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Friends or foes in virus-host interactions: Cell regulation of HIV-1 replication

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Dr. Jaume Farrés

Al Joan, per la seva il·lusió i companyia en tot moment.
A la Júlia i als meus pares.
Al Jaume, a la Meritxell i al Jordi.

SUMMARY

HIV is an obligate intracellular pathogen and its replication is entirely reliant on intracellular resources, depending on its capacity of recruitment and utilization of a wide array of host proteins to complete the viral life cycle.

Current antiretroviral treatments generally suppress viral replication but do not avoid persistence and proliferation of latently infected cells over time, thus not curing HIV/AIDS and evidencing the need to develop new strategies that may finally converge in eliminating the virus. The discovery of human cellular factors could provide new targets for the development of these strategies. Previous siRNA-based screenings and whole-genome studies published a high amount of alleged HIV-1 host factors but their functional mechanisms need to be addressed to translate scientific knowledge into the clinic.

This dissertation explores a hypothetical therapeutic intervention and discusses the concrete application of studying HIV host factors for chronically infected patients, based on results concerning both the HIV dependency on a protein complex from the cellular transcriptional machinery called Mediator (MED) and the HIV restriction mediated by SAMHD1, a recently recognized antiviral factor whose mechanism of regulation is still under debate. A friend and a foe in viral-host interactions that converge in their implication on cell metabolism, thus suggesting that identified host factors that either enhance or inhibit HIV-1 are in turn modulated by cell regulation-associated mechanisms that finally govern viral replication.

Concretely, we provide an evaluation of the MED activity on HIV replication identifying nine out of 28 human MED proteins significantly affecting HIV replication, all pointing at a post-integration step. Moreover, the identified MED proteins modulating the generation of new viral transcripts were differentially affecting early or late stages of HIV-1 transcription, apart from compromising particularly the HIV transcription induced by the HIV-1 protein Tat. Interestingly, co-immunoprecipitation experiments also suggested physical interaction between MED and Tat. We speculate on whether

specific MED proteins may be used as therapeutic targets in order to repress HIV transcription from persistently infected cells.

Among the high number of cellular proteins modulating HIV, only a few actively inhibits viral replication. SAMHD1 achieves it by blocking the viral reverse transcriptase. Here, SAMHD1 regulation and activity in relation with intracellular dNTP levels is investigated. The influence of SAMHD1 activity on nucleoside reverse transcriptase inhibitors was demonstrated, showing that a reduction of SAMHD1 levels significantly decreased HIV sensitivity solely on thymidine-analog inhibitors.

Proliferating primary $CD4^+$ T cells or macrophages were then observed to express a phosphorylated form of SAMHD1 that corresponded with susceptibility to infection in cell culture and that was not present in non-cycling cells. Identified phosphorylation sites in cycling cells are mainly driven by Cyclin-Dependent Kinases (CDK). In fact, we identified CDK6 and its associated cyclin D3 as an upstream regulator of CDK2 controlling SAMHD1 phosphorylation in primary T cells and macrophages susceptible to infection by HIV-1. In turn, CDK2 was strongly linked to cell cycle progression and coordinated SAMHD1 phosphorylation and inactivation. Knockdown of the cellular CDK2 inhibitor CDKN1A (p21) led to a relief of the SAMHD1 restriction, increasing virus replication. Confirming the findings, the CDK6 inhibitor palbociclib blocked SAMHD1 phosphorylation, intracellular dNTP levels, HIV-1 reverse transcription and reduced CDK2 activation; the antiviral activity was lost when SAMHD1 was degraded by Vpx. Given both the selectivity for proliferating cells and its antiviral effect, we speculate that a controlled-dose of a particular CDK inhibitor in a model of viral latency would reduce the viral reservoir by preferentially targeting persistently infected, proliferating T cells.

All together, our results reinforce the idea that in the following years the deeper study of HIV-1 cellular factors will probably raise the possibility of a new class of “anti-HIV” therapeutics targeting the host rather than the virus, collectively giving new proposals to the unresolved challenge to cure HIV.

RESUM

El VIH és un patogen intracel·lular obligat i per tant està totalment subjecte als recursos intracel·lulars disponibles, depèn de la seva capacitat de reclutar i utilitzar un ampli ventall de proteïnes de l'hoste per a completar el cicle viral.

El tractament antiretroviral actual suprimeix la replicació viral però no evita la persistència i la proliferació de les cèl·lules infectades de forma latent, per tant no cura de la infecció per VIH i la progressió a la SIDA, posant en evidència la necessitat de desenvolupar noves estratègies que aconsegueixin eliminar de forma definitiva el virus. El descobriment de nous factors cel·lulars del VIH pot aportar noves dianes per al desenvolupament d'aquestes estratègies. Diversos estudis de *screening* basats en tècniques de silenciament gènic i d'identificació de gens a partir del rastreig de tot el genoma humà han identificat un número molt elevat de factors cel·lulars del VIH. Per poder traslladar el coneixement científic a la clínica, però, cal un estudi molt més acurat que defineixi els mecanismes funcionals i la regulació de cadascun d'aquests factors.

En aquesta tesi doctoral s'especula sobre una hipotètica intervenció terapèutica amb l'aplicació de l'estudi dels factors cel·lulars del VIH per a pacients infectats de forma crònica, partint dels mecanismes de regulació de dos factors amb funcions antagòniques sobre el VIH. En primer lloc s'estudia la dependència del VIH al complex proteic Mediator (MED) que forma part de la maquinària de transcripció cel·lular; i en segon lloc la restricció del VIH mitjançada per SAMHD1, un factor antiviral identificat recentment el mecanisme del qual és encara controvertit. Un amic i un enemic en les interaccions virus-hoste que comparteixen, però, un comú denominador en quant a la seva implicació en el metabolisme cel·lular. Aquest tret comú suggereix que els factors cel·lulars del VIH, tot i que en la seva relació estreta amb el virus el potenciïn o l'inhibeixin, alhora sempre són modulats per mecanismes complexes associats a la regulació cel·lular que finalment són els què governen la replicació viral.

En l'estudi s'inclou una avaluació de l'activitat MED sobre la replicació del VIH amb la identificació de 9 sobre 28 proteïnes del complex MED que de forma significativa afecten la replicació del VIH, sempre en una etapa posterior a la integració. D'entre les subunitats MED identificades algunes tenen un rol predominant en la transcripció de gens virals inicials i d'altres en la generació de transcrits virals més tardans, comprometent la transcripció del VIH que és induïda per la proteïna viral Tat. Els assaigs de coimmunoprecipitació, a més, han suggerit per primera vegada que aquesta dependència passa per una interacció física entre MED i Tat. Els resultats permeten especular sobre la utilització d'alguna d'aquestes subunitats MED com a diana terapèutica per tal de reprimir la transcripció de cèl·lules latentment infectades.

D'entre l'ampli grup de proteïnes cel·lulars que modulen el VIH, només algunes inhibeixen el virus de forma activa. SAMHD1 ho aconsegueix bloquejant l'enzim del VIH transcriptasa inversa. La seva regulació i activitat sobre els nivells de dNTPs intracel·lulars s'investiga al llarg del segon capítol. L'activitat de SAMHD1 influencia la potència dels inhibidors de la transcriptasa inversa anàlegs a nucleòtids, de manera que una reducció intracel·lular dels nivells de SAMHD1 porta a una disminució significativa de la sensibilitat del VIH enfront els inhibidors anàlegs a Timidina.

Un estudi molecular més extens ha permès identificar que en els limfòcits T $CD4^+$ proliferants i en els macròfags s'expressa una forma fosforilada de SAMHD1 que correspon amb la susceptibilitat a la infecció. La fosforilació no s'observa en cèl·lules que no han entrat en etapes proliferants del cicle cel·lular. Les posicions aminoacídiques susceptibles de fosforilació identificades en SAMHD1 són principalment regulades per quinases dependents de ciclines (CDK) i de forma específica s'ha identificat CDK6 i la parella catalítica ciclina D3 com un complex regulador de CDK2, que alhora controlaria la progressió a cicle cel·lular i la fosforilació de SAMHD1 per tal d'inactivar-lo en cèl·lules T primàries i en macròfags, fent-los susceptibles a la infecció per VIH-1. La inhibició de CDKN1A (ó p21) que és un inhibidor natural de CDK2, porta a un alleujament en la restricció de VIH modulada per SAMHD1, generant més replicació viral. L'inhibidor de CDK6 palbociclib confirma la via de senyalització proposada ja que bloqueja efectivament la fosforilació de SAMHD1, els nivells intracel·lulars de dNTP, la transcripció inversa i l'activació de CDK2. L'especificitat de la droga queda demostrada quan perd l'acció antiviral amb l'addició al cultiu de partícules Vpx que degraden SAMHD1. La selectivitat de palbociclib per

les cèl·lules proliferants i el seu efecte antiviral suggereixen que una dosi controlada d'un inhibidor particular de CDK en un model de latència podria reduir el reservori viral tot dirigint-se de forma específica als limfòcits T $CD4^+$ infectats de forma latent que proliferen de forma persistent.

En els propers anys l'estudi acurat del factors cel·lulars del VIH-1 permetrà el desenvolupament d'estratègies terapèutiques "anti-VIH" basades en fer diana a l'hoste més que en el virus, donant així noves propostes que facin front a les limitacions de l'actual tractament antiretroviral.

RESUMEN

El VIH es un patógeno intracelular obligado y por lo tanto está totalmente sujeto a los recursos intracelulares disponibles, dependiendo de su capacidad para reclutar y utilizar un amplio abanico de proteínas del huésped para completar el ciclo viral.

El tratamiento antiretroviral actual suprime la replicación viral pero no evita la persistencia y la proliferación de las células infectadas de forma latente, por lo tanto no cura de la infección por VIH y la progresión al SIDA, poniendo en evidencia la necesidad de desarrollar nuevas estrategias que consigan eliminar de forma definitiva el virus. El descubrimiento de nuevos factores celulares del VIH puede aportar nuevas dianas para el desarrollo de esas estrategias. Varios estudios de *screening* basados en técnicas de silenciamiento génico y de identificación de genes a partir del rastreo de todo el genoma humano han identificado un número muy elevado de factores celulares del VIH. Pero para poder trasladar el conocimiento científico a la clínica hace falta un estudio mucho más detallado que defina los mecanismos funcionales y la regulación de cada uno de estos factores.

En esta tesis doctoral se especula sobre una hipotética intervención terapéutica acerca de la aplicación del estudio de los factores celulares del VIH para pacientes infectados de forma crónica, partiendo de los mecanismos de regulación de dos factores con funciones antagónicas sobre el VIH. En primer lugar se estudia la dependencia del VIH al complejo proteico Mediator (MED) que forma parte de la maquinaria de transcripción celular; y en segundo lugar la restricción del VIH mediada por SAMHD1, un factor antiviral identificado recientemente cuyo mecanismo es aún controvertido. Un amigo y un enemigo en las interacciones virus-huésped que, sin embargo, comparten un común denominador en cuánto a su implicación en el metabolismo celular. Este rasgo común sugiere que los factores celulares del VIH, aunque en su relación estrecha con el virus lo potencien o lo inhiban, a la vez siempre son modulados por mecanismos complejos asociados a la regulación celular que finalmente son los que gobiernan la replicación viral.

En el estudio se incluye la evaluación de la actividad MED sobre la replicación del VIH con la identificación de 9 sobre 28 proteínas del complejo MED que de forma significativa afectan la replicación del VIH, siempre en una etapa posterior a la integración. De entre las subunidades MED identificadas algunas tienen un rol predominante en la transcripción de genes virales iniciales y otras en la generación de transcritos virales más tardanos, comprometiendo la transcripción del VIH inducida por la proteína viral Tat. Los ensayos de coimmunoprecipitación además sugieren por primera vez que esta dependencia pasa por una interación física entre MED y Tat. Los resultados permiten especular sobre la utilización de alguna de éstas subunidades MED como diana terapéutica para reprimir la transcripción de células latentemente infectadas.

De entre el amplio grupo de proteínas celulares que modulan el VIH, sólo algunas inhiben el virus de forma activa. Es el caso de SAMHD1 que bloquea la enzima del VIH transcriptasa reversa. Su regulación y actividad sobre los niveles de dNTPs intracelulares se investiga a lo largo del segundo capítulo. La actividad de SAMHD1 influencia la potencia de los inhibidores de la transcriptasa reversa análogos a nucleótidos, de manera que una reducción intracelular de los niveles de SAMHD1 conduce a una disminución significativa de la sensibilidad del VIH frente a los inhibidores análogos a Timidina.

Un estudio molecular más extenso ha permitido identificar en los linfocitos T $CD4^+$ proliferantes y en los macrófagos una forma fosforilada de SAMHD1 que corresponde con la susceptibilidad a la infección. La fosforilación no se observa en células que no han entrado en etapas proliferantes del ciclo celular. Las posiciones aminoacídicas susceptibles de fosforilación identificadas en SAMHD1 son principalmente reguladas por quinasas dependientes de ciclinas (CDK) y de forma específica se ha identificado CDK6 y su pareja catalítica ciclina D3 como un complejo regulador de CDK2, que a su vez controlaría la progresión a ciclo celular y la fosforilación de SAMHD1 con tal de inactivarlo en células T primarias y macrófagos, haciéndolos susceptibles a la infección por VIH-1. La inhibición de CDKN1A (ó p21) que es un inhibidor natural de CDK2, conlleva a un alivio en la restricción de VIH mediada por SAMHD1, generando así más replicación viral. El inhibidor de CDK6 palbociclib confirma la vía de señalización propuesta ya que efectivamente bloquea la fosforilación de SAMHD1, los niveles intracelulares de dNTP, la transcripción reversa y la activación de CDK2. La especificidad de la droga queda demostrada cuando pierde la acción antiviral

al añadir al cultivo partículas Vpx que degradan SAMHD1. La selectividad de palbociclib por las células proliferantes y su efecto antiviral sugieren que una dosis controlada de un inhibidor particular de CDK en un modelo de latencia podría reducir el reservorio viral dirigiéndose de manera específica a los linfocitos T $CD4^+$ infectados de forma latente que proliferan de forma persistente.

En los próximos años el estudio de los factores celulares del VIH permitirá el desarrollo de estrategias terapéuticas “anti-VIH” basadas en hacer diana al huésped en vez de al virus, ofreciendo así nuevas propuestas que hagan frente a las limitaciones del tratamiento antiretroviral actual.

ABBREVIATIONS

3TC	Lamivudine
ABC	Abacavir
ABCE-1	ATP-binding cassette protein family E member 1
AIDS	Acquired immunodeficiency syndrome
AGS	Aicardi-Goutières Syndrome
AP1	Activator protein 1
APC	Antigen-presenting cells
ART	Antiretroviral treatment
ATF	Cyclic AMP-dependent transcription factor 1
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad-3 related kinase
AZT	Zidovudine
BAF	Barrier-to-autointegration factor
BST-2	Bone Stromal tumor protein 2
CA	HIV capsid protein
CBFβ	Core-binding factor subunit beta
CC50	50% cytotoxic concentration
CCR5	Chemokine receptor 5
CDC	Center for Disease Control
CDK	Cyclin Dependent Kinase
CDKI	Cyclin Dependent Kinase Inhibitor
CoIP	CoImmunoPrecipitation
CREB	cAMP response element-binding protein
CRL4DCAF1	Cullin-RING E3 ubiquitin ligase complex
CXCR4	CXC Chemokine receptor 4
CypA	Cyclophilin A
d4T	Stavudine
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule
dCTP	DeoxyCytidine
ddC	Zalcitabine
ddI	Didanosine
DDX3	ATP-dependent DEAD box RNA helicase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
dThD	DeoxyThymidine
EC	Elite Controllers
EC50	50% effective concentration
EFV	Efavirenz
ELISA	Enzyme-lynked immunoSorbent assay
Env	HIV envelope glycoprotein
ESCRT	Endosomal sorting complexes required for transport
FA	Formaldehid
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HD	Healthy Donors
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HMG-I/Y	High mobility group protein-I/Y
hnRNP	Heterogeneous nuclear ribonucleoproteins
HP1	Heterochromatin protein 1
HRP-2	Hepatoma-derived growth factor related protein 2
Hsp	Heat shock protein
HTLV	Human T-cell leukaemia virus
IC50	Half maximal inhibitory concentration
IFN	Interferon
IL	Interleukin
IN	HIV integrase enzyme
LAP2α	Lamina-associated polypeptide 2 α
LAV	Lymphadenopathy associated virus
LEDGF	Lens-epithelium derived growth factor
LTR	Long terminal repeat
M-CSF	Monocyte-colony stimulating factor
MA	HIV matrix protein
MDM	Monocyte-derived macrophage
MED	Mediator
mRNA	Messenger RNA
MTT	Methyl Tetrazolium-based colorimetric assay
N-MLV	N-tropic murine leukemia virus

NC	HIV nucleocapsid
ND	No Drug
Nef	HIV viral negative effector
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NL4-3-GFP	Envelope-deficient HIV-1 NL4-3 clone encoding IRES-GFP
NLS	Nuclear Localization Sequence
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NPC	Nucleopore complexes
NRTI	Nucleoside reverse transcriptase inhibitor
NT	Non-targeting
NVP	Nevirapine
P-TEFb	Positive Transcription Elongation factor b
PAPSS1	PAPS synthetase 1
PBMCs	Peripheral Blood Mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PHA	Phytohaemmagglutinin
PIC	Pre-Integration Complex
Pol	HIV polymerase enzyme
PR	HIV protease enzyme
PURα	Purine-rich element binding protein A
qPCR	Quantitative real-time PCR
Ralt	Raltegravir
RanBP1	Ran-binding protein 1
Rb	Retinoblastoma protein
Rev	HIV Regulator of virion protein expression
RNA	Ribonucleic acid
RNAi	RNA interference
RNAPII	RNA Polymerase 2
RNR2	Ribonucleotide Reductase 2
RRE	Rev response element
RT	HIV reverse transcriptase enzyme
SAMHD1	Sterile α motif and C-terminal dNTP phosphohydrolase domain containing conserved histidine and aspartate residues
SF1	Splicing Factor 1

shRNA	Short-hairpin RNA
siRNA	Small-interfering RNA
SIV	Simian Immunodeficiency virus
SMNSIP1	Survival motor neuron interacting protein 1
SOCS1	Suppresor of cytokine signaling 1
Sp1	Specificity protein 1
Spt5	Supressor of Ty protein 5
SWI/SNF	SWItch/Sucrose NonFermentable proteins
T20	Enfuvirtide
TAR	Transactivation Response element
Tat	HIV viral transactivator protein
TFAP4	Transcription factor-activating enhancer binding protein 4
TFV	Tenofovir Disoproxil Fumarate
TIP47	Tail interacting protein-47
TNPO3	Transportin 3
TRIM	Tripartite Motif
Tsg101	Tumor susceptibility gene 101
Vif	HIV viral infectivity factor
VL	Viral Load
VLP	Viral-like particles
VLPVpx	Viral-like particles carrying Vpx
VP	Viral Progressors
Vpr	HIV viral protein R
Vpu	HIV viral protein U
Vpx	HIV viral protein X
VSV	Vesicular stomatitis virus
WCL	Whole Cell Lysate
WT	Wild type
ZNRD1	Zinc Ribbon domain-containing 1

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INTRODUCTION

1 History of the AIDS epidemic

In June 1981, the USA Center for Disease Control and Prevention (CDC) described what is considered the first reported case of acquired immune deficiency syndrome (AIDS)[1]. In 1983, only two years later, its etiological agent was first identified[2] and then confirmed[3,4] as a virus belonging to the general family of T-lymphotropic retroviruses and as the causative agent of several pathological syndromes affecting the normal cellular immune function, including AIDS[2]. In 1986, the International Committee on the Taxonomy of Viruses designated the previously named LAV or HTLV-III as human immunodeficiency virus (HIV)[5].

According to the UNAIDS Reports on the global AIDS epidemic, globally, an estimated 35.3 (32.2–38.8) million people were living with HIV in 2012. An increase from previous years as more people are receiving the life-saving antiretroviral therapy (ART). There were 2.3 (1.9–2.7) million new HIV infections globally, showing a 33% decline in the number of new infections from 3.4 (3.1–3.7) million in 2001. At the same time the number of AIDS deaths is also declining with 1.6 (1.4–1.9) million AIDS deaths in 2012, down from 2.3 (2.1–2.6) million in 2005. Although global new infections and deaths are declining, the AIDS epidemic is still one of the most serious health challenges of the world[6].

2 The HIV replication cycle

As an obligatory intracellular parasite HIV can only replicate inside human cells. The steps of the HIV life cycle are described below (Figure 1):

2.1. HIV Viral Entry and uncoating

The principal targets for HIV-1 infection are T lymphocytes, and to a lesser extent macrophages and dendritic cells (DC). This tropism is determined at the level of viral entry by the use of CD4 as the primary receptor and the use of one of the two co-receptors that define two different viral strains. R5 strains of HIV use CCR5 as their co-receptor and can, therefore, enter macrophages, DCs and T lymphocytes, whereas X4 strains of HIV use CXCR4 as a co-receptor and can infect only T

cells. The engagement of the HIV envelope glycoprotein Env (whose cleavage products are gp120 and gp41) with cell receptors triggers conformational changes that culminate in viral and host cell membrane fusion and release of the viral core into the cytoplasm. Inhibitors of the different steps of HIV-1 entry into target cells have been identified including co-receptor antagonists such as Maraviroc or AMD3100 for CCR5- and CXCR4- using viruses respectively and fusion inhibitors such as enfuvirtide (T-20)[7–9].

2.2. HIV reverse transcription, nuclear import and integration

Once internalized and uncoated, the HIV genome is reverse transcribed by the viral reverse transcriptase (RT)[5]. HIV-1 RT utilizes both RNA and DNA as templates and has two enzymatic activities: DNA polimerase and RNase H, that cooperate to convert the linear single-stranded RNA into a linear double-stranded DNA molecule (called provirus), by incorporating nucleotides from the host cell in a template-dependent manner. Initially, the reverse transcriptase reaction requires a cellular tRNA primer that allows a conformational change into the HIV genome to form a stable initiation complex between the RT, the viral RNA and the tRNA primer. Then the synthesis starts by copying the positive sense RNA into a negative sense DNA strand in a process that is dependent on the available deoxyribonucleotides triphosphate (dNTP) pool in the host cytoplasm, thus generating RNA/DNA hybrids. In a second step of the reaction, the negative sense DNA is copied into a positive DNA strand, generating double-stranded DNA molecules. The RNase H activity of RT degrades the template RNA from the nascent negative sense DNA strand, making it available for annealing to the homologous region of the acceptor strand. The end product of the reverse transcription is the proviral DNA, which is associated with other viral elements like Vpr, matrix (MA) and some host proteins to enter the nucleus[10]. Reverse transcription can be inhibited by nucleoside and non-nucleoside RT inhibitors (NRTIs and NNRTIs, respectively). NRTIs such as zidovudine (AZT) mimic natural dNTPs and are incorporated into the viral DNA by the RT. NNRTIs bind at a hydrophobic pocket adjacent to the polymerase active site. After reverse transcription and once nuclear import is accomplished, integration of HIV proviral DNA into the host

genome is catalyzed by integrase (IN), a process that could be in turn inhibited by integrase inhibitors such as raltegravir (Ralt)[5].

2.3. HIV transcription and translation

The HIV transcriptional activity is dependent on cellular factors including the host cell RNA polymerase II (RNAPII) machinery but also viral factors that allow the transcription of the integrated viral genome. The small mRNAs produced during the early transcription phase are directly exported to the cytoplasm and encode for the regulatory proteins Nef, Tat and Rev. Regulator of the viral gene expression (Rev) acts as an adaptor protein which binds to the Rev response element (RRE) and mediates cytosolic export of unspliced and singly spliced mRNAs. The viral transactivator protein (Tat) binds newly transcribed mRNAs and promotes recruitment of other cellular factors that stimulate transcription elongation. Viral structural and enzymatic proteins are synthetized in the cytoplasm and transported to the plasma membrane. Negative effector (Nef) facilitates viral assembly[5].

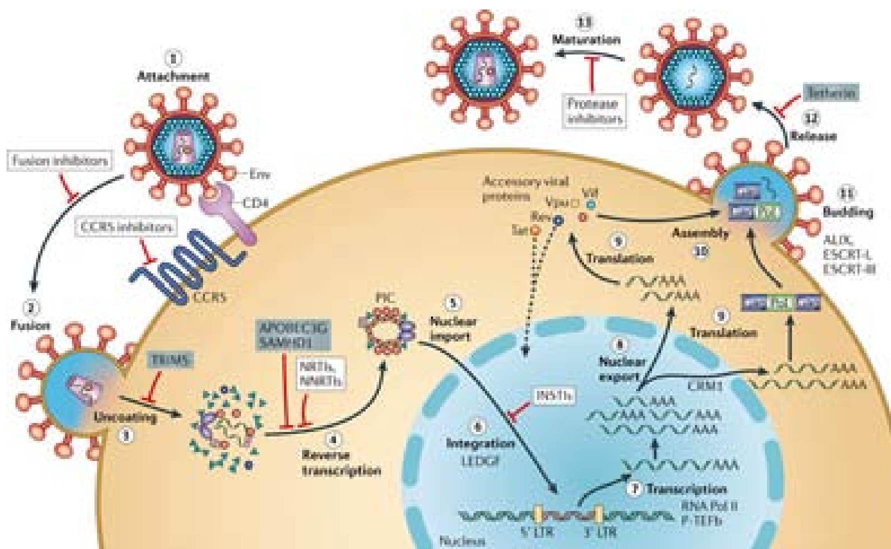


Figure 1. The HIV-1 life cycle. After binding to CD4 and one co-receptor (CCR5 or CXCR4), viral fusion with the cell membrane results in entry of the viral core into the cytoplasm. Following reverse transcription, the viral cDNA is transported to the nucleus to form the integrated provirus. Genomic viral transcripts exported from the nucleus and newly transduced viral proteins are packaged to form new virions. After budding, particle maturation occurs by protease cleavage[10].

2.4. HIV assembly, budding and maturation

The formation of new HIV virions occurs at the plasma membrane by packaging two copies of genomic viral RNA, the Env viral protein, the Gag polyprotein and the three viral enzymes: protease (PR), RT and IN. Conversion of the immature virus into its mature infectious form requires the activation of PR, which cleaves Gag precursor releasing three structural proteins: MA, Capsid (CA) and Nucleocapsid (NC). Protease inhibitors can block HIV replication at this step[10].

3 Pathogenesis and Virulence of HIV-1 infection

The natural course of untreated HIV infection varies widely with some rare HIV-positive individuals able to maintain high CD4 cell counts and/or suppressed viral load (VL) in the absence of ART, and others, the great majority, that progress to AIDS defining condition more rapidly. Different factors affect HIV pathogenesis and virulence. HIV genetics and virulence heritability likely contribute to disease severity[11], as well as host factors, predominantly linked to immune system[12]. Although HIV infection is a complex disease and the different rates of disease progression may be also due to a combination of multiple factors, the existence of individuals who resist infection, delay the disease outcome or control viral replication without the need of ART demonstrates that prevention of infection and long-lasting disease remission are attainable objectives.

4 Host Factors supporting HIV life cycle

The HIV-1 genome has 9700 base pairs (bp) and codes 9 genes. There are three structural genes (*gag*, *pol* and *env*) that code for protein precursors Gag, Pol and Env. *Gag* codes the precursor of capsid proteins (MA, CA, NC and p6), *pol* codes enzymes precursor (RT, IN and PR) and *env* the envelope glycoproteins (gp41 and gp120). *gag-pol* and *env* are transcribed separately, translated and processed by the viral PR to provide the necessary elements for the virus assembly. Two genes code for regulatory proteins Tat and Rev. Tat enhances transcriptional elongation performed by RNAPII, while Rev is necessary for transport of

viral RNA from the nucleus to the cytoplasm. Finally, there are four genes (*vif, vpr, nef* and *vpu*) coding for accessory proteins not essential for viral replication[13].

Apart from those 15 viral proteins coded in the HIV-1 genome, HIV-1 must take advantage of multiple cellular proteins. As an obligate intracellular pathogen, HIV-1 depends upon the recruitment and utilization of a wide array of host proteins for successful replication. Still today, important viral-host relationships likely remain to be discovered. Towards this goal, large-scale small-interfering RNA (siRNA) and short-hairpin RNA (shRNA) screens have been performed to identify host factors involved in a broad array of cellular functions and implicated during HIV-1 infection[14–22] (Figure 2). The identification of host factors affecting HIV replication may allow searching for new therapeutic targets for the future[23]. While the current repertoire of ART had great success in improving and extending the lives of HIV infected patients, they are inherently limited by the mutable nature of their viral target. Instead, targeting host factors as an antiviral strategy could be a solution against viral diversity and escape mutations because HIV would be hard-pressed to evolve resistance to drugs or other personalized treatments targeting cellular proteins, having to evolve a new capability, not simply mutating a drug-binding site[24].

Although over 1000 candidate host factors have been identified during the last years, just a subgroup has been validated through independent studies or mechanistically investigated after the initial screen. Those confirmed factors that assist HIV-1 in every step during its life cycle and thus support viral replication are briefly described.

More than 10 chemokine receptors have been shown to affect HIV attachment and entry apart from the widely known CCR5 and CXCR4 HIV co-receptors or the CD4 receptor. Other cellular factors like Galectin-1[25], DC-SIGN[26] or gp340[27] among others[15] also promote viral attachment by mediating intercellular contacts and enhancing trans-infection of susceptible cells.

After the fusion of the viral envelope with the cell membrane, uncoating[28] and reverse transcription[29] are processes also assisted by factors including Pin1[30] or Cyclophilin A (Cyp A) that destabilizes capsid formation affecting also several viral infections[31]; tRNA(Lys)3[32], survival motor neuron (SMN) interacting protein 1 (SIP1)[33,34] and CD63[35] have been shown to have a role in an early, post-entry event prior to or at the reverse transcription step.

After viral entry and during uncoating and reverse transcription steps, virions must travel from the point of cell entry to the nucleus, where small proteins can passively diffuse through the nuclear pores but larger proteins are gated if not having specific nuclear localization signals (NLS). HIV-1 makes use of several cellular proteins to be transported inside the nucleus by taking part of the pre-integration complex (PIC), consisting of both viral and cellular proteins[24,36] including hepatoma-derived growth factor related protein 2 (HRP-2) and LEDGF/p75 that together with importins like Imp A3 or TNPO3, kariopherins, nucleoporins like Nup153[37] and others, move the PIC across the nuclear membrane and subsequently HIV-1 gains access to the cellular genomic DNA[29,38].

Once inside the nucleus, proviral DNA must integrate into the host chromosome, with LEDGF/p75 playing an important role binding to both HIV-1 IN and DNA[39], and other cellular proteins like barrier-to-autointegration factor (BAF)[40], lamina-associated polypeptide 2α (LAP2α)[41], emerin[42], high mobility group protein-I/Y (HMG-I/Y)[43] or HRP2[44] also enhancing HIV-1 integration.

Once integrated, proviral DNA can be transcribed or enter a latency phase. Latent cells are a major obstacle in successfully treating HIV-1 infections[45]. Certain cellular factors have a role during this process by suppressing gene expression, for example histone deacetylases (HDACs)[46] and associated factors like heterochromatin protein 1 (HP1), Suv39H1[47] and G9a[48], recruited to the HIV-1 promoter LTR and responsible of eliminating acetylation of histones and thus blocking transcription. Latency phase is still poorly understood and probably some factors already known to mediate transcription of HIV genes may also play a role in latency reactivation.

