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CELL-HOST INTERACTION: ASSESSING THE ROLE OF HELPER FACTORS IN HIV-1 REPLICATION

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Aos meus Pais

Preface

“The greatest grand challenge for any scientist is discovering how to prevent the spread of HIV and finding the cure or an effective vaccine for AIDS.”

Philip Emeagwali

The first recognized cases of AIDS, although not yet identified, occurred in the USA in the early 1980s. A number of homosexual men in New York and California suddenly began to develop rare opportunistic infections, such as pneumocystis pneumonia and Kaposis sarcoma, and cancers that seemed stubbornly resistant to any treatment. From these occurrences, a number of theories were developed included infection with cytomegalovirus, the use of amyl nitrite or butyl nitrate "poppers", and "immune overload", but it was of common sense that these facts were due to a common syndrome.

In January 1983, Montagnier group obtained a lymph node biopsy from a young homosexual man with a lymphadenopathy in the neck. They analysed the lymph node, dissociated it into single cells, and cultured the T lymphocytes with interleukin-2 and antiserum to human interferon. Surprisingly, Françoise Barré-Sinoussi found traces of reverse transcriptase viral enzyme in the supernatant of the lymphocyte culture, indicating the presence of a retrovirus. However, after testing if the viral proteins in the supernatant were recognized by antibodies against HTLV (the only type of retrovirus known at the time, discovered by Gallo´s group), the

labelled viral supernatant did not precipitated with the HTLV antibodies, but could be precipitated with a protein of the patient's own serum. This protein with a molecular mass of about 25 kD seemed to be the equivalent to the p24 protein of HTLV-1. However they were not able to isolate the virus from the lymphocytes. Afterwards, several blood samples from other infected patients were analysed in Montagnier laboratory and other laboratories, and the first publications came out at the end of 1983 were Montagnier and Gallo's groups identified a new retrovirus as the causative agent of AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1983). Gallo claimed that the virus they isolated from an AIDS patient was strikingly similar in shape to other HTLVs and designated by HTLV-III. In contrast, Montagnier's group demonstrated that the isolated virus was distinct from HTLV-1 and named it LAV from lymphadenopathy-associated virus (LAV). HIV was chosen as a compromise between the two claims (LAV and HTLV-III).

Soon after the identification of AIDS in humans, similar symptoms were identified in captive colonies of Asian rhesus macaques in USA primate centres. The sera from these animals showed cross-reactivity to HIV-1 antigens, which led to the identification of the related lentivirus, termed simian immunodeficiency virus (SIV) (Daniel *et al.*, 1985). Subsequently, it was isolated a virus from West African AIDS patients from Guinea-Bissau and Cape Verde, more closely related to SIV than HIV-1. This virus, referred to as lymphadenopathy-associated virus type 2 was then denominated as HIV-2 (Clavel *et al.*, 1986).

From 1983 until nowadays, the scientific achievements in HIV research have been outstanding. Thousands of scientist articles have been published and a great knowledge was achieved at diverse areas as etiology, molecular virology, natural history, epidemiology and virus pathogenesis. Moreover, the identification of infected patients through blood tests and the development of anti-retroviral drugs have helped the infected patients to be better controlled and having a prolonged life. Nevertheless, despite the effort, a cure for HIV-1 has not been achieved and AIDS pandemic continues to be a reality, being the underdeveloped countries the most affected.

Therefore it is imperative to continue to study HIV-1 infection giving close attention to the mechanisms underlying the complex battle between HIV-1 and its host. It is important to develop new antiviral strategies and new therapies that can be effective against HIV-1.

With these goals in mind, I have performed my PhD research, focused on the interplay between HIV and its host. The presented thesis is divided in five chapters: Chapter 1 provides a general introduction and review of HIV-1 biology and the cellular proteins and pathways known to be involved in HIV-1 infection; Chapter 2 describes an iterative shRNA screen in Jurkat T cells that we have developed to identify helper factors for HIV-1 infection. Here it is described the screen itself and the identification and validation of newly cellular proteins essential for HIV-1 replication; Chapter 3 presents the characterization of the identified proteins function during the major steps of HIV-1 life cycle; Chapter 4 focus on the characterization of DNA-PKcs function in HIV-1 replication, compared with CIB2, identified in the shRNA screen; and Chapter 5 integrates our overall findings in a general conclusion.

With this thesis we hope to contribute to a better knowledge of HIV-1 infection, through the study of mechanisms involved in the interaction between HIV-1 and its cell host and ultimately by providing evidences for new therapeutic targets against HIV-1.

Abstract

Much progress has been made in the past twenty-seven years to understand the complexity of human immunodeficiency virus type 1 (HIV-1) infection and to develop an efficient strategy that can eliminate the virus from its host. Despite all efforts this strategy has not been achieved mainly due to the emergence of resistant viruses and to the persistence of latently infected viral reservoirs. Hence, it is crucial to identify novel drug targets and new therapeutic strategies to combat the acquired immunodeficiency disease syndrome (AIDS). It is conceivable that the use of cellular proteins as antiviral targets instead of viral proteins could be a good alternative strategy, once cellular proteins are less variable than those from viral origin, avoiding effectiveness fluctuations of drug efficacy. Therefore it is important to understand HIV-1 and host interaction, by studying the exploitation of the cellular machinery by HIV-1.

The work described in this thesis was focused on the interaction between HIV-1 and its host cell and on the discovery of new co-factors for HIV-1 infection. We have developed an iterative shRNA screen in Jurkat T $CD4^+$ cells to identify co-factors for HIV-1 infection, focusing on kinases and phosphatases, a very druggable class of proteins. With this innovative screen we were able to identify 14 new cellular proteins essential for HIV-1 replication in T lymphocytes but that do not interfere with cell survival. We have identified two phosphatases, PTPN9 and PTPRE; five kinases, PRKD1, MAP3K2, MAPK9, SGK and STK24; one hypothetical kinase-binding-protein, CIB2; two phosphatase-binding-proteins, PPFIA2 and

PPFIBP1; and four other proteins with diverse functions, RAD23B, EZH2, WT1 and ELA1. The role of these proteins in HIV-1 replication was validated through replication assays with T cell lines-expressing-shRNA for each gene in study and the role of the identified co-factors in different steps of the HIV-1 life cycle was then evaluated. It was verified that none of the studied proteins have a relevant role in HIV-1 proviral integration. Instead, all proteins seemed to play an important role before viral integration, in an early step of HIV-1 life cycle. Moreover, our results indicate that PRKD1, MAP3K2, MAPK9, RAD23B, EZH2, PPFIA2, PPFIBP1, WT1 and STK24 have an additional effect on HIV-1 LTR transcription.

The identification of CIB2, a hypothetical DNA-PKcs binding protein, in the initial screen led us to study the cellular protein DNA-PKcs and its importance in HIV-1 replication. We assessed its role in different steps of HIV-1 life cycle and verified that DNA-PKcs is essential for HIV-1 replication. Our results indicated that this co-factor does not have a role in the early steps of HIV-1 life cycle until viral cDNA integration but it is crucial to HIV-1-LTR driven transcription, having dramatic effects in the expression of Tat viral protein levels.

This study brings new insights for the complex interplay of HIV-1/host cell, showing additional knowledge on cellular proteins and pathways that are essential for HIV-1 replication but non-important for cell viability and opens new possibilities for antiviral strategies.

Keywords: HIV-1; shRNA; kinases; phosphatases; DNA-PKcs.

Resumo

Desde a descoberta em 1983 do vírus da imunodeficiência humana (VIH) como o agente etiológico da síndrome da imunodeficiência adquirida (SIDA), que tem sido feito um enorme esforço para compreender a complexidade da infecção com o VIH e desenvolver estratégias eficientes que consigam neutralizar o vírus no seu hospedeiro. Actualmente 33,4 milhões de pessoas vivem infectadas com o VIH, tendo sido descritos 2,7 milhões de novos casos em 2008.

O VIH é um retrovírus do género lentivírus, sendo conhecidos dois tipos de VIH, o vírus da imunodeficiência humana tipo 1 (VIH-1) e de tipo 2 (VIH-2), ambos identificados como responsáveis pela SIDA. No entanto o VIH-1 é o mais infeccioso e prevalente na população em geral.

A terapia antiviral actualmente aceite pela comunidade científica e médica, HAART (de *Highly Active AntiRetroviral Treatment*), embora consiga aumentar a esperança de vida dos indivíduos infectados e diminuir a carga viral, não consegue eliminar o VIH-1 do seu hospedeiro. Uma das principais razões para a persistência da infecção é o aparecimento de mutantes de resistência nos indivíduos infectados devido à baixa fidelidade da enzima viral transcriptase reversa, que gera mutações durante a replicação do genoma viral. Assim sendo, é peremptório o estudo e desenvolvimento de novas terapias antivirais para combater o VIH/SIDA.

O uso de proteínas celulares como alvos terapêuticos poderá ser uma estratégia alternativa para uma terapia antiviral mais abrangente. As proteínas celulares são muito menos variáveis comparativamente com as proteínas virais, diminuindo assim a probabilidade do aparecimento de mutantes de resistência. Deste modo, as proteínas celulares são consideradas um alvo mais fácil para o desenvolvimento de fármacos antivirais. Por esta razão é imperativo estudar a interacção do VIH-1 com o hospedeiro, compreendendo melhor as estratégias usadas pelo vírus para explorar as vias bioquímicas celulares para seu próprio benefício.

Duas das famílias de proteínas celulares consideradas como bons alvos para o desenvolvimento de fármacos são as cinases e as fosfatases. Estas proteínas devido à sua actividade catalítica e à sua capacidade de controlar as cascatas de sinalização na célula são um promissor alvo terapêutico. Estas proteínas são actualmente usadas como alvos terapêuticos para diversas doenças, como o cancro, sendo comercializados vários inibidores químicos.

Neste contexto o trabalho desenvolvido nesta tese focou-se na identificação e estudo de proteínas celulares importantes para a replicação do VIH-1. Para isso, desenvolvemos um método inovador baseado numa biblioteca de "*short hairpin RNAs*" (shRNA) que tem como alvo principal as cinases e fosfatases. As moléculas shRNAs, são um tipo de RNA de interferência (RNAi), que quando transcritas numa célula activam a maquinaria do RNAi e inibem especificamente o RNA menssageiro celular alvo. O nosso trabalho baseou-se numa biblioteca de shRNAs clonada em plasmídeos lentivirais. Estes vectores permitiram a produção de lentivírus que expressam os shRNAs e que após transdução levam à integração do shRNA no genoma da célula, tornando a sua expressão constitutiva. Neste trabalho foi utilizada a linha celular Jurkat, uma linha celular imortalizada de linfócitos T de modo a mimetizar o hospedeiro natural do VIH-1. Durante o processo, as células Jurkat previamente transduzidas com os shRNA lentivirais foram infectadas com VIH-1 e, as resistentes à infecção, foram recuperadas e isoladas. Deste modo, com este procedimento obtivemos células que devido à expressão do shRNA adquiriram resistência à infecção com VIH-1. Após sequenciação dos alvos dos shRNA nas células isoladas, conseguimos identificar 14 proteínas celulares essenciais para a replicação do VIH-1 em linfócitos T, que não tinham sido descritas anteriormente. Estas proteínas embora essenciais para o VIH-1 não interferem com a

sobrevivência da célula. Dentro deste grupo identificámos duas fosfatases, PTPN9 e PTPRE; cinco cinases, PRKD1, MAP3K2, MAPK9, SGK e STK24; uma proteína que hipoteticamente se liga a uma cinase, CIB2; duas proteínas de ligação a fosfatases, PPFIA2 e PPFIBP1; e outras quatro proteínas, RAD23B, EZH2, WT1 e ELA1 de diferentes funções na célula. As proteínas indentificadas estão envolvidas em várias vias de sinalização celular, nomeadamente nas vias de sinalização das MAPK/JNK, MAPK/ERK e NFκB.

Após a identificação das 14 novas proteínas celulares essenciais para a replicação do VIH-1 em linfócitos T procedemos à confirmação da sua importância na replicação do VIH-1 com a constituição de linhas celulares Jurkat a expressarem shRNAs contra cada proteína em estudo. Observámos que a regulação negativa das proteínas por parte dos shRNAs leva a uma drástica inibição da replicação do VIH-1 sem afectar a viabilidade celular. Posteriormente, continuamos o estudo com ensaios de replicação para avaliar o efeito das proteínas identificadas no ciclo de vida do vírus. Nestes ensaios observou-se que a inibição da replicação do VIH-1 nos shRNA clones é progressiva e acumulativa ao longo do tempo. Ao analisarmos especificamente algumas das principais etapas do ciclo de vida do VIH-1, observámos que as proteínas em estudo não têm uma função importante aquando da integração do genoma viral no cromossoma do hospedeiro, tendo uma função anterior, numa fase inicial do ciclo de vida.

Além das fases iniciais de entrada e integração, estudámos também o papel destas proteínas na transcrição do provírus sob o controlo do promotor viral, LTR. Os nossos resultados indicam que as proteínas PRKD1, MAP3K2, MAPK9, RAD23B, EZH2, PPFIA2, PPFIBP1, WT1 e STK24 além de serem importantes logo numa fase inicial do ciclo de replicação, também são importantes para a transcrição dos RNAs virais.

Numa fase final do trabalho, avaliámos a função da cinase celular, DNA-PKcs, durante a replicação do VIH-1, devido à identificação da proteína CIB2 na selecção inicial. CIB2 é uma proteína muito pouco conhecida e com grande homologia com CIB1, outra proteína celular descrita como parceira de interacção com DNA-PKcs. DNA-PKcs é uma proteína muito importante para a célula desempenhando diversas funções, sendo a mais crucial na reparação não homóloga de DNA após quebras na cadeia dupla de DNA. DNA-PKcs já tinha sido anteriormente descrito como um factor celular importante na infecção do VIH-1 mas a sua função é bastante controversa. No nosso trabalho estudámos o efeito da regulação negativa

de DNA-PKcs na replicação do VIH-1. Para isso constituímos, linhas celulares Jurkat com uma cassete de shRNA contra DNA-PKcs e avaliámos a replicação viral. Os nossos resultados indicam que a inibição de DNA-PKcs faz com que as células sejam resistentes à replicação com o VIH-1 indicando a importância desta proteína para a replicação viral. A função de DNA-PKcs no ciclo de vida do VIH-1 também foi avaliada e verificámos que, contrariamente a CIB2, DNA-PKcs não possui um papel nem na entrada do vírus na célula nem na integração do genoma viral no cromossoma da célula hospedeira. A importância de DNA-PKcs na replicação do VIH-1 parece ocorrer aquando da transcrição dos RNAs virais a partir do promotor viral LTR, afectando significativamente os níveis transcripcionais da proteína viral Tat.

Em resumo, o trabalho desenvolvido nesta tese contribuiu de forma significativa para um melhor conhecimento da complexa interligação entre o VIH-1 e a célula hospedeira, uma vez que foram identificados novos factores celulares de ajuda para VIH-1 e que nunca tinham sido directamente relacionados com o vírus. Estas proteínas demonstraram ser importantes para o vírus mas inócuas para a célula. A identificação e caracterização destas proteínas no contexto da replicação do VIH-1 juntamente com o estudo funcional de DNA-PKcs contribuiram para uma melhor compreensão dos mecanismos virais de sobrevivência e replicação, na célula hospedeira. Estes estudos além de contribuirem para um aumento de conhecimento nesta área podem levar também ao desenvolvimento de novas terapias antivirais mais abrangentes, tendo como alvos as proteínas celulares identificadas.

Palavras-chave: VIH-1; shRNA; cinases; fosfatases, DNA-PKcs.

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Abbreviations

Reagents and Techniques

CPRG	ChloroPhenolRed-β-D-Galactopyranoside
DMEM	Dulbecco's modified Eagle's Medium
BLAST	Basic Local Alignment Search Tool
ECL	Enhanced ChemiLuminescence reagent
ELISA	Enzyme-Linked ImunoSorbent Assay
MOI	Multiplicity Of Infection
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
HEK293T	Human Embryonic Kidney 293T cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RIPA	Radio-Immunoprecipitation Assay buffer
RPMI	Roswell Park Memorial Institute medium
qPCR	quantitative Real Time PCR
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

General

μg	microgram
β-TrCP	β-Transducin repeat-Containing Protein
AGO2	ArGOnaute 2
AIDS	Acquire Immunodeficiency Syndrome
AIP1/ALIX	Actin Interacting Protein 1
AP	Adaptor Protein
APJ	Apelin receptor
APOBEC	APOlipoprotein B mRNA Editing enzyme, Catalytic polypeptide-like

APOBEC3B	APOlipoprotein B mRNA Editing enzyme, Catalytic polypeptide-like 3B
APOBEC3C	APOlipoprotein B mRNA Editing enzyme, Catalytic polypeptide-like 3C
APOBEC3F	APOlipoprotein B mRNA Editing enzyme, Catalytic polypeptide-like 3F
APOBEC3G	APOlipoprotein B mRNA Editing enzyme, Catalytic polypeptide-like 3G
ARP	Actin Related Protein
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 related
BAF	Barrier-to–Autointegration Factor
BCA2	Breast cancer-associated gene 2
CA	Capsid
CBP	CREB Binding Protein
CCR2	C-C chemokine Receptor type 2
CCR3	C-C chemokine Receptor type 3
CCR5	C-C chemokine Receptor type 5
CCR8	C-C chemokine Receptor type 8
CCR9	C-C chemokine Receptor type 9
CD28	Cluster of Differentiation 28
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD45	Cluster of Differentiation 45
CDK9	Cyclin Dependent Kinase 9
cDNA	complementary DNA
ChemR23	G protein-coupled receptor CMKLR1
CIB1	Calcium and Integrin Binding family 1
CIB2	Calcium and Integrin Binding family 2
CMV	CytoMegaloVirus
CRM1/XPO1	Exportin 1
Cul5	Cullin 5
CXCR1	C-X-C chemokine Receptor type 1
CXCR4	C-X-C chemokine Receptor type 4
CXCR6	C-X-C chemokine Receptor type 6
CXCR7/RDC1	C-X-C chemokine Receptor type 7
CypA	Cyclophilin A
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DDX1	DeaD boX protein RNA helicase 1
DDX3	DeaD boX protein RNA helicase 3
DNA	DeoxyriboNucleic Acid
DNA-PK	DNA-dependent Protein Kinase
DNA-PKcs	DNA-dependent Protein Kinase catalytic subunit
dsRNA	double-stranded RNA
EED	Embryonic Ectoderm Development protein
EF	Elongation factor
EGFP	Enhanced Green Fluorescent Protein
ELA1	ELAstase 1
EloB	Elongin B
EloC	Elongin C
endo-siRNA/esiRNA	endogenous siRNA

Env	Envelop polyprotein
ER	Endoplasmic Reticulum
ERK 1	Extracellular signal-Regulated Kinase 1
ERK 2	Extracellular signal-Regulated Kinase 2
ERK 5	Extracellular signal-Regulated Kinase 5
ESCORT	EndoSomal-associated COmplex Required for Transport
EZH2	Enhancer of Zeste Homolog 2
FIV	Feline Immunodeficiency Virus
Fv1	Friend Virus susceptibility-1 protein
Gag	Group specific antigen polyprotein
GAPDH	GlycerAldehyde 3-Phosphate DeHydrogenase
GR	Glucocorticoid Receptor
h	hour
HAART	Highly Active Antiretroviral Therapy
HAT	Histone AcetylTransferase
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HLA	Human leukocyte Antigen
HLE	Human Leukocyte Elastase
HMG	High-Mobility Group
HMG I (Y)/HMGA1	Chromosomal protein A1
HRP	HorseRadish Peroxidase
HRP2	Hepatoma-derived growth factor Related Protein 2
HSA	Heat Stable Antigen
HSP60	Heat shock Protein 60
hTRIM5α	human TRIM5α
IL-1	InterLeukine 1
IL-7	InterLeukine 7
IN	Integrase
IRES	Internal Ribosome Entry Site
JAK	Janus Kinase
JNK	Jun N-terminal Kinase
kD	kiloDalton
LAP2 α	Lamina-Associated Polypeptide 2 alpha
LEDGF	Lens Epithelium-Derived Growth Factor
LFA-1	Lymphocyte function-associated Antigen 1
lhRNA	long harpin RNA
LTR	Long Terminal Repeat
MA	MAtrix
MAP3K2/MEKK2	Mitogen Activated Protein Kinase Kinase 2
MAPK	Mitogen-Activated Protein Kinase
MAPK9	Mitogen Activated Protein Kinase 9
MEK	MAP kinase kinase
MHC I	Major Histocompatibility Complex class I
MHC II	Major Histocompatibility Complex class II
min	minute
miRNA	microRNA
ml	millilitre

MLV	Murine Leukemia Virus
mM	miliMolar
mRNA	messenger RNA
NC	NucleoCapsid
NCBI	National Center for Biotechnology Information
NDR1	Nuclear Dbf2-Related kinase 1
NDR2	Nuclear Dbf2-Related kinase 2
Nef	NEgative Regulatory Factor
NES	Nuclear Export Signal
NFκB	Nuclear Factor-Kappa B
NFAT	Nuclear Factor of Activated T-cells
Ng	nanogram
NHEJ	Non-Homologous End Joining
NIAID	National Institute of Allergy and Infectious Diseases
NLS	Nuclear Localization Signal
nm	nanometer
NPC	Nuclear Pore Channel
PACS-1	Phosphofurin Acidic Cluster Sorting protein 1
PAK2	p21 Activated Kinase 2
PARP-1	Poly [ADP-Ribose] Polymerase 1
PBS	Primer Binding Site
PCAF	P300/CBP-Associated Factor
PCE	Post-transcriptional Control Element
PI3K	PhosphatidylInositol-3-Kinase
PIC	Post-Integration Complex
PIP2	PhosphatidylInositol 4,5-bisPhosphate
PIP3	PhosphatidylInositol (3,4,5)-trisPhosphate
piRNA	PIWI-interacting RNA
PKB	Protein Kinase B
PKC	Protein Kinase C
PKR	Protein Kinase R
Pol	Polymerase
PP1	Protein Phosphatase 1
PP6	Protein Phosphatase 6
PPFIA2	Protein tyrosine Phosphatase, receptor type F polypeptide Interacting (liprin) Alpha 2
PPFIBP1	PTPRF Interacting protein, Binding Protein 1 (liprin beta 1)
PR	PRotease
PRKD1	Protein Kinase D1
PtdIns(3,4)P_2	PhosphatidylInositol 3,4-bisPhosphate
P-TEFb	positive Transcription Elongation Factor b
PTEN	Phosphatase TENsin homolog
PTPN9	Protein Tyrosine Phosphatase Non Receptor type 9
PTPRE	Protein Tyrosine Phosphatase Receptor type E
PTPRF	Protein tyrosine Phosphatase, Receptor type F
Rab/hRIP	human Rev-Interacting Protein
RAD23A	RAD23 homolog A(*S. Cerevisiae*)
RAD23B	RAD23 homolog B(*S. Cerevisiae*)
ra-siRNA	repeat-associated siRNA

RBX1	RING-BoX protein 1
Ref1	Redox factor-1
Rev	Regulator of virus protein expression
RHA	RNA Helicase A
rhTRIM5α	rhesus macaque TRIM5α
RISC	RNA-Induced Silencing Complex
RNA	RiboNucleic Acid
RNAi	RNA interference
RNAPol II	RNA Polymerase II
RNase H	RiboNuclease enzyme H
RRE	Rev Responsive Element
RT	Reverse Transcriptase
RTC	Reverse Transcription Complex
SAM68	Src-Associated in Mitosis, 68 kDa
SCID	Severe Combined ImmunoDeficiency
scnRNA	scan RNA
SGK	Serum/Glucocorticoid regulated Kinase
SHIP	Src Homology 2 domaincontaining Inositol-5-Phosphatase
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIV	Simian Immunodeficiency Virus
SIVagm	SIV from african green monkeys
SIVmac	SIV from macaques
SKIP	Splicing-associated c-SKi-Interacting Protein
SKP1	S-phase-Kinase-associated Protein 1
SP1	Specificity Protein 1
STAT	Signal Transducers and Activator of Transcription
STK24	Serine/Threonine Kinase 24
SU	SUrface
TAR	TransActivation Response
ta-siRNA	*trans*-acting siRNA
Tat	Transcriptional transactivator protein
TF	Transcription Factor
TFIID	Transcription Factor II D
TM	TransMembrane
tncRNA	tiny non-conding RNA
TNF-α	Tumor Necrosis Factor-alpha
TRBP1	TAR RNA Binding Protein 1
TRBP2	TAR RNA Binding Protein 2
TRC	The RNAi Consortium
TRIM5α	Tripartite Motif Protein 5 alpha
tRNA	transference RNA
TSG101	Tumor Susceptibility Gene 101 protein
U3	Unique 3´ sequence
U5	Unique 5´sequence
UNAIDS	United Nations Joint Programme on HIV/AIDS
UNG2	Uracil DNA glycosilase 2
Vif	Viral infectivity factor
Vpr	Viral protein R

Vpu	Viral protein U
VSV-G	Glicoprotein G from Vesicular Stomatitis Virus
WT1	Wilms Tumor 1
XRCC4	X-Ray Repair Cross-Complementing protein 4

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CHAPTER 1

General Introduction

1.1. Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS)

Human immunodeficiency virus (HIV) is the causative agent of the progressive disease acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983), one of the leading causes of death worldwide (UNAIDS, 2008). At the present, approximately 33.4 million of people live with HIV, with 2.7 million new cases of HIV infection described in 2008 (UNAIDS, 2009). The natural HIV infection results in a continuous process where progressive depletion of cluster differentiation 4 $(CD4)^+$ T cells and immune dysfunctions occur. This leads to a final stage of the disease where individuals are more susceptible to opportunistic infections or malignancies that constitute the clinically defined AIDS (Fauci *et al.*, 1985; Phillips *et al.*, 1991). There are two types of human immunodeficiency virus described, type 1 (HIV-1) (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983) and type 2 (HIV-2) (Clavel *et al.*, 1986). Both types are known to cause AIDS, although HIV-2 represents a significant minority off all HIV infections. Furthermore, HIV-2 is characterized by a slow disease progression and transmission, a lower plasma viral load and a low rate of CD4-T cell decline (Marlink *et al.*, 1994; Whittle *et al.*, 1994; Jaffar *et al.*, 1997).

Since HIV discovery until our present days many efforts have been made to counteract AIDS. Several drugs were developed and efficient HIV therapies were settled. The current antiviral therapy accepted by scientific and medical community is the highly active anti-retroviral therapy (HAART) and consists of the administration of a combination of antiviral drugs that act against HIV proteins or viral RNA (De Clercq, 2009). HAART is able to reduce viral loads to almost undetectable levels. Still it has several drawbacks such as toxicity and the appearance of viral resistant mutants. Despite the substantial progress in the development of these anti-retroviral drugs, eradication of HIV-1 and consequently the cure of AIDS has still not been achieved. Therefore, HIV-1 research is still a very active field of research, where the study of HIV-1 biology and more precisely the interaction between the virus and its host cell can provide new insights for the development of new antiviral therapeutic strategies.

1.2. HIV-1 taxonomy

HIV-1 belongs to the genus Lentivirus of the *Retroviridae* family. Retroviruses are characterized by their single strand positive RNA diploid genome and by the virion polymerase that is capable of RNA-directed DNA synthesis (reverse transcription) generating a DNA intermediate that is integrated in the host genome (Bishop, 1978; Coffin, 1997).

Lentiviruses differ from other retroviruses mainly due to their long incubation period before manifestations of clinical illness. Moreover, lentiviruses share a common morphogenesis and morphology, a tropism for macrophages, extensive genetic and antigenic variability, and additional regulatory genes not found in the other groups of retroviruses (described below) (Narayan and Clements, 1989; Coffin, 1997).

Phylogenetic studies have distinguished 3 major groups among HIV-1 isolates: group M (Major), group O (Outlier) and group N (non-M, non-O). M group includes more than 90 % of HIV/AIDS cases and it is further subdivided into eleven clades (from A to K) (Geretti, 2006). In 2009, a newly-analyzed HIV sequence was reported and a new group, Group P, was proposed (Plantier *et al.*, 2009).

1.3. HIV-1 structure

HIV-1 mature virions are spherical shaped, with a diameter of approximately 100 nm. Like the other lentiviruses, HIV-1 has an outer membrane and an inner membrane. The outer viral membrane is derived from the host cell membrane, which consist of a lipid bilayer where the viral surface (gp120 or SU) glycoprotein is anchored via interaction with the viral transmembrane (gp41 or TM) glycoprotein (Turner and Summers, 1999; Wang *et al.*, 2000). The outer viral membrane is also constituted by cellular proteins derived from host cell, namely the histocompatibility antigens, actin and ubiquitin (Arthur *et al.*, 1992). The

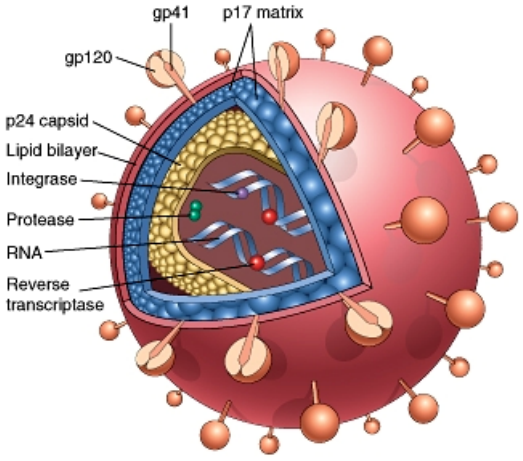

Figure 1.1. HIV-1 structure (Elsevier Science : Kumar *et al.*, 2009).

viral inner membrane is composed by several matrix proteins (p17 or MA) that involve the conical capsid core, formed by the capsid protein (p24 or CA). Subsequently, the capsid core encompasses two RNA double strand molecules of approximately 9 Kb, which are stabilized as a ribonucleoprotein complex. The ribonucleoprotein complex is formed by several copies of the nucleocapsid protein (p7 or NC) and by the viral enzymes integrase (p31 or IN), reverse transcriptase (p66/p51 or RT) and protease (p11 or PR). The viral proteins, negative regulatory factor (Nef), virion infectivity factor (Vif) and viral protein R (Vpr) are also incorporated in the viral particle (reviewed in Turner and Summers, 1999; Hoffmann *et al.*, 2007) (Figure 1.1).

1.4. HIV-1 genome

HIV-1 carries its genetic information in double strand RNA molecules that are synthesized by the host DNA-dependent RNA polymerase II (RNAPol II). Similarly to eukaryotic cellular mRNAs, HIV-1 RNA has post-translational modifications such as 5´ cap and 3´poly A tract (Whitcomb and Hughes, 1992). During HIV-1 life cycle, RNA molecules are reverse transcribed by RT to viral complementary DNA (cDNA) and then integrated into the host chromosome. Conceptually, HIV genome organization is discussed in terms of viral DNA after integration (i.e. provirus). Provirus representation places viral promoter, RNA start site and polyadenylation site in the same positions as they are typically found in host genes (Coffin, 1997).

HIV-1 genome, common to all replication-competent retroviruses, has three major genes, *gag,* for *group specific antigen polyprotein* (coding for structural proteins), *pol,* for *polymerase* (coding for viral enzymes) and *env, for* envelop (coding for envelope glycoproteins) flanked by long terminal repeats (LTR) sequences (figure 1.2) (Frankel and Young, 1998; Wang *et al.*, 2000). LTR regions are composed by short directed repeat sequence (R) flanked by unique 5´ (U5) and 3´ (U3) sequences (U5-R-U3). These regions are formed after duplication of U3 e U5

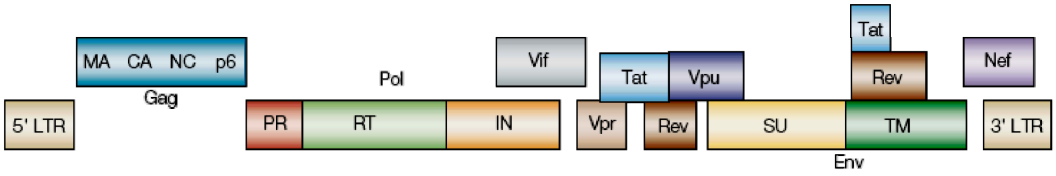

Figure 1.2. **HIV-1 genome** (adapted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol], Robinson, 2002 copyright (2002).

in both ends during reverse transcription (Whitcomb and Hughes, 1992). LTR sequence contains functional regions essential for HIV-1 transcription regulation, such as the transactivation response (TAR) element, the basal promoter and the core enhancer (Pereira *et al.*, 2000).

HIV-1 genes expression is mediated by viral proteins through regulatory mechanisms with overlapping reading frames and through alternative messenger RNA (mRNA) splicing (Wang *et al.*, 2000). The *gag* gene encodes a precursor protein of 55 kDa ($p55^{Gag}$) that during virus maturation is cleaved by the viral protease into the structural proteins matrix, capsid and nucleocapsid, and into the peptides p1, p2 and p6. The *pol* gene codifies three enzymes: protease, reverse transcriptase and integrase that are essential during HIV-1 life cycle (discussed bellow). These proteins are originated from a fusion polyprotein precursor $P160^{GagPol}$ that is synthesized through a rare frameshift event that occurs during $P55^{Gag}$ translation. The *env* gene encodes the polyprotein Env precursor gp160, which is cleaved by a cellular protease originating the surface (gp120) and the transmembrane (gp41) glycoproteins (Frankel and Young, 1998) (figure 1.2).

Differently from other retroviruses, HIV-1 codifies for six additional proteins: two regulatory proteins, transcriptional transactivator (Tat) and regulator of virus protein expression (Rev); and four accessory proteins Nef, Vif, Vpr and viral protein U (Vpu) (Frankel and Young, 1998). The regulatory proteins are essential for virus replication by controlling HIV-1 expression in host cells. On the other hand, the so called “accessory proteins” have this designation since they are often dispensable for virus replication *in vitro*. However, these proteins play essential roles in virus persistence, spread and pathogenesis *in vivo* and carry out many important functions during HIV-1 life cycle by interacting with cellular proteins (Li *et al.*, 2005a)

Tat is a multifunctional protein that acts mainly as a transactivating protein inducing a variety of effects through modulation of cellular and viral genes expression levels. Tat functions include chromatin remodelling, induction of phosphorylation of RNAPol II, transactivation of viral genes and binding to specific structures of HIV-1 mRNA (reviewed in Romani *et al.*, 2010).

Rev is a regulator of viral mRNA production that binds to rev responsive element (RRE) in viral RNA and facilitates the nuclear export of the single spliced viral RNAs (Hope, 1999).

Vif is essential for *in vivo* infectivity and pathogenesis. Its main function is to counteract the innate antiretroviral defences mediated by cytidines deaminases such as the apoliprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) (Sheehy *et al.*, 2002) and the apoliprotein B-editing catalytic polypeptide 3F (APOBEC3F) (Liddament *et al.*, 2004; Wiegand *et al.*, 2004; Zheng *et al.*, 2004) by different mechanisms (discussed below and reviewed in Henriet *et al.*, 2009).

Nef, Vpr and Vpu act as multiple-functional proteins in regulating HIV-1 infectivity and pathogenesis. Nef induces downregulation of CD4, major histocompatibility complex class I (MHC I) and II (MHC II), human leukocyte antigen (HLA) and cluster differentiation 28 (CD28) from HIV-1 surface of infected cells; enhances virion infectivity; stimulates viral replication; and modulates T cell activation state of its cell host (reviewed in Jere *et al.*, 2010). Vpr modulates transcription of the virus genome, promotes nuclear transport of the HIV-1 pre-integration complex (PIC), facilitates reverse transcription, causes G2 cell cycle arrest, induces apoptosis, induces defects in mitosis, suppresses immune activation and balances HIV-1 mutation rate (reviewed in Romani and Engelbrecht, 2009). Vpu is responsible for CD4 degradation, induction of apoptosis, enhancement of viral particle release and downregulation of MHC I and MHC II (reviewed in Nomaguchi *et al.*, 2008). More recently, it was reported that Vpu counteracts the cellular protein Tetherin, that specifically inhibits virion release from the host cells (Neil *et al.*, 2008).

1.5. HIV-1 life cycle

HIV-1 life cycle, commonly to all retroviruses, is divided into two distinct phases: 1) an early phase that begins with the recognition of the target cell by the infectious virion and involves all following steps until integration of the genomic DNA into the chromosome of the host cell; 2) a late phase, which begins with the expression of the integrated proviral genome, and involves all processes up to and including virus budding and maturation of the progeny virions (Turner and Summers, 1999) (represented in Figure 1.3). All these processes depend not only on viral proteins but also on the host cell machinery, which is exploited by the virus during its replication.

1.5.1 Early phase

1.5.1.1. Virus entry

HIV-1 life cycle begins when an infectious particle encounters its cell host. HIV host cells are mostly helper T cells, monocytes and macrophages. Other cells, such as Langerhans, follicular dendritic, glial, and certain colon tumor cell lines, are susceptible to HIV-1 infection (Vaishnav and Wong-Staal, 1991; Weiss, 2002). When a typical helper T cell is infected, virion attaches to cell surface through a high-affinity interaction between the viral glycoprotein gp120 and the primary cell receptor CD4 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985). This binding causes structural alterations in gp120 enhancing its affinity to a

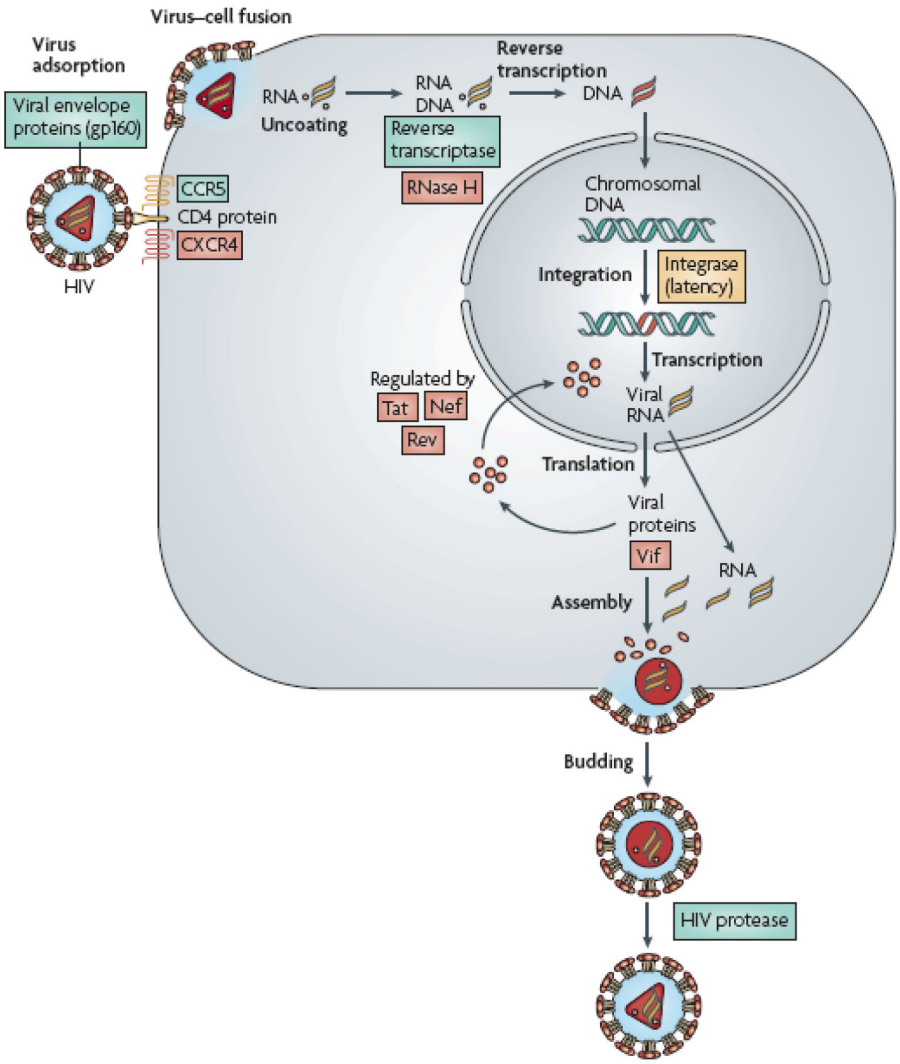

Figure 1.3. Schematic representation of HIV-1 life cycle. See main text for detailed description. Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Drug Discov], Flexner, 2007, copyright (2007).

coreceptor, that trigger fusion of the viral envelope to the cellular membrane (reviewed in Melikyan, 2008). HIV-1 co-receptors were identified such as G protein-coupled receptor superfamily of seven-transmembrane domains proteins (Berger *et al.*, 1999). The two major co-receptors for HIV-1 infection in vivo are C- X-C chemokine receptor type 4 (CXCR4) (Feng *et al.*, 1996) and C-C chemokine Receptor type 5 (CCR5) (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996). Based on the CD4/chemokine receptors paradigm, HIV-1 strains were classified as R5 viruses (isolates that uses only CCR5 co-receptor), X4 viruses (isolates that use only the CXCR4 coreceptor) and R5X4 viruses (isolates that use both co-receptors (Berger *et al.*, 1998). The coreceptor binding triggers conformational changes in gp120 and gp41 viral glycoproteins, namely the exposure of the gp41 fusion peptide and consequently insertion into the target cell membrane (Doms and Moore, 2000). This process leads to the fusion of the viral envelope with the target cell membrane and to the delivery of viral core into the cytoplasm of the target cell.

