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Functional aspects of new helper factors for HIV replication

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MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada pelo Prof. Doutor João Gonçalves (Faculdade de Farmácia da Universidade de Lisboa) e pela Prof. Doutora Maria Filomena Caeiro (Faculdade de Ciências da Universidade de Lisboa)

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Abbreviations

Ago	Argonaute protein
Ago2	Argonaute protein 2
AIDS	Acquired Immunodeficiency Syndrome
Atg12	Autophagy related protein 12
Atg5	Autophagy related protein 5
BAF	Barrier-to-Autointegration Factor
BlaM	Beta-lactamase
CA	Capsid
CARD	Caspase activation and recruitment domain
Cardif	CARD adaptor inducing IFN-β
Cdk9	Cyclin-dependent protein kinase-9
CIB1	Calcium- and integrin-binding protein 1
CIB2	Calcium- and integrin-binding protein 2
CTD	Carboxy-terminal domain
DICER1	Double-stranded RNA-specific endoribonuclease type III
DCP1A	mRNA-decapping enzyme 1A
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EDC4	Enhancer of mRNA deccaping 4
ELISA	Enzyme-Linked Imunosorbent Assay
Env	Envelope protein
Gag	Group-specific-antigen protein
GW182	Glycine(G)-tryptophan(W) repeats of 182 kD
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HMG I(Y)	High mobility group proteins
IFN	Interferon
IN	Integrase
IPS-1	IFN-β promoter stimulator 1
IRF	IFN regulatory factor
ISG15	IFN-stimulated gene 15
LGP2	Laboratory of genetics and physiology 2
LSm1	Sm-like protein, homolog 1
LTRs	Long-terminal repeats
MA	Matrix
MAVS	Mitochondrial antiviral signaling adaptor
MDA-5	Melanoma differentiation associated gene-5
miRNAs	MicroRNAs
mRNA	Messenger RNA
NC	Nucleocapsid
Nef	HIV negative factor
NF-κB	Nuclear factor kappa B
NRLX1	Nucleotide-binding domain and leucine-rich-repeat-containing family 1
OTUD5	OTU domain containing 5 or deubiquinating enzyme A

P-bodies	mRNA processing-bodies
PIC	Pre-integration complex
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1
Pol	Polymerase
PPP1R14D	Protein phosphatase 1 regulatory subunit 14D
PR	Protease
RCK/p54	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 6 (RNA helicase, 54kD)
Rev	Regulator of Virion
RIG-I	Retinoic acid inducible gene-I
RISC	RNA-induced silencing complex
RLH	RIG-like family of helicases
RNA	Ribonucleic Acid
RNAi	RNA interference
RNAP	RNA polymerase
RNASEN	RNase III nuclear or Drosha
RNF125	Ring finger protein 125
RRE	Rev responsive element
RT	Reverse transcriptase
SGK	Serum/glucocorticoid regulated kinase or Serine/threonine-protein kinase
shRNAs	Short hairpins RNAs
siRNAs	Small interfering RNAs
SnK	Serum-inducible kinase
Sp1	Specificity protein 1
ssRNA	Single-stranded RNA
SU	Surface
TAR	Trans-activating response element
Tat	Trans-activator of Transcription
TLR	Toll-like receptors
TM	Transmembrane
TNRC6A	Trinucleotide repeat-containing gene 6A protein or human related GW182
TRAF	Tumor necrosis factor (TNF) receptor associated factor (TRAF)
TRIM25	Tripartite motif-containing 25
Vif	Viral Infectivity Factor
VISA	Virus-induced signaling adaptor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-g	Vesicular Stomatitis Virus glycoprotein
XRN1	5'-3' exoribonuclease 1

Reagents and Simbols

Abs	Absorbance
CPRG	Chlorophenol red-β-D-galactopyranoside
DMEM	Dulbecco's minimal essential médium
HEK	Human Embryonic Kidney
HeLa-P4	HeLa-CD4-LTR-β-gal
LB	Luria-Bertani Broth
MOI	Multiplicity of infection
PBS	Phosphate-Buffered Saline solution
PCR	Polymerase chain reaction

Abstract

Human immunodeficiency virus (HIV) depends on the host cell machinery to complete its life cycle. Several host proteins may help the virus to replicate and others have the ability to suppress its replication. These helper and restriction factors might be involved in many different pathways, such as RIG-I like helicases (RLH) signaling or microRNA (miRNA)-mediated silencing pathways, or could be a member of a specific group of proteins, like kinases and phosphatases. Here, we conducted a shRNA screen focused on innate antiviral defenses. Our study discovered 4 factors involved in HIV-1 replication: two participate in miRNA silencing, RNASEN and TNRC6A; and two proteins are regulators of RLH signaling pathway, ISG15 and OTUD5. RNASEN and ISG15 showed a helper factor nature regarding HIV-1, while TNRC6A and OTUD5 appear to have a restriction effect in HIV-1 replication. We also proceeded to the characterization of previously identified helper factors in other shRNA screen performed by Rato *et al*. For this purpose, we evaluated the effect of 13 proteins from the 14 identified in HIV-2 cycle, which exhibited a similar outcome from the one observed in HIV-1. From all kinases and phosphatases identified, we observed that one protein, CIB2, when overexpressed led to an enhanced LTR-driven expression, suggesting a role for CIB2 in HIV-1-LTR transcription. Moreover, we assessed that two of the identified proteins, SGK and CIB2, are important in HIV-1 entry, since their knockdown reduces the number of fusion events. In conclusion, this study highlights the power of small scale RNAi screens, providing new insights for the complex host-HIV interactions and instigating new possibilities for antiviral strategies.

Key words: HIV-1; HIV-2; RNAi; miRNA silencing; RLH signaling; CIB2; SGK

Resumo

Introdução

O Vírus da Imunodeficiência Humana do tipo 1 (*Human Immunodeficiency Virus type 1, HIV-1*) é do género *Lentivirus* da família *Retroviridae*. O seu genoma é formado por duas moléculas de RNA com cerca de 9 kb, flanqueadas por repetições terminais longas (*long terminal repeats, LTR*) e com 9 grelhas de leitura abertas (*open reading frame, ORF*). As 4 proteínas Gag – matrix (MA), cápside (CA), nucleocápside (NC) e p6 – e as 2 proteínas Env – glicoproteína de superfície gp120 (SU) e glicoproteína transmembranar gp41 (TM) – são componentes estruturais. As proteínas Pol – protease (PR), transcriptase reversa (RT) e integrasse (IN) – providenciam as funções enzimáticas. Adicionalmente, 6 ORF originam as proteínas acessórias Rev, Tat, Vif, Vpr, Nef, e Vpu, que regulam a expressão dos genes virais e que participam no processo de encapsidação, aumentando a eficiência da infecção.

A fase precoce do ciclo de replicação viral inicia-se com a ligação de um virião infeccioso ao receptor celular de superfície CD4, através da glicoproteína de superfície do vírus, gp120 (SU). Esta sofre alterações conformacionais que permitem o reconhecimento dos co-receptores, em particular CCR5 e CXCR4 no HIV-1, possibilitando a fusão do invólucro viral com a membrana celular e consequentemente a entrada viral. No citoplasma, o RNA genómico viral em cadeia simples é convertido pela transcriptase reversa (RT) num intermediário de DNA de cadeia dupla, que é então transportado para o núcleo na forma de complexo de pré-integração (PIC), e integrado no genoma da célula, por acção da integrase. Na fase tardia do ciclo, os genes do DNA proviral são transcritos e traduzidos pela maquinaria da célula hospedeira em proteínas virais. As proteínas estruturais são transportadas para a membrana plasmática onde se acumulam. A poliproteína Gag liga-se ao RNA genómico do vírus e então interage com a membrana plasmática, que é forçada a se projectar e eventualmente a se separar, formando-se uma partícula que é libertada para o exterior da célula infectada. A maturação do virião, iniciada pela protéase, resulta na reorganização nuclear e a aquisição da infecciosidade viral.

Vários autores focaram o seu estudo em vias celulares, que envolvem imunidade inata e defesas antivirais. Uma das vias inatas antivirais é a detecção de RNA viral através de receptores intracelulares que pertencem à família das helicases do tipo RIG-I (*RIG-I like helicases, RLH*). Após a ligação ao RNA, a RLH RIG-I ou MDA-5 sofre uma mudança conformacional que permite a interacção entre os domínios CARD da RLH e da proteína mitocondrial VISA. Desta interacção resulta a rápida indução de citocinas antivirais incluindo o interferão (*interferon, IFN*) tipo I, através da activação de IRF3-IRF7 ou NF-κB mediada por TRAF6 e TRAF3, respectivamente. Várias moléculas reguladoras participam nesta

sinalização, incluindo ISG15 (*IFN-stimulated gene 15*), RNF125 (*Ring finger protein 125*), ATG5 (*Autophagy related protein 5*), ATG12 (*Autophagy related protein 12*), LGP2 (*Laboratory of genetics and physiology 2*), OTUD5 (*OTU domain containing 5*), e PIN1 (*Peptidylprolyl cis/trans isomerase, NIMA-interacting 1*).

As possíveis interacções entre transcritos virais e a maquinaria da célula hospedeira para a tradução, o armazenamento e a degradação de mRNAs surgem como um aspecto significante da resposta antiviral. Este processo de *turnover* do RNA mediado por microRNAs (miRNAs) pode ocorrer em focos citoplasmáticos chamados *P-bodies* (*mRNA processing bodies*). Vários estudos revelam que os intervenientes da via miRNA que são simultaneamente componentes dos *P-bodies* causam supressão da replicação do HIV-1, nomeadamente a proteína TNRC6A (também conhecido por GW182), a endorribonuclease do tipo III DICER1 (ds*RNA-specific endoribonuclease type III*), a RNA helicase RCK/p54, e a proteína LSm1 (*Sm-like protein 1*). Outros componentes dos *P-bodies* que podem interferir são a RNase nuclear do tipo III RNASEN (*RNase III nuclear*), a enzima DCP1A (*mRNA-decapping enzyme 1A*) e o factor EDC4 (*Enhancer of mRNA deccaping 4*).

Como todos os vírus, o HIV-1 depende da maquinaria celular para conseguir replicar-se. Este facto impulsionou estudos cujo objectivo era a identificação de factores celulares associados à replicação do HIV-1. Recentemente, vários autores exploraram como tecnologia um fenómeno biológico no qual pequenas moléculas de RNA de cadeia dupla (*double stranded RNA, dsRNA*) levam à degradação de mRNAs. Este processo chamado de RNA de interferência (*RNA interference, RNAi*) tornou-se uma ferramenta útil, podendo ser usados pequenas moléculas de RNAs de interferência (small interfering RNAs, siRNAs) ou pequenos RNAs com formato em *hairpin* (*short hairpin RNAs, shRNAs*). Neste contexto foi recentemente realizado um trabalho por Sylvie Rato e colaboradores que, através de um *screen* de shRNA focado em cinases e fosfatases, identificou 14 proteínas importantes para a replicação do HIV-1, nomeadamente CIB2 e SGK. Ambas as proteínas demonstraram serem importantes numa fase inicial ciclo do HIV-1, antes da integração viral e muito provavelmente na entrada do vírus. SGK (*serum/glucocorticoid regulated kinase*) é uma cinase que activa certas vias de potássio, sódio e cloro, também dependentes de alguns coreceptores, sugerindo um envolvimento na fusão vírus-célula. CIB2 é uma proteína regulatória de ligação ao cálcio (*calcium and integrin binding regulatory protein 2*), que parece interagir com integrinas e pode ter um papel importante nos canais iónicos, sugerindo uma influência na fusão vírus-célula. Uma vez que todas as funções de CIB2 são suportadas pela elevada homologia entre CIB2 e CIB1, pensa-se que CIB2 pode também interagir com DNA-PKcs (*DNA-dependent protein kinase*). DNA-PKcs é a unidade catalítica

de um complexo proteico da cinase nuclear DNA-PK que parece estar envolvida na integração e na transcrição do HIV-1.

Objectivos

Neste estudo pretendeu-se: 1) Identificar novos factores celulares adjuvantes essenciais à replicação do vírus HIV-1 associados às duas vias da imunidade inata, i.e o silenciamento de genes pela via de miRNAs e a via de sinalização de RLH; 2) Avaliar o efeito das proteínas identificadas por Rato e colaboradores no ciclo viral do HIV-2 e; 3) investigar o mecanismo pelo qual CIB2 e SGK influenciam a fase precoce do HIV-1, mais concretamente na fusão e na transcrição do vírus.