Sp1, NF-kB, AP1, TFAP4, CREB, cMyc or NF-AT are cellular transcription factors involved in HIV-1 gene transcription, a complex process with an extensive network of proteins having diverse functions[49–51]. The best-known transcription factor used to generate HIV-1 progeny viruses from integrated proviral DNA is the positive transcription elongation factor b (P-TEFb) consisting of CDK9 and cyclin T1, that binds HIV-1 protein Tat and recruits it to the transactivation response (TAR) RNA structure, consequently stimulating transcriptional elongation[52,53]. However, HIV transcription is far from being a process with a main protagonist leading the story. On contrast, lots of cellular proteins cooperate

together with RNAPII to transcribe proviral DNA into mRNA, including purine-rich element binding protein A (PURα), suppressor of Ty protein 5 (Spt5), Tat-SF1, Rap30, ZNRD1[54], PAPS synthetase 1 (PAPSS1), the SWI/SNF complex, ATF, BRG-1[24] or some subunits from the Mediator complex[16]. The role of some Mediator complex subunits in HIV replication, although still not explored, has been attributed during viral transcription, due to its RNAP II-related function[15] or its link to the Tat-mediated transcription through the HIV LTR[21].

It is specially relevant for this dissertation the introduction of this protein complex, named Mediator complex (MED), whose effect on HIV-1 replication is demonstrated and discussed throughout the first chapter, and also recently published[55]. In humans, MED is composed by 28 proteins assembled in four distinct modules called “Head”, “Middle”, “Tail” and the “CDK8 module”, that can contact various transcriptional regulators and act together as an adaptor to convey transcription signals from activators to the general transcription machinery, helping the initiation of transcription by the RNAP II[56–58]. Mediator complex is considered a global regulator of gene expression although is not required for transcription *per se*, operating in mechanistically distinct ways

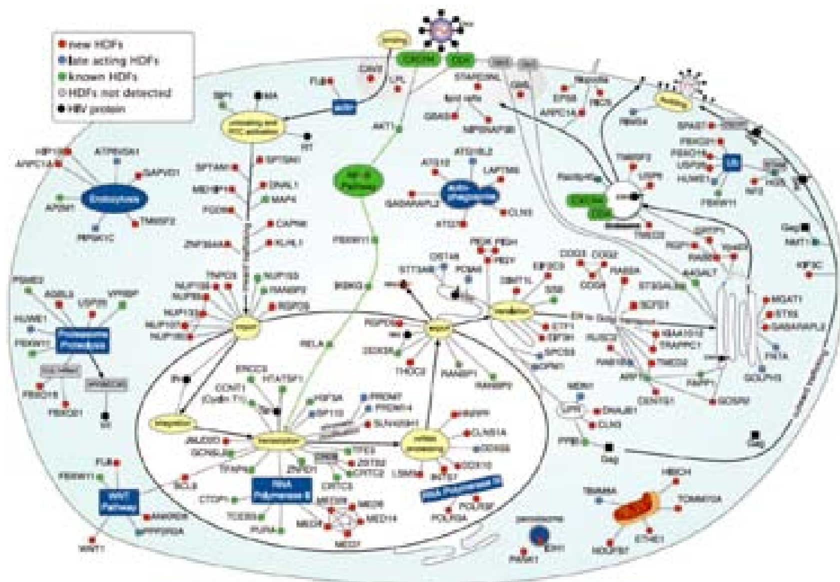


Figure 2. Model of the role of HIV cellular factors during the viral life cycle. With the stages of the HIV life cycle as a framework, HIV cellular factors were placed at the position most likely to elicit HIV dependency. HDF: HIV dependency factors[15].

at different genes or different cell types including those susceptible to HIV infection, with a high degree of structural flexibility and variable subunit composition[59,60].

Accessory HIV-1 proteins like Vpr also enhance viral transcription by activating the ataxia telangiectasia and Rad-3 related kinase (ATR), which is a master regulator of a DNA damage checkpoint and controls the G2/M checkpoint arresting the cell at G2 phase[61,62].

The HIV-1 Rev protein promotes the nuclear export of unspliced and partially spliced viral mRNA by bridging viral RNA and the export receptor human CRM1 (hCRM1)[63], while Ran-binding protein 1 (RanBP1) dissociates those nuclear export complexes from the nucleopore complexes (NPC) to release them on the cytoplasm[64]. Rev/hCRM1 complexes utilize the ATP-dependent DEAD box RNA helicase (DDX3) to export incompletely spliced HIV-1 RNAs and direct the synthesis of all HIV-1 proteins[65,66]. Splicing of viral unspliced transcripts to the cytoplasm is also regulated by cellular proteins like the splicing factor (ASF/SF2) associated protein (P32) or heterogeneous nuclear ribonucleoproteins (hnRNPs)[67,68].

HIV-1 mRNA encodes for its 15 proteins that must be translated and directed to the site of assembly by using host ribosomes and protein trafficking signals. Once there, HIV-1 incorporates all necessary proteins as well as two copies of its RNA genome into a new virion, followed by budding of the virion from the membrane to conclude its replication cycle[69]. Proteins from the Rab family[70,71], the adaptor-protein family (AP), suppressor of cytokine signaling 1 (SOCS1) or the tail interacting protein-47 (TIP47) are required for viral protein trafficking to the site of assembly, play a role in HIV-1 export from the nucleus and participate in vesicular trafficking in the post-Golgi network[72]. Other cellular proteins have a main role facilitating HIV-1 assembly, like the ATP-binding cassette protein family E member 1 (ABCE-1). Cells have endosomal sorting complexes required for transport (ESCRT) and some proteins from those complexes, as well as Hrs, Tsg101, annexin and AIP1/ALIX have been shown to regulate budding and release of new HIV viral particles, helping the viral protein Vpu[73]. A large system of protein trafficking and signaling pathways into the cell are also related to ubiquitin, an important signal for sorting specific proteins, including translated HIV proteins, that can be then transported to be assembled and released to the extracellular environment as new infective particles[74].

Taken together, the discovery of human cellular factors involved in HIV-1 replication could provide new targets for the development of new anti-HIV-1 agents to respond to ART limitations[75]. Although chemically synthetized seven years ago with another perspective, as a proof of concept Maraviroc was an approved-drug that targets a cellular cofactor, the CCR5 co-receptor[76]. Other under-study targets include LEDGF[77,78] among others. However, the first step to make translational research in this area is putting more efforts to better characterize and discern between the myriad host factors already identified in genome-wide association studies and whole-genome RNA interference-based screenings.

5 Host Restriction Factors inhibiting HIV life cycle

An incredibly high number of cellular proteins modulate HIV infection[16,79,80], lots of them by supporting and enhancing viral replication, as described above. However, only a few of them have been unequivocally identified as genuine restriction factors, that is, cellular proteins that actively inhibit retrovirus replication and so protect cells from infection[81]. Human cells probably possess only a few HIV restriction factors, compared with hundreds of HIV dependency factors (essential host proteins for HIV replication) and thousands of proteins that are not particularly essential to HIV but have an enhancing resultant effect on viral replication[82].

Restriction factors have at least four defining characteristics: first and foremost, must directly and dominantly cause a significant decrease in HIV infectivity; second, the HIV predecessors will evolve an equally potent counter-restriction that will persist in the present day virus; third, restriction factors often show signatures of rapid evolution and mutations that are maintained in a population only if they confer a selective advantage; fourth, the expression of a restriction factor is often hard-wired to the innate immune response and strongly induced by interferon (IFN)[82].

APOBEC3 DNA deaminase subfamily, the BST-2 (bone stromal tumor protein 2)/tetherin integral membrane protein, TRIM5α and the recent identified sterile α motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1 (SAMHD1) are some of the most notable restriction factors targeting primate lentiviruses, briefly described in the following paragraphs.

APOBEC3 represents a seven-member subfamily of cell proteins with the capacity to catalyze DNA Cytosines to Uracils deamination. The four HIV-relevant APOBEC3 proteins (APOBEC3D, APOBEC3F, APOBEC3G, APOBEC3H) can encapsidate into HIV virions and result in the deamination of C-to-U in viral cDNA upon initiation of reverse transcription in target cells. Uracil is template for Adenine while Cytosine templates Guanine upon second-strand synthesis, thus resulting in a G-to-A mutation[83]. This proviral cDNA is subsequently degraded or integrated to follow HIV cycle, with many nonfunctional new viruses as a result. APOBEC3 have a viral counterpart, the HIV-1 protein Vif that overcomes the restriction by binding CBFβ and recruiting an E3 ubiquitin ligase complex, targeting APOBEC3 proteins for degradation by the 26S proteasome after polyubiquitinating them[84]. All APOBEC3 proteins that restrict HIV share some properties: expression in nonpermissive $CD4^+$ T cells, capacity to package into the nucleic acid-containing viral core, potent virus restriction activity, ability to inflict G-to-A mutations, susceptibility to HIV Vif and functional conservation with the homologous proteins of rhesus macaque (Figure 3 upper left squeme)[85,86].

Tetherin, also known as BST-2, acts to physically tether budding virions to the cell surface of productively infected T cells. The topology of tetherin, with two opposite ends in the molecule that allow the simultaneous association with both viral and cellular membranes, accounts for its broad antiviral activity not only against HIV-1 but also against many other enveloped viruses[87]. As viruses attempt to bud from infected cells, tetherin becomes incorporated into the viral envelope and physically bridges nascent virions to the cell. Captured virions are subsequently internalized for degradation in lysosomes. HIV-1 Vpu overcomes tetherin restriction by internalizing and sequestering tetherin in compartments away from sites of viral budding[88]. Restriction can be counteracted also by Vpu recruiting an E3 ubiquitin ligase complex that ubiquitinates tetherin and targets it for lysosomal degradation, or even sequestrating tetherin in a perinuclear compartment without degradation. Vpu-mediated tetherin blockage by recycling, internalization or degradation requires other cellular proteins like dynamin-2, components from the ESCRT machinery or Rab7a[89]. Most primate lentiviruses do not have Vpu and therefore depend on other viral proteins to counteract tetherin. In the case of HIV-2, Env leads to internalization and sequestration of

tetherin away from sites of virus release at the plasma membrane by a clathrin-dependent pathway (Figure 3 upper right squeme)[82].

TRIM family includes approximately 100 proteins characterized by a highly conserved tripartite motif (TRIM) structure on their amino terminal region, called RBCC motif. This motif is constituted by a RING (*Really Interesting New Gene*) domain, one or two B-box domains and a coiled-coil domain (CC). TRIM proteins are involved in several biological processes such as innate immunity, cell differentiation and transcriptional regulation[90]. Several TRIM family members have been shown to restrict HIV-1, including TRIM5, TRIM11, TRIM15, TRIM19, TRIM22, TRIM31 and TRIM32. However, TRIM5 proteins are the best studied. TRIM5 is expressed along the primate lineage and is encoded by the *TRIM5* gene, which codes for different TRIM5 isoforms, amongst which TRIM5α and TRIM5Cyp, with structural differences on their C-terminal end, show antiviral properties. TRIM5 proteins block HIV-1 infection at an early-stage of reverse transcription, binding to the HIV-1 CA and inducing its premature disassembly before reverse transcription can occur. The biochemical interactions between CA and TRIM5 proteins are complex and important for their restriction activity. They involve the binding of their C-terminal domain to the CA lattice, and the dimerization and higher-order multimerization of TRIM5, which ultimately leads to the formation of a hexameric protein lattice. Both coiled-coil and B-box domains of TRIM5 are required for its dimerization and multimerization[91]. In addition to a direct antiviral mechanism, it has been suggested that TRIM5 acts as a pattern recognition receptor that "senses" the CA lattice, leading to the activation of the innate immune response. This CA sensing triggers the E3-ubiquitin ligase activity of the RING domain of TRIM5, leading to a signaling pathway involving ubiquitin, the TAK1 kinase complex and the subsequently NF-kB and AP-1 signaling[92]. Primate TRIM5α orthologs inhibit several retroviruses and lentiviruses but are ineffective against their own host-specific viruses. For example, while human TRIM5α (huTRIM5) strongly restricts N-tropic murine leukemia virus (N-MLV), it only weakly restricts HIV-1 infection, and rhesus monkey TRIM5α (rhTRIM5) efficiently blocks HIV-1 but not the infection by the autologous simian immunodeficiency virus (SIV) (Figure 3 lower right squeme)[93].

SAMHD1 is the last known among the HIV restriction factors. Like the others, SAMHD1 has found its nemesis in the HIV-2-encoded Vpx[94].

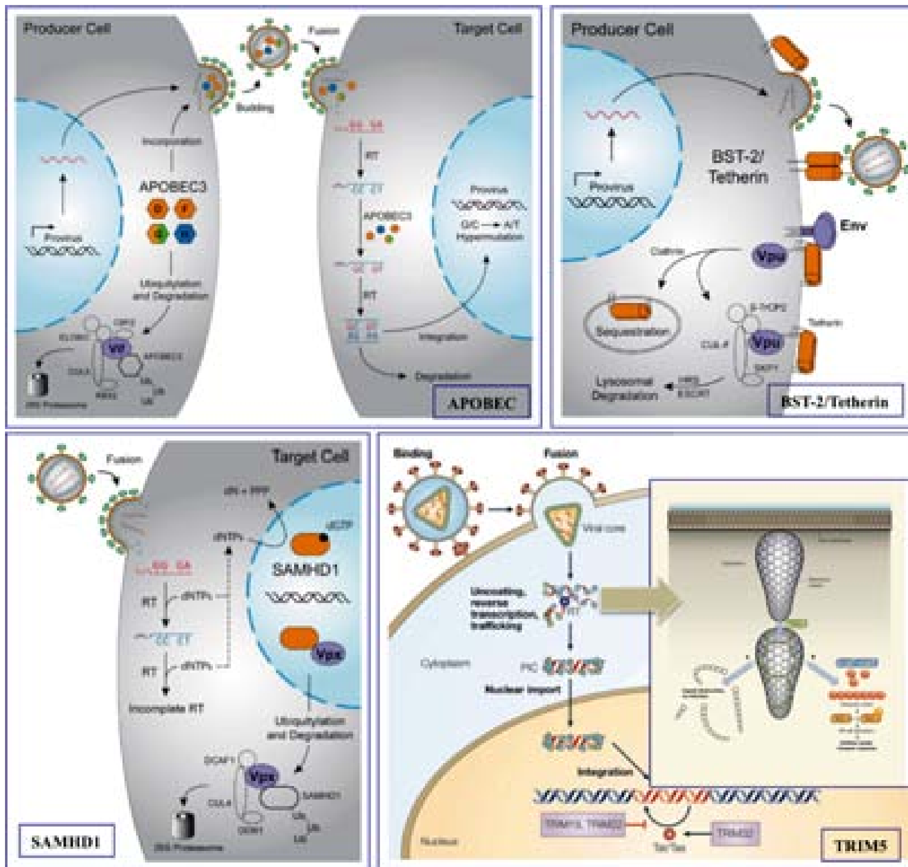


Figure 3. The Restriction Factors of HIV-1. Adapted schemes of the main HIV-1 restriction factors from references [91,106].

Vpx is an accessory protein encoded in the HIV-2 genome and in its simian ancestor of sootey mangabey (SIVsmn). On the contrary, HIV-1 and its simian ancestor of chimpanzees (SIVchz) do not encode the *vpx* gene, but encodes the accessory viral protein Vpr, also present in HIV-2. However, Vpr cannot degrade SAMHD1. Therefore, it seems that Vpr from the HIV-1 lentiviral lineage never acquired the ability to degrade SAMHD1, in contrast with HIV-2 ancestors[95].

SAMHD1 has been discovered in the past few years, with the year 2011 signaling the date when two independent groups identified SAMHD1 as the restriction factor targeted by Vpx in cells from the myeloid lineage (monocytes, macrophages and dendritic cells)[96,97]. Even so, SAMHD1 discovery must be explained starting from some major previous clues showing that Vpx protein from HIV-2 or SIV, naturally absent in HIV-1, improved susceptibility of myeloid cell types to HIV-1 infection

when delivered by pre-infection with Vpx-containing virus-like particles (VLPVpx). Moreover, Vpx-mediated suppression of HIV-1 restriction was shown to be dependent on the proteasomal degradation system[98–101]. Vpx was subsequently identified as capable of interacting with the enzymatic cullin-RING E3 ubiquitin ligase complex (CRL4DCAF1) leading to its ubiquitylation and degradation by the proteasomal machinery[102,103]. As described above, other HIV accessory proteins like Vif or Vpu, have similar ability in interacting with different E3 ligase complexes inducing degradation of restriction factors (APOBEC3 and tetherin, respectively). When Vpx protein, which is directly loaded into SIVsmn or HIV-2 viral particles, is delivered into the host cytoplasm, SAMHD1 becomes ubiquitylated and is degraded by the cell proteasome, relieving the retroviral restriction.

The current accepted SAMHD1 function that leads to HIV restriction during reverse transcription is dNTP depletion, although it has been recently shown a link attributing antiviral properties derived from its exonuclease function, in particular through its RNase activity[118]. Nevertheless, it is well established that SAMHD1 is a dNTP triphosphohydrolase enzyme[104], i.e., it hydrolizes all four dNTPs to deoxynucleosides and inorganic triphosphate, controlling the size of the dNTP pool inside the cell. Once delivered into the cytoplasm, single-stranded viral RNA is reverse transcribed to DNA, a step that is dependent on the cytoplasmic availability of dNTPs. SAMHD1 has been found to control dNTP levels in myeloid cells below the Michaelis constant (Km) of the HIV-1 RT, preventing proviral DNA formation and HIV-1 replication[105]. Delivery of Vpx degrades SAMHD1, thus increasing dNTP concentrations and allowing higher infection efficacy (Figure 3 lower left squeme)[106]. In fact, early observations already suggested that dNTP availability for the HIV-1 RT was a restriction step in viral replication, as the addition of dNTPs to the media led to increased HIV replication[107]. The biological active structure of SAMHD1 is a tetramer, which is formed through a mechanism of activation of two inactive dimers induced by dGTP[108]. Binding of dGTP to four allosteric sites promotes tetramerization and induces a conformational change in the substrate-binding pocket to yield the catalytically active enzyme[109]. However, cellular regulation of active SAMHD1 is not determined by GTP alone but also the levels of all dNTPs, that globally regulate and equilibrate SAMHD1 structural states apart from being its own substrates[110].

As all good restriction factor, SAMHD1 was found related with the innate immune response in people suffering from the Aicardi-Goutières syndrome (AGS), a rare autoimmune disease characterized by an IFN-stimulated gene expression signature that resembles a congenital viral infection[111,112]. Given its capacity to degrade dNTPs, it is thought that SAMHD1 avoids accumulation of nucleotides that otherwise would trigger the innate immune sensors leading to undesired IFN production and chronic inflammation[94].

The following study after SAMHD1 identification proved that dNTP-mediated restriction also existed in resting $CD4^+$ T lymphocytes with similar evidences as those used to prove it in myeloid cells, a demonstration that was importantly linked to the undiscovered mechanism explaining the differences on HIV susceptibility between activated and resting $CD4^+$ T cells. In that work, a genetically modified HIV-1 clone carrying Vpx was shown to infect resting lymphocytes; downregulation of SAMHD1 through RNA interference allowed virus replication in resting cells; and resting lymphocytes obtained from a patient with AGS encoding a mutation in SAMHD1 could be infected with HIV-1[113]. Notably, Vpx-induced degradation of SAMHD1 in activated lymphocytes did not increase virus replication, suggesting that SAMHD1 restriction on the dNTP pool was absent in activated lymphocytes. Indeed, as previously indicated[107], nucleotide levels were found higher in activated lymphocytes when compared to resting lymphocytes. Moreover, the observation that SAMHD1 mRNA expression was not altered in HIV resistant resting T cells as compared to HIV susceptible activated cells[113], suggested that unidentified regulatory mechanisms may activate/deactivate SAMHD1 function in T cells.

Since then, several reports have tried to describe the molecular mechanism of SAMHD1[108,109,114–120], together with our work that is exposed throughout chapter 2 in the results section and extensively discussed while mirrored to those publications that were simultaneously published in the literature during the last two years and that included the identification of the signaling pathway by which this HIV restriction factor regulates viral replication through starving the viral RT in myeloid and resting $CD4^+$ T cells.

For sure additional HIV restriction factors await discovery. For instance, $CD4^+$T cells are much more susceptible to infection by Nef-expressing compared with Nef-deficient viruses, suggesting that this

accessory protein may serve to counteract at least other non identified cellular restriction factor[106].

6 The cyclin dependent kinase-mediated cell regulation

The cell cycle of higher eukaryotes is mainly controlled by a set of cyclin-dependent kinases (CDK) and associated regulators. The classical view of CDKs role in cell cycle progression includes the following phases where a series of sequential CDK-directed phosphorylation events enable the cell to pass crucial cell cycle checkpoints, resulting in cell growth, DNA replication and mitosis. Mitogenic stimuli drive cells from G0 phase into G1 phase by inducing the expression of D-type cyclins, which activate CDK4 and CDK6 by competing for their binding to the CDK inhibitors. CDK4 and CDK6 phosphorylate and inactivate retinoblastoma protein (Rb), a negative regulator of E2F transcription factor, leading to the induction of proteins required for DNA replication, and to increased levels of cyclin E, the partner of the major G1/S phase kinase CDK2. CDK2/cyclin E complex targets other proteins and cooperate to enter synthesis or S phase. During S phase, CDK2 partners also with cyclin A, to inhibit further E2F activity and replication origin assembly. CDK1 then partners with cyclin B, which is induced during the S phase to promote nuclear envelope breakdown, chromatin de-condensation, mitotic spindle assembly and finally cell division[121].

CDK role in controlling cell cycle progression is well established for CDK1, CDK2, CDK4 and CDK6, although CDK family includes other members having diverse functions like the regulation of cell development in specific cell-types (CDK5), a driving role in RNAPII-dependent transcription (CDK7, CDK8 and CDK9), mRNA splicing (CDK11) or non-cell cycle roles in DNA damage repair and signal transduction (CDK3, CDK10, CDK12 and CDK20). Pan-CDK inhibitors have been shown to inhibit HIV-1 transcription, therefore, CDK have also been associated generally to HIV-1 transcription[122].

HYPOTHESIS AND OBJECTIVES

HYPHOTESIS

HIV is an obligate intracellular pathogen and its replication is entirely reliant on intracellular proteins. In turn, cells have developed strategies to combat viral infections by using restriction factors that block viral replication or trigger immune recognition of incoming pathogens.

Host factors that either enhance or inhibit HIV-1 are modulated by cell regulation-associated mechanisms that finally govern viral replication. Thus, the discovery of human cellular factors involved in viral replication and the identification of their functional mechanisms in relation to cell metabolism is important to understand HIV pathogenesis and could provide new strategies to face unresolved challenges like viral persistence or long-term immune activation by therapeutically targeting the host rather than the virus.

OBJECTIVES

Objective 1:
Mediator complex is a global regulator of gene expression and was suggested as HIV-1 factor although its role was not defined. Based on the extensive characterization of all Mediator subunits for its effect on HIV-1 cycle, we aimed to determine the functional protein cluster that essentially affects viral replication.

Objective 2:
SAMHD1 has been identified as a HIV-1 restriction factor blocking reverse transcription, whose regulatory mechanism is still under debate. Therefore, we aimed at investigating the functional mechanism of SAMHD1 and assessing its impact in the susceptibility to nucleoside analog reverse transcriptase inhibitors.

Objective 3:
To explore a hypothetical therapeutic intervention from results answering objectives 1 and 2; to discuss the relevance and the concrete application of studying HIV-1 cellular factors for chronically HIV infected patients.

MATHERIALS AND METHODS

Cells. Peripheral Blood Mononuclear cells (PBMC) were obtained using a Ficoll-Paque density gradient centrifugation and used for fresh purification of $CD4^+$ T lymphocytes by negative selection (StemCell Technologies) or purification of monocytes using a negative selection antibody cocktail (StemCell Technologies). Purity of the populations was confirmed by flow cytometry. $CD4^+$ T lymphocytes and monocytes were stained using fluorochrome-conjugated antibodies against CD4 (>95%) or CD14 (>80%), respectively (1:20; BD Biosciences). $CD4^+$ T lymphocytes were kept in complete RPMI 1640 (Gibco), with or without IL-2 (16 U/ml) and PHA (4 µg/ml; Sigma-Aldrich). Monocytes were resuspended in complete culture medium: RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), penicillin and streptomycin (Gibco). Monocytes were differentiated for 4-5 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech) at 100 ng/ml or a combination of IL-12 (100 ngr/ml; Peprotech) and IL-18 (100 ngr/ml; Bionova, Madrid, Spain). The work was approved by the scientific committee of Fundació IrsiCaixa. PBMC were isolated from 'buffy coats' of healthy blood donors. Buffy coats were purchased anonymously from the Catalan Banc de Sang i Teixits (http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for disease. Cells from patients were used; all patients participating in the study gave informed consent. The work was approved by the ethical committee of Hospital Germans Trias i Pujol. Criteria for selection of Elite Controllers (EC) (n:12) was confirmed HIV-1 infection with sustained plasma viral load (VL) under the limit of detection in the absence of antiretroviral treatment (non-consecutive blips of <2,000 copies/mL were allowed if present only in <20% of VL determinations). Mean VL and $CD4^+$ T-cell count of EC were <40 RNA copies/ml and 706 cells/mm3 (range: 498-880). Cells from viremic progressors (VP) corresponded to a set point previous to antiretroviral treat-

ment. (n:10, mean VL: 491,531 copies/ml, $CD4^+$ T-cell count: 398 cells/mm3 (range: 370-544)).

The human cell lines Jurkat, MT-4, TZM-bl and HEK293T (AIDS Reagent Program, National Institutes of Health, Bethesda, MD) were grown in complete RPMI medium (for Jurkat and MT-4) or in Dulbecco modified Eagle Medium (DMEM; Gibco) (for TZM-bl and HEK293T), supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco).

Compounds. 3-Azido-3-deoxythymidine (Zidovudine, AZT), AMD3100, roscovitine and purvalanol A were purchased from Sigma-Aldrich (Madrid, Spain). Stavudine (d4T), lamivudine (3TC), zalcitabine (ddC), didanosine (ddI), tenofovir disoproxil fumarate (TFV), Efavirenz (EFV) and Nevirapine (NVP) were obtained from the NIH AIDS Research and Reference Reagent Program. Abacavir (ABC) and CDK inhibitors AT7591 and 0332991 were purchased from Selleckchem (Munich, Germany). Raltegravir (Ralt) was obtained from Merck. All compounds were resuspended in DMSO and stored at -20 ºC until use. Nucleotides (thymidine and 2'-deoxycytidine hydrochloride) were purchased from Sigma-Aldrich. Thymidine (ThD) was dissolved in RPMI (pH 4.1) and 2'-deoxycytidine hydrochloride in RPMI (pH 7.4).

RNA interference. siRNAs targeting 4 non-overlapping mRNA sequences for every gene were purchased from Dharmacon (SMARTpool; Dharmacon, Thermo-Scientific). For *MED14*, *CDK2* and *CDK6* genes, confirmatory siRNA were purchased from Sigma-Aldrich or Qiagen (FlexiTube Gene Solution). Monocytes were transfected with 50 pmol of the corresponding siRNA using the Monocyte Amaxa Nucleofection kit (Lonza) as previously described[55,123]. Transfected monocytes were left untreated overnight and then differentiated to macrophages as described above. TZM-bl cells were siRNA interfered by mixing 12.5 pmol or 50 pmol of the corresponding siRNA pool with Lipofectamine 2000 reagent (Life technologies) in serum-free medium (Opti-MEM I, Life Technologies) and added onto $2x10^4$ or $8x10^4$ TZM-bl cells for 96-well plates or 24-well plates format, respectively. The same siRNA retrotransfection protocol was used for HEK293T by scaling-up the experiment in a 6-well plate format. In all cases, 48 hours post-transfection, cells were used for gene expression assay (mRNA or protein), Tat transactivation assay or HIV-1 infection.

Plasmids and DNA transfection. Tat-expressing plasmid pcTat has been previously described[124,125]. To generate the p3XFLAG-CMV-Tat, RNA from TZM-bl cells infected with an HIV-1 NL4-3 strain was extracted using the NucleoSpin RNA II kit (Magerey-Nagel) in accordance with the manufacturer's instructions. After reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Life technologies), Tat was amplified using the following primers: forward 5'-ATGGAGCCAGTAGATCCT-3' and reverse 5'-CTATTCCTTCGGGCCTGT-3'. PCR products were cloned into the pGEM-T Easy Vector (Promega) and transformed into competent cells (One Shot OmniMAX 2 T1 R E.coli, Life Technologies). Plasmid DNA was extracted using HiSpeed kits (Qiagen) and the purified plasmid was then digested with EcoRI before being introduced into the EcoRI-digested p3XFLAG-CMV-7.1 vector (Sigma). Sequence integrity of all plasmids was checked by sequencing. For DNA transfections, plasmids were transfected into TZM-bl cells using Lipofectamine 2000 reagent. HEK293T cells were transfected using polyethylenimine (PEI) (Polysciences) as previously described[123]. In all cases, 48 hours post-transfection, cells were used for gene expression assay (mRNA or protein), Tat transactivation assay or HIV-1 infection.

For identification of SAMHD1 phosphorylation sites, HEK293T cells were transfected with an empty expression plasmid (empty) or the plasmid encoding the wild type (WT) form or mutated forms of SAMHD1. Serines at position 18 and 33 and threonines at positions 21, 25 and 592 were mutated to alanines (S18A, T21A, T25A, S33A and T592A). A plasmid with a combined mutations of S18A, T21A, T25A and S33A (S18A/T21A/T25A/S33A) was also transfected.

Viruses. R5-tropic HIV-1 strain BaL was grown in stimulated PBMC and titrated for its use in monocyte-derived macrophages (MDM). NL4-3 virus was grown in lymphoid MT-4 cells. Envelope-deficient HIV-1 NL4-3 clone encoding IRES-GFP (NL4-3-GFP) was pseudotyped with VSV-G by cotransfection of HEK293T cells using PEI (Polysciences). A plasmid encoding ROD9 HIV-2 GFP-expressing virus, the corresponding ΔVpx mutant and a NL4-3-GFP expressing clone modified to bind Vpx (NL4-3*GFP) were used to transfect HEK293T cells to produce viral stocks. For the production of viral-like particles carrying Vpx (VLPVpx), HEK293T cells were cotransfected with pSIV3^{+} [125] and a VSV-G expressing plasmid. Three days after transfection, supernatants were harvested, filtered and stored at -80 °C. In some

cases, viral stocks were concentrated using Lenti-X concentrator (Clontech). Viruses were titrated by infection of lymphoid MT-4 cells or TZM-bl in the case of Mediator complex screenings, except for BaL and NL4-3, in which a specific titration was performed for its use in assays of proviral DNA formation.

Viral fusion. The virus-cell membrane fusion was quantified as described before[127]. Briefly, HEK293T cells were cotransfected with NL4-3 HIV provirus plasmid and a plasmid carrying the *vpr* gene fused with β-lactamase (Vpr-BlaM; NIH AIDS Reagents Program) and then cocultured overnight with primary $CD4^+$ T lymphocytes. Cells were loaded with the CCF2-AM loading kit (Invitrogen) following the protocol provided by the manufacturer. Cells were incubated 1 h at room temperature then washed and immediately fixed. The change in emission of the cleaved CCF2 generated by the BlaM-Vpr chimera was measured by flow cytometry.