1.5.1.2. Post-entry: Uncoating and Reverse Transcription

Immediately after release of the viral core into the cell host cytoplasm, HIV-1 undergoes a partial and progressive process of uncoating, where viral core is rearranged into a new structure, the reverse transcription complex (RTC) (Lehmann-Che and Saib, 2004). The RTCs are large dense complexes that contain a different viral protein composition from the original viral core, like MA and RT; and several cellular host proteins necessary for reverse transcription, translocation and forward processes, such as actin, barrier-to-autointegration factor (BAF) and lens epithelium-derived growth factor (LEDGF/p75) (McDonald *et al.*, 2002; Warrilow and Harrich, 2007). Exposure of RTC to a significant concentration of deoxyribonucleotides in the cytoplasm is thought to trigger the initiation of reverse transcription (Lehmann-Che and Saib, 2004). Reverse transcription, although being a very complex process, is very well established (reviewed in Gotte *et al.*, 1999). During this reaction, viral RNA genome is converted into a double-stranded DNA molecule, by the action of the viral heterodimer RT protein (p51/p66). The p66 subunit contains both polymerase and RiboNuclease enzyme H (RNase H) enzymatic activities, essential for the reverse transcription process, while the role of p51 is mainly structural (Kohlstaedt *et al.*, 1992).

Reverse transcription starts with the binding of a cellular tRNA(Lys3), present in the RTC, to the primer binding site (PBS) sequence of the viral RNA and continues with a series of steps that involve several *cis*-acting elements of the viral genome, deoxyribonucleotides addition and RT enzymatic activity. The newly synthesized viral DNA remains associated with viral and cellular proteins, leading to the maturation of RTC and generation of post-integration complex (PIC) (Miller *et al.*, 1997; Gotte *et al.*, 1999). PIC differs from RTC mainly by enclosing viral cDNA instead of viral RNA, nevertheless loss of viral proteins, and reduction of capsid molecules are also observed (Warrilow *et al.*, 2009).

1.5.1.3. Nucleus import and Integration

Upon viral DNA synthesis, HIV-1 has to deliver the recently retrotranscribed viral DNA, enclosed in PIC, into the nucleus, crossing the nucleus membrane, in order to integrate the viral DNA into the host chromosome. In the cytoplasm, PIC was shown to connect with cytoskeleton components, that seem to favour its proximity to the nuclear membrane (Bukrinskaya *et al.*, 1998). It is generally accepted that PIC nuclear import is made in an active form (Suzuki and Craigie, 2007). PIC has a Stoke diameter of 56 nm that greatly exceeds the central channel of the nuclear pore channel (NPC) of 25 nm, thus excluding passive diffusion as a viable mechanism (Miller *et al.*, 1997; McDonald *et al.*, 2002). Moreover, some HIV-1 PIC proteins have been identified for having karyophilic signals like MA, IN, CA and Vpr, and/or for interacting with cellular proteins like importin LEDGF/p75. These karyophilic signals and cellular proteins allow the direct transport across the intact nuclear envelope through the NPCs (reviewed in Lehmann-Che and Saib, 2004; Suzuki and Craigie, 2007). In addition, a small viral DNA derived from reverse transcription, the DNA flap, has also been implicated in PIC nuclear import acting as a *cis*-acting determinant of HIV-1 genome nuclear import (Zennou *et al.*, 2000).

Following nuclear import of PIC, viral DNA is covalently integrated into the host chromosome through the viral IN catalytic activity. This process seems not to be random, occurring preferentially in transcription units that are transcriptionally active (Ciuffi and Bushman, 2006). Briefly, IN catalyzes endonucleolytic cleavage of viral genome extremities, cleaves cellular target DNA and promotes a strand transfer reaction leading to the insertion of viral DNA into the cellular target DNA (reviewed in Van Maele and Debyser, 2005; Delelis *et al.*,

2008). Integration is completed when the opposite short gaps in the complementary strands, resulted from the integration process, are repaired. This process is designated by post-integration repair and seems to be accomplished by host cellular proteins (Yoder and Bushman, 2000; Sakurai *et al.*, 2009).

Although IN is necessary and sufficient for all basic catalytic activities during the integration process, several cellular proteins have been implicated as important partners for IN and/or integration process (reviewed in Van Maele and Debyser, 2005 and be forward discussed).

After integration, the viral DNA, referred to as provirus, persists in the host cell and serves as template for the transcription of viral genes and replication of the viral genome (Freed, 2001).

1.5.2. Late phase

1.5.2.1. HIV-1 LTR Transcription

HIV-1 provirus may either remain latent (post-integration latency) or become transcriptionally active. Provirus acts as a template for the synthesis of viral RNA that can be subsequently used as genomic RNA for virions progeny or can be translated into viral proteins. HIV-1 transcription is controlled by the inducible viral promoter located at U3 of 5´LTR (Sodroski *et al.*, 1985). HIV-1 LTR is a prototypic promoter of the cellular RNAPol II. LTR enclosing a consensus TATA motif, where the host cell transcription factor II D (TFIID) binds; Sp1 and NFκB binding sites, that are recognized by endogenous host cell transcription factors and control LTR expression in the absence and in the presence of the transcription transactivator, Tat; and an additional DNA sequence-binding sites for several further cellular proteins (Garcia *et al.*, 1989; Harrich *et al.*, 1990; Steffy and Wong-Staal, 1991).

Tat protein acts as a transactivator of LTR by triggering viral transcription more than 100 fold than its basal expression. Tat function is dependent of its binding to a stem-loop element of the nascent RNA transcript, TAR (for trans-activating response element). TAR tethers Tat and brings it upstream of the promoter, endorsing TAT interaction to transcription factors and other cellular proteins (Steffy and Wong-Staal, 1991). Tat recruits the heterodimer positive transcription elongation factor b (P-TEFb) formed by cyclin T and cyclin-dependent kinase 9 (CDK9) proteins (Wei *et al.*, 1998) leading to the phosphorylation of RNAPol II and consequently, to an increase in viral RNA transcription (Romano *et al.*, 1999). The first rounds

of proviral transcription and translation occur independently of Tat, resulting in basal amounts of Tat, Rev and Nef. Once sufficient amounts of Tat have been produced, Tat further controls transcription of HIV-1 genes (reviewed in Sierra *et al.*, 2005).

1.5.2.2. Gene expression, Assembly and Budding

Transcribed viral RNAs are divided into three groups according to their splicing processes: 1) the unspliced RNAs that are precursors for Gag and Gag-Pol polyproteins and can ve used as genomic RNA, which is subsequently packaged into progeny virions; 2) the partially spliced RNAs that encode Env, Vif, Vpu and Vpr proteins; and 3) the multiple spliced RNAs that are translated into Tat, Rev and Nef. Initially, only multiple spliced RNAs are produced and the regulatory proteins Tat, Rev and Nef are expressed. When sufficient amounts of Rev protein are produced, Rev binds to the *cis*-acting RNA element, the Rev-responsive element (RRE) of the unspliced or single-spliced RNAs, leading to the formation of a protein complex that interacts with the cellular nuclear export machinery, which consequently promotes RNA transportation out of the nucleus, where translation occurs (Wang *et al.*, 2000; Freed, 2001).

In contrast to all the other viral proteins, the Env precursor polyprotein (gp160) is synthesized in the endoplasmic reticulum (ER) using the single spliced *env* mRNA, and suffers post-translationally modifications in the ER and in the Golgi apparatus. During trafficking through the Golgi, gp160 is cleaved by a host cell protease generating the mature gp120 and gp41 proteins that subsequently form trimeric complexes (gp120-gp41). These complexes are transported to the cell surface via the secretory pathway and gp41 protein anchors the complexes in the cell membrane for virus assembly (Turner and Summers, 1999).

Gag polyprotein is synthesized in the ribosomes from unspliced RNAs and a translation frameshift leads to the formation of smaller amounts of Gag-Pol precursor proteins (Turner and Summers, 1999). Gag polyprotein is the major responsible for the assembly of new immature viral particles. During assembly, Gag is targeted to the plasma membrane. The anchored Gag leads to the induction of Gag multimerization and subsequently incorporation of the viral genomic RNA, Env glycoproteins and Gag-pol precursor into the viral particle. MA is responsible for the targeting of Gag to the plasma membrane and for the incorporation of gp120-gp41 complexes and Gag-Pol precursor into the viral particle, whereas C-terminal domain of CA (CA-CTD) and NC are responsible for Gag multimerization. In addition, NC due

to its zinc-finger motifs, it also mediates the specific recognition of viral RNA genome for packaging into the virions (Ono, 2010). Viral enzymes, accessory viral proteins, the cellular tRNAlys3 primer and cellular proteins also associate to the immature core for incorporation into the viral particle (reviewed in Jewell and Mansky, 2000; Adamson and Freed, 2007; Bieniasz, 2009). Subsequently, the immature core associated to the plasma membrane suffers budding through the plasma membrane. The viral P6 protein helps in this process through the recruitment of the endosomal sorting complex required for transport (ESCRT), facilitating fission of virions from the plasma membrane to the extracellular medium (Demirov and Freed, 2004; Morita and Sundquist, 2004).

1.5.2.3. Maturation

After the release of the immature budded virus from the host cell, Gag and Gag-Pol polyproteins are cleaved by the viral PR to produce the independent enzymes, as well as the MA, CA and NC structural proteins. The structural proteins rearrange via a process called maturation to form the infectious virus particle. Cleavage of Gag appears to occur via an ordered, sequential cleavage process that is controlled by different intrinsic proteolysis rates at the different cleavage sites (Wiegers *et al.*, 1998).
After maturation, HIV-1 life cycle is completed with the formation of newly infectious viral particle ready to infect a new host cell (Freed, 2001).

1.6. Host factors for HIV-1 replication

HIV-1 uses its cell-host machinery to promote replication and also to evade cellular antiviral responses to assure progeny spread (Sorin and Kalpana, 2006; Goff, 2007). The interest in studying cellular factors that could regulate HIV-1 replication started soon after characterization of the virus. Several research studies have indicated that the interaction between the virus and the host is dynamic and very tight, being observed at every step of HIV-1 life cycle (Komano *et al.*, 2005; Sorin and Kalpana, 2006; Goff, 2007). HIV-1-host interaction is extremely complex as it can be observed in NIADS HIV Protein Interaction Database, where it is represented a extensive list of cellular proteins that interact directly and

indirectly with HIV-1 (Fu *et al.*, 2009). However, and despite the effort, many cellular factors are yet to be discovered.

Cellular proteins can either act as helpers factors (or co-factors) during HIV replication, being explored by the virus to complete its life cycle or as restriction factors presenting anti-viral functions and hampering viral replication (Sorin and Kalpana, 2006; Goff, 2007).

Clearly understanding the complex battle between HIV-1 and its host will provide us new insights to a better understanding of the mechanisms underlying the various steps of retroviral replication and consequently can lead to the development of novel antiviral therapeutic strategies.

1.6.1 Restriction factors

In addition to the conventional innate and acquired immune responses, mammalian have evolved to efficiently counteract retroviruses infection (Bieniasz, 2004). This inhibitory effect can be due to: genetic factors, mainly polymorphisms in host response genes or in receptors genes; or to inhibitory gene products that, when expressed in the host cell, prevent efficiently the progression of particular steps of the retroviruses life cycle (Rowland-Jones *et al.*, 2001; Goff, 2004b).

Genetic outcomes of HIV-1 infection are well recognized and can indicate potential mechanisms in the pathogenesis of infection. One of the best studied polymorphism is in the CCR5 chemokine-receptor gene (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996) The CCR5Δ32 allele expresses a truncated protein that is no longer transported to the cell surface. Therefore, the homozygotes for this polymorphism are resistant to HIV-1 infection (Benkirane *et al.*, 1997). Moreover, many other genetic polymorphisms have been described to affect susceptibility to HIV infection (reviewed in Lama and Planelles, 2007).

The cellular proteins named host restriction factors function as inhibitors of viral replication and are constitutively expressed or enhanced by interferons (Neil and Bieniasz, 2009). Although restriction factors can be enhanced by viral infection, they do not require virus-triggered signaling. These proteins are active in the host cell and are an important part of the innate immunity against viral infection (Bieniasz, 2004). Nevertheless, as host cells have evolved to block viral infection, retroviruses have co-evolved to counteract these restrictions,

being described several viral proteins that can efficiently neutralize the inhibitory effect of some restriction factors (reviewed in Strebel *et al.*, 2009).

1.6.1.1. Fv1

The first and probably the most studied restriction factor described against retroviruses is the friend virus susceptibility-1 protein (Fv1). Fv1 gene is naturally encoded in mouse and its expression promotes mice resistance to murine leukemia virus (MLV) infection (Lilly, 1967). Fv1 gene has two different alleles, $Fv1^n$ and $Fv1^b$ that confer different MLV tropism. $Fv1^n$ allows replication of N-tropic strains of MuLV and blocks B-tropic MuLV viruses; Fv^b allele permits replication of B-tropic viruses and blocks N-tropic viruses. $Fv1^{n/b}$ heterozygotes animals are resistance to both N- and B-tropic virus. The aminoacid 110 of the CA domain in gag gene has been shown to be responsible of MLV virus tropism, with an arginine specifying the N-tropism and a glutamate the B-tropism (Kozak and Chakraborti, 1996). The Fv1 restriction mechanism is thought to be after reverse transcription, where Fv1 somehow detects the CA protein of the incoming PIC and blocks virus replication (Goff, 2004a).

1.6.1.2. TRIM family

Like in mouse, other mammalian cells were demonstrated to have restriction activities against retroviruses capsid. Members of the tripartite motif family of proteins have been described to have a restriction activity against several retroviruses. This very large family of proteins is characterized by their structure composed by a RING finger, B-box, and coiled-coil domains (Nisole *et al.*, 2005). The most studied family member is the tripartite motif 5-alpha (TRIM5α) that was demonstrated to be an important restriction factor in mammals blocking infection by retroviruses in a species-specific manner (figure 1.4) (reviewed in Luban, 2007; Towers, 2007). TRIM5α was first identified in rhesus monkey (rh) as a blocking factor for HIV-1 replication (Stremlau *et al.*, 2004). Shortly after, several groups demonstrated that the restriction factors previously described in human, redox factor 1 (Ref1), and in primates, Lv1, restricting MuLV and HIV-1 and/or SIV from macaques (SIVmac) respectively, were species-specific variants of TRIM5α protein (Hatziioannou *et al.*, 2004; Keckesova *et al.*, 2004).

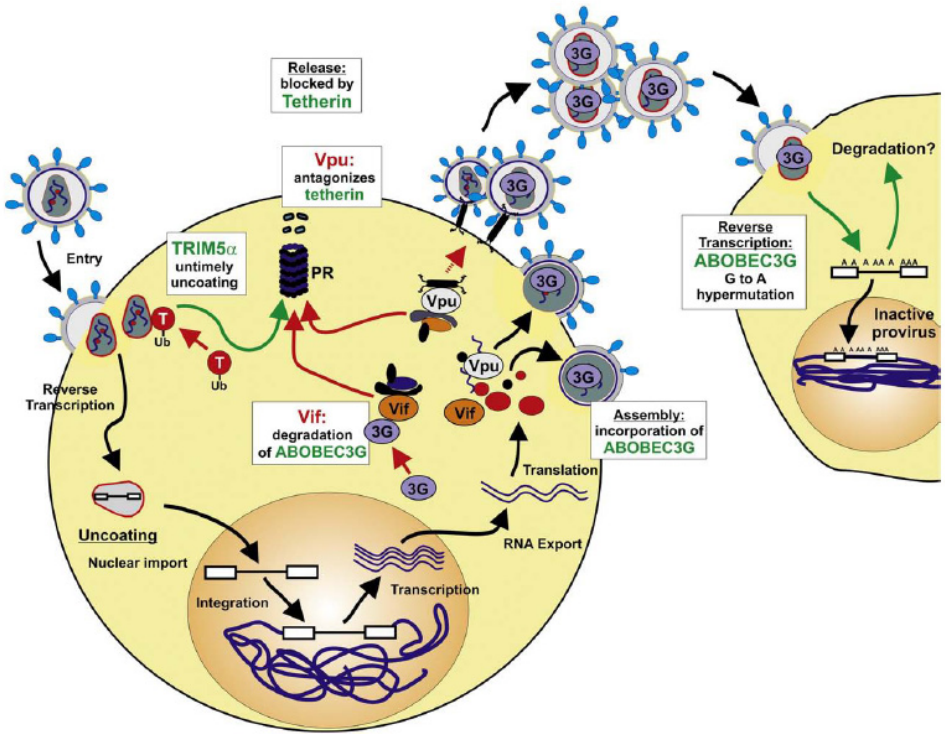

Figure.1.4. Host-restriction factors for HIV-1 and their viral antagonists. As schematically indicated, TRIM5α interacts with the incoming HIV-1 capsids and may induce accelerated uncoating by proteosomal degradation. APOBEC3G, in the absence of Vif, is incorporated into the budding virions and causes lethal G-to-A hypermutations at the retroviral genome in the newly infected cell. Vif counteracts APOBEC3G by directly binding to it and promoting its proteosomal degradation via Cullin5-based ubiquitin ligase complex or by inhibiting apobec3g translation. Tetherin blocks the release of newly formed virions from the cell surface and is antagonized by Vpu. The exact mechanism by which Vpu counteracts tetherin remains to be identified but involves direct interaction of Vpu and tetherin and maybe activation of proteosomal degradation (adapted from Arhel and Kirchhoff, 2009: Reprinted from Publication, Biochim Biophys Acta, 1802, Arhel, N., Kirchhoff, F, Host proteins involved in HIV infection: new therapeutic targets, 313., Copyright (2009), with permission from Elsevier).

Although rhTRIM5α has a potent inhibitory effect against HIV-1, the human TRIM5α (hTRIM5α) appears to only modestly inhibit HIV-1 replication (Stremlau *et al.*, 2004). The exact antiviral mechanism of TRIM5α is so far not clear. It is known that TRIM5α trimers interact with hexameric capsids and block replication. TRIM5α is ubiquitinylated within cells and is rapidly turned over by the proteasome in a RING-domain-dependent way, suggesting that auto-ubiquitination might promote CA degradation (Diaz-Griffero *et al.*, 2006a). Other studies indicate that TRIM5α can just disrupt the rearrangement/uncoating of the capsid

before reverse transcription; or alternatively can somehow act after reverse transcription blocking PIC access to the nucleus (Towers, 2007).

TRIM5α restriction activity has also been associated to the cyclophilin A (CypA) isomerase expressed in primates. CypA is a peptidyl poryl isomerase that performs cis/trans isomerisation of proline peptide bonds in the target proteins. CypA was first described as interacting with viral CA within the target cell soon after viral infection, promoting or inhibiting infection (Franke *et al.*, 1994; Thali *et al.*, 1994). The restriction of viral infection by CypA was proven to be associated to TRIM5α (Berthoux *et al.*, 2005). In the New World species owl monkey TRIM5 has CypA pseudogene in place of the viral binding B30.2 domain, designated by TRIMCyp (Nisole *et al.*, 2004; Sayah *et al.*, 2004). This restriction factor strongly restricts HIV-1, simian immunodeficiency virus from African green monkey (SIVagm) and feline immunodeficiency virus (FIV) by recruitment of the incoming capsid, promoted by the interaction between the CypA domain and the capsid (Diaz-Griffero *et al.*, 2006b).

Other members of TRIM family have been described to have an anti-retroviral activity (Nisole *et al.*, 2005). TRIM19, TRIM22 and TRIM32 have been identified as having restriction activity against HIV-1. TRIM19 acts in early steps of HIV-1 replication (Turelli *et al.*, 2001), while TRIM22 (Tissot and Mechti, 1995) and TRIM32 (Fridell *et al.*, 1995) were shown to repress HIV-1 transcription.

1.6.1.3. APOBEC family

Another very important group of restriction factors are included in Apoliprotein B-editing catalytic polypeptide-like (APOBEC) family of cytidine deaminases, being APOBEC3G the most studied in HIV-1 context (Conticello *et al.*, 2005). APOBEC3G is a strong inhibitor of HIV-1 and other retroviruses infections, acting after uncoating of the viral capsid, during reverse transcription, and is counteracted by the viral Vif protein (Sheehy *et al.*, 2002) (figure 1.4). In the absence of Vif, APOBEC3G can be incorporated into HIV-1 particles in the producer cell. Upon infecting a new cell, during reverse transcription, APOBEC3G deaminates the cytosine bases of the viral minus-strand DNA to uracil resulting in G to A hypermutations in the complementary positive sense DNA strand (Mangeat *et al.*, 2003; Mariani *et al.*, 2003; Zhang *et al.*, 2003). These U-rich transcripts are either degraded by activation of the cellular

uracil-DNA-glycosilase causing the failure of reverse transcription or originates proviruses that are largely non-functional due to G-to-A hypermutation (Goff, 2003). HIV-1 Vif protein counteracts APOBEC3G by blocking its incorporation into the viral particles (Mariani *et al.*, 2003; Stopak *et al.*, 2003). Vif interacts directly with APOBEC3G and via a conserved SOCS box-like sequenced, recruits a RING-finger E3 ubiquitin complex containing elongin B and C, cullin 5, and ring-box protein 1 (Rbx1), inducing the poly-ubiquitination of APOBEC3G and subsequently, its degradation by the proteasome machinery (Conticello *et al.*, 2003; Marin *et al.*, 2003; Sheehy *et al.*, 2003; Stopak *et al.*, 2003; Mehle *et al.*, 2004; Yu *et al.*, 2004b). Vif also impairs APOBEC3G mRNA translation by a not well-known mechanism (Stopak *et al.*, 2003; Mercenne *et al.*, 2010).

APOBEC3F, other protein from APOBEC family has been also demonstrated to be an efficient inhibitor of HIV-1 in human cells (Liddament *et al.*, 2004; Wiegand *et al.*, 2004; Zheng *et al.*, 2004). APOBEC3F is also efficiently inhibited by HIV-1 Vif in a similar mode as APOBEC3G and excluding it from the viral particles (Liddament *et al.*, 2004; Wiegand *et al.*, 2004; Zheng *et al.*, 2004; Liu *et al.*, 2005). Other members of the APOBEC family, such as Apoliprotein B mRNA editing enzyme, catalytic polypeptide-like 3B (APOBEC3B) and 3C (APOBEC3C) have also been shown to have a potent antiviral activity against simian immunodeficiency birus (SIV), although not efficient against HIV-1 (Yu *et al.*, 2004a).

1.6.1.4. Tetherin

Recently, tetherin, also known as BST-2, CD317, or HM1.24, was identified as a potent antiviral factor, inhibiting HIV-1 replication by a novel mechanism (Neil *et al.*, 2008; Van Damme *et al.*, 2008). Tetherin expression and its antiviral activity can be induced by interferon α in T cells (Neil *et al.*, 2007). Tetherin inhibits the release of newly formed virions from the host cell surface by binding to the cell surface at the near site were the virions bud (figure 1.4). However, the mechanism of this restriction is still unclear. It was recently discovered that tetherin associates with a RING-type E3 ubiquitin ligase breast cancer-associated gene 2 (BCA2) that can accelerate internalization and degradation of tethered HIV-1 virions (Miyakawa *et al.*, 2009). However, it is not yet understood if the virions hijacked by tetherin are generally degraded or remain intact within the cell.

Retroviruses have also evolved to counteract tetherin action. Specifically, HIV-1 counteracts tetherin action through the viral Vpu protein. Vpu interacts directly with the transmembrane domain of tetherin, promoting the reduction of tetherin levels at the cell surface and a decrease of the total amount of this protein in the cell (Van Damme *et al.*, 2008). Although the exact mechanism of tetherin down-modulation is not unclear, it has been hypothesized that Vpu targets tetherin to the trans-Golgi network or to the early endosomes for proteosomal or lysosomal degradation (Douglas *et al.*, 2009; Goffinet *et al.*, 2009; Mangeat *et al.*, 2009; Mitchell *et al.*, 2009).

Similarly to other restriction factors, tetherin anti-viral activity is also effective against other retroviruses. The antiviral mechanism seems to be common but the viral counteracting protein differs. Most primate lentiviruses do not contain vpu gene and use Nef protein to antagonize tetherin, in a similar way as Vpu (Jia *et al.*, 2009; Sauter *et al.*, 2009; Zhang *et al.*, 2009). HIV-2 uses Env protein to counteract tetherin by sequestering it away from virus assembly sites (Le Tortorec and Neil, 2009).

1.6.2 Helper factors

HIV-1, has an obligate intracellular parasite, takes advantage from the host cell machinery to replicate and to assure its progeny. The virus during its life cycle, “uses and abuses” of cellular proteins from which it can takes advantage for its own benefit, being this close interaction observed in all steps of HIV-1 replication. The field of HIV-1 co-factors research has increased enormously in the last few years. In spite of the numerous studies performed, there exists some controversy about several cellular proteins, remaining their functions as helper factors for HIV-1 unclear. These differences can be justified by the type of model used, like in vitro versus in vivo models. This section will focus on the main advances in the discovery, understanding and characterization of HIV-1 helper factors during the course of HIV-1 life cycle, their interaction with viral accessory proteins and the importance of kinases and phosphatases during HIV-1 replication. The highlighted helper factors are described in table 1.1.

Table 1.1. Highlighted Helper factors for HIV-1 replication

Gene name	Cellular function	Function in HIV-1 replication	Step of HIV-1 life cycle	Viral protein aided	References
CD4	Receptor that assists T cell receptor (TCR) to activate T cell following an interaction with an antigen-presenting cell	HIV-1 primary receptor	Entry	gp120	Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; McDougal *et al.*, 1985
CCR5	β-chemokine receptors family of integral membrane proteins; plays a role in inflammatory responses to infections	HIV-1 co-receptor	Entry	gp160	Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996
CXCR4	α-chemokine receptor specific for stromal-derived-factor-1 (SDF-1)	HIV-1 co-receptor	Entry	gp160	Feng *et al.*, 1996
DC-SIGN	C-type lectin present in macrophages and DCs; binds to mannose type carbohydrates, typically found on viruses, bacteria and fungi; modulates immune response by activating intracellular signaling pathways	HIV-1 envelope ligand. Allows DC to bind and capture viral particles	Entry	gp120	Geijtenbeek *et al.*, 2000; Burleigh *et al.*, 2006
Heparan sulphate proteoglycan	Cell surface molecule. Regulates a wide variety of biological activities	Favours attachment of HIV-1 to cell surface	Entry	-	Mondor *et al.*, 1998
LFA-1	Cell surface molecule; adhesion molecule promoting T-cells binding to antigen-presenting cells	Favours attachment of HIV-1 to cell surface	Entry	-	Fortin *et al.*, 1998
Nucleolin	Cell surface molecule	Favours attachment of HIV-1 to cell surface	Entry	-	Nisole *et al.*, 1999
BAF	Barrier to auto-integration factor. Thought to facilitate nuclear reassembly by binding to both DNA and inner nuclear membrane proteins and thereby recruiting chromatin to the nuclear periphery.	PIC´s component; promotes integration	Integration	-	Chen and Engelman, 1998; Lin and Engelman, 2003
HMG I (Y)	High mobility group protein. Nonhistone DNA-binding protein; modulates transcriptional regulation and chromatin structure	PIC component; may promote formation of active integrase-cDNA complexes; may stabilize active conformation of IN by modulating DNA structure; role in HIV-1 expression: interacts with HIV LTR 5´ and indirectly recruiting hSWI/SNF complex	Nuclear import & integration	-	Farnet and Bushman, 1997; Li *et al.*, 2000; Van Maele and Debyser, 2005
Ku 70 & Ku 80	Forms a heterodimer required for the non-homologous end joining (NHEJ) pathway of DNA repair	Component of PIC; important during formation of circular DNA.	Integration	-	Li *et al.*, 2001
LAP2α/TMPO	Lamina-associated polypeptide 2α; direct the assembly of the nuclear lamina and thereby helps the maintenance of the structural organization of the nuclear envelope	PIC component	Integration	-	Van Maele and Debyser, 2005

Table 1.1. Highlighted Helper factors for HIV-1 replication (cont.)

Gene name	Cellular function	Function in HIV-1 replication	Step of HIV-1 life cycle	Viral protein aided	References
LEDGF/P75	Transcriptional regulator	PIC component; binds to integrase; may help IN nuclear import; tethers IN and thereby PICs to chromatin and directs HIV DNA integration, affecting HIV integration site distribution; protects IN from proteosomal degradation	Nuclear import; integration	IN	Cherepanov *et al.*, 2003; Maertens *et al.*, 2003; Llano *et al.*, 2004a; Llano *et al.*, 2004b; Ciuffi and Bushman, 2006; Vandegraaff *et al.*, 2006
HRP2	Hepatoma-derived growth factor related protein 2; hepatoma-derived growth factor (HDGF) family	Binds to IN and stimulate its integration activity *in vitro*	Integration	IN	Vandegraaff *et al.*, 2006
Microtubules (α- and β-Tubulin)	Cytoskeleton components	Aids PIC translocation to the nucleus periphery; facilitates Gag transport for viral assembly	Nuclear import; assembly	Gag	McDonald *et al.*, 2002; Arhel *et al.*, 2006
Actin	Cytoskeleton	Helps PIC translocation, possibly through a direct interaction with MA	Nuclear import	MA	Bukrinskaya *et al.*, 1998; Arhel *et al.*, 2006
Dynein	Motor protein	PIC uses the cytoplasmatic dynein motor to travel along the microtubule network to migrate towards the nucleus.	Nuclear import	-	McDonald *et al.*, 2002
Importin β	Involved in the importin pathway through the nucleus	Important for PIC's nuclear import; enhances Rev nuclear import; binds to Vpr	Nucleus Import; gene expression	Rev; Vpr	Henderson and Percipalle, 1997; Vodicka *et al.*, 1998
Importin-α	Forms a heterodimer with importin β; involved in the importin pathway through the nucleus	Interacts with Vpr, MA and IN; favours nuclear import	Nuclear import	Vpr, MA, IN	De Rijck *et al.*, 2007
Importin 7	Involved in the importin pathway through the nucleus	Maybe involved in PIC import into the nucleus in macrophages through interaction with IN	Nuclear import	IN	Fassati *et al.*, 2003
Nucleoporins (e.g. Nup 98, Nup 153)	Nucleoporin (component of nuclear pore)	Docking PIC in the nuclear envelope	Nuclear import	Vpr, IN	Fouchier *et al.*, 1998; Vodicka *et al.*, 1998; Ebina *et al.*, 2004; Woodward *et al.*, 2009
INI1/hSNF5	Integrase interactor1; component of the chromatin remodeling SWI/SNF complex	Facilitates PIC's nuclear import as well as integration reaction: activates IN DNA joining activity; it is incorporated into the virions.	Nuclear import; integration	PIC, IN	Kalpana *et al.*, 1994; Yung *et al.*, 2004
Emerin	Member of the nuclear lamina-associated protein family; mediates membrane anchorage to the cytoskeleton	Facilitates PIC binding to chromatin; it does not occurs in all types of cells	Integration	PIC	Jacque and Stevenson, 2006

Table 1.1. Highlighted Helper factors for HIV-1 replication (cont.)t

Gene name	Cellular function	Function in HIV-1 replication	Step of HIV-1 life cycle	Viral protein aided	References
DNA-PKcs	DNA-dependent protein kinase catalytic subunit; NHEJ component; involved in V(D)J recombination	Controversial role: important for HIV integration; in pos-repair integration process; in removing pro-apoptotic signal of free dsDNA ends; binds to Tat	Integration	IN, Tat	Chun *et al.*, 1998; Daniel *et al.*, 1999; Van Maele and Debyser, 2005
ATM	Kinase activated and recruited by double strand breaks; component of NHEJ	Responsible for post-repair integration process?. Role in removing pro-apoptotic signal of free dsDNA ends	Integration?	-	Van Maele and Debyser, 2005
ATR	Protein kinase involved in sensing DNA damage and activating the DNA damage checkpoint, leading to cell cycle arrest. NHEJ component	Responsible for post-repair integration process; role in removing pro-apoptotic signal of free dsDNA ends	Integration?	-	Van Maele and Debyser, 2005
XRCC4	DNA repair protein; function together with DNA ligase IV and DNA-PKcs in V(D)J recombination; NHEJ component	Responsible for post-repair integration process? Role in removing pro-apoptotic signal of free dsDNA ends	Integration?	-	Van Maele and Debyser, 2005
DNA Ligase IV	Joins double-strand breaks during NHEJ; important for V(D)J recombination	Responsible for post-repair integration process? Role in removing pro-apoptotic signal of free dsDNA ends	Integration?	-	Van Maele and Debyser, 2005
HSP60	Ubiquitous chaperone; plays an essential role in cells by binding newly synthesized proteins and facilitating their folding	Interacts with IN; stimulates the *in vitro* 3' processing and strand- transfer activities of IN and protected IN from thermal denaturation	Integration	IN	Parissi *et al.*, 2001
PARP-1	Nuclear enzyme activated by ssDNA and dsDNA breaks to attach ADP-ribose groups to nuclear proteins	Implicated in HIV-1 integration; also described with a putative role in HIV-1 transcription through regulation of HAT expression	Integration; transcription	-	Ha *et al.*, 2001; Kameoka *et al.*, 2004
RAD18	Component of the post-replication/translation repair	Interacts with IN. Proposed to destabilize PIC proteins to facilitate the access of other cellular protein to viral DNA or to regulate IN stability	Integration	IN	Mulder *et al.*, 2002; Van Maele and Debyser, 2005
RNAPol II	Responsible for mRNA transcription	Transcribes viral RNA from the integrated provirus	Transcription	Tat	Garcia *et al.*, 1989; Harrich *et al.*, 1990
Sp1	Transcription Factor	Enhances LTR transcription; interacts with Vpr; interacts with Tat for optimal transactivation and HIV-1 transcription	Transcription	Tat, Vpr	Wang *et al.*, 1995; Rohr *et al.*, 2003
NFκB	Protein complex that acts as a transcription Factor	Transcription activator for HIV-1 LTR	Transcription	Tat	Demarchi *et al.*, 1996
hGCN5	Histone acetyltransferase; transcription activator	Acetylates of Tat protein, facilitating transcription elongation	Transcription	Tat	Col *et al.*, 2001

Table 1.1. Highlighted Helper factors for HIV-1 replication (cont.)

Gene name	Cellular function	Function in HIV-1 replication	Step of HIV-1 life cycle	Viral protein aided	References
p300/CBP complex	Histone acetyltransferase; transcriptional co-activating proteins	Acetylates Tat; is also recruited by Vpr to enhance viral expression through LTR; also known to interact with IN	Transcription; integration	Tat, Vpr, IN	Ott *et al.*, 1999; Kino *et al.*, 2002; Cereseto *et al.*, 2005
PCAF	p300/CREB binding protein-associated factor	Recruited by Tat to the elongating RNAPol II complex for transcription elongation	Transcription	Tat	Dorr *et al.*, 2002; Mujtaba *et al.*, 2002
Cyclin T1/CDK9	Forms P-TEFb complex; is a transcription elongation regulator	Bridges activation domain of Tat and TAR loop	Transcription	Tat	Wei *et al.*, 1998
PP1	Phosphatase protein involved in the regulation of a variety of cellular processes	Important for initiation of transcription; activated by Tat	Transcription	Tat	Ammosova *et al.*, 2005
SKIP	Splicing-associated c-Ski-interacting protein: splicing factor	Stimulates HIV-1 transcription elongation, by association with with P-TEFb and Tat:P-TEFb complexes	Transcription	Tat	Bres *et al.*, 2005
Nucleolar phosphopprotein B23	Possibly involved in the assembly and/or transport of ribosomes	Interacts with importin-β and Rev. The B23-Importin β-rev complex recruits Rev to the nuclear pore	Nuclear export	Rev	Szebeni *et al.*, 1997
CRM1 or Exportin1	Mediates leucine-rich nuclear export signal (NES)-dependent protein transport	Enhances Rev nuclear export	Nuclear export	Rev	Pollard and Malim, 1998; Li *et al.*, 2002a; Cullen, 2003; Dayton, 2004
eIF-5A	Eukaryotic initiation factor 5A	Nuclear transport activity of Rev	Nuclear export	Rev	Bevec *et al.*, 1996
Sam68	Functions as an adapter protein in signal transduction cascades by binding to SH2 and SH3 domain-containing proteins	Facilitates nuclear transport activity of Rev; Sam68:RNA enhances translation machinery	Nuclear export	Rev	Reddy *et al.*, 1999; Soros *et al.*, 2001; Li *et al.*, 2002a; Li *et al.*, 2002b; McLaren *et al.*, 2004; Modem *et al.*, 2005
DDX1	EAD box protein RNA helicase implicated in several cellular processes, involving alteration of RNA secondary structure	Favours nuclear transport activity of Rev: maintains proper subcellular distribution of Rev	Nuclear export	Rev	Fang *et al.*, 2004; Fang *et al.*, 2005; Li *et al.*, 2005a
DDX3	EAD box protein RNA helicase	Favours nuclear transport activity of Rev; promotes protein shuttling; promotes the localization of the nuclear membrane pores	Nuclear export	Rev	Yedavalli *et al.*, 2004; Li *et al.*, 2005a
Rab/hRIP	Human Rev-interacting protein; related to nucleoporins	Nuclear transport activity of Rev by promoting the release of HIV-1 RNAs from the perinuclear region	Nuclear export	Rev	Bogerd *et al.*, 1995; Fridell *et al.*, 1996; Sanchez-Velar *et al.*, 2004; Yu *et al.*, 2005
RanBP1	Ran-specific GTPase-activating protein	Enhances Rev nuclear import and export	Nuclear export	Rev	Zolotukhin and Felber, 1997

Table 1.1. Highlighted Helper factors for HIV-1 replication (cont.)