Materiais e Métodos

Para a construção de clones shRNA, procedeu-se à transdução de células Jurkat por *spinoculation*, utilizando vectores lentivirais que expressam shRNAs individuais para os genes selecionados das duas vias de imunidade inata em estudo. Os clones individuais foram obtidos pelo método de ClonaCell-TCS e selecionados em meio suplementado com 2 µg/mL de puromicina. Os ensaios de replicação de HIV-1 e HIV-2 foram efectuados após a infecção dos diferentes clones por *spinoculation* com uma multiplicidade de infecção (*Multiplicity of Infection, MOI*) de 1. O HIV-1 e o HIV-2 foram produzidos por transfecção, pelo método de fosfato de cálcio dos plasmídeos pHIV-1_{NL4-3} e pHIV-2_{ROD} em HEK 293T, respectivamente. A transcrição pelo HIV-LTR foi avaliada pela expressão de β-galactosidase após transfecção transiente em células HeLa-P4 com pCMV-Tat, pCMV-CIB2, pLKO.1 shRNA CIB2, pLKO.1 shRNA DNA-PKcs, pLKO.1 shSCRAM, e pcDNA3.1ZEO(+) em diferentes combinações. Para avaliar a fusão vírus-célula, os clones shRNA CIB2 e SGK foram infectados com HIV-1_{NL4-3} com a enzima Beta-lactamase (BlaM)-Vpr incorporada e posteriormente incubados com o flurocromo CCF2-AM. A degradação do corante pela enzima BlaM após a fusão vírus-célula foi detectada por citometria de fluxo.

Resultados

Dos 152 shRNA clones obtidos, 19 foram viáveis e destes, 10 foram submetidos a ensaios de replicação. Desta forma foram identificados 4 factores importantes na replicação do HIV-1: RNASEN e ISG15, cuja supressão induziu uma redução da quantidade de vírus detectada; e OTUD5 e TNRC6A cuja supressão induziu um aumento da carga viral.

As proteínas previamente identificadas por Rato e colaboradores como factores adjuvantes do HIV-1 também demonstraram ter um papel importante no ciclo replicativo do

HIV-2, como se pôde observar pela redução da quantidade viral detectada no sobrenadante dos clones infectados.

Em relação a CIB2, os níveis de β-galactosidase obtidos na co-transfecção com CIB2 e Tat foram superiores ao controlo, evidenciando que a sobre-expressão de CIB2 leva ao aumento da transcrição de LTR. Quando quantidades crescentes de CIB2 são transfectadas com quantidades crescentes de shRNA de DNA-PKcs, observa-se uma forte diminuição dos níveis de β-gal que corresponde a uma redução da transcrição do LTR pela Tat.

No ensaio de fusão vírus-célula observou-se uma diminuição da percentagem de células que sofreram fusão viral de 20% nas células Jurkat, para 7,65% e 8,10% nos clones shRNA de CIB2 e SGK, respectivamente.

Conclusões

Os resultados apresentados nesta tese mostram as potencialidades da tecnologia do RNAi para a compreensão da interacção entre factores celulares e o HIV-1. A identificação de factores adjuvantes e de restricção envolvidos na via de silenciamento pelos miRNAs e a via de sinalização de RLH é um passo importante para aprofundar o conhecimento relativamente às defesas antivirais inatas. Além disso, a caracterização de proteínas relevantes no ciclo de replicação do HIV-1, assim como do HIV-2, pode estimular novos estudos relativos à interacção HIV-célula. Desta forma, estes estudos poderão propor novas estratégias antivirais contra a infecção por HIV-1, importantes para encontrar tratamentos mais eficientes para a SIDA.

Palavras-chave: *HIV-1; HIV-2; RNAi; silenciamento por miRNAs; via de sinalização RLH; CIB2; SGK*

Introduction

1. Acquired Immunodeficiency Syndrome

Acquired Immunodeficiency Syndrome (AIDS) was recognized in early 1980s as the decline of immune system characterized by opportunistic diseases appearance in previously healthy individuals [1]. In 1983, the virus responsible for this syndrome was identified as a retrovirus capable of infecting $CD4^+$ lymphocytes [2, 3] and consequently death with a half-life of less than two days [4]. Nowadays, the virus is known as Human Immunodeficiency Virus (HIV) and there are two types of HIV known until now: type 1 (HIV-1) and type 2 (HIV-2). The former is more virulent and is more worldwide prevalent than type 2.

Highly active antiretroviral therapy (HAART), although powerful, effective against HIV and capable of prolonging life and health of the infected individuals, is still not able to cure AIDS [5]. For this reason, several studies started to focus on the host-virus interactions involved in various steps of retroviral replication, becoming more evident that understanding the dynamic interplay of host cell and virus is essential to the effort to eradicate HIV [6].

2. HIV-1 characterization

2.1. Genome and structure of HIV-1

HIV-1 is a member of *Lentivirus* genus from the *Retroviridae* family, which is characterized by a long incubation period [4].

The HIV-1 genome encodes nine open reading frames (ORF) (Figure 1). Three of these encode the group specific-antigen (Gag), polymerase (Pol), and envelope (Env) polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses [7].

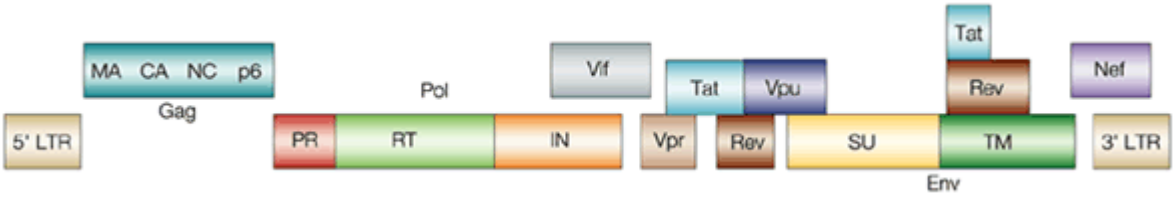

Figure 1. HIV-1 genome. Adapted from Robinson, H. L. Nature Reviews Immunology 2, 239-250 (April 2002)

The four Gag proteins – matrix (MA), capsid (CA), nucleocapsid (NC), and p6 – and the two Env proteins – glycoproteins gp120 (surface or SU) and gp41 (transmembrane or TM) – are structural components that make up the core of the virion and outer membrane envelope.

The three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN), provide essential enzymatic functions and are also encapsulated within the particle (Figure 2). HIV-1 encodes six additional proteins, often called accessory proteins, three of which (Vif, Vpr, and Nef) are found in the viral particle. Two other accessory proteins, Tat and Rev, provide essential gene regulatory functions, and the last protein, Vpu, indirectly assists in assembly of the virion. The retroviral genome is encoded by a ~9-kb RNA flanked by 5' and 3' long-terminal repeats (LTRs), and the 5' LTR contains the promoter for transcription. Two genomic-length RNA molecules are also packaged in the particle [4, 7-9].

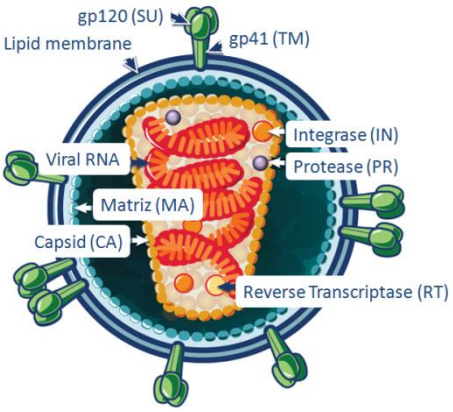

Figure 2. HIV-1 virion. Adapted from NIAHI.

2.2. HIV-1 replication cycle

In the early phase of HIV-1 replication, viral particles bind specifically to cells bearing CD4 receptor, a protein that normally functions in immune recognition (Figure 3). Binding occurs via specific interactions between the viral envelope glycoprotein gp120 and the amino-terminal immunoglobulin domain of CD4 [4, 8, 9]. The gp120-CD4 interaction is sufficient for binding but not for infection. Instead, a group of CC and CXC chemokine receptors, particularly CCR5 and CXCR4, which mobilize intracellular calcium and induce leukocyte chemotaxis serve as essential viral coreceptors to trigger membrane fusion [4, 7]. After the engagement of HIV coreceptor, gp41 undergoes conformational changes exposing a region of the viral transmembrane protein, the "fusion peptide". This peptide promotes directly the fusion event between the viral and the host membranes, allowing the viral core to gain access to the cytoplasm [4, 8, 9]. Once inside the cell, the HIV-1 core endures a poorly understood uncoating process that involves the core disassembly by the dissociation of the capsid (CA) [4, 9], which appears to be essential for the progress of reverse transcription [10, 11]. Uncoating is followed by the activation of reverse transcriptase (RT) to copy the single-stranded positive-sense viral RNA genome into double-stranded linear DNA [4, 7-10, 12]. This DNA is delivered into the nucleus in the form of a pre-integration complex (PIC) containing several proteins. Components of PIC include viral proteins, such as reverse transcriptase (RT), matrix (MA), Vpr, and integrase (IN), as well as host proteins including BAF and HMG I(Y) [9, 12]. Following nuclear import of the viral PIC, the integrase (IN) catalyzes the insertion of the linear, double-stranded viral DNA into the host cell

chromosome, completing the early phase of HIV-1 replication cycle [4, 7, 8, 12]. The unintegrated DNA can also follow non-productive pathways, in which it becomes circularized or degraded, creating in some cases 2-LTR circles [13, 14].

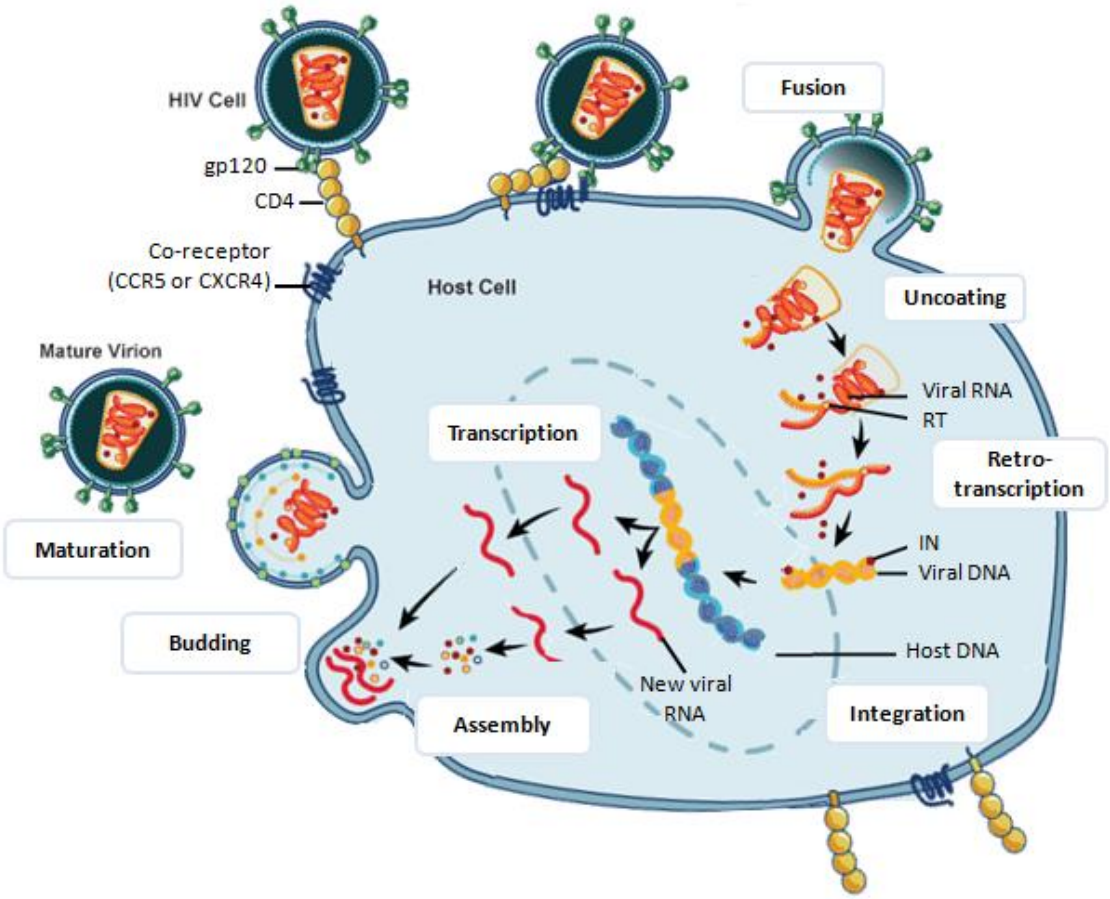

Figure 3. HIV-1 replication cycle. After fusion with the host membrane, HIV-1 suffers uncoating, followed by retro-transcription of viral ssRNA genome into dsDNA by reverse transcriptase (RT). This DNA is delivered into the nucleus and is then inserted into the host genome by the viral integrase (IN) to form the integrated provirus. Afterwards, the viral DNA is transcribed and the viral RNAs are processed and exported out to the cytoplasm. New viral RNA is used as genomic RNA and to make viral proteins. New viral RNA and proteins move to cell surface and a new, immature, HIV virus is formed. The virus matures by protease releasing individual HIV proteins. Adapted from NIAHI.