Viral infections. TZM-bl cells were infected with a NL4-3 strain HIV-1 stock. 24 h after infection samples were lysed and DNA was extracted for integrated HIV DNA quantification. 48h after infection samples were collected for β-galactosidase assay and quantification of viral transcripts.

For MDM infections, MDM were pre-treated with drugs or VLPVpx (2-4 hours before infection). MDM were then infected with NL4-3-GFP cells, detached using a commercial PBS-EDTA solution, fixed and analyzed by flow cytometry two days later (LSRII, BD Biosciences). Infection range showed variability between donors, ranging from 2% to 35% GFP^+ cells in the absence of treatment. To measure early events of viral infection (reverse transcription) in MDM, NL4-3-GFP or BaL HIV-1 infections were stopped after 16h.

For p24 antigen detection, MDM were infected with the R5 strain between day 3 and day 7 after stimulation. 3 days post-infection, l00 µl of culture supernatant were replaced by 100 µl of fresh medium containing the corresponding stimuli. HIV production was analyzed at 7 or 14 days after infection by ELISA HIV-p24 antigen detection (Bio-Rad Laboratories, Hercules, USA) in culture supernatants.

$CD4^+$ T lymphocytes and CD3/CD8 stimulated PBMCs were spinoculated in the presence of the corresponding virus (HIV-1*GFP or HIV-2 for $CD4^+$ T lymphocytes and PBMCs, respectively) for 90 minutes at 1,200g. Viral replication was measured in all cases two days later by flow cytometry.

MT-4 and Jurkat were pretreated with VLPVpx for 4h before infection or left with fresh media as a control. Cells were then infected with VSV-pseudotyped NL4-3-GFP and antiviral drugs and/or nucleotides were added immediately before infection.

The anti-HIV activity of the different compounds was determined by infection of cells in the presence of different concentrations of the drug and 50% effective concentrations (EC50) were calculated, as previously described[127].

Viability assays. Measurement of cell cytotoxicity was performed by a methyl tetrazolium-based colorimetric assay (MTT method) for TZM-bl cells or MDM during antiviral activity of analyzed compounds. For siRNA-derived toxicity in MDM or in the case of lymphocytes, viability assays were done by relative quantification of live cells by flow cytometry.

RNA extraction. RNA from TZM-bl cells, HEK293T, MT-4 cells, Jurkat, PBMC, monocytes, MDM, $CD4^{+}$T cells and commercially available Lymph Node, Adipose, Small Intestine and Thymus RNA (First Choice from Life-Technologies) were used to analyze mRNA expression of different genes. RNA was extracted using the NucleoSpin RNA II or XS kits (Magerey-Nagel) in accordance with the manufacturer's instructions. Some analyzed data from extracted RNA were used for a clustering analysis of all MED genes by using the GEPAS software (www.gepas.org).

Quantitative real-time PCR (qPCR). For quantification of proviral DNA in primary cells, total cellular DNA was extracted using QIAamp DNA extraction kit (QIAamp DNA Blood mini kit; Qiagen) as recommended by the manufacturer. Quantitative amplification of LTR for viral entry detection was performed using the following primers and probe (forward, 5'-GACGCAGGACTCGGCTTG-3'; reverse, 5'-ACTGACGCTCTCGCACCC-3' and probe 5'-TTTGGCGTACTCACCAGTCGCCG-3' labeled with the fluorophore FAM and the quencher TAMRA). To normalize HIV copy values/cell, amplification of cellular RNaseP gene was performed using TaqMan® RNaseP Control Reagents Kit (Applied Biosystems). Ct values for proviral DNA were normalized using the ΔΔCt method, and infections were normalized to an untreated control. For proviral DNA quantifications, samples treated with RT inhibitor AZT (3 μM) were run in parallel to ensure that proviral DNA measured was product of productive infection and not result from DNA contamination of the viral stocks. As well, Ralt (2 μM) was used to

ensure that no post-RT steps were being quantified by the assay. For integrated DNA quantification in non-primary cells like TZM-bl cells, total cellular DNA was extracted and integrated viral DNA quantification was performed. Briefly, an Alu-Gag preamplification was performed by using the following primers: forward 5'-GCCTCCCAAAGTGCTGGGATTACAG-3', reverse 5'AGGGTTCCTTTGGTCCTTGT-3'; samples were then followed by a Gag amplification by using the following primers and probe: forward 5'-CAAGCAGCCATGCAAATGTT-3', reverse 5'-TGCACTGGATGCAATCTATCC-3' and probe FAM'5-AAAGAGACCATCAATGAGGAAGCTGCAGA-3'TAMRA. For viral transcripts quantification, RNA was extracted as described above. After reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Life Technologies), the following primers and probe amplifying *tat/rev/nef* mRNA were used: forward 5'-GGATCTGTCTCTGTCTCTCTCTCCACC-3', reverse 5'-ACAGTCAGACTCATCAAGTTTCTCTATCAAAGCA-3' and the dual-labeled fluorescent probe: FAM'5-TTCCTTCGGGCCTGTCGGGTCCC3'TAMRA. TAR transcripts were quantified with the following primers and probe: forward 5'-GGGTCTCTCTGGTTAGA-3'; reverse 5'-GGGTTCCCTAGTTAG-3', and the probe that is complementary to the TAR loop region of the HIV-1 LTR and also dual-labeled: FAM'5-GCCTGGGAGCTCTCTGG-3'TAMRA[128].

For relative mRNA quantification to check transfection efficiency in all siRNA treated cells RNA was extracted and reverse transcriptase was performed as described above. mRNA relative levels of different genes were measured by two-step quantitative RT-PCR and normalized to GAPDH mRNA expression using the ΔΔCt method. Primers and DNA probes were purchased from Life Technologies (TaqMan gene expression assays).

Flow Cytometry. For intracellular Ki-67 staining, cells were fixed for 3 min with Fixation Buffer (Fix & Perm, Life Technologies) before adding precooled 50% methanol for 10 min at 4ºC. Cells were washed in PBS with 5% FBS and incubated for 30 min with the Ki-67 FITC (1:10; clone B56, BD Biosciences) antibody diluted in permeabilization buffer. For intracellular SAMHD1 staining, cells were fixed for 20 minutes with FA 4% followed by permeabilization for 15 minutes with 0.5% Triton X-100 in PBS. After incubation for 1 hour in PBS containing 2% BSA, cells were stained with the rabbit polyclonal anti-SAMHD1 (1:100; 12586-1-AP, Proteintech) for 1 hour followed by incubation for 20 minutes with the goat anti-rabbit Alexa 633 antibody (1:1,000; Life Technologies) both diluted in the blocking media.

For cell-cycle sorting experiments, $CD4^+$ T cells were incubated in 10 μg/ml of Hoechst 33342 (Life Technologies) for 45 min at 37ºC. Pyronin Y (Sigma-Aldrich) was added to a final concentration of 1.5 μg/ml and cells were further incubated at 37ºC for 45 min. The cell-cycle subpopulations were identified by FACS and immediately sorted using FACS Aria II (BD Biosciences). Cell-cycle subpopulations were suspended in 0.03% saponin (Sigma-Aldrich) in PBS and then incubated in 20 μM 7-amino-actinomycin D (7AAD; Sigma-Aldrich) for 30 min at room temperature in the dark. Cells were kept on ice for 5 min before addition of pyronin Y (Sigma-Aldrich) to a final concentration of 5 μM. After incubation for 10 min on ice cells were directly analyzed by flow cytometry. Flow cytometry was performed in a FACS LSRII flow cytometer (BD Biosciences). Data were analysed using the FlowJo software (BD Biosciences).

Immunoprecipitation and immunoblotting. Treated cells were rinsed in ice-cold PBS and extracts prepared in lysis buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM Na β-glycerophosphate, 50 mM NaF, 5 mM Na Pyrophosphate, 270 mM sucrose and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride. For immunoprecipitation assays, lysates were incubated with the antibody of interest overnight at 4ºC. Next day lysates were incubated with Fast flow Sepharose (Sigma-Aldrich) for one hour and beads washed three times with lysis buffer. Lysates or beads were then subjected to SDS-PAGE and transferred to a PVDF membrane (ImmunolonP, Thermo) followed by an overnight incubation with the antibody of interest; or subjected to an enzyme treatment like phosphatases (denaturalizing with Laemmli buffer or incubating for 30 min at 30ºC with 80 U of λ-phosphatase (New England Biolabs) in the presence or absence of phosphatase inhibitors) or benzonases at 600U/ml (Sigma). Benzonase treatment was performed in cell lysates before the immunoprecipitation by incubating for 2 hours at 37ºC with 2mM $Mg2^+$). For FLAG immunoprecipitation, anti-FLAG antibodies are already covalently attached to agarose (anti-Flag M2 Affinity Gel, Sigma) and are used by following same procedure.

The following antibodies were used for immunoblotting: anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5,000; Pierce); anti-human Hsp90 (1:1000; 610418, BD Biosciences); anti-SAMHD1 (1:2,500; ab67820, Abcam); anti-phospho(Thr)-Pro (2321), phospho-CDK2 (Thr160; 2561), anti-CDK2 (2546), anti-CDK6 (3136) and

anti-CDK1 (9116) (all 1:1,000; Cell Signalling Technologies), anti β-Actin (1:5,000; A5316, Sigma-Aldrich), horseradish peroxidase-conjugated secondary antibody (Pierce), anti-MED14 (ab72141, Abcam) and anti-TAT (LS-C68064, Abyntek). Anti-phospho-SAMHD1 Thr592 (pSAMHD1 T592) was obtained by immunization of rabbit using a phosphorylated peptide as described before[115]. Density of the bands corresponding to the phosphorylated SAMHD1 and the bands corresponding to the unphosphorylated form were quantified with Adobe Photoshop software. Ratio between these two densities (pSAMHD1/SAMHD1) was used to quantify SAMHD1 phosphorylation.

Determination of dNTP intracellular levels. MDM were rinsed and lysed with trichloroacetic acid (TCA, 0.5M). Cellular proteins were cleared by centrifugation and supernatant was neutralized with 0.5 M Tri-n-octylamine in 1,1,2-trichlorotrifluoroethane (Sigma-Aldrich). Aquose phase was recovered and dried in a SpeedVac. Pellets were resuspended in Tris-HCl buffer (40mM, pH7.4) and dNTP content determined using a polymerase-based method[129] with minor modifications. Briefly, 20 μL of reaction mixture contained 5 μL of dNTP extract in 40 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.25 μM oligoprimer, 0.75 μM [8-3H]dATP, 12–21 Ci/mmol (or [methyl-3H]dTTP for the dATP assay) and 1.7 units of Thermo Sequenase DNA Polymerase (GE Healthcare). Reaction mixtures with aqueous dNTP standards were processed in parallel. After incubation at 48°C for 60 min, 18 μL of the mix were spotted on a Whatman DE81 paper and left to dry. The filters were washed 3 times for 10 min with 5% Na_2HPO_4, once with water, once with absolute ethanol, and left to dry again. The retained radioactivity was determined by scintillation counting and dNTP amounts calculated from interpolation on the calibration curves. To ensure the reliability of the results, triplicates of 2 different dilutions of each dNTP extract (usually undiluted and 1:3 water-diluted) were processed in each independent experiment.

Kinase assays. Recombinant His-cdk1/GST-CycB, His-cdk2/GST-CycA and His-cdk6/GST-CycD3 complexes purified from Sf21 insect cells were used (Millipore). HA-SAMHD1 was immunoprecipitated from HEK293T transfected cells. 0.5 μg of the indicated CDK/cyclin complex was incubated with HA-SAMHD1 or 1 μg histone H1 (Roche Diagnostics). Phosphorylation reactions were carried out in kinase assay buffer (50 mM Tris HCl

pH 7.5, 10 mM $MgCl_2$, 2 mM DTT) in the presence of 50 μM cold ATP and 1μCi/assay of radiolabeled ^{32}P-γ-ATP (3,000 Ci/mmol from Perkin Elmer) in a final volume of 40 μl/assay for 15 min at 30ºC. Reactions were stopped by adding SB5X (250 mM Tris HCl pH 6.8, 0.5 M DTT, 10% SDS, 20% glycerol, 0.5% bromophenol blue) and boiling at 100ºC for 5 min. Phosphorylated proteins were subjected to SDS-PAGE and transferred to a PVDF membrane and exposed to Biomax XAR films (Kodak).

Tat transactivation assay. The *trans*-activation activity of HIV Tat was analyzed by monitoring the production of β-galactosidase after activation of *lacZ* expression in TZM-bl cells that were previously transfected both with siRNA and pcTat, a Tat expression plasmid. In brief, $8x10^4$ TZM-bl cells were retrotransfected with siRNA and seeded into 24-well plates as described above; 24 hours later, 200 ngr of a plasmid encoding HIV-1 Tat (pcTat) was transfected. 48 hours after DNA transfection, samples were processed for β-galactosidase assay as following described.

β-Galactosidase detection assay. β-galactosidase activity was quantified by a colorimetric assay, as described elsewhere[124]. Background from non-infected cells was subtracted from the rest of the samples and absorbance expressed as percentage of β-galactosidase activity relative to the control treated without siRNA.

Statistical Methods. Data were analyzed with the PRISM statistical package. If not stated otherwise, all data were normally distributed and expressed as mean ± SD and p-values were calculated using an unpaired, two-tailed, t-student test and represented as *, $p<0.05$; **, $p<0.005$; ***, $p<0.0005$. For normalized data from different donors, one-sample t-test against hypothetical value of 1 or 100 was applied. For the siRNA screen with all Mediator subunits, siRNA pools were considered for further analysis when HIV replication was inhibited at least 50%, together with conserved viability values as measured by MTT method above 70% compared to mock transfected cells. P values less than 0.05 were considered significant.

RESULTS

1 Role of Mediator complex in HIV-1 transcription

1.1. Summary

siRNA-based screenings have identified lots of HIV factors implicated in different pathways of the viral cycle that participate in a broad range of cellular functions, among them the human Mediator complex. Mediator complex (MED) is composed by 28 elements and represents a component of the transcription machinery, interacting with the RNAPII enzyme and regulating its ability to express genes[56,59,60] (Figure 4).

In this chapter, we provide an extensive evaluation of the MED activity on HIV replication. Knockdown of nine of the 28 human MED proteins significantly impaired viral replication without affecting cell viability, including MED6, MED7, MED11, MED14, MED21, MED26, MED27, MED28 and MED30. Impairment of viral replication by MED subunits was at a post-integration step. Inhibition of early HIV transcripts was observed by siRNA-mediated knowckdown of MED6, MED7, MED11, MED14 and MED28, specifically affecting the transcription of the nascent viral mRNA TAR. In addition, MED14 and MED30 were shown to have a special relevance during the formation of unspliced viral transcripts ($p<0.0005$). Knockdown of the selected MED factors compromised HIV transcription induced by Tat, with the strongest inhibitory effect shown by siMED6 and siMED14 cells. Co-immunoprecipitation experiments suggested physical interaction between MED14 and HIV-1 Tat protein.

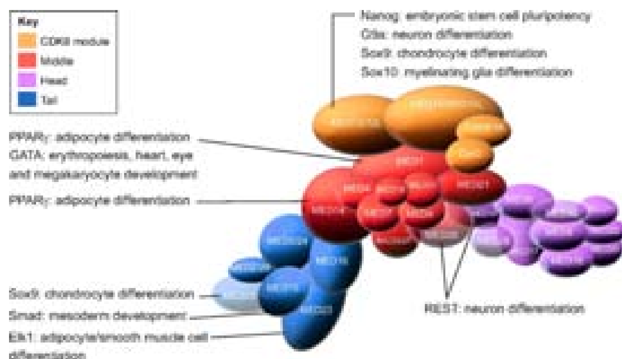


Figure 4. Mediator complex in Humans. Mediator complex interactions with gene-specific transcription factors that promote diverse gene expression[56].

1.2. RNA interference of Mediator complex subunits impaired HIV-1 replication

We first characterized the expression pattern of the different MED subunits in primary cells including PBMC, monocytes and macrophages, two human cell lines MT-4 and HeLa TZM-bl and HIV-susceptible tissues like lymph node, thymus and small intestine, as well as adipocytes to include a non HIV-susceptible tissue.

Expression profiling analysis indicated the highest MED expression in adipocytes and PBMCs, whereas the lowest expression was found in monocytes and MT-4 cells. Hela TZM-bl cells and macrophages had a similar mRNA profile with medium levels of expression on average. MED genes were also clustered in different groups to easily distinguish those MED subunits related in terms of mRNA profile (Figure 5).

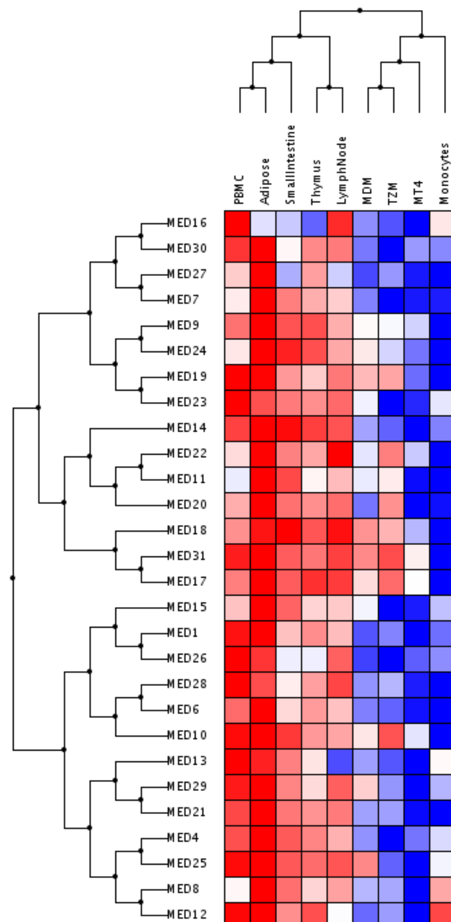


Figure 5. Expression profile of Mediator complex mRNA in different cell types and tissues. mRNA expression of each MED gene was quantified by qPCR in samples of RNA extracted from TZM-bl cells, MT-4 cells, PBMC, monocytes, MDM and commercial Lymph Node, Adipose, Small Intestine and Thymus RNA. Normalized expression of different mRNAs was calculated using GAPDH quantification as reference. Results from gene expression assays were uploaded to the GEPAS software in order to obtain a cluster image representing gene expression from low (blue) to high (red) expression and functional profiling of the Mediator complex. Mean of two MED expression profile assay is shown.

The presented Mediator mRNA profile (Figure 5) is useful to compare analyzed cells and tissues to Hela TZM-bl cells, which were chosen for being highly susceptible to HIV infection and easily transfectable

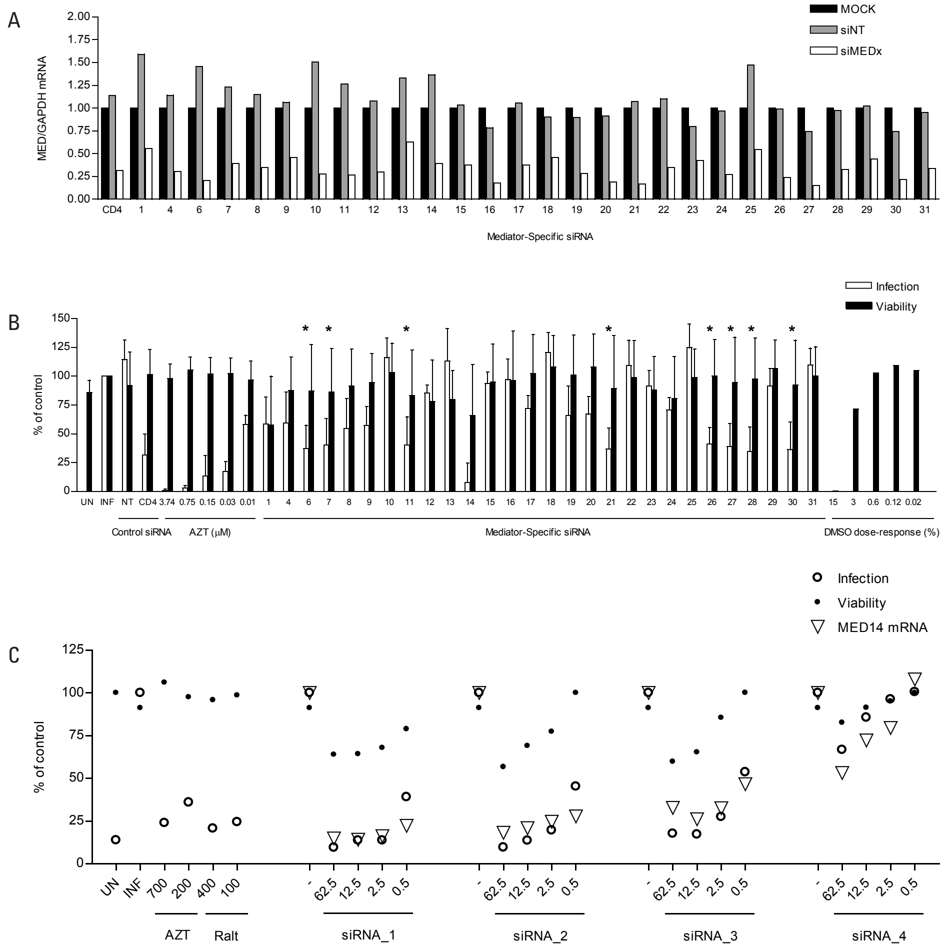


Figure 6. Inhibition of HIV-1 replication after siRNA-mediated interference of Mediator complex subunits. (A) mRNA knockdown efficiency in TZM-bl cells was quantified by qPCR 48h post transfection. mRNA expression of each MED gene was normalized to a sample treated without siRNA (Mock). Results from one of three independent experiments are shown. (B) TZM-bl cells were transfected with the indicated siRNA and infected 48h later with HIV-1 NL4-3 strain for 72h. A non-targeting siRNA pool (NT) and a siRNA targeting CD4 were used as a control. Infection was measured by quantifying expression of β-gal reporter and validated with a dose-response effect of the reverse transcription inhibitor AZT (white bars). Viability of transfected cells was monitored by the MTT method and validated with a DMSO dose-response effect (black bars). Mean±SD of at least three independent experiments is shown. (C) Different siRNA targeting MED14 confirmed the antiviral effect at subtoxic concentrations. HeLa TZM-bl cells were transfected with four different siRNA targeting MED14 at different molarities and mRNA levels were quantified by qPCR 48h later. Transfected cells were then infected with HIV-1 NL4-3 strain and 72h later infection was quantified, or cells were assayed for siRNA-derived toxicity by MTT assay. A result of one representative experiment of three is shown.

with RNA interference, as the model for a comprehensive analysis of the relative contribution of each MED subunit in HIV-1 replication by RNAi. Therefore, efficient and specific inhibition of each of the different MED subunits was achieved at the mRNA level compared to mock-treated cells (Figure 6A). siRNA treated cells were then infected with the NL4-3 HIV-1 strain, and three days later HIV-1 replication was assessed (Figure 6B; white bars). Cell viability was assessed in parallel in uninfected cells (Figure 6B; black bars).

As expected, RNA interference of CD4 or AZT led to decreased HIV replication in the absence of significant cytotoxicity. We selected for further study those siRNA that inhibited HIV-1 replication by >50% compared to untreated infected cells and their associated cell viability did not vary more than 30% compared to mock transfected cells. The strongest blockade of HIV replication was achieved by siRNA-mediated inhibition of MED14 (β-gal values were reduced by 90% compared to mock-transfected cells, $p<0.005$), although partly compromising cell viability in the assayed conditions (reduced viability by 35% of control). The use of confirmatory siRNA targeting MED14 (Figure 6C) confirmed its specificity and potency in impairing HIV replication in the absence of off-target effects or siRNA-derived toxicity, pointing to MED14 as one of the most relevant MED subunits for HIV replication.

The siRNA-based screen of MED genes showed that silencing of 9 out of the 28 human MED genes significantly impaired viral replication without affecting cell viability including: MED6, MED7, MED11, MED14, MED21, MED26, MED27, MED28 and MED30, thus confirming previous reports linking Mediator to HIV[21,22,130].

We also silenced mRNA expression of selected MED in human MDM (Figure 7A) and HIV-1 infection and cell viability were quantified by flow cytometry in either infected or uninfected siRNA treated cells (Figure 7B). siMED14 interfered cells showed the strongest reduction in viral replication (58% of control, $p<0.01$), followed by siMED27 cells (63% of control, $p=0.01$). Nevertheless, other tested MED subunits did not significantly affect HIV-1 replication in MDM.

As well, we could not identify any association between HIV replication and MED proteins associated to the CDK8 module[130] but we could replicate the effect of four subunits that appeared in at least two siRNA screens before: MED6, MED7, MED14 and MED28 (Table 1). MED subunits identified in this work belong to different functional domains in

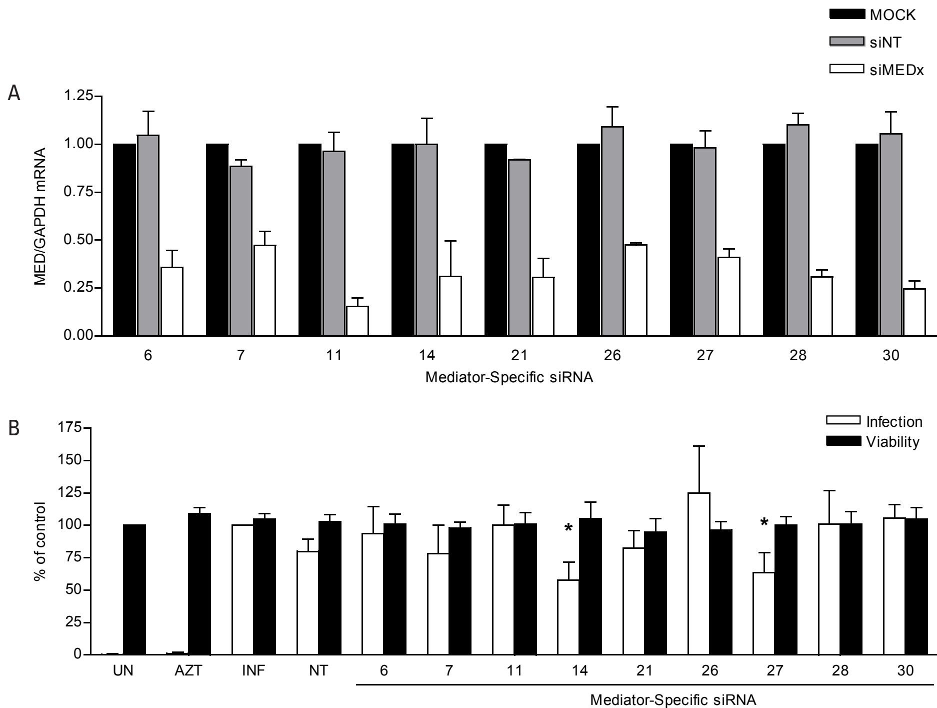


Figure 7. MED14 and MED27 impaired HIV-1 replication in monocyte-derived macrophages (MDM). (A) mRNA knockdown efficiency in MDM cells was quantified by qPCR 72h post transfection. mRNA expression of each MED gene was normalized to a sample treated without siRNA (Mock). Mean±SD of three independent experiments is shown. (B) MDM were transfected with the indicated siRNA and infected 72h later with VSV-pseudotyped NL4-3-GFP virus. Infection was measured as the percentage of GFP positive cells in siRNA treated MDM and expressed as the percentage to mock-treated cells. AZT was used as a control (white bars). In parallel, cell viability was assayed in uninfected siRNA treated macrophages by counting live cells using flow cytometry (black bars). Mean±SD of three independent donors is shown.

the whole MED complex, including four head-module factors (MED6, MED11, MED28 and MED30) and five middle module factors (MED7, MED14, MED21, MED26 and MED27).

1.3. Effect of Mediator complex on viral transcription

Since we identified a group of MED subunits whose activity is required for efficient HIV-1 viral replication we then investigated the role of these subunits before and during viral transcription. For this purpose, the levels of integrated proviral DNA and viral transcripts were quantified by qPCR.

Table 1. Comparison of MED subunits that have been previously linked to HIV-1 replication. Labeled cells show MED subunits identified as "*hits*" by the indicated papers. Last column shows the MED subunits identified as "*hits*" by the current work. See references at [15,21,22].

	Brass *et al.*	Zhou *et al.*	König *et al.*	This work
MED 1				
MED 4	■	■		
MED 6	■	■	■	■
MED 7	■	■	■	■
MED 8		■		
MED 9				
MED 10				
MED 11		■		■
MED 12				
MED 13				
MED 14	■		■	■
MED 15			■	
MED 16				
MED 17		■		
MED 18				
MED 19		■	■	
MED 20		■		
MED 21				■
MED 22				
MED 23				
MED 24				
MED 25				
MED 26		■		■
MED 27		■		■
MED 28	■	■		■
MED 29				
MED 30				■
MED 31		■		

Mediator-interfered (siMED) TZM-bl cells were infected with a NL4-3 HIV-1 strain and viral integration 24h after infection was quantified (Figure 8, grey bars). No differences were observed between siMED and control cells, indicating that Mediator down-regulation did not affect HIV-1 integration or earlier steps in viral replication cycle. As expected, the inhibitor of HIV-1 reverse transcriptase AZT and the inhibitor of HIV integration raltegravir effectively inhibited HIV integration.

Evaluation of viral mRNA transcription and formation of TAR was then evaluated. Early viral mRNA transcripts and specifically viral nascent RNA sequences named TAR are formed when HIV promoter elements assemble the PIC and RNAPII clears the promoter, initiating the transcription of HIV genes[131]. Impairment in the formation of TAR forms was found in knockdown cells for MED6, MED7, MED11, MED14 and MED28 (Figure 8, white bars; p-value range 0.01-0.0001), suggest-

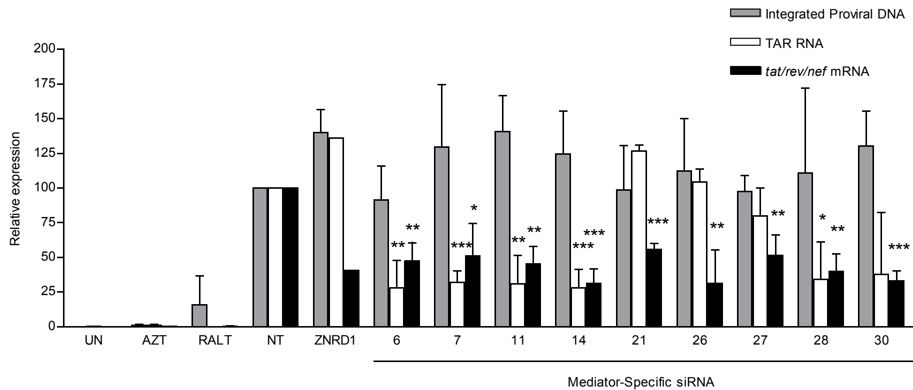


Figure 8. Selected Mediator subunits have a role in HIV-1 cycle at the early and late transcription level. Integrated proviral DNA (grey bars), TAR RNA (white bars) and *tat/rev/nef* late viral transcripts (black bars) were compared by qPCR in siRNA treated TZM-bl cells infected for 24h (for integrated proviral DNA) or 48h (for TAR and late viral transcripts) with NL4-3 HIV-1 strain. Values are expressed as percentage of the control cells treated with a non-targeting siRNA (NT). The RT inhibitor AZT, the integrase inhibitor Ralt and a siRNA targeting ZNRD1 gene (ZNRD1) were used as controls. Mean±SD of three independent experiments is shown.

ing that these MED subunits are required particularly during initiation steps in HIV-1 transcription.