Gene name	Cellular function	Function in HIV-1 replication	Step of HIV-1 life cycle	Viral protein aided	References
RHA or DHX9	RNA helicase A; involved in translation initiation; nuclear and mitochondrial splicing; and ribosome and spliceosome assembly	Enhances HIV mRNA translation through binding to post-transcriptional control element (PCE) in the viral RNA	Translation	-	Bolinger *et al.*, 2010
9G8 or SFRS7	Splicing regulatory protein; involved in alternative splicing	Increases the translation of HIV-1 gag-pol RNA	Translation	-	Bolinger and Boris-Lawrie, 2009
eEF21A, eEF1B, Ef2, eEF5	Elongation factors; involved in elongation of RNA translation	Favour viral RNA elongation and Ribosomal frameshift	Translation	-	Bolinger and Boris-Lawrie, 2009
PKR	Protein kinase R; protects cells against viral infections by detecting viral dsRNA	Activated by TAR; favours ribosome frameshift	Translation	-	Gendron *et al.*, 2008
TRBP1 &TRBP2	TAR binding proteins; unknown function in cell	Bind to TAR RNA and increase viral expression	Translation	-	Dorin *et al.*, 2003
HP68	RNAse L inhibitor	Promotes assembly of Gag, by association with Gag, Gag-Pol and Vif	Assembly	Gag, Gag-pol and Vif	Zimmerman *et al.*, 2002
TSG101	Tumor susceptibility gene 101 protein; ubiquitin-conjugating enzyme	Recruits the pinch-off machinery endosome-associated complex required for transport (ESCORT) to the site of budding.	Budding	P6	Garrus *et al.*, 2001; VerPlank *et al.*, 2001
AIP1/ALIX	Hypothetical fucntion in programmed cell death	Binds to p6 and plays a similar role to TSG101, recruiting the ESCORT complex at the cell surface to promote the pinch-off process	Budding	P6	Strack *et al.*, 2003
ESCORT complex	Vesicular sorting machinery	Promotes budding	Budding	P6	Garrus *et al.*, 2001
β-TrCP	Member of the F-box protein family; component of the ubiquitin ligase complex.	Is recruited by Vpu-CD4 for degradation	-	Vpu	Margottin *et al.*, 1998
SKP1	Member of the ubiquitination machinery	Is recruited by Vpu-CD4- β-TrCP to promote CD4 degradation	-	Vpu	Li *et al.*, 2005a
Cul5-EloBC complex	Ubiquitin system for protein degradation	Cul5, EloB and EloC bind to Vif and lead to the recruitment of the complex for A3G ubiquitination and proteosomal degradation	-	Vif	(Yu *et al.*, 2003; Mehle *et al.*, 2004; Liu *et al.*, 2005
UNG2	Uracil DNA glycosilase; prevents mutagenesis by eliminating uracil from DNA molecules	Is incorporated in the virions to act in the accuracy of reverse transcription. Interacts with Vpr and IN	-	Vpr; IN	Le Rouzic and Benichou, 2005
hCG1	Human nucleoporin CG1	Interacts with Vpr; participates in the Vpr docking at the nuclear envelope that may contribute to the disruption of the nuclear envelope and to the nuclear import of the viral DNA	Nuclear Import	Vpr	Le Rouzic *et al.*, 2002
Karyopherin α	Cellular receptor for nuclear-targeted proteins	Interacts with Vpr.; promotes nuclear import	Nuclear import	Vpr	Popov *et al.*, 1998

Table 1.1. Highlighted Helper factors for HIV-1 replication (cont.)

Gene name	Cellular function	Function in HIV-1 replication	Step of HIV-1 life cycle	Viral protein aided	References
AP1 complex	Adaptor protein (AP) complex	Recruited by Nef to induce endosomal degradation of CD4 and CD28	-	Nef	Janvier *et al.*, 2003
PAK2	Serine/threonine-protein kinase from PAK family ; serves as target for the small GTP binding proteins	Recruited by Nef; induces activation of infected cells	-	Nef	Raney *et al.*, 2005

1.6.2.1. Role in HIV-1 Entry

As soon as HIV-1 encounters its host cell, it interacts with its first co-factor, the CD4 molecule. CD4 is the primary receptor for HIV-1 replication and is expressed in T helper cells, regulatory T cells, monocytes, macrophages, and dendritic cells (DCs), determining HIV-1 tropism for these cells (Maddon *et al.*, 1987). As previously described, the efficient entry of HIV-1 only occurs when the viral glycoproteins interact with a co-receptor triggering the entry step. The most common co-receptors for HIV-1 are CXCR4 and CCR5 although others like c-c chemokine receptor-type 2 (CCR2),-type 3 (CCR3), -type8 (CCR8), -type 9 (CCR9), C-X-C chemokine receptor-type 1 (CXCR1), type 6 (CXCR6), G protein-coupled receptor CMKLR1 (ChemR23), Apelin recceptor (APJ) and C-X-C chemokine receptor type 7 (CCR7/RDC1) can also be used as co-receptors for HIV-1 replication (reviewed in Lama and Planelles, 2007).

Other cell surface molecules were also described to be important for HIV-1 infection by favouring the virus attachment to cells. A well-known helper-factor for HIV-1 present in some host-cells surface is the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). This molecule is expressed in DCs and macrophages and promotes HIV-1 transmission from DCs to T lymphocytes–trans-enhancement (Geijtenbeek *et al.*, 2000). HIV transmission requires binding of HIV-1 particles to DC-SIGN via high mannose glycans present in gp120 and internalization of HIV. Viruses are targeted into multivesicular body of DCs and upon contact with T cells HIV-1 is transmitted to T cells (Burleigh *et al.*, 2006). This transfer is used by the virus to spread from the mucosa (where DCs are major present) to the lymph nodes for T cell infection (Lama and Planelles, 2007).

1.6.2.2. Role in HIV-1 Post-entry until Nucleus import

After cell entry and uncoating processes, viral core is rearranged into reverse transcription complexes (RTCs), which in turn, after reverse transcription, leads to the formation of PIC structures. Both RTC and PIC are composed by viral and cellular proteins. The cellular components of PIC were extensively studied and they seem to be important to nuclear import of PIC and/or PIC's attachment to chromatin and/or integration of the viral DNA into the host chromosome. Among these group of cellular proteins there is BAF, the high mobility group (HMG), the chromosomal protein A1 [HMG I (Y) or HMGA1], the Ku70 and the Ku80 proteins, the lamina-associated polypeptide 2α (LAP2α) and the lens-epithelium-derived growth factor (LEDGF/p75) (reviewd in Van Maele and Debyser, 2005; Suzuki and Craigie, 2007).

The import of PIC into the nucleus is also dependent on cell machinery. Indeed, HIV-1 uses the cytoskeleton to translocate PIC close to the nucleus envelope. Microtubules, actin and dynein have been described as HIV-1 co-factors for PIC´s movement within the cell (Bukrinskaya *et al.*, 1998; McDonald *et al.*, 2002; Arhel *et al.*, 2006). Although the exact mechanism of PIC import into the nucleus is unclear, it is accepted that HIV-1 PIC crosses the intact nuclear envelope in an active way using cellular transport machinery. Various viral proteins known to be part of PIC such as MA, Vpr and IN were shown to interact with the components of the importin pathways, the importin-α and -β. Importin-α forms a heterodimer with importin-β and transports cargo molecules with a nuclear localization signal (NLS) through the nuclear envelope in an energy-dependent manner (GTP/GDP) (Suzuki and Craigie, 2007). Moreover, Vpr was also shown to interact directly with nucleoporins promoting its accumulation at nuclear pore channels (NPCs). Other reports refer that importin 7 (Fassati *et al.*, 2003) and Nup98 (Ebina *et al.*, 2004) could also be positively involved in HIV-1 infection.

1.6.2.3. Role in HIV-1 Integration

As described above some of the PIC's components have been proposed to be required for HIV-1 integration step, like BAF, HMG I(Y), Ku and LEDGF/p75 (Van Maele and Debyser, 2005; Suzuki and Craigie, 2007). LEDGF/p75 is one of the most studied PIC's proteins, and it has been described to bind tightly to IN and to have a function in import of IN into the nucleus (Maertens *et al.*, 2003; Llano *et al.*, 2004b). Moreover, several studies indicate that

LEDGF/p75 enhances IN function, contributing for HIV-1 viral integration into the host chromosome enhancing the tethering of IN, and thereby PIC, to chromatin (Maertens *et al.*, 2003; Llano *et al.*, 2004b; Emiliani *et al.*, 2005; Vanegas *et al.*, 2005). LEDGF/p75 can also regulate the integration-site selection of the HIV IN (Ciuffi and Bushman, 2006; Suzuki and Craigie, 2007). Furthermore, another study has also demonstrated that this cellular protein protects IN form proteosomal degradation (Llano *et al.*, 2004a).

In addition to cellular PIC proteins, other cellular proteins were described has being co-factors for HIV-1 IN, like Ini1/hSNF5 and Emerin. Ini1/hSNF5, a component of the SWI/SNF complex is present in HIV-1 virions and interacts with IN (Kalpana *et al.*, 1994). Various functions have been proposed for this cellular protein, such as helping in PIC's nuclear import and in integration reaction, and activating the IN DNA joining activity (Yung *et al.*, 2004; Van Maele and Debyser, 2005). Emerin was described to promote the association of PIC with chromatin and its localization is mediated by the cellular protein BAF. Emerin function was just described in some cells like macrophages and HeLa cells (Jacque and Stevenson, 2006).

Proteins involved in cell DNA damage response pathway were also described to be important for HIV-1 integration. Integration process involves cleavage of cellular and viral DNA and insertion of viral DNA into the host chromosome (reviewed in Van Maele and Debyser, 2005; Delelis *et al.*, 2008), which leads to the formation of short gaps on the opposite strands of complementary host DNA 5´ends that have to be repaired to assure provirus expression. To assure the completion of the pos-integration repair process, these gaps have to be filled and therefore, their corresponding ends have to be jointed and the chromatin structure reconstituted (Yoder and Bushman, 2000). In this context, component of the non-homologous end jointing (NHEJ) pathway have been proposed to be responsible for the post-integration repair process (Sakurai *et al.*, 2009). NHEJ pathway is mediated by DNA-dependent protein kinase (DNA-PK), which is activated by double strand DNA (dsDNA) ends and is composed by a 450-kDa catalytic subunit, DNA-PKcs and the dsDNA-binding Ku70/80 heterodimer (Gottlieb and Jackson, 1993). DNA-PK and other components of NHEJ, like ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia mutated AND Rad3-related (ATR), X-ray Repair Cross-Complementing protein 4 (XRCC4) and ligase IV were described to be important for HIV-1 integration (reviewed in Smith and Daniel, 2006). In this model the retroviral integration intermediate seems to be detected as DNA damage by the host cell and therefore the

completion of the integration process would need the NHEJ-mediated repair pathway to avoid the pro-apoptotic signals mediated by DNA gaps (Van Maele and Debyser, 2005). Nevertheless, the role of NHEJ pathway and in particular DNA-PK function during HIV-1 integration has been questioned. Other groups have shown that DNA-PK is not required for HIV-1 integration and alternative functions have been proposed to NHEJ pathway component during HIV-1 infection (Baekelandt *et al.*, 2000; Van Maele and Debyser, 2005). In fact, Ku 70 and Ku 80 were described as associated with the HIV-1 PICs without being necessary for the integration activity and the other components of NHEJ system were proposed to be important in avoiding pro-apoptotic signals, not by repairing the gaps in the pos-integration step but rather by inducing the 2-LTR circles formation, from the non integrated viral DNA in the nucleus (reviewed in Smith and Daniel, 2006).

Other cellular proteins have been hypothesized to be important for HIV-1 integration such as Poly-ADP-ribose-polymerase-1 (PARP-1) (Ha *et al.*, 2001; Kameoka *et al.*, 2004), RAD18 (Mulder *et al.*, 2002; Van Maele and Debyser, 2005), HSP60 (Parissi *et al.*, 2001), and p300 (Cereseto *et al.*, 2005) (see table 1.1 for proteins functions).

1.6.2.4. Role in HIV-1 Transcription

After viral DNA integration, provirus can remain silent (latency) or can be activated by the binding of viral Tat protein and its cellular co-factors to the HIV-LTR, promoting viral transcription. The HIV-1-LTR driven expression is dependent on a large number of cellular proteins, mainly transcriptional activators, repressor proteins and the entire RNAPol II machinery (reviewed in Rohr *et al.*, 2003). The RNAPol II complex is constitutively bound to HIV-1 promoter and specificity protein 1 (Sp1) and nuclear factor-kappa B (NFκB) transcription factors are known to promote transcription of short viral transcripts in the absence of Tat. However, since Tat is absent, RNAPol II efficiency is very low, resulting in the production of abortive short viral transcripts (Rohr *et al.*, 2003; Romani *et al.*, 2010). Once expressed, Tat enhances HIV-LTR transcription by recruiting cellular proteins to relieve the repression of the LTR and promoting HIV-1 RNA transcription. Tat recruits various histone acetyltransferases (HATs), like hGCN5 and CREB-binding protein (CBP)/p300 complex, to the HIV-1 promoter region, to acetylate the nucleosomes located at the promoter (Ott *et al.*, 1999; Col *et al.*, 2001; Deng *et al.*, 2001; Cereseto *et al.*, 2005). This acetylation relieves the

repression of LTR promoter, leading to a non-abortive transcription by RNAPol II and transcription of the viral genes (Pumfery *et al.*, 2003). It was also shown that Tat itself is acetylated by the p300/CBP-associated factor (PCAF) in an essential domain for HIV-1 transcription (Dorr et al., 2002; Mujtaba et al., 2002). In the current model, Tat, to initiate transcription, binds do TAR RNA and recruits the positive transcription elongation factor b (P-TEFb complex), which is composed by the CyclinT1 and CDK9 proteins, promoting RNAPol II phosphorylation (Wei *et al.*, 1998). These events lead to an enhancement of RNAPol II transcription (Romani *et al.*, 2010). Tat can also trigger phosphorylation of some transcription factors, including Sp1 and NFκB, leading to an up-regulation of the LTR promoter (Demarchi *et al.*, 1996; Demarchi *et al.*, 1999; Romani *et al.*, 2010). Other studies demonstrated that the splicing factor, splicing-associated c-Ski-interacting protein (SKIP) (Bres *et al.*, 2005), COUP-TF and protein phosphatase-1 (PP1) are also required for transcription (Ammosova *et al.*, 2005).

1.6.2.5. Role in HIV-1 mRNA Processing and Nuclear export

Following transcription, spliced and unspliced viral mRNAs have to be exported from the nucleus to cytoplasm. The export of unspliced and single-spliced RNAs is mediated by Rev with the help of cellular factors. Rev binds to the RRE element present in viral mRNA that directs the viral RNA to the cellular RNA export pathway. Later, Rev protein has to be imported again into the nucleus to proceed with the mRNA exporting (reviewed in Wang *et al.*, 2000; Freed, 2001). Rev interacts directly with importin-β and with the nucleolar phosphoprotein B23. The Rev-importin-β-B23 complex is recruited to the nuclear pore by direct interaction between importin-β and nucleoporin (Henderson and Percipalle, 1997; Szebeni *et al.*, 1997). During RNA export, Rev protein connected with the viral RNA binds to CRM1 (also named as exportin 1), through its nuclear export signal (NES). CRM1 forms a complex with the Ran protein (GTP bounded) and mediate the viral RNA transport from the nucleus through the nuclear pore (Pollard and Malim, 1998; Li *et al.*, 2002a; Dayton, 2004). Besides CRM1, other cellular proteins are recruited by Rev to aid viral RNA export such as eukaryotic initiation factor 5A (eIF-5A) (Bevec *et al.*, 1996), 68-kDa-Src-associated protein (Sam68) (Reddy *et al.*, 1999; Li *et al.*, 2002a; Li *et al.*, 2002b), certain DEAD box protein RNA helicases (DDX3, DDX1) (Fang *et al.*, 2004; Yedavalli *et al.*, 2004; Fang *et al.*, 2005; Li *et al.*, 2005a), human Rev-interacting protein (Rab/hRIP) (Bogerd *et al.*, 1995; Fridell *et al.*, 1996;

Sanchez-Velar *et al.*, 2004; Yu *et al.*, 2005) and Ran-specific GTPase-activating protein (RanBP1) (Zolotukhin and Felber, 1997).

1.6.2.6. Role in HIV-1 Translation

HIV mRNA translation uses the cellular ribosomal machinery to promote viral protein production. Viral mRNAs have 5´cap and internal ribosome entry site (IRES) sequences, so either cap 5´-dependent or 5´-independent (IRES) initiation of translation can occur from the viral RNAs. Some co-factors have been described to help in initiation of HIV-1 RNA translation including: RNA helicase A (RHA) that interacts with a 5' terminal post-transcriptional control element (PCE) enhancing viral RNA translation (Bolinger *et al.*, 2010); 9G8, a splicing regulatory protein, which seems to increase the translation of HIV-1 gag-pol RNA (Bolinger and Boris-Lawrie, 2009); and Sam68 afar from having a role in viral transcription, has also a helper function in translation, since Sam68-RNA interaction seems to facilitate and enhance the translation machinery in the cytoplasm (Soros *et al.*, 2001).

During elongation process, ribosomal frameshift has to occur for Gag-Pro-Pol polyprotein synthesis. The cellular elongation factors 1A e and 1B (eEF21A and eEF1B), elongation factor 2 (eEF2) and probably the elongation factors 5 (eEF5) help in this elongation process (Zanelli and Valentini, 2007; Bolinger and Boris-Lawrie, 2009).

Additionally, protein kinase R (PKR) is known to be activated by TAR and this activation controls the balance between translation initiation and ribosomal frameshift. When PKR is activated, the rate of initiation is slower and the ribosomal frameshift is higher or *vice-versa* (Gendron *et al.*, 2008). TAR-RNA-binding proteins (TRBP), TRBP1 and TRBP2 also bind to TAR RNA and promote viral RNA translation (Dorin *et al.*, 2003).

1.6.2.7. Role in HIV-1 Assembly and Budding

The latest steps of HIV-1 life cycle that include assembly and budding of viral particles are also assisted by cellular factors. HP68, an RNAse L inhibitor, was demonstrated to promote HIV-1 assembly through association with Gag, Gag-pol and Vif (Zimmerman *et al.*, 2002). Other cellular protein, such as tumor susceptibility gene 101 protein (TSG101), component of the endosomal sorting machinery, was demonstrated to bind to Gag-p6 protein and recruit the

endosomal-associated complex required for transport (ESCORT) to the site of budding promoting the release of the viral particles (Garrus *et al.*, 2001; VerPlank *et al.*, 2001). The efficient release of viral particles requires binding of TG101 with other cellular proteins, like PTAP and ubiquitin and furthermore, the affinity of TG101 for Gag is enhanced when Gag is ubiquitinated (Goff *et al.*, 2003). Similarly to TG101, actin interacting protein 1 (AIP1/ALIX) was also described to bind to p6 and recruit the ESCORT complex at cell surface to enhance budding process (Strack *et al.*, 2003). The INI1/hSNF5 cellular protein was also hypothesized to have a function in viral assemble and release (Yung *et al.*, 2001).

1.6.2.8. Role of Helper factors towards viral accessory proteins

During HIV-1 replication, viral accessory proteins develop several functions that are enhanced or dependent from cellular proteins. Vpu, for degradation of CD4, binds to the cellular β-transducin repeat-containing protein (h-βTrCP) (Margottin *et al.*, 1998). CD4-Vpu-h-βTrCP ternary complex then recruits S-phase-kinase-associated protein 1 (SKP1), another member of the ubiquitination machinery. As a result, CD4 is ubiquitinated and targeted to proteasomes for degradation (Li *et al.*, 2005a).

Vif also recruits the cellular ubiquitin complex, Cullin5 (Cul5)-ElonginB (EloB)-ElonginC (EloC) E3 ubiquitin ligase through binding to EloC and Cul5. This interaction promotes ubiquitination of APOBEC3G/3F and subsequently degradation, mediated by proteasomes (Yu *et al.*, 2003; Mehle *et al.*, 2004; Liu *et al.*, 2005).

Vpr also plays an important role in viral replication and pathogenesis and associates to some helper factors. Vpr interacts with uracil DNA glycosilase 2 protein (UNG2) in virus-producing cells promoting UNG2 incorporation into the viral particles. Subsequently, UNG2 may directly influence the reverse transcription accuracy by preventing the accumulation of uracil mis-incorporation in viral DNA strands during reverse transcription (Le Rouzic and Benichou, 2005). The Vpr function as a transcription activator is also assisted by cellular factors. Vpr seems to interact with Sp1, TFIIB and p300 to enhance HIV-LTR driven transcription (Wang *et al.*, 1995; Agostini *et al.*, 1996). Vpr also binds to nucleoporins, like p54, p58 and CG1 promoting Vpr docking in NPCs. The exact function of Vpr in NPCs is not clear but it is thought to enhance PIC's entry into the nucleus.

Downregulation of CD4 and CD28, mediated by the viral protein Nef is performed by inducing endocytosis via clathrin-coated pits, followed by lysosomal degradation. Nef connects with the adaptor protein (AP) complex, a part of the cellular endocytosis machinery (Janvier *et al.*, 2003). Down-regulation of MHC class I also involves Nef-mediated connection in the endosomes through recruitment of cluster sorting protein-1 (PACS-1) (Piguet *et al.*, 2000). In addition, Nef binds do PAK2, a serine threonine/kinase, to induce activation of infected cells (Raney *et al.*, 2005).

1.6.2.9. Role of Kinases/Phosphatases and Cell signaling in HIV-1 infection

Besides the helper factors, important during HIV-1 life cycle and for the viral accessory proteins, there are other cellular proteins with broader function that are also very important for HIV-1 replication, namely kinases and phosphatases. During HIV-1 replication, kinases and phosphatases can act, directly through specific interactions with viral proteins (as described above) or indirectly, in a broader way, by modulating signal transduction pathways that favour HIV-1 infection. Kinases and phosphatases regulate phosphorylation, a reversible process essential in cell signaling (or signal transduction), where kinases add a phosphate group to their substrate and phosphatases remove a phosphate group from the substrate, promoting changes in enzymatic activity and protein conformations (Hunter, 1995). Therefore, signal transduction processes occur in eukaryotic cells and permit cells to adjust their metabolism, growth and differentiation in response to a specific stimulus (Evans and Hemmings, 1998). This mechanism converts a mechanical/chemical stimulus into a specific cellular response, by triggering a cascade of reactions that promote changes in the cell functioning (e.g. altering metabolism, gene expression, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis and differentiation) (Berg *et al.*, 2002; Cornell and Shanley, 2005). Communication between the extracellular environment and the cell normally leads to change in the transcriptional machinery. Therefore, the signal has to pass from the extracellular medium through plasma membrane to cytosol and then to the nucleus compartment to achieve the transcriptional machinery (Cornell and Shanley, 2005). In order to accomplish this goal, the cell has evolved a broad array of pathways mainly regulated by protein phosphorilation events, where kinases and phosphatases are essential. Some of the most studied signal pathways, such as mitogen activated protein kinase (MAPK), and PKB/Akt

can be involved in various signal pathways like T cell receptor, Jak/Stat and NFκB pathways, which depending on the cell and in the initial stimulus can promote different activities within the cell (for review Johnson and Lapadat, 2002; Kisseleva *et al.*, 2002; Zingarelli, 2005; Manning and Cantley, 2007).

HIV-1, like other viruses, to ensure its own survival and propagation, manipulates host kinases and phosphatases and their signal pathways. The cell signaling triggered during HIV-1 infection depends on the phase of the HIV-1 infection and/or the cell host that is infected. Cell signaling pathways can be triggered by the virus to activate cells, to bypass virus latency, to favour HIV-1 gene expression, to avoid immune responses and to enhance or repress apoptosis.

Several kinases and phosphatases were described to be important for HIV-1 replication, being some of them involved in the most described signalling pathways. For example, MAP kinases control many cellular events from complex programs, such as embryogenesis, cell differentiation, cell proliferation, and cell death (figure 1.5) (reviewed in Chen *et al.*, 2001).

Some of these cascades have been reported to be important for HIV-1 replication. For

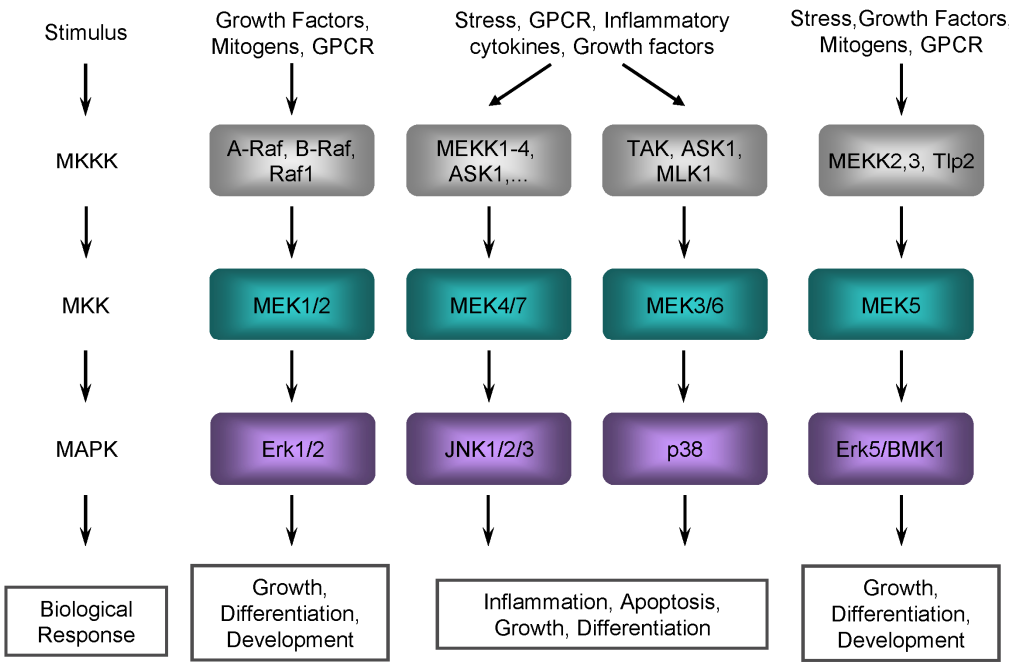

Figure 1.5. MAPK pathway. Schematic representation of MAPK pathway components and their connections. Left column is a general representation of the pathway. The subsequent columns represent the Erk, JNK and p38 subfamilies pathways, respectively. A different stimulus triggers different proteins, leading to different biological responses (adapted and modified from Johnson and Lapadat, 2002)

example, phosphorylation of p38MAPK by p53 was demonstrated to be critical step of Env-induced apoptosis (Perfettini *et al.*, 2005). Other study indicated that PPA2 activates MAPK pathway during HIV infection triggering MAPK pathway to activate other kinases like protein kinase C (PKC) in order to activate HIV-1 provirus (Faulkner *et al.*, 2003). Indeed, HIV-1-LTR driven transcription have been described by several groups as dependent of MAPK pathway: Adaptor protein (AP1) transactivation is dependent of SAPK/JNK signaling pathway activation (Chen *et al.*, 2000); NFκB motif seems to be a Raf-responsive element in fibroblasts and in T-lymphocytes, where Raf pathways were demonstrated to be important for transactivation of the HIV-1-LTR, for synthesis and release of HIV particles (Flory *et al.*, 1998). These results confirmed previous observations that indicated that activation of the p21Ras protein may be one of the signals that regulate LTR driven transcription during HIV infection (Lembo *et al.*, 1995). Tat itself was described to activate Jun N-terminal kinase (JNK) protein for its benefit leading to AP1 activation (Kumar *et al.*, 1998a).

MAPK proteins have also been described to phosphorylate and activate a subset of HIV proteins, increasing viral infectivity. Prior to HIV-1 replication, HIV-1 MA must be phosphorylated by ERK2 to allow translocation of HIV pre-integration complex into the nucleus. Viral replication may also require the phosphorylation of Vif protein by ERK1 or ERK2 (Barraud *et al.*, 2008). Nef and Tat proteins have also shown to induce the extracellular signal-regulated kinase 1 and 2 (ERK 1/2) cascade (Schrager *et al.*, 2002; Toschi *et al.*, 2006). ERK1/2 has been shown to phosphorylate HIV-1 Nef, Rev, and Tat *in vitro* (Yang and Gabuzda, 1999), but the roles of these phosphorylation events in HIV infectivity remain unknown (Yang and Gabuzda, 1998). ERK1/2 pathway was also described to be important for HIV-1 reverse transcription via GαI receptors (Mettling *et al.*, 2008).

Other very important protein kinase in signal transduction is serine/threonine kinase Akt also known as protein kinase B (PKB). Akt/PKB is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli, having an important role in cell survival, growth, proliferation, angiogenesis, metabolism, and migration (reviewed in Manning and Cantley, 2007). Akt acts downstream of phosphatidylinositol-3-kinase (PI3K), a tyrosine kinase protein activator, and is activated by recruitment to the plasma membrane through direct contact with PIP3, and phosphorylation at Thr308 and Ser473. Thr308 is phosphorylated by the 3-phosphoinositide-dependent protein kinase PDK1, whereas Ser473 is phosphorylated by

mTOR2 (Vivanco and Sawyers, 2002) Activation of Akt triggers transduction signals to a series of downstream regulators of cell survival (figure 1.6). PI3K/Akt pathway can be negatively regulated by the phosphatase tensin homolog protein (PTEN), which converts phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) (Stambolic *et al.*, 1998) or by Src homology 2 domaincontaining inositol-5-phosphatase (SHIP) that converts PIP3 into Phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) (Liu *et al.*, 1999).

The role of Akt and PI3K/Akt pathway during HIV-1 infection is controversial. Akt activation in HIV-1 context leads to different outcomes, depending of the activated downstream pathway, being related to cell survival or apoptosis. In Jurkat T cell lines, Tat expression was shown to activate PI3K and Akt/PKB kinases leading to an increased cell survival (Borgatti *et al.*, 1997). In macrophages, Tat protein was also related to PI3K/Akt pathway activation by establishing a

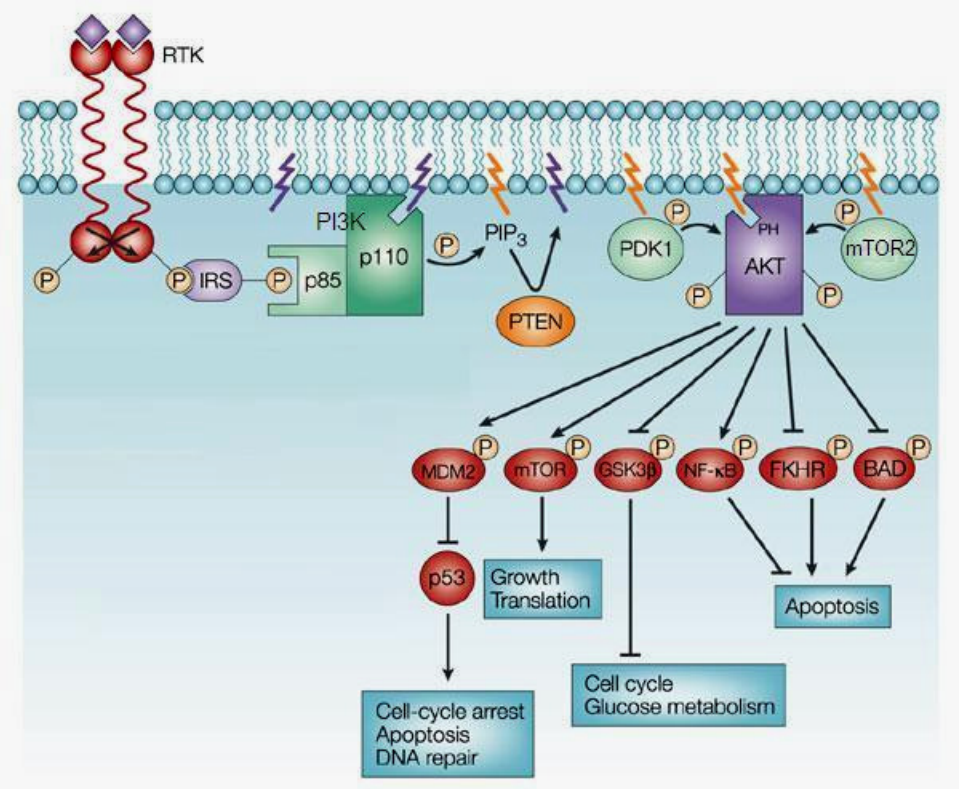

Figure 1.6. PI3K/Akt signaling. Activation of PI3K occurs through stimulation of receptor tyrosine kinases (RTKs) and the concomitant assembly of receptor–PI3K complexes. These complexes trigger conversion of PIP2 to PIP3 that helps Akt activation. Through phosphorylation, activated Akt mediates the activation and inhibition of several targets, resulting in cellular growth, survival and proliferation through various mechanisms (adapted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol], Vivanco and Sawyers, 2002, copyright (2002).

cytoprotective effect during HIV-1 infection, which extends the lifespan of infected macrophages. It was shown that HIV-1 infection activates PI3K/Akt pathway in human primary macrophage, by decreasing PTEN protein expression and thus increasing Akt kinase activity. Additionally the same group have observed that Tat interaction with p53 contribute to the downregulation of PTEN expression, since HIV-1 Tat was found to compete with PTEN for p53 binding (Chugh *et al.*, 2008). gp120 protein was also associated to Akt. One study showed that HIV-1 envelope induces early activation of the PI3K/Akt pathway in primary $CD4^+$ T lymphocytes (Francois and Klotman, 2003). Another study indicated that gp120 triggers apoptosis through inhibition of Akt phosphorylation via cluster differentiation 45 (CD45) (Anand and Ganju, 2006).

Signalling pathways are networks where cascades like MAPK and Akt interconnect with other cascades upon a stimulus. During HIV-1 infection some of these cascades seem to favour HIV-1 infection, like T cells receptor signaling, JAK/Stat pathway or NF-κb signaling, leading to cell proliferation, cell survival or apoptosis.

T cell receptor signaling is the major signaling pathway that characterizes T lymphocytes. The main characteristic of T cells is their ability to respond to external signals through synthesis and secretion of a broad spectrum of soluble factors, together with the expression of certain surface molecules. Various stimuli can cause T cell activation—antigens, cytokines, receptor contacts, or mitogens. T cell activation leads to proliferation and ultimately to the generation of effector cells which mediate helper, suppressor and cytotoxic T-cell functions (Linch *et al.*, 1987).

HIV-1 is dependent on T cell activation. The virus infects activated and quiescent primary T cells but needs a prior activation in quiescent cells for a productive HIV-1 infection (Bukrinsky *et al.*, 1991; Spina *et al.*, 1995). Therefore, to replicate, HIV-1 alters T cell signaling to favour T cell activation. This activation is triggered by the gp120 binding to CD4, when HIV-1 encounters a cell host, and also when latent viruses are set off. HIV-1 gp120 direct binding to CD4 receptor and consequently coreceptor binding triggers a broad spectrum of signaling pathways, leading to T cell activation and to a series of events as cytokine expression, HIV-1 coreceptor expression, enhancement of HIV-LTR promoter activity, which can favour HIV-1 infectivity and also apoptosis induction (reviewed in Popik and Pitha, 2000).

The activation of latent HIV proviruses through T cell activation can be induced by a wide range of T cell stimuli, including T cell receptor ligation by anti-cluster differentiation 3 (CD3) antibodies, cytokines (IL-1b, IL-7, and TNF-α), and mitogens (e.g., PMA and prostratin). Each of these stimuli induces broad changes in T cell activation status and affects cellular and viral gene expression, through both distinct and shared signaling pathways. Such signaling ultimately drives HIV-1 transcription through the induction of activating cellular transcription factors, including NF-κB, nuclear factor of activated T-cells (NFAT), and AP-1, which bind to cognate enhancer sequences within the HIV-1 LTR (Williams and Greene, 2007).

1.6.2.10. Genome-wide screenings for identification of Helper factors for HIV-1

In the past few years the field of HIV-1-helper factors has deeply increased. Numerous cellular factors were described and characterized has essential for an accurate HIV-1 infection. Nevertheless, due to the extremely dependence of the virus from its host it is certain that much co-factors are yet to be discovered. With this pursuit of find out new helper factors important for HIV-1 infection, several genome-wide studies were performed using the RNA interference (RNAi) machinery as a main tool. RNAi is a powerful technique for studying loss-of-function phenotypes by specific down-regulation of gene expression, allowing the evaluation of its function during HIV-1 infection (discussed further in more detail). Four genome-wide RNAI screens have been performed to discover essential genes for HIV-1 replication (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009). From these screen hundreds of human proteins were described as important for HIV-1 replication, acting as co-factors during HIV-1 infection. Nevertheless, when the different studies were compared, a low overlap was observed (Goff, 2008). This can be justified by the different experimental design, the cell lines used and the criteria for analysing data. Despite the low overlap between genes, their function analysis indicated they belong to common pathways, like ubiquitination, SUMOylation, DNA repair, vesicular transport, and energy production (Goff, 2008; Kok *et al.*, 2009). Moreover, among the identified pathways, there were described pathways already identified as important for HIV-1 but also new pathways that were not known (Kok *et al.*, 2009). In the new group of pathways, the mediator complex that regulates transcription (MEDs) have been implicated in activation of HIV-1-LTR transcription;

Golgi proteins were hypothesized as critically involved in the intracellular transport and processing of viral nucleic acids and proteins; and many genes encoding proteins that are involved in mitochondrial function and energy production were also identified (Goff, 2008; Kok *et al.*, 2009).

The RNAi screen studies have opened a new area of research in HIV-1. The discovery of these new proteins and pathways important for HIV-1 replication was a very important accomplishment in HIV research, but we are still far from the end. It is evident that the search for host factors is not over yet. New screens with new conditions and new readouts can be performed in order to address more important players of this intrinsic battle between HIV-1 and its host.

1.7. RNA Interference (RNAi)

RNA interference (RNAi) was first observed in plants in the late 1980s (Ecker and Davis, 1986), but its mechanism remained unknown until 1998, when Fire *et al.* observed that exogenous double-stranded RNA (dsRNA) could induce potent and sequence-specific silencing of endogenous gene expression in Caenorhabditis elegans (Fire *et al.*, 1998). Years later, the same mechanism was proved to exist in mammalian cells by using a synthetic small interfering RNA (siRNA) that was able to knockdown genes (Elbashir *et al.*, 2001). RNAi was proven to be an evolutionarily conserved mechanism that leads to a sequence-specific gene silencing and is involved in processes as diverse as defence against viral infection, mobilization of transposable genetic elements, cell fate specification, and regulation of developmental timing (Rao and Sockanathan, 2005).

Initially, two types of natural occurring small dsRNAs acting as gene silencers were described: the short interfering siRNAs (siRNAs) and the microRNAs (miRNAs) (figure 1.7). siRNAs, derive from RNA long duplexes that are often produced during the course of viral infection within cells (foreign dsRNA) or by hybridization of overlapping transcripts from repetitive sequences in the genome, such as transposons or latent viruses (Rao and Sockanathan, 2005). Each long dsRNA is processed by Dicer, a ribonuclease III family (RNase III) enzyme, into 21–25 base pairs (bp) dsRNAs called small siRNAs (Zamore *et al.*, 2000). The siRNA has two nucleotides (nt) 3′ overhangs that allow them to be recognized by the enzymatic machinery of RNAi.

Consequently, the resulting small RNAs are incorporated into the RNAi-induced silencing complex (RISC), where Argonaute 2 (AGO2), a multifunctional protein contained within RISC, cleaves the passenger (sense) strand. The activated RISC, which contains the guide (antisense) strand of the siRNA is directed to the mRNA target, recognizes it and degrades the mRNA complementary to the antisense strand (cleavage carried out by the catalytic domain of AGO2) (Whitehead *et al.*, 2009).

