P., and Van Lint, C. (1993). Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. EMBO J. *12*, 3249–3259.

Wong, J.K., Hezareh, M., Günthard, H.F., Havlir, D.V., Ignacio, C.C., Spina, C.A., and Richman, D.D. (1997). Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science *278*, 1291–1295.

Yadav, S.S., Wilson, S.J., and Bieniasz, P.D. (2012). A facile quantitative assay for viral particle genesis reveals cooperativity in virion assembly and saturation of an antiviral protein. Virology *429*, 155–162.

Yang, H.-C., Xing, S., Shan, L., O'Connell, K., Dinoso, J., Shen, A., Zhou, Y., Shrum, C.K., Han, Y., Liu, J.O., et al. (2009). Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. J. Clin. Invest. *119*, 3473–3486.

Yarosh, C.A., Tapescu, I., Thompson, M.G., Qiu, J., Mallory, M.J., Fu, X.-D., and Lynch, K.W. (2015). TRAP150 interacts with the RNA-binding domain of PSF and antagonizes splicing of numerous PSF-target genes in T cells. Nucleic Acids Res. *43*, 9006–9016.

Yedavalli, V.S.R.K., and Jeang, K.-T. (2011). Matrin 3 is a co-factor for HIV-1 Rev in regulating post-transcriptional viral gene expression. Retrovirology *8*, 61.

Yedavalli, V.S.R.K., Neuveut, C., Chi, Y.-H., Kleiman, L., and Jeang, K.-T. (2004). Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. Cell *119*, 381–392.

Zapp, M.L., Hope, T.J., Parslow, T.G., and Green, M.R. (1991). Oligomerization and RNA binding domains of the type 1 human immunodeficiency virus Rev protein: a dual function for an arginine-rich binding motif. Proc. Natl. Acad. Sci. U. S. A. *88*, 7734–7738.

Zeitz, M.J., Malyavantham, K.S., Seifert, B., and Berezney, R. (2009). Matrin 3: chromosomal distribution and protein interactions. J. Cell. Biochem. *108*, 125–133.

Zhang, Z., and Carmichael, G.G. (2001). The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. Cell *106*, 465–475.

Zhang, L., Chung, C., Hu, B.S., He, T., Guo, Y., Kim, A.J., Skulsky, E., Jin, X., Hurley, A., Ramratnam, B., et al. (2000). Genetic characterization of rebounding HIV-1 after cessation of highly active antiretroviral therapy. J. Clin. Invest. *106*, 839–845.

Zhang, S., Pointer, D., Singer, G., Feng, Y., Park, K., and Zhao, L.J. (1998). Direct binding to nucleic acids by Vpr of human immunodeficiency virus type 1. Gene *212*, 157–166.

Zhong, H., May, M.J., Jimi, E., and Ghosh, S. (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol. Cell *9*, 625–636.

Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M.B., and Price, D.H. (1997). Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. Genes Dev. *11*, 2622–2632.

Zolotukhin, A.S., Michalowski, D., Bear, J., Smulevitch, S.V., Traish, A.M., Peng, R., Patton, J., Shatsky, I.N., and Felber, B.K. (2003). PSF acts through the human immunodeficiency virus type 1 mRNA instability elements to regulate virus expression. Mol. Cell. Biol. *23*, 6618–6630.