We also quantified the unspliced *tat/rev/nef* mRNA transcripts, transcribed during the elongation process that follows the initial transcription process[128]. A reduction in viral elongated transcripts was observed after silencing of all MED subunits (Figure 8, black bars; p-value range 0.05-0.0005), compared to cells treated with a non-targeting siRNA pool.

AZT-treated as well as raltegravir-treated cells were used as a negative controls of infection. siRNA targeting the HIV cofactor zinc ribbon domain–containing 1 (ZNRD1) was used as a control of impaired HIV-1 transcription, as it has been previously shown to have a role in late HIV mRNA formation[54].

Taken together, these results demonstrate a role for Mediator complex in HIV transcription and indicate the Mediator subunits implicated in the transcription initiation or elongation.

1.4. Tat-mediated HIV-1 transcription is modulated by Mediator Subunits

To determine the requirement of MED subunits in Tat-dependent viral expression of the HIV-1 LTR promoter, an HIV-1 Tat transactivation assay was performed in HeLa TZM-bl cells, previously treated with

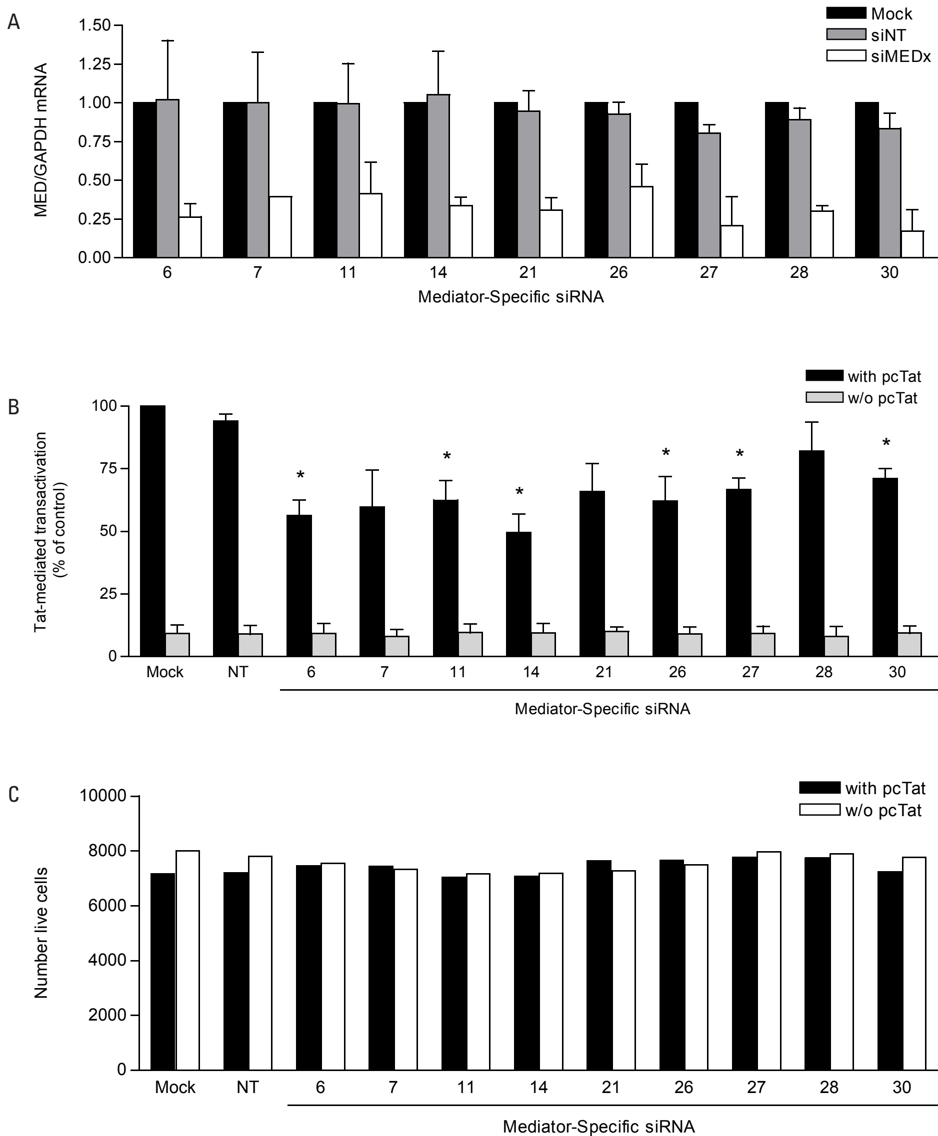


Figure 9. Mediator complex has a role in Tat-driven expression of HIV-1 LTR promoter. (A) Indicated siRNA were transfected into HeLa TZM-bl cells. After 48h, an expression vector of HIV-1 Tat was also transfected. After an additional 24h RNA samples were obtained to quantify MED mRNA levels by qPCR. Expression levels were normalized to the Mock-transfected sample and a non-targeting siRNA (NT) was used as a control. Mean±SD of three independent experiments is shown. (B) β-gal assay was performed in samples treated as in (A), either transfected with a Tat-expressing plasmid (black bars) or transfected in the absence of Tat plasmid (white bars). Values were normalized to the mock control. Non-targeting siRNA (NT) was used as control. Mean±SD of three independent experiments is shown. (C) Samples treated as in (A) were recovered and viability was monitored by counting viable cells by flow cytometry in pcTat-transfected cells (black bars) or pcTat-untransfected cells (white bars).

siRNA targeting MED subunits. After successfully knockdown expression of Mediator genes (Figure 9A), a Tat-expressing vector was transfected and β-galactosidase expression under the control of the HIV-1 LTR promoter was monitored. siRNA knockdown of MED6 and MED14 showed significant differences in Tat-mediated HIV-1 transcription levels (up to 40% inhibition, $p<0.05$), as well as a more modest inhibition for MED11, MED26, MED27 and MED30 subunits. In contrast, MED7, MED21 and MED28 interfered cells did not significantly changed compared to mock-transfected cells (Figure 9B).

Viability in mock transfected, siRNA transfected and siRNA+DNA transfected cells was monitored by counting live cells by flow cytometry after single or double transfection (Figure 9C).

1.5. The role of Mediator complex in HIV-1 transcription is mediated by Tat-Mediator interactions

Mediator complex interacts with a variety of activators/repressors through its “Tail” module. Received regulatory signals are then transmitted to the RNAPII via the “Middle” and the “Head” modules to start transcription of specific genes[132]. Based on previous results, where MED14 showed the highest inhibition in HIV replication during early and late stages of viral transcription and in Tat-transactivation assays, we explored the possibility of Tat-MED14 interactions.

To investigate possible interactions between MED14 and the viral LTR-transactivator Tat, a plasmid expressing a fusion protein (3x)Flag-Tat was transfected into HEK293T cells and Tat was immunoprecipitated using Flag-specific agarose beads. Interestingly, MED14 co-immunoprecipitated with Flag-Tat (Figure 10A, lane 2), but it was not detected when using lysates from mock-transfected cells (Mock, lane 1), cells transfected with un-tagged Tat (pcTat plasmid, lane 3) or when beads alone were used (lane 4). Intracellular Flag-Tat or Tat expression did not affect MED14 protein levels compared to the loading control Hsp90 (Figure 10A, lanes 6 and 7). Flag-Tat and Tat expression in whole cell lysates (WCL) were verified by immunoblotting analysis and compared to β-actin (Figure 10B, lanes 6 and 7). Conversely, MED14 immunoprecipitation with anti-MED14 antibodies attached to sepharose led to the detection of co-immunoprecipitated Tat protein when cells had been

transfected with either Flag-Tat plasmid or un-tagged Tat (pcTat) (Figure 10B, lanes 2 and 3, compared with lanes 1 and 4).

To rule out that DNA may be the bridging factor mediating MED14/Tat interaction, cell lysates were treated with or without benzonase prior

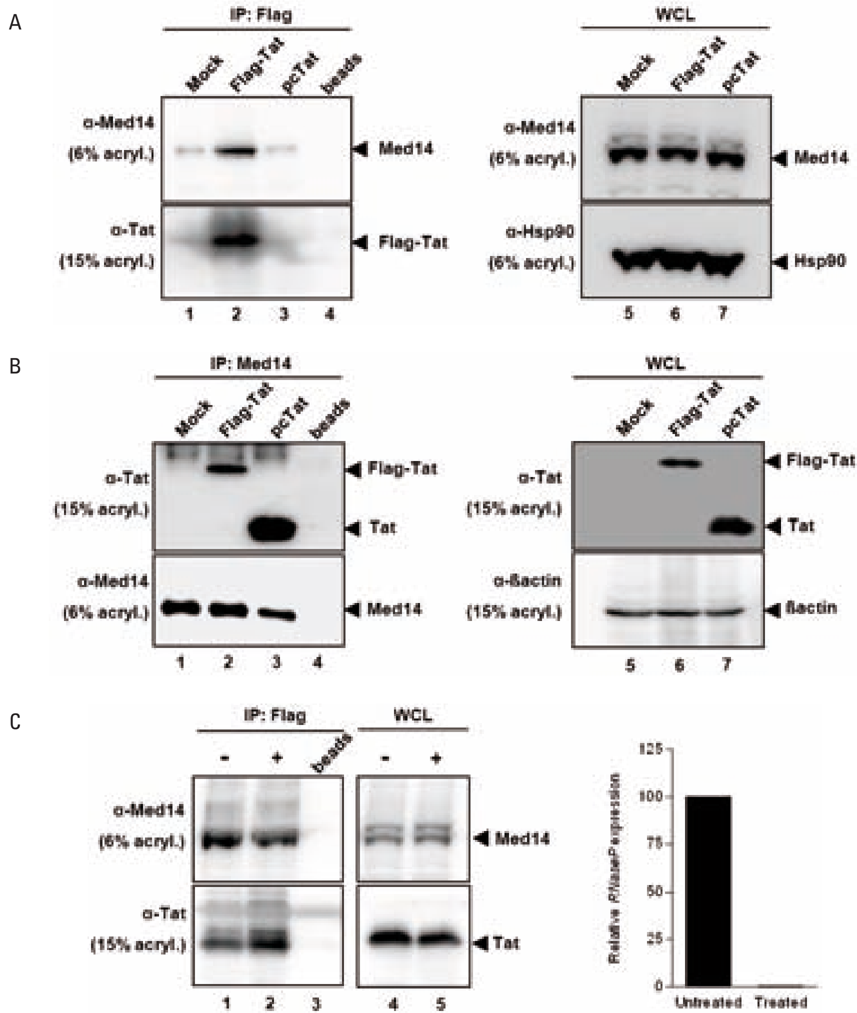
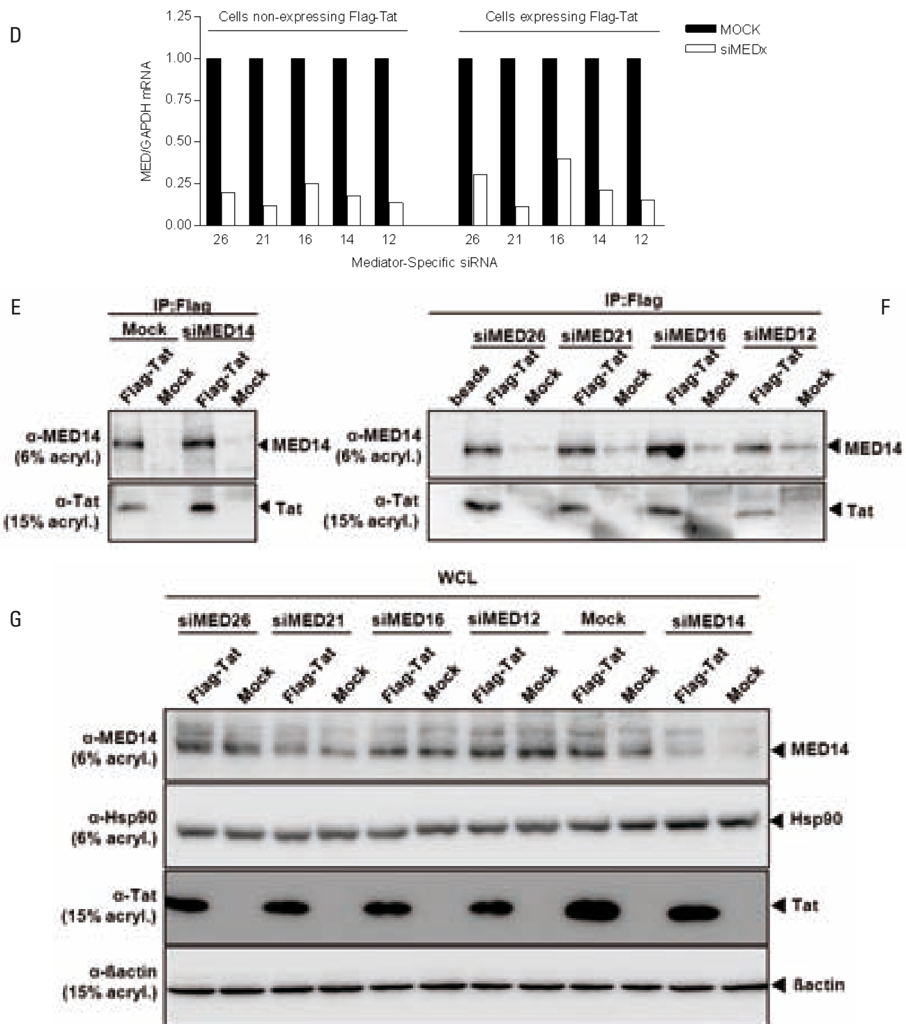


Figure 10. Co-immunoprecipitation of HIV-1 Tat with MED14. (A) Lysates from untransfected (mock) HEK293T cells, transfected with a Flag-Tat expression plasmid (Flag-Tat) or transfected with an untagged Tat expression plasmid (pcTat) were subjected to immunoprecipitation with anti-Flag antibodies attached to agarose (lanes 1-3) or sepharose alone (lane 4). Immunoprecipitates were then blotted with an anti-MED14 antibody or anti-Tat antibody. WCL (lanes 5-7) were blotted with anti-MED14 or anti-Hsp90 antibodies as a control. (B) Lysates used in (A) were subjected to immunoprecipitation with anti-MED14 antibody attached to sepharose (lanes 8-11). Immunoprecipitates were probed by immunoblotting analysis with an anti-Tat antibody or anti-MED14 antibody. WCL were subjected to SDS-PAGE and blotted with anti-Tat or anti-β-actin antibodies (lanes 12-14). One representative experiment of three is shown. (C) Benzonase treatment was performed before

to CoIP. MED14 immunoprecipitated with Flag-Tat either in the absence (Figure 10C, lane 1) or presence (lane 2) of benzonase, thus excluding DNA as a bridging factor for the described protein-protein interaction. WCL from Flag-Tat expressing cells after benzonase treatment showed



the immunoprecipitation following same procedure as in (A). DNA from untreated or treated protein lysates was extracted and quantified by qPCR. Values were relativized and expressed as 2^ (-RNaseP amplification cycle). (D) HEK293T cells, previously transfected with a Flag-Tat expression plasmid (Flag-Tat) or mock-transfected, were then retrotransfected with siRNA targeting mRNA from indicated MED subunits. mRNA expression of each MED gene was normalized to a sample treated without siRNA (mock). Results from one of two independent experiments are shown. (E) Cell lysates treated as described in (D) were then subjected to immunoprecipitation with anti-Flag antibodies attached to agarose (beads). Immunoprecipitates were then blotted with an anti-MED14 antibody or anti-Tat antibody. (F) WCL from (E) were blotted with anti-MED14, anti-Hsp90, anti-Tat or anti-β-actin antibodies as a control.

no differences on Tat or MED14 expression (Figure 10C, lanes 4 and 5). Efficacy of DNA removal was controlled by qPCR amplification of genomic RNaseP gene and graphed in Figure 10C. MED14 is in contact with other MED subunits when organized into the complex that could be, in turn, mediating its interaction with Tat. To test this, apart from MED14 we also interfered MED subunits that interact with MED14 either in the same module (MED21 and MED26) or in a different module (MED16). As a negative control, MED12, which is in the CDK8 module, was also interfered (Figure 10D).

Although significantly inhibiting MED14 mRNA and protein by siRNA, the remaining protein also could be immunoprecipitated with Flag-Tat, thus reinforcing our results (Figure 10E).

In all siRNA interfered Flag-Tat expressing cells, the absence of other MED subunits did not affect the interaction between Tat and MED14 (Figure 10F). MED14, Tat and Hsp90 protein levels were compared in whole cell extracts (Figure 10G), corresponding to the lysates used in Figure 10E and F.

These results indicate that Tat associates with MED14 in cultured cells demonstrating for the first time a possible physical interaction between the viral protein Tat and Mediator complex.

2 The HIV-1 restriction factor SAMHD1

2.1. Summary

In this chapter, we obtained confirmation of SAMHD1 degradation by Vpx and HIV-1 restriction in primary cells including IL12/18 differentiated MDM. We also showed how SAMHD1 function is influencing HIV-1 sensitivity to NRTIs and which is its functional mechanism.

The most commonly agents used in ART include viral RT inhibitors that compete with intracellular dNTPs as substrate for viral reverse transcription. A panel of different RT inhibitors was analyzed for their different antiviral efficacy depending on SAMHD1. Reduction of SAMHD1 levels significantly decreased HIV sensitivity to thymidine but not other nucleotide RT analog inhibitors, pointing towards a differential effect of SAMHD1 activity on thymidine.

The dNTP pool is *per se* a limiting factor for retroviral reverse transcription in non-cycling cells, mainly driven by the SAMHD1 capacity to degrade nucleotides, while proliferating cells have higher dNTP intracellular levels and are preferentially susceptible to infection by retroviruses. We showed that proliferating (Ki67^{+}) primary CD4^{+} T cells or macrophages express a phosphorylated form of SAMHD1 that corresponds with susceptibility to infection in cell culture and that is not present in non-cycling cells, although SAMHD1 mRNA expression do not change between HIV-resistant cells compared to HIV-susceptible cells.

SAMHD1 was previously identified as a substrate for CDK. We thus studied all CDKs implicated in cell cycle control, identifying CDK6 as well as its catalytic partner cyclin D3, as upstream regulators of CDK2 controlling SAMHD1 phosphorylation in primary T cells and macrophages susceptible to infection by HIV-1. In turn, CDK2 was strongly linked to cell cycle progression and coordinated SAMHD1 phosphorylation and inactivation. CDK inhibition specifically blocked HIV-1 infection at the reverse transcription step in a SAMHD1 function and thus, reducing the intracellular dNTP pool. To dilucidate the entire cellular signaling pathway guiding viral replication by regulating intracellular dNTP availability through SAMHD1 phosphorylation, we then knocked down the cellular CDK2 inhibitor CDKN1A (p21), observing a relieve of the SAMHD1 restriction and thus increasing viral replication.

We explored at the last part of the chapter how current available drugs targeting CDKs can be used to inhibit HIV-1 infection by regulating intracellular dNTP availability. Pan-CDK inhibitors reduced SAMHD1 phosphorylation and blocked HIV-1 replication at subtoxic concentrations. Palbociclib, a potent and selective CDK6 inhibitor blocked SAMHD1 phosphorylation, intracellular dNTP levels, HIV-1 reverse transcription and HIV-1 replication in primary macrophages and $CD4^+$ T lymphocytes. Notably, treatment of macrophages with palbociclib led to reduced CDK2 activation. The antiviral effect was lost when SAMHD1 was degraded by Vpx, providing further evidence for a role of SAMHD1 in mediating the antiretroviral effect.

2.2. SAMHD1 is degraded by Vpx and restricts HIV-1 in primary cells

SAMHD1 expression is variable in distinct human cell lines and tissue types[133,134], although high levels of expression have been reported for HIV-1 target cells, such as MDM and $CD4^+$ T lymphocytes. mRNA and protein expression of SAMHD1 were assessed in two HIV-1 highly susceptible T cell lines (MT-4 and Jurkat) and primary HIV-1 target cells (MDM and activated $CD4^+$ T cells), with the aim to identify cell lines with different degrees of SAMHD1 expression. Jurkat T cells expressed very low levels of SAMHD1 mRNA and protein expression was undetectable, as previously reported[134]. On the contrary, MDM or activated $CD4^+$ T cells expressed high levels of both SAMHD1 mRNA and protein, whereas expression levels were intermediate in MT-4 cell line (Figure 11A and 11B).

Despite the observed differences in SAMHD1 mRNA expression, both MT-4 and Jurkat T cells were equally susceptible to HIV-1 infection (Figure 11C, white bars) and infection was not enhanced by degradation of SAMHD1 with VLPVpx in MT-4 cells (Figure 11B, lanes 1 and 2 and Figure 11C, black bars), confirming that SAMHD1 is not restricting viral replication in transformed cell lines, where availability of dNTPs is presumably high. In contrast, resting $CD4^+$ T lymphocytes that are resistant to HIV-1 infection can be infected with a NL4.3 HIV-1 strain expressing Vpx (Figure 11D) that has previously degraded SAMHD1 (Figure 11E).

As well, in MDM, VLPVpx induced the degradation of SAMHD1 (Figure 11F) and a 5-fold and significant ($p<0.0001$) enhancement of HIV-1 replication (Figure 11G).

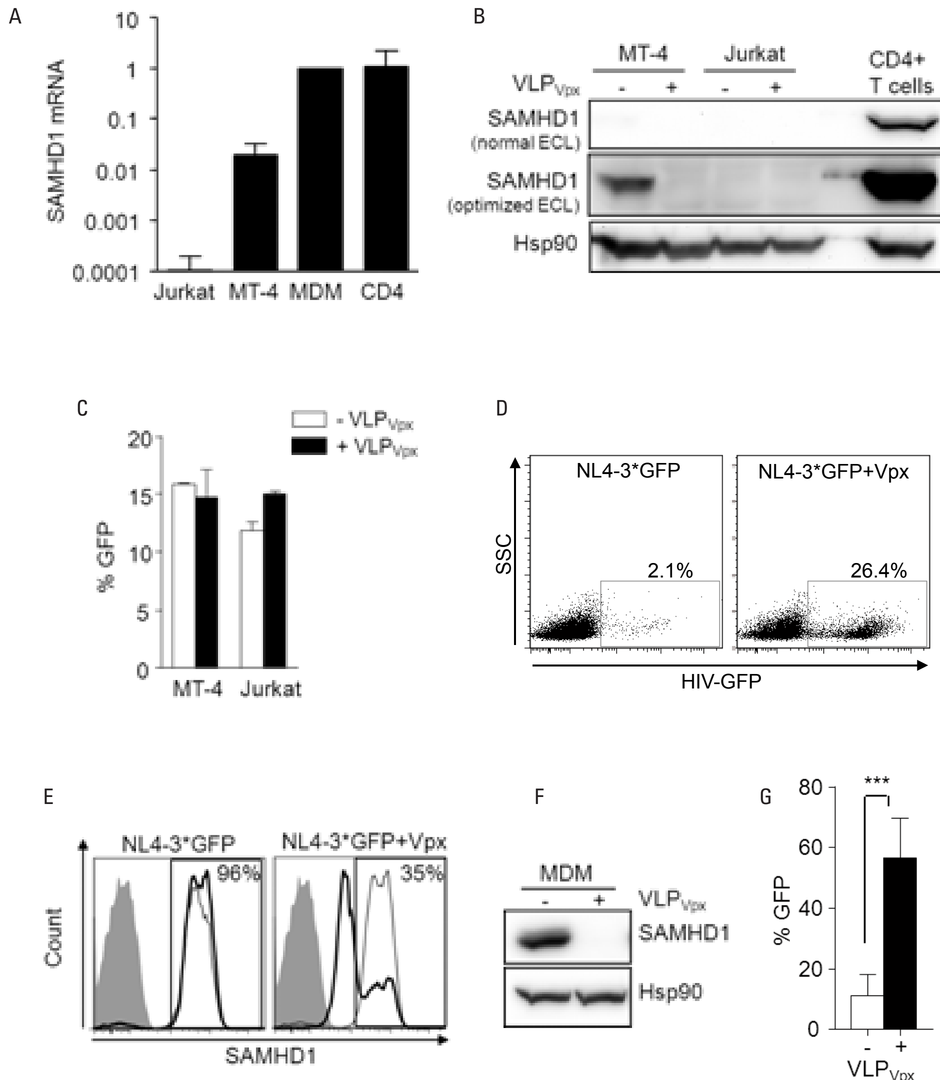


Figure 11. SAMHD1 is degraded with VLP carrying Vpx and HIV-1 restriction is bypassed in primary cells. (A) Relative mRNA expression of *SAMHD1* gene in T cell lines and primary cells. (B) Western blot of SAMHD1 expression in MT-4 and Jurkat T cell lines treated or not with VLPVpx for 24 hours. Activated CD4+ T cells are included as a reference to illustrate the different degree of expression. SAMHD1 protein was detected only in MT-4 cells and after optimizing band detection by enhanced chemiluminescence (ECL). (C) MT-4 and Jurkat T-cell lines previously treated or not with VLPVpx for 4 hours. Percentage of infected cells during 48 hours after treatment with VLPVpx was assessed by flow cytometry. (D) Resting CD4+ T cells were infected with an NL4-3*GFP (left panel) or NL4-3*GFP with Vpx (right panel). After 72 hours, infection was assessed by flow cytometry. (E) Flow cytometry histograms showing intracellular staining of SAMHD1 in resting T cells infected as in (D). Grey line histogram shows uninfected cells; black line histogram, infected cells. The secondary antibody alone was used as a control (shadowed histogram). (F) Western blot of SAMHD1 expression in MDM treated or not with VLPVpx. (G) MDM previously treated or not with VLPVpx were infected with a VSV-pseudotyped HIV-1 GFP virus and replication assessed 2 days later by measuring GFP expression. Mean±SD of at least six independent donors performed in duplicate is shown.

Monocytes and macrophages have different susceptibility to infection by HIV depending on their origin, stage of differentiation and environment. Apart from M-CSF differentiated monocytes showed in Figure 11F and 11G, we also showed how stimulation of macrophages with a combination of IL-12 and IL-18 prevented or blocked a productive infection by HIV-1 (Figure 12A). Furthermore, IL12/IL18 blocked HIV-1 replication in already differentiated, HIV-1 susceptible, M-CSF MDM suggesting that IL12/IL18 triggered an antiviral restriction although treatment of IL12/IL18 MDM with M-CSF did not fully restore the capacity of HIV-1 to infect macrophages (Figure 12B). Protein levels of SAMHD1 were found upregulated in IL12/IL18 MDM (Figure 12C) and importantly, the downregulation of this restriction factor with RNA interference (Figure 12E) or by treatment with VLP carrying Vpx protein (Figure 12D and 12F) restored HIV-1 infectivity of IL12/IL18 MDM, thus demonstrating that the main restriction in IL12/IL18 MDM is effectively due to SAMHD1.

2.3. SAMHD1 specifically affects the antiviral potency of thymidine analog HIV reverse transcriptase inhibitors

SAMHD1 activity is able to modify HIV-1 replication in primary cells by controlling the limitation of intracellular dNTPs[94]. Given that some antiretroviral drugs targeting the viral RT are chemically designed to be nucleoside analogs, we hypothesized that SAMHD1 activity could be affecting the potency of these NRTI for incorporation into viral DNA. The antiviral activity of a panel of NRTI and NNRTI were evaluated in MDM in the presence or not of VLPVpx.

After incubation with VLPVpx, macrophages were treated with NRTIs (AZT, d4T, 3TC, ddC, ABC, ddI, and TFV), NNRTIs (EFV and NVP) or the integrase inhibitor Ralt as a control, at different concentrations prior to infection with VSV-pseudotyped HIV-GFP. As shown in Figure 13, macrophages transduced with VLPVpx, showed a significantly reduced viral sensitivity to NRTI thymidine analogs (AZT and d4T), compared to untreated macrophages ($p<0.0001$ and $p=0.03$ at highest concentrations tested for AZT and d4T respectively; Figure 13, firsts two panels). No differences were observed for the cytidine, guanosine and adenosine analogs tested, NNRTI or raltegravir. Calculation of 50% effective concen-

trations (EC50) of macrophages expressing or not Vpx, showed a fold change of 26 and 7 for AZT and d4T, respectively, whereas no change in EC50 was observed for any other drug (data not shown, see [135]).

Importantly, knockdown of SAMHD1 expression by RNA interference (Figure 14A) showed similar results, i. e., an increase in viral replication consequence of SAMHD1 absence and decreased sensitivity to

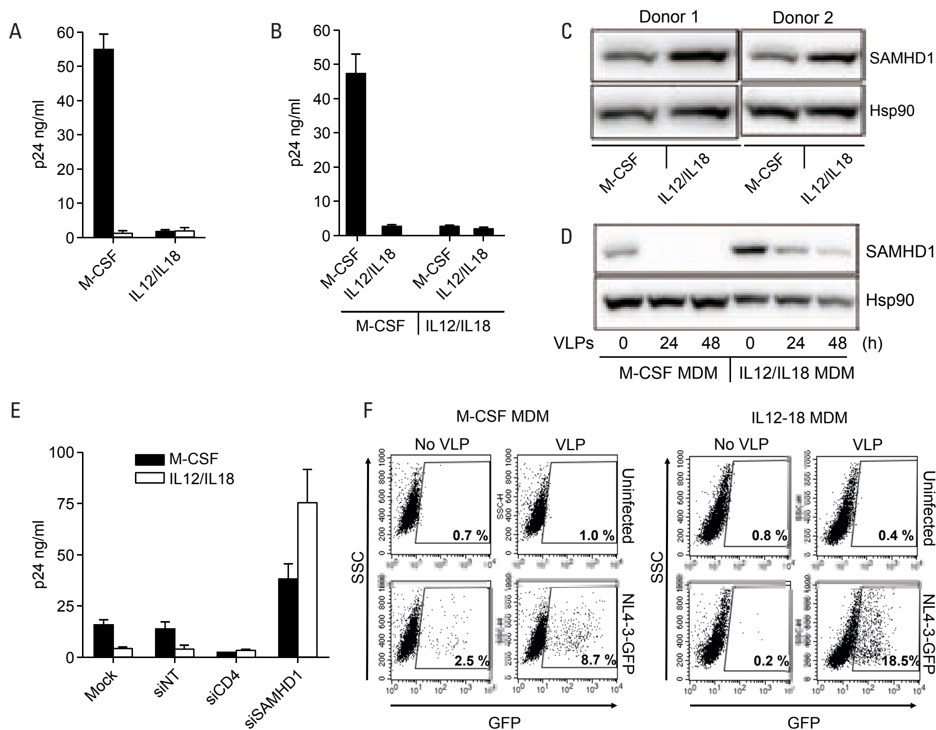


Figure 12. The HIV-1 restriction in IL12/IL18 differentiated MDM is mediated by SAMHD1. (A) Monocytes were differentiated into macrophages for 7 days in the presence of M-CSF or a combination of IL-12 and IL-18 (IL12/IL18). MDM were infected with R5 HIV-1 strain BaL and replication quantified by ELISA measurement of p24 antigen 7 days post-infection. Mean±SD of one representative experiment is shown. (B) Monocytes differentiated as in (A) were then further treated 7 days with both stimuli and simultaneously infected with HIV-1 BaL. Viral replication was quantified by ELISA measurement of p24 antigen. Mean±SD of one representative experiment is shown. (C) Immunoblot of the differentiated monocytes. The expression of a housekeeping gene (Hsp90) is used as control. Blots from two independent donors are shown. (D) M-CSF and IL12/IL18 differentiated macrophages were left untreated or treated for 24 and 48h with VLPVpx. Cells were lysed and SAMHD1 and Hsp90 levels assessed by western blotting. (E) Monocytes were left untreated (mock-transfected) or transfected with the indicated siRNA and then differentiated for 3 days with M-CSF (black bars) or IL12/IL18 (white bars) before being infected with HIV-1 BaL for 7 days. HIV-1 replication was quantified as p24 antigen. Data represent the mean±SD of one representative experiment. Three independent experiments were performed. siRNA interference was confirmed by qPCR (see [123]). (F) MDM treated as in (E) were infected with a VSV-pseudotyped NL4-3-GFP virus. Percentage of GFP positive cells was measured by flow cytometry. One of two independent experiments is shown.