Human miRNAs are derived from cellular dsRNA harpin precursors and are known to modulate gene expression at the post-transcriptional level, having a role in a variety of cellular processes, including differentiation, development, and metabolism. miRNAs precursors are originated by transcripts of 20-50 bp inverted repeats of complementary sequences. These transcripts fold back on themselves and base pair along complementary

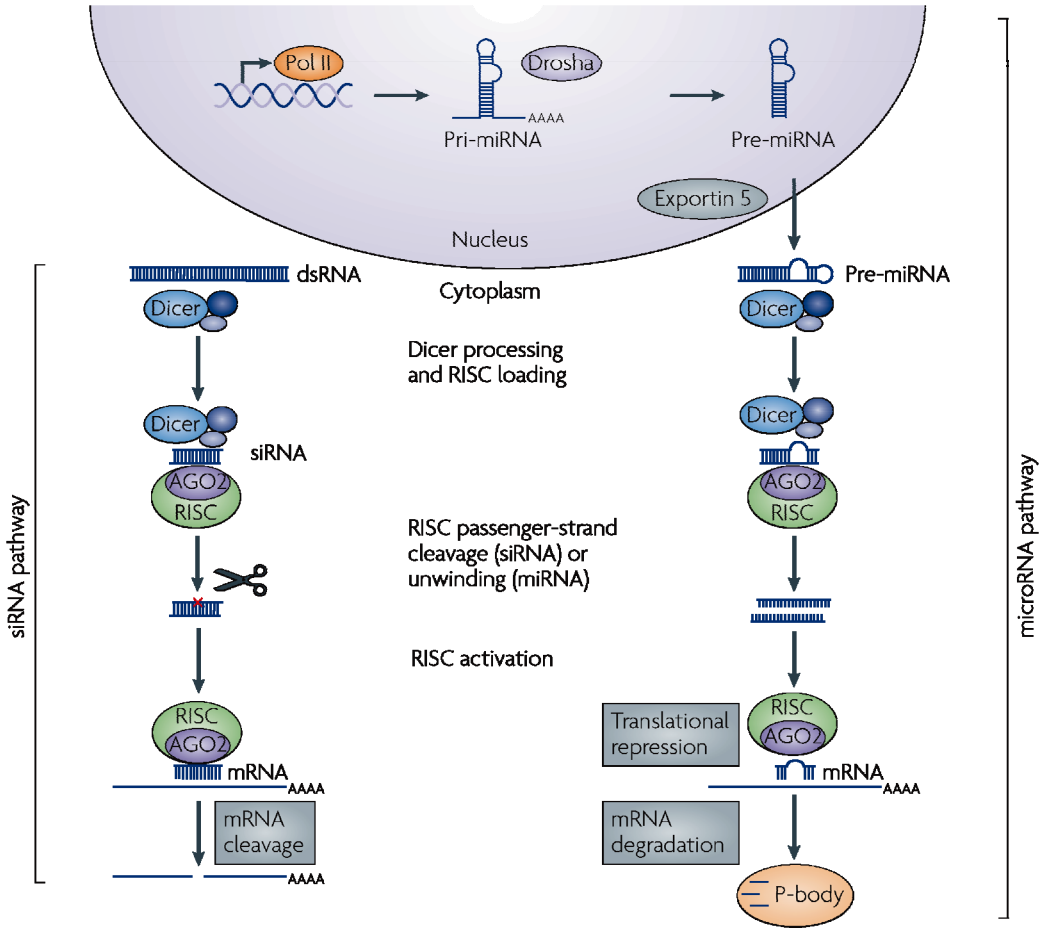

Figure 1.7. RNAi mechanism in mammalian cells. Schematic representation of the RNAi pathway in mammalian cells, guided by small RNAs that include siRNA and miRNAs that lead ultimately to knockdown of the target gene (Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Drug Discov] de Fougerolles *et al.*, 2007, copyright (2007).

regions to form dsRNA hairpins. Hairpin precursors are first processed into shorter pre-miRNAs (about 70 nt long) within the nucleus by the Drosha (a ribonuclease III similar to Dicer) enzyme complex. These precursors are then exported to the cytoplasm by exportin 5 and subsequently bind to the Dicer enzyme complex, which processes the pre-micro RNA (miRNA) for loading onto the AGO2–RISC complex. When the RNA duplex loaded onto RISC has imperfect sequence complementarity, the sense strand is unwound leaving a mature miRNA bound to active RISC. The mature miRNA recognizes target sites in the mRNA, leading to direct translational inhibition. Binding of miRNA to target mRNA may also lead to mRNA target degradation in processing (P)-bodies (Winter *et al.*, 2009).

Recently, many other endogenous small RNAs have been identified in different species: PIWI-interacting RNAs (piRNAs), originate from transposons, viruses and repetitive sequences that are characterized by their interactions with the PIWI subfamily or Argonaute proteins and were identified in germlines; a new class of endogenous siRNAs (endo-siRNAs or esiRNAs) of small RNAs, including repeat-associated siRNAs (ra-siRNAs), tiny non-coding RNAs (tncRNAs), *trans*-acting siRNAs (ta-siRNAs) and scan RNAs (scnRNAs), that were found in fungi, plants and animals. Nevertheless, so far none of these has been observed in mammals (Siomi and Siomi, 2009).

RNAi discovery led to a revolution in RNA biology. The discovery of this mechanism has not only revolutionized our ability to study biology but has challenged some of the most basic assumptions behind regulation of gene expression. Due to this discovery the biology studies grew enormously, and RNAi was adopted for analysing gene function in mammalian systems and ultimately for gene specific therapeutics (Leung and Whittaker, 2005).

The RNAi machinery became a powerful tool and synthetic RNAi molecules were rapidly, constructed. Synthetic siRNAs were constructed and were efficiently delivered into cells. Their low size compared with dsRNA precursors lead to a lower probability of triggering the innate immune system (Elbashir *et al.*, 2001). siRNAs are normally transfected in cells, activate RNAi machinery and effectively promote gene silencing. Although siRNAs were proven to be effective, their expression is transient and their effect is dependent on the number of siRNA molecules that are transfected and their persistence in the cell (Leung and Whittaker, 2005). One way to overcome this problem was the construction of short hairpin RNAs (shRNAs) expressed vectors. shRNAs construction was based in cellular miRNA and are constituted by

perfectly double-stranded stems of 19–29 bp that have an identical sequence to the target mRNA. The two strands of the stem are connected by a loop of 6–9 bases, which is removed in vivo by Dicer to generate effective siRNAs (Paddison *et al.*, 2002a; Paddison *et al.*, 2002b). These shRNA vectors are plasmid-based shRNAs that have a constitutive efficient promoter, like U6 and a RNAPol III promoter that is active in all cell types and efficiently directs the synthesis of small, non-coding transcripts (Mittal, 2004). Transfection can be easily performed with these plasmids and stable cell lines can be generated through a drug resistance marker selection. These vectors lead to an efficient and stable knockdown of target genes. Nevertheless, plasmid vectors also have limitations in terms of transfection efficiency. Consequently, retroviral, lentiviral and adenoviral vector systems for shRNA delivery were constructed, permitting the efficient introduction and stable integration of these shRNA-expression cassettes into the host genome (Bos *et al.*, 2009). Lentivirus-mediated delivery has advantages over the others systems once the virus can efficiently integrate into the genome of dividing and non-dividing cells, and transgenes carried by lentiviral vectors are resistant to silencing (Mittal, 2004).

The discovery of RNAi functionality in mammalian cells, all developments in "RNAi technology", together with the complete genome sequencing of a large number of organisms lead to the construction of siRNA and shRNA libraries. These libraries allow the targeting of any gene of the studied genome and permit the development of wide-genome screens to evaluate individual gene function through loss-of-function analyses and interaction between genes and biological pathways (Cullen and Arndt, 2005; Root *et al.*, 2006).

1.7.1. RNAi and HIV-1

The potential of RNAi machinery and all the powerful tools designed to silence gene expression were overtaken to study HIV-1 genes functions, HIV-cell interaction and, ultimately, to be used as a powerful tool against HIV-1 infection, by developing new and non-conventional antiviral strategies.

The continuous search for a cure to HIV-1 infection motivates researchers to look for new strategies to develop anti-HIV therapies. RNAi, being a based silence mechanism that is described in mammals seems a very powerful candidate to overcome some viral infections including HIV-1. RNAi can be used against HIV-1 using as target the HIV-1 provirus integrated

in the host genome, the viral RNA transcripts, or cellular genes essential for HIV-1 replication (Singh and Gaur, 2009; Soejitno *et al.*, 2009). Several studies were performed by targeting HIV-1 genes, involved in early and late infection. Effective siRNAs were developed against *gag* (Novina *et al.*, 2002; Chang *et al.*, 2005; Lee *et al.*, 2005; Morris *et al.*, 2005; Song *et al.*, 2005; Cave *et al.*, 2006), *pol* (Chang *et al.*, 2005; Morris *et al.*, 2005; Lau *et al.*, 2007), *vif* (Barnor *et al.*, 2005; Lee *et al.*, 2005), *tat* (Coburn and Cullen, 2002; Lee *et al.*, 2003; Dave and Pomerantz, 2004; Li *et al.*, 2005b), *rev* (Coburn and Cullen, 2002; Lee *et al.*, 2002; Dave and Pomerantz, 2004; Lee *et al.*, 2005; Li *et al.*, 2005b), *env* (Park *et al.*, 2003; Hayafune *et al.*, 2006a; Hayafune *et al.*, 2006b) and *nef* (Dave and Pomerantz, 2004; Chang *et al.*, 2005; Yamamoto *et al.*, 2006). Viral RNA was also targeted with RNAi by targeting LTR promoter or by inducing transcriptional gene silencing, through TAR inhibition (Barnor *et al.*, 2005; Puerta-Fernandez *et al.*, 2005; Barichievy *et al.*, 2007; Christensen *et al.*, 2007).

Alternatively, anti-HIV strategies with RNAi were constructed to inhibit host genes that have an essential role during HIV-1 replication. siRNAs targeting the receptor CD4 (Novina *et al.*, 2002) and the co-receptors CXCR4 and CCR5 (Martinez *et al.*, 2002; Lee *et al.*, 2003; Qin *et al.*, 2003; An *et al.*, 2007) were developed. Host factors that are important for HIV-1 LTR driven transcription were also targeted and it was demonstrated that its knockdown led to an efficient inhibition of HIV-1 replication. Other proteins that influence HIV-1 replication, like actin related protein (ARP) 2/3 complex (Komano *et al.*, 2004), PARP1 (Kameoka *et al.*, 2004), P-TEFb (Chiu *et al.*, 2004) and TSG101 (Garrus *et al.*, 2001) were also used for RNAi targeting, having an effect during HIV-1 infection.

The RNAi engineering seems to be effective against HIV-1, however it has some drawbacks. Indeed, although siRNAs have a smaller size then dsRNA, several studies have indicated that siRNA and shRNA expressed by vectors can trigger interferon's pathway, triggering host innate immunity (Robbins *et al.*, 2009). Another concern is the propensity of HIV-1 to emerge with rapid mutation, leading to RNAi resistance (Westerhout *et al.*, 2005). The use of cellular factors overpasses this problem. Nevertheless, cellular target selection as to be made carefully, taking into account that RNAi effect can affect both virus and cell host. To counteract these weaknesses co-expression of multiple siRNAs targetting different HIV-1 RNA sequences or HIV-1 and cellular RNA sequences was used and combinatory shRNA strategies were developed with the construction of long hairpin RNAs (lhRNA). These RNAi molecules

are transcribed by RNAPol III and are processed by Dicer into multiple siRNAs (Saayman *et al.*; Konstantinova *et al.*, 2006).

Other RNAi molecules that can be efficient against HIV-1 are cellular and native miRNAs. Cellular miRNAs have already been described to target HIV-1, namely against Nef (Ahluwalia *et al.*, 2008). Cellular miRNA against viral proteins together with miRNAs that target cellular genes essential for HIV-1 replication could also be used for an antiviral strategy (Corbeau, 2008).

Despite the drawbacks, RNAi against HIV-1 can be used in therapeutics against HIV-1 and it seems a very promising strategy. The continuous development of this technology could lead to future treatments for HIV-1 infection.

Beyond the potential of the RNAi as a therapeutic approach to combat HIV-1, this mechanism is also very powerful to study HIV-1 replication, viral proteins function and HIV-host interaction. The development of artificial siRNA/shRNA against viral and cellular proteins as described above has helped to identify the importance of those proteins in HIV-1 life cycle.

Nevertheless, maybe the most striking utility of RNAi as a tool for loss-of-function studies in HIV-1 was the construction of the siRNA and shRNA libraries that permitted the emergence of RNAi screens to identify host proteins required for HIV-1 replication. As described above, several siRNA and shRNAs genome wide screens were performed (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009), and hundreds of new human genes were identified as important for HIV-1 replication (Goff, 2008).This screening approach expanded enormously the field of HIV-host interaction and provided new hints for cellular pathways involved in HIV-1 replication.

In conclusion, despite the tremendous progress that has been made in HIV research, the discovery of HIV/AIDS cure was not yet achieved. It is important to proceed with research in HIV-1 not forgetting the restrict interplay between HIV-1 and host cell. Drawing all background information that it was gathered until nowadays together with new technologies that have been developed it is imperative to develop new antiviral strategies in order to be closer to HIV elimination.

Aims

The research studies presented in this thesis focused on the interplay between HIV-1 and its host, mainly on the study of cellular factors that favour HIV-1 infection. The lack of an effective therapy that can eliminate HIV-1 from infected patients, due to the emergence of resistance mutants, together with the knowledge that in the complex interplay between HIV-1 and its host, most of these interactions and have not yet been unravelled, it becomes imperative the identification of new cellular co-factors for HIV-1 infection. Indeed, cellular factors due to their less variability when compared to viral proteins are promising alternative targets for an antiviral strategy. In particular, kinases and phosphatases are considered a good druggable group of proteins. Therefore, the main goal of this thesis is to identify new helper factors for HIV-1 infection, particularly kinases and phosphatases, and to study the viral and cellular underlying mechanisms responsible for this interplay.

To achieve our main goal, the specific questions addressed in this thesis were:

- Can we develop an innovative and feasible screen in T lymphocytes based in a shRNA library to identify cellular proteins essential for HIV-1 replication?
- Can we identify new helper factors for HIV-1 infection, particularly kinases and phosphatases that could be essential for HIV-1 but innocuous for its host cell?

- In which step(s) of the HIV-1 life cycle the identified protein can act to promote HIV-1 infection?

- Does DNA-PKcs has also a positive role in HIV-1 replication, having a similar function as CIB2, one of the identified proteins, in the HIV-1 life cycle?

Ultimately, our encompassing goal was to contribute to a better knowledge of the complex interplay between HIV-1 and its host through the characterization of cellular proteins and their signal pathways that act in benefit to HIV-1 replication. This contribution could open doors for new therapeutic approaches using cellular factor as antiviral targets.

CHAPTER 2

Development of a Long-Term Iterative shRNA Screen in Jurkat T-cells to identify kinases/phosphatases important for HIV-1 replication

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2.1. Abstract

HIV-1 is a complex retrovirus that uses the host machinery to promote its replication. Understanding cellular proteins involved in the multistep process of HIV-1 infection may result in the discovery of more adapted and effective therapeutic targets. Kinases and phosphatases are a druggable class of proteins critically involved in regulation of signal pathways of eukaryotic cells. In these work we performed a newly iterative screen in Jurkat T-cells with a short-hairpin-RNA (shRNA) library highly enriched for human kinases and phosphatases. With this screen we sought to discover kinases and phosphatases that were essential for HIV-1 replication but dispensable for cell viability. We identified new 14 proteins essential for HIV-1 replication that do not affect cell viability. These proteins are described to be involved in MAPK, JNK and ERK pathways, vesicular traffic and DNA repair. This study brings new insights for the complex interplay of HIV-1/host cell and opens new possibilities for antiviral strategies.

2.2. Introduction

Despite all the efforts in the last three decades for the development of new drugs for acquired immune deficiency syndrome (AIDS) treatment, human immunodeficiency virus type 1 (HIV-1)/AIDS continue to be one of the major human health setbacks of our days (Fauci, 2008). HIV therapies (HAART) developed so far (reviewed in Flexner, 2007) although powerful, effective against HIV and capable of prolonging life and health of the infected individuals, are still not able to cure AIDS (Stevenson, 2008). The ability of HIV to establish latent reservoirs early on the course of infection and its capacity to mutate at a high rate, leading to the emergency of resistant viruses, are the major concerns for the current therapies (Clavel and Hance, 2004). Therefore, it is crucial to identify novel drug targets and new therapeutic strategies to combat AIDS. A better understanding of the virus and host-cell interplay could hopefully provide valuable insights into the molecular interactions involved in various steps of retroviral replication. The knowledge of these novel critical players can lead to the development of more adapted and effective therapeutic approaches for eradication of HIV-1 infection (Greene and Peterlin, 2002).

During the past years, several studies have been focused in the identification of host factors that assist HIV-1 during the different steps of its replication cycle (Greene and Peterlin, 2002; Goff, 2007). Nevertheless, due to the complexity of the interaction between the virus and the host cell, numerous proteins and mechanisms are yet to be discovered. Recently, different studies using genome-wide RNA interference (RNAi) screens were performed to discover new cellular proteins important for HIV-1 replication (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009). Three of these screens used siRNA libraries and were transiently expressed in HeLa or HEK293T cells (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008). Recently, Kuan-Teh Jeang and co-workers performed 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-cells (Yeung *et al.*, 2009). All these screens are based in RNAi libraries that cover all human genes. Nonetheless, despite using similar strategies, the degree of functional overlap between the identified proteins in the different screens was very low. Importantly, these studies brought noteworthy knowledge on HIV-1/host interaction by identifying many cellular proteins that had not yet been related to HIV-1 infection. Moreover, the diversity of identified proteins suggests a vast complexity of host-virus interplay.

Differently than previous studies, in this work we used a smaller library enriched for human kinases and phosphatases, narrowing down the heterogeneity and possible off-target genes that could result from a genome-wide RNAi screen. Similarly to the work of Kuan-Teh Jeang and co-workers, we used Jurkat cells to access specific T-cell genes important for HIV-1 replication but with the additional goal of identifying cellular drug targets for an antiviral strategy. Furthermore, in contrast to the previous study, selection of HIV-1 resistant cells was dependent on viral expression (direct readout) instead of cell death due to viral infection (indirect readout). These differences are expected to complement and improve the goal of discovering novel HIV-1 knockdown targets**.**

Amongst all proteins considered, kinases and phosphatases are probably the most important regulators of biological and signal pathways. These proteins are key complementary players in protein phosphorylation, a well-characterized biochemical process for reversible regulation of protein activity (Hunter, 1995). Moreover, since kinases and phosphatases are enzymes whose catalytic activity can be effectively and specifically turned off by active site-directed inhibitors, they constitute nowadays the largest subset of the druggable genome, the so-called “kinome” and “phosphatome”. Thus, we can envision that kinase/phosphatase modulation is a promising approach for the development of novel therapeutic strategies to overcome antiviral drug resistance (Hopkins and Groom, 2002; Russ and Lampel, 2005). In this context, the study of kinase and phosphatases genes and their function during HIV-1 replication may not only contribute to a better knowledge of HIV-cell interaction but also may lead to the discovery of new cellular targets for HIV-1 therapy.

With this iterative shRNA screen in Jurkat cells we identified 14 different cellular proteins, involved in several cellular pathways that are essential for HIV-1 replication.

Our results bring not only new insights to the complexity of the HIV-1/host interaction but also open possibilities of exploring novel therapeutic strategies for the treatment of AIDS by targeting kinases and phosphatases.

2.3. Material and Methods

2.3.1. Cell Lines and culture conditions

Jurkat E6-1 T-cells obtained through the NIH AIDS Research and Reference Reagent Program (MD, USA, contributor Dr. Arthur Weiss) were cultured in Roswell Park Memorial Institute medium (RPMI)-1640, supplemented with 10 % of fetal bovine serum (FBS) (RPMI-10). Human embryonic kidney 293T (HEK293T) (ATCC, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS (DMEM-10). Jurkat cells expressing shRNA (shRNA clones) were cultured in RPMI-10 supplemented with 2 μg/ml of puromycin (Sigma, MO, USA). All cell cultures were maintained at 37 °C in 5 % CO_2. All cell culture media and reagents, otherwise indicated, were from Lonza (Basel, Switzerland).

2.3.2. Viral production

HEK293T cells were transfected, by calcium phosphate method, with pNL4-3-r-HSAS (AIDS reagent, contributors Drs. Beth Jamieson and Jerome Zack) or pHIV-1_{NL4-3} plasmids (AIDS reagent, contributor Dr. Malcolm Martin) to produce HIV-HSA or HIV-1_{NL4-3} virions, respectively. After 48 h, virions were collected from supernatant cultures, measured by $p24^{CA}$ ELISA (AIDS & Cancer Research Program, NCI Frederick, MD, USA) and used to infect Jurkat cells.

2.3.3. Infection assays

Jurkat cells, shRNA library and individual cell clones were infected with HIV-HSA or HIV-1_{NL4-3} at the indicated multiplicity of infection (MOI). For this purpose, cells were ressuspended in a viral preparation and subject to spinoculation (as described in O'Doherty *et al.*, 2000). After 6 h, cells were washed with phosphate buffer saline (PBS) (1 x) 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 & Cancer Research Program).

2.3.4. Lentiviral shRNA Library Screen

Lentiviral shRNA library composed by 3-5 shRNA for each gene was enriched for human kinases and phosphatases by rearraying LKO.1 shRNA constructs obtained from the RNAi Consortium (TRC) (Broad Institute, MA, USA). High-titer lentiviral production was obtained after transfection of HEK293T cells with the shRNA-encoded library. The plasmids included in the lentiviral packaging mix encode the key structural viral packaging genes and a heterologous viral envelope gene in a three-plasmid lentivirus packaging system (Amendola *et al.*, 2005) (lentiviral library production was performed by the Luis Moita Laboratory). We collected the cell supernatant containing a highly infectious pool of VSV-G pseudotyped shRNA-encoding lentiviral particles and used it for transduction of Jurkat cells.

Jurkat cells were transduced with the lentiviral shRNA library at MOI of 1 and enhanced by spinoculation. Two independent transductions were performed with the same pool, leading to two populations of transduced Jurkat cells. Medium was replaced 24 h later and after 48 h, cells were challenged with HIV-HSA at a MOI of 1, washed 6 h later and cultured in RPMI-10 for 7 days as aforementioned. Medium was replaced every 2 days.

Transduced Jurkat cells challenged with HIV-HSA were negatively selected using a biotinylated anti-HSA antibody (BD Pharmingen, CA, USA) and CELLection Biotin magnetic beads (Invitrogen Dynal, Oslo, Norway), according to the manufacturers' protocol. This iterative procedure was repeated for 3 more rounds. Cells were recovered, cultured in RPMI-10 supplemented with 2 μg/ml of puromycin and grown for 7 days. Procedures of viral infection, negative selection and cell culture with puromycin were performed 3 times. The negatively-selected puromycin resistant-cells were cloned with a ClonaCell™-TCS semi-solid medium (StemCell Technologies, Vancouver, Canada) 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.

2.3.5. shRNA sequence identification

shRNAs of the HIV-1 resistant clones were PCR amplified from genomic DNA. Approximately $5x10^6$ Jurkat cells were lysed in 500 μl of DNA extraction buffer (10 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.2 % SDS, 0.2 M NaCl, 100 μg/ml of proteinase K), incubated at 37 °C during 3 h

with agitation, precipitated with one volume of isopropanol, recovered, washed with ethanol (70 %), and dissolved in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). shRNAs sequences were amplified with the following primers: 5'-GGATGAATACTGCCATTTGTCTCG-3' and 5'-AGGCCCGAAGGAATAGAAGA-3'. The resultant PCR products were sequenced using an internal primer: 5´-CAGGGCTGTTAGAGAGATAATTGGA-3´ (MACROGEN, Seoul, Korea). To identify the target gene of each shRNA, computation was performed at National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) network service.

2.3.6. Development of individual shRNA Jurkat cell clones

After identifying target genes, we selected 3-5 shRNA constructions for each gene from the TRC and generated Jurkat cells expressing a specific shRNA. Briefly, we produced shRNA lentiviral particles and transduced Jurkat cells with each shRNA as described in Material and Methods. Transduced cells were isolated with ClonaCell™-TCS semi-solid medium (StemCell Technologies) and clones were expanded and maintained in RPMI-10 supplemented with 2µg/ml of puromycin (Sigma, MO, USA). shRNA sequenced are described in table 2.1

2.3.7. Quantitative Real-Time PCR analysis

Total RNA was extracted from shRNA clones using Trizol (Invitrogen, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, CA, USA) according to the manufacturer's protocol. qPCR was performed on an ABI Prism 7300 PCR system (Applied Biosystems, CA, USA), using the Maxima™ SYBR Green qPCR Master Mix (Fermentas, Ontario, Canada). Primer sequences are described in Table 2.2. For PRKD1, ELA1 and PPFIA2 genes, qPCR was performed using a Maxima™ Probe qPCR Master Mix (Fermentas) and TaqMan® Gene Expression Assays (Applied Biosystems). The relative amount of target gene mRNA was normalized to glycerAldehyde 3-phosphate deHydrogenase (GAPDH) mRNA. Specificity was confirmed by melt curve analysis for SYBR Green qPCR .

Table 2.1. shRNA sequences used for each gene in study.

Gene Target	shRNA sequence
PTPN9	CCGGGACTATGTTAATGCCAGGCAACTCGAGTTGCCTGGCATTAACATAGTCTTTTT
	CCGGCGAAGGAAGGAAGGCATTGTACTCGAGTACAATGCCTTCCTTCCTTCTTT
	CCGGCCATACTCAGACAGATTACATCTCGAGATGTAATCTGTCTGAGTATGGTTTTT
	CCGGGCTATCAAACTGTGCCTTATTCTCGAGAATAAGGCACAGTTTGATAGCTTTTT
PRKD1	CCGGCCCACGCTCTCTTTGTTCATTCTCGAGAATGAACAAAGAGAGCGTGGGTTTTT
	CCGGCTAAGGAACAAGGGCTACAATCTCGAGATTGTAGCCCTTGTTCCTTAGTTTTT
	CCGGCCATCTCCTATAATCTGTCAACTCGAGTTGACAGATTATAGGAGATGGTTTTT
	CCGGCGGCACTATTGGAGATTGGATCTCGAGATCCAATCTCCAATAGTGCCGTTTTT
	CCGGCCAGAGCACATAACGAAGTTTCTCGAGAAACTTCGTTATGTGCTCTGGTTTTT
MAP3K2	CCGGGCAACGTCAAACTAGGAGATTCTCGAGAATCTCCTAGTTTGACGTTGCTTTTT
	CCGGCCAGATGAATTACACCAGGTTCTCGAGAACCTGGTGTAATTCATCTGGTTTTT
	CCGGCCTTTGGATGGAGAGAGCTATCTCGAGATAGCTCTCTCCATCCAAAGGTTTTT
	CCGGCATATCATCATCAAGAGGTAACTCGAGTTACCTCTTGATGATGATATGTTTTT
MAPK9	CCGGGCTGTCGATGATAGGTTAGAACTCGAGTTCTAACCTATCATCGACAGCTTTTT
	CCGGGATGTGTATTTGGTTATGGAACTCGAGTTCCATAACCAAATACACATCTTTTT
	CCGGCTGTGAGGAATTATGTCGAAACTCGAGTTTCGACATAATTCCTCACAGTTTTT
	CCGGAGGGATTGTTTGTGCTGCATTCTCGAGAATGCAGCACAAACAATCCCTTTTTT
ELA1	CCGGGTACGTGAGTGTGCAGAAGATCTCGAGATCTTCTGCACACTCACGTACTTTTT
	CCGGGAGTGTGCAGAAGATCGTGGTCTCGAGACCACGATCTTCTGCACACTCTTTTT
	CCGGCGTTACCCTCAATAGCTATGTCTCGAGACATAGCTATTGAGGGTAACGTTTTT
	CCGGCTGAAAGACTATTGAGCCATTCTCGAGAATGGCTCAATAGTCTTTCAGTTTTT
	CCGGCTGAAAGACTATTGAGCCATTCTCGAGAATGGCTCAATAGTCTTTCAGTTTTT
RAD23B	CCGGCCAGCGTTACTACAGCAGATACTCGAGTATCTGCTGTAGTAACGCTGGTTTTT
	CCGGGTGTACTAGATCCAGAAACTTCTCGAGAAGTTTCTGGATCTAGTACACTTTTT
	CCGGAGAAGCTGGAAGTGGTCATATCTCGAGATATGACCACTTCCAGCTTCTTTTTT
	CCGGCTCCAGCATCAGCGACAGCATCTCGAGATGCTGTCGCTGATGCTGGAGTTTTT
CIB2	CCGGCAACTACCAGGACTGCACCTTCTCGAGAAGGTGCAGTCCTGGTAGTTGTTTTTG
	CCGGCTGACTTCGAGGACATGATTGCTCGAGCAATCATGTCCTCGAAGTCAGTTTTTG
	CCGGCCTCCTTCACAATGTGAAGCTCTCGAGAGCTTCACATTGTGAAGGAGGTTTTTG
EZH2	CCGGCAACACAAGTCATCCCATTAACTCGAGTTAATGGGATGACTTGTGTTGTTTTTG
	CCGGTATGATGGTTAACGGTGATCACTCGAGTGATCACCGTTAACCATCATATTTTTG
	CCGGAAACAGCTGCCTTAGCTTCACTCGAGTGAAGCTAAGGCAGCTGTTTCTTTTTG
PPFIA2	CCGGGCTGAGAAGGATCGAAGACTACTCGAGTAGTCTTCGATCCTTCTCAGCTTTTT
	CCGGCCTACCACAATGATGCTCGAACTCGAGTTCGAGCATCATTGTGGTAGGTTTTT
	CCGGCCTCCATTACTGCCTCTGTTACTCGAGTAACAGAGGCAGTAATGGAGGTTTTT
	CCGGCTAAGAAGACGAGCAGTGAAACTCGAGTTTCACTGCTCGTCTTCTTAGTTTTT
PPFIBP1	CCGGCGGTTAGAGCAGATGGAAGATCTCGAGATCTTCCATCTGCTCTAACCGTTTTT
	CCGGCCAGAGTGTTTCCATTCATATCTCGAGATATGAATGGAAACACTCTGGTTTTT
	CCGGCCTCAATATAAGACCCAGTTTCTCGAGAAACTGGGTCTTATATTGAGGTTTTT
	CCGGGCGTGGATTGTTAGAGATGATCTCGAGATCATCTCTAACAATCCACGCTTTTT
	CCGGGCCAAAGTGAAGCCAAAGAAACTCGAGTTTCTTTGGCTTCACTTTGGCTTTTT
WT1	CCGGCACTCATTCAAGCATGAGGATCTCGAGATCCTCATGCTTGAATGAGTGTTTTTG
	CCGGATGAACTTAGGAGCCACCTTCTCGAGAAGGTGGCTCCTAAGTTCATCTTTTTG
	CCGGTATAAGTACTAGATGCATCACCTCGAGGTGATGCATCTAGTACTTATATTTTTG

Table 2.2. Oligonucleotide sequence for target-gene cDNA amplification by qPCR

Gene target	Primers (5´- 3´)
PTPN9	agctgaccccggaggag gacagccacattccaagaca
MAP3K2	cgtcagattctggagggtgt ggccccaaaatctcctagtt
MAPK9	tctttaccagatgctttgtgg gtcaaggatcttcagggtgc
RAD23B	atgcaggtcaccctgaagac gacctgctactggaaaggca
CIB2	accaggactgcaccttcttc tctggcatctggatgatgag
EZH2	aaaggatacagacagtgatagg cgagaatttgcttcagagga
PPFIBP1	acgtgtggatcactttgctg gtcaggggcaaattccaac
WT1	gaaatggacagaagggcaga cacatcctgaatgcctctga
STK24	aacaagaaatcacagtgctgagtc gcctccaccaagatattcca
SGK	tcggactctgcaaggagaac atacaagacagctcccaggc
GAPDH	ggtggtctcctctgacttcaaca gttgctgtagccaaattcgttgt

2.3.8. Immunoblotting

Intracellular Gag protein expression was evaluated by western blot. Briefly, cells were washed in ice-cold PBS and lysed in RIPA lysis buffer (50 mM Tris-Cl [pH=7,2], 150 mM NaCl, 1 % Triton X-100) for 30 min at 4 ^{o}C. Protein concentration was quantified by Bradford colorimetric assay (BioRad, CA, USA). Equal amounts of protein were analyzed by 12 % SDS-PAGE, transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK), blotted with anti-p24 primary antibody (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH , #530, from Dr. Susan Zolla-Pazner) and anti-GAPDH primary antibody (6C5; Santa Cruz Biotechnology, CA, USA), followed by incubation with HRP-conjugated secondary antibodies (BioRad) and developed using the ECL (GE Healthcare) or Femto (Pierce, IL, USA).

2.3.9. Assessment of cell viability

After 7 days of infection, shRNA clones viability was determined using the Cell Proliferation Reagent WST-1 (Roche) according to manufacturer's instructions.

2.3.10. Bioinformatic analysis

The protein network constructions and core analysis was generated through the use of ingenuity Pathway Analyses (IPA) software (Ingenuity Systems, Inc., CA, USA). The identified genes were uploaded into the IPA application and each gene was mapped to its corresponding gene object in the IPA Knowledge base. For network generation, our genes were overlaid onto a global molecular network developed from information contained in the IPA Knowledge Base. Networks were algorithmically generated based on their connectivity. Parameters were restricted for molecules and/or relationships from human species and direct and indirect relationships were taken into account. The core functional analysis identified the biological functions that were most significant to the data set. Fischer's exact test was used to calculate a score determining the likelihood that each biological function assigned to that data set was due to chance alone. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. The association significance between data set and the canonical pathway was measured by the ratio of the number of genes from the data set that mapped to the pathway divided by the total number of genes that mapped to the canonical pathway is displayed.

2.3.11. 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).

2.4. Results

2.4.1. 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 subset of the RNAi consortium (TRC) lentiviral library highly enriched for human kinases and phosphatases This library includes 2855 clones corresponding to 622 human kinase genes and 735 clones corresponding to 180 human phosphatase genes, together with 1693 clones corresponding to other human genes. As described in figure 2.1, shRNA encoding lentiviral particles were produced and used as a pool to transduce Jurkat cells at an MOI of 1 ($1x10^6$ TU/ml quantified by $p24^{CA}$ ELISA). Lentiviral library transduction was performed twice in $5x10^6$ cells to increase the odds of having all shRNAs transduced in our Jurkat population. Due to high transduction efficiency and the reduced number of individual clones in this library

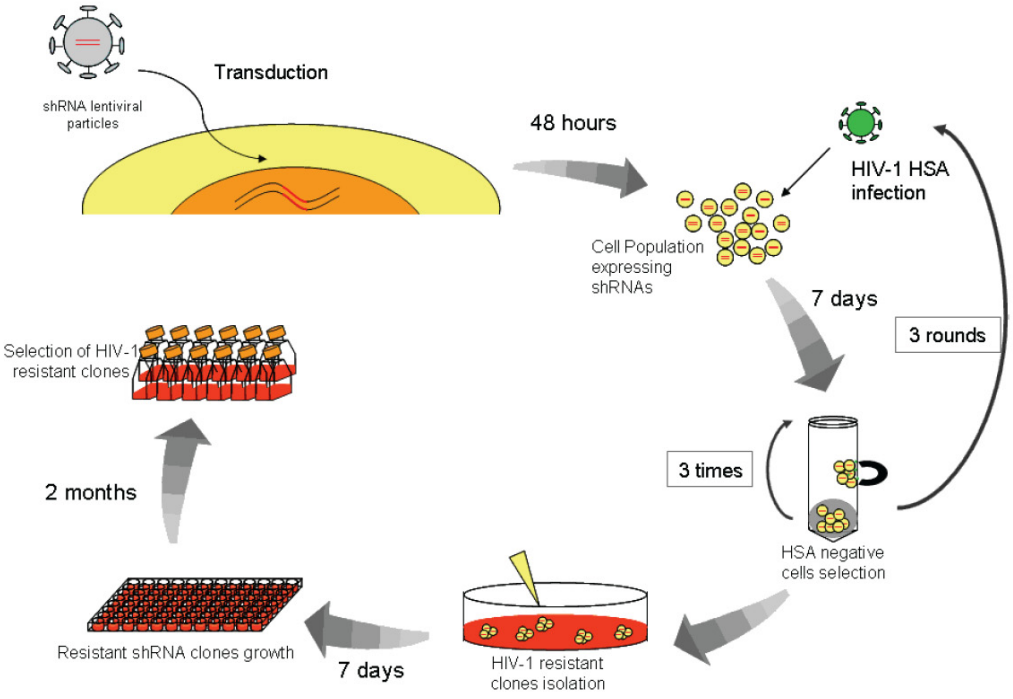

Figure 2.1. shRNA screen in Jurkat cells. Schematic representation of the shRNA screen. A pool of shRNA-encoding-lentiviral particles was used to transduce Jurkat cells and after 48 h they were challenged with HIV-HSA. After 7 days of infection, the shRNA transduced cells were negatively selected with magnetic beads conjugated with biotinylated anti-HSA. After 3 rounds of infection/selection, the HIV-1 resistant clones were recovered and isolated. Seven-hundred shRNA Jurkat clones were obtained, expanded and allowed to growth for 2 months to identify cellular proteins essential for HIV-1 replication in Jurkat cells but not essential for the cell viability. We obtained 180 viable shRNA clones.

compared to genome-wide representation, we found that the probability of all shRNA clones to be transduced is P(A') = 0,998. Subsequently, transduced cells were challenged with a replication competent HIV-1 encoding murine heat stable antigen (HSA) used as a cell surface marker to discriminate between infected and non-infected cells. After 7 days of HIV-1 infection, we selected shRNA-transduced cells HSA-surface-negative and potentially resistant to HIV-1 replication. This procedure was performed in 3 consecutive rounds to assure an enrichment of cells resistant to HIV-1 with the removal of HSA-expressing cells from the system. The remaining HSA-negative cells were cultured in the presence of puromycin to select expressing shRNA Jurkat cells. Following negative and puromycin selections, cells were individually cloned and expanded to evaluate resistance to HIV-1 infection.

We obtained 700 individual shRNA-transduced Jurkat cell clones resistant to HIV-1 replication. To identify kinases and phosphatases 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 period, the number of Jurkat shRNA clones that survived was reduced to 184, which may be due to cytotoxic effects resulting from gene knockdown in cells cultured for 60 days.

To further confirm that viable shRNA clones were resistant to HIV-1 replication, an infection assay was performed for each individual clone. All 184 shRNA clones were infected with HIV-1_{NL4-3} at a MOI of 1. After 7 days of infection, viral replication was measured by $p24^{CA}$ ELISA from supernatant of infected cultures and resistance to HIV-1 replication was determined. As shown in figure 2.2, the majority of shRNA clones were highly resistant to HIV-1 replication. Indeed, when compared to wild-type Jurkat cells, 136 out of 184 shRNA clones exhibited more than 80 % reduction in HIV-1 replication, indicating that our original shRNA screen was able to efficiently isolate T cells clones resistant to HIV-1 replication.

2.4.2. Identification of the shRNAs targets

To identify HIV-1 dependent host-factors targeted by the shRNA that were responsible for viral resistance, we selected 30 shRNA Jurkat T cell clones with highest resistance to HIV-1 replication and sought to identify their shRNA sequences after genomic DNA extraction and PCR amplification. Primers for PCR amplification and subsequently sequencing were specific for a common sequence of the pLKO.1 backbone present in the shRNA cassette integrated in

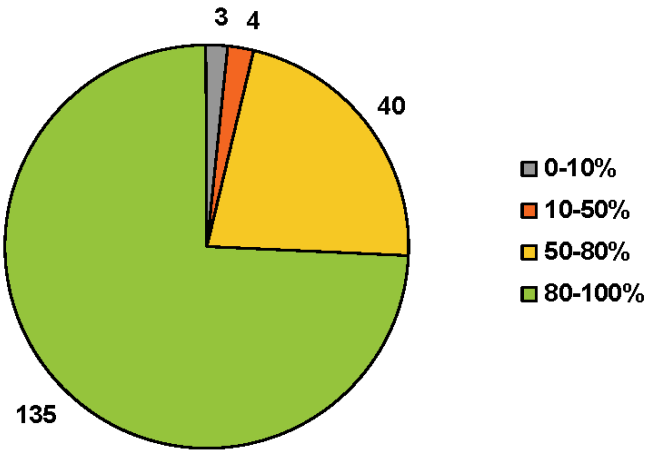

Figure 2.2. Resistance of shRNA clones to HIV-1 replication, measured by $p24^{CA}$ expression in the cell culture supernatant after 7 days of infection with HIV-1_{NL4-3} (MOI of 1). Percentage values are relative to Jurkat cells infected with HIV-$1_{NL4-3.}$ Values indicated in graph represent the number of clones isolated in each subgroup.

the shRNA clones genome (figure 2.3A). Results from sequence analysis allowed us to identify the different regions that compose a shRNA: the sense strand, loop, antisense strand and the terminal sequence (example of a sequence result is represented in figure 2.3B). From the sense strand of each sequence we performed a BLAST analysis (NCBI using the BLAST network service) to identify the target genes of each shRNA sequence. The 30 shRNA clones analyzed lead to the identification of 14 different gene targets, as described in table 2.3. From the 30 sequences we have identified cellular host-factors with only one shRNA gene-specific sequence and genes that were targeted by more than one shRNA (table 2.4). As shown in table 2.3, amongst the 14 host proteins identified as essential for HIV-1 replication we found 2 phosphatases, 5 kinases, 1 hypothetical kinase-binding-protein, 2 phosphatase-binding-proteins and 4 other proteins with various functions. By performing biochemical functional analysis with the IPA software (IPA–Ingenuity Systems) the identified proteins were clustered into groups algorithmically generated and based on their connectivity. From this analysis two networks were originated: Network1: Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry (figure 2.4); and Network 2: Cell Cycle, Cell Signaling, Cellular Growth and Proliferation (figure 2.5). The possible biological functions that our set of genes could perform are represented at figure 2.6. These functions are related to the networks described above being Amino Acid Metabolism, Post-Translational Modification

and Small Molecule Biochemistry the most predominant. We have also performed a Canonical pathways analysis to identify the canonical pathways from the IPA library that were most significant to our gene set. Several pathways were pulled out being JNK, MAPK, and extracellular signal-regulated kinase 5 (ERK5) signaling some of the most relevant (figure 2.7 and table 2.5).