In the late phase of the life cycle, the viral DNA is transcribed by the host RNA polymerase II (RNAP) system into unspliced and spliced mRNA transcripts [4, 12]. Initially, short spliced RNA species that encode the regulatory proteins Tat, Rev and Nef are synthesized. Tat is an essential transcriptional activator that binds to a stem-loop element (trans-activating response element, TAR), located at the 5' end of the nascent RNA transcript. This protein requires interactions with other cellular proteins to improve its function. Thus, enhanced transcription by Tat implies the recruitment of cellular proteins such as cyclin T and cyclin-dependent protein kinase-9 (Cdk9) [4, 7, 8, 12]. Transcription from the

HIV-1 LTR leads to the generation of different viral RNAs. These fall into three major classes: 1) unspliced RNAs, which function as the mRNAs for the Gag and Gag-Pol polyprotein precursors, and are packaged into progeny virions as genomic RNA; 2) partially spliced mRNAs, which encode the Env, Vif, Vpu, and Vpr proteins; and 3) small multiply spliced mRNAs, which are translated into Rev, Tat, and Nef [4, 8, 12]. Since most cellular mRNAs are fully spliced before their transport out of the nucleus, the need for unspliced and partially spliced RNAs in the cytoplasm is a problem for HIV. This problem has been overcome through the evolution of a novel viral protein, Rev, and a *cis*-acting RNA element, the Rev responsive element (RRE) [4, 7-10, 12]. Three viral structural protein precursors — group-specific-antigen protein (Gag), Gag-polymerase (Gag-Pol) and the envelope protein (Env) — are translated in the cytoplasm, and transported to the plasma membrane by vesicular, cytoskeletal or other routes. Nascent virions are assembled from these proteins on host membranes, and immature particles are released from the cells. Finally, maturation of the virions, which is triggered by the viral protease, results in a drastic reorganization of the core and the acquisition of virus infectivity [4, 8, 12].

3. HIV-2 Characterization

Less consideration has been given to HIV type 2 comparing to HIV-1, due to its restricted endemicity. Also a lentivirus, HIV-2 is a closely related retrovirus to HIV-1, presenting ~60% similarity to HIV-1 at the aminoacid level in Gag and Pol, and 30-40% in the regions encoding the Env [15]. Besides these structural genes, HIV-2 also comprises the accessory proteins Tat, Rev, Nef, Vif, Vpr and Vpx (instead of Vpu as in HIV-1). In HIV-2, the molecular weight of some proteins varies from those on HIV-1. This is particularly true for surface glycoprotein gp125 (gp120 for HIV-1) and transmembrane glycoprotein gp32 (gp41 in HIV-1) [15-17].

In addition to genomic and structural similarity, HIV-2 reveals a parallel replication cycle with HIV-1. The main difference relies on the early phase, more precisely in the fusion even, since HIV-2 can use a much wider repertoire of both CC and CXC chemokine receptors including CCR1 to CCR5, CXCR2, and CXCR4 [18].

Henceforth, only HIV-1 will be referred on this work, since every aspect of HIV-2, otherwise indicated, is considered to be analogous to HIV-1.

4. HIV-Cell Interaction

Like all viruses, HIV-1 depends on the host cell machinery to support its replication. This fact prompted studies aimed at identifying host cell factors associated with HIV-1 replication since the virus exploits many different cellular factors and pathways during its cycle [12]. Some cellular factors can help HIV-1 to complete its life cycle, named helper factors, and others can defend cells from viral infection by reducing or suppressing HIV-1 replication, called restriction factors [12, 19]. Certain host factors have been demonstrated to participate in different pathways of innate immunity and antiviral defenses [20-28]. Also, several authors recently turned to RNA interference (RNAi) screenings to uncover new host proteins important in HIV-1 cycle [29-34].

4.1. Innate antiviral defenses

Viruses that invade mammalian cells are recognized by different innate responses through pattern-recognition receptors from different pathways: TLR-dependent pathway characterized by Toll-like receptors [20-23]; and RIG-I like helicases (RLH)-dependent recognition, which is associated to mitochondrial antiviral pathway [22-24]. Mitochondrial antiviral immunity involves the detection of viral RNA by intracellular pattern-recognition receptors (PRRs) belonging to the RLH family. The convergence of these and other signaling molecules to the outer mitochondrial membrane results in the rapid induction of antiviral cytokines including type-1 interferon (IFN) [22-24].

RLH signaling pathway begins with the recognition of the viral intermediates, double-stranded RNA (dsRNA) or 5'triphosphate single stranded RNA (ssRNA), by RIG-like family of helicases, retinoic acid inducible gene-I (RIG-I) or melanoma differentiation associated gene-5 (MDA-5). Upon ligand binding, RIG-I or MDA5 undergoes a conformation change allowing for the interaction of the RLH Caspase activation and recruitment domain (CARD) with the CARD-containing virus-induced-signaling adapter (VISA), also known as MAVS, IPS-1, and Cardif (Figure 5). VISA signals through TNF receptor associated factor (TRAF)3 or TRAF6 to activate kinases leading to the nuclear translocation of IFN regulatory factor (IRF)3-IRF7 and Nuclear factor kappa B (NF-κB), respectively, resulting in the transcription of type-1 IFN [22, 24]. Several regulatory molecules regulate this response, in a positive or negative manner. Laboratory of genetics and physiology 2 (LGP2) was thought to inhibit RIG-I and augment MDA-5-mediated responses [24], but more recently evidences showed that LGP2 facilitates viral RNA recognition by RIG-I and MDA5 through its ATPase domain [25]. Ligand-activated RIG-I is regulated through proteosomal degradation after ISGylation by IFN-stimulated gene

15 (ISG15) or after lysine 48 ubiquitination by Ring finger protein 125 (RNF125). On the other hand, lysine 63 ubiquitination of RIG-I by tripartite motif-containing 25 (TRIM25) augments RIG-I-VISA interactions and enhances downstream signaling, whereas RIG-I-VISA interactions are inhibited by an autophagy regulator (Atg5-Atg12). The protein nucleotide-binding domain and leucine-rich-repeat-containing family 1 (NLRX1) functions as an inhibitor of VISA signaling from within the mitochondria. Also, the removal of ubiquitin moieties from TRAF3 by the deubiquitinase OTU domain-containing 5 (OTUD5) prevents TRAF3 associations with downstream kinases, thereby inhibiting RLH signaling. At the most distal level, phoshorylated IRF3 is targeted for proteosomal degradation by the peptidyl-prolyl isomerase 1 (PIN1) [22, 24]. Understanding the role of these regulators could bring some light into innate antiviral responses regarding HIV-1.

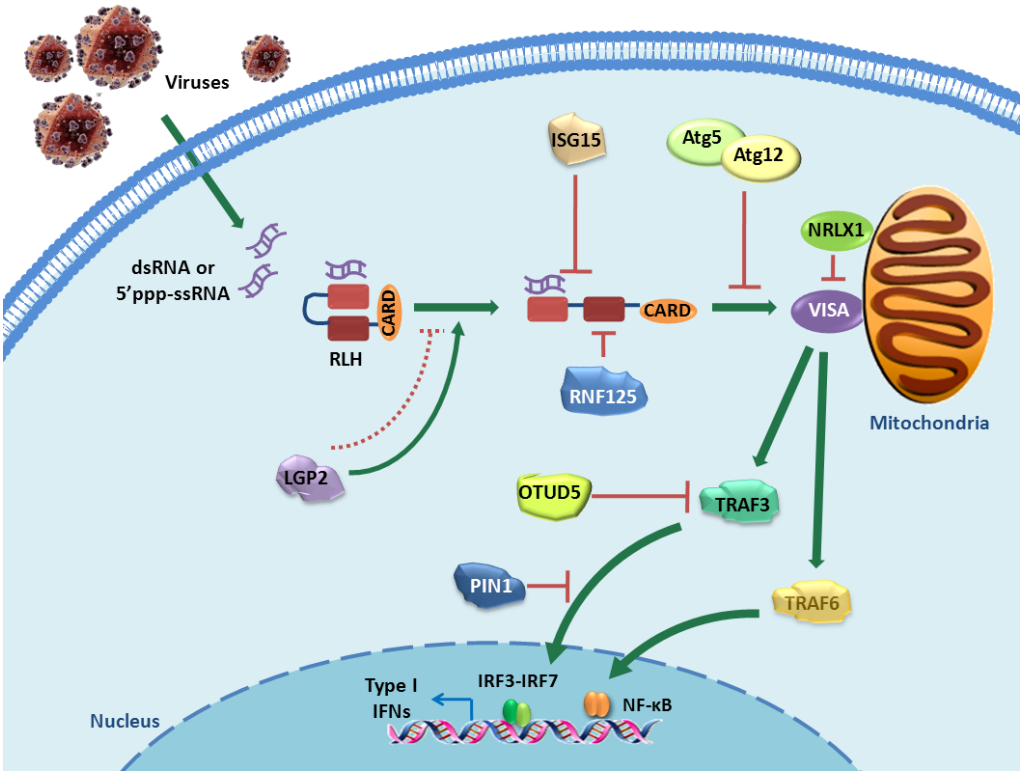

Figure 5. Regulators of RLH signaling. Intracellular signaling responses to viral infection begins with the recognition of the viral intermediates by the RIG-like family of helicases (RLH). After ligand binding, RIG-I or MDA5 undergo conformational changes, allowing the interaction between the VISA's CARD and the CARDs of RIG-I or MDA5. VISA-mediated signaling results in transcription of type-I IFNs through activation of IRF3-IRF7 or NF-κB mediated by TRAF6 or TRAF3, respectively. Several regulatory molecules (shown in red) regulate this response and the inhibitory (red lines) and stimulatory (green arrows) effects of these molecules on RLH signaling are illustrated. Adapted and modified from Moore, C. B. and J. P. Ting (2008). Immunity 28(6): 735-9.

Another integral component of innate antiviral defense is post-transcriptional regulation mediated by microRNAs (miRNAs) [35]. Since viruses are dependent on cellular machinery for their survival and replication, interactions between viral transcripts and host machinery for

translation, storage and degradation of mRNAs emerge as a significant aspect of antiviral response. This process of RNA turnover can occur in cytoplasmic foci named mRNA processing bodies (P-bodies), also associated with translational repression and RNA-mediated silencing [36-38]. Recent studies reveal that miRNA effectors that are simultaneously P-bodies components cause suppression of HIV-1 replication. In fact, some of these factors like RNA helicase RCK/p54 and protein GW182 were demonstrated to repress HIV-1 gene expression by preventing viral mRNA translation, as well as other P-bodies components such as protein LSm1 and 5'-3' exoribonuclease XRN1 [26]. In fact, knockdown of RCK/p54 and LSm1, since it leads to P-bodies disruption [36], results in enhanced virus production [26]. Furthermore, HIV-1 mRNAs physically associate with Argonaute 2 (Ago2), a central component of RNA-induced silencing complex (RISC), and co-localize with cellular proteins required for miRNA-mediated silencing such as RCK/p54 and Ago2 in P-bodies [26]. Others proteins such as decapping enzyme DCP1A, nuclear RNase type III RNASEN (also known as Drosha) and enhancer of mRNA deccaping EDC4 have also been pointed out as key players in RNA turnover [36-38].

Despite all efforts, the knowledge about the interactions between RNA silencing and HIV-1 is minor. There are four kinds of interactions that seems to occur in HIV-1 infection: 1) RNAi of viral origin blocking viral RNA; 2) RNA silencing of viral origin down-regulating cellular mRNA; 3) cellular miRNAs targeting viral RNA; and 4) cellular miRNA down-regulating cellular mRNAs encoding for cellular proteins necessary for virus replication [27]. Moreover, HIV-1 infection has been suggested to exert a suppressive effect on miRNA processing and RNA-silencing, by producing viral suppressors of this pathway [35, 27]. Regarding the use of RNA interference (RNAi) technology, the forced overexpression of short hairpins RNAs (shRNAs) and small interfering RNAs (siRNAs) in cells might also have the potential to exhaust the cellular machinery for RNA silencing, complicating the interpretation of biological outcomes. Whether these influences might significantly affect the siRNA and shRNA screens for HIV-1 cofactors remains to be determined. Moreover, when shRNA-cell clones are infected by HIV-1, the shRNA-silencing of targeted mRNAs would have already been completed and would be unlikely influenced by the effects of infection on RNAi activities [39].

The increasing knowledge of the interactions between viral transcripts and host cell factors involved in RNA turnover will be essential for the discovery of new targets that could be used to design new therapeutic strategies.

4.2. RNA interference and gene silencing

RNA interference (RNAi) is a biological phenomenon in which small dsRNA molecules present in the cytoplasm of a cell lead to the destruction of cognate mRNAs. The substrate of this pathway is small interfering RNAs (siRNAs) which consist on 21-23 nucleotides duplexes capable of direct sequence-specific inhibition of gene expression by mRNA degradation [36, 40-42]. Others dsRNAs have also been described to trigger small RNA-guided gene silencing pathways in mammals, such as microRNAs (miRNAs) [37, 41-43]. miRNAs are 18-23 nucleotide RNA molecules which bind usually with imperfect complementarity to their target mRNAs, leading to their repression [38, 44, 45]. Although siRNAs and miRNAs differ in their mechanism of biogenesis, their functions are connected by members of the conserved family of Argonaute (Ago) proteins, associated to RNA-induced silencing complexes (RISCs) [37, 41, 43, 44].