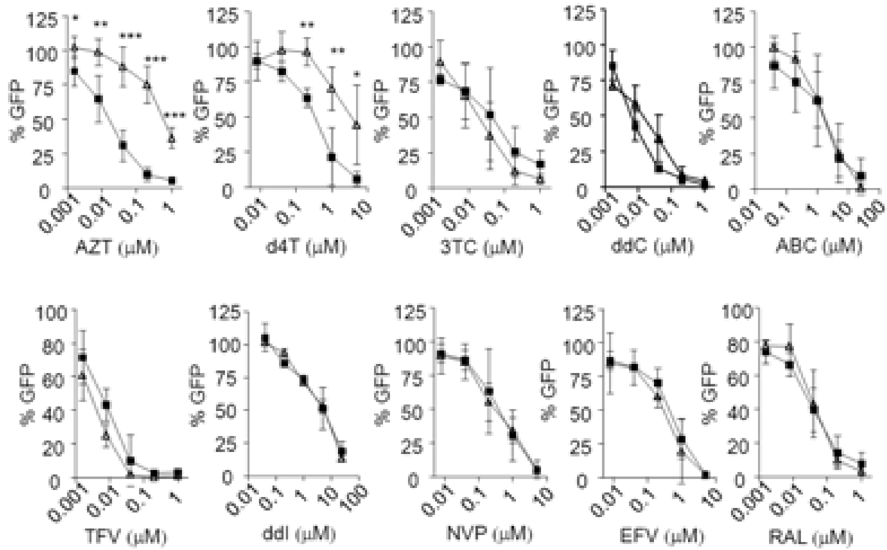


Figure 13. Decreased sensitivity of thymidine analogs NRTI after Vpx-mediated SAMHD1 degradation in MDM. Dose response of NRTI (AZT, d4T, 3TC, ddC, ABC, TFV and ddI), NNRTIs (NVP and EFV) and integrase inhibitor Ralt as control. Inhibition of HIV infection was measured as the percentage of GFP positive cells relative to the no drug condition. Mean±SD of at least three independent donors performed in duplicate is shown.

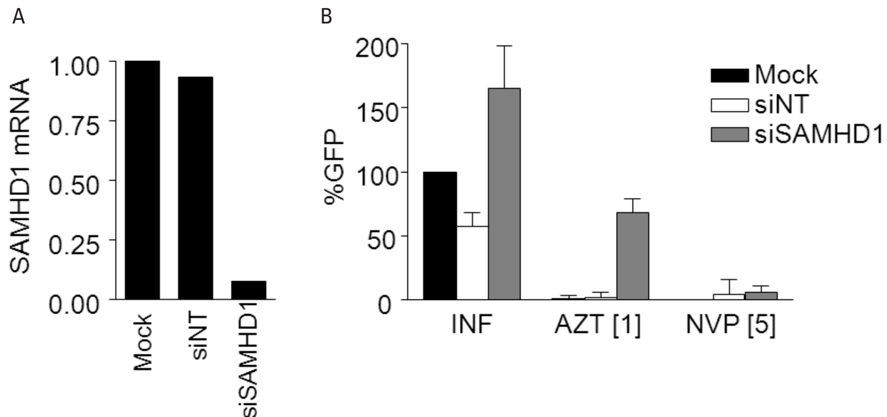


Figure 14. siRNA-mediated knockdown of SAMHD1 in MDM affects AZT antiviral potency. (A) siRNA-mediated knockdown of SAMHD1 in MDM is quantified by qPCR by using the ΔΔCt method. (B) Specific siRNA-mediated inhibition of SAMHD1 expression led to an increase in HIV-1 replication compared to mock-transfected or MDM transfected with a control siRNA (siNT). Absence of SAMHD1 correlated with a decreased sensitivity of AZT (1μM), whereas no changes in NVP sensitivity (5 μM) were observed. MDM were infected with a VSV-pseudotyped NL4-3-GFP virus. Percentage of GFP positive cells was measured by flow cytometry. Values from a representative donor performed in duplicate are shown.

AZT (Figure 14B), discarding that the observed effect is due to the presence of Vpx.

To further study the effect of Vpx-induced degradation of SAMHD1 in viral sensitivity to thymidine analogs, the intracellular dNTP pool availability for reverse transcription was measured. As previously reported, treatment of macrophages with VLPVpx led to an increase of all intracellular dNTP (Figure 15), suggesting that the reduced efficacy of thymidine analogs observed upon degradation of SAMHD1 may be the result of direct competition with intracellular dNTPs.

Competition with intracellular pool of dNTPs is the most plausible hypothesis underlying the reduced efficacy of NRTI thymidine analogs. Therefore, addition of exogenous thymidine may mimic the effect observed by SAMHD1 degradation. 1mM of thymidine (dThD) was added to macrophages together with NRTI thymidine analogs AZT and d4T, as well as to cytosine analog 3TC and adenosine analog TFV as controls, prior to HIV-1 infection (Figure 16A). No differences in viral replication were observed as a result of adding dThD except for AZT and d4T, that completely lost their antiviral activity, therefore mimicking the effect of SAMHD1, arguing in favor of the competition with intracellular dNTPs as the mechanism underlying the lost of antiviral sensitivity. No changes were observed in viral sensitivity to 3TC and TFV. As expected, dTTP intracellular levels were significantly increased compared to untreated macrophages (5-fold increase, $p<0.0001$, Figure 16B) whereas no differences were observed for any other dNTP. Similarly, exogenous addition

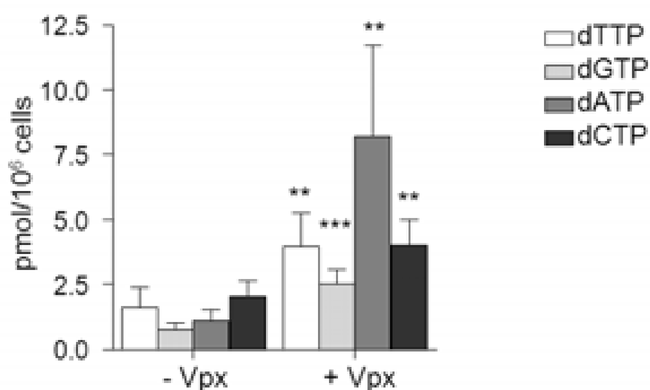


Figure 15. Intracellular dNTP levels in MDM. MDM were transduced with VLPVpx for 24 hours and intracellular dNTPs were extracted. dNTP content was determined using a polymerase-based method. Mean±SD of five independent donors is shown.

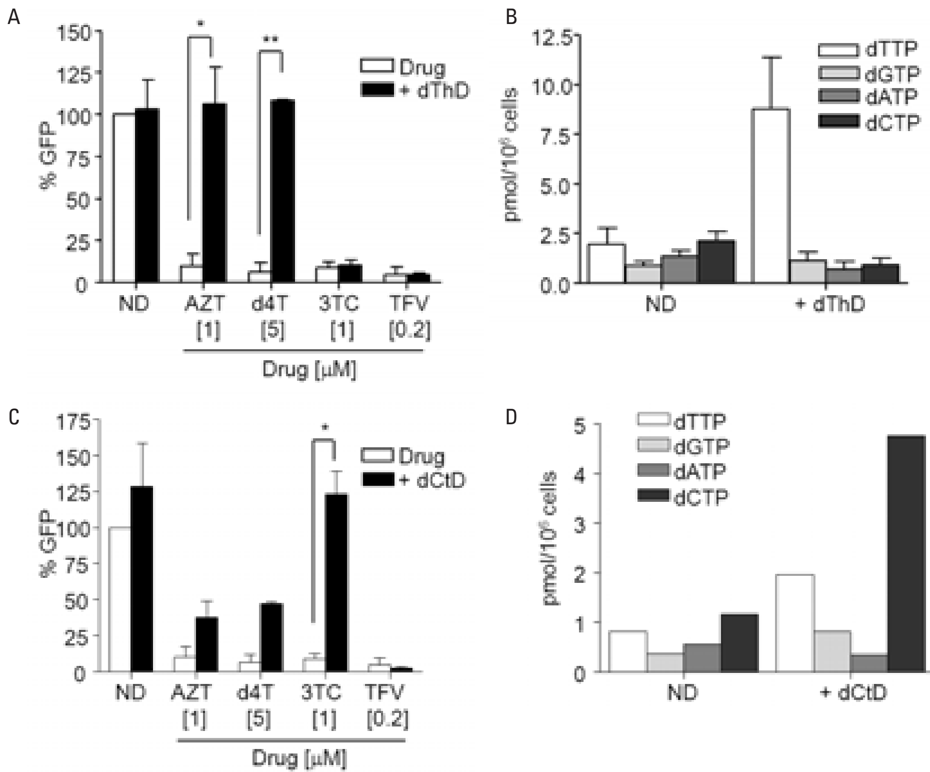


Figure 16. Imbalance of the dNTP intracellular pool mimics SAMHD1-mediated decrease in viral sensitivity to NRTI thymidine analogs. (A) Thymidine (1mM) was added to the culture media together with the corresponding drug 4 hours prior to infection with VSV-pseudotyped HIV-1 GFP virus and replication was assessed 2 days later. Mean±SD of two independent donors performed in duplicate is shown. (B) dThD (1mM) was added to the culture media for 24 hours before dNTPs were extracted and measured. Mean±SD of three independent donors is shown. (C-D) MDM were treated as in (A-B) but adding Cytidine instead of Thymidine. dThD; deoxythymidine, dCtd; deoxycytidine, ND; no drug.

of cytidine led to the lost of antiviral activity of the NRTI cytidine analog 3TC (Figure 16C), consequence of the increase in dCTP intracellular levels (Figure 16D). The addition of exogenous cytidine also partially affected dTTP intracellular levels which was also reflected in the infection outcome where AZT and d4T partly loss its antiviral potency (Figures 16C and 16D). However, these effects were not observed after degradation of SAMHD1 (Figure 13), although intracellular concentrations of both nucleotides were similarly affected after Vpx-mediated SAMHD1 degradation (Figure 15), further supporting a preferential effect of SAMHD1 activity on thymidines. As seen for macrophages, we also found a decreased sensitivity of thymidine NRTI in activated $CD4^+$ T lymphocytes infected with HIV-1 together with VLPVpx (data not shown, see [135]).

As it is already described, in contrast to HIV-1, HIV-2 encodes for Vpx protein and consequently it harbors the ability to overcome the restriction imposed by SAMHD1. Therefore, HIV-2 Vpx-mediated degradation of SAMHD1 may play a role on the decreased AZT viral sensitivity observed. Although data from HIV-2 infected patients treated with AZT and other NRTIs are limited to small cohorts of patients, differences in antiviral activity of AZT on HIV-2 infection have been previously reported[136], but the underlying mechanisms are still under debate.

Here, to evaluate a possible decreased potency of AZT in HIV-2 infected PBMCs depending on SAMHD1, PBMCs from healthy donors were activated with a CD3-CD8 bispecific antibody for five days, prior to infection with WT HIV-2 or HIV-2 defective for Vpx (HIV-2ΔVpx).

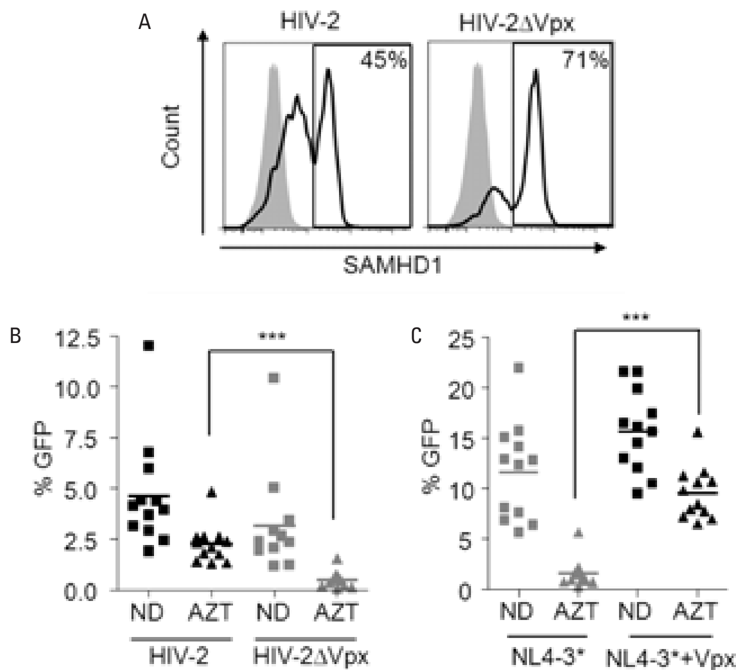


Figure 17. Decreased potency of AZT in HIV-2-infected PBMCs depends on SAMHD1. (A) Flow cytometry histograms showing intracellular staining of SAMHD1 in PBMCs infected with WT HIV-2 (left panel) or HIV-2 defective for Vpx protein (right panel) during three days. Cells were stained using a primary specific SAMHD1 antibody followed by an APC-conjugated secondary antibody (grey line histogram, uninfected cells; black line histogram, infected cells). The secondary antibody alone was used as a control (shadowed histogram). (B) CD3/CD8 activated PBMCs from donors (n=12) were infected with WT GFP-expressing HIV-2 or HIV-2 defective for Vpx protein. Antiviral activity of AZT (3 μM) was assessed. (C) CD3/CD8 activated PBMCs from same donors as in (B) (n=12) were infected with NL4-3*GFP carrying or not Vpx protein and antiviral activity of AZT was assessed. ND; no drug.

Infection with WT HIV-2 was able to partially induce SAMHD1 degradation (from 71% SAMHD1 expressing cells in HIV-2ΔVpx infected cells, to 45% after infection with HIV-2, Figure 17A). Viral replication was not significantly altered by Vpx (mean of 4.6% vs. 3.1% GFP^+ cells after infection with WT HIV-2 and HIV-2ΔVpx, respectively, Figure 17B). Importantly and in accordance with previous results, a significant decrease of AZT antiviral potency against HIV-2 was observed when compared to HIV-2ΔVpx infection ($p<0.0001$, Figure 17B). As expected, similar results regarding infection and limited AZT antiviral potency were obtained in parallel infections using HIV-1 virus modified to incorporate or not Vpx (Figure 17C). In summary, these results point to SAMHD1 as a contributor to the limited antiviral activity of AZT in HIV-2 infections.

2.4. The HIV-1 restriction mediated by SAMHD1 is regulated by phosphorylation

As it was shown before, SAMHD1 is a key element in the restriction of HIV-1 infection in myeloid cells and suppression of SAMHD1 in quiescent $CD4^+$ T lymphocytes enables HIV-1 infection. Nevertheless, SAMHD1 expression levels are similar in HIV-1 resistant cells such as monocytes or quiescent lymphocytes compared to HIV-1 susceptible cells like MDM or activated lymphocytes[120], suggesting that SAMHD1 deactivation is controlled by a post-transcriptional mechanism.

As shown in Figure 18, viral fusion and proviral DNA formation were measured in resting (black bars) and activated (white bars) $CD4^+$ T lymphocytes in the presence of the RT inhibitor AZT or the CXCR4 antagonist AMD3100. Results indicated that resting $CD4^+$ T lymphocytes are resistant to HIV-1 infection by a mechanism independent of viral fusion but prior to proviral DNA formation, contrasting to PHA/IL2 activated $CD4^+$ T lymphocytes (Figure 18A and Figure 18B). As previously described (Figure 11D and 11E), resting $CD4^+$ T lymphocytes can be infected by HIV-1 carrying Vpx, which is able to degrade SAMHD1. Only a percentage of the activated $CD4^+$ lymphocytes enter at a cell dividing stage, correlating with high levels of Ki67 staining, whereas quiescent $CD4^+$ T lymphocytes are Ki67 negative (Figure 18C). While the expression of SAMHD1 in resting and activated lymphocytes is not significantly different, slow migrating forms of SAMHD1 can be observed in 6%

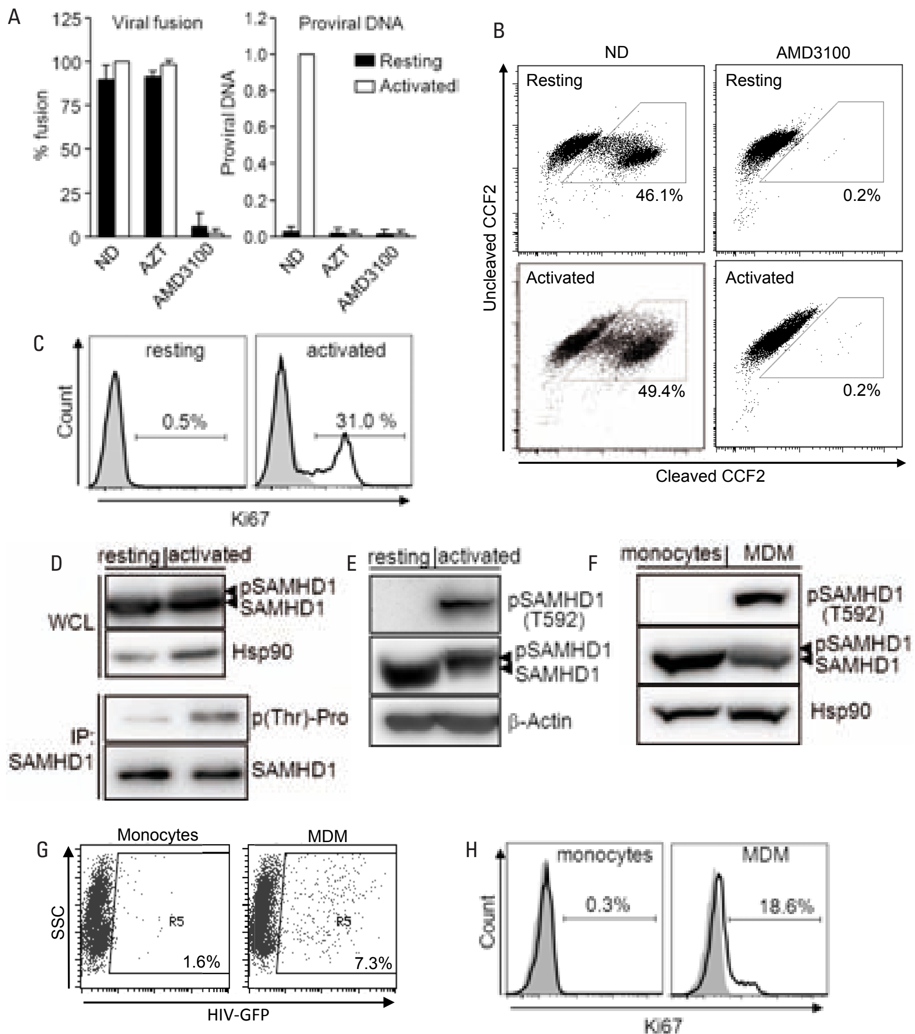


Figure 18. SAMHD1 is phosphorylated in cycling primary cells. (A) Viral fusion and proviral DNA formation measured in CD4+ T lymphocytes without drug (ND) or in the presence of the RT inhibitor AZT (3 μM) or the CXCR4 antagonist AMD3100 (1 μM). Results were normalized to the infection of activated CD4+ cells in the absence of drug. Graphs represent the mean±SD of three independent experiments. (B) Dot blots measuring viral fusion in resting and activated CD4+ T lymphocytes in the presence or not of AMD3100 (1 μM). One experiment of three is shown. (C) Histograms quantifying the percentage of Ki67 positive cells in resting (left) or PHA/IL2 activated (right) CD4+ T lymphocytes (black line). Isotype staining was used as a control (grey histogram). Representative histograms of one donor out of three are shown. (D) Lysates of resting and activated CD4+ lymphocytes were subjected to SDS-PAGE and blotted with anti-SAMHD1 and anti-Hsp90 antibodies (top blots) or subjected to overnight immunoprecipitation of SAMHD1 followed by blotting with and anti-(p(Thr)-Pro) antibody and SAMHD1 antibody (bottom blots). (E) As in (D), a specific phospho-antibody recognizing the Thr592 of SAMHD1 (pSAMHD1(T592)) was used. A representative experiment of two is shown in (D) and (E). (F) As in (E), lysates of monocytes and macrophages were used. A representative experiment of three is shown. (G) Monocytes before and after 3 days stimulation with M-CSF were infected with a VSV-pseudotyped NL4-3-GFP virus. Infection measured by GFP positive cells two days post-infection is shown in dot-blots from flow cytometry analysis. (H) As in (C), percentage of Ki67 positive cells in monocytes (left) or M-CSF derived macrophages (right) was quantified.

acrylamide gels (Figure 18D upper panel). We found that SAMHD1 has several phosphorylation sites that are responsible for the observed protein shift which were further confirmed by a specific phospho-threonine-proline (p(Thr)-Pro) antibody (Figure 18D lower panel). Lysates of activated CD4$^+$ T lymphocytes were also positive for immunoblotting with a specific antibody recognizing the phospho-threonine at the position 592 of SAMHD1 (pSAMHD1(T592), Figure 18E). M-CSF differentiated MDM were also stained for the proliferation marker Ki67 and a relation between Ki67$^+$ and HIV-1 susceptible MDM was found as previously suggested[137] compared to the original population of monocytes (Figures 18G and 18H). Positive staining of Ki67 also correlated with the phosphorylation of SAMHD1 as shown with the pSAMHD1(T592) antibody (Figure 18F).

To further confirm that observed migrating forms in western blot analysis corresponded to phosphorylated SAMHD1, we immunoprecipitated samples with an anti-SAMHD1 antibody attached to sepharose, and we treated them with λ-phosphatase in the presence or absence of phosphatase inhibitors. Lysates were then immunoblotted with an anti-phospho(Thr)-Pro antibody, demonstrating that either in activated CD4$^+$ T cells or in the human monocytic cell line THP-1 cells, SAMHD1 has several detected phosphorylated threonine residues followed by proline (or phospho-serine-containing sequences because of antibody cross-reactivity) that were effectively eliminated in the presence of phosphatase (Figure 19A).

As seen in Figure 19B, SAMHD1 has several phosphorylation sites located in the Vpx-interaction domain or in the Nuclear Localization Sequence (NLS) domain. Using cloning strategies, cells expressing indicated SAMHD1 mutations were generated and subjected to the same immunoprecipitation and immunoblotting techniques as in Figure 19A to confirm those residues as the responsible for the protein shift. In Figure 19C, it is shown how combined mutations in the N-terminal cluster of SAMHD1 led to the total disappearance of the slow migrating forms, suggesting a major contribution to the SAMHD1 phosphorylation.

Phosphorylations on serine residues are the most common among proteins, followed by threonine residues, representing major substrates for protein kinases. Although biological function of SAMHD1 is not well understood, we found phosphorylated forms of SAMHD1 that can be eliminated when treating with phosphatases, in Ki67$^+$ primary cells

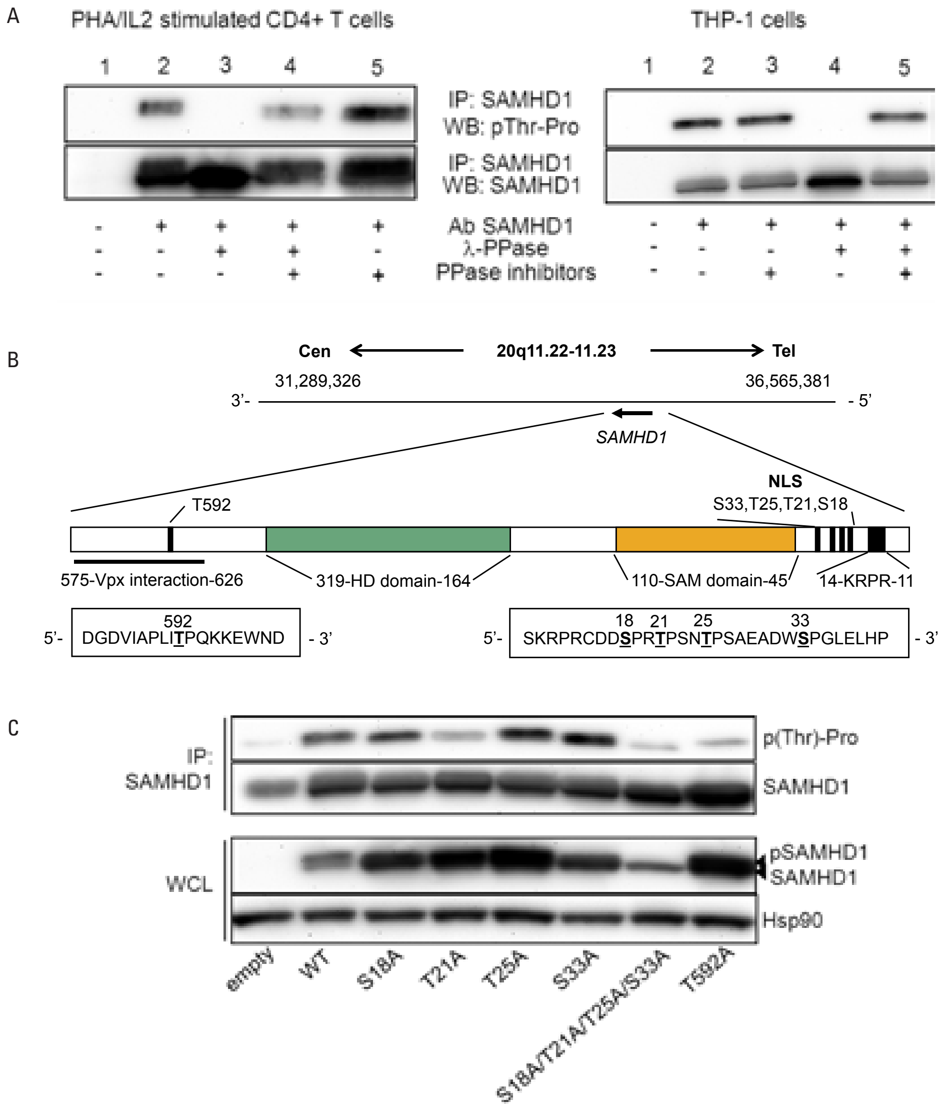


Figure 19. Phosphorylation of SAMHD1. (A) SAMHD1 was immunoprecipitated from lysates of PHA/IL-2 activated CD4+ T cells or THP-1 cells and treated with -phosphatase in the presence or absence of phosphatase inhibitors as indicated. One representative blot of three independent experiments is shown. (B) Schematic representation of human *SAMHD1* gene indicating previously identified phosphorylation sites. Aminoacid sequences were obtained from UniProtKB database. Potential phospho-serines (S) or -threonines (T) are shown in the graph indicating the aminoacid position. (C) HEK293T cells were transfected with an empty expression plasmid (empty) or the plasmid encoding the WT form of SAMHD1 (see materials and methods section). SAMHD1 was immunoprecipitated from cell lysates and blotted using a generic phospho-threonine followed by proline antibody (pThr-Pro; upper blot). Total SAMHD1 was used as control of immunoprecipitation. Cell lysates were also run in 6% polyacrylamide gels and SAMHD1 phosphorylation estimated by the appearance of slow migrating bands when immunoblotting with a specific anti-SAMHD1 antibody. Hsp90 immunoblotting was used as loading control. One representative blot of three is shown. (bottom blot).

that are susceptible to HIV-1 infection (Figures 18 and 19). Proliferating cells are characterized by increased levels of total RNA and DNA content, as well as high levels of Ki67 staining, that corresponds to the entrance at a cell dividing stage, where biological signaling pathways are extensive networks of different cellular proteins acting coordinately. A major role in controlling cell cycle is generally attributed to several Cyclins and Cyclin-dependent Kinases (CDK). SAMHD1 was reported to interact with CDK2 in a large-scale mass spectrometry approach[138], and CDK1 and CDK2 are closely related kinases that share many *in vitro* substrates[139,140]. Moreover, CDKs are active during cell cycle stages, phosphorylating other proteins in order to control their functions during cell cycle, where intracellular dNTPs also play an important role.

Thus, we decided to explore those mechanistic insights by which SAMHD1 is a substrate for phosphorylation and how is this related to cell activation and cell cycle in primary cells.

2.5. Cell cycle control and HIV-1 susceptibility are linked by a CDK6-dependent CDK2 phosphorylation of SAMHD1 in myeloid and lymphoid cells

To better understand the effect of SAMHD1 on HIV-1 infection, the phosphorylation-dependent regulation of SAMHD1 activity was further investigated using small interfering RNAs (siRNA) against well-characterized CDK known to regulate cell-cycle progression. We effectively downregulated (>60 %) CDK1, CDK2, CDK4, CDK5 and CDK6 mRNA in primary MDM (Figure 20A). Protein downregulation was further confirmed by western blot for CDK1, CDK2 and CDK6 (Figure 20B). siRNA-mediated knockdown of CDK2 and CDK6, and to a lesser (not significant) degree by CDK4 or CDK5, but not CDK1 led to reduced SAMHD1 phosphorylation as measured by the disappearance of the slow-migrating form of SAMHD1, the reduction of the p(Thr)-Pro signal after SAMHD1 immunoprecipitation and confirmed using the specific pSAMHD1(T592) antibody (Figure 20C-E).