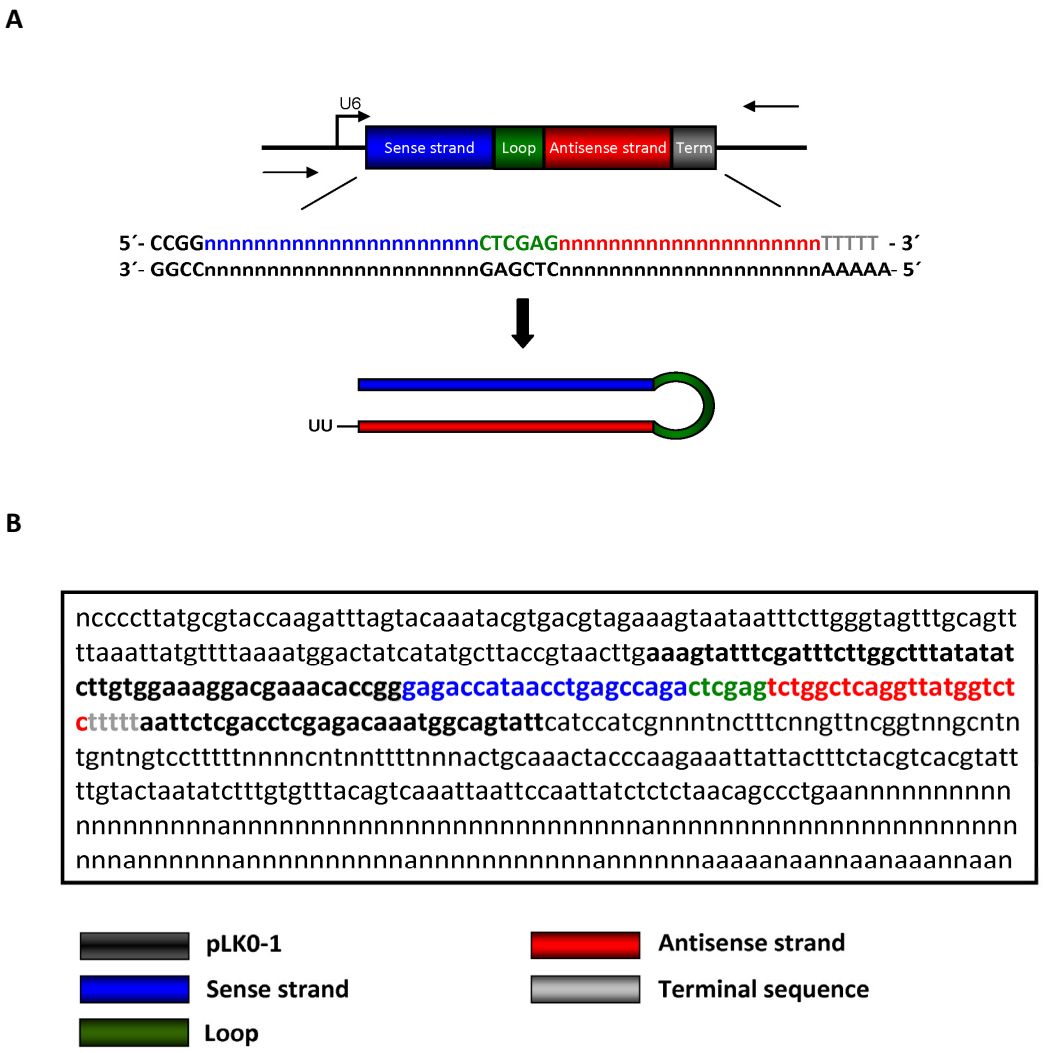

Figure 2.3. Representation of shRNA sequence cassette. A. Schematic representation of shRNA sequence in pLKO.1 and shRNA conformation after transcription. **B.** Example of shRNA cassette sequencing result where are indicated vector and shRNA sequence.

Table 2.3. Proteins identified in the shRNA screen important for HIV-1 replication

Gene symbol	Name	Functional category	# shRNA clones
PTPN9/PTP-MEG2	Protein tyrosine phosphatase, non-receptor type 9	Member of the protein tyrosine phosphatase (PTP) family	10
PTPRE	Protein tyrosine phosphatase, receptor type, E	Member of the protein tyrosine phosphatase (PTP) family	1
PRKD1/PKD/ PKC-MU	Protein kinase D1	Member of the protein kinase C (PKC) family Cytosolic serine/threonine kinase	2
MAP3K2/MEKK2	Mitogen-activated protein kinase kinase kinase 2	Serine/threonine protein kinase Member of MEK kinase family	1
MAPK9/JNK2	Mitogen-activated protein kinase 9	Member of the MAP kinase family	1
SGK/SGK1	Serum/glucocorticoid regulated kinase	Serine/threonine protein kinase	1
STK24/MST3	Serine/threonine kinase 24 (STE20 homolog, yeast)	Upstream of the mitogen-activated protein kinase (MAPK) cascade	1
CIB2	Calcium and integrin binding family member 2	Ca^{2+}-binding regulatory protein that potentially interacts with DNA-dependent protein kinase catalytic subunit (DNA-PKcs)	1
PPFIA2	Protein tyrosine phosphatase, receptor type, f polypeptide interacting protein (liprin), alpha 2	Member of the LAR protein-tyrosine phosphatase-interacting protein (liprin) family	1
PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	Member of the LAR protein-tyrosine phosphatase-interacting protein (liprin) family	1
RAD23B	RAD23 homolog B (*S. cerevisiae*)	Protein involved in the nucleotide excision repair (NER)	1
EZH2	Enhancer of zeste homolog 2	Member of the Polycomb-group	1
WT1	Wilms tumor 1	Transcription factor	3
ELA1/CELA1	Elastase 1, pancreatic	Serine protease	4

In this screen we have identified proteins other than kinases or phosphatases. This is due to the fact that we started with an enriched shRNA library that contained other target genes besides kinases and phosphatases. The significant number of non-kinase and non-phosphatase genes in our output compared with its percentage in the initial library could be justified by the strategy of long-term screening. After two months of selection with puromycin and infection with HIV-1, a large number of shRNA clones were not detected. These clones may constitute a mixture of shRNA that induced different levels of cytotoxicity in the cell. During more than 60 days in culture, viable shRNA clones may outgrowth other Jurkat T cell clones that showed decreased cytotoxicity over time.

Table 2.4. Sequences identified from the 30 clones analysed

Gene	shRNA Sequence
PTPN9/PTP-MEG2	gactatgttaatgccaggcaa
	gatatctaacagaggcttatt
	gctatcaaactgtgccttatt
PTPRE	tggacacatacaaggaacttt
PRKD1/PKD/ PKC-MU	cccacgctctctttgttcatt
MAP3K2/MEKK2	gcaacgtcaaactaggagatt
MAPK9/JNK2	agggattgtttgtgctgcatt
SGK/SGK1	gaaatgtacgacaacattctg
STK24/MST3	tggacagaaataagatgaaag
CIB2	cctccttcacaatgtgaagct
PPFIA2	caagactattactggagcatt
PPFIBP1	cctcaatataagacccagttt
RAD23B	agaagctggaactggtcatat
EZH2	gaaacagctgccttagcttca
WT1	tataagtactagatgcatcac
ELA1/CELA1	gagaccataacctgagccaga

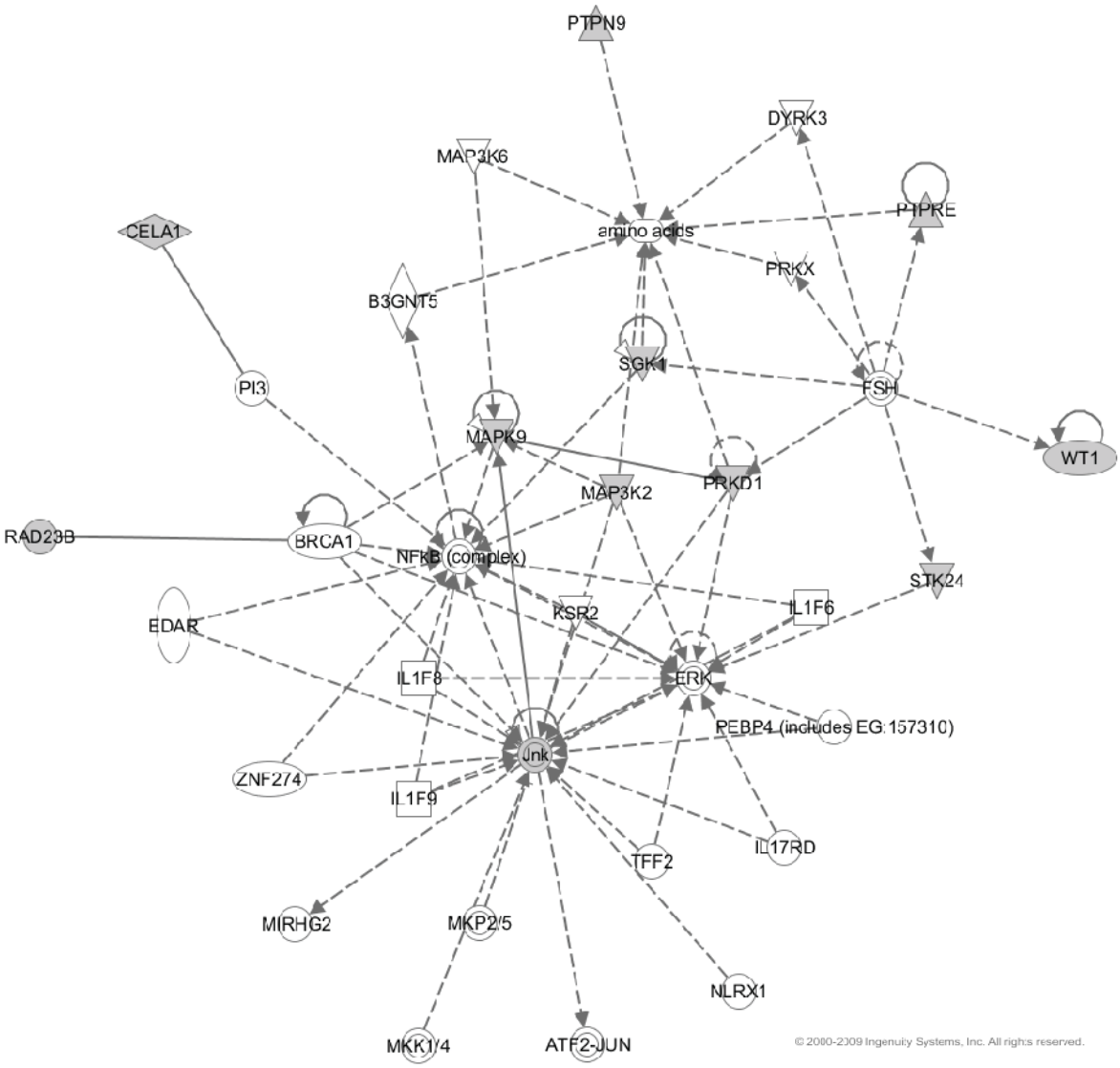

Figure 2.4. Biochemical relationships between identified proteins. Network 1 corresponds to Amino Acid Metabolism, Post-Translational Modification, and Small Molecule Biochemistry, and includes ELA1 (CELA1), MAP3K2, MAPK9, PRKD1, PTPN9, PTPRE, RAD23B, SGK1, STK24 and WT1. Core analysis was performed with Ingenuity Pathway Analyses (IPA) software (Ingenuity Systems, Inc., CA, USA) to analyse putative relationships between all genes identified in our shRNA screen. The analysis includes only molecules and/or relationships from human specie. Direct (continuous lines) and indirect (dashed lines) relationships are taken into account.

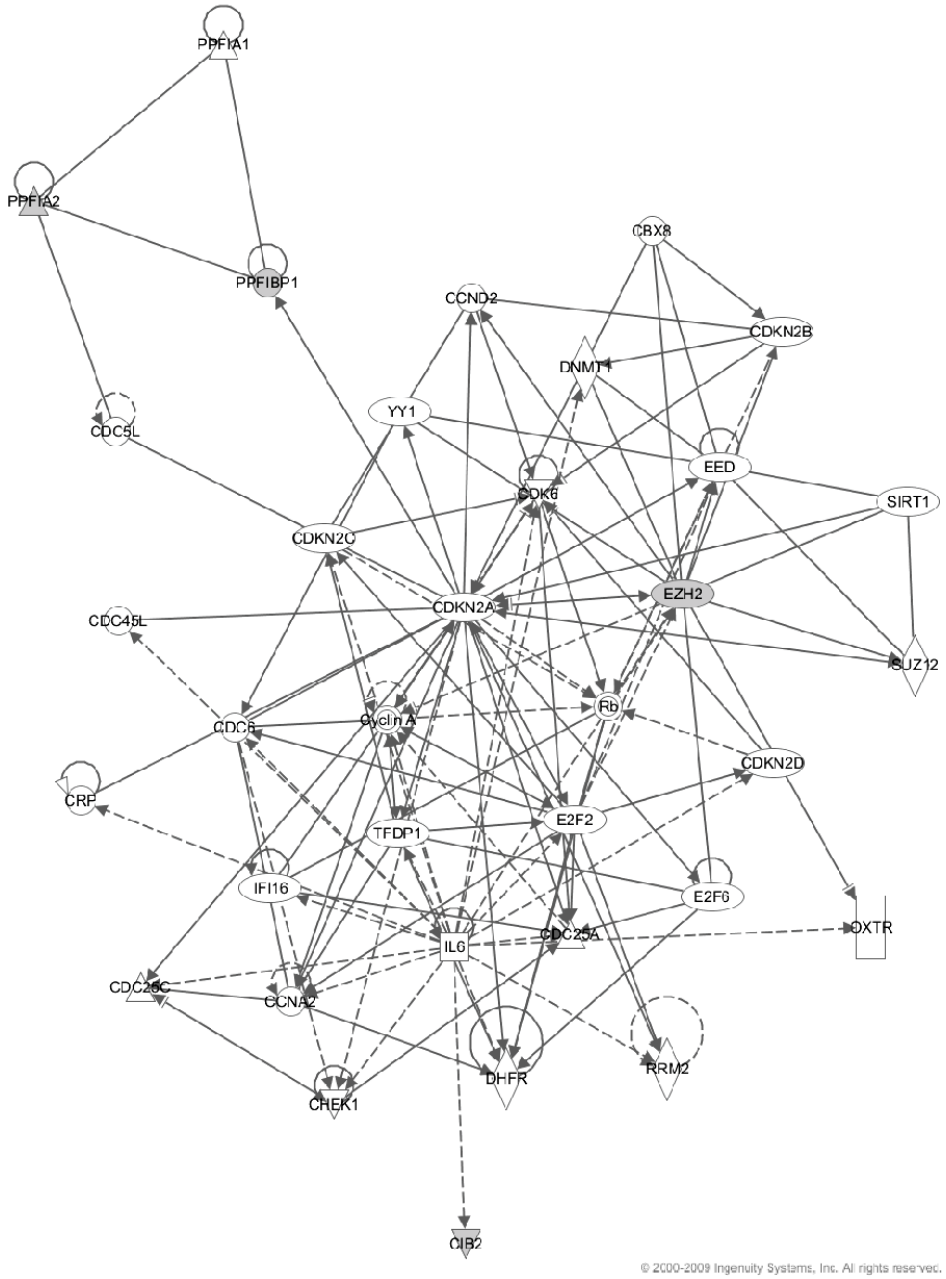

Figure 2.5. Biochemical relationships between identified proteins. Network 2: corresponds to Cell Cycle, Cell Signaling, Cellular Growth and Proliferation and includes CIB2, EZH2, PPFIA2 and PPFIBP1. Core analysis was performed with Ingenuity Pathway Analyses (IPA) software (Ingenuity Systems, Inc., CA, USA) to analyse putative relationships between all genes identified in our shRNA screen. The analysis includes only molecules and/or relationships from human specie. Direct (continuous lines) and indirect (dashed lines) relationships are taken into account.

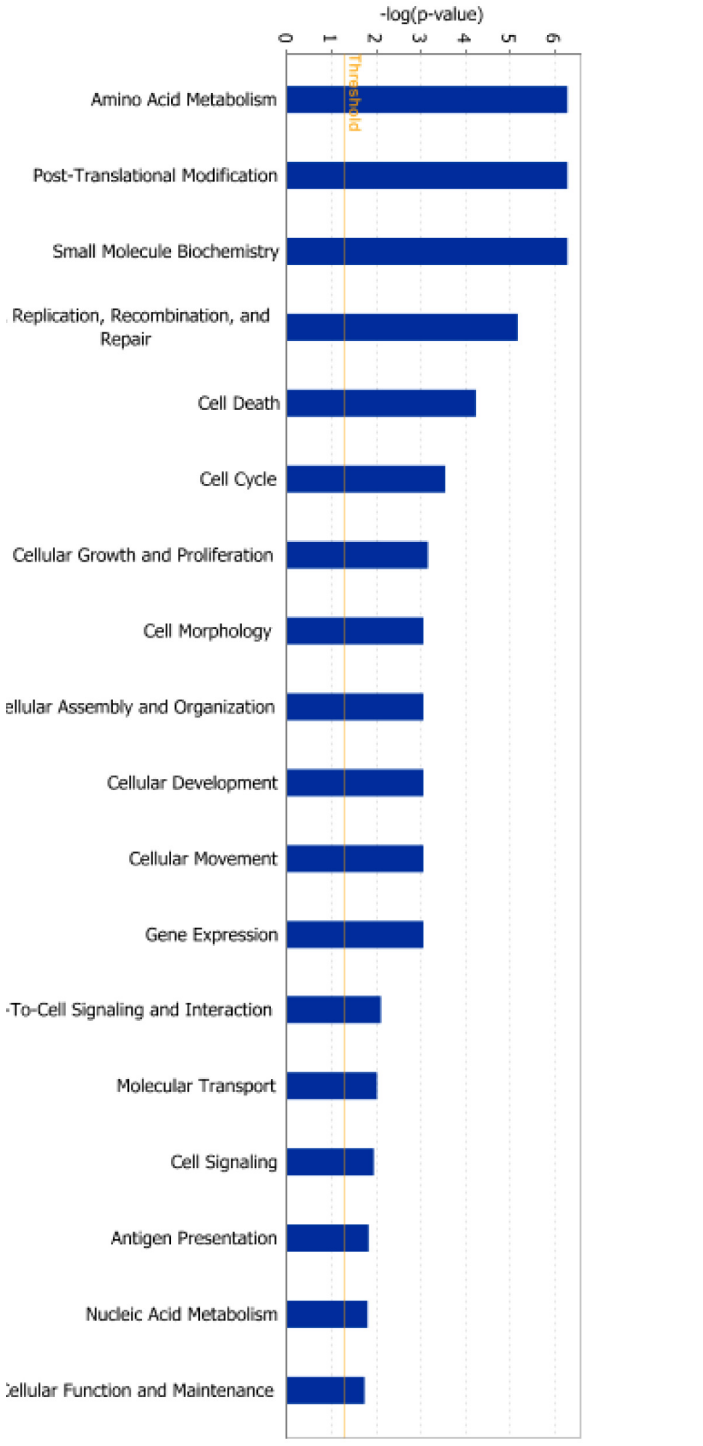

Figure 2.6. Molecular and cellular functions of identified genes. Bars indicate the representativeness of genes described in this study. Line represents ratio values between the genes present in each pathway and its representativeness in all canonical pathways. Threshold value is 0.05. Fisher's Exact Test-P value was performed with IPA software.

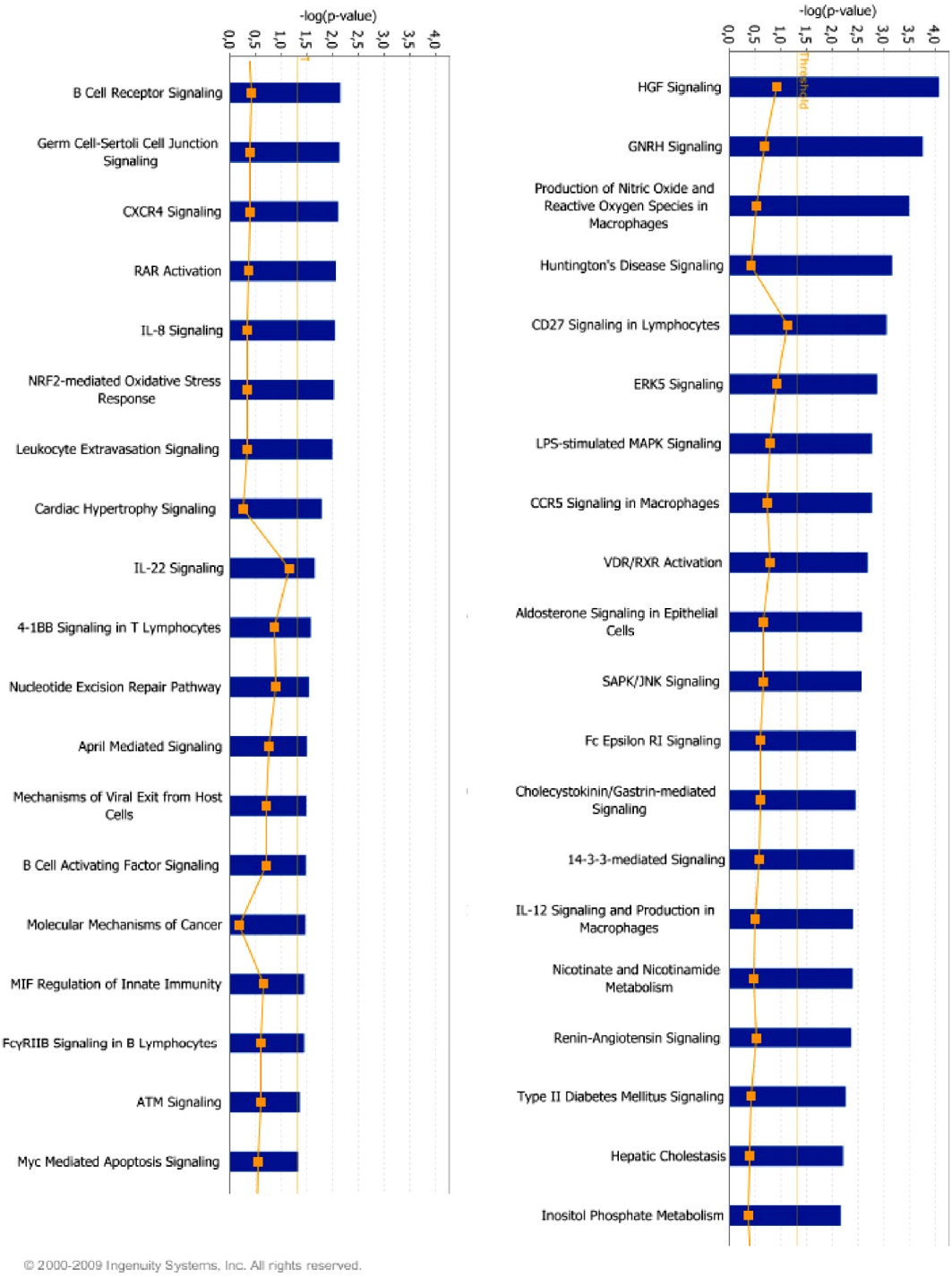

Figure 2.7. Representation of the different canonical pathways wherein the identified genes are present. Bars indicate representativeness in the canonical pathways of genes described in this study. Line represents ratio values between the genes present in each pathway and its representativeness in all canonical pathways. Threshold value is 0.05. Fisher's Exact Test-P value was performed with IPA software.

Table 2.5. Canonical Pathways involving identified genes.

Ingenuity Canonical Pathways	-Log(P-value)	Ratio	Molecules
HGF Signaling	4,11E00	2,88E-02	MAPK9, PRKD1, MAP3K2
GNRH Signaling	3,8E00	2,1E-02	MAPK9, PRKD1, MAP3K2
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3,53E00	1,61E-02	MAPK9, PRKD1, MAP3K2
Huntington's Disease Signaling	3,18E00	1,27E-02	SGK1, MAPK9, PRKD1
CD27 Signaling in Lymphocytes	3,08E00	3,51E-02	MAPK9, MAP3K2
Xenobiotic Metabolism Signaling	2,91E00	1,02E-02	MAPK9, PRKD1, MAP3K2
ERK5 Signaling	2,88E00	2,9E-02	SGK1, MAP3K2
LPS-stimulated MAPK Signaling	2,79E00	2,5E-02	MAPK9, PRKD1
CCR5 Signaling in Macrophages	2,78E00	2,2E-02	MAPK9, PRKD1
VDR/RXR Activation	2,71E00	2,5E-02	WT1, PRKD1
Aldosterone Signaling in Epithelial Cells	2,59E00	2,04E-02	SGK1, PRKD1
SAPK/JNK Signaling	2,58E00	2,04E-02	MAPK9, MAP3K2
Fc Epsilon RI Signaling	2,48E00	1,9E-02	MAPK9, PRKD1
Cholecystokinin/Gastrin-mediated Signaling	2,47E00	1,92E-02	MAPK9, PRKD1
14-3-3-mediated Signaling	2,43E00	1,75E-02	MAPK9, PRKD1
IL-12 Signaling and Production in Macrophages	2,41E00	1,52E-02	MAPK9, PRKD1
Nicotinate and Nicotinamide Metabolism	2,41E00	1,47E-02	SGK1, MAPK9
Renin-Angiotensin Signaling	2,39E00	1,67E-02	MAPK9, PRKD1
Type II Diabetes Mellitus Signaling	2,27E00	1,27E-02	MAPK9, PRKD1
Hepatic Cholestasis	2,21E00	1,21E-02	MAPK9, PRKD1
Inositol Phosphate Metabolism	2,18E00	1,14E-02	SGK1, MAPK9
B Cell Receptor Signaling	2,17E00	1,29E-02	MAPK9, MAP3K2
Germ Cell-Sertoli Cell Junction Signaling	2,16E00	1,24E-02	MAPK9, MAP3K2
CXCR4 Signaling	2,12E00	1,19E-02	MAPK9, PRKD1
RAR Activation	2,08E00	1,12E-02	MAPK9, PRKD1
IL-8 Signaling	2,06E00	1,07E-02	MAPK9, PRKD1
NRF2-mediated Oxidative Stress Response	2,04E00	1,08E-02	MAPK9, PRKD1
Endothelin-1 Signaling	2,03E00	1,08E-02	MAPK9, PRKD1
Leukocyte Extravasation Signaling	2,02E00	1,03E-02	MAPK9, PRKD1
Cardiac Hypertrophy Signaling	1,8E00	8,23E-03	MAPK9, MAP3K2
IL-22 Signaling	1,66E00	3,7E-02	MAPK9
4-1BB Signaling in T Lymphocytes	1,58E00	2,78E-02	MAPK9
Nucleotide Excision Repair Pathway	1,54E00	2,86E-02	RAD23B
April Mediated Signaling	1,51E00	2,38E-02	MAPK9
Mechanisms of Viral Exit from Host Cells	1,5E00	2,27E-02	PRKD1
B Cell Activating Factor Signaling	1,48E00	2,27E-02	MAPK9
Molecular Mechanisms of Cancer	1,48E00	5,38E-03	MAPK9, PRKD1
MIF Regulation of Innate Immunity	1,45E00	2,08E-02	MAPK9
FcγRIIB Signaling in B Lymphocytes	1,45E00	1,79E-02	MAPK9
ATM Signaling	1,37E00	1,92E-02	MAPK9
Myc Mediated Apoptosis Signaling	1,33E00	1,67E-02	MAPK9
Thrombopoietin Signaling	1,32E00	1,61E-02	PRKD1
Induction of Apoptosis by HIV1	1,31E00	1,54E-02	MAPK9
CD40 Signaling	1,29E00	1,45E-02	MAPK9

Table 2.5. Canonical Pathways involving identified genes (cont)

Ingenuity Canonical Pathways	-Log(P-value)	Ratio	Molecules
Calcium-induced T Lymphocyte Apoptosis	1,29E00	1,54E-02	PRKD1
Activation of IRF by Cytosolic Pattern Recognition Receptors	1,28E00	1,37E-02	MAPK9
Macropinocytosis Signaling	1,27E00	1,39E-02	PRKD1
Erythropoietin Signaling	1,25E00	1,32E-02	PRKD1
IL-3 Signaling	1,24E00	1,37E-02	PRKD1
Growth Hormone Signaling	1,24E00	1,37E-02	PRKD1
IL-17 Signaling	1,23E00	1,37E-02	MAPK9
Agrin Interactions at Neuromuscular Junction	1,23E00	1,39E-02	MAPK9
Caveolar-mediated Endocytosis Signaling	1,22E00	1,22E-02	MAP3K2
NF-κB Activation by Viruses	1,22E00	1,23E-02	PRKD1
Reelin Signaling in Neurons	1,22E00	1,3E-02	MAPK9
Prolactin Signaling	1,21E00	1,28E-02	PRKD1
BMP signaling pathway	1,21E00	1,25E-02	MAPK9
Melatonin Signaling	1,19E00	1,28E-02	PRKD1
TGF-β Signaling	1,18E00	1,16E-02	MAPK9
Factors Promoting Cardiogenesis in Vertebrates	1,17E00	1,12E-02	PRKD1
p53 Signaling	1,14E00	1,12E-02	WT1
IL-6 Signaling	1,13E00	1,05E-02	MAPK9
Virus Entry via Endocytic Pathways	1,13E00	1,04E-02	PRKD1
HMGB1 Signaling	1,13E00	1,02E-02	MAPK9
Neuregulin Signaling	1,12E00	1E-02	PRKD1
FXR/RXR Activation	1,11E00	1E-02	MAPK9
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1,11E00	9,62E-03	PRKD1
HIF1α Signaling	1,1E00	9,52E-03	MAPK9
G Beta Gamma Signaling	1,09E00	8,55E-03	PRKD1
α-Adrenergic Signaling	1,09E00	9,35E-03	PRKD1
Glioma Signaling	1,09E00	8,93E-03	PRKD1
Neuropathic Pain Signaling In Dorsal Horn Neurons	1,08E00	9,62E-03	PRKD1
Pancreatic Adenocarcinoma Signaling	1,07E00	8,62E-03	MAPK9
Type I Diabetes Mellitus Signaling	1,07E00	8,77E-03	MAPK9
Natural Killer Cell Signaling	1,05E00	8,7E-03	PRKD1
Gα12/13 Signaling	1,04E00	8,13E-03	MAPK9
Cdc42 Signaling	1,04E00	7,58E-03	MAPK9
fMLP Signaling in Neutrophils	1,04E00	7,87E-03	PRKD1
CD28 Signaling in T Helper Cells	1,03E00	8E-03	MAPK9
Synaptic Long Term Potentiation	1,03E00	8,7E-03	PRKD1
CCR3 Signaling in Eosinophils	1,03E00	8,2E-03	PRKD1
Androgen Signaling	1,02E00	6,9E-03	PRKD1
Corticotropin Releasing Hormone Signaling	1E00	7,35E-03	PRKD1
p70S6K Signaling	9,97E-01	7,63E-03	PRKD1
Insulin Receptor Signaling	9,74E-01	7,25E-03	SGK1
mTOR Signaling	9,47E-01	6,41E-03	PRKD1
Synaptic Long Term Depression	9,32E-01	6,1E-03	PRKD1
Mitochondrial Dysfunction	9,13E-01	5,81E-03	MAPK9
Dendritic Cell Maturation	9,13E-01	5,78E-03	MAPK9
Acute Phase Response Signaling	8,72E-01	5,65E-03	MAPK9
ILK Signaling	8,7E-01	5,38E-03	MAPK9

Table 2.5. Canonical Pathways involving identified genes (cont)

Ingenuity Canonical Pathways	-Log(P-value)	Ratio	Molecules
CREB Signaling in Neurons	8,63E-01	5,15E-03	PRKD1
Thrombin Signaling	8,25E-01	4,9E-03	PRKD1
LPS/IL-1 Mediated Inhibition of RXR Function	8,14E-01	4,88E-03	MAPK9
Colorectal Cancer Metastasis Signaling	7,51E-01	4,05E-03	MAPK9
Glucocorticoid Receptor Signaling	7,13E-01	3,57E-03	MAPK9
Axonal Guidance Signaling	5,72E-01	2,47E-03	PRKD1

Therefore, since kinases and phosphatases are involved in biochemical and cell-cycle pathways essential for proliferation and cell survival, the long-term selection method could have eliminated the less viable clones from the final output. In this study, we have evaluated all resultant genes including non-kinases and non-phosphatases, except for PTPRE that was not studied due to technical reasons.

2.4.3. Identified host-proteins are essential for HIV-1 replication

Next, we wanted to exclude the possibility that integration of shRNA lentiviral vector showed off-target effects and confirm the importance of the identified genes for HIV-1 replication. Thus, we recloned 3-to-5 shRNA for each host-protein identified in the previous assay, creating Jurkat T cell lines stably expressing the corresponding shRNA (each shRNA sequence is reported in table 2.1). The resistance of these shRNA clones to HIV-1 replication was evaluated by replication assays. shRNA clones were infected with HIV-1_{NL4-3} at MOI of 1 and after seven days of infection viral production was evaluated by quantification of $p24^{CA}$ (Figure 2.8). For each gene, we selected the two most efficient shRNA clones. To confirm effectiveness of the selected shRNA clones we performed a quantitative real-time PCR (qPCR) to assess mRNA downregulation for each target gene (figure 2.9). The real-time assays were performed for all genes in study. In the majority of the shRNA clones a denoting decrease is observed in gene-specific mRNA levels indicating a down-modulation of the genes by the shRNAs. However, for some shRNA clones the mRNA level reduction was not so evident. For ELA1 gene both shRNA clones showed a diminution in mRNA levels (figure 2.9E), but the replication assays indicate a strong reduction in HIV-1 replication. These results may indicate that a slight alteration in ELA1 mRNA levels reflects a considerable alteration in phenotype, as observed in figure 2.8E. For PPFIA2-2 and SGK-2 shRNAs, the results showed a minor

reduction in mRNA levels, although for PPFIA2-1 and SGK-1 shRNA the knockdown is stronger (figures 2.9I and 2.9M).

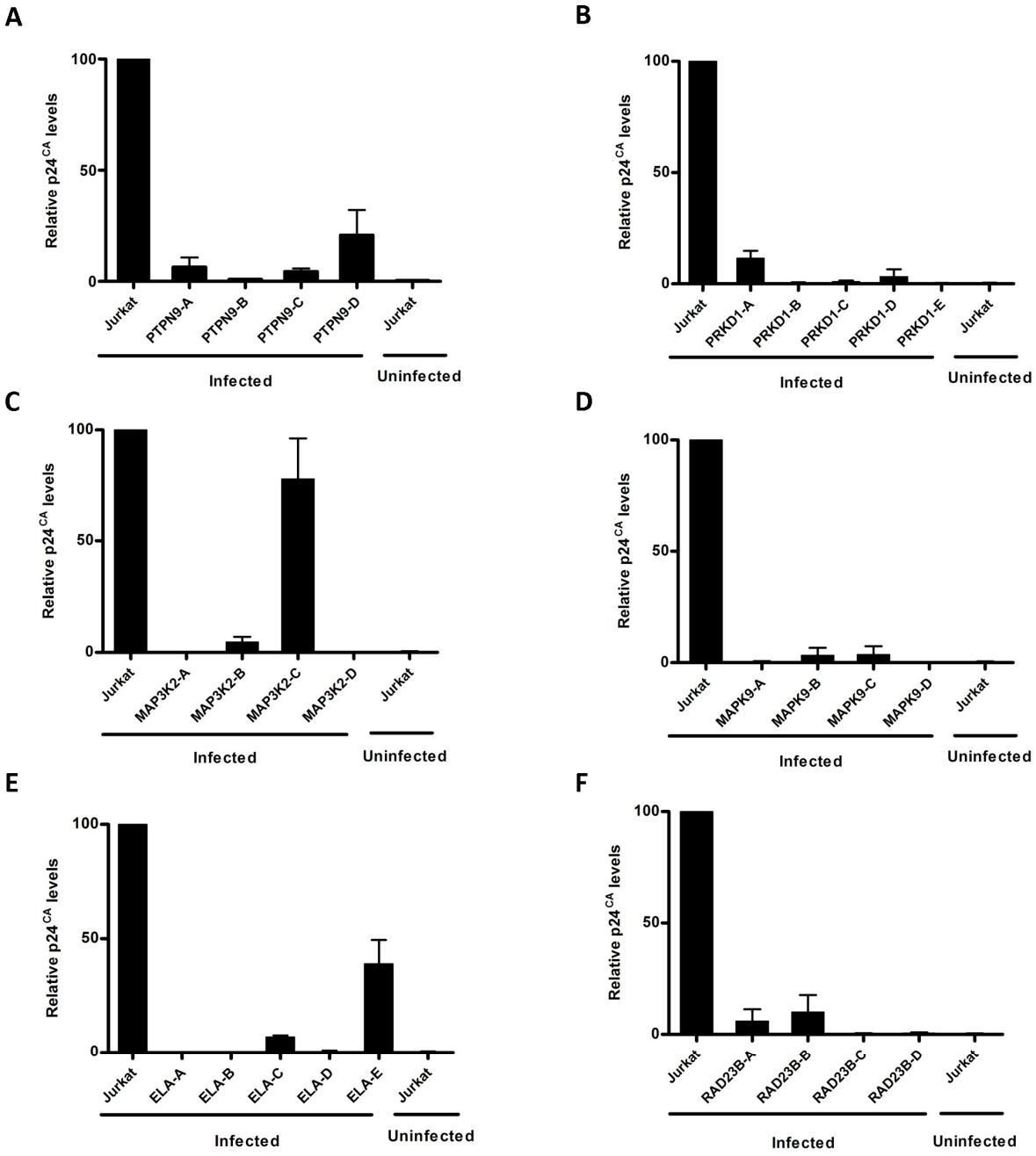

Figure 2.8. Resistance of shRNA Jurkat clones to HIV-1 replication (Continues next page).

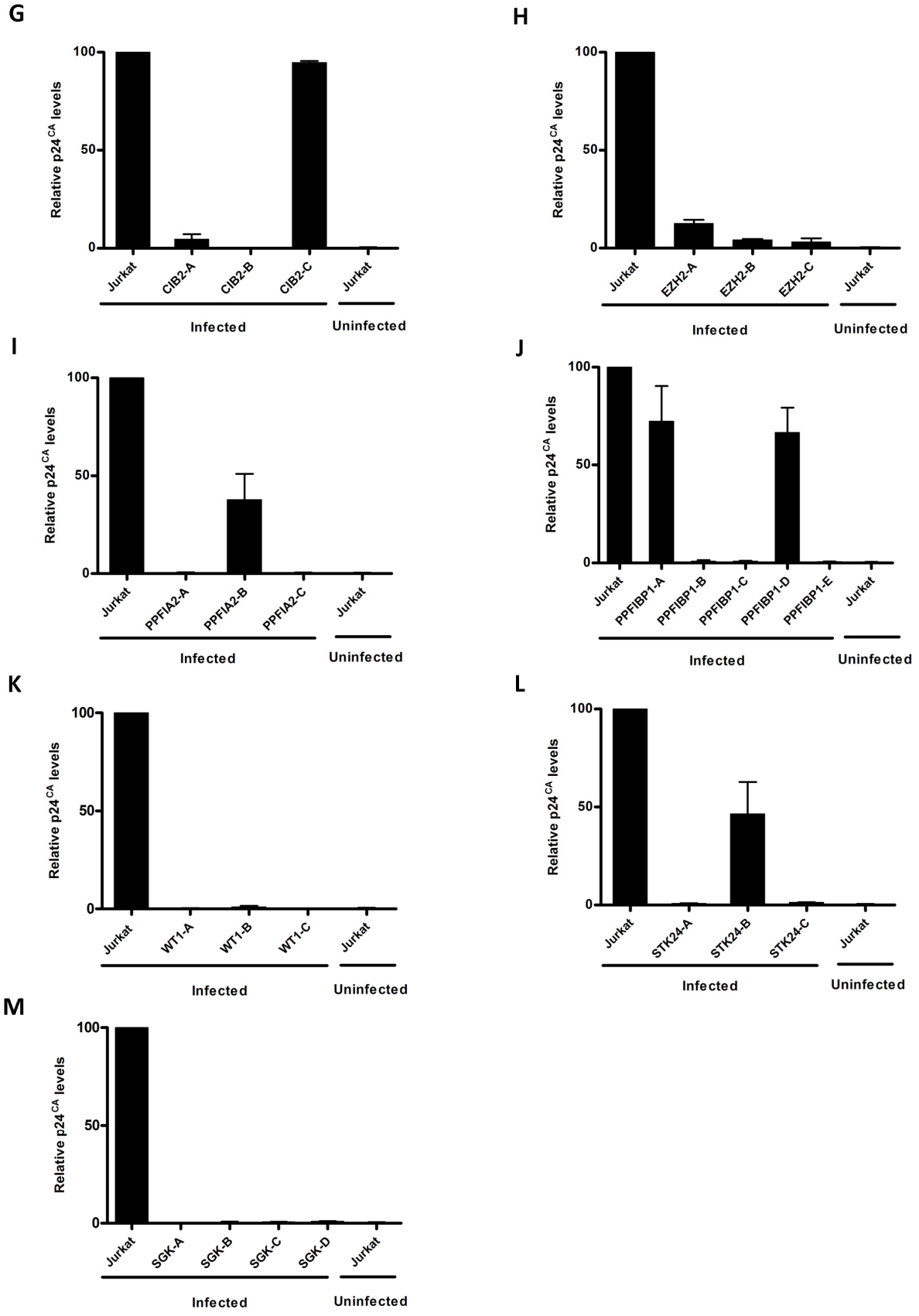

Figure 2.8. Resistance of shRNA Jurkat clones to HIV-1 replication (Legend next page).