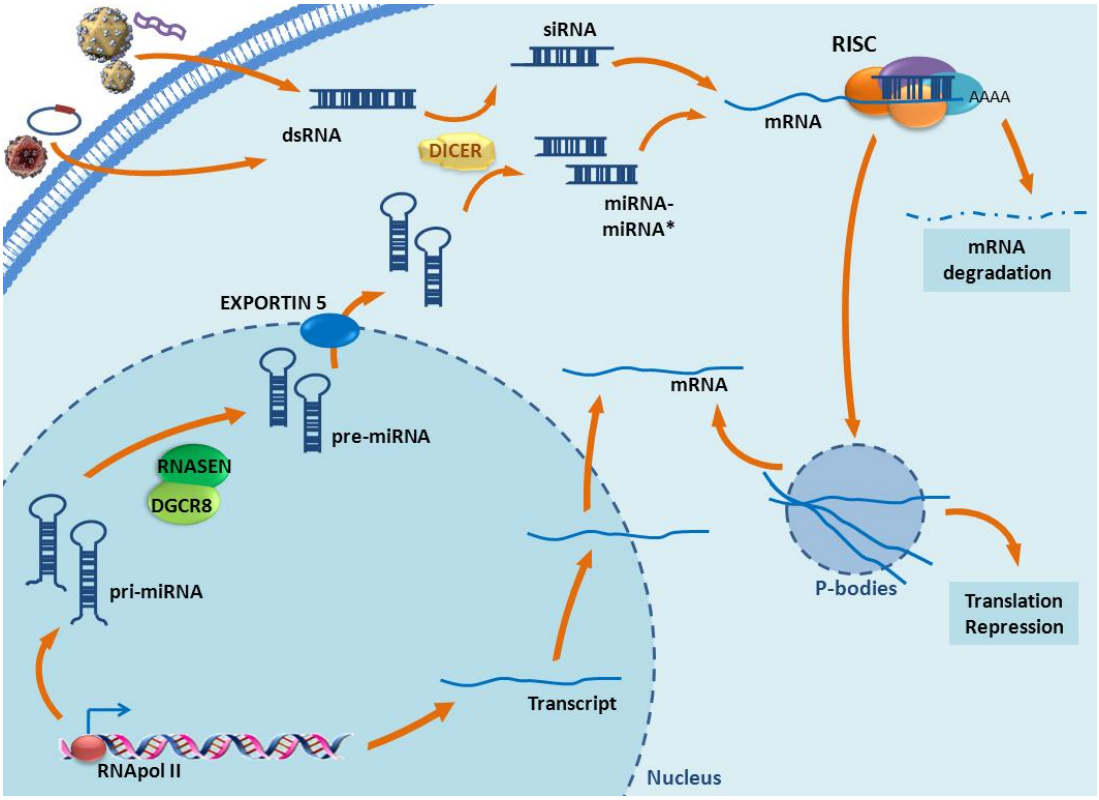

Figure 4. RNA silencing by miRNAs and siRNAs. MicroRNAs (miRNAs) are transcribed by RNA Polymerase II as primary-miRNAs (pri-miRNAs), which are processed by Drosha into pre-miRNAs. These pre-miRNAs, after exported to cytoplasm by Exportin 5, are cleaved by Dicer, leading to the formation of duplexes miRNA/miRNA*. In steps shared with siRNA (small interfering RNA) pathway, one of the strands is incorporated to RNA-induced silencing complex (RISC). This complex leads to mRNA targeting and subsequent degradation in cytoplasmic foci named P-bodies. RNAs assembled in P-bodies can become available for translation. Adapted and modified from Scaria, et al. (2006). Retrovirology 3:68

The gene silencing mediated by miRNAs begins with the miRNAs processing by RNA Polymerase II as primary microRNAs (pri-miRNAs) which are imperfect RNA hairpins of hundreds to thousands of base pairs (Figure 4). Subsequently, pri-miRNAs are processed in the nucleus by the microprocessor complex comprising DGCR8 and RNA III endonuclease RNASEN to a stem loop structure, the pre-miRNA. The exportation of pre-miRNA to the cytoplasm is mediated by the nuclear export factor Exportin-5. Cytoplasmic pre-miRNAs are then cleaved by DICER, another RNA III endonuclease, leading to the formation of 18-23-base pair duplexes miRNAs/miRNAs*. The miRNA/miRNA* is then incorporated in RISC, where the miRNA* strand is degraded and miRNA serves as a mature strand or guide strand. The incorporation of the mature strand promotes the activation of RISC, which is not possible without the catalytic activator Ago2. RISC is then capable of targeting a mRNA with complementary sequence to anti-sense strand of the miRNA. The grade of sequence complementarity will determine the final step of the pathway: with perfectly complementary sequence, the mRNA will be degraded; with partially complementary sequence, there will be translation repression, which is the most common result in miRNA pathway [37, 40, 42-44].

The siRNA-mediated silencing is parallel to the process above, differing in the beginning which initiates with the cleavage of long dsRNAs into siRNAs by the RNA III endonuclease Dicer (Figure 4). The duplex siRNA is unwound and one of the two strands is incorporated into RISC. This complex, along with Ago2, supports mRNA targeting in a similar manner as in miRNA-mediated gene silencing, but the result is always cleavage and subsequent degradation of the transcript [35, 40, 41, 44, 46-48].

RNAi, for its characteristics, became a useful lab tool for understanding viral-host interactions. Researchers take advantage of this pathway by directly transfecting siRNAs or by delivering transgenes encoding short hairpins RNAs (shRNAs), which are processed by Dicer into siRNAs [27, 49].

4.2.1. siRNAs and shRNAs screenings

Three screens that used siRNA pools to identify cellular proteins important in HIV-1 replication were reported in 2008 [29-31]. Intriguingly, the identities of the gene candidates discovered in these reports are highly divergent with very little overlap, as illustrated at [32]. Also, some well-known HIV-1 cofactors such as Sp1 transcription factor were not identified [39]. These evidences offer credibility to the notion that these approaches were not extensive as initially thought. However, many of those host factors can alter in different contexts of cellular metabolism, changing their importance in HIV-1 cycle. Moreover, differences in cell types, and how primary data sets and off-target effects are filtered have also been suggested

to account for the discovery of the many different HIV-1 cofactors in the screens [39]. Despite all these discrepancies, the technology of short-RNA-based screening is a developing and crucial tool to uncover all putative host factors required for HIV-1 replication.

One limitation to the reported screens is the use of HeLa or HEK293T cells that are not physiological substrates for infection by HIV-1. Human T-cell lines, such as Jurkat cells, are better models, although the latter cells cannot be efficiently transfected with siRNAs. In addition, the use of a pseudotyped virus or a mutated strain of HIV-1 also limits the interpretability of some of the results. To overcome these limitations, Yeung *et al*. and Rato *et al*. performed recently a loss of function screen with short-hairpin-RNA (shRNAs) cloned in lentiviral vectors to allow the constitutive expression of the shRNAs in Jurkat T lymphocytes [33, 34]. This approach is remarkable not only for the advantage of employing cells physiologically infected by HIV-1, including primary T cells and macrophages [33, 39], but also for the selection of shRNA transduced Jurkat cells for extended duration before being subject to HIV-1 infection [33, 34]. This pre-infection selection for shRNA cell clones serves to eliminate clones affected in cell growth or survival, reducing the number of false positive genes identified in the screening. Jurkat T-cell clones, each expressing a single discrete shRNA, were subjected to infection by HIV-1, and then genes responsible for any alteration in viral replication were identified, completing the screening [33, 34, 39]. For the above reasons, shRNA screens have certain inherent and nuanced advantages over siRNA screening selected settings.

RNAi screenings can also be employed in recognizing targets in a specific group of proteins, like kinases and phosphatases, as described by Rato *et al*. [34]. Protein phosphorylation is a well-characterized biochemical process for reversible regulation of protein activity. Both kinases and phosphatases are required for this crucial process that regulates many cellular processes in eukaryotes [50]. Moreover, they constitute nowadays the largest subset of the druggable genome, sustaining kinase/phosphatase modulation as approach for the development of novel therapeutic strategies [51]. For this purpose, Rato *et al*. performed an iterative shRNA screen in Jurkat cells which allowed the identification of 14 different cellular proteins, involved in several cellular pathways that are essential for HIV-1 replication. Outcome results demonstrated that the majority of these proteins are involved in steps prior to viral integration, maybe during entry into the cell and/or uncoating and also affecting viral transcription [34]. One of the identified proteins is serum/glucocorticoid regulated kinase (SGK), a serine/threonine protein kinase that activates certain potassium, sodium, and chloride channels, suggesting an involvement in the regulation of processes such as cell survival [52]. For the same reason, it could be implicated in virus-cell fusion [53, 54]. Additionally, SGK is transcriptionally activated by the glucocorticoid receptor (GR) [55],

which has been demonstrated to interact with HIV viral protein Vpr within a complex integrating VIP-1. Moreover, it has been suggested that the Vpr-GR interaction could induce apoptosis since NF-kB inhibition by Vpr seemed to be GR-dependent [56], implicating SGK as an important HIV-1 cofactor.

Another protein also identified by Rato *et al.* screen is CIB2, calcium and integrin binding family member 2. Little is known about this calcium binding regulatory protein, and almost everything is supported by its high homology with CIB1 [57]. CIB1 not only has a role in ion channels, but also interacts with integrins, proposing a role in cell-to-cell interactions, as well as in viral entry [53, 54, 58, 59]. Furthermore, CIB1 has been described to interact with several proteins involved in hemostasis, DNA damage response and apoptosis [60], such as Snk, inositol 1,4,5-triphosphate receptor and DNA-PKcs [61-64].

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated upon DNA damage. It is a three-protein complex consisting of a 470-kDa catalytic subunit (DNA-PKcs) and the regulatory DNA binding subunits, Ku heterodimer (Ku70 and Ku80) [65]. Although its role in HIV-1 integration has been strongly suggested [66], this retroviral step is not the only one affected. DNA-PK has been identified as an Sp1 kinase [67] and has also been shown to phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II [68]. In fact, Chun *et al*. proposed that Tat and DNA-PK interact to increase the phosphorylation state of Sp1, resulting in upregulated expression of the HIV-1 LTR [67]. These findings suggest a function for DNA-PK in transcription.

5. Aim of the present work

Human Immunodeficiency Viruses (HIV-1 and HIV-2) rely on host proteins to facilitate their replication, which may affect several cellular pathways. The study of some regulators of innate immune responses could add some light into HIV-1 infection, as well as clarifying the function of kinases and phosphatases during HIV-1 and HIV-2 replication at the level of viral entry and transcription. Both directions may contribute to a better knowledge of HIV-cell interaction and also lead to the discovery of new cellular targets for HIV-1 therapy.

This work combined three main goals: 1) identification of new helper factors essential for HIV-1 replication related to innate immunity, more precisely, to miRNA-mediated silencing and RLH signaling pathways; 2) characterization of previously identified proteins by Rato and co-workers in HIV-2 replication cycle; and 3) evaluation of the mechanism by which CIB2 and SGK affect the early phase of HIV-1 life cycle, more precisely in viral fusion and transcription.

Methods

Plasmid propagation in *Escherichia coli* competent cells

E.coli JM109 (New England Biolabs) (genotype: F´*traD36 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/Δ(lac-proAB) ginV44 e 14*$^{-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17*) was used for plasmid propagation of pHIV-1_{NL4-3} (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), pLKO.1 which contains a shSCRAM sequence or shRNA for each gene (Sigma, USA); pHEF-VSVg (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), pCMV-BlaM-Vpr (gift from Dr. Olivier Schwartz, Pasteur Institute) and pHIV-2_{ROD} (gift from Francois Clavel, INSERM). *E.coli* TOP10F' (Invitrogen, USA) (genotype: F'[lacI^{q}Tn10(Tet^{R})] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 deoR nupG araD139 Δ(ara-leu)7697 galU galK rpsL (Str^{R}) endA1 λ-) was transformed to propagate pcDNA3.1/ZEO(+) (Invitrogen, USA), pCMV-Tat (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), and pCMV-CIB2 (cloned in João Gonçalves Lab). All plasmids are displayed in annexes.

Plasmid DNA Extraction

Transformed bacteria were grown in LB medium (for 1 L: 10 g triptone; 5 g yeast extract; 10 g NaCl in 1 L ddH_2O; pH=7,2) supplemented with 100 µg/mL ampicilin (USB, Cleveland, USA) at 37°C *o.n*. with agitation for pLKO.1, pcDNA3.1/ZEO(+), pCMV-Tat and pCMV-CIB2 or at 30°C used for pHIV-1_{NL4-3}, pHIV-2_{ROD}, pHEF-VSVg and pCMV-BlaM-Vpr.

The pHIV-1_{NL4-3} was used for production of HIV-1 viral particles (NL4-3 strain without VSV) with replication and infection ability. All plasmids extraction was carry out by midipreps preparation via JETStar 2.0 Plasmid Purification MIDI Kit (Genomed, Portugal) or via Invisorb Plasmid Midi Kit (Invitek, Berlin). pLKO.1 plasmids were extracted by minipreps preparation via Invisorb Spin Plasmid Mini Two Kit (Invitek, Berlin), according to manufacturers' instructions.