CDK2 and CDK6 knockdown in MDM led to a reduction in the $Ki67^+$ staining (Figure 21A), suggesting that CDK2 and CDK6 control cell cycle progression and SAMHD1 deactivation, linking cell cycle control to SAMHD1 deactivation. Moreover, inhibition of SAMHD1 phospho-

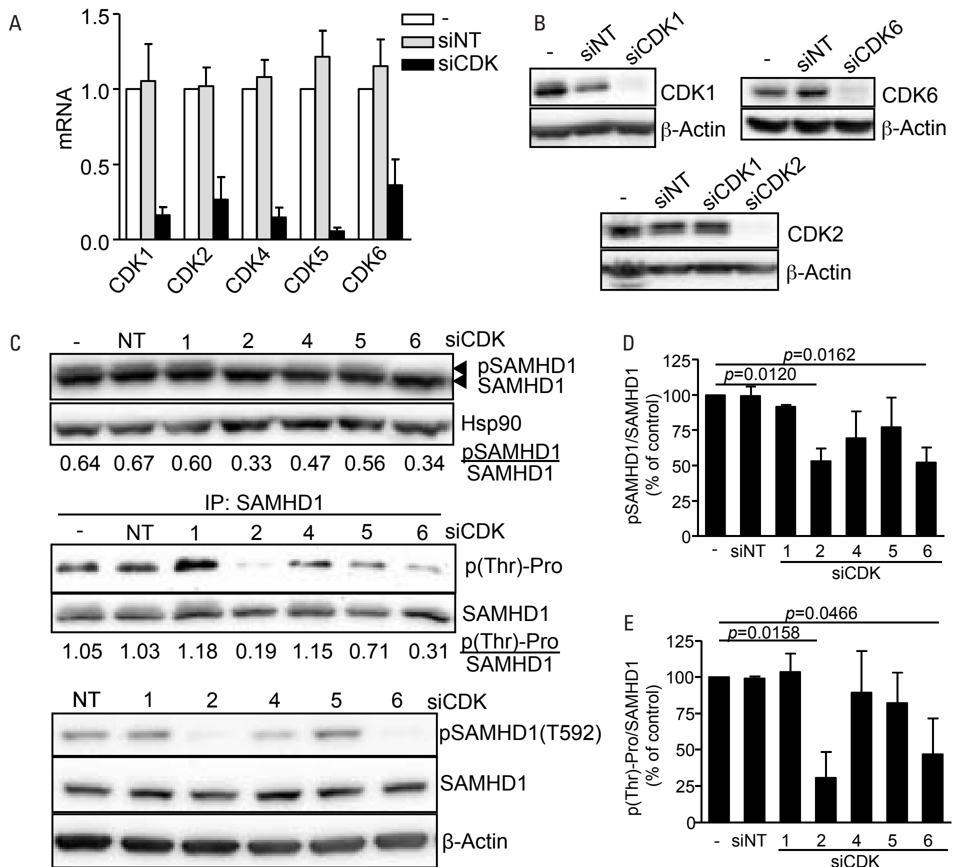


Figure 20. RNA interference of CDK2 and CDK6 inhibits SAMHD1 phosphorylation. (A) mRNA of CDK1, 2, 4, 5 and 6 was quantified in samples of macrophages treated with siRNA targeting each of the CDK and normalized to samples transfected without siRNA (-). Macrophages treated with a non-targeting siRNA (siNT) were also included. Mean±SD of the combined data of three independent experiments are shown. (B) Lysates of the siRNA treated macrophages were subjected to SDS-PAGE and blotted with an anti-CDK1, anti-CDK2, anti-CDK6 or anti-β-actin antibodies. One representative blot of two is shown. (C) Lysates of macrophages treated with siRNA targeting CDK (siCDK), a non-targeting siRNA (siNT) or mock-trasfected (-) were run, transferred and blotted with SAMHD1 and Hsp90 antibodies (top panel). Lysates were subjected to overnight immunoprecipitation of SAMHD1 followed by blotting with and anti-phospho-threonine followed by proline antibody (p(Thr)-Pro) and SAMHD1 antibody (middle panel). Lysates were run, transferred and blotted with the phospho-specific Thr592 antibody (pSAMHD1(T592)), total SAMHD1 and Hsp90 antibodies (bottom panel). Representative blots of three independent donors are shown. (D) Quantification of SAMHD1 phosphorylation relative to the mock-transfected control (-) as measured by the ratio between the density of the phosphorylated band and the unphosphorylated SAMHD1. (E) Quantification of SAMHD1 phosphorylation relative to the mock-transfected control (-) as measured by the ratio between (p(Thr)-Pro) antibody and total immunoprecipitated SAMHD1. For (D) and (E) graphs represent the mean±SD of three independent experiments.

rylation correlated with an effect on viral replication, as knockdown of CDK2 and CDK6 significantly reduced HIV-1 infection with a VSV-pseudotyped NL4-3 GFP-expressing virus (p=0.0021 and p=0.0060, respec-

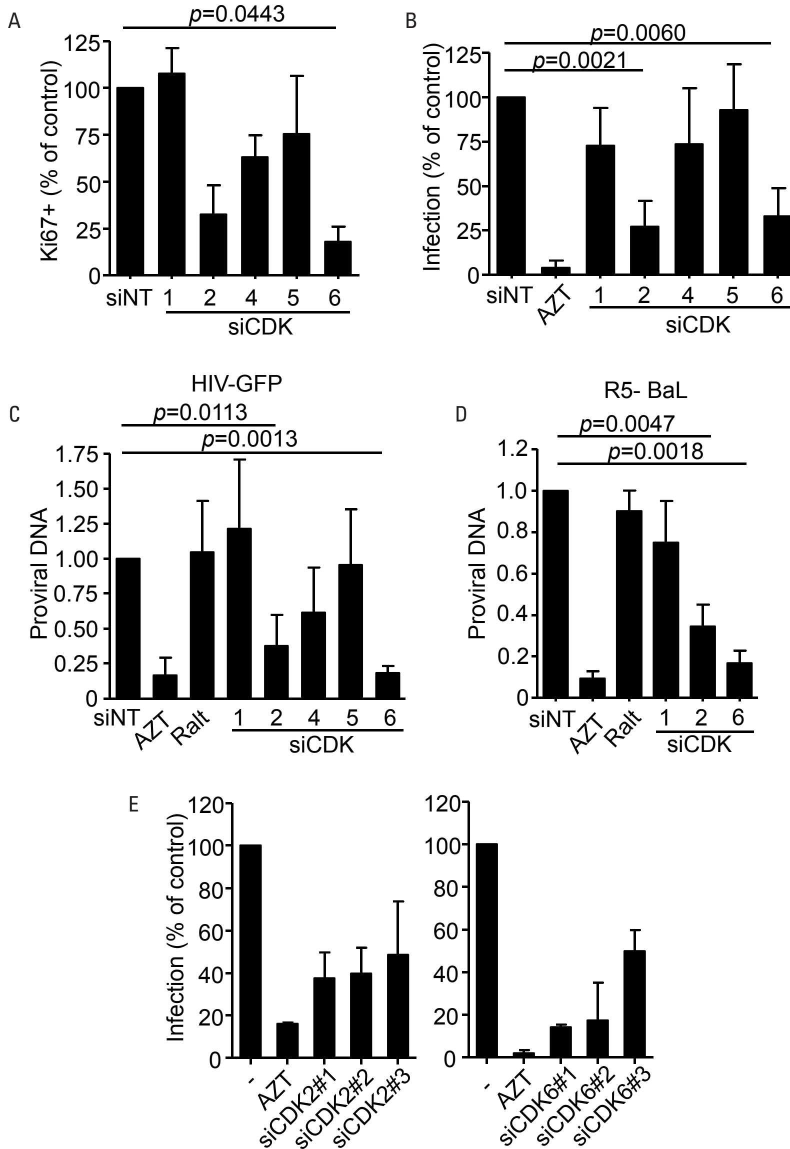


Figure 21. Knockdown of CDK2 and CDK6 blocks HIV-1 reverse transcription. (A) Percentage of Ki67 positive cells quantified by flow cytometry and normalized to the macrophages treated with siNT. Mean±SD of three independent donors is shown. (B) HIV-1 infection was measured as the percentage of GFP positive cells in siRNA treated macrophages and expressed as the percentage to siNT-treated cells. AZT (3 μM) was used as a control. Mean±SD of three independent donors is shown. (C) Transfected macrophages were infected with VSV-pseudotyped NL4-3-GFP virus and proviral DNA was measured 16h post-infection. Proviral DNA was normalized to the sample treated with non-targeting siRNA (siNT). As a control, non-transfected cells were treated with 3 μM of AZT and 2 μM Ralt. (D) As is (C), R5-tropic HIV-1 strain BaL was used to infect treated macrophages. Mean±SD of the normalized data from at least three independent donors are shown. (E) As in (B), macrophages were transfected with other commercially available siRNA targeting CDK2 or CDK6, infected and measured by flow cytometry and expressed as the percentage to non-transfected cells.

tively) (Figure 21B). However, inhibition of CDK1, CDK4 and CDK5 had a minor (not significant) effect.

To identify the viral replication step affected by CDK2 and CDK6 inhibition, proviral DNA formation after overnight infection was measured. Knockdown of CDK2 ($p = 0.0113$) and CDK6 ($p=0.0013$) and, to a lesser extent CDK4 and CDK5 ($p>0.05$), but not CDK1 significantly inhibited proviral DNA formation (~60% and ~80%, for CDK2 and CDK6, respectively) in MDM infected with a VSV-pseudotyped NL4-3 GFP-expressing virus (Figure 21C) or the fully replicative HIV-1 R5-tropic strain BaL (Figure 21D). As expected, the HIV-1 reverse transcriptase inhibitor AZT completely blocked proviral DNA formation, while the HIV-1 integrase inhibitor Ralt did not have an effect on proviral DNA formation. Confirmatory siRNA sequences targeting CDK2 and CDK6 showed similar inhibitory effects on HIV-1 infection (Figure 21E).

We have shown that SAMHD1 is fully active in resting, quiescent $CD4^+$ T lymphocytes but partially inactive in activated $CD4^+$ T cells (Figure 11). To address the role of CDK2 in this process, we obtained four different populations of $CD4^+$ T lymphocytes consisting of resting $CD4^+$ (Figure 22A, top left), PHA/IL-2 activated $CD4^+$ (Figure 22A, top right), sorted quiescent $CD4^+$ from the PHA/IL-2 activated population (G0, Figure 22A, bottom left) and $CD4^+$ at G1, M or G2 cell-cycle stage (Figure 22A, bottom right) as seen by RNA (Pyronin A) and DNA (7AAD) staining. Activation status of the populations correlated with the percentage of $Ki67^+$ cells (Figure 22B, top bar graph). Correspondingly, CDK2 expression was almost not detectable by Western blot in both unstimulated, resting $CD4^+$ T lymphocytes or in G0 cells sorted from the stimulated $CD4^+$ T lymphocytes (Figure 22B, lanes 1 and 3). Upon activation, CDK2 was expressed and activated, seen as phosphorylation at threonine 160 (Thr160) in the T-loop of the ATP binding site of CDK2 in cells that have entered the cell cycle (G1/M/G2) (Figure 22B, lane 4). Notably, expression and activation of CDK2 correlated with the phosphorylation of SAMHD1 indicating that CDK2 links cell cycle progression and SAMHD1 deactivation. As expected, only cells that have entered the cell cycle, showing >80% Ki67 positive staining (Figure 22C) were susceptible to HIV-1 infection measured as the formation of proviral DNA (Figure 22D) in contrast to G0, Ki67 negative cells (Figure 22C) sorted from the same population of PHA/IL2 activated $CD4^+$ T lymphocytes.

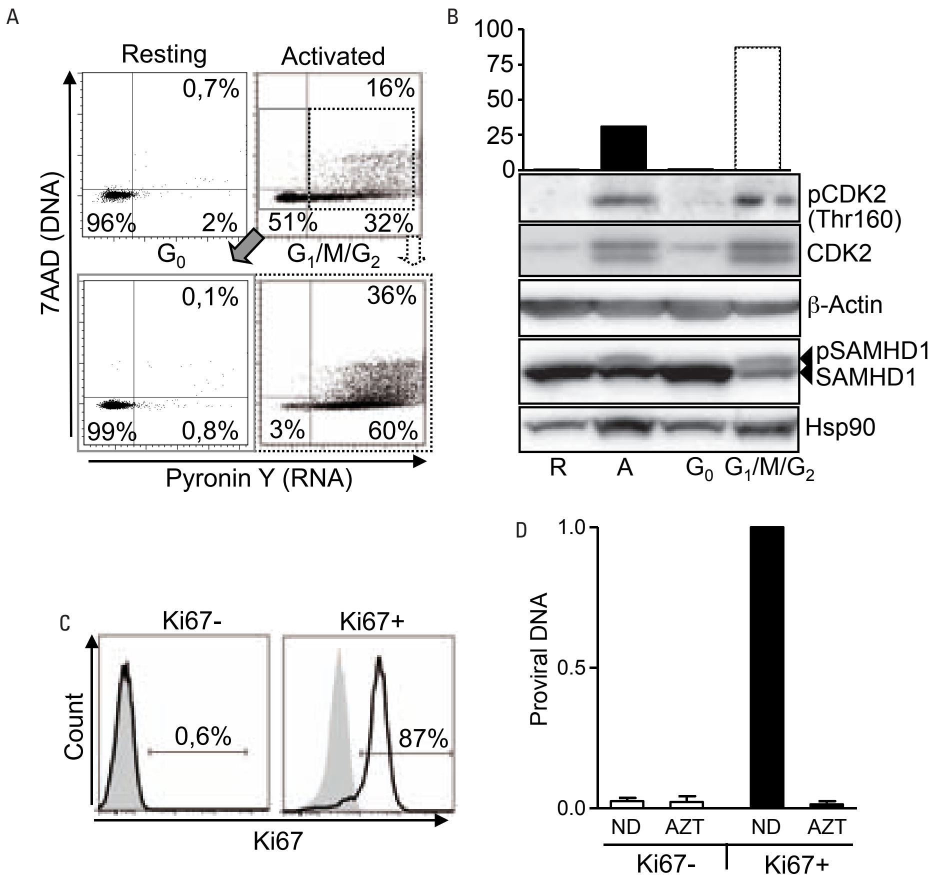


Figure 22. CDK2 links cell-cycle progression to G1 and SAMHD1 phosphorylation in primary CD4+ T cells. (A) Shown are representative flow cytometry dot plots of DNA and RNA content of resting (left top panel) or activated (top right panel) CD4+ T lymphocytes stained with 7AAD and pyronin. Cells at G0 stage of cell cycle (grey square) or at G1, G2 or M stage (dotted square) were sorted by flow cytometry and analyzed for DNA/RNA content (bottom dot plots). (B) Percentage of Ki67 positive cells (top bar graph) and western blot analysis (bottom blots) of the populations depicted in (A). Resting (R), activated (A) and sorted (G0 and G1/M/S) populations were stained with anti-Ki67 antibody and analyzed by flow cytometry. Cell lysates were immunoblotted with anti-phospho-CDK2 antibody raised against phospho-Thr160 (pCDK2(Thr160)), anti-SAMHD1, anti-CDK2, anti-Hsp90 and anti-β-actin antibodies. Representative results for one donor of three are depicted in the figure. (C) Histograms quantifying the percentage of Ki67 positive cells in G0 (left) and G1/M/S populations (right) sorted from PHA/IL2 activated CD4+ T lymphocytes as indicated in (A). Isotype staining was used as a control (grey histogram) in each population. Representative histograms of one experiment are shown. (D) Cells were then infected with NL4-3 and proviral DNA measured by qPCR. Data were normalized to the proviral DNA detected in untreated Ki67 positive (black bars) lymphocytes. AZT (3 μM) was used as control. Data represent mean±SD of two donors.

In sight of the results shown above, we addressed whether SAMHD1 was a substrate of CDK2 *in vitro*. We found that CDK1 and CDK2 were able to phosphorylate SAMHD1. As expected, both kinases phosphorylated histone H1 (Figure 23A, lanes 2 and 3). Phosphorylation by

CDK2 was drastically reduced but not completely abolished when the SAMHD1-T592A mutant was evaluated, indicating a prominent role for T592 (Figure 23B). Nevertheless, as it was shown in Figure 19, we also observed that SAMHD1 has several phosphorylation sites that are responsible for the protein shift. Moreover, CDK2 was co-immunoprecipitated with SAMHD1 when a HA-tagged SAMHD1 construct was overexpressed in 293T cells (Figure 23C).

On the other hand, we could not detect CDK6-mediated phosphorylation of SAMHD1, despite similar phosphorylation activity on histone H1 by CDK2 and CDK6 (Figure 23A, lane 4). However, we observed that CDK6 siRNA knockdown led to a slight reduction of CDK2 expression

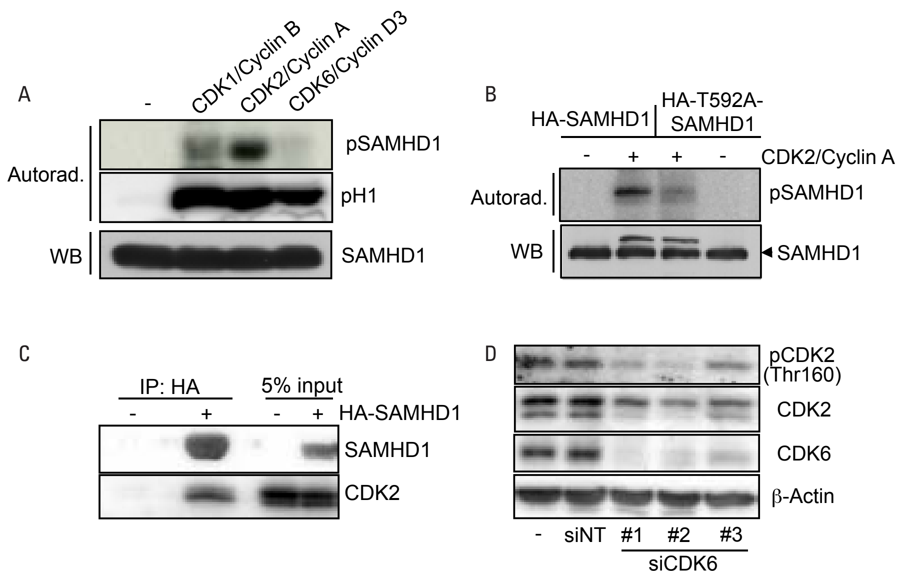


Figure 23. CDK6 acts upstream of CDK2, which directly phosphorylates SAMHD1. (A) HA-tagged SAMHD1 immunoprecipitated from HEK293T transfected cells was subjected to an *in vitro* kinase assay with recombinant complexes cdk1/cyclin B, cdk2/cyclin A and cdk6/cyclin D3 in the presence of radioactive ATP. SAMHD1 phosphorylation was monitored by autoradiography of the incorporated radioactive ATP. Histone H1, a known substrate of CDK, was used to monitor total CDK activity. An anti-HA antibody was used to blot for total SAMHD1. (B) HA-tagged WT SAMHD1 and Thr592Ala SAMHD1 mutant were immunoprecipitated from HEK293T transfected cells and subjected to an *in vitro* kinase assay with recombinant complex cdk2/cyclin A in the presence of radioactive ATP. (C) Lysates of 293T cells transfected (+) or not (-) with HA-SAMHD1 were subjected to immunoprecipitation with anti-HA agarose and analyzed by SDS-PAGE followed by immunoblotting with anti-CDK2 and anti-SAMHD1 antibodies. Approximately 5% of the amount of lysate used to perform the coimmunoprecipitation was run in parallel as an input control (5% input). (D) Western blot of lysates of untreated macrophages (-), macrophages transfected with a non-targeting siRNA (siNT) or treated with different siRNA targeting CDK6 (siCDK6#1, 2 and 3). Membranes were blotted with anti phospho-CDK2, anti-CDK2, anti-CDK6 and anti-β-actin antibodies. One of three independent experiments is shown for Figure 23A-D.

and a marked reduction of activation of CDK2 measured as phosphorylation at Thr160 (Figure 23D), indicating that CDK6 function affects CDK2 and therefore, tracing an indirect effect of CDK6 on SAMHD1, mediated by CDK2.

Cell cycle is coordinated by a series of CDKs and their inhibitory partners, with CDK1 as the ancestral mitotic kinase, and CDK2, CDK4 and CDK6 regulating cell cycle progression through the interphase (G1, S and G2 phases). Their activity is controlled by the availability of their cyclin partners and by physical interactions with members of the CDK-inhibitor family, like CDKN1A (p21).

We previously observed that RNA interference of CDK6 and CDK2 regulated SAMHD1 phosphorylation and HIV-1 reverse transcription, linking cell cycle progression during the G0/G1/S phases with SAMHD1 deactivation and therefore release of the HIV-1 restriction. In turn, CDK6 and CDK2 are regulated by cyclins D and E, respectively[141]. We consequently evaluated whether SAMHD1 activity was also regulated by those cyclins. We effectively downregulated in primary MDM all cyclins type D (cyclin D1, D2 and D3) and type E (cyclin E1 and E2), as well as mRNA of cyclins A and B types, whose function together with CDK2 and CDK1 in a posterior step during cell cycle should serve as a negative control for SAMHD1 activity regulators (Figure 24A). As expected, siRNA-mediated knockdown of D-type cyclins and also cyclin E2, but not cyclins A or B, led to a significant blockage of HIV-1 replication (Figure 24B).

CDK2 expression depends on the E2F transcription factor, which in turn promotes gene expression if it is released by the Cyclin D/CDK6-mediated phosphorylation of Rb[121]. As we have shown before, CDK2 co-immunoprecipitated with SAMHD1 and was able to phosphorylate it (Figure 23). On the other hand, we did not detect CDK6-mediated phosphorylation of SAMHD1 but we observed a CDK6-mediated reduction of phosphorylated CDK2. Such a relation between CDK6 affecting CDK2-dependent SAMHD1 function must be governed by cyclin D, so we investigated cyclin D role in SAMHD1 activity and HIV-1 replication.

D-type cyclins are usually referred as cyclin D; indeed, their expression varies in different cell types and species in order to be more efficient in driving Rb phosphorylation but its functions are closely related[142]. In MDM, cyclin D3 showed the strongest effect on HIV-1 replication followed by cyclin D1, while cyclin D2 showed a slight effect (Figure 24B). We assayed two different siRNA targeting cyclin D3 gene (*CCND3*) to

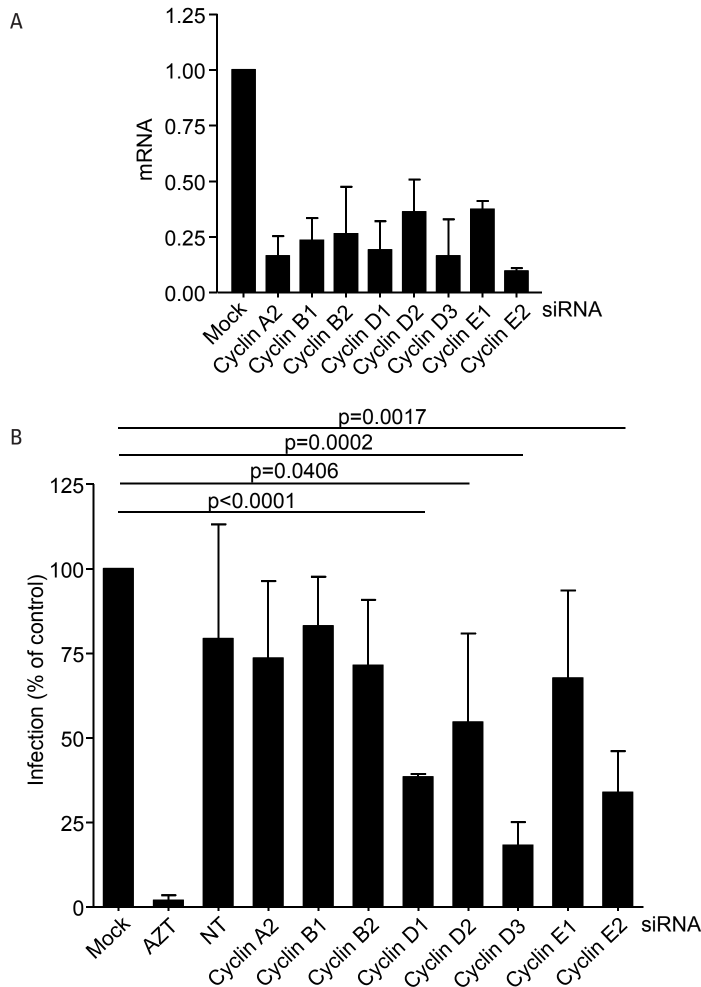


Figure 24. RNA interference effect of cell cycle-related cyclins on HIV replication. (A) A panel of siRNA were transfected in MDM and mRNA of the corresponding cyclin was measured by quantitative PCR and normalized to GAPDH expression. mRNA levels are shown compared to a non-transfected sample (Mock). Mean±SD of four independent donors is shown. (B) MDM were infected with a VSV-pseudotyped NL4-3-GFP virus and infection measured 72h later by flow cytometry and expressed as the percentage to non-transfected cells. Mean±SD of four independent donors is shown.

confirm the results. Again, cyclin D3-interfered MDM led to the disappearance of SAMHD1 phosphorylation but not SAMHD1 total expression (Figure 25A), and the consequent effect on HIV-1 at the proviral DNA level was a strong reduction of the viral replication at the reverse transcription step with both siRNA (Figure 25B). As expected, cyclin-D3 interfered MDM caused the loss of CDK2 phosphorylated forms, which is the active form of CDK2 that would lead to SAMHD1 phosphoryla-

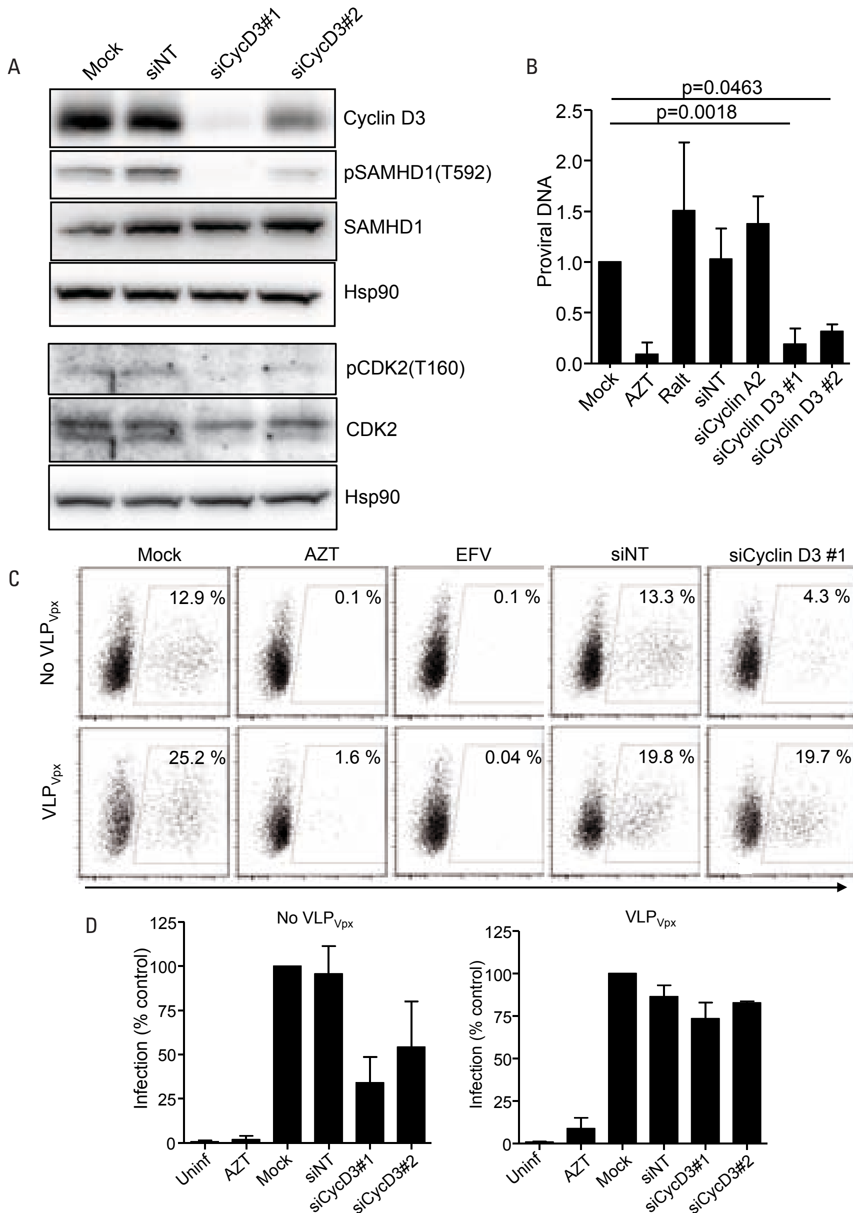


Figure 25. RNA interference of cyclin D3 blocks SAMHD1, CDK2 phosphorylation and inhibits HIV-1 reverse transcription in macrophages. (A) Western blots of untreated macrophages (Mock) or transfected with siRNA targeting indicated cyclins or a non-targeting sequence (siNT). Hsp90 antibody blot was used as loading control. One blot of three independent donors is shown. (B) Proviral DNA formation after 16h infection with HIV-1 BaL of macrophages transfected with the indicated siRNA or treated with AZT (3 μM) or Ralt (2 μM). Data were normalized to the proviral DNA detected in macrophages treated in the absence of siRNA (Mock). Data represent mean±SD of at least two donors. (C) siRNA-transfected MDM were treated or not with VLPVpx and then infected with VSV-pseudotyped NL4-3-GFP virus. Infection was measured 72h later by flow cytometry and dot-blots are shown. (D) Infection of MDM treated as in (C) was expressed as the percentage to non-transfected cells. Mean±SD of three independent donors is shown.

tion, as suggested in previous experiments. Importantly, when SAMHD1 was degraded using VLPVpx (Figure 25C and 25D), the inhibitory effect on HIV-1 replication due to *CCND3* gene silencing was completely lost, indicating that cyclin D3 effect on HIV-1 was mediated by SAMHD1.

2.6. Deciphering the pathway to SAMHD1 function: p21

We have shown that SAMHD1 is constitutively expressed in myeloid and lymphoid cells and participates in the tight control of their dNTP levels; SAMHD1 was shown to be deactivated in proliferating cells by a post-transcriptional mechanism that requires phosphorylation, therefore allowing virus replication.

In a recent publication, it was suggested that CDKN1A (p21) restricts HIV-1 replication in MDM by controlling the expression of the ribonucleotide reductase subunit R2 (RNR2), enzyme that, in turn, controls the intracellular dNTP pool required for HIV-1 reverse transcription[143]. p21 belongs to the Cip/Kip family of CDK inhibitors (CDKIs)[121] specifically controlling cell cycle progression through binding of cyclin-CDK1 or –CDK2 complexes[144]. Together with our previous results, the aim of the following part of the study was to determine a possible role of p21 on the CDK2-mediated SAMHD1 regulation, which in turn controls dNTP availability.

In this context, efficient RNA interference of p21 expression in MDM (Figure 26A) led to an increase in the number of $Ki67^+$ cells (Figure 26B). The siRNA-induced downregulation of p21 strongly enhanced the phosphorylation of SAMHD1 (Figure 26C) without affecting total SAMHD1 expression (Figures 26C and 26D).

When siRNA treated MDM silencing p21 transcripts were infected, the result was an increase in HIV-1 proviral DNA formation (Figures 27A and 27B) and virus replication (Figures 27C and 27D). These results strongly indicate that p21 affects SAMHD1-mediated HIV-1 restriction. In their experiments, Allouch *et al.* did not control SAMHD1 deactivation and, therefore, cannot exclude a role for SAMHD1 in p21-mediated lentivirus restriction[143]. Notably, the effect of p21 downregulation in HIV-1 replication disappeared when SAMHD1 was degraded by VLPVpx (Figure 27E), providing additional support to a role for SAMHD1 in p21-mediated HIV-1 restriction.