Figure 2.8. Resistance of shRNA Jurkat clones to HIV-1 replication. To evaluate the effect of the different shRNAs to HIV-1 replication, shRNA clones were infected with HIV-1_{NL4-3} and after 7 days of infection viral replication was measured by $p24^{CA}$ ELISA. **A**. Evaluation of PTPN9 shRNA clones resistance to HIV-1 replication. shRNA clones PTPN9-B and PTPN9-C were selected to perform the subsequent studies and from than on designated as PTPN9-1 and PTPN9-2, respectively. **B**. Evaluation of PRKD1 shRNA clones resistance to HIV-1 replication. shRNA clones PRKD1-C and PRKD1-E were selected to perform the subsequent studies and from than on designated as PRKD1-1 and PRKD1-2, respectively. **C**. Evaluation of MAP3K2 shRNA clones resistance to HIV-1 replication. shRNA clones MAP3K2-A and MAP3K2-D were selected to perform the subsequent studies and from than on designated as MAP3K2-1 and MAP3K2-2, respectively. **D**. Evaluation of MAPK9 shRNA clones resistance to HIV-1 replication. shRNA clones MAPK9-A and MAPK9-D were selected to perform the subsequent studies and from than on designated as MAPK9-1 and MAPK9-2, respectively. **E**. Evaluation of ELA1 shRNA clones resistance to HIV-1 replication. shRNA clones ELA1-A and ELA-D were selected to perform the subsequent studies and from than on designated as ELA1-1 and ELA1-2, respectively. **F**. Evaluation of RAD23B shRNA clones resistance to HIV-1 replication. shRNA clones RAD23B-C and RAD23B-D were selected to perform the subsequent studies and from than on designated as RAD23B-1 and RAD23B-2, respectively. **G**. Evaluation of CIB2 shRNA clones resistance to HIV-1 replication. shRNA clones CIB2-A and CIB2-B were selected to perform the subsequent studies and from than on designated as CIB2-1 and CIB2-2, respectively. **H**. Evaluation of EZH2 shRNA clones resistance to HIV-1 replication. shRNA clones EZH2-A and EZH2-C were selected to perform the subsequent studies and from than on designated as EZH2-1 and EZH2-2, respectively. **I**. Evaluation of PPFIA2 shRNA clones resistance to HIV-1 replication. shRNA clones PPFIA2-A and PPFIA2-C were selected to perform the subsequent studies and from than on designated as PPFIA2-1 and PPFIA2-2, respectively. **J**. Evaluation of PPFIBP1 shRNA clones resistance to HIV-1 replication. shRNA clones PPFIBP1-C and PPFIBP1-E were selected to perform the subsequently studies and from than on designated as PPFIBP1-1 and PPFIBP1-2, respectively. **K**. Evaluation of WT1 shRNA clones resistance to HIV-1 replication. shRNA clones WT1-A and WT1-C were selected to perform the subsequent studies and from than on designated as WT1-1 and WT1-2 respectively. **L**. Evaluation of STK24 shRNA clones resistance to HIV-1 replication. shRNA clones STK24-A and STK24-C were selected to perform the subsequent studies and from than on designated as STK24-1 and STK24-2, respectively. **M**. Evaluation of SGK shRNA clones resistance to HIV-1 replication. shRNA clones SGK-C and SGK-D were selected to perform the subsequent studies and from than on designated as SGK-1 and SGK-2 respectively. All values are relative to infected Jurkat cells and represent mean $\pm$ SEM ($n \geq 3$).

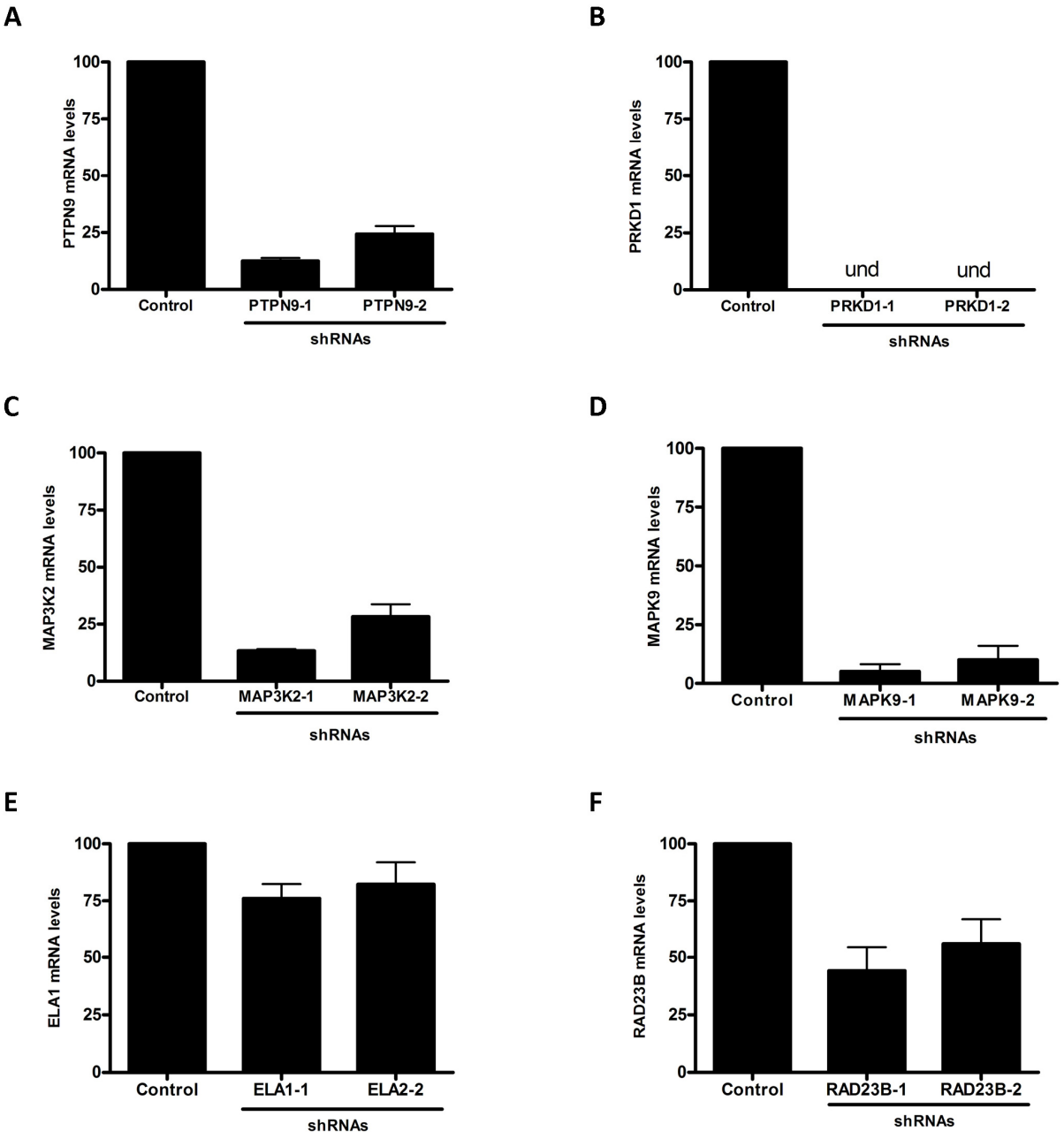

Figure 2.9. mRNA knockdown in individual clones stably expressing shRNA (Continues next page).

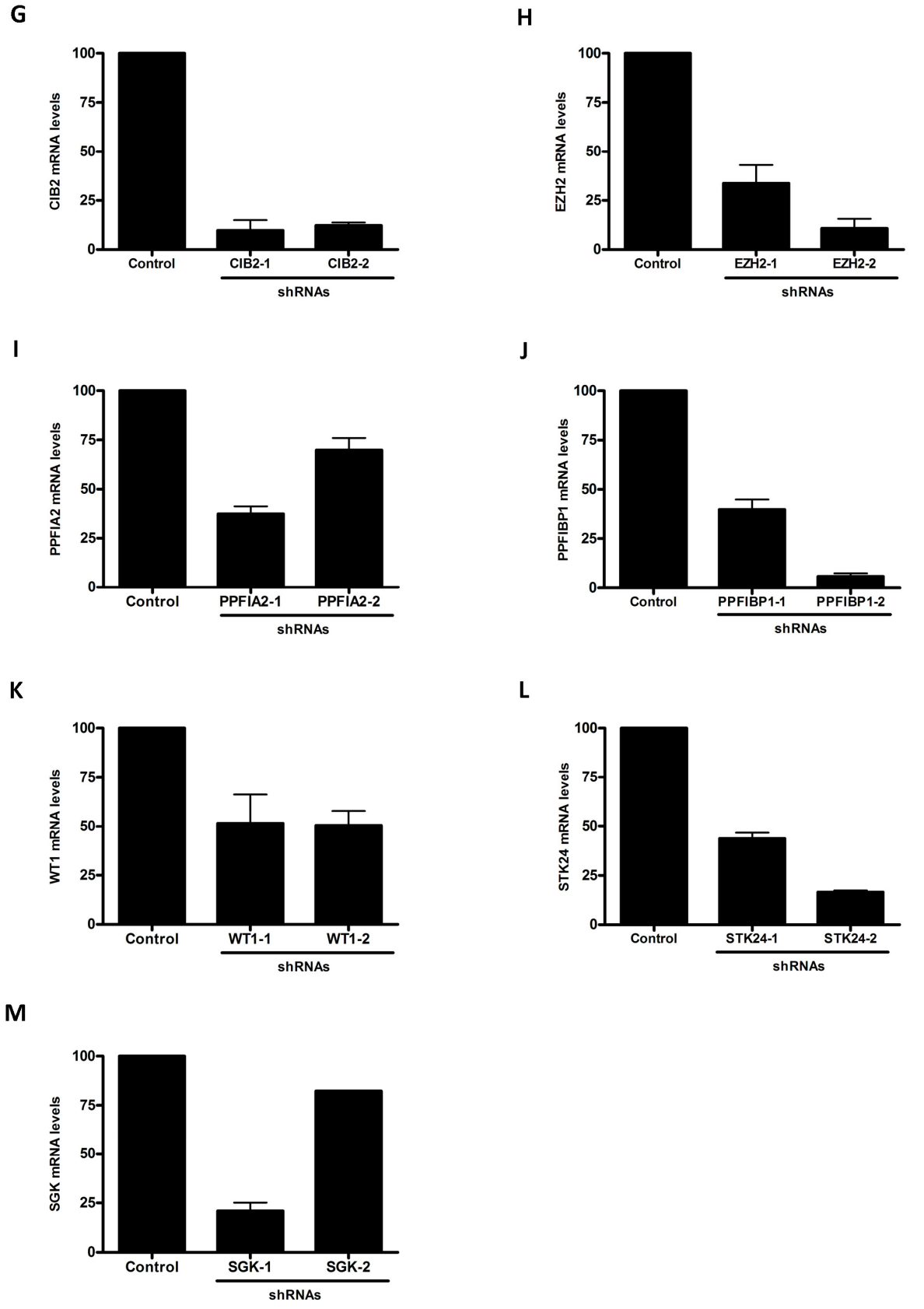

Figure 2.9. mRNA knockdown in individual clones stably expressing shRNA (Legend next page).

Figure 2.9. mRNA knockdown in individual clones stably expressing shRNA. After recloning shRNA in Jurkat cells, mRNA was extracted from the different shRNA clones and cDNA was purified for posterior quantification by real-time PCR. All graphs represent the two more efficient shRNAs for each gene and mRNA levels are relative to Jurkat cells (control). Values represent mean ± SEM. **A**. Reduction of mRNA levels in PTPN9 shRNA clones (n = 2). **B**. Reduction of mRNA levels in PRKD1 shRNA clones (n = 2). *und* indicate that mRNA levels for these gene were undetectable by qPCR assay. **C.** Reduction of mRNA levels in MAP3K2 shRNA clones (n = 2). **D.** Reduction of mRNA levels in MAPK9 shRNA clones (n = 3). **E**. Reduction of mRNA levels in ELA1 shRNA clones (n = 3). **F**. Reduction of mRNA levels in RAD23B shRNA clones (n = 3). **G**. Reduction of mRNA levels in CIB2 shRNA clones (n = 2). **H.** Reduction of mRNA levels in EZH2 shRNA clones (n = 3). **I.** Reduction of mRNA levels in PPFIA2 shRNA clones (n = 2). **J.** Reduction of mRNA levels in PPFIBP1 shRNA clones (n = 3). **K.** Reduction of mRNA levels in WT1 shRNA clones (n=3). **L.** Reduction of mRNA levels in STK24 shRNA clones (n = 2). **M.** Reduction of mRNA levels in SGK shRNA clones (n = 2).

The following studies assessing the inhibitory effect on HIV-1 replication were performed with the chosen shRNA clones. Subsequent to clone expansion, shRNA clones were infected with HIV-1_{NL4-3} at a MOI of 1 to determine the extent of viral replication in these cells. After 7 days of infection we determined viral replication by quantifying the amount of virion capsid protein $p24^{CA}$ in cell culture supernatant by ELISA. 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 showed in figure 2.10A, HIV-1 replication was strongly inhibited in all shRNA clones tested (over 80 % of inhibition), supporting the relevance of these host-proteins during HIV-1 replication. Importantly, inhibition of HIV-1 replication was not due to an outcome of decrease cell viability as all shRNA clones exhibited viability values similar to the control (Figure 2.10B).

To test if inhibition of HIV-1 replication was a result of lower viral expression or instead a reduction in viral release, we analyzed the intracellular gag gene expression of shRNA T-cell clones by western-blot. As shown in Figure 2.10C, after 7 days of infection with HIV-$1_{NL4-3,}$ neither $P55^{Gag}$ nor $p24^{CA}$ were detected in any of the shRNA Jurkat clones except for shSCRAM (control). These results corroborate the $p24^{CA}$ ELISA data and strengthen the important role of targeted host-proteins in a step(s) prior to virus expression. Therefore, this results validate the shRNA screen performed, verifying the importance of the identified proteins for HIV-1 replication.

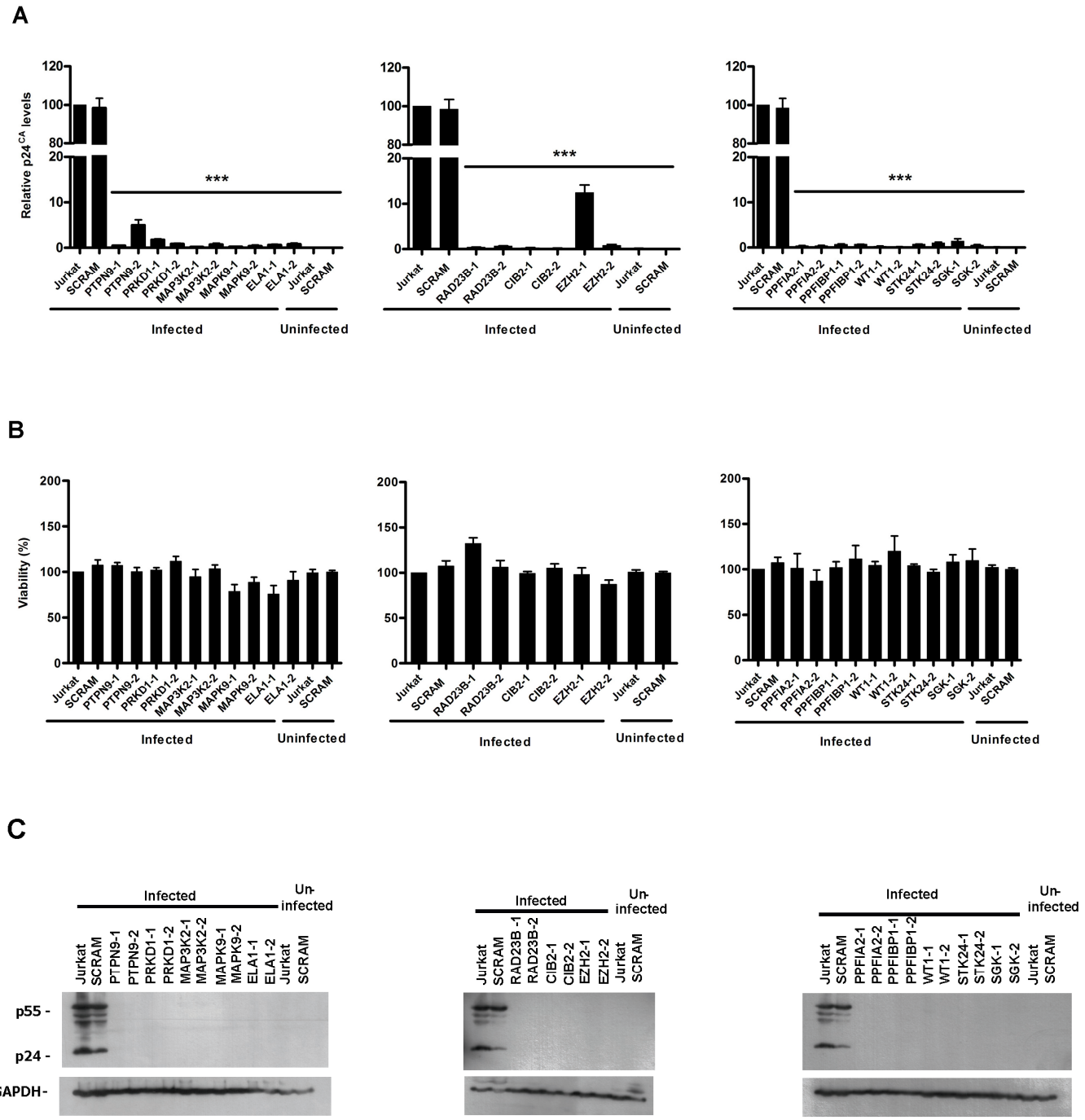

Figure 2.10. shRNA clones are resistant to HIV-1 replication. A. Two different shRNA clones for each target gene were infected with HIV-1NL4-3 (MOI of 1) and after 7 days of infection, HIV-1 replication was measured by $p24^{CA}$ levels in the cell culture supernatant. Values are relative to Jurkat cells infected with HIV-1_{NL4-3} and represent the mean ± SEM (*n* = 6). *** corresponds to $P < 0{,}0001$. **B.** Viability of shRNA clones after 7 days of infection with HIV-1_{NL4-3}. Values are relative to Jurkat cells infected with HIV-1NL4-3 and correspond to mean ± SEM (*n* = 4). **C.** Immunoblotting of intracellular Gag protein in different shRNA clones after 7 days of HIV-1_{NL4-3} infection (MOI of 1). This figure is representative of three independent experiments.

2.5. Discussion

Current anti-HIV therapies targeting viral proteins have significant constraints. One strategy to overcome current limitations is to target cellular proteins that are less variable than viral proteins. Kinases and phosphatases are key drug targets and subject of intense scrutiny due to their wide role in cell signalling and other biochemical activities. Hence, the aim of this study was to identify cellular proteins, in particular kinases and phosphatases that would be essential for HIV-1 replication but innocuous for the cell. Consequently, these proteins can potentially be used as antiviral targets. For this purpose, we explored a shRNA library enriched for all human kinases and phosphatases and performed an iterative shRNA screen in Jurkat T-cells. Previous studies have performed RNAi screens at a genome-wide-scale to identify possible host factors that could assist HIV-1 infection (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009). These genetic screens were all performed with the same goal but the resultant proteins did not overlap in great extent. These results can be justified by the different approaches chosen in each study. In the first three studies using siRNA libraries (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008), where the knockdown is transient, different host cell lines were used, different timings of siRNA treatment were employed, and different readout experiments were performed. In addition, in all studies different criteria were chosen for the bottlenecks after the primary screen which could have accentuated the differences in the final results (Goff, 2008). The more recent screen (Yeung *et al.*, 2009) used a different approach that is closest to the study described in this manuscript. The work of Kuan-Teh Jeang and co-workers performed a more extensive knockdown screen with a genome-wide shRNA library in Jurkat T cell line. This last work seems to be more advantageous compared to previous studies (Kok *et al.*, 2009). Despite the differences, all screens applied a broad approach with an extensive RNAi library that covers all human genes.
Our work focused on a more restricted strategy using a smaller library, enriched against kinases and phosphatases instead of a generalized genome-wide library. With this strategy not only we ensured that this druggable class of proteins was the major focus of the screening but also we bias it representation in the final results diminishing the probability of off-target proteins. Like Yeung *et al.* we decided to perform a screen with a shRNA library to constitutively knockdown the target genes and used Jurkat T cells to better mimic the HIV-1 natural host. The main differences between our study and Yeung *et al.* reside on the use of a

restricted shRNA library and a different screen platform. We selected HIV-1 resistant cells with a reduction in viral expression (reduced expression of HSA at cell surface) instead of cell survival after HIV-1 infection. With this strategy we assure that HIV-1 resistant Jurkat cells express shRNA that knockdown cellular proteins directly contributing to productive HIV-1 replication. Instead, selection by cell survival can recover shRNA that may interfere with apoptosis, or other mechanisms that promote cell viability. These differences are expected to complement and improve the goal of discovering novel knockdown targets for HIV-1 that can be used as antiviral targets. With this work we identified 14 different proteins that strongly assist HIV-1 replication. They are PTPN9, PTPRE, PRKD1, MAP3K2, MAPK9, SGK, STK24, CIB2, PPFIA2, PPFIBP1, RAD23B, EZH2, WT1 and ELA1.

None of the 14 proteins identified in this study have been previously reported as directly involved in HIV-1 replication. Although several kinases and phosphatases have been described to contribute to productive HIV-1 replication (Nekhai *et al.*, 2007; Chugh *et al.*, 2008; Dayton, 2008), we have not selected them in this screening. Moreover, even when we introduced a bias for selecting kinases and phosphatases, only 57 % of resulting proteins belong to this class, when initially 68 % were present in the library. This might have occurred due to cell cytotoxicity during the period of more than two months in culture when these proteins were knocked down by shRNA. The relative weak viability of this knockdown cells could have been outcompeted by other shRNA clones against proteins which function was less cytotoxic for the cell. This explanation is particularly important for kinases and phosphatases since these classes of proteins are crucial in processes of cell death and survival. Therefore, interfering with HIV-1 replication by knockdown kinases/phosphatases function can have deleterious effects on cell viability particularly when important kinases are targeted.

Nevertheless, when we overlap our screen with the previous studies (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009), we observed that two proteins were already present in early screens. PTPN9 was identified by Konig *et al.* and CIB2 was reported by Zhou *et al.* Although, when these authors used more restricted conditions in subsequent screens, these proteins were not identified. The different selection strategies used between these studies and ours can justify this incongruity. Moreover, when we searched our proteins in the NIAID HIV Protein Interaction Database (Fu *et al.*, 2009) two other important proteins emerge from our results, MAPK9 and PRKD1. In addition, a recent meta-analysis study where

several reports of cellular proteins essential for HIV-1 replication and protein databases were intersected, identifies CIB2 as a potential druggable protein important for viral replication (Bushman *et al.*, 2009). Importantly, in a core analysis to our set of genes aiming to evaluate their biochemical relationships and possible function in the cell, we observed that the more represented canonical pathways in our screen (figure 2.7 and table 2.4), are also clearly represented in the previous studies (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009), indicating a possible overlap of signal pathways instead of a direct overlap of genes.

Even though our shRNA screen in Jurkat cells did not select highly-known HIV-1 helper-factors, the links described above validate the strategy presented in this study as efficient to identify important helper factors that assist HIV-1 replication. As mentioned above, the 14 proteins that we have identified have not yet been described as directly involved in HIV-1 replication. Nevertheless, the putative involvement of these proteins into different cellular pathways could lead to a better knowledge of their function during HIV-1 life cycle (Table 2.6). Their role in cell host can give us hypothesis for hypothetical functions of these proteins in HIV-1 infection.

The identification of these 14 proteins essential for HIV-1 replication opens perspectives for new research. One of the rising questions is the mechanism of action of these proteins on the HIV-1 life cycle and if they interact with viral proteins. Therefore, replication studies have to be made to uncover more about these proteins and to understand its importance in HIV-1 replication.

This work with recent RNAi technology had improved our knowledge about HIV-1 replication and the interplay between the virus and the host. Furthermore the identification of essential proteins in HIV replication, which are not so well known in cell, can increase the knowledge for these proteins in the host and also can give us insights for new antiviral strategies based in cellular proteins that are innocuous for the cell.

Table 2.6. Genes identified in this screen as important for HIV-1 replication

Symbol	Synonyms	Name	Gene ID	Subcellular Localization	Molecular class	Molecular function	Biological Importance	References
PTPN9	MEG2 ; PTPMEG2	Protein Tyrosine Phosphatase, non-receptor type 9	NM_002833	Cytoplasm	Tyrosine phosphatase.	Protein tyrosine phosphatase activity	Involved in intracellular traffic of secretory pathways and regulation of vesicular fusion	Huynh *et al.*, 2004; Saito *et al.*, 2007
PTPRE	PTPE; HPTPE; FLJ57799; FLJ58245; DKFZp313F1310; R-PTP-EPSILON	PTPRE protein tyrosine phosphatase, receptor type, E	NM_006504	Plasma membrane/cytoplasm	Receptor tyrosine phosphatase	Receptor signaling protein tyrosine phosphatase activity	Negative regulator of cell proliferation in endothelial cells, of Erk and MAPK pathway in fibroblasts and of Jak-STAT singnalling in M1 myeloid cells	Thompson *et al.*, 2001; Toledano-Katchalski *et al.*, 2003 Tanuma *et al.*, 2000
PRKD1	PKD; PKCM; PRKCM; PKC-MU	Protein kinase D1	NM_002742	Plasma membrane/cytoplasm/Golgi apparatus/Mitochondrion	Serine/threonine kinase. Member of the protein kinase C (PKC) family	Protein serine/threonine kinase activity	Targeted by DAG that regulates PRKD1 intracellular localization. Activated by PKC. Involved in cell growth, gene expression, survival, motility, protein trafficking and lymphocyte biology	Wang, 2006
MAP3K2	MEKK2; MEKK2B	Mitogen-activated protein kinase kinase kinase 2	NM_006609	Cytoplasm/nucleus	Serine/threonine kinase. MEK kinase family	Protein serine/threonine kinase activity	MAPK pathway. Leads to ERK1/2, JNK, p38MAPK and ERK5 activation	Chen *et al.*, 2001
MAPK9	JNK2; SAPK; p54a; JNK2A; JNK2B; PRKM9; JNK-55; JNK2BETA; p54aSAPK; JNK2ALPHA;	Mitogen-activated protein kinase 9	NM_139069	Cytoplasm/nucleus	Serine/threonine kinase. MAPK family	Protein serine/threonine kinase activity	MAPK cascade. With a controversial role: Studies indicate that is a negative player in cell proliferation; others refute, presenting it as a positive regulator of c-Jun.	Chen *et al.*, 2001; Sabapathy and Wagner, 2004; Jaeschke *et al.*, 2006
SGK	SGK1	serum/glucocorticoid regulated kinase 1	NM_005627	Cytoplasm/Endoplasmic Reticulum/nucleus	Serine/threonine kinase	Protein serine/threonine kinase activity	Like PKB, SGK is activated by phosphorilation in response to signals that stimulate PI3K, and this is mediated by PKD1 and other unknown kinases. Various proteins were identified as SGK substrates such as GSK3, the transcription factor FOXO3, Raf kinase, the MEKK319 and recently NF-kB20.	Loffing *et al.*, 2006; Tai *et al.*, 2009
STK24	MST3; STK3; MST-3; MST3B; STE20	Serine/threonine kinase 24 (STE20 homolog, yeast)	NM_003576	Cytoplasm/nucleus	Serine/threonine kinase	Protein serine/threonine kinase activity	Regulation of cell cycle by phosphorilation and subsequent activation of NDR protein, leading to NDR pathway activation which control cell shape and cell cycle	Stegert *et al.*, 2005
CIB2	KIP2	Calcium and integrin binding family member 2	NM_006383	Cytoplasm/nucleus (?)	Calcium binding protein	Calcium ion binding	Protein with unknown function. Isolog of KIP/CIB gene (DNA-PKcs interacting protein)	Seki *et al.*, 1999
PPFIA2	FLJ41378; MGC132572	Protein tyrosine phosphatase, receptor type, f polypeptide interacting protein (liprin), alpha 2	NM_003625	Plasma membrane/Cytoplasm	Anchor protein	Cytoskeletal anchoring activity	Alter PTPRF cellular localization and induces clustering	Serra-Pages *et al.*, 1998
PPFIBP1	L2; hSGT2; hSgt2p	PTPRF interacting protein, binding protein 1 (liprin beta 1)	NM_003622	Plasma membrane/Cytoplasm	Anchor protein	Cytoskeletal anchoring activity	Role in tumorigenesis	Kriajevska *et al.*, 2002
RAD23B	P58; HR23B; HHR23B; RAD23B	RAD23 homolog B (S. cerevisiae)	NM_002874	Cytoplasm/nucleus	DNA repair protein	DNA binding	RAD23A homologous. Has a function in DNA repair (NER pathway) and protein stability by ubiquitin-proteosome system regulation.	Sweder and Madura, 2002; van Hoffen *et al.*, 2003
EZH2	ENX1; EZH1; KMT6; ENX-1; MGC9169	Enhancer of zeste homolog 2	NM_004456	Nucleus	Transcript on regulatory protein. Member of the Polycomb-group (PcG) family	DNA binding	Involved in maintaining the transcriptional repressive state of human genes by DNA methylation over sucessive cell generations to assure cellular identity	Vire *et al.*, 2006
WT1	GUD; WAGR; WT33; WIT-2	Wilms tumor 1	NM_024424	Nucleus	Transcription factor	Transcription regulator activity	Involved in: regulation of cell proliferation ; regulation of gene expression, epigenetic ; cell differentiation ; hemopoiesis	Yang *et al.*, 2007; Morrison *et al.*, 2008
ELA1	CELA1	Elastase 1, pancreatic	NM_001971	Extracellular region/Endoplasmic reticulum/Cytoplasmic vesicle Secretory granule	Serine Protease	Serine-type peptidase activity	Hydrolyses many other protein than Elastase. Seems to activate -kB, AP-1, and NFAT in human myeloid cell.	Hietaranta *et al.*, 2004

2.6. Acknowledgments

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CHAPTER 3

Discovery of Novel Helper Factors essential for HIV-1 entry and/or HIV-LTR driven Transcription

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3.1. Abstract

The complex interplay between HIV-1 and its host cell is reflected in each step of HIV-1 life cycle. HIV-1 to replicate needs both viral and cellular proteins. Co-factors have emerged as essential apparatus for HIV-1 and seem to be hijacked by the virus. Many cellular factors have already been discovery to help HIV-1 but an enormous number is yet to be revealed. Previously we have developed an iterative shRNA screen to identify cellular proteins, mainly kinases and phosphatases, which could be important for HIV-1 infection. We have discovered 14 different proteins that were proven to be essential for HIV-1 replication.

In this study we evaluated the role of each protein in the major steps of HIV-1 life cycle. We showed that the proteins under study are important in an early step of HIV-1 infection before viral integration whereas some of them also affect viral transcription/translation.

This study brings knowledge for the intrinsic relationship between the virus and its host, revealing new co-factors proteins and their function in the HIV-1 life cycle that can give new insights for the development of new antiviral strategies.

3.2. Introduction

For an efficient infection, HIV-1 has to complete its life cycle. Its journey is initiated with the attachment and fusion of the viral glycoproteins with the cell surface, followed by uncoating, reverse transcription of the RNA genome and nuclear entry of the pre-integration complex. In the nucleus, viral DNA is integrated into the host chromosome, transcribed into viral transcripts that are translocated from nucleus. In the cytoplasm, viral proteins are produced and assembled with viral RNA genome to generate viral particles that bud from the cell and mature in infectious particles (reviewed in Sierra *et al.*, 2005). For each of these steps to successfully occur, several viral and host proteins have to operate. In fact, a dynamic interaction between the virus and the host machinery is observed, being cellular proteins hijacked by the virus to act as co-factor during HIV-1 replication (Sorin and Kalpana, 2006). The identification of cellular proteins that help HIV-1 to complete its replication cycle can helps us to understand the pathogenesis of the virus and ultimately can lead us to the development of new antiviral strategies to combat the virus (Greene and Peterlin, 2002).

Much progress has been made in recent years to investigate the interplay between HIV-1 and its host cells. Several proteins have been identified as important for HIV-1 infection and much effort is being done to characterize its function in the HIV-1 replication context (Trkola, 2004). Nevertheless, our current knowledge on HIV-1-host interplay indicates that much is yet to be revealed. Recently with the development of RNAi technology, several genome wide screens were performed to identify cellular proteins that are required for HIV-1 infection (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009). From these screens hundreds of cellular proteins were pulled out. Our group has also performed an RNAi based screen to identify important proteins for HIV-1 replication. Based on a shRNA library against mainly kinases and phosphatases, we performed an iterative screen and identified and validated 14 different proteins essential for HIV-1 replication. In this group there were 2 phosphatases, 5 kinases, 1 hypothetical kinase-binding-protein, 2 phosphatase-binding-proteins and 4 other proteins with various functions.

In the present work we focused in the assessment of where in the HIV-1-life cycle these proteins could be involved. Early steps of HIV-1 replication, integration and LTR transcription were evaluated. We concluded that all studied proteins have an early function in HIV-1 replication and some were demonstrated so also be important for HIV-1 LTR driven

transcription. None of these proteins seemed to have a function in proviral integration. The identification and characterization of these co-factors during HIV-1 replication opens new perspectives for the complex interplay between HIV-1 and its host. Mainly, the identification and characterization of kinases and phosphatases lead to future studies for understanding signal pathways that can be triggered by virus. This work gives insights for further investigations and to the development of novel therapeutic strategies having cellular proteins as its throw weapon.

3.3. Material and Methods

3.3.1. Cell lines and culture conditions

Jurkat E6-1 T-cells were obtained through the NIH AIDS Research and Reference Reagent Program (MD, USA, contributor Dr. Arthur Weiss) and were cultured in RPMI-1640, supplemented with 10 % FBS (RPMI-10). HEK293T (ATCC, VA, USA) and HeLa-P4 cells (HeLa-CD4-LTR-β-gal, AIDS Reagent, MD, USA, contributor Dr. Richard Axel) were cultured in DMEM supplemented with 10% FBS (DMEM-10). Jurkat cells expressing shRNA (shRNA clones) were cultured in RPMI-10 supplemented with 2 μg/ml of puromycin (Sigma, MO, USA). All cell cultures were maintained at 37 °C in 5 % CO_2. All cell culture media and reagents, otherwise indicated, were from Lonza (Basel, Switzerland).

3.3.2. Viral production

HEK293T cells were transfected, by calcium phosphate method, pHIV-1_{NL4-3} plasmids (AIDS reagent, contributor Dr. Malcolm Martin) or with pNL4-3-r-HSAS (AIDS reagent, contributors Drs. Beth Jamieson and Jerome Zack) to produce HIV-1_{NL4-3} or HIV-HSA virions, respectively. After 48 h, virions were collected from supernatant cultures, measured by $p24^{CA}$ ELISA (AIDS & Cancer Research Program, NCI Frederick, MD, USA) and used to infect Jurkat cells.

3.3.3. Infection assays

Jurkat cells and individual cell clones were infected with HIV-HSA or HIV-1_{NL4-3} at the indicated MOI. For this purpose, cells were ressuspended in a viral preparation and subject to spinoculation (as described in O'Doherty *et al.*, 2000). After 6 h, cells were washed in PBS (1 x) 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.

3.3.4. Immunoblotting

Detection of Vif protein expression intracellularly was evaluated by western blot. After 48 h of infection with HIV-1_{NL4-3} cells were collected and cell extracted were performed. Briefly, cells

were washed in ice-cold PBS and lysed in RIPA lysis buffer (50 mM Tris-Cl [pH=7,2], 150 mM NaCl, 1 % Triton X-100) for 30 min at 4 ^{o}C. Protein concentration was quantified by Bradford colorimetric assay (BioRad, CA, USA). Equal amounts of protein were analyzed by 12 % SDS-PAGE, transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK), blotted with anti-Vif primary antibody (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH , #2221, from Dr. Dana Gabuzda) and anti-GAPDH primary antibody (6C5; Santa Cruz Biotechnology, CA, USA), followed by incubation with HRP-conjugated secondary antibodies (BioRad) and developed using the ECL (GE Healthcare) or Femto (Pierce, IL, USA).

3.3.5. EGFP-encoding lentiviral particles production and shRNA clones transduction

To produce eGFP-encoding lentiviruses particles, HEK293T cells were co-transfected with pGagPol (Amendola *et al.*, 2005), pRev (Amendola *et al.*, 2005), pFugW (Lois *et al.*, 2002) and pHEF-VSVG (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Lung-Ji Chang) or pSVIIIexe7pA-HxB2 (Sullivan *et al.*, 1998) in the proportion of 2:1:1:0.2. After 48 h, lentiviral particles were collected and quantified by $p24^{CA}$ ELISA. shRNA clones were transduced with the eGFP-encoding lentiviral particles at a MOI of 1 and enhanced by spinoculation. After 6 h, cells were washed and medium was replaced. After 48 h post-transduction cells were collected for flow cytometry analysis.

3.3.6. Flow Cytometry analysis

Cells transduced with FUGW-VSV or FUGW-GP120 were harvested after 48 h, washed with 1 % BSA-PBS and fixed with 0.1 % formaldehyde in PBS. Cells transduced with HIV-HSA were harvested at 2, 4 and 7 days. Afterwards, washing with 1 % BSA-PBS, HIV-HSA-transduced cells were stained for HSA surface expression, with anti-HSA-PE (BD Pharmingen) for 30 min at 4 ^{o}C. After staining, cells were fixed with 0.1 % formaldehyde in PBS. Cells infected with HIV-1_{NL4-3} were harvest at day 0, 2, 4 and 7, washed 1 % BSA-PBS, stained for CD4 surface expression with anti-CD4-FITC (RPA-T4 BD Pharmigen) and fixed with 0.1 % formaldehyde in PBS. Labeled untransduced cells were used as negative controls. BD FACS Calibur (BD

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

3.3.7. Transient transfection assays

HeLa-P4 cells were co-transfected with 300 ng of pLKO.1 shRNA for each target gene and 100 ng of pHIV-1_{NL4-3}. Transfections were performed with FuGENE® HD (Roche, IN, USA) according to manufacturers' protocol. After 48 h, viral particles were collected and quantified by $p24^{CA}$ ELISA. Cells were collected to evaluate *β-galactosidase* expression by a colorimetric assay, based on the cleavage of chlorophenolred-β-D-galactopyranoside (CPRG; Roche) as described in Mammano *et al.*, 2000.