Cell culture conditions

Human embryonic kidney (HEK) 293T (ATCC, VA, USA) and HeLa-P4 (HeLa-CD4-LTR-β-gal, AIDS Reagent, MD, USA, contributor Dr. Richard Axel) cell lines were cultivated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL Penicillin, 100µg/mL Streptomycin, and 0.25 µg/mL Amphotericin B (DMEM-10). Cells were grown throughout 70-80% of confluence in tissue culture flasks (75 cm^3) (Orange Scientific, Belgium), at 37°C with 5% CO_2.

Jurkat E6-1 T-cells obtained through the NIH AIDS Research and Reference Reagent Program (MD, USA, contributor Dr. Arthur Weiss) were cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL Penicillin, 100µg/mL Streptomycin, and 0,25 µg/mL Amphotericin B (RPMI-10). Jurkat cells expressing shRNA (shRNA clones) were cultured in RPMI-10 supplemented with 2 µg/mL of puromycin (Sigma, MO, USA). Cells were grown in tissue culture flasks (25 cm^3) (Orange Scientific, Belgium), at 37°C with 5% CO_2. All cell culture media and reagents, otherwise indicated, were from Lonza (Basel, Switzerland).

Construction of lentiviral shRNA clones

Jurkat cells were seeded at 5 x 10^4 per well in 96-well plates (6.9 mm Ø) (Orange Scientific, Belgium), 20-24 hours prior transduction. Transduction was performed by spinoculation [69] using lentiviruses-based vectors that express RNA-inducing shRNAs for the elected genes (contribution from Dr. Luís Moita, UBCSI, IMM). The conditions of spinoculation were 1 h, 200 rpm, 20°C. Each well suffered a medium change 6 hours after the spinoculation using RPMI-10. In the following 96 hours, all cells were challenged gradually with increasing concentrations of puromycin ranging 0.5 and 1 µg. In order to isolate individual clones for each shRNA, puromycin resistant-cells were cloned with a ClonaCell™-TCS semi-solid medium (StemCell Technologies, Vancouver, Canada). For this purpose, 500 cells were collected from each population expressing a different shRNA and placed in 200 µL of semi-solid medium supplemented with 0.5 µg/mL of puromycin. After 7 days in culture, individual clone clusters were recovered and grown in 96-well plates with RPMI-10 supplemented with 2 µg/mL of puromycin. The resistant cell clones were expanded and cells were allowed to growth for 2 months for phenotype stabilization.

Viral production

For transfection assays, 5 x 10^5 HEK 293T cells were distributed in 6-well plates (34.7 mm Ø) (Orange Scientific, Belgium), 20-24 hours before. Cells were transfected by calcium phosphate transfection method [70, 71] with 5 µg of pHIV-1_{NL4-3}, 5 µg of pHIV-2_{ROD}, 5 µg of pHIV-1_{NL4-3} plus 0.5 µg of pHEF-VSVg, or 3.2 µg of pHIV-1_{NL4-3} plus 1.1 µg of pCMV-BlaM-Vpr, regarding the different assays.

Afterwards, transfected cells were incubated for 20-24 hours, followed by a medium change (DMEM-10). Viral production quantification for HIV-1_{NL4-3} was performed 48 hours after transfection by $p24^{CA}$ ELISA, according to manufacturer's recommendations using NCI-Frederick Cancer Research and Development Center – AIDS Vaccine Program kit (NIH,

USA). For HIV-2_{ROD} quantification, INNOTEST™ HIV Antigen mAb kit (INNOGENETICS, Belgium) was used 48 h post-transfection.

Infection Assays

Jurkat cells and shRNA clones were infected with HIV-1_{NL4-3}, HIV-1_{NL4-3} with VSVg, and HIV-2_{ROD} at the indicated Multiplicity of Infection (MOI), by spinoculation [69]. After 6 h, cells were washed in phosphate-buffered saline solution (PBS) (1×) and medium was replaced. During the 7 day-infection assay, medium was replaced at day 4. HIV-1 replication was monitored in all experiments by $p24^{CA}$ ELISA (AIDS Vaccine Program), whereas HIV-2 replication was measured by INNOTEST™ HIV Antigen mAb kit (INNOGENETICS).

Assessment of Cell Viability

After 7 days of infection, Jurkat cells and shRNA clones viability was assessed by determining the cellular reducing capacity through the extent of AlamarBlue® (resazurin) reduction to resorufin. The amount of resorufin produced is proportional to the number of living cells and can be quantified at 570 nm by spectrophotometry. Briefly, cells were incubated with AlamarBlue® (Invitrogen, USA) for 4 h at 37°C and the absorbance was read in Infinite M200 plate reader (Tecan). Results were represented as % of AlamarBlue reduction, comparing to control cells.

Virus-cell Fusion Assay and Flow Cytometry Analysis

Jurkat cells and shRNA clones were submitted to infection to evaluate fusion event. All the experiment was carried on as demonstrated in [72]. Briefly, Jurkat cells and shRNA clones were seeded at 5 x 10^5 per well in 24-well plates (Orange Scientific, Belgium) and were incubated with virions containing BlaM-Vpr (50-500 ng $p24^{CA}$) at 37°C for 3 h. Following 3 h of incubation, cells were washed in CO_2-independent medium (GibCo, Invitrogen) and then loaded with CCF2/AM dye as described by the manufacturer (Aurora Biosciences). Cells were incubated with CCF2/AM dye for 1 h at room temperature. After two washes with CO_2-independent medium, the BlaM reaction was allowed to develop for 7 h at room temperature in 200 μL of CO_2-independent medium supplemented with 10% FBS and 2.5 mM probenecid, a nonspecific inhibitor of anion transport (Sigma, MO, USA). Finally, infected and uninfected cells were washed and fixed with 1.2% paraformaldehyde in PBS. Uninfected cells were used as negative controls, as well as HIV-1_{NL4-3}-infected cells. The change in emission fluorescence of CCF2 after cleavage by Blam-Vpr chimera from green (520 nm) to blue (447 nm) was monitored by flow cytometry, indicating the percentage of cells where fusion

occured. BD FACSAria (BD Bioscience, CA, USA) was used to acquire 10,000-gated events from each sample. Data were analyzed using FlowJo software (Tree Star, OR, USA).

Expression Assays

The extent of transcription was determined as a measure of β-*galactosidase* expression by Chlorophenol red-β-D-galactopyranoside (CPRG) colorimetric assay, according to the manufacturers' recommendation (Roche, Germany) and as described in [73]. For this purpose, HeLa-P4 cells containing the β-gal gene under the control of HIV-LTR were seeded at 5 x 10^4 cells per well in 24-well plates (14,5 mm Ø) (Orange Scientific, Belgium), 20-24 hours prior transfection with pCMV-Tat alone or in several combinations with pCMV-CIB2, pLKO.1 shRNA CIB2, pLKO.1 shRNA DNA-PKcs, pLKO.1 shSCRAM, pcDNA3.1ZEO(+) and several concentrations (up to 2 μg of total DNA), as indicated in the legend of the figures. Total amount of DNA was normalized with pcDNA3.1ZEO(+). Transfections were performed with FuGENE® HD Transfection Reagent (Promega Corporation, USA) according to manufacturers' protocol. After 48h, β-*galactosidase* expression was measured by Infinite M200 plate reader (Tecan).

Statistical Analysis

Statistical significance was determined using the Paired t-test. Differences were considered statistically significant when $p \leq 0.05$. Analyses were performed using the Graphpad Prism 4.0 software (GraphPad Software, CA, USA).

Results

1. RNAi screening towards antiviral responses

1.1 shRNA screening to isolate HIV-1 resistant Jurkat T-Cells

To identify host factors essential for HIV-1 replication we developed a shRNA screen in Jurkat T-cells using a RNAi lentiviral library. This library includes 25 shRNAs corresponding to 5 human genes regarding miRNA-mediated silencing, and 35 shRNAs corresponding to 7 human genes responsible for regulating the mitochondrial antiviral signaling pathway, composing a total of 5 shRNAs for each gene. The chosen genes concerning miRNA pathway encode for DICER, RNASEN (also known as Drosha), EDC4 (enhancer of mRNA deccaping 4), DCP1A (mRNA-decapping enzyme 1A), and TNRC6A (GW182-related). For the RLH signaling, the following regulators were selected: ISG15, ATG5, ATG12, RNF125, OTUD5, LGP2, and PIN-1. All of these regulators are involved in innate immune responses, particularly in IFN production (see Figure 5).

As described in Figure 6, lentiviral-based vectors that express RNA-inducing shRNAs for each gene were used to transduce Jurkat cells at an MOI of 1. For 96 hours, transduced cells were challenged gradually with increasing concentrations of puromycin and then cloned with a ClonaCell™-TCS semi-solid medium (StemCell Technologies, Vancouver, Canada). This method was chosen for its ability to simultaneously select and clone. We obtained 152 individual shRNA-transduced Jurkat cell clones after this procedure. To identify regulators essential for HIV-1 replication but innocuous for T-cell viability, individual shRNA clones were expanded and cultured for 2 months in medium supplemented with puromycin. At this stage, the number of Jurkat shRNA clones that survived was reduced to 19 (Table 1), which may be due to cytotoxic effects resulting from gene knockdown in cells cultured for 60 days.

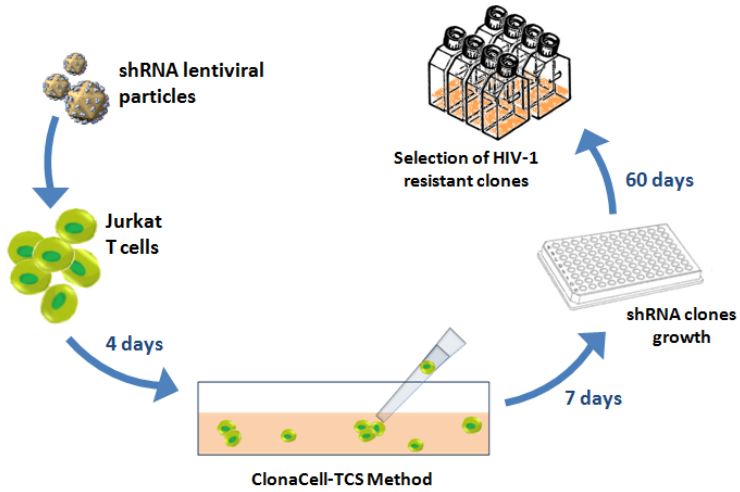

Figure 6. shRNA screen in Jurkat cells. shRNA encoding lentiviral particles were used to transduce Jurkat T cells. After ClonaCell-TCS Method, shRNA Jurkat clones were obtained, expanded and allowed to growth for 2 months to identify cellular proteins essential for HIV-1 replication but not essential for the cell viability.

Gene Symbol	Name and Features	shRNA	# shRNA clones
DICER1	**Double-stranded RNA-specific endoribonuclease type III** Required for formation of RISC and to process precursor miRNAs and siRNAs.	E6 E8	3 2
RNASEN	**DROSHA, Double-stranded RNA-specific endoribonuclease type III** Core nuclease that executes the initiation step of miRNA processing in the nucleus by cleaving pri-miRNA to release pre-miRNA.	B5 C5	2 1
EDC4	**Enhancer of mRNA decapping 4 (or Hedls)** Component of a decapping complex containing DCP1A and DCP2, seems to play a role mRNA decapping. Component of P-bodies.	-	0
DCP1A	**mRNA decapping enzyme 1A** Part of a cytoplasmic complex in P-bodies containing proteins involved in mRNA decay, including XRN1 and LSm1.	-	0
TNRC6A	**Trinucleotide repeat containing 6A protein (or GW182)** Plays a role in RNA-mediated gene silencing by both miRNAs and siRNAs. The protein associates with mRNAs and Argonaute proteins in P-bodies.	F3 F5	1 2
ISG15	**Interferon-stimulated protein or ISG15 ubiquitin-like modifier** Seems to display antiviral activity during viral infections by conjugating to intracellular target proteins after IFN-α or IFN-β stimulation.	B4	1
ATG5	**ATG5 autophagy related 5-like** May play an important role in the apoptotic process, being essential for autophagy when conjugated to ATG12.	-	0
ATG12	**ATG5 autophagy related 12 homolog** Required for autophagy. An autophagy regulator complex containing Atg5-Atg12 inhibits RIG-I-MAVS interactions.	-	0
RNF125	**Ring finger protein 125** E3 ubiquitin ligase that acts as a positive regulator in the T-cell receptor signaling pathway. Promotes degradation of ligand-activated RIG-I.	-	0
OTUD5	**OUT domain containing 5 (or DUBA)** Suppresses the type I interferon-dependent innate immune response by cleaving the polyubiquitin chair from TRAF3.	G2	2
LGP2	**RNA helicase likely ortholog of mouse D11LGP2** Facilitates viral RNA recognition by RIG-I and MDA5 through its ATPase domain.	D6 D10	2 3
PIN1	**Peptidyl-prolyl cis/trans isomerase NIMA-interacting 1** Essential isomerase involved in regulation of mytosis. Inhibits IRF3 activity during RLH signaling.	-	0

Table 1. Genes selected to the construction of shRNA clones.