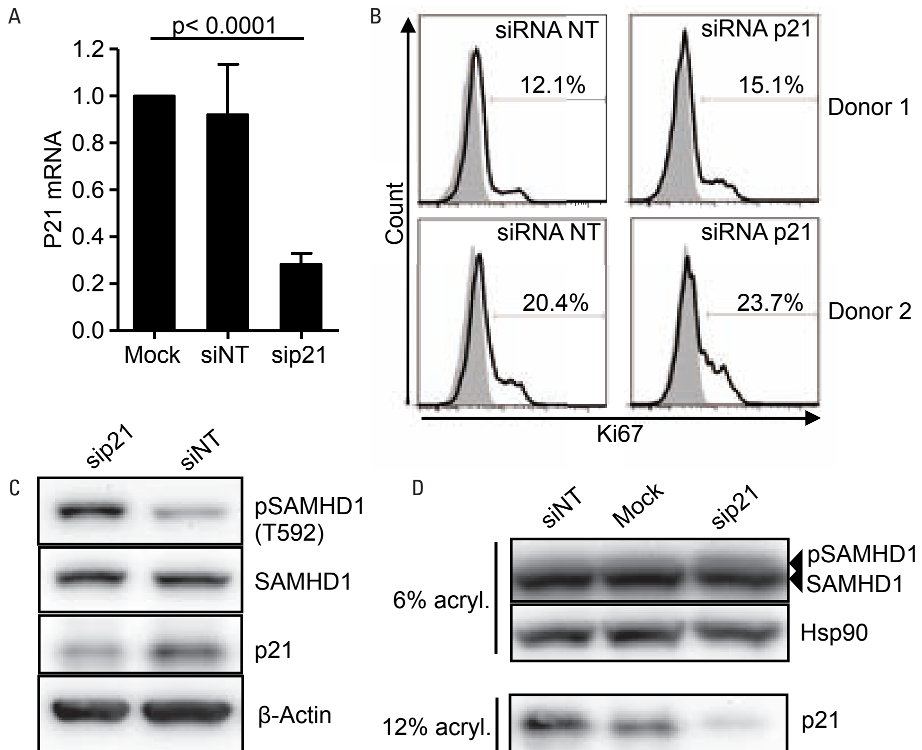


Figure 26. RNA interference of p21 induced SAMHD1 phosphorylation. (A) MDM were transfected with a siRNA targeting p21 (sip21) or a non-targeting siRNA control (siNT) and mRNA was quantified by qPCR. Mean±SD of four independent experiments is shown. (B) MDM were transfected as described in methods section and Ki67 positive cells were evaluated by flow cytometry. Histograms corresponding to two representative donors are shown. (C) Lysates of cells treated with the indicated siRNA were immunoblotted with antibodies recognizing the phosphorylated form of SAMHD1 at position T592, anti-SAMHD1, anti-p21 and anti β-actin. One representative blot of three is shown. (D) Alternatively, cell lysates were run in 6% polyacrylamide gels and SAMHD1 phosphorylation can be compared between samples as the appearance of slow-migrating forms.

As a resume, cell cycle control must include a coordinated regulation of RNR2 and SAMHD1 function as both tightly regulate dNTP availability. We envision that CDK activity may be the underlying mechanism explaining p21-mediated control of both RNR2 and SAMHD1 function and their control of the dNTP pool required for cell proliferation and virus replication (Figure 28).

Apart from regulating dNTP availability either through the RNR2 or SAMHD1 functions and its reflection on HIV-1 reverse transcription, increased p21 levels have been associated to elite control of HIV-1[145] and might be important in maintaining HIV-1 latency[146]. Therefore, we aimed at evaluating the expression of p21 in different HIV^{+} phenotypic groups.

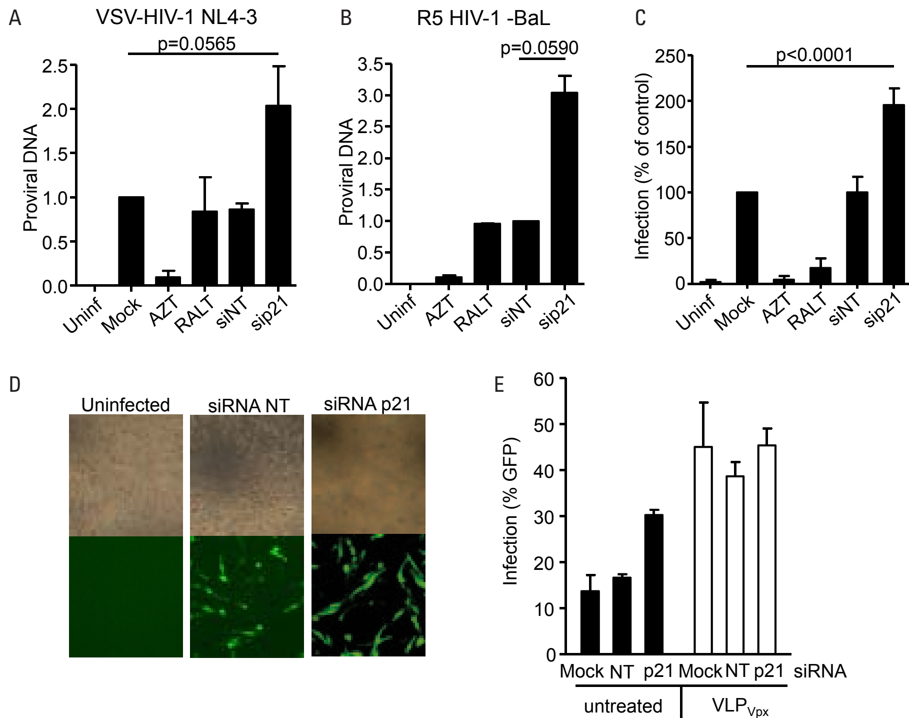


Figure 27. RNA interference of p21 induced increased HIV-1 replication. (A) MDM treated with the indicated siRNA were infected with a VSV-pseudotyped NL4-3 virus and proviral DNA was quantified by qPCR after 16h and normalized to the proviral DNA detected in macrophages transfected in the absence of siRNA (Mock). AZT (3 μM) and Ralt (2 μM) were used as control. Ct values for proviral DNA were normalized using RNaseP as housekeeping gene using the ΔΔCt method. Mean±SD of three independent experiments is shown. (B) As in (A), MDM were infected with the R5-tropic strain BaL. (C-D) Virus replication as measured by percentage of GFP positive cells at 48h post-infection with a VSV-pseudotyped NL4-3-GFP virus, evaluated by (C) flow cytometry or (D) immunofluorescence microscopy. For (C), mean±SD of six independent experiments is shown. (E) Infection of siRNA treated macrophages in the absence (black bars) or presence (white bars) of VLPVpx. Mean±SD of three independent infections of the same donor is shown. Three independent donors showed similar results.

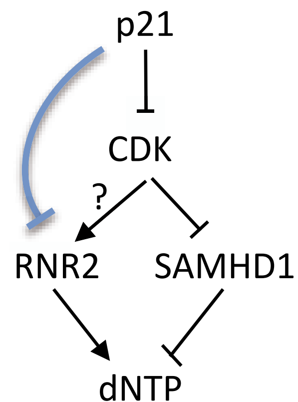


Figure 28. Model by which CDK and CDKI regulate dNTP pool through SAMHD1 and RNR2 function. p21 is a known inhibitor of cyclin-dependent kinases shown to regulate CDK1/2 function in cell culture and *in vivo*. In turn, it has been shown that SAMHD1 activity is controlled by CDK through phosphorylation leading to SAMHD1 deactivation and increased dNTP levels required for infection. Conversely, RNR2 increases dNTP. As shown in [143] p21 may directly affect RNR2 function. We envision that RNR2 may be controlled by p21 upstream of CDK.

The degree of cellular proliferation in all analyzed subjects (see Materials and Methods for study criteria) was quantified by intracellular Ki67 staining, as previously reported[147]. Elite Controllers (EC) patients showed similar levels of cell proliferation to healthy donors (HD). Conversely, the mean of $Ki67^{+}$ cells was significantly different between viremic patients (VP) and HD (p=0,0002) or VP and EC (p=0,0003) confirming increased T-cell activation and proliferation as a marker of disease progression in HIV^{+} individuals, a feature not present in the EC, who show cell proliferation values undistinguishable from uninfected subjects (Figure 29A).

In contrast with previous reports, we could not identify significant differences on the mRNA levels of the *CDKN1A* gene when comparing EC to HD and only a slight but significant (p=0,0298) decrease in p21 expression was observed in VP compared to HD or EC (Figure 29B). After stimulation with an anti-CD3/CD8 bi-specific antibody as described in [145], cells from both HIV^{+} groups (VP and EC) reached similar T-cell proliferation capacity, slightly higher than HD (Figure 29C). p21 levels were decreased in EC group after stimulation (p=0,0186) (Figure 29D), thus not being able to demonstrate the relationship between high p21 expression and elite control of HIV-1 infection.

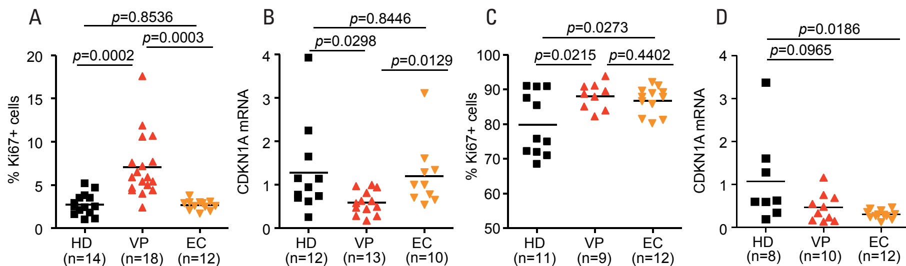


Figure 29. Increased p21 levels do not correlate with a better control of the HIV-1 infection. Percentage of Ki67 positive cells of unstimulated (A) or stimulated (C) PBMC from seronegative healthy donors (HD), viremic patients (VP) or elite controllers (EC), measured by flow cytometry. Relative mRNA expression of *CDKN1A* (p21) gene in unstimulated (B) and stimulated (D) cells from HD, VP and EC, respectively. Horizontal bars indicate mean values.

2.7. Research Application: Palbociclib, a selective inhibitor of CDK4/6, blocks HIV-1 reverse transcription through the control of SAMHD1 activity

As shown above, SAMHD1 phosphorylation may be directly regulated by CDK2. AT7519, roscovitine and purvalanol A are wide-spectrum CDK inhibitors that include CDK2 between their targets. Antiviral activity of these compounds was tested in MDM. All three compounds partially inhibited HIV replication in MDM infected with GFP-expressing NL4-3 HIV-1 pseudotyped with VSV-G protein (Figure 30A, left panel) at subtoxic concentrations (Figure 30B). Cellular toxicity observed with these compounds did not allow us to use higher concentrations. EC50 and CC50 could only be calculated for purvalanol A (EC50 = 2.21 ± 1.82 and CC50 = 18.77 ± 5.93), indicating a selectivity index (CC50/EC50) of 8. Importantly, HIV-1 inhibition was lost in the absence of SAMHD1, after treatment of macrophages with VLPVpx, while Ralt kept its activity (Figure 30A, right panel). Moreover, AT7519 ($p=0.0009$), roscovitine ($p=0.0099$) and purvalanol A ($p=0.0073$) significantly reduced the formation of proviral DNA after 4h infection of $CD4^+$ T lymphocytes (Figure 30C) at subtoxic concentrations (Figure 30D), which indicates that a preintegration step in viral life cycle is being affected by CDK inhibition. As expected, AZT blocked proviral DNA formation, while the integrase inhibitor Ralt had no effect. Consistent with our hypothesis, all three compounds partially decreased SAMHD1 phosphorylation at T592 or reduced the appearance of slow migrating forms of SAMHD1 (Figure 30E).

The CDK6 inhibitor PD-0332991 (palbociclib)[148,149] potently inhibited (EC50: 0.12 ± 0.076 μM) a VSV-pseudotyped NL4-3 HIV-1 infection in MDM in a SAMHD1-dependent manner (Figure 31A and 31B) at subtoxic concentrations (Figure 31C). CC50 was calculated in 21.4 ± 19.0 μM, indicating a selectivity index of 178. Moreover, palbociclib inhibited the formation of early viral DNA in MDM but not when SAMHD1 was degraded with VLPVpx (Figure 31D). Inhibition of HIV-1 infection in MDM correlated with a strong inhibition of the phosphorylation of SAMHD1 at T592 or the disappearance of slow migrating (phosphorylated) forms of SAMHD1 (Figure 31E).

Palbociclib also inhibited HIV-1 infection of $CD4^+$ T with VSV-pseudotyped NL4-3 GFP expressing HIV-1 (Figure 32A and 32B, EC50 was

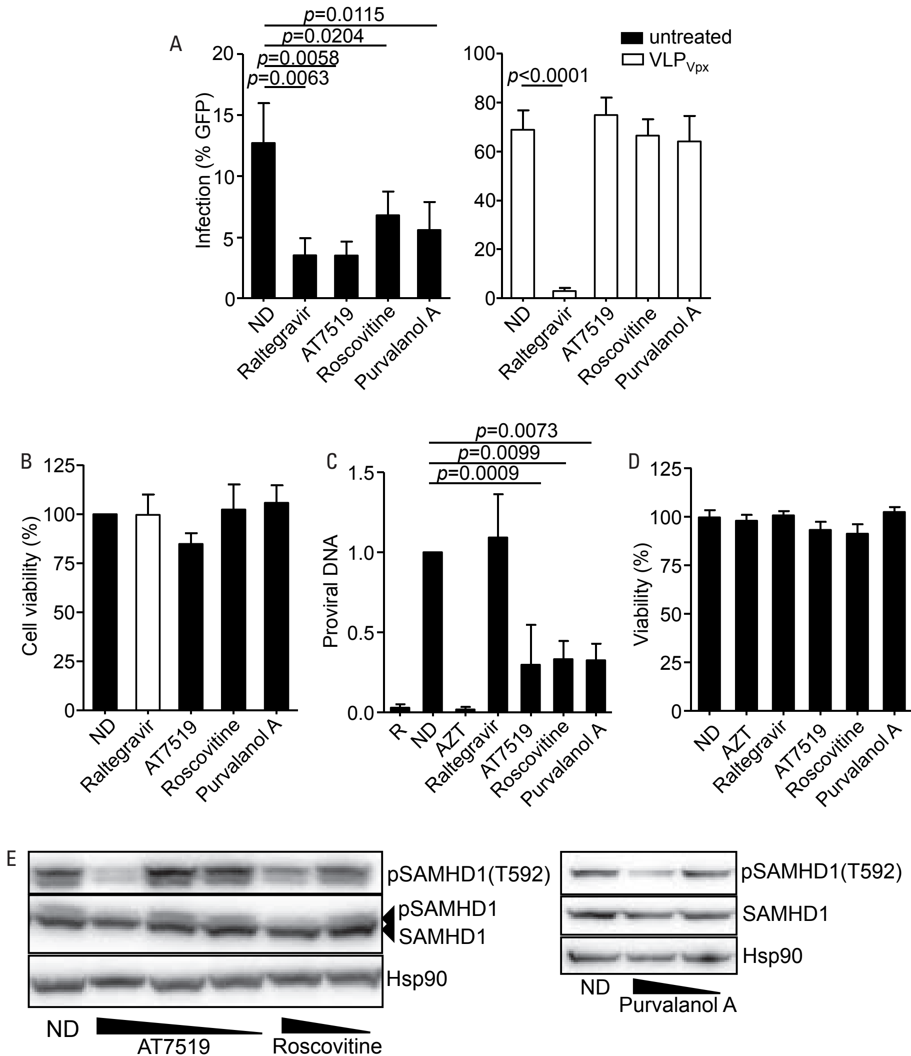


Figure 30. Pan CDK inhibitors regulate SAMHD1 phosphorylation and HIV-1 infection. (A) HIV-1 infection measured as percentage of GFP positive cells of macrophages treated without drug (ND), raltegravir (2 μM), AT7519 (0.5 μM), roscovitine (4 μM) or purvalanol A (4 μM) in the absence (left panel) or presence of VLPVpx (right panel). Mean±SD of four donors tested is shown. (B) MTT assay was performed in macrophages treated for two days with the concentrations indicated in (A). OD550/620 was measured and values normalized to the sample treated without drug (ND) to calculate the percentage of cell viability. Mean±SD of three donors is shown. (C) Proviral DNA measured 4h post-infection of $CD4^+$ T lymphocytes pre-treated with AZT (3 μM), raltegravir (2 μM), AT7519 (4 μM), roscovitine (20 μM) or purvalanol A (20 μM). Proviral DNA was normalized to the value of untreated lymphocytes (ND). Mean±SD of three donors is shown. (D) Percentage of live $CD4^+$ T lymphocytes quantified by flow cytometry after exposure to the concentrations of compounds indicated in (C) and normalized to the sample in the absence of drug treatment (ND). Mean±SD of three donors is shown. (E) Lysates of MDM treated with five-fold dilutions of the indicated compounds (starting concentration: AT7519, 0.5 μM; roscovitine, 4 μM and purvalanol A, 4 μM) were subjected to SDS-PAGE, transferred and immunoblotted with anti-phopho-SAMHD1, anti-SAMHD1 and anti-Hsp90 antibodies. Representative blots of three independent donors are shown.

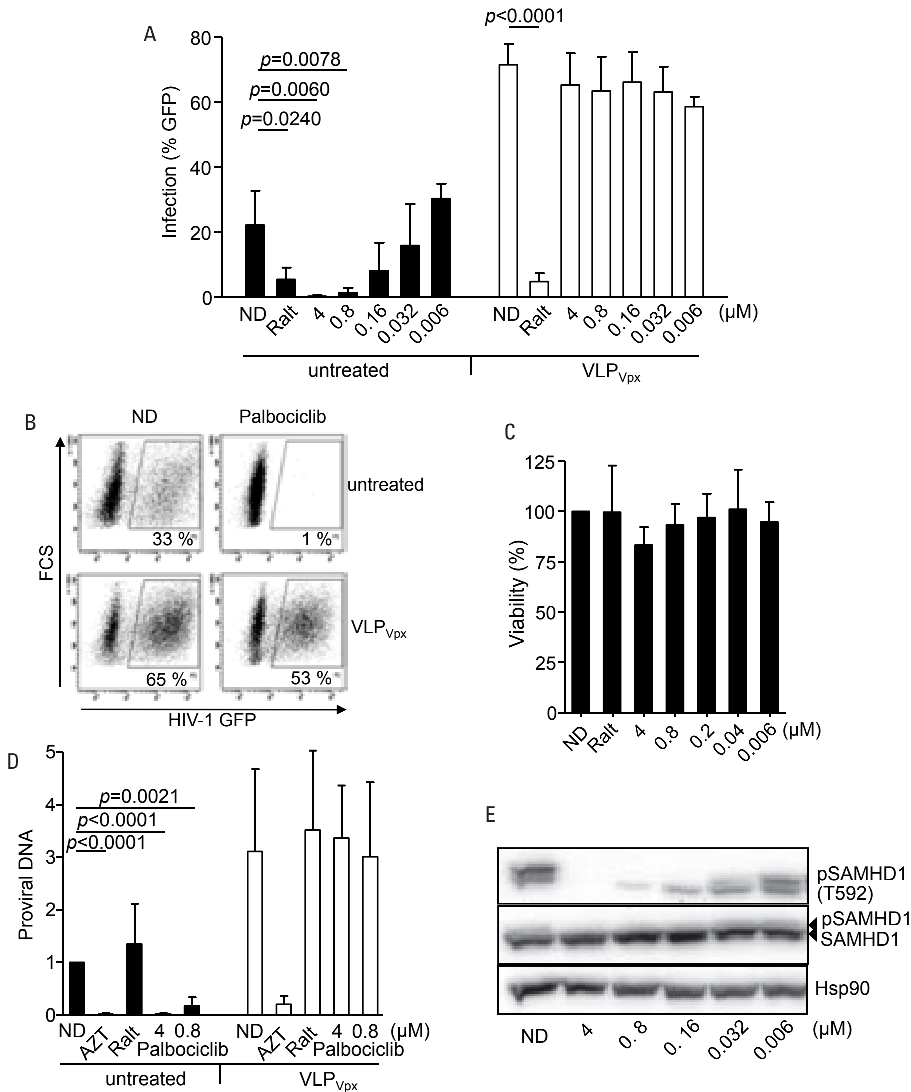


Figure 31. Palbociclib inhibits SAMHD1 phosphorylation, proviral DNA formation and HIV-1 replication in MDM. (A) Macrophages were pre-treated with the indicated concentrations of Ralt at 2 μM or palbociclib in the absence (untreated) or presence of VLPVpx and then infected with VSV-pseudotyped NL4-3-GFP virus. Infection was measured by flow cytometry. Mean±SD of three independent donors is shown. (B) Representative dot plots showing percentage of infected GFP positive cells measured by flow cytometry are depicted. (C) MTT assay was performed in macrophages treated for two days with the indicated concentrations of the drug. OD550/620 was measured and values normalized to the sample treated without drug (ND) to calculate the percentage of cell viability. Mean±SD of three donors is shown. (D) Proviral DNA formation after 16h infection with HIV-1 BaL of macrophages untreated (black bars) or treated (white bars) with VLPVpx in the presence of AZT (3 μM), Ralt (2 μM) or palbociclib (4 and 0.8 μM). Data were normalized to the proviral DNA detected in untreated macrophages (ND) in the absence of VLPVpx. Data represent mean±SD of three donors. (E) Lysates of MDM treated with the indicated concentrations of palbociclib were subjected to SDS-PAGE, transferred and immunoblotted with anti-phopho-SAMHD1, anti-SAMHD1 and anti-Hsp90 antibodies. Representative blots of three independent donors are shown.

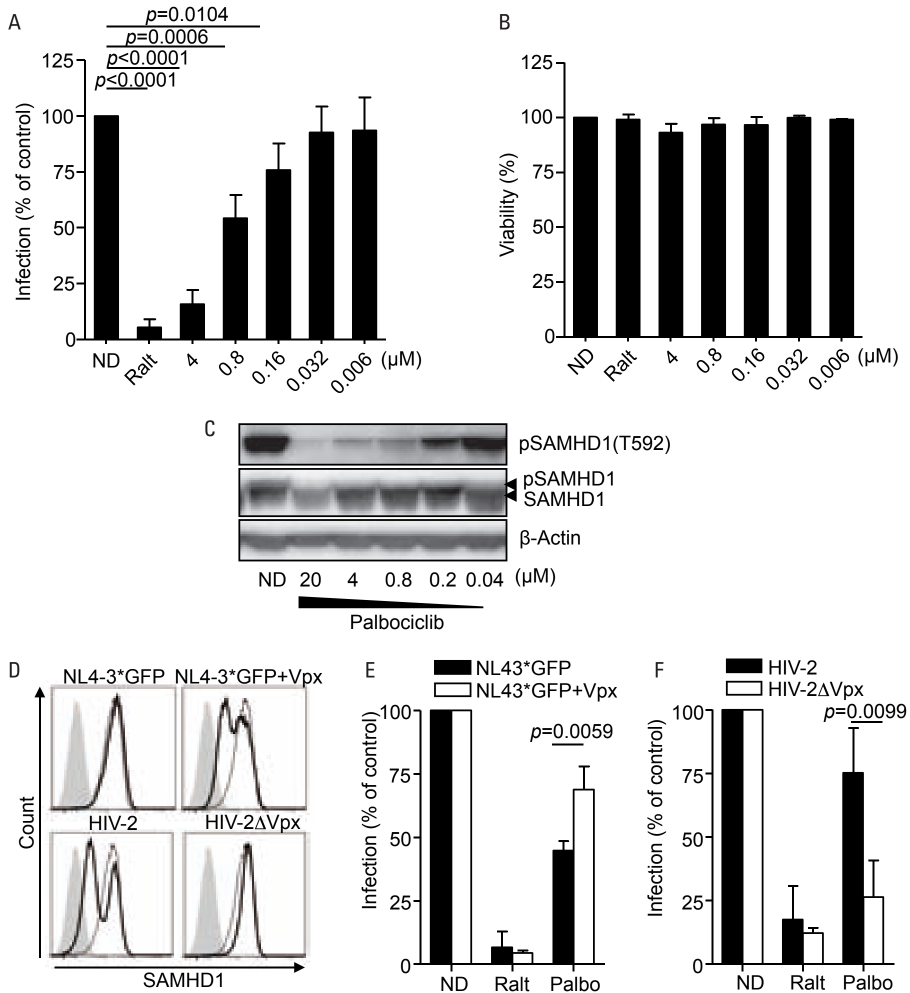


Figure 32. Palbociclib inhibits HIV-1 infection of CD4⁺ lymphocytes. (A) Activated CD4⁺ T lymphocytes were left untreated (ND), treated with Ralt (2 μM) or treated with serial dilutions of palbociclib and then infected with VSV-pseudotyped, NL4-3 virus and infection measured 48h later by flow cytometry. Infection was normalized to the ND control. Mean±SD of four donors is shown. (B) Percentage of live CD4⁺ T lymphocytes quantified by flow cytometry after exposure to the concentrations of compounds indicated and normalized to the sample in the absence of drug treatment (ND). Mean±SD of three donors is shown. (C) Western blots analysis of untreated CD4⁺ T lymphocytes (ND) or treated overnight with serial dilutions of palbociclib at indicated concentrations. Samples were blotted using anti-phospho-SAMHD1, anti-SAMHD1 and anti-β-actin antibodies. Representative blots of three donors are shown. (D) CD4⁺ T lymphocytes were infected with NL4-3*GFP carrying (NL4-3*GFP-Vpx) or not Vpx protein (NL4-3*GFP), HIV-2 GFP virus or the same HIV-2 virus lacking Vpx (HIV-2ΔVpx). Two days after infection, SAMHD1 was quantified by flow cytometry (heavy black line; upper histograms). Secondary antibody (grey filled histogram) and uninfected cells (black line) were used as controls. Representative histograms of one experiment are shown. Three independent donors were tested. (E) Activated CD4⁺ T lymphocytes were pre-treated in the absence of drug (ND) or the presence of Ralt (2 μM) or palbociclib (Palbo, 4 μM) and then infected with NL4-3*GFP carrying (white bars) or not Vpx protein (black bars) (F) As in (E), HIV-2 (black bars) and HIV-2ΔVpx (white bars) were used to infect activated CD4⁺ cells. Infections were normalized to the corresponding control in the absence of drug (ND). Mean±SD of four donors is shown.

0.98±0.417 μM). Palbociclib treatment led to a dose-response inhibition of SAMHD1 phosphorylation in PHA/IL2 activated CD4+ T lymphocytes (Figure 32C). As shown in macrophages, early viral DNA formation was also blocked by palbociclib incubation in CD4+ T lymphocytes (data not shown, see [122]). Importantly, when activated CD4+ T lymphocytes were challenged in the presence of fully-replicative NL4-3 carrying Vpx (NL4-3*GFP-Vpx) or a GFP-expressing HIV-2, both of which degrading SAMHD1 (Figure 32D), the antiviral effect of palbociclib was significantly lost (p=0.0059 and p=0.0099, respectively) compared with the control virus lacking Vpx (Figures 32E and 32F).

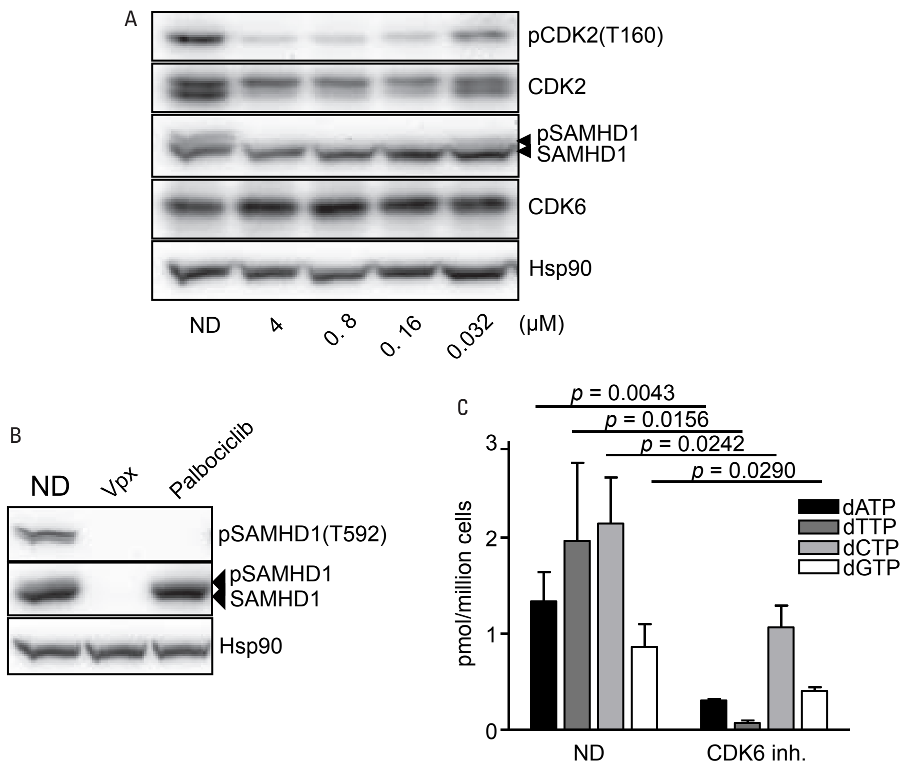


Figure 33. Palbociclib blocks CDK2 activation and decreases the intracellular pool of dNTP. (A) Western blot analysis of lysates of untreated MDM (ND) or macrophages treated with palbociclib at the indicated doses. Membranes were blotted with an anti phospho-CDK2 antibody raised against phospho-Thr160 (pCDK2(Thr160)). Anti-CDK2, anti SAMHD1, anti-CDK6 and anti-Hsp90 antibodies were used as controls. Representative blots of three independent experiments performed are shown. (B) Western blot analysis of lysates of untreated MDM (ND), macrophages treated with VLPVpx or treated with palbociclib (4 μM). Anti-phospho-SAMHD1, anti-SAMHD1 and anti-Hsp90 antibodies were used. One representative experiment of three is shown. (C) MDM treated as in (B) were lysed, dNTP extracted and measured by a PCR-based protocol. Data represent mean±SD of three independent donors.

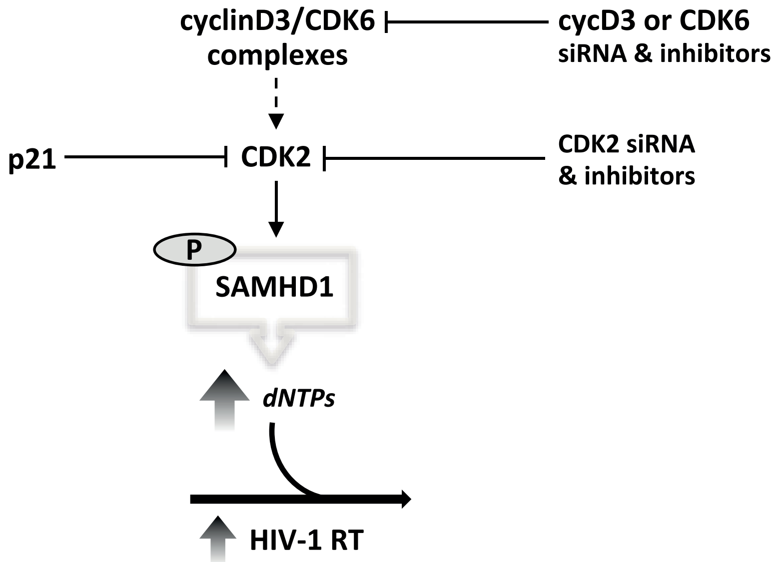


Figure 34. Proposed model leading to SAMHD1 phosphorylation in macrophages and CD4+ T lymphocytes. CDK activation is tightly controlled depending on the moment of the cell cycle. Mammalian cell cycle progression throughout the G1 phase is controlled by signalling pathways regulated by the cyclin-dependent kinases complexes CDK4/6-cyclinD and CDK2-cyclinE/A. The cell nucleotide pool required for cell division and HIV-1 infection is tightly controlled during the cell cycle. Phosphorylation of SAMHD1 by CDK2 is responsible for deactivation of the restriction activity on HIV-1 replication in primary myeloid and lymphoid cells. CDK6/cyclinD3 may directly or indirectly regulate CDK2 activity. RNA interference of CDK6/cyclinD3 or CDK2, pharmacological inhibition of CDK6 or CDK2 and the natural CDK2 inhibitor CDKN1A (p21) prevent SAMHD1 phosphorylation leading to increased dNTP available for reverse transcription and viral replication.