3.4. Results

3.4.1. Efficient knockdown is of HIV-1 replication is maintained overtime

Previously we have performed a shRNA based screen where we have identified 14 different proteins essential for HIV-1 replication. Afterwards, the importance of these proteins for HIV-1 infection was validated by the construction of individual cell Jurkat cell clones expressing a shRNA cassette that knockdown each gene expression. All clones were demonstrated to inhibit HIV replication, being viral particles production and viral proteins expression almost abolished.

To evaluate whether inhibition of HIV-1 replication in the shRNA clones occurred early in viral replication or rather if it would reflect a cumulative effect, we followed HIV-1 infection over time and assessed viral spread in culture. We infected shRNA T-cell clones with HIV-1_{NL4-3} and monitored viral production overtime by measuring the $p24^{CA}$ in cell culture supernatant at day two, four and seven (Figure 3.1A). As represented in the three panels of Figure 3.1A, we can observe a pattern of viral replication throughout time for all different shRNA clones. Compared to HIV-1 replication in shSCRAM cells normalized to 100 % at all time points, we observed a continuous reduction in the amount of virus in supernatant of shRNA infected clones. $p24^{CA}$ levels were lower at day 2 (25 % to 75 % depending the shRNA clone) and were constantly reduced until day 7 where nearly no $p24^{CA}$ was detected.

To determine the effect of shRNAs in the expression of *de novo* viral protein we evaluated by western-blot the expression of Vif protein after 48h of HIV-1_{NL4-3} infection. As shown in figure 3.1B, the expression levels of Vif were barely undetectable compared to control (shSCRAM). The greater reduction of Vif expression (figure 3.1B) compared with $p24^{CA}$ levels in figure 3A can be explained by the detection of residual input $p24^{CA}$ from virus still present at 48 h and not resulting from a *de novo* replication. Therefore, when considered together, these results indicate that inhibition of HIV-1 replication by gene-specific shRNAs is very effective and initiates early in HIV-1 replication. Nevertheless, for EZH2-1 shRNA and with minor importance for EZH2-2 and PPF1A2-2 shRNAs, expression of Vif was higher than for other knockdowns. Taking in consideration the reduction in viral replication observed with these gene-specific shRNA, this fact may reflect a mechanism of HIV-1 inhibition that is subsequent to viral expression.

To get better understanding on the blocking effect of host-protein expression by shRNA in HIV-1 infection and dissemination in culture, we challenged the different shRNA clones with the HIV-HSA reporter virus. This system allows HIV-1 infection to be followed at single-cell level by enumerating HSA^+ cells by flow cytometry after surface staining. Seven-day time course assay demonstrated that while the percentage of shSCRAM infected cells steadily increased from less than 10 % at day 2 to approximately 60 % at day 7, the percentage of HSA^+ of all shRNA clones remained relatively unchanged through time (figure 3.1C).

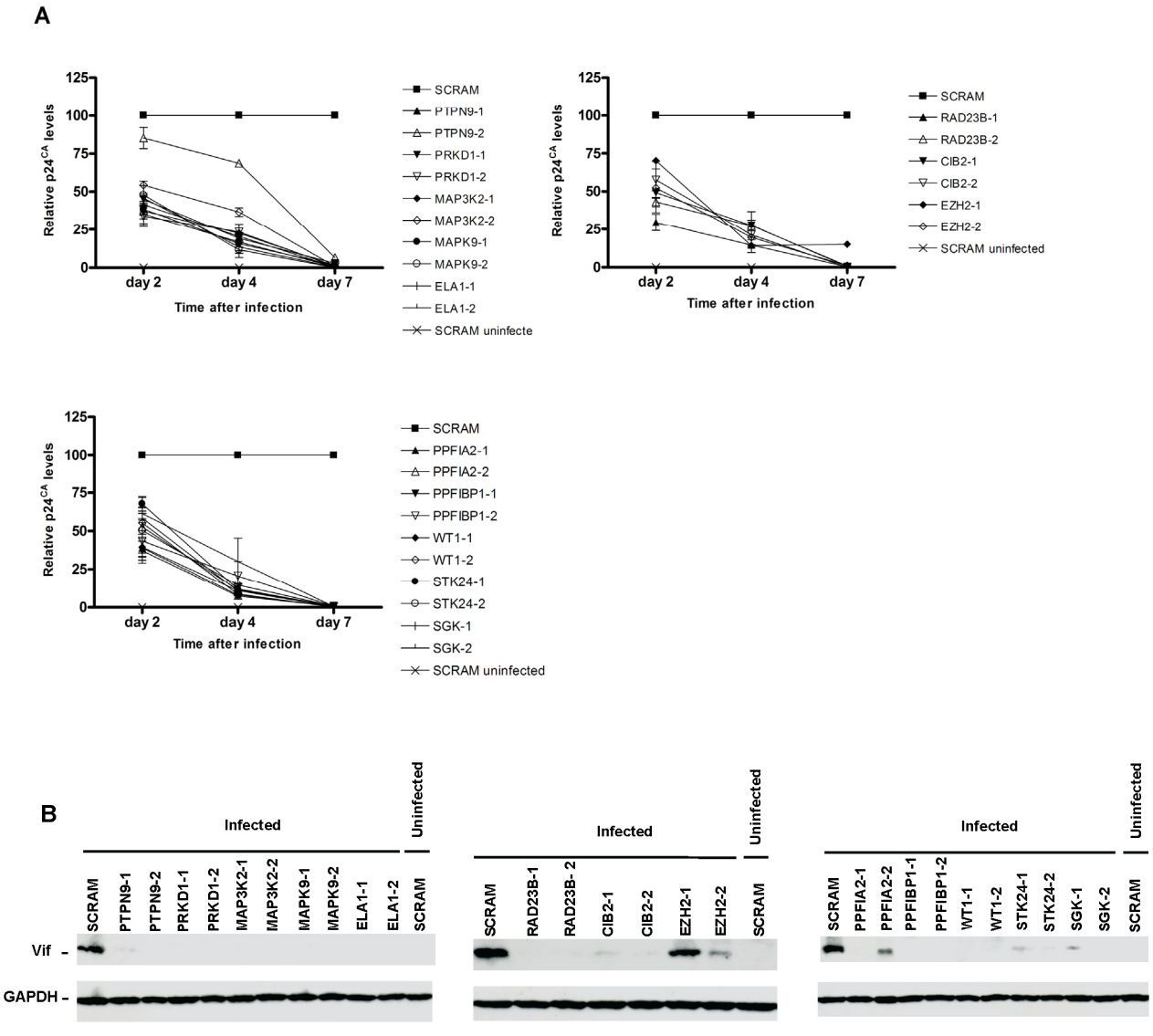

Figure 3.1. Neutralization of HIV-1 replication by shRNAs is cumulative over time. (Continues next page).

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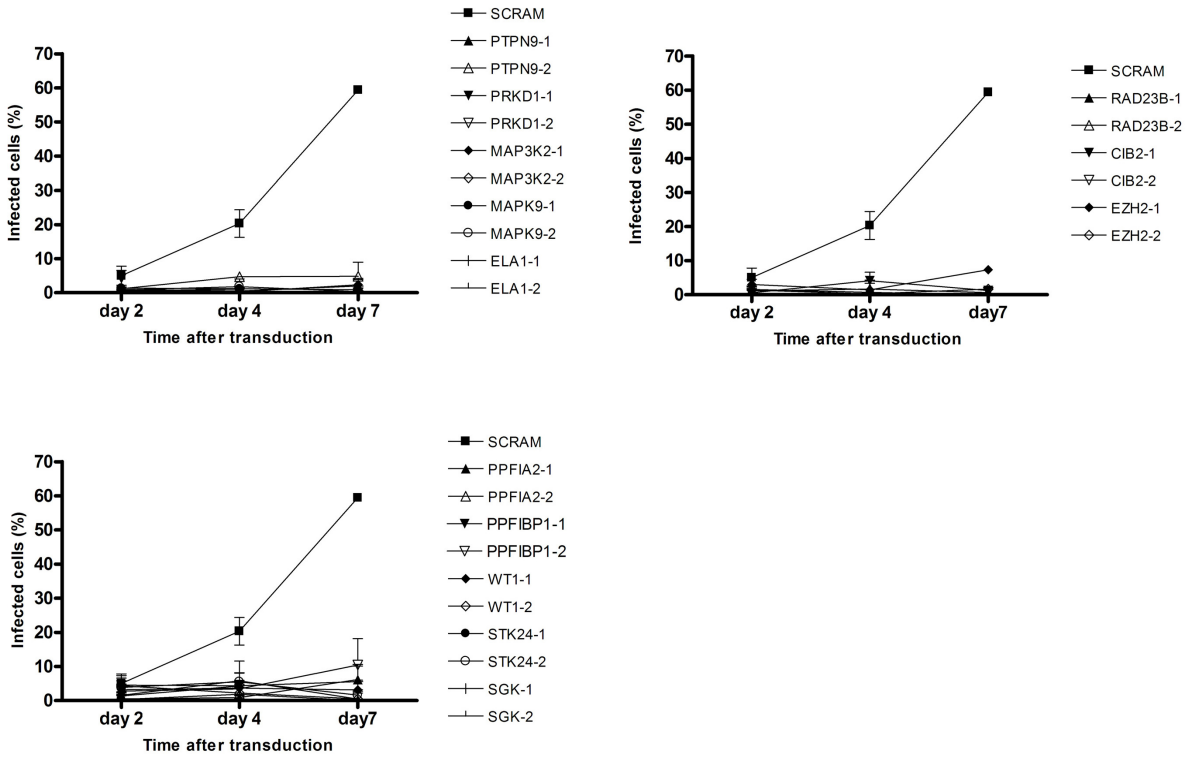

Figure 3.1. Neutralization of HIV-1 replication by shRNAs is cumulative over time. A. Kinetics of HIV-1_{NL4-3} replication in shRNA Jurkat clones during 7 days of infection. shRNA clones were infected with HIV-1_{NL4-3} (MOI of 1) and $p24^{CA}$ antigen was measured at day 2, 4 and 7. Values are relative to control shSCRAM cells infected with HIV-1 (■) and represent mean ± SEM (*n* = 3). **B**. Immunoblotting of Vif protein in the different shRNA clones after 48 h of HIV-1_{NL4-3} infection (MOI of 1). This figure is representative of three independent experiments. **C**. Flow cytometry analysis of HSA surface expression in shRNA clones infected with HIV-HSA (MOI of 1) during a time course assay of 7 days of infection. Cells were membrane stained with anti-HSA antibody for detection of HIV-1 infection. Percentage of infected cells was analysed by flow cytometry. Values correspond to mean ± SEM (*n* = 2).

In this experiment we also monitored HIV-1-HSA replication by $p24^{CA}$ ELISA and observed a similar pattern in all shRNA clones compared to replication of the parental HIV-1 (Figure 3.2) indicating that both HIV-1_{NL4-3} and HIV-HSA replication in the shRNA clones were performed in an akin way.To evaluate if the lower percentage of infected cells over time could result from the loss of CD4 expression at the cell surface, we monitored the CD4 positive cells during 7 days of infection with HIV-1_{NL4-3} (Figure 3.3). We observed a lack of significant decrease of CD4 surface expression in shRNA clones compared with wild-type Jurkat cells or shSCRAM clone.

Despite the observation that shRNAs do not completely knockdown host-proteins gene expression (was demonstrated previously), viral replication was strongly reduced as shown in figure 3. 1.

These results support the hypothesis that an effect of host-proteins on viral replication is highly sensitive to small variations in protein expression which is reflected in an immediate effect of shRNA.

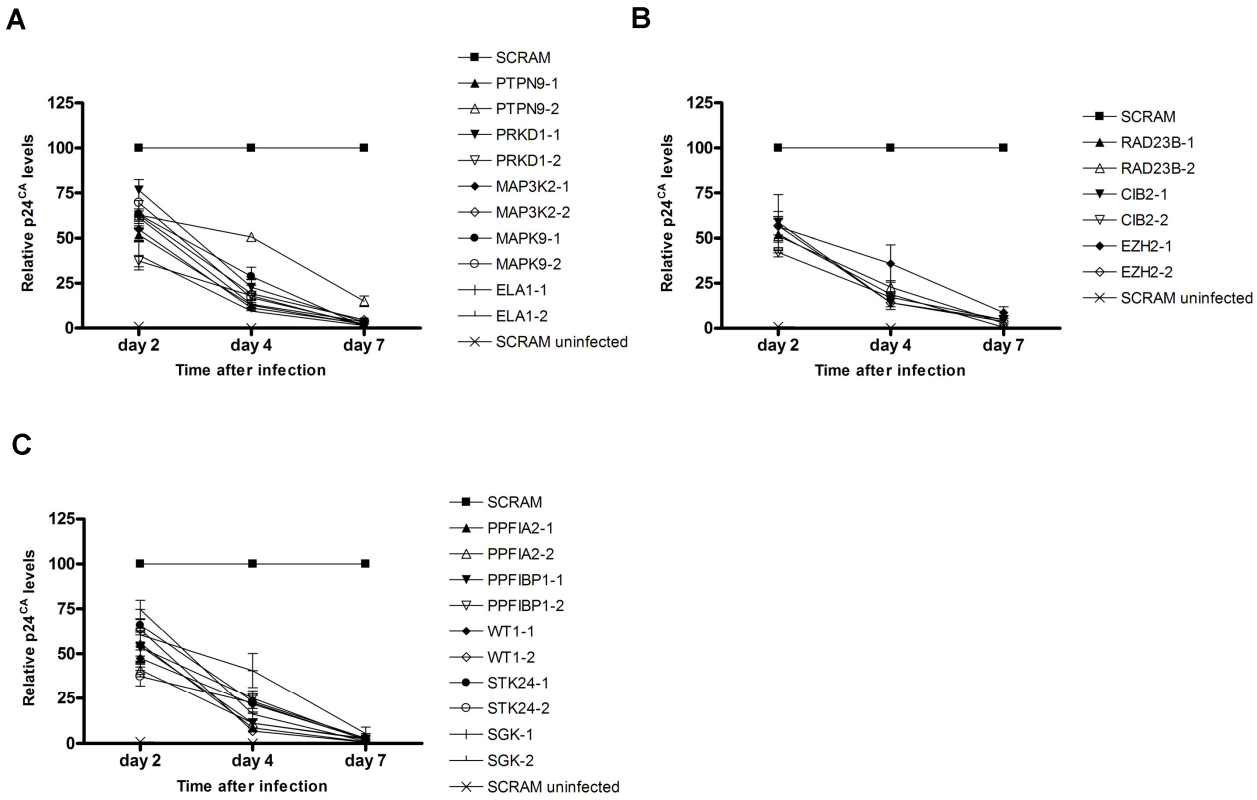

Figure 3.2. Monitoring HIV-HSA infection in shRNA Jurkat clones. HIV-1 replication kinetics in shRNA clones during 7 days of infection. shRNA clones were infected with HIV-HSA and $p24^{CA}$ expression was measured at day 2, 4 and 7. Values are relative to control shSCRAM infected cells (■) and represent mean ± SEM (*n* = 3). **A**. Evaluation of HIV-HSA replication in shRNA clones for PTPN9, PRKD1, MAP3K2, MAPK9 and ELA1. **B.** Evaluation of HIV-HSA replication in shRNA clones for RAD23B, CIB2 and EZH2. **C**. Evaluation of HIV-HSA replication in shRNA clones for PPFIA2, PPFIBP1, WT1, STK24 and SGK.

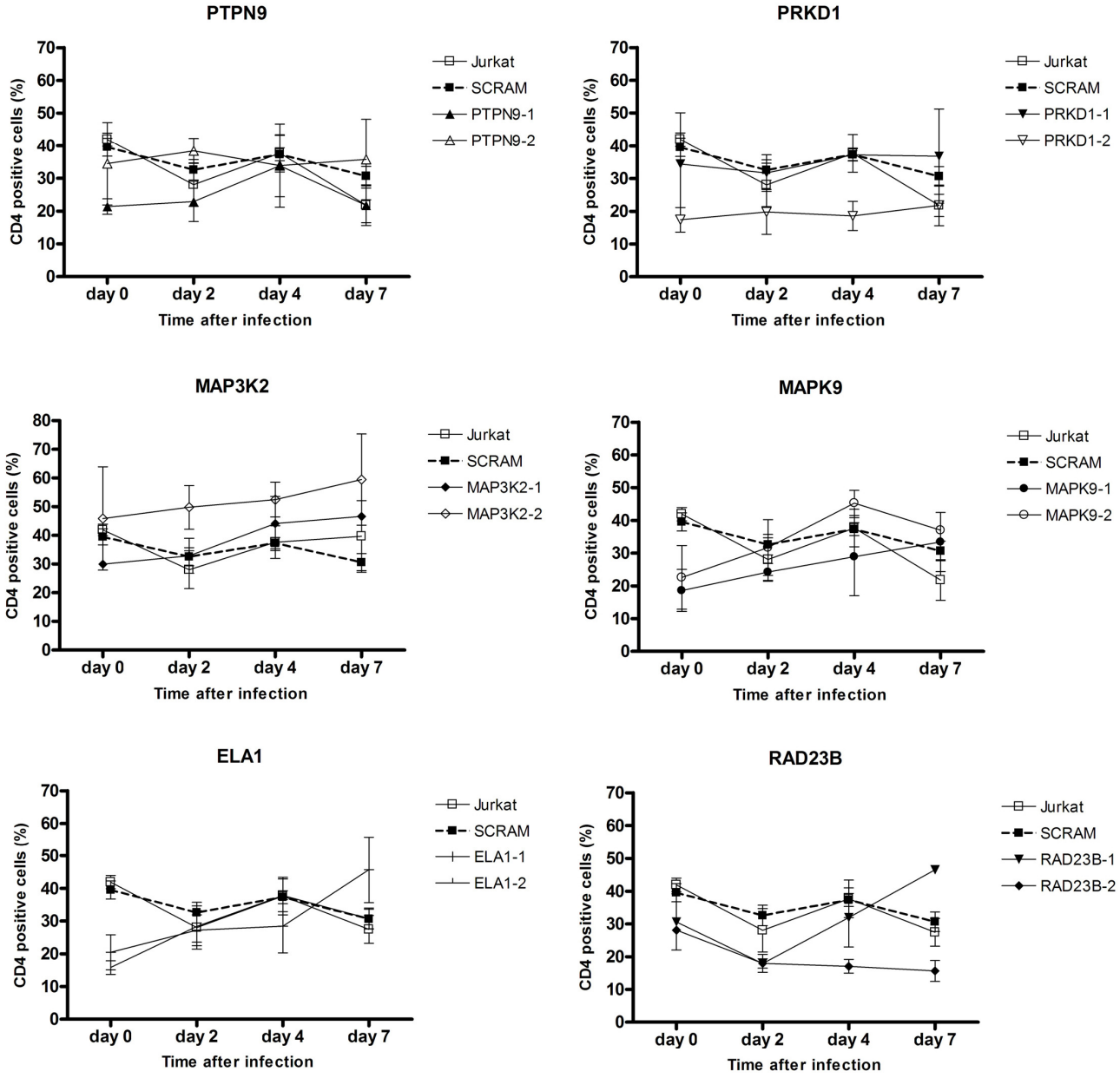

Figure 3.3. CD4 surface expression in Jurkat shRNA clones during HIV-1 (Continues nest page).

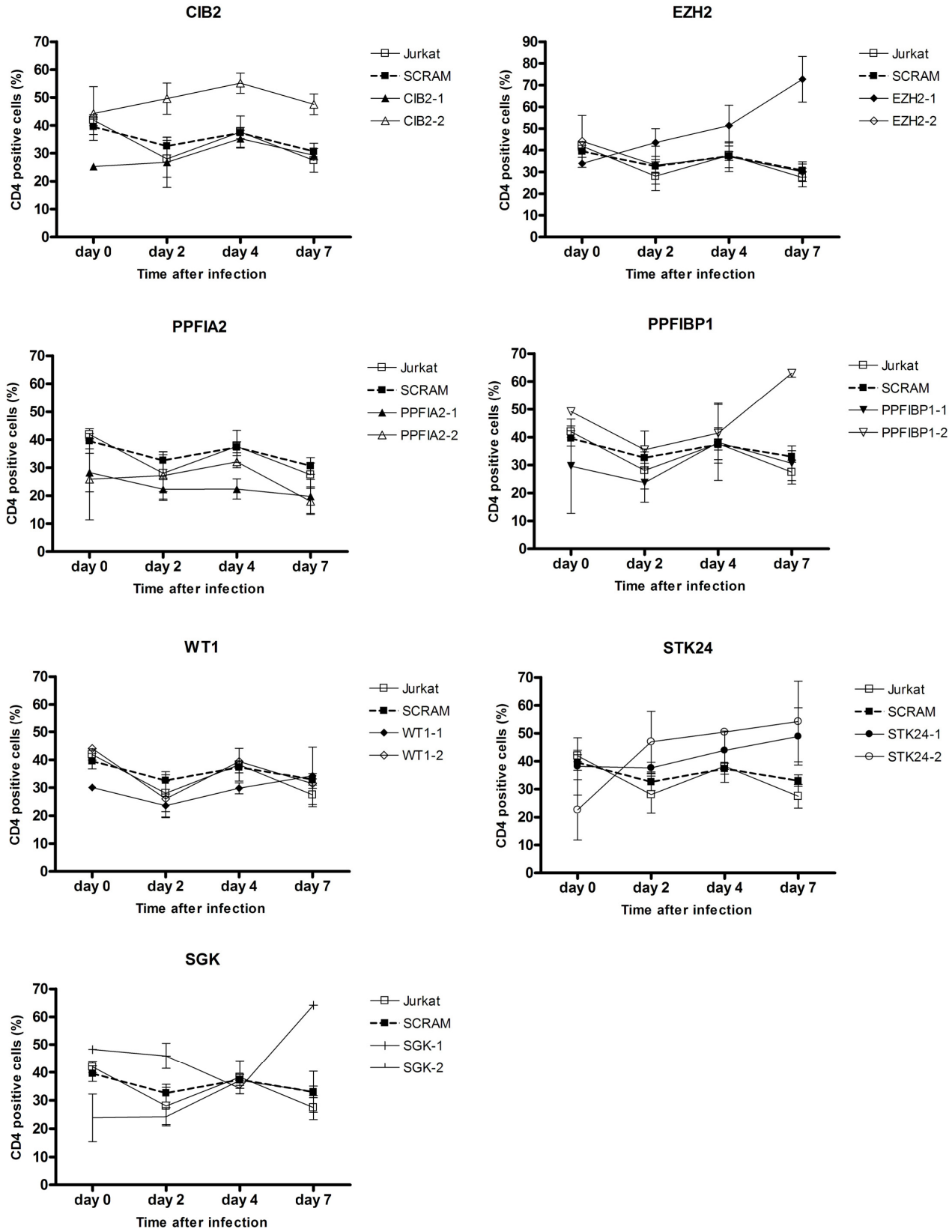

Figure 3.3. CD4 surface expression in Jurkat shRNA clones during HIV-1. Jurkat shRNA clones were infected with HIV-1_{NL4-3} and CD4 positive cells were measured by flow cytometry at day 0, 2, 4 and 7. Dash lines indicate the values for shSCRAM clone. Values represent mean ± SEM (n= 3).

3.4.2. Knockdown of host-proteins does not affect integration but rather affect an early step in HIV-1 replication

The observation in our previous work that Gag products were not detected intracellularly, lead to the hypothesis that the identified host-proteins would be important in an early stage of HIV-1 life cycle, before Gag expression (chapter 2, figure 2C). To further investigate whether HIV-1 replication cycle was affected before or after viral integration knockdown-Jurkat cells were transduced with an HIV-1-based lentiviral vector, carrying the EGFP transgene, and pseudotyped with a VSV-G envelope. Expression of the fluorescent protein was under the control of human ubiquitin-C promoter to avoid a bias effect that host-proteins might be involved in LTR-driven expression. Analysis of EGFP expression at 48 h post-transduction by flow cytometry showed similar fluorescence results compared to the control shSCRAM indicating that in all Jurkat-knockdown-cells HIV-1 proviral vector was efficiently integrated (Figure 3.4, black bars). To assess whether by using VSV-G-pseudotyped HIV-1 vectors we were overcoming an entry defect in Jurkat knockdown clones, similar experiments were performed with a gp120-pseudotyped lentiviral vector. Results from transduction experiments showed that all shRNA Jurkat clones express low levels of EGFP compared to Jurkat shSCRAM (Figure 3.4, white bars). Taken together, these results indicate that these newly identified proteins do not affect viral integration but have a role during HIV-1 entry.

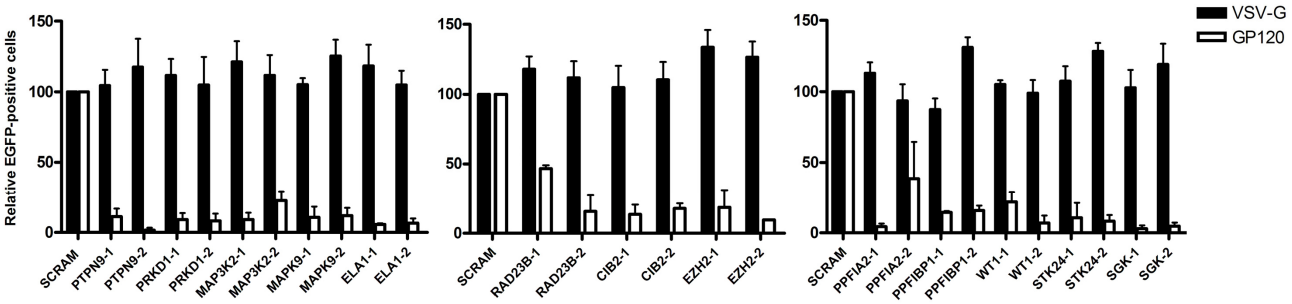

Figure 3.4. Knockdown of host-proteins do not affect integration but affect an early step in HIV-1 replication. Jurkat shRNA clones were transduced with EGFP-expressing lentiviral particles (FugW-EGFP) pseudptyped with VSV-G and HIV-gp120. After 48h, EGFP expression was measured by flow cytometry. Values are relative to the percentage of shSCRAM EGFP positive cell and represent mean ± SEM (*n* =3). Black bars indicate values for shRNA clones transduced with a VSV-G-lentivirus and white bars indicate values to shRNA clones transduced with a GP120-lentivirus.

3.4.3. Knockdown of some host-proteins affects HIV-1 LTR driven replication

Since HIV-1 LTR-driven transcription is affected by host-proteins, we sought to assess whether its knockdown could affect Tat transactivation of HIV-1 expression. The effect of knockdown host-proteins was monitored in HeLa-P4 cells containing the *β-galactosidase* gene under the control of the HIV-LTR. HeLa-P4 cells were transiently cotransfected with pHIV-1_{NL4-3} and an individual gene-specific shRNA. All previous proteins were studied except for ELA1 due to technical reasons. The efficiency of LTR-driven expression was determined by β-Galactosidase activity 48h post-transfection. By examining β-Galactosidase activity (figure. 3.5, black bars), the relative expression of shRNA against PRKD1, MAP3K2, MAPK9, RAD23B, EZH2, PPFIA2, PPFIBP1, WT1 and STK24 exhibited a decrease on LTR-directed transcription compared with control shSCRAM. However, no noteworthy effect could be observed for shRNA against PTPN9, CIB2 and SGK. Concomitantly, levels of $p24^{CA}$ antigen in supernatant were also determined to assess viral production in one cycle of infection (Figure 3.5, white bars). Our data showed that shRNAs with an effect on HIV-1 transcription were associated with a significant decrease in the $p24^{CA}$ levels. Taken together, our results strongly suggest that the decrease in viral production in this context is a direct consequence of reduced LTR-driven expression when PRKD1, MAP3K2, MAPK9, RAD23B, EZH2, PPFIA2, PPFIBP1 and WT1 and

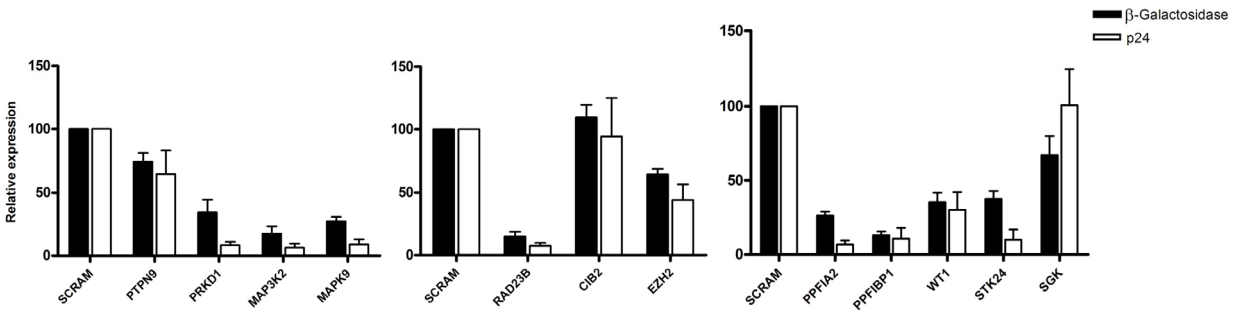

Figure 3.5. HIV-1 LTR-driven transcription is affected by host-proteins. Transient assays were performed in HeLa-P4 cells co-transfected with pHIV-1_{NL4-3} and the different shRNA plasmids. After 48h, cells were harvested and LTR transcription was measured by quantification of the β-Galactosidase activity in cell lysates. Cell supernatant was also collected to measure viral production by $p24^{CA}$ ELISA. Black bars indicate values for measurements of β-Galactosidase activity in cell lysates and white bars indicate values for measurements of $p24^{CA}$ in the supernatant. Values are relative to the control shSCRAM and represent mean ± SEM (*n* = 3).

STK24 are knockdown. Furthermore, for PTPN9, CIB2 and SGK shRNAs, neither β-Galactosidase nor $p24^{CA}$ levels were affected by shRNA expression, suggesting that these proteins do not play an important role in HIV-1 transcription.

3.5. Discussion

We have previously developed a newly shRNA screen to identify mainly kinases and phosphatases that could be important during HIV-1 infection. With this screen we identified 14 proteins that after validation were proven to be essential for HIV-1 replications. From these 14 proteins, we identified two phosphatases, PTPN9 and PTPRE; five kinases, PRKD1, MAP3K2, MAPK9, SGK and STK24; one hypothetical kinase-binding-protein, CIB2; two phosphatase-binding-proteins PPFIA2 and PPFIBP1; and four other proteins with diverse function RAD23B, EZH2, WT1 and ELA1. After identifications of these co-factors we questioned its functions during HIV-1 infection and where in the HIV-1 life cycle these proteins could be involved. Therefore, in this work we accessed the effect of down-modulation of these proteins during infection time and verified that the inhibition of replication occurs early in infection and is cumulative over time having a drastic effect after seven days of infection. These results permitted us to validate the efficiency of the shRNA in a stable cell lines. We then assessed critical steps in HIV-1 life cycle, entry, integration and viral transcription. Using the same individual shRNA Jurkat T clones as previously, we concluded that none of these proteins seem to a have a relevant role in HIV-1 proviral integration. Instead, all proteins seem to play an important role before viral integration in an early step of HIV-1, entry and/or uncoating. Moreover, transient assays performed with HeLa cells demonstrated that PRKD1, MAP3K2, MAPK9, RAD23B, EZH2, PPFIA2, PPFIBP1, WT1 and STK24 have an effect on HIV-1 LTR transcription. On contrary, PTPN9, CIB2 and SGK do not show an important outcome on this viral replication step. Therefore, we can infer that PRKD1, MAP3K2, MAPK9, RAD23B, EZH2, PPFIA2, PPFIBP1, WT1 and STK24 may be important for HIV-1 entry (or uncoating) and transcription. Conversely, PTPN9, CIB2 and SGK are only involved in entry/uncoating. This double functionality could be the result of multifunctional protein acting on different replication steps of HIV-1 replication or instead, can be an indirect effect of these proteins on the HIV-1 LTR promoter and other cellular promoters leading to the inhibition of additional proteins involved HIV-1 entry.

Understanding the cellular function of the identified proteins and the pathways were they can be involved could help us to better realize the interaction of these proteins and the virus. For example, EZH2 was described to interact with embryonic ectoderm development protein (EED), a member of the PcG family, at the EED–EZH2 complex in mammals (Han *et al.*, 2007).

In turn, EED was described to interact with HIV-1 matrix, integrase and Nef (Peytavi *et al.*, 1999; Violot *et al.*, 2003; Witte *et al.*, 2004). Until this date, the EED role in HIV-1 life cycle is not well understood. Data suggests that EED could be involved in cellular function(s) necessary in early steps of HIV-1 life cycle (Violot *et al.*, 2003) or that EED may function as a negative regulator of HIV-1 assembly and release (Rakotobe *et al.*, 2007). Our results seem to indicate that EZH2 acts as a positive factor in HIV-1 entry. Other cellular proteins demonstrated to be important for HIV-1 are Nuclear Dbf2-Related kinase 1 (NDR1) and 2 (NDR2). A previous work showed that NDR1 and NDR2 are incorporated into HIV-1 virions and that the viral protease cleaves these proteins altering its enzymatic activity to favour HIV-1 replication (Devroe *et al.*, 2005). In addition, NDR1 phosphorylation leads to activation of STK24 (Tamaskovic *et al.*, 2003). Therefore, it is conceivable that STK24 could also be incorporated in HIV-1 and could have an important role in HIV-1 replication. SGK is transcriptionally activated by the Glucocorticoid receptor (GR) (Loffing *et al.*, 2006), which has been demonstrated to interact with HIV viral protein Vpr within a complex integrating VIP-1. A study suggested that the interaction Vpr-GR could induce apoptosis since NF-kB inhibition by Vpr seemed to be GR-dependent (Muthumani *et al.*, 2005). In addition, a recent work has also demonstrated that Vpr-GR interactions increases LTR-mediated transactivation, most likely prior to the presence of Tat (Schafer *et al.*, 2006). The GR recruitment by Vpr into the nucleus could activate SGK that would induce LTR-driven expression. However this hypothesis does not correlate with our results where SGK do not seem to influence HIV-1 transcription.

Furthermore, a report using chemical inhibitors indicated that ERK pathway via PRKD1 was dramatically activated by Tat in monocytes (Hui *et al.*, 2006). Moreover, PRKD1 together with PI3K has a role in presenting the catalytically active form of CDK9 to the HIV-1 promoter. It was also demonstrated that LTR-activation by PRKD1 is Tat-independent (Choudhary *et al.*, 2008), corroborating with our findings.

RAD23B is homologous to RAD23 homolog A (RAD23A or HHR23A) and both are described to have the same function in Nucleotide Excision Repair (NER) pathway (van Hoffen *et al.*, 2003). RAD23A is important for HIV-1 replication, it interacts physically with VPR, which seems to be critical for cell cycle arrest (Withers-Ward *et al.*, 1997; Gragerov *et al.*, 1998). Hence, RAD23B could be as important to HIV-1 as RAD23A or have a different function in HIV-1 entry and/or HIV-1 transcription as our results indicate.

Finally, the human leukocyte elastase (HLE), also known as ELA2, has been shown to interact with HIV-1 glycoprotein gp41. HLE is proposed to be a rate-limiting receptor for viral entry potentially by a mechanism involving the phenomenon of receptor co-patching (Bristow *et al.*, 2003). The structural similarities between ELA1 and ELA2 could lead to the hypothesis that ELA1 could have similar function to ELA2. Indeed, our results identify ELA1 as important for HIV-1.

Regarding MAP3K2 and MAPK9, although they were not shown to be directly involved in HIV-1 replication, many reports have described MAPK pathway to be important for HIV-1 replication in several cell types (Jacque *et al.*, 1998; Mischiati *et al.*, 1999; Yang *et al.*, 1999; Popik and Pitha, 2000; Greenway *et al.*, 2003; Mishra *et al.*, 2007).

Our study brings new perspectives for HIV-1-host interplay. The exact mechanism and pathway(s) where these proteins are involved is a continuous open question. The indication of possible network(s) (as observed previously in chapter 2) between genes and the putative overlap in some signal pathways, will lead us to future work in the cross-talk between HIV-1 replication and cell signalling, and now that a picture is emerging from these studies, more work with primary cells should be implemented to better understand the importance of these genes in primary T-lymphocytes.

In summary, the results presented in this report bring new insights for the complex interplay between HIV-1 and its cellular host leading to a novel perspective for the multifunctional role of the cellular proteins in HIV-1 replication. The identification of new kinases and phosphatases essential for viral replication emphasizes the cell signalling complexity and instigates for further studies involving cellular pathways and HIV-1 replication. Moreover, we show the feasibility to identify host factors that are both essential to the virus and non-essential to the cell. Importantly, these HIV-1 helper-factors being druggable can have a significant impact for new antiviral approaches when traditional strategies fail.

3.6. Acknowledgments

We greatly appreciate the gift of pGagPol, pRev by Dr. Luigi Naldini, pFugW by Dr. David Baltimore, pHEF-VSVG by Dr. Lung-Ji Chang and pSVIIIexe7pA-HxB2 by Dr. Joseph Sodroski. We thank Pedro Simões for some of the lentiviral preparations used in this report. This work was supported by Fundação para Ciência e Tecnologia (PTDC/SAU-MII/65346/2006, PTDC/SAU-MII/69280/2006 and PTDC/SAU-MII/78333/2006) and Fundação Luso-Americana para o Desenvolvimento. SR is supported by FCT fellowship SFRH/BD/24129/05, SM and PMB are supported by a post-doctoral fellowship.

CHAPTER 4

DNA-PKcs, an Helper factor in HIV-LTR driven Transcription

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(Manuscript in preparation)

4.1. Abstract

Recently, our group has developed a shRNA screen in Jurkat T cells to identify helper factors for HIV-1 replication. In this screen, 14 proteins were described as essential for HIV-1 infection. Among these proteins, we have identified CIB2, a poorly known protein that has a high homology to the calcium and integrin binding family 1 (CIB1) protein. CIB1 was described as interacting with the DNA-dependent kinase catalytic subunit (DNA-PKcs), one of the crucial proteins involved in DNA repair after double strand breaks. Due to the homology of CIB2 with CIB1, we sought to evaluate the role of DNA-PKcs in HIV-1 replication and if it correlates with function of CIB2 in the entry steps of HIV-1 life cycle.

Our results indicated that DNA-PKcs is essential for HIV-1 replication. Down-regulation of DNA-PKcs led to an inhibition of viral transduction and to a consequent decrease in the production of viral particles. Furthermore, our results showed that DNA-PKcs does not act as a co-factor for HIV-1 neither in the early steps of viral infection or in integration but instead plays a crucial role during HIV-1-LTR driven transcription. The observed DNA-PKcs function during HIV-1 life cycle differs from CIB2, indicating that these two proteins have distinct and independent functions during HIV-1 infection.

This study brings new insights for the role of DNA-PKcs in HIV-1 infection and contributes for a better knowledge of the intrinsic interplay between HIV-1 and its host.

4.2. Introduction

HIV-1 to replicate depends on its host machinery, recruiting cellular proteins to help it during viral replication. Cellular proteins can play a positive role, being modulated by the virus in all steps of the HIV-1 life cycle. Several studies have been performed to identify these co-factors. Our group has recently developed a shRNA screen in Jurkat T cells where 14 different proteins were identified as essential for HIV-1 replication (Rato *et al.*, 2010). One of the identified proteins was CIB2, a calcium binding protein that has close homology to CIB1 (46 % identity, 64 % similarity) (Seki *et al.*, 1999). CIB1 and CIB2 belong to a gene subfamily, coding homologous EF-hand proteins named *cib*, which consists in four genes in humans and several genes in other vertebrate and invertebrate species (Gentry *et al.*, 2005).

CIB1 is known to bind to Ca^{2+}, undergoes N-myristoylation and interacts with various protein targets, such as integrin αIIb, Snk, inositol 1,4,5-triphosphate receptor and DNA-PKcs (Naik *et al.*, 1997; Wu and Lieber, 1997; Ma *et al.*, 2003; White *et al.*, 2006). The exact CIB1 function is unclear, however has already been implicated in haemostasis, DNA damage response, and apoptosis (Yamniuk *et al.*, 2006).

Similarly to CIB1, CIB2 function is also not known. Its homology with CIB1 may indicate that CIB2 can also be a calcium binding protein. In fact, a recent study in mouse and human brain have associated CIB2 to Ca^{2+} signaling involving ERK1/2 and PKC and also localized CIB2 at Ku70 and Ku80 originating a complex, commonly named as DNA-PK. This complex recognizes and binds to the damaged DNA, targeting other protein repair activities to the site of DNA damage.