Subsequently, to screen which viable shRNA clones were resistant to HIV-1 replication, an infection assay was performed for each individual clone. Since several clones were originated from the same lentiviral particles, only 10 different shRNA clones were infected with HIV-1_{NL4-3} VSVg with a MOI of 1. After 7 days of infection, viral replication was measured by $p24^{CA}$ ELISA in supernatant of infected cultures and resistance to HIV-1 replication was determined. To assure that the RNAi pathway was activated by the stable expression of shRNAs and did not interfere with HIV-1 replication leading to off-target effects, we used scrambled shRNA (shSCRAM) as control. This is a non-specific shRNA that activates the RNAi pathway, without targeting any human genes. As shown in Figure 7, shRNA RNASEN C5 and ISG15 B4 clones were resistant to HIV-1 replication without compromising cell viability. Indeed, when compared to wild-type Jurkat cells, these 2 shRNA clones exhibited more than 20% reduction in HIV-1 replication, indicating that our original shRNA screen was

able to efficiently isolate T-cells clones resistant to HIV-1 replication. However, we could also identify two clones that showed an 80% increase in HIV-1 replication, implicating a restriction nature in TNRC6A and OTUD5 proteins.

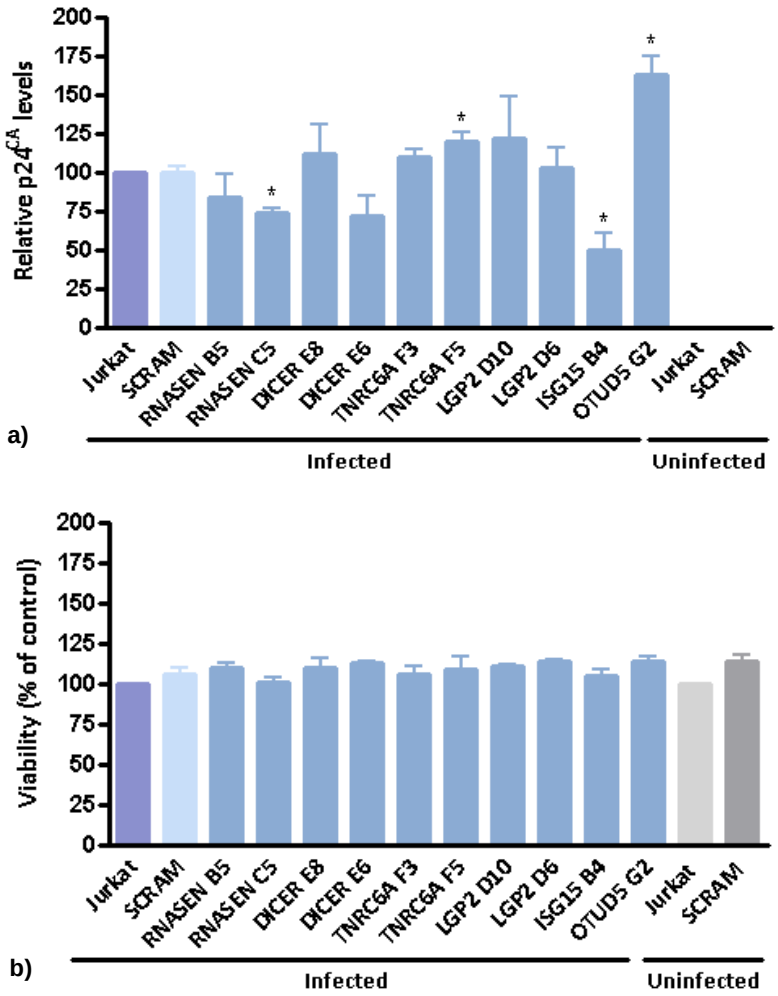

Figure 7. HIV-1 infection of resistant shRNA clones. a) One to two shRNA clones for each target gene were infected with HIV-1_{NL4-3} VSVg (MOI of 1) and after 7 days of infection, HIV-1 replication was measured by $p24^{CA}$ levels in the supernatant. **b)** Viability of shRNA clones after 7 days of infection with HIV-1_{NL4-3} VSVg. Values are relative to infected Jurkat cells, and results are shown as mean ± SD of 4 (a) or 3 (b) independent experiments. *P < 0,05 versus control.

2. Searching for more evidences

In the second part of this dissertation, the work developed focus on 13 of the 14 proteins identified by Rato *et al.* and their effect in HIV-2 replication cycle. In addition, several assays regarding LTR transcription and virus fusion were performed in order to establish the exact step of HIV-1 early phase in which SGK and CIB2 play their function.

2.1 Effect of identified proteins on HIV-2 replication cycle

First, we evaluated if the host factors previously identified by Rato *et al*. as helper factors in HIV-1 could also have an inhibitory effect in HIV-2 replication. For this purpose, we challenged shRNA clones with HIV-1_{NL4-3} and HIV-2_{ROD} using an MOI of 1 for 7 days and assessed both viral amounts in the supernatant and cellular viability (Figure 8).

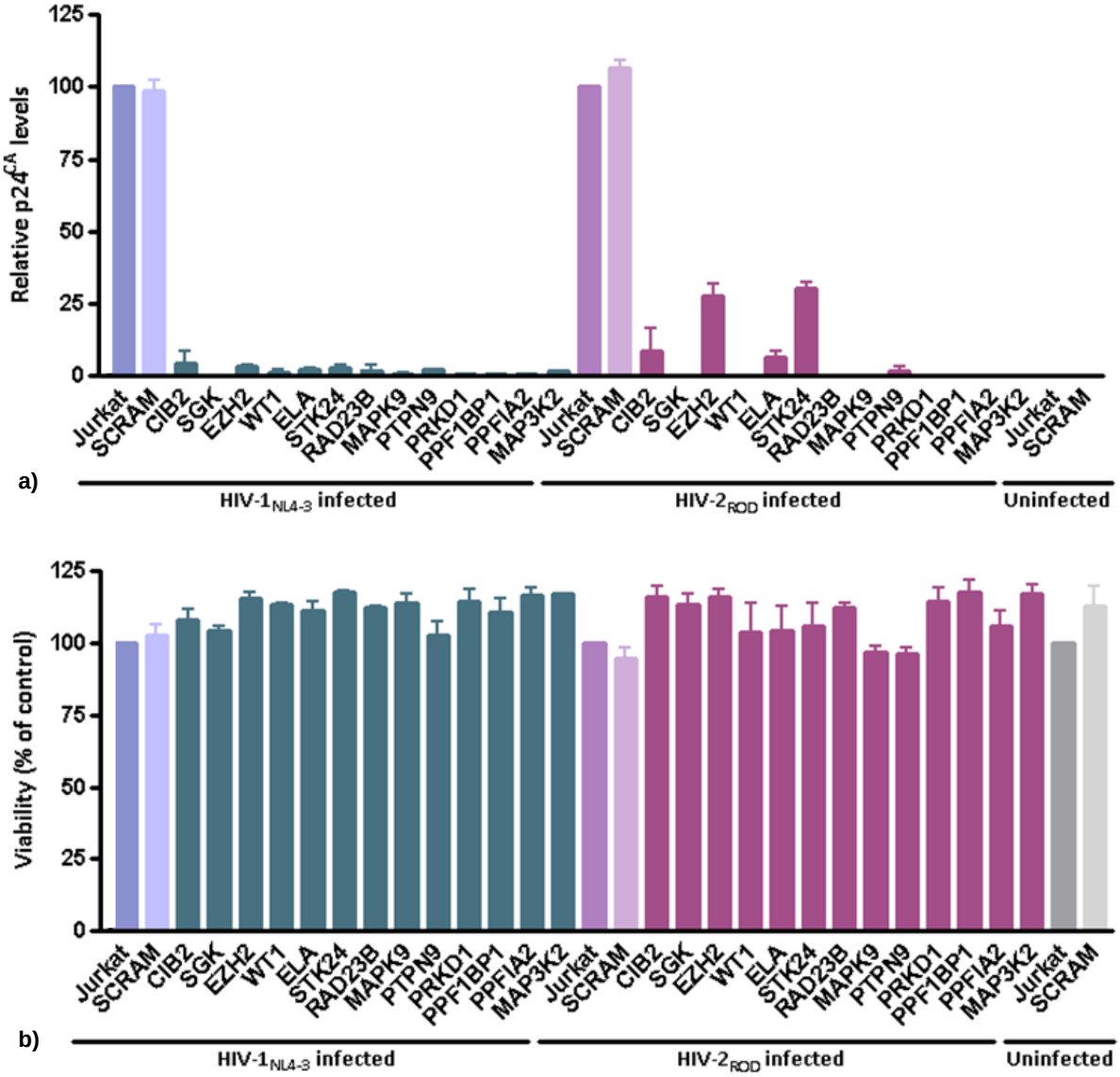

Figure 8. shRNA clones resistant to HIV-1 and HIV-2 infection. a) Each shRNA clone was infected with HIV-1_{NL4-3} and HIV-2_{ROD} (MOI of 1) and after 7 days of infection, HIV replication was measured by $p24^{CA}$ (HIV-1) or $p27^{CA}$ (HIV-2) levels in the supernatant. Values are relative to infected Jurkat cells. **b)** Viability of shRNA clones after 7 days of infection with HIV-1_{NL4-3} and HIV-2_{ROD}. Values are relative to infected Jurkat cells. This figure is representative of two independent experiments.

As represented in Figure 8a, we can observe a reduction in the amount of virus in all tested shRNA clones with both HIV-1, as already reported by Rato *et al*., and HIV-2. However, the decrease in viral amount was less dramatic for some clones in HIV-2

replication, particularly CIB2, EZH2 and STK24, when comparing to HIV-1 infection. Moreover, these clones show a higher amount of HIV-2 when compared to other shRNA clones. Since all shRNA clones exhibited viability values similar to the control (Figure 8b), these results were not due to an outcome of cell viability decrease.

2.2 Virus-cell fusion is affected by CIB2 and SGK

Since CIB2 potentially interacts with integrins [58, 58] and both SGK and CIB2 are involved in regulation of several ion channels [52-54], we inquired if these proteins could affect virus-cell fusion. To get a better understanding on the blocking effect of host-protein expression by shRNA in HIV-1 entry, we challenged Jurkat cells, shSCRAM, CIB2 and SGK shRNA clones with HIV-1_{NL4-3} particles with β-lactamase-Vpr chimeric proteins (BlaM-Vpr) incorporated. As a result of virion fusion, BlaM-Vpr is delivered into the cytoplasm of target cells and this transfer is detected by enzymatic cleavage of the CCF2 dye. BlaM cleaves the β-lactam ring in CCF2 loaded in target cells, changing its fluorescence emission spectrum from green (520 nm) to blue (447 nm) and thereby allowing fusion to be detected by flow cytometry.

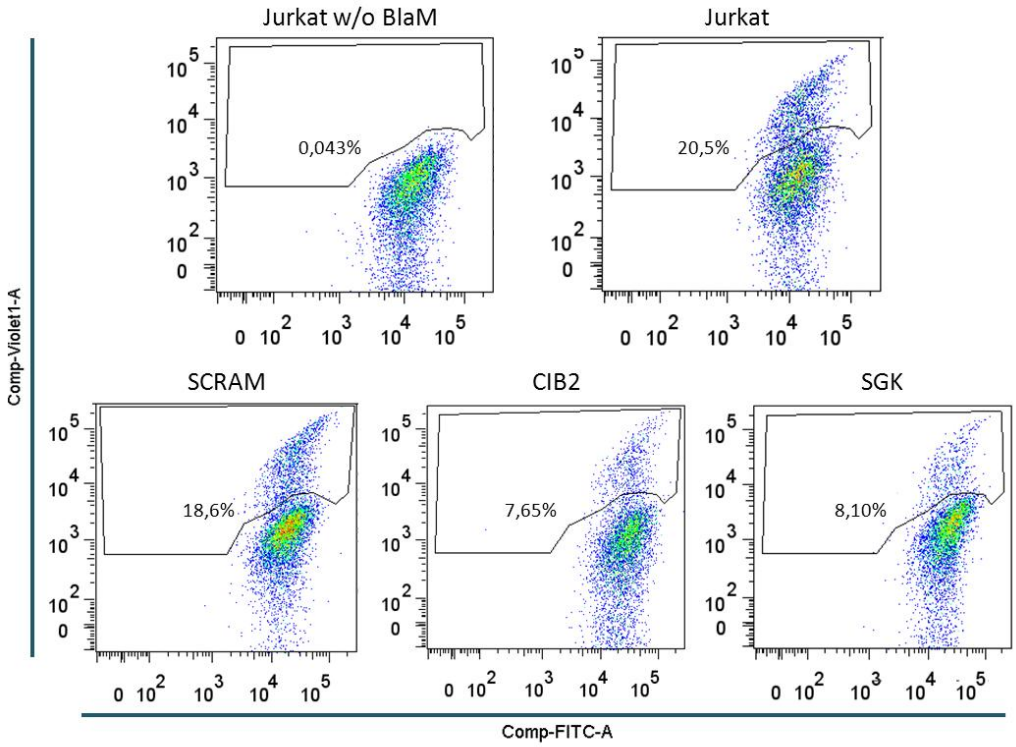

Figure 9. CIB2 and SGK shRNA clones lead to reduced fusion events. Jurkat cells, shSCRAM, CIB2 and SGK shRNA clones were infected with HIV-1_{NL4-3} with BlaM-Vpr (50-500 ng $p24^{CA}$) and after 3h of infection, cells were submitted to incubation with CCF2 substrate. Fusion was analyzed by flow cytometry using a violet laser for excitation of CCF2. Values are relative to Jurkat cells infected with HIV-1_{NL4-3} with BlaM-Vpr. This figure is representative of the two assays performed.