Palbociclib is a potent CDK4 and CDK6 inhibitor with an *in vitro* IC50 of 11 nM and 16 nM respectively, but completely inactive against CDK1, 2 or 5 (>10 μM)[148]. We were unable to see direct *in vitro* phosphorylation of SAMHD1 by recombinant purified CDK6/cyclinD3 (data not shown). However, palbociclib treatment led to a marked reduction of activation of CDK2 and a slight reduction of CDK2 expression (Figure 33A), without affecting CDK6 expression. These results suggest again that CDK6 functions upstream of CDK2 in controlling T cell proliferation that, in turn, controls SAMHD1 phosphorylation and HIV-1 replication.

To confirm the role of CDK6 in the SAMHD1–mediated restriction we evaluated the effect of palbociclib treatment in the intracellular dNTP pool availability for reverse transcription. As previously reported, VLPVpx-induced degradation of SAMHD1 in MDM led to increased intracellular dNTP levels (Figure 15). Importantly, inhibition of SAMHD1

phosphorylation by palbociclib (Figure 33B) was concomitant to a marked decrease in the intracellular dNTPs (Figure 33C).

Taken together, our results show that a potent inhibitor of macrophage and lymphocyte proliferation altering CDK6 function, blocks HIV-1 replication through the inhibition of SAMHD1 deactivation by CDK2-mediated phosphorylation (Figure 34), suggesting that inhibition of the cell cycle at specific stages could represent a novel antiviral strategy aimed to the combined effect of targeting viral replication and interfere with the proliferation of persistently infected cells.

DISCUSSION AND PERSPECTIVES

Current antiviral therapy has improved the life quality and expectancy of HIV+ individuals. Nevertheless, antiretroviral therapy continues to have limitations, including insufficient immunological reconstitution, selection of drug resistance, ongoing abnormal immune activation despite effective suppression of HIV-1 viremia and the inability to eliminate latently infected cells that are responsible for long-term viral persistence[11]. These challenges call for new approaches to be added to an effective treatment to finally cure HIV infection. An interesting approach is to identify unique markers between the myriad cellular proteins suggested as HIV host factors, impairing ongoing viral persistence in ART treated HIV infected patients.

From the viral point of view, inside the host there are "friends and foes" participating in cell metabolism processes and directly or indirectly determining virus replication. Two seemingly distinct elements of the cell metabolism, one controlling transcription and one severely altering the cell cycle have been used as examples of how virus replication is tightly dependent on the cell machinery. In fact, I have attempted to provide with two exceptional examples that host factors, either those that promote HIV infection or those restricting it, may be identified and modulated as to thwart virus replication and possibly HIV-induced disease. Moreover, the results may allow to speculate on alternative ways to prevent and even eliminate persistent HIV-1 infection.

In the first chapter, I provided a detailed analysis of the role of MED subunits in HIV replication and specifically on viral transcription, proving that Mediator complex is a relevant factor affecting HIV-1 replication. Among the high number of cellular factors modulating HIV infection[23,24], isolated Mediator subunits have been previously identified as potential hits in whole genome siRNA-based screenings[15,22,130]. Here, there were identified nine MED subunits affecting HIV-1 replication, belonging to different modules from the whole MED complex, although none of them from the CDK8

module[130]. I could replicate the effect of four subunits that appeared in at least two siRNA screens before: MED6, MED7, MED14 and MED28[16]. Of note, it was shown herein that siRNA mediated interference of MED subunits MED14 and MED27 significantly affected HIV-1 replication in primary cells (macrophages). However, not all the MED subunits identified to affect viral replication in HeLa cells appeared to have impact in HIV infection of MDM, suggesting either that additional posttranscriptional control mechanisms may regulate MED function[150] in MDM or that differences at the level of transcriptional regulation between cell types may exist.

Identified MED subunits were shown to control HIV-1 transcription by significantly reducing viral mRNA without affecting HIV-1 integration. Importantly, the four subunits previously identified as host factors affecting HIV replication in whole-genome siRNA screens affected particularly the early stage of HIV-1 transcription, limiting the formation of the TAR element. In contrast, newly identified MED subunits showed prevalent roles in the elongation stage of the transcription of viral genes, probably when P-TEFb recruitment via Tat and TAR takes over and results in abundant HIV replication. This suggests that Mediator complex may act as an interacting activator of P-TEFb, mediating RNAPII function and increasing rates of initiation as well as elongation of transcription as suggested[131].

Interestingly, MED14, MED23 and MED26 have been recently identified as important proteins for the transcription of early genes of human adenovirus 5[151] suggesting a broader role for Mediator complex in viral infections and a possible target for intervention. In addition to the effect on initiation and elongation steps during HIV transcription, Tat transactivation assays revealed that depletion of Mediator complex effectively inhibited Tat mediated activation of the HIV-1 LTR, as Zhou et. al showed for a subgroup of MED[21].

MED is a coactivator complex acting as a bridge between transcription factors that are mainly linked to the Tail module and the transcription machinery interacting strongly with RNAP II via its Head-module factors[152]. Although the whole complex is flexible in changing its conformation and is involved in many other molecular mechanisms[150], these results served me to select MED14 as the candidate for coimmunoprecipitation analysis to prove a possible interaction with the HIV-1 protein Tat that could signal downstream in the MED complex the initiation of HIV transcription. MED14 was found to co-immunoprecipitate with Tat HIV-1 protein and the inverse co-immunoprecipitation was also observed. This interaction was not mediated by other interacting MED subunits or DNA, although the presence of other

subunits cannot be excluded. These results indicate for the first time a direct *in vitro* interaction between MED14 subunit and HIV-1 Tat. More studies will be needed in order to define the intricate pattern of interactions within the three main Mediator modules in relation to Tat, although our study offers the starting point to identify the Mediator cluster mediating the HIV transcription process.

Although preliminary, the role of Mediator complex in virus replication and particularly on transcription of viral genes suggests new prospects for drug development. Whether the lack of a Mediator subunit could minimize or prevent expression of a specific set of genes remains to be established, but a growing number of studies have proposed Mediator as a therapeutic target for several diseases such as cancer, cardiovascular disease, metabolic and neurological disorders[57,60]. Targeting a single Mediator subunit might block a specific pathway yet allowing a majority of cellular transcription to function normally, and this effect could vary in depending both on gene and on cell-type.

Non-T cell activating latency reversing agents appear not to disrupt the latent reservoir *ex vivo* at all[153]. Future discoveries would establish whether specific Mediator complex subunits could be selectively inhibited and used as therapeutic targets in order to exert control on HIV latency. A promising prospect would be targeting specific MED proteins in order to repress HIV transcription from persistently infected cells, thus avoiding the long-term HIV propagation inside the reservoirs when combined with effective antiretroviral therapies to suppress circulating virus.

In the recent past years, numerous host factors have been identified for HIV by small-scale and high-throughput experiments, perhaps lightening more than ever the HIV dependency on its host due to its obligate parasitism nature. Patterns determining rates of evolutionary change in HIV and other viruses in order to develop such host dependency along with the common evolution include diverse aspects of viral biology, such as polymerase fidelity, genomic architecture and replication speed[154].

The identification and characterization of HIV host factors may serve also to understand host-viral coevolution, representing a model to reveal viral evolutionary dynamics. The increasing availability of HIV-host interaction datasets including physical, functional and genetic interactions would be able to indirectly explain parameters driving drug resistance and treatment failure. However, meta-analysis and software tools created to integrate and

visualize published data just found little overlap between the various sources[16,130]. Therefore, a more detailed analysis is required for the already incredibly high number of cellular proteins identified to modulate HIV infection.

Interestingly, only a few HIV factors have been unequivocally identified as genuine restriction factors, that is, cellular proteins that actively inhibit retrovirus replication and so protect cells from infection[81], compared with hundreds of essential HIV dependency factors and thousands of non-essential factors enhancing viral replication[82]. Their identification represents the best example of viral adaptation to host acquired antiviral responses, particularly to innate immune responses. Indeed, several studies indicated that mammalian cells have mechanisms for responding to HIV-1 through activation of innate sensors, but such responses are usually minimized by virus-specific and general host physiological processes[155].

Throughout the second chapter of this dissertation, we aimed at understanding SAMHD1-mediated restriction of HIV-1 infection. Beyond the technical findings on the role of SAMHD1 in HIV infection, our findings allowed us to understand the existence of a delicate line between HIV enhancing factors and HIV restriction factors. The balanced force into the cell to drive cell cycle progression in equilibrium with a sensing of viral forms that must activate innate immune responses requires a strict and well controlled feedback between all participating cellular proteins, whose particular and isolated effect on HIV replication sometimes could be antagonistic.

Our results allowed us to suggest how dNTPase activity of SAMHD1 is not only starving HIV but also contributing to the complex network governing the normal growth of the cell, where dNTP levels are extremely regulated in a template-dependent manner to complete cell cycle stages. In fact, both dNTP synthesis and destruction regulate the replication of both cell and virus genomes[190].

Competition with dNTP intracellular levels for incorporation into viral DNA during reverse transcription was the first effective pharmacological strategy to combat HIV infection[156]. NRTIs remain at the cornerstone of antiretroviral treatment after 30 years. Thus it is intuitive to think that SAMHD1 dNTPase activity may be influencing HIV-1 sensitivity to RT inhibitors. Similar to a previous report[120], our results revealed that SAMHD1 function did not affect viral sensitivity to NNRTI (that bind to an allosteric site of the HIV-1 RT and therefore do not compete against cellular dNTPs) but it reduced NRTI efficacy. Specifically, we identified a SAMHD1-dependent dif-

ference in viral sensitivity to thymidine but not to other NRTI analogs in both macrophages and $CD4^+$ T cells. In contrast, previous results showed differences in various analogs tested (AZT, ABC, ddC and TDF) in GM-CSF differentiated macrophages, but not in activated T cells[120] or in the THP1 cell line[120,157]. Although we showed similar results in different cell types[135] and a more extended panel of NRTI as well as relevant controls such as various NNRTI and the integrase inhibitor raltegravir that allowed us to better demonstrate the specificity of the effect, the discordance between studies using macrophages might be explained by the method used for differentiation in cell culture that affects the susceptibility to HIV-1 infection[158].

GM-CSF or M-CSF differentiated MDM are differently infected by HIV-1 and also have different dNTP intracellular levels, both parameters being higher in M-CSF-differentiated MDM[135]. In the same line, we also observed changes in HIV-1 susceptibility for IL12/IL18 differentiated MDM that became resistant to HIV-1 infection. Interestingly, SAMHD1 mRNA levels were found elevated and probably in a dephosphorylated state (data not shown) in IL12/IL18 MDM, rendering them with very low dNTPs levels when compared to M-CSF-differentiated MDM, thus explaining the acquired HIV non-permissiveness. Moreover, RNA interference or Vpx-mediated degradation of SAMHD1 completely reverted the restriction in the IL12/IL18 MDM, thus demonstrating that the main HIV-1 restriction in IL12/IL18 MDM is due to SAMHD1.

Considering that SAMHD1 is not able to efficiently hydrolyze NRTI[120,157] and the observation that exogenous addition of nucleosides mimicked SAMHD1 activity, our results are in accordance with the idea that reduced efficacy of thymidine analogs observed upon degradation of SAMHD1 may be the result of direct competition with increased intracellular dNTPs but not to the NRTI activation pathway[157]. Moreover, the HIV-1 RT has similar binding dissociation constants (Kd) in the low μM range for all dNTPs[159] but it may have differential affinity for each of the four dNTPs[160].

Taking all these considerations, our results suggest that SAMHD1 degradation or loss of function, may preferentially affect dTTP availability and therefore, significantly affecting the antiviral potency of thymidine analogs.

In agreement, enhanced viral replication or not when SAMHD1 is degraded can be determined by enzymatic RT constants Km and Kd below or above dNTP cell type-specific levels[159]. This is, in cell lines having significantly higher intracellular dNTPs levels, for example rapidly dividing cancer cells and transformed cell lines[161,162] like the MT-4 cell line, no differences in viral

replication were observed after SAMHD1 degradation. In contrast, in T cell lines and primary cells with lower dNTPs intracellular levels and resistance to HIV-1 infection, the Vpx mediated degradation of SAMHD1 induced an increase in viral replication, rendering cells susceptible to HIV-1.

Over the last three years, the mechanism controlling SAMHD1 activity has been a major issue[94]. Our group has been actively working to shed light on the cell-cycle-dependent post-translational modifications that regulate SAMHD1 antiviral activity in T cell and primary cell lines.

In September 2012 we first observed a change in the mobility of SAMHD1 when the protein was run in low-percentage acrylamide gels, when comparing resting and activated lymphocytes. This observation led us to hypothesize that a phosphorylation event could be controlling SAMHD1 activity.

Phosphorylation of SAMHD1 by CDKs, whose activation is tightly controlled depending on the moment of the cell cycle[121], have been finally demonstrated as the regulatory mechanism of SAMHD1-mediated viral restriction in cycling cells[114–116].

We showed that in primary macrophages and T cells, CDK6-dependent CDK2 phosphorylation of SAMHD1 appeared to control SAMHD1 restriction of HIV-1, although CDK1 was initially pointed as the kinase responsible for SAMHD1 phosphorylation in immortalized cell lines[114,115].

While CDK1 can execute all the events that are required to drive cell division in immortalized cells[163,164], other specialized cells may have unique requirements of specific CDK for controlling cell cycle and proliferation[165]. On the other hand, CDK1 and CDK2 are closely related kinases that share many *in vitro* substrates[139,140], suggesting that other CDK, different from CDK1, may play a role in regulating SAMHD1 phosphorylation.

SAMHD1 has been reported to interact with CDK2 in a large-scale mass spectrometry approach[138] and more recently in small scale experiments in lysates of stable cell lines[166] in agreement with our results. CDK dominant-negative mutants as those used by White et. al[115] may be problematic if they share the same activating partner, because associated cyclins could activate other CDK to replace its function[115,163]. Moreover, immortalized cell lines present deregulated cell cycle and abnormally rapid proliferation, so we found relevant to address the role of SAMHD1 phosphorylation in non-stable primary human cells susceptible to HIV infection.

It has been suggested that phosphorylation at residue T592 of SAMHD1 does not significantly affect the catalytic dNTPase activity of SAMHD1[114,115,167,168].

Our work indicates that phosphorylation at T592 correlates with the overall status of SAMHD1 phosphorylation measured as the appearance of slow migrating forms or as the staining with the specific phospho-Thr-Pro antibody, suggesting that this is a good indicator of SAMHD1 restriction. However, future studies should be addressed to determine the phosphorylation sites that may be required to clarify the role of phosphorylation in the function of SAMHD1.

Generated SAMHD1 mutants that incorporated diverse mutations in the N-terminal cluster of SAMHD1 led to the total disappearance of the slow migrating forms corresponding to SAMHD1 phosphorylation, as we shown. An evaluation of both dNTPase activity and HIV-1 restriction in SAMHD1-defective cells like pro-monocytic U937 expressing SAMHD1 single mutants was published, and no relation between phosphorylation and activity was found[115]. However, cells expressing our combination of SAMHD1 mutations would be expected to increase the dNTPase activity of SAMHD1 and consequently generate a decrease in intracellular dNTPs levels and HIV-1 restriction.

Moreover, it has been shown that the C-terminus of SAMHD1 (aa 596-626) was not required for dNTPase activity *in vitro* but it was required for full depletion of dNTPs *in vivo*, suggesting *in vitro* catalytic activity of SAMHD1 may not always directly correlate with its ability to decrease cellular dNTP pools[108]. In this line, Ryoo *et al.* suggested that phosphorylation of SAMHD1 at T592 is a mechanism that negatively regulates its RNase activity *in vivo* and that impedes HIV-1 restriction[168]. Despite having point mutants that cause loss of SAMHD1 function, in this study *in vitro* evidences are not shown.

Collectively, it seems clear that *in vitro* observations do not reflect SAMHD1 *in vivo* activity, either regarding dNTPase or RNAse functions, as mutations in SAMHD1 at T592 site, while showing differential phenotype in U937 cells, do not reveal *in vitro* differences. All together, I suggest that SAMHD1 activity in the cellular context may be regulated through additional factors or may require phosphorylation of multiple unidentified phosphosites.

The dNTPs usage by HIV to achieve a non-abortive reverse transcription was already described in the early 90's, when describing the 3'-azido-2', 3'-dideoxythymidine or AZT pharmacology[169]. Since then, it was known that size of the dNTP pool represents a major limiting factor for HIV-1 reverse transcription[107,170] and therefore drugs capable of decreasing dNTP pool size could be proposed as therapeutic alternatives[171]. Numerous reports have de-

scribed SAMHD1 capacity to modulate the concentration of intracellular dNTPs limiting its availability for the RT of incoming viruses[94,104,172–175].

The recent association between the already described RNase function of SAMHD1[176,177] to HIV-1 restriction independently of dNTPs, by enzymatically characterizing AGS-associated SAMHD1 mutations and mutations in the allosteric dGTP-binding site of SAMHD1[168] demonstrated that more studies are needed to decipher the link between SAMHD1 enzymatic activity and HIV-1 restriction. Nevertheless, different affinities to HIV-1 genomic RNA between SAMHD1 WT or mutated at T592 site are needed to confirm dissociation between dNTPs and HIV-1 restriction, as well as a complete quantification of all dNTPs, not only by assessing dGTP hydrolysis. Moreover, control drugs must be used to avoid false positives in every step during viral replication, either when quantifying proviral RNA or DNA before or after the reverse transcription, for example including inhibitors specifically blocking HIV entry, HIV reverse transcriptase and HIV integrase.

New perspectives are also opened in relation to a possible SAMHD1 restriction in other viruses, as it was already demonstrated for DNA viruses that do not employ reverse transcription during infection, but replication of their viral genomes is also dependent on intracellular dNTP concentrations, like Hepatitis B Virus (HBV) in liver cells[178] and Herpes Simple Virus 1 (HSV-1) in macrophages, where the SAMHD1 mediated regulation by phosphorylation does not seem to affect its antiviral activity[179].

Our evidences showing that a SAMHD1-related imbalance in the intracellular dNTP pool decreases the sensitivity to RT inhibitors like AZT or other thymidine analogs, or even the capacity of Palbociclib to decrease levels of dNTPs when inhibiting SAMHD1 phosphorylation offer an important contribution to underlie regulation of dNTPs as the mechanism of SAMHD1-mediated HIV-1 restriction.

During the cell cycle, I hypothesized that SAMHD1 deactivation by phosphorylation must occur prior to DNA synthesis (S phase of the cell cycle) when an elevated dNTP pool is required. Progression to early G1 phase is required for completion of HIV-1 reverse transcription and viral replication in T cells[180], suggesting that SAMHD1-restriction must be suppressed when cells enter G1.

According to the classical model of cell cycle control, cell cycle transition from G0 to G1 by CDK4/CDK6 leads to CDK2 activation during G1 that

triggers S phase[163,165]. Correspondingly, we have observed that upon activation, CDK2 is expressed and activated when resting, quiescent CD4+ T cells (Ki67-) enter G1 and start proliferating (Ki67+), and this correlates with the phosphorylation of SAMHD1. Moreover, CDK6 and CDK2 knockdown led to reduction in Ki67 staining providing further evidence that both CDK control cell cycle progression and SAMHD1 phosphorylation, linking cell cycle control to viral replication through SAMHD1 deactivation.

Early inactivation of SAMHD1 by CDK2 and CDK6 also suggests that other factors controlling cell cycle progression may impact on SAMHD1 activity. Confirming our results, the identification of cyclin D3 as the responsible partner governing CDK6 activity which in turn regulates CDK2-dependent pSAMHD1 also clarified the role of CDK in SAMHD1 deactivation in MDM, in contrast to previous *in vitro* results pointing to cyclin A2/CDK1 or cyclin B/CDK1[114,139].

As well, cyclin/CDK complexes have associated CDK inhibitors to better regulate cell entry or exit throughout cell cycle phases. p21 regulates the cell cycle through inhibition mainly of CDK2[181]. p21 has been associated to ribonucleotide reductase control of dNTP and HIV-1 restriction[143]. In addition, IFN-α, associated to SAMHD1 HIV-1 restriction, induces the expression of p21 and arrests macrophage cell cycle[144]. We have observed that knockdown of p21 induced an increase in SAMHD1 phosphorylation, concomitant to elevated proviral DNA formation and HIV-1 replication in MDM. This effect disappeared when SAMHD1 was degraded.

We do not exclude that CDK2 or other CDK that regulate cell cycle progression and transcription can control other steps of the HIV-1 replication cycle, neither the association between high levels of p21 and elite control of the HIV infection could be due to other molecular mechanisms (although we failed to observe that correlation). However, taken together, our results provide additional support to the role of CDK2-dependent phosphorylation of SAMHD1 in mediating restriction of HIV-1, acting coordinately with the cyclinD3/CDK6 complex and the CDK inhibitor p21, a model that is schematized in Figure 34.

The use of CDK inhibitors widely studied as anti-cancer drugs by their ability to inhibit cell proliferation may help to unravel the role of specific CDKs in HIV infection and viral restriction. Pan-CDK inhibitors have been shown to have anti-HIV-1 activity, an effect generally attributed to inhibition on viral transcription[182].

Of interest, it should be collectively appreciated that the control of RNAPII-based transcription is analogous to the regulation of the cell cycle, whereby a series of CDK/cyclin complexes, activities of which are restricted during each phase of the transcription cycle, are required to achieve the dynamic patterns of phosphorylation marks on the RNAPII C-terminal domain[141], and drive the step-wise progression from pre-initiation, initiation, elongation to termination[141].

Although CDK1 was the first identified CDK mediating RNAPII phosphorylation[183] to activate cell transcription, today a growing number of CDK have been added to this group, including CDK7, CDK9, CDK11 or the Mediator-associated CDK8, an important part of the puzzle during RNAPII-based transcription[132,150]. Here we showed that independently of additional anti-HIV activity, CDK blockade leads to inhibition of early viral DNA formation attributed to an effect over SAMHD1 phosphorylation. Moreover, we showed that CDK inhibitors specifically inhibited HIV-1 infection at the reverse transcription step in an exquisite SAMHD1-dependent manner. The observation that CDK inhibitors lost their effect when SAMHD1 was absent (after Vpx-induced degradation) strongly indicates the specificity of their effect.

Palbociclib is a specific inhibitor of CDK4 and CDK6[148,149] currently in Phase II/III clinical trials for treatment of advanced breast cancers. Treatment of primary cells with palbociclib led to inhibition of: SAMHD1 phosphorylation, CDK2 activation measured as the phosphorylation at Thr160, early viral DNA formation and virus replication. These results were in accordance to the model we had proposed, in which CDK6 induces the activity and stability of CDK2, allowing cell-cycle progression[121,141,165] and consequently SAMHD1 deactivation by phosphorylation.

It is well accepted that proliferating cells are preferentially susceptible to infection by HIV-1. Proliferating $CD4^{+}Ki67^{+}$ lymphocytes in HIV-infected patients are effector T cells accumulated in the G1 phase of the cell cycle but not in the S and G2/M phases[184] and homeostatic proliferation of latently infected cells is a major hurdle in eradicating infection in patients under active ART[185]. Moreover, the latent HIV reservoir may be integrated at sites that speed cell proliferation such as cancer genes[186], whose worst prognosis is the uncontrolled acceleration of cell division associated with elevated dNTPs levels, considered a biochemical marker of transformed/cancerous cells[187].

Anti-cancer drugs that inhibit dNTP synthetic pathways by targeting ribonucleotide reductases have been suggested to have antiretroviral activity[107,170,171,188,189], although the appearance of serious side effects outweighed its benefits for HIV patients. Previous studies analyzing the transcriptome of cells from long-term non-progressor HIV-1 patients have found changes in several clusters of genes, including those controlling the cell cycle[191–193] suggesting that differences in how cell cycle is regulated may play a role in the natural control of HIV infection. In relation to a possible toxicity and associated cell death related with cell cycle deficiencies, the added observation that cdk2-/- lymphocytes in mice may show similar proliferating capacity[165,194] collectively suggests that CDK2 or its upstream control by CDK6 could represent a potential target for antiviral treatment.

In the following years the deeper study of HIV-1 cellular factors will probably raise the possibility of a new class of "anti-HIV" therapeutics targeting the host[195]. By focusing their research on the pathway causing HIV-associated CD4 T-cell death in lymphoid organs, which contain more than 98% of the body's CD4 T cells representing the primary sites of HIV replication, whose extensive cellular contacts also mediate immune responses critical to HIV disease progression, Doitsh *et al.* suggested that it may be possible to break the pathogenic cycle of cell death and inflammation with safe and effective caspase-1 inhibitors. Interestingly, the permissively status of the host cell dictates the pathway through which lymphoid CD4 T cells die following HIV infection[195].

In agreement with those perspectives and speculating on the relevance of our results for HIV patients, a controlled-dose of palbociclib in chronically HIV-1 infected patients with controlled CD4 cell counts and receiving an effective ART treatment would reduce the viral reservoir by targeting only persistently infected proliferating T cells, indirectly avoiding the intensely inflammatory form of programmed cell death in HIV infected quiescent CD4 T cells from lymphoid tissue, and globally preventing HIV-1 pathogenesis and immune activation.

The development of chronic inflammation is a main disease-promoting process related with multiple interconnected mechanisms, including an improper metabolism of host nucleic acids that triggers chronic stimulation of the innate immune response in retrovirus-infected cells or also in autoimmune diseases such as systemic lupus erythematous or AGS[196]. It has been suggested that SAMHD1 could act as nucleotide sensor because SAMHD1 mutations associated with AGS exhibited both impaired nucleic acid-bind-

ing to ssRNA and ssDNA and complex formation[176]. Moreover, SAMHD1 restriction triggers the activation of the type-I IFN response after infection with the retrovirus human T cell leukemia virus type 1[197].

No evidences so far have been able to functionally explain the relevance of SAMHD1 as a sensor. However, the demonstration that death of abortively infected quiescent CD4 T cells was triggered by premature termination of viral DNA elongation during reverse transcription[198] suggests that SAMHD1 is related with a sensing activity in non cycling cells having the SAMHD1-dependent phosphorylation pathway inactivated, thus controlling the accumulation of intracellular viral DNA in relation to the dNTP availability and modulating an innate cellular response.

Although big efforts were collectively done to elucidate SAMHD1 mechanism, lots of questions continue arising to cover whether SAMHD1 acts as a DNA sensor in coordination with other host proteins; to understand the physiological relevance of SAMHD1 activity; to answer whether cell cycle progression and innate immunity converge in elements like SAMHD1 and how is this phenotypically reflected depending on cell type; or even which is the mechanistic link between SAMHD1 restriction of reverse transcription, sensing of retroviral reverse transcription intermediates and the initiation of antiviral and apoptotic responses.

To conclude, retroviruses have evolved to take profit of extremely complex cellular networks. There is a significant period of common history that extends back millions of years, which had a significant impact on host-gene evolution, as dated from mammalian genome sequencing and bioinformatics[199]. In particular, the reliance of HIV-1 upon numerous cellular host factors for nearly every step of viral replication is well appreciated for various authors[15,16,21,22]. In some cases the roles of viral proteins include functions that serve as countermeasure to host restriction factors.

Strikingly, although HIV-1 gained an apparent evolutionary advantage compared to HIV-2 all over the world, HIV-1 accessory proteins did not evolve a specific function to counter SAMHD1 activity similar to HIV-2 or SIV, presumably because by avoiding the activation of cytoplasmic sensors and thereby limiting the induction of inflammatory cytokines, HIV-1 acquired the capacity of reducing the global impact of IFN production in certain cell types[200]. In other cases, HIV proteins recruit already assembled cellular machinery to perform essential roles in the virus life cycle, as it has been suggested for Tat and Mediator complex.

Advances in antiretroviral combination therapy lasting the past two decades have transformed HIV-1 infection from a fatal disease into a chronic medical condition that in many cases does not compromise life quality. But not everything is done with HIV research. Persistent infection in reservoirs such as latently infected $CD4^+$ lymphocytes and cells of the macrophage-monocyte lineage impedes the total remission of HIV infection, affecting at the long-term selection of drug resistances and the derived immune activation, representing a risk for present and future ART treated HIV infected patients.

The basic research on the mechanism of HIV cellular factors is important for understanding HIV pathogenesis and must be considered for future "anti-HIV" drug approaches that may target the host rather than the virus to cooperatively cover ART limitations.

CONCLUSIONS

1 The Mediator complex is required during HIV-1 transcription. Identified Mediator subunits affect Tat-mediated transactivation of the HIV-1 promoter LTR and this effect seems to require a direct interaction with Tat. Once HIV-1 transcription is ready to initiate, a MED subgroup (MED6, MED7, MED11, MED14 and MED28) participate in the transcription of the nascent viral mRNA TAR. The elongation phase of HIV-1 transcription should be mediated by MED26, also helped by other MED subunits like MED14 and MED30 that had a major role during the formation of unspliced viral transcripts.

2 Although the biological mechanism of Mediator complex is still not completely understood, as a proof of concept it seems reasonable that controlling non-essential MED subunits to repress or activate viral genes, it may be possible to specifically control HIV transcription in susceptible cells to modulate HIV infection.

3 The control of HIV replication by SAMHD1 is tightly regulated by cell cycle progression, mediated by cyclin-dependent kinases that phosphorylate to finally control dNTPs intracellular levels through SAMHD1 dNTP hydrolase activity.

4 CDK6 and its cyclin partner D3 acts upstream of CDK2 that phosphorylates and activates SAMHD1, while p21 is a CDK2 inhibitor that has an opposite function. Although other participating elements are not excluded, this represents an equilibrium of forces in the cell- signaling pathway that allows a tight control of the cellular dNTP pool.

5 SAMHD1 determines the antiviral potency of Thymidine analog RT inhibitors that depends on a direct competition with intracellular dNTP levels during reverse transcription and underlies that regulation of dNTPs is the mechanism of SAMHD1-mediated HIV-1 restriction.

6 The SAMHD1 signalling pathway may be susceptible for its use in the development of new therapeutic approaches against HIV-1 infection. The activity of the CDK6-inhibitor palbociclib suggests that inhibition of the cell cycle at specific stages could represent a novel antiviral strategy.

7 Although current therapy for HIV infection represents a triumph for modern medicine, remained challenges like viral persistence in reservoirs and long-term immune activation must be addressed with new approaches. The study of HIV host factors may provide insights into mechanisms of cell regulation of viral infection and offers new potential targets for combined antiviral therapies.

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