After infection with HIV-1_{NL4-3} virions containing BlaM-Vpr, ~20% of the shSCRAM and Jurkat cells displayed a higher ratio of green to blue fluorescence, indicating that fusion occurred. The shift to increased blue fluorescence induced by viral infection was reduced to 7,65% and 8,10% in CIB2 and SGK clones, respectively (Figure 9). This decrease by half suggests an involvement of CIB2 and SGK in viral fusion.

2.3 CIB2 as an enhancer of Tat transcription

Rato *et al*. demonstrated that the knockdown of CIB2 did not affect HIV-1 transcription; however no overexpression assays were ever done. For this reason, we sought to investigate whether CIB2 overexpression could affect Tat transactivation of HIV-1-LTR expression. To determine the most suitable concentration to use in subsequent assays, HeLa-P4 cells were transiently cotransfected with increasing concentrations of Tat, and the concentration 0.2 µg was chosen (data not shown). Then, Tat was either cotransfected with 0.5 µg or 1 µg of CIB2, using the same concentrations of pcDNA as a control. By examining β-Galactosidase activity (Fig. 10a), the relative expression of CIB2 exhibited a significant increase on LTR-directed transcription compared with control pcDNA. Additionally, a knockdown assay was performed using a shRNA for CIB2 (0.5 µg or 1 µg) to transfect with Tat, and shSCRAM was used as control. Here, no noteworthy effect could be observed for shRNA against CIB2 (Fig. 10b).

Next, we wanted to evaluate if the increase in transcription by CIB2 could be a result from an interaction with DNA-PKcs, since several findings suggest a function for DNA-PK in transcription, as well as other proteins that interact with this kinase [67]. Thus, we proceeded to the cotransfection of Tat, with increasing concentrations of CIB2 and shRNA DNA-PKcs, as shown in Figure 10c. As a control, increasing concentrations of CIB2 were transfected with Tat. Simultaneously, Tat was cotransfected with the corresponding concentrations of shRNA DNA-PKcs, and shSCRAM was used as control. As demonstrated in Figure 10c, knockdown of DNA-PKcs with overexpression of CIB2 led to a decrease in transcription by Tat. This result appears to be more significant than the reduction observed with the knockdown of DNA-PKcs. Taken together, our results suggest that CIB2 leads to increased LTR-driven expression, possibly via interaction with DNA-PKcs.

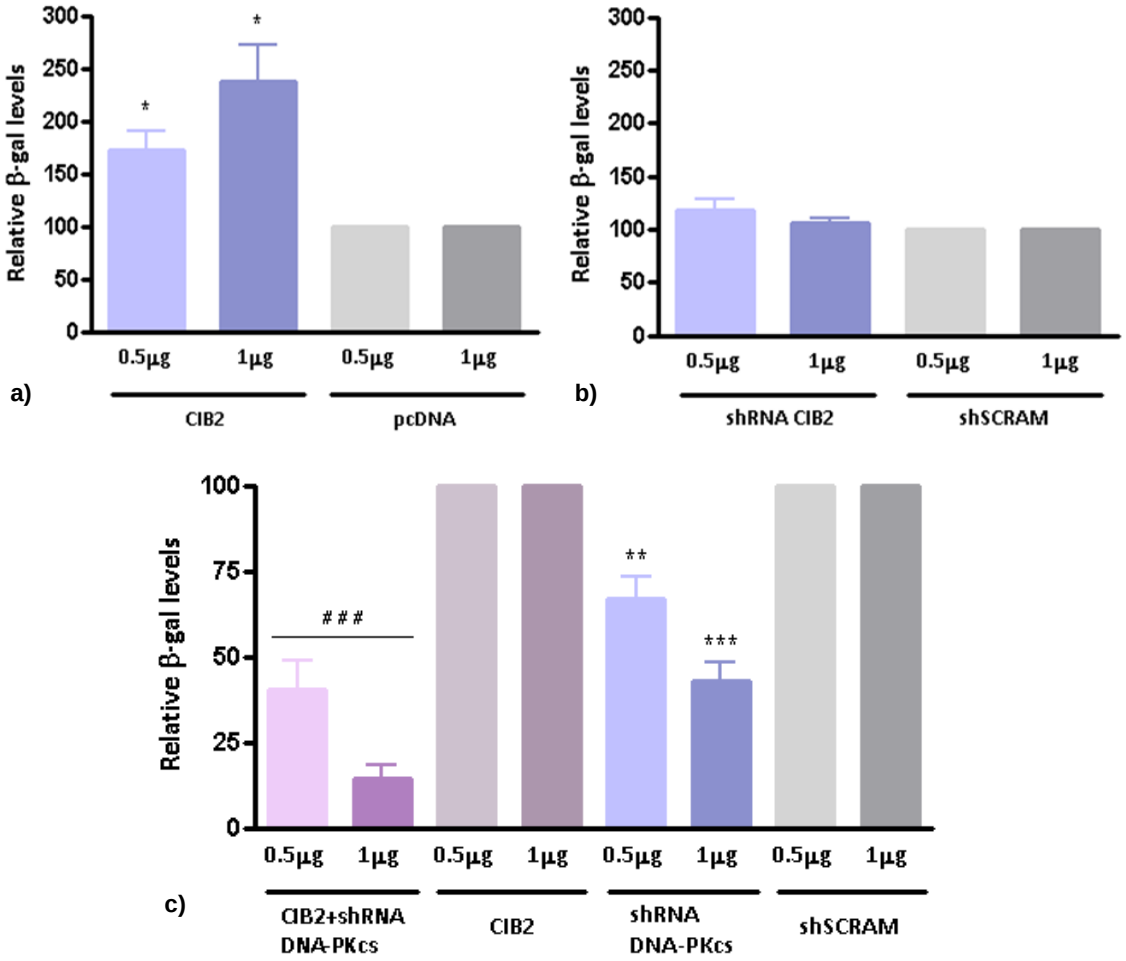

Figure 10. CIB2 has an effect in LTR-driven transcrition. a) pCMV-CIB2 or pcDNA3.1ZEO(+) was cotransfected with 0.2 µg of pCMV-Tat, using the concentrations 0.5 and 1 µg. **b)** shRNA CIB2 or shSCRAM was cotransfected with 0.2 µg of pCMV-Tat, using the concentrations 0.5 and 1 µg. **c)** pCMV-CIB2 was cotransfected with shRNA DNA-PK in the same proportion. Transfection of pCMV-CIB2 was used as control. shRNA DNA-PK was transfected with the same concentrations, and shSCRAM was used as control. All wells were also transfected with 0.2 µg of pCMV-Tat. LTR-driven expression was determined by β-Galactosidase activity. Results are shown as mean ± SD of 5 (a, b) or 9 (c) independent experiments. *P<0,05 versus pcDNA (n=5). $^{\#\#\#}$P<0.001 *versus* CIB2 (n=9). **P<0.05 *versus* 0,5µg shSCRAM (n=5). ***P<0.001 *versus* 1µg shSCRAM (n=5).

Discussion

Understanding the role of cellular proteins in cell types targeted by HIV during infection, such as T-cells, is critical to uncover the contribution of these factors towards disease progression. Since cellular proteins are less variable than viral proteins, the identified host proteins can potentially be used as antiviral targets, leading to therapeutic promise. To identify new HIV-1 cofactors, we performed a shRNA screen in Jurkat T-cells using a RNAi lentiviral library focused on two specific pathways, instead of a generalized genome-wide library, emphasizing the diversity of RNAi technology. These pathways are both involved in antiviral innate immunity: miRNA-mediated silencing pathway and RLH signaling pathway. Regarding miRNA pathway, we obtained clones for three genes, specifically RNASEN, DICER, and TNRC6A, failing this task for genes EDC4 and DCP1A. As respect to RLH signaling pathway, from the seven genes under study we only got clones for ISG15, LGP2 and OTUD5 genes. Both EDC4 and DCP1A are involved in specific decay pathways that recognize several mRNAs and promote their decapping [36, 74]. The failure of these pathways possibly assures a continuous process of translation, which may implicate a problematic cost of energy, conceivably leading to cellular arrest and/or cell death. Since all mRNAs are available for protein synthesis and are not retained for later return into translation, cellular machinery of transcription could also be overexpressed and uninterruptedly synthesizing new mRNAs. The deregulation of these mechanisms might be responsible for the cytotoxic effect observed in EDC4 and DP1A shRNA clones. Some genes encoding for regulators of RLH signaling are also associated to cell development and homeostasis, particularly RNF125, PIN1 and the complex ATG5-ATG12. RNF125 is a positive regulator in T-cell receptor signaling pathway [75] whereas PIN1 is involved in regulation of mitosis [76] and in degradation of inducible nitric oxide synthase [77]. In addition, the complex ATG5-ATG12 plays an important role in autophagy, an essential process for physiological homeostasis [78]. Therefore, the knockdown of any of these proteins could implicate a deregulation of essential cellular mechanisms, suggesting an explanation for our inability to obtain positive clones without compromising cellular viability.

According to our results for miRNA pathway, RNASEN C5 shRNA clone showed a significant decrease in HIV-1 replication, indicating a positive role in HIV-1 cycle. RNASEN, as the RNA endonuclease III responsible for the cleavage of pri-miRNA into pre-miRNA, plays an important role in miRNA-mediated silencing [46]. Down-regulation of this protein leads to a suppression of miRNA pathway which impairs interaction between RNA silencing and HIV-1. Although earlier evidences reported that the inhibition of RNASEN by siRNA increases HIV-1 replication [79], our results could also implicate a deregulation of RNA

silencing but in a different direction. Indeed, this suppression of RNASEN can affect specifically one kind of interaction, viral miRNAs down-regulation of cellular mRNA [27], suggesting a manipulation of immune response by HIV-1. Our results can also be explained by the use of different cell lines, as Nathans et al. used 293T cells and we returned to T lymphocytes, more precisely Jurkat cells. Although the aim of this screen was to identify helper factors, we obtained unexpected results suggesting that two genes could be restrictions factors. One of these genes is TNRC6A. As shown in Figure 7, the knockdown of TNRC6A led to an increase in HIV-1 replication, proposing a viral repressor role for this gene probably by preventing HIV-1 mRNA translation. Our work confirms previous evidences of enhanced viral replication when TNRC6A is down-regulated [26], while using Jurkat T cells, a physiological model for infection by HIV-1.

Regarding RLH signaling, we identified two proteins, ISG15 and OTUD5, with opposite effects in HIV-1 replication. While ISG15 B4 shRNA clone showed a robust decrease in HIV-1 replication indicating that it could act as a helper factor, OTUD5 exhibited an increase suggesting it as a restriction factor. When challenged with HIV-1_{NL4-3}, ISG15 B4 shRNA clone showed a robust decrease in HIV-1 replication, proposing ISG15 as a helper factor. ISG15 seems to display a role during viral infections by conjugating to intracellular target proteins after IFN-α or IFN-β stimulation [80]. In fact, the evidence that IFN-α stimulates the induction of ISG15 [81] prompted a study by Okumura and colleagues [80] in which they show that overexpression of ISG15 inhibits HIV-1 release from infected cells. This effect was reverted when ISG15 expression was inhibited by siRNA [80]. For this reason, we would expect an enhanced level of viral replication with ISG15 knockdown. Instead, we observed a decrease in HIV-1 amount. This result, although controversial, could lead to a different direction towards understanding HIV-1 infection and innate immunity. Previous studies by Okumura *et al*. only focused on HIV-1 production, leaving a question concerning viral entry. Our results could indicate that ISG15 affect viral infectivity, influencing subsequent infections during a 7 day-infection assay, and therefore reducing HIV-1 replication. OTUD5 is a deubiquitinase that prevents TRAF3-mediated activation of downstream kinases, thereby inhibiting RLH signaling and consequently type I IFN response [24]. Since a type I IFN response leads to an antiviral cellular state [81], OTUD5 down-regulation could positively regulate the IFN signaling, enhancing the antiviral response against HIV-1. This increased antiviral response was expected to have a negative effect in HIV-1 cycle, but instead we observed an opposite effect when we down-regulate OTUD5. This could mean a possible negative feedback by other regulator from RLH signaling, perhaps PIN1, mimicking OTUD5 function in order to guarantee a lower IFN response and therefore allowing a higher rate of HIV-1 replication.

RNAi screening performed by Rato *et al*. allowed the identification of 14 different proteins that assist HIV-1 replication, possibly playing an important role before viral integration in an early step of HIV-1 cycle, such as entry or uncoating. Development of small molecules that modulate the activity of these proteins may provide novel strategies for treatment of HIV/AIDS, particularly since the inhibition of these stages in the viral life cycle has already proven to be therapeutically effective. For this purpose, we attempted to assess two topics: 1) the effect of 13 of the 14 identified proteins in HIV-2 replication cycle; and 2) the mechanism by which CIB2 and SGK affect the early phase of HIV-1 life cycle, more precisely in viral fusion and transcription.

Since HIV-2 has a reduced pathogenicity and slower disease progression than HIV-1 [15, 82], it provides an important tool to study immune control during lentiviral infections. Identifying the host factors that account for controlled immunity will probably clarify the mechanisms responsible for the pathogenic nature of HIV-1 and may help uncover immune factors responsible for the control of HIV-2 infection. In order to establish the effect of the proteins identified by Rato *et al*. in HIV-2 life cycle, we compared the amount of virus in all tested shRNA clones challenged with both HIV-1 and HIV-2. Our results demonstrate that all proteins have an inhibitory effect in HIV-2 replication, as well as in HIV-1 cycle. This evidence could indicate that both HIV-1 and HIV-2 share some cellular pathways while hijacking these host factors to assure its survival. Since previous data demonstrated that all identified proteins have an effect on HIV-1 early phase, and presuming the same for HIV-2 cycle, any difference in viral inhibition could highlight some knowledge into HIV-2 replication cycle. Indeed, some shRNA clones, particularly CIB2, EZH2 and STK24, showed a less drastic reduction in viral amount in HIV-2 replication, when comparing to HIV-1. Both CIB2 and EZH2 could be involved in viral entry, due to either their connection to ion channels and integrins [53, 54, 58, 59] or their association to EED [34], respectively. The lower contribution of these proteins to viral entry could rely on the fact that HIV-2 can use a much wider repertoire of both CC and CXC chemokine receptors including CCR1 to CCR5, CXCR2, and CXCR4 [18]. Because STK24 is activated upon NRD1 phosphorylation, it has been proposed that STK24 could be incorporated in HIV-1 along with NRD1 and NRD2 [34]. This incorporation may not occur with the same efficiency in HIV-2 cycle, leading to the possibility that other cellular factors could perform a similar role. Further studies regarding the role of all 13 proteins in HIV-2 replication might bring new perspectives in HIV-cell interactions.

The characterization of the identified targets by Rato *et al*. is a succeeding tread, guiding to intrinsic knowledge of their role in specific steps of HIV-1 replication cycle. To

accomplish this, we performed virus-cell fusion and LTR-driven transcription assays against CIB2 and SGK shRNA clones.

First, we attempted to assess whether CIB2 and SGK affect the specific step of the fusion event in HIV-1 early phase. Our results indicate a decrease in the percentage of cells subjected to viral fusion from ~20%, in Jurkat cells, to 7,65% and 8,10% in CIB2 and SGK clones, respectively. This reduction suggests an involvement of CIB2 and SGK in viral fusion. Since CIB2 interacts with integrins [83], and integrins have alrealdy been associated to HIV-1 replication [58], the involvement of CIB2 probably focuses on this interaction. Knockdown of CIB2 could lead to a deregulation of integrin signaling, as a cytoplasmic effector in integrin α7Bβ1D signaling pathway [83], which could reduce HIV-1 replication. CIB2 possibly modulates the function of other integrins, and integrin modulation has been demonstrated to inhibit HIV infection, more precisely virus-to-cell and cell-to-cell transmission [84]. Also, this viral suppression could result from a decrease in NF-κB activation due to inhibition of integrins, as shown in macrophages [83]. Moreover, CIB2 is also related to ion channels [61], as well as SGK. The activation of certain potassium, sodium, and chloride channels by SGK suggests an involvement in the regulation of processes such as cell survival [52], which may control other cellular elements possible incompatible with HIV replication, such as intracellular pH and calcium levels [83]. Furthermore, ion channeling is strongly connected to chemokine signaling, which could affect HIV-1 entry although without blocking gp120/chemokine interaction [53, 85]. Moreover, both CXCR4 and CCR5 were demonstrated to transduce intracellular signals in monocyte-derived macrophages, activating K^+ and Cl^- ion channels and elevating intracellular calcium in response to their chemokine ligands [52]. The HIV-1 entry coreceptor activity of a chemokine receptor does not simply result from its expression in conjunction with CD4, but involves cell-specific determinants that enable fusion and productive infection [52]. For these reasons, we propose that both CIB2 and SGK could affect HIV-1 entry by deregulating chemokine signaling through inhibition of certain ion channels.

Regarding CIB2, we perfomed the cotransfection with Tat and CIB2 which revealed an increase in β-gal level, implicating CIB2 as an enhancer of LTR transcription by Tat. As already stated by Rato *et al*., the knockdown of CIB2 did not affect transcription, which could demonstrate that CIB2 is not essential in HIV-1 transcription but nonetheless important to enhance expression by LTR. Since CIB2 potentially interacts with DNA-PKcs, we investigated a putative contribution of DNA-PK on CIB2 overexpression LTR-driven transcription. Outcome results from the cotransfection with CIB2, shRNA for DNA-PKcs, and Tat showed a strong inhibition of LTR transcription, even more significant than the one observed with the knockdown of DNA-PKcs. These data could indicate that CIB2 has an

enhancing effect in LTR-driven transcription by Tat, possibly due to DNA-PKcs. Indeed, our findings suggest a putative interaction between CIB2 and DNA-PKcs that might result in upregulated expression of the HIV-1 LTR.

In conclusion, the results presented in this dissertation bring new insights for the complex interaction between HIV-1 and its cellular host. The identification of helper and restriction factors involved in miRNA-mediated silencing and RLH signaling pathways is an important step for a better understanding of innate antiviral immunity, particularly in T lymphocytes. Moreover, uncovering new targets through characterization of proteins in HIV-2 replication cycle or either by assessing the specific step of HIV-1 cycle affected by each identified protein could instigate further studies involving cellular pathways and HIV-1 replication. More importantly, these new studies could lead to novel antiviral strategies towards HIV-1 infection and maybe accomplish a more efficient treatment for AIDS.

Concluding Remarks

RNAi technology constitutes an important tool to uncover cellular factors important for HIV-1 replication and to enrich the knowledge of HIV-cell interactions. However, several limitations are present and only future screens will improve the stringency of the selection, expand the cell types under study, and devise better strategies to address false positive/negative candidates.

Since viruses are dependent on host machinery, all cellular mechanisms or its interveners may be important as antiviral factors. For this reason, short-RNA-mediated silencing and every related innate pathway have become relevant for understanding mammalian antiviral defenses and viral interference on them. The involvement of some P-body components in RNA silencing seems clear, although it remains to be established the functional connection between miRNA-mediated regulation and P-bodies. A significant future challenge will be to uncover the steps that underlie RNA silencing of viral origin and its connection to HIV-1 life cycles. One step to accomplish that is to determine all HIV-1 encoded miRNAs and then establish their role in evading cellular defenses. Another interesting point for future studies is to understand how some regulators of innate immunity could offer different outcomes regarding type I IFN signaling, and more precisely the mechanisms by which they affect HIV-1 infection.

A better understanding of the mechanism by which the identified clones by Rato *et al.* affect HIV-2 replication may be important to uncover specific pathways hijacked by HIV-2. Although chemokine signaling has been reported to be involved in HIV-1 entry, the exact role chemokine-mediated activation of ion channels in fusion event remains unclear. To achieve this, it will be essential to discover the mechanism by which CIB2 and SGK regulate ion signaling. Even more, it is crucial to establish if the interaction between CIB2 and integrin α7Bβ1D has in fact an effect in HIV-1 replication. CIB2 was recognized as an enhancer of Tat activity in LTR-driven expression, but its role appears to be unnecessary to complete viral transcripion. The presumable interaction between CIB2 and DNA-PKcs given by our results is still a hypothesis that needs to be assessed. Further studies will be imperative to confirm this theory. Moreover, assessing other possible roles of CIB2 and SGK in HIV-1 early phase could provide other therapeutic approaches.

The work presented in this dissertation proposes several functions of the identified proteins in HIV pathogenesis, but more important, incite further studies regarding HIV-cell interaction.

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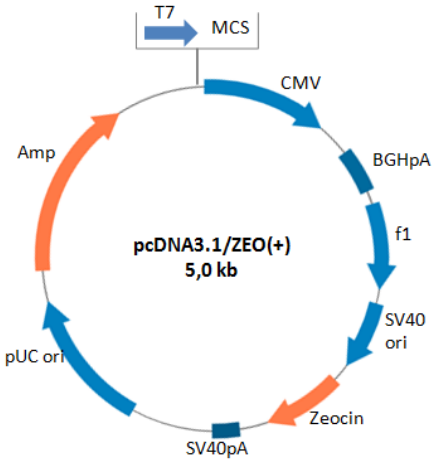
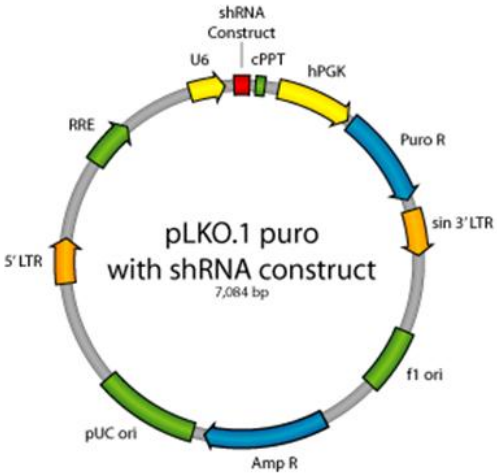
Annexes

Plasmids



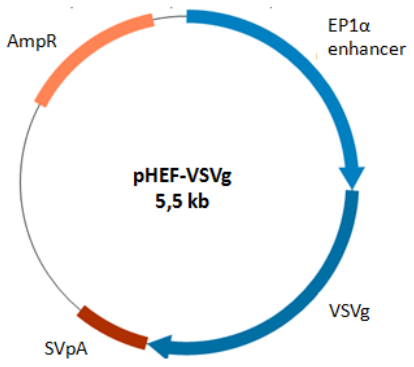
Plasmid Features
CMV promoter
T7 promoter priming site
Multiple cloning site
BGH reverse priming site
BGH polyadenylation signal
f1 origin
SV40 promoter and origin
EM7 promoter
Zeocin™ resistance gene
SV40 polyadenylation
pUC origin
bla promoter
Ampicillin (bla) resistance gene

Figure 11. Plasmid features and genomic map of pcDNA3.1/ZEO(+). This plasmid was used with CIB2 construct in MCS (BamHI and XbaI).



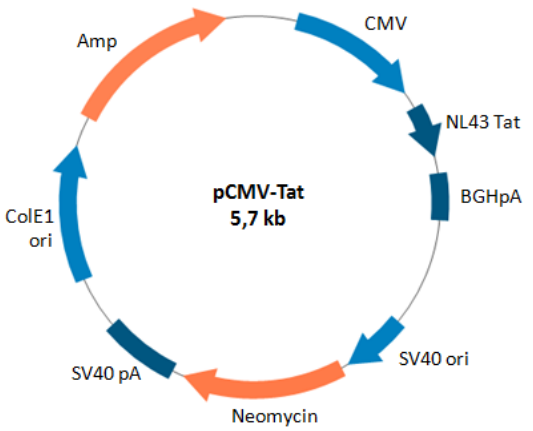
Plasmid Features
hPGK
puroR
SIN/3'LTR
f1 ori
ampR
pUC ori
5' LTR
RRE
hU6 promotor
cppt

Figure 12. Plasmid features and genomic map of pLKO.1 puro. This plasmid was used with shRNA constructs for SCRAM, CIB2 and DNA-PKcs. Adapted from MISSION® shRNA (Sigma Aldrich).



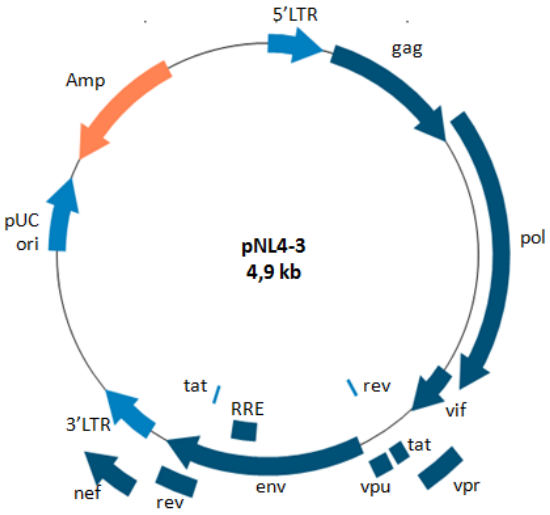
Plasmid Features
EP1α enhancer
VSVg
SVpA
Ampicillin resistance gene

Figure 13. Plasmid features and genomic map of pHEF-VSVg.



Plasmid Features
CMV promoter
T7 promoter priming site
NL43 Tat
BGH polyadenylation signal
SV40 promoter and origin
Neomycin resistance gene
SV40 polyadenylation
ColE1 origin
Ampicillin resistance gene

Figure 14. Plasmid features and genomic map of pCMV-Tat.



Plasmid Features
5'LTR
Gag
Pol
Vif
Vpr
Tat
Vpu
Env
Rev
Nef
3'LTR
pUC origin
Ampicillin resistance gene

Figure 15. Plasmid features and genomic map of pHIV-1_{NL4-3}.