Beyond the interaction with Ku70 and Ku80, DNAPKcs has been implicated to interact with several other proteins and is involved in several signalling pathways. Studies have shown that DNA-PKcs interacts with C1D, a nuclear matrix associated factor (Yavuzer *et al.*, 1998), Lyn tyrosine kinase (Kumar *et al.*, 1998b), c-abl tyrosine kinase (Jin *et al.*, 1997), protein phosphatase 6 (PP6) (Mi *et al.*, 2009), Akt (Feng *et al.*, 2004) and CIB1 (Wu and Lieber, 1997).

Several reports have described DNA-PKcs as a co-factor for HIV-1 replication. However its role is very controversial. Skalka group demonstrated that DNA-PKcs was important for retroviral DNA integration, together with other components of the NHEJ pathways (Ku, XRCC4 and DNA ligase IV), using as a study model, the DNA-PK-deficient murine SCID cells (Daniel *et al.*, 1999).

This work brought the hypothesis that the viral integration intermediate could be detected by the host cell as DNA damage, activating the post-integration repair through the NHEJ pathway. With this mechanism, HIV-1 could avoid the pro-apoptotic signal mediated by the DNA gaps and efficiently complete the provirus integration (Daniel *et al.*, 2004). However, this hypothesis was refuted by a different study with DNA-PKcs and Ku-deficient cells, showing that DNA-PKcs is not required for an efficient HIV-1 integration together with NHEJ pathways (Baekelandt *et al.*, 2000). Later, a new study arguing that NHEJ pathway usually repairs double-strand breaks, but not single-stranded gaps, proposed a different function for NHEJ pathway during the early phase of lentiviral infection. The dsDNA ends produced during reverse transcription could serve as a pro-apoptotic signal during retroviral infection and that could be implicated in the removal of this pro-apoptotic signal by DNA circularization (Van Maele and Debyser, 2005). This hypothesis was corroborated by two other studies where the lack of the NHEJ pathway components (Ku80, XRCC4 or ligase IV) was directly related to a decrease in 2-LTR circles production (Li *et al.*, 2001; Jeanson *et al.*, 2002). Nevertheless, DNA-PKcs in particularly, seemed to have modest effects on 2-LTR circle formation (Kilzer *et al.*, 2003). Despite the controversy, whether DNA-PKcs is involved or not in HIV-1 integration, this kinase has also been associated with HIV-1 Tat. It was demonstrated that DNA-PKcs binds to Tat protein to increase the phosphorylation state of Sp1, resulting in upregulated expression of the HIV-1 LTR (Chun *et al.*, 1998; Rossi *et al.*, 2006).cellular membranes and Golgi apparatus (Blazejczyk *et al.*, 2009). Furthermore, CIB2 has also been showed to interact with integrins (Hager *et al.*, 2008).

Due to the homology between CIB2 and CIB1 and since CIB1 interacts with DNA-PKcs we hypothesized that DNA-PKcs, as CIB2, could have an assistant role during HIV-1 replication.

DNA-PKcs is a kinase DNA-dependent, member of the PI3K family. It is involved in signal transduction cascades related to NHEJ pathway, V(D)J recombination, apoptosis cell death, telomere maintenance and other pathways of genome surveillance (reviewed in Dip and Naegeli, 2005). The best studied function of DNA-PKcs is its central role in the double strand breaks (DSBs) repair in order to maintain the chromosomal integrity. In higher eukaryotes, NHEJ is the primary pathway responsible for DSBs, which rejoins free DNA ends without a homologous template (reviewed in Shrivastav *et al.*, 2008). Six core proteins are required for NHEJ: Ku70, Ku80, DNA-PKCS, XRCC4, DNA ligase IV, and Artemis. DNA-PKcs interacts with.

Ku70 and Ku80 originating a complex, commonly named as DNA-PK. This complex recognizes and binds to the damaged DNA, targeting other protein repair activities to the site of DNA damage.

Beyond the interaction with Ku70 and Ku80, DNAPKcs has been implicated to interact with several other proteins and is involved in several signalling pathways. Studies have shown that DNA-PKcs interacts with C1D, a nuclear matrix associated factor (Yavuzer *et al.*, 1998), Lyn tyrosine kinase (Kumar *et al.*, 1998b), c-abl tyrosine kinase (Jin *et al.*, 1997), protein phosphatase 6 (PP6) (Mi *et al.*, 2009), AKT (Feng *et al.*, 2004) and CIB1 (Wu and Lieber, 1997).

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In the present work we sought to study DNA-PKcs function during HIV-1 infection in Jurkat T cells and evaluate a possible correlation with CIB2. Here we demonstrate that DNA-PKcs is essential for HIV-1 replication. Our results indicate that when DNA-PKcs is down-modulated, viral production is rather inhibited. This effect was demonstrated not to be due to a positive function in HIV-1 entry or integration but due to a role in HIV-1-LTR driven transcription. These results suggest that DNA-PKcs and CIB2 act independently during HIV-1 infection.

Our results strengthen the importance of DNA-PKcs in HIV-1 infection and bring new perspectives for DNA-PKcs function during HIV-1 life cycle.

4.3. Material and Methods

4.3.1. Cell lines and culture conditions

HEK293T (ATCC, VA, USA) and HeLa-P4 cells (HeLa-CD4-LTR-β-gal, AIDS Reagent, MD, USA, contributor Dr. Dr. Michael Emerman) were grown in DMEM supplemented with 10 % FBS (DMEM-10). Jurkat E6-1 T-cells (obtained AIDS Reagent, MD, USA, contributor Dr. Arthur Weiss) were cultured in RPMI-1640, supplemented with 10 % FBS (RPMI-10). Jurkat cells expressing shRNA (shRNA clones) were cultured in RPMI-10 supplemented with 2 μg/ml of puromycin (Sigma, MO, USA). All cell cultures were maintained at 37 °C in 5 % CO_2. All cell culture media and reagents, otherwise indicated, were from Lonza (Basel, Switzerland).

4.3.2. shRNA Jurkat cell lines construction

To produce Jurkat cell lines expressing shRNA cassettes, transducing particles containing shRNAs were provided from Luis Moita (IMM, Portugal). Briefly, to produce lentiviral particles HEK293T cells were co-transfected with pHEF-VSVG, pLKO.1, and pCMVΔR8.9 plasmid DNAs (plasmids were obtained from the RNAi consortium (TRC)), by calcium-phosphate method. After 48 h lentiviral particles were collected and tittered via a p24 antigen ELISA assay.
The viral particles were used for transducing Jurkat E6-1 cells. Cells were ressuspended in a viral preparation and subject to spinoculation (O'Doherty *et al.*, 2000). 48 h after, transduced cells were isolated with ClonaCell™-TCS semi-solid medium (StemCell Technologies, Grenoble, France), expanded and maintained in RPMI-10 supplemented with 2 μg/ml of puromycin (Sigma, MO, USA).

4.3.3. Viral production and infection assays

HEK293T cells were transfected, by calcium phosphate method, with pHIV-1_{NL4-3} plasmid (AIDS reagent, contributor Dr. Malcolm Martin) and after 48 h, HIV-1_{NL4-3} virions were collected from supernatant cultures and measured by $p24^{CA}$ ELISA (AIDS & Cancer Research Program, NCI Frederick, MD, USA). These viruses were used to infect Jurkat cells and shRNA-Jurkat clones at an MOI of 1. For this purpose, cells were ressuspended in a viral preparation and subject to spinoculation. After 6 h, cells were washed in PBS (1x) and medium was replaced.

This procedure was repeated at day 4. Upon 7 days of infection, supernatant was collected and used to measure virus production by $p24^{CA}$ ELISA (AIDS & Cancer Research Program) and cells were extracted to perform western blot analysis.

4.3.4. Immunoblotting

Intracellular DNA-PKcs and Gag proteins expression was evaluated by western blot. Briefly, cells were washed with ice-cold PBS (1x) and lysed in RIPA lysis buffer (50 mM Tris-Cl [pH=7,2], 150 mM NaCl, 1 % Triton X-100) for 30 min at 4 ^{o}C. Protein concentration was quantified by Bradford colorimetric assay (BioRad, CA, USA). Same amounts of protein were analyzed by an SDS-PAGE (7 % for DNA-PKcs and 12 % for Gag protein detection) and transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). DNA-PKcs blotting was performed with the anti-DNA-PKcs primary antibody (Ab-4, Thermo Scientific, CA, USA), followed by incubation with anti-mouse HRP-conjugated secondary antibody (BioRad). For Gag protein detection, anti-p24 primary antibody (AIDS Reagent, #530, from Dr. Susan Zolla-Pazner) was used succeeded by anti-human HRP-conjugated secondary antibody (SC-2453, Santa Cruz Biotechnology, CA, USA). Endogenous controls were blotted with anti-β-actin (AC-15, Abcam, Cambridge, UK) and anti-GAPDH primary antibody (6C5, Santa Cruz Biotechnology, CA, USA), followed by incubation with anti-mouse HRP-conjugated secondary antibody (BioRad). All blots were developed using the ECL (GE Healthcare) or Femto (Pierce, IL, USA).

4.3.5. Quantitative Real-Time PCR analysis

Total RNA was extracted from shRNA T cell clones using Trizol (Invitrogen, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using SuperScript^{TM} III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, CA, USA) according to the manufacturer's protocol. qPCR was performed on an ABI Prism 7300 PCR system (Applied Biosystems, CA, USA), using the Maxima™ SYBR Green qPCR Master Mix (Fermentas, Ontario, Canada). Primer sequences are described in Table 4.1. The relative amount of target gene mRNA was normalized to GAPDH mRNA. Specificity was verified by melt curve analysis for SYBR Green qPCR .

Table 4.1. Oligonucleotides used for qPCR

Gene target	Primers (5´- 3´)	References
DNA-PKcs	gctatattggatggaattgtggac	-
	gcgatttggtgtttactgga	-
Tat	actcgacagaggagagcaag	Williams *et al.*, 2006
	gagtctgactgttctgatga	Williams *et al.*, 2006
TAR	gttagaccagatctgagcct	Williams *et al.*, 2006
	gtgggttccctagttagcca	Williams *et al.*, 2006
GAPDH	ggtggtctcctctgacttcaaca	Rato *et al.*, 2010
	gttgctgtagccaaattcgttgt	Rato *et al.*, 2010

4.3.6. Lentiviral transduction and Flow Cytometry analysis

To produce eGFP-encoding lentiviruses particles, HEK293T cells were co-transfected with pGagPol (Amendola *et al.*, 2005), pRev (Amendola *et al.*, 2005), pFugW (Lois *et al.*, 2002) and pHEF-VSVG (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Lung-Ji Chang) or pSVIIIexe7pA-HxB2 (Sullivan *et al.*, 1998) in the proportion of 2:1:1:0.2.

After 48 h, lentiviral particles were collected and quantified by $p24^{CA}$ ELISA. shRNA clones were transduced with the eGFP-encoding lentiviral particles at a MOI of 1 and enhanced by spinoculation. Upon 6 h, cells were washed and medium was replaced. 48 h after post-transduction, cells were collected for flow cytometry. BD FACS Calibur (BD Bioscience, CA, USA) was used to acquire at least 20.000-gated events from each sample. Data were analyzed with FlowJo software (Tree Star, OR, USA).

4.3.7. Transient transfection assays

HeLa-P4 cells were co-transfected with 300 ng of pLKO.1 shRNA for DNA-PK gene or with shRNA control and 100 ng of pHIV-1_{NL4-3}. Transfections were performed with FuGENE® HD (Roche, IN, USA) according to manufacturers' protocol. Viral particles were collected after 48 h, and quantified by $p24^{CA}$ ELISA. In parallel, cells were collected to evaluate *β-galactosidase* expression CPRG (Roche) as described in Mammano *et al.*, 2000 and RNA was extracted for cDNA production and subsequent qPCR analysis (as described above).

4.3.8. Assessment of cell viability

To determine the viability of the shRNA clones, live and dead cells were counted by the trypan blue exclusion method. For each experimental condition a total of $\geq$ 250 cells were considered.

4.3.9. 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).

4.4. Results

4.4.1. DNA-PKcs is efficiently knockdown in Jurkat T cell using shRNAs

Upon identification in our previous screen of CIB2 as a hypothetic DNA-PKcs binding protein (Rato *et al.*, 2010), we sought to evaluate the role of DNA-PKcs in HIV-1 replication and its correlation with CIB2 function. For this purpose, we generated five shRNA Jurkat cell clones constitutively expressing a different shRNA that target DNA-PKcs mRNA. Down-modulation of DNA-PKcs was evaluated by qPCR (mRNA levels) (figure 4.1A) and immunoblotting (protein expression) (figure 4.1B).

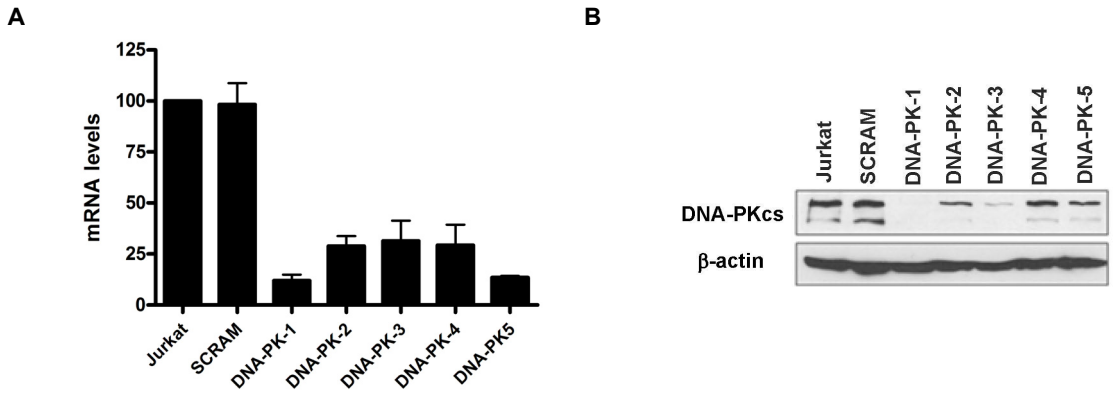

Figure 4.1. DNA-PKcs is efficiently knockdown in shRNA clones. **A.** Evaluation of DNA-PKcs mRNA levels in the different shRNA clones. mRNA levels were detected by qPCR. Values are relative to Jurkat cells and represent mean ± SEM (*n* = 3) **B.** Immunoblotting of DNAPKcs to evaluate the efficiency of the shRNA knockdown in the Jurkat shRNA clones. This figure is representative of two different experiments.

Both experiments indicate that all shRNA clones efficiently knockdown DNA-PKcs expression. Low levels of DNA-PKcs mRNA and protein were detected in the shRNA clones when compared with Jurkat cells alone or with control shSCRAM. The lowest protein levels detected by western blot were in DNA-PK-1 and DNA-PK-3 clones. Neverthleless, the mRNA levels of DNA-PKcs in clone DNA-PK-3 was not as drastic as in DNA-PK-1. In fact, DNA-PK-3 differs in both experiment having about 31 % of mRNA expression and 5 % of protein expression (after quantification with Image J software), compared to Jurkat and shSCRAM. These observations could be justified by DNA-PKcs half-life that has been described to be greater than 5 days (Ajmani *et al.*, 1995). Therefore, the partial inhibition of mRNA levels can enable some protein expression that due to proteins half-life fosters an imbalance of mRNA: protein ratio.

Analysing both experiences, our results indicate that we have constructed shRNA T cell clones that efficiently inhibit DNA-PKcs expression.

4.4.2. DNA-PKcs protein is crucial for HIV-1 replication

After generating shRNA T cell clones that efficiently knockdown DNA-PKcs expression we evaluated if down-modulation of DNA-PKcs affected HIV-1 replication. For this purpose, we infected the five shRNA clones, the shSCRAM clone and Jurkat cells with HIV_{NL4-3} at a MOI of 1 and seven days after infection, HIV-1 replication was measured through quantification of $p24^{CA}$ levels in cell culture supernatant by ELISA, and intracellularly by Western Blot analysis. As shown in figure 4.2, all shDNA-PKcs clones presented very low levels of $P24^{CA}$ in the supernantant (figure 4.2A) as well as intracellulary, with $P24^{CA}$ being barely detected by Western Blot (figure 4.2B).

These results indicate that when shDNA-PKcs is down-modulated, a strong inhibition of viral production occurs, demonstrating an essential role of DNA-PKcs in Jurkat T cells during HIV-1 replication in a step before viral expression.

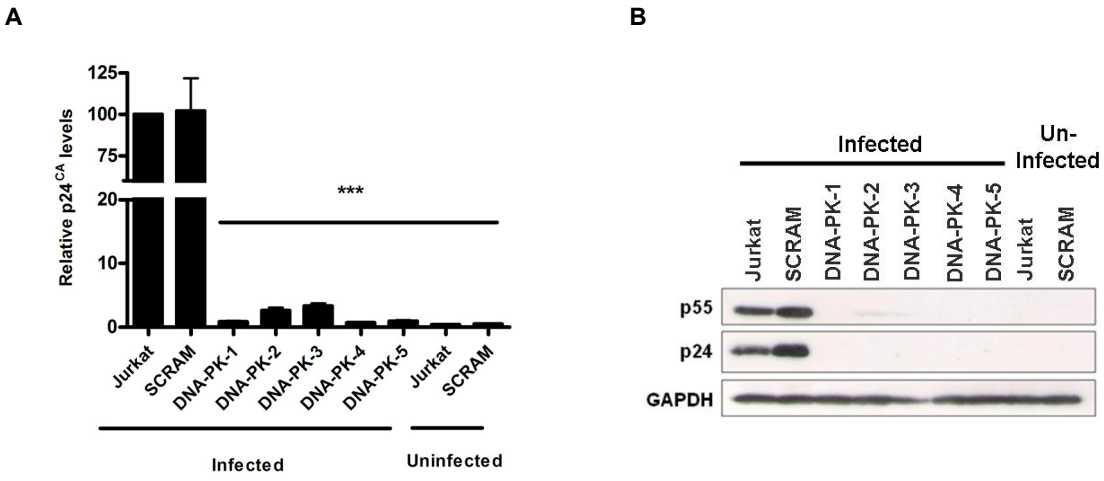

Figure 4.2. DNA-PKcs knockdown inhibits HIV-1 replication. **A.** shRNA clones against DNAPKcs were infected with HIV_{NL4-3} and 7 days after infection, HIV-1 replication was measured by p24 quantification in the cell culture supernatant. Values are relative to Jurkat cells infected with HIV_{NL4-3} and represent the mean ± SEM (n = 6). *** correspond to P < 0,0001. **B.** Immunoblotting of intracellular Gag protein in the different shRNA DNA-PK clones after 7 days of HIV-1 infection. This figure is representative of three independent experiments.

4.4.3. DNA-PKcs-knockdown affects HIV-1 replication without influencing host-cell viability

To exclude the hypothesis that the low levels of HIV-1 replication observed in the shRNA clones was not due to a decrease of cell viability, we analysed the viability of infected and non-infected cells. Trypan blue assay was performed to assess the percentage of viability in infected and non-infected cells.

From the previous experiments we observed that the shDNA-PK-1 T cell clone was the most reliable, once it presented the most consistent values in all experiments. Therefore, the subsequent experiments were performed only with the shDNA-PK-1 clone that came to be designated as shDNA-PK.

As observed in figure 4.3, both shRNA control and shDNA-PK clones exhibited a small decrease in cell viability in either infected or non-infected cells when compared with Jurkat cells. The shRNA integrated cassette by promoting the continuous activation of the RNAi machinery, can in somehow influence the viability of Jurkat cells.

When comparing shDNA-PK clone with shSCRAM clone, we do not observe a significant difference between infected and non-infected cells.

These results indicate that the drastic reduction of HIV-1 replication observed in the shDNA-PKs clones is not due to an effect in viability of infected cells but rather due to a direct effect in HIV-1 infection.

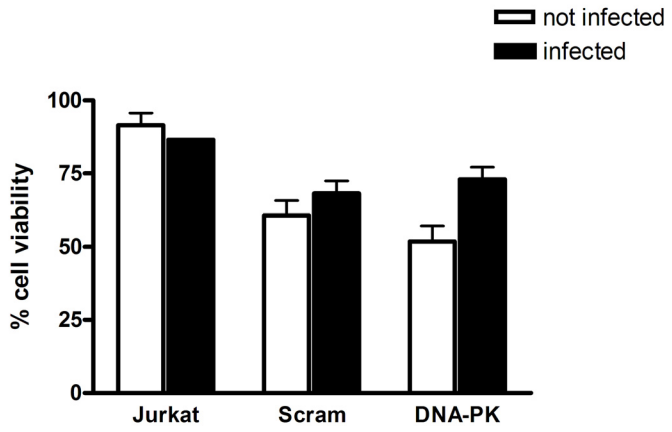

Figure 4.3. shRNA clones viability. After 7 days of infection with HIV-1_{NL4-3}, viability was evaluated in the shRNA clones. Values are correspond to mean ± SEM (n = 3).

4.4.4. shDNA-PKcs do not affect integration or early steps of HIV-1 replication

The observation that DNA-PKcs down-modulation promotes a dramatic reduction of HIV-1 replication, led us to further study the function of DNA-PKcs in different steps of the HIV-1 life cycle. Experiments were performed to evaluate if DNA-PKcs affects viral integration or an earlier step during HIV-1 infection. Thus, we transduced shRNA clones with lentiviruses expressing EGFP cassette, pseudotyped with a VSV-G envelope or with HIV-1 gp120. EGFP gene is under control of ubiquitin-C promoter, allowing the expression of EGFP independently of HIV-LTR. After transduction in the shRNA clones, EGFP is only expressed after viral integration in host cells occurs. EGFP positive cells were detected by flow cytometry.

Transduction with VSV-G pseudotyped lentiviruses allowed the bypass of the entry step of the HIV-1 life cycle and thus only HIV-1 integration was evaluated. Instead, the entry step was assessed after cells transduction with gp120 pseudotyped lentiviruses.

Analysis of EGFP expression at 48 h post-transduction showed that shDNA-PK do not lead to a decrease in fluorescence, compared with shSCRAM, during transduction with VSV-G- and gp120-lentiviral particles (figure 4.4, black bars and white bars, respectively). Indeed, results indicate an increase of EGFP expression when comparing shDNA-PK with shSCRAM.

These results indicate that the restriction effect of shDNA-PK in HIV-1 replication is not reflected neither in integration nor in the early steps if HIV replication.

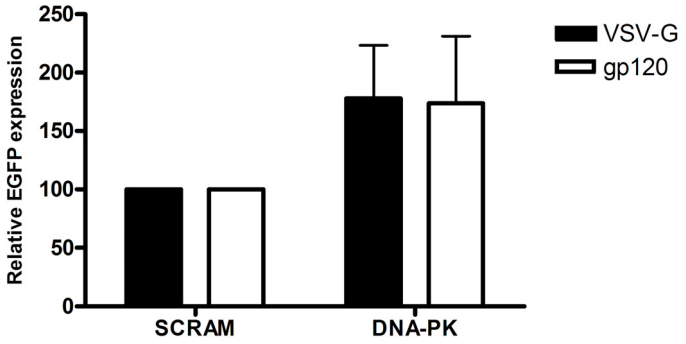

Figure 4.4. Knockdown of DNA-PKcs do not affect integration or entry steps during HIV-1 life cycle. Jurkat shRNA clones were transduced with EGFP-expressing lentiviral particles (FugW-EGFP) pseudotyped with VSV-G and HIV-1 gp120. After 48 h, EGFP expression was measured by flow cytometry. Values are relative to the percentage of shSCRAM EGFP positive cell and represent mean ± SEM (n = 3). Black bars indicate values for shRNA clones transduced with a VSV-G-lentivirus and white bars indicate values to shRNA clones transduced with a gp120-lentivirus.

4.4.5. DNA-PKcs acts as a co-factor in HIV-1-LTR driven transcription

From the previous results we observed that DNA-PKcs is important for HIV-1 infection, once DNA-PKcs knockdown affected viral proteins expression and viral production. Nevertheless, results indicate that DNA-PKcs is not essential for entry or integration of HIV-1. Taken together, we hypothesized that DNA-PKcs could have a role after integration, during HIV-1-LTR driven transcription.

To evaluate if down-regulation of DNA-PKcs interferes with HIV-1-LTR driven transcription, we co-transfected HeLa-P4 cells with a plasmid containing the shDNA-PK and $pHIV_{NL4-3}$. HeLa-P4 cells have the *β-galactosidase* gene under the control of the HIV-1 promoter that is activated in the presence of Tat protein. Thus when cells are transfected and Tat is expressed β-Galactosidase is also produced, being detected by a colorimetric assay (CPRG). β-Galactosidase activity and virus production were evaluated after 48h of co-transfection (figure 4.5). It was observed that cells transfected with pLKO.1shDNA-PK have about 20 % of β-Galactosidase expression compared with pLKO.1shSCRAM transfection (black bars). The decrease of β-Galactosidase suggest that shRNA lead to an inhibition of Tat expression through HIV-1-LTR. Additionally, $p24^{CA}$ levels detected in cells culture supernatant corroborate the previous results, demonstrating a significant decrease in viral production in cells with pLKO.1shDNA-PK transfected when compared the control (white bars).

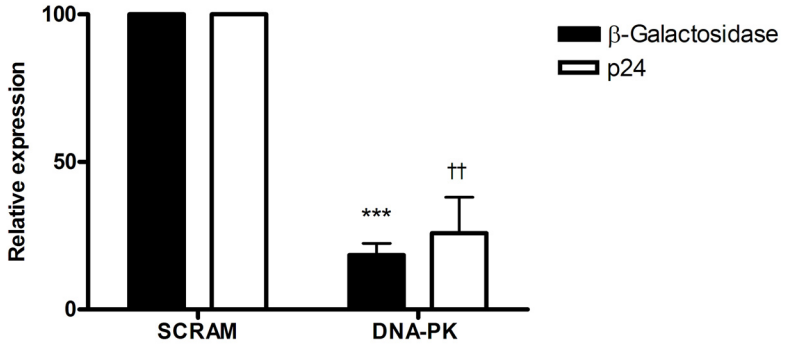

Figure 4.5. DNA-PKcs is important for HIV-1 LTR-driven transcription. Transient assays were performed in HeLa-P4 cells co-transfected with $pHIV\text{-}1_{NL4-3}$ and the different shRNA plasmids. After 48 h, cells were harvested and HIV-1-LTR transcription was measured by quantification of the β-Galactosidase activity in cell lysates. Cell supernatant was also collected to measure viral production by $p24^{CA}$ ELISA. Black bars indicate β-Galactosidase activity in cell lysates and white bars indicate $p24^{CA}$ in the supernatant. Values are relative to the control shSCRAM and represent mean ± SEM (n = 6). *** correspond to $P < 0{,}0001$ and †† correspond to $P < 0{,}001$.

Altogether these results suggest that DNA-PK has a significant effect in HIV-1-LTR driven transcription, acting as co-factor.

4.4.6. shDNA-PKcs affects expression of viral RNA transcripts

The observation that DNA-PKcs acts as a co-factor for HIV-1-LTR transcription led us to question on the underlying mechanism of this protein. To answer this question, we evaluated the production of HIV-1 transcripts by measuring *tat* levels in HeLaP4 cells after 48 h of pLKO.1shRNA and $pHIV_{NL4-3}$ co-transfection. Our data (figure 4.6) showed that down-modulation of DNA-PK led to a decrease in the mRNA *tat* levels and this is reflected in β-Galactosidase expression. In this system, HIV-LTR driven transcription occurs in $pHIV_{NL4-3}$ and in LTR-β-Galactosidase construction. As observed in the previous results, the decrease of β-Galactosidase expression is a consequence of the decrease of Tat levels from $pHIV_{NL4-3,}$ as β-Galactosidase can only be expressed after activation of HIV-LTR by Tat.

These results indicate that DNA-PKcs is important for HIV-1-LTR driven transcription of the viral elongated transcripts.

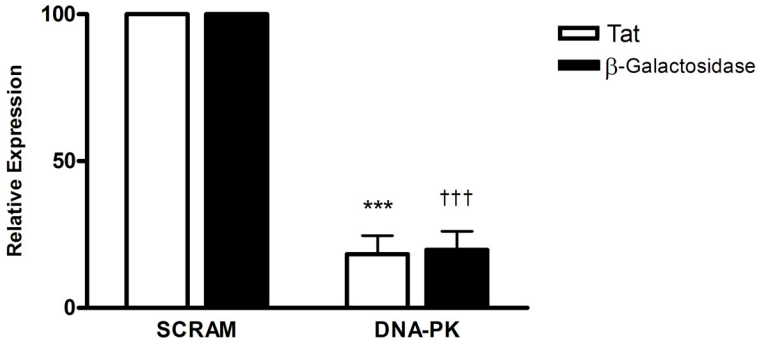

Figure 4.6. DNA-PKcs knockdown affect late LTR-driven transcription. Transient assays were performed in HeLa-P4 cells co-transfected with p_{NL4-3} and DNA-PKcs plasmid and after 48h Tat levels were evaluated by qPCR. Cells lysates were collected to measure β-Galactosidase activity at the same time-point. Values represent mean ± SEM (*n* = 6). *** correspond to *P* < 0,0001. and ††† correspond to *P* < 0,0001.

4.5. Discussion

During HIV-1 life cycle, from entry to viral particles production, HIV-1 hijacks cellular proteins to successfully replicate in its host cell. This intrinsic interplay led to the expansion of the viral co-factors research, and in the last few years, the emergence of new helper factors for HIV-1 replication has been enormously. The development of genome wide screens aided by advances in the application of RNAi has contributed enormously to this growth. Therefore these screens permitted the identification of new co-factors important for HIV-1 replication (Brass *et al.*, 2008; Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009). Concomitantly, our group has also recently performed an RNAi based screen in Jurkat T cells where we were able to identify several newly cellular proteins that were shown to be essential for HIV-1 replication (Rato *et al.*, 2010). Our screen gave a new approach, having identified mainly kinases and phosphatases and proteins related to this class of proteins.

One of the identified proteins from the previous screen was CIB2, a poorly known protein with homology to CIB1. Our previous results indicated that this protein has a role in early steps of HIV-1 life cycle, not affecting integration or viral transcription. CIB2, due to its homology to CIB1, has also been hypothesized to be a DNA-PKcs interacting protein. Based in these homologies we have decided to evaluate the role of DNA-PKcs during HIV infection. Some studies have implicated DNA-PKcs in HIV-1 replication. DNA-PKcs role seems to be positive and has been identified by several research groups as a cofactor for HIV-1 (reviewed in Van Maele and Debyser, 2005), including in one of the genome wide screens described above (Konig *et al.*, 2008). Nevertheless, DNA-PKcs function during HIV-1 replication is controversial.

In this work, we studied the effect of DNA-PKcs down-modulation during HIV-1 infection. The use of shRNAs allowed us to create Jurkat T cell lines that stable express shRNAs against DNA-PKcs. Our results indicated that down-regulation of DNA-PKcs led to a resistance of T-cell clones to HIV-1 replication. This was observed by a decrease in viral proteins expression and consequently, viral particles production. In contrast with previous reports, HIV-1 infection in DNA-PKcs-deficient cells did not led to a decrease in cell viability (Daniel *et al.*, 1999; Baekelandt *et al.*, 2000; Daniel *et al.*, 2004). Differences in the experimental design, including the use of different cell lines, lentiviral vectors and MOI could explain this discrepancy. In our

work, we use a human cell line, Jurkat T cell, constitutively activated that could have masked the apoptotic effect that DNA-PKcs deficiency could induce.

Beyond confirming that DNA-PKcs was essential for HIV-1 replication we addressed the question of in which step of the HIV-1 life cycle could this protein be involved. Our results indicate that DNA-PKcs is dispensable for virus entry and for HIV-1 genome integration, but seems to have an important role in HIV-1-LTR driven transcription. These results flare up the controversy around DNA-PK role during HIV-1 integration. Our study is in agreement with Baekelandt *et al.*, 2000 and Ariumi *et al.*, 2005 studies, where they show that DNA-PKcs is dispensable for HIV-1 integration step. These independent studies together with ours, although using different types of cells and different experimental designs have converged in the conclusion of a dispensable role of DNA-PKcs in HIV-1 integration. Nevertheless, these studies diverge from two other studies from another group, where they identify DNA-PKcs as important for HIV-1 integration (Daniel *et al.*, 1999; Daniel *et al.*, 2004). The differences between this antagonistic data were already described without being conclusive, being the most plausible explanation the differences in the experimental approaches (Ariumi *et al.*, 2005).

Interestingly, when we evaluated the role of DNA-PKcs in HIV-1 integration we observed that not only HIV-1 integration when DNA-PKcs is down-modulated was not inhibited, as the levels of EGFP expression were even though higher that the control shSCRAM. These results corroborate the data reported by Ariumi *et al.*, 2005, where they detected a two fold increase when transduced deficient DNA-PKcs cells with lentiviruses particles compared with non-deficient. These results strengthen the concordance of the two studies and lead to the hypothesis of a positive role of DNA-PKcs in HIV-1 integration.

Our results pointed out a new and different role for DNA-PKcs in HIV-1 infection. Our data indicated that DNA-PKcs plays a critical role in HIV-1-LTR driven transcription and can influence the formation of the elongated transcripts.

HIV-1 LTR driven transcription is dependent on transcription factors as Sp1 and NFκB and initially promotes the formation of short viral transcripts in the absence of Tat. However because Tat is absent, RNAPol II efficiency is very reduced, resulting in the production of abortive short viral transcripts (Rohr *et al.*, 2003; Romani *et al.*, 2010). When expressed, Tat

enhances HIV-LTR transcription endorsing the completion HIV-1 RNA transcription (elongated transcrips).

In 1998, Chun *at al.* observed that DNA-PK interacts with Tat and with Sp1 transcription factor (Chun *et al.*, 1998). In their study, Tat augmented significantly the phosphorylation of purified Sp1 in a DNA-PK dependent manner, indicating a possible positive function of DNA-PKcs in HIV-1 transcription. Therefore, our results together with previous studies can lead to the hypothesis that during HIV-1 replication DNA-PK can play a different role from the DNA break repair. DNA-PKcs could phosphorylate Sp1 transcription factor, enhancing HIV-1 transcription. Concomitantly with these results, DNA-PKcs was already described to phosphorylate Sp1 in HeLa cells (Jackson *et al.*, 1990). Our results, when quantifying Tat levels in HeLa-P4 cells in the presence of shRNA indicate a role of DNA-PKcs in elongated viral late transcripts formation. Therefore, in our model DNA-PKcs can phosphorylate Sp1, influencing viral RNA elongated transcription. Future studies should be performed to evaluate if the crucial role of DNA-PKcs in HIV-1 LTR transcription is due to direct binding to Tat, being recruited to phosphorylate Sp1 and triggering the viral elongated RNA transcription or if DNA-PKcs is responsible for Sp1 activity even when HIV-1-LTR transcription independent of Tat occurs.

With this work we also intended to understand if a correlation exists between CIB2 and DNA-PKcs during HIV-1 replication. Comparing the results from this studies and from our previous study (Rato *et al.*, 2010), we can conclude that these two proteins may have different functions during HIV-1 replication. CIB2 was observed to be important in early steps of the virus life cycle such as entry, having no significant role in viral transcription. In contrast, DNA-PK data showed no significant importance during viral entry but instead demonstrated a strong effect in HIV-1 transcription. Taken together and not excluding the possibility of a hypothetic interaction between CIB2 and DNA-PKcs, these two proteins have separate roles during viral infection.

In conclusion, our study revealed a different role of DNA-PKcs during HIV-1 infection, in HIV-1-LTR driven transcription. This work not only contributed to a better knowledge of DNA-PKcs function during HIV-1 replication but also brought new perspectives for regulation of HIV-1 transcription.

4.6. Acknowledgments

We greatly appreciate the gift of pGagPol, pRev by Dr. Luigi Naldini, pFugW from Dr. David Baltimore, pHEF-VSVG by Dr. Lung-Ji Chang and pSVIIIexe7pA-HxB2 by Dr. Joseph Sodroski. We thank to João Ferreira for its theoretical contribution for this work. This work was supported by Fundação para Ciência e Tecnologia (PTDC/SAU-MII/65346/2006, PTDC/SAU-MII/69280/2006 and PTDC/SAU-MII/78333/2006) and Fundação Luso-Americana para o Desenvolvimento. SR is supported by FCT fellowship SFRH/BD/24129/05 and PMB is supported by a post-doctoral fellowship.

CHAPTER 5

Concluding Remarks

Since HIV-1 discovery in 1983 and subsequent association as the etiologic agent of AIDS, an enormous effort has been made to prolong life of infected patients and to find a cure. However AIDS is still considered an incurable disease. HAART, the current therapy accepted by UNAIDS and WHO, is considered the most effective until now and can prolong the infected patients survival (UNAIDS, 2008). This therapy is based in a combination of antiretroviral drugs that act directly against the virus. Combinations usually comprise three antiretroviral drugs, usually two nucleoside analogues and either a protease inhibitor or a nonnucleoside reverse-transcriptase inhibitor (Clavel and Hance, 2004). Despite the use of different antiviral drugs acting differently against HIV, the emergence of viral mutant persists, leading to antiretroviral agents' resistance. The high mutation rate of is due to reverse transcription notoriously prone to error that introduces on average one mutation for each three viral genome transcribed (Chen *et al.*, 2004). Furthermore, HIV has the ability to persist latently in cellular reservoirs, from where it is triggered (by unknown mechanism(s)) to produce viral particles that infect new cells (reviewed in Richman *et al.*, 2009). Combination of the high mutation rate with the high level of viral production and infection result in a deadly diverse viral population that is very difficult to eliminate.

Facing the difficulties to eradicate HIV-1, new antiviral strategies have to emerge. One possible approach is to target cellular factors instead of viral proteins. HIV-1 is completely dependent on its host machinery, both to promote its replication and, at the same time, to subvert and evade the antiviral responses of the cell (Stevenson, 2003). Moreover, cellular proteins compared to viral proteins are much less variable and some are easily druggable. Therefore, the use of cellular proteins with a positive effect on HIV-1 replication could be a very promising approach for the development of new antiviral strategies. Nevertheless, the only anti-HIV-1 drug that as been clinical approved against a cellular protein is Maraviroc (Pfizer's Sutent), which binds to CCR5 and blocks its interaction with the viral envelope gp120, preventing membrane fusion events necessary for viral entry (Dorr *et al.*, 2005). Three other antagonists for CCR5 are in clinical trials, in phase II and III (Hughes, 2010). The lack of antiviral therapies based in cellular factors offers an opportunity to reveal potential helper factors for HIV-1 replication.

The work presented in this thesis pursuited the identification and characterization of new co-factors for HIV-1 that ultimately could be good targets for new antiviral therapeutics. In

this work we focused on studying kinases and phosphatases, a class of proteins well known for being effectively druggable (Russ and Lampel, 2005). Their catalytic activity and ability to control signal pathways in a "switch on/off" system make them good targets for therapy (Hopkins and Groom, 2002; Russ and Lampel, 2005). In fact, a considerable progress has been made to understand the function of kinases and phosphatases and novel drugs are currently used. Several inhibitors have been developed against these proteins and some of them are already commercialized (Norman, 2010). The first kinase inhibitor commercialized was Imatinib (Novartis' Gleevec) that is used to treat certain types of cancer. It is active against the tyrosine kinase Abl (V-abl Abelson murine leukemia viral oncogene homolog 1). Others like Erlotinib (OSI/Roche's Tarceva), against epidermal growth factor receptor (EGFR) and Sunitinib (Pfizer's Sutent), receptor tyrosine kinase (RTK) inhibitor have been also successful applied against several kinds of cancer. The number of inhibitors, mainly for kinases is growing fast. In 2009, 201 small-molecule kinase inhibitors were in active clinical development, mainly against cancer. Inhibitors against kinases and phosphatases to treat diseases like cancer provide us the hypothesis that these molecules can also be used for HIV-1 therapeutic strategy. Even though the role of kinases and phosphatases during HIV-1 infection has been well described, some aspects are still unclear. Therefore the work presented in this thesis gave an important contribution to the understanding of the puzzling interplay between cellular proteins, specially kinases and phosphatases, and HIV-1.

In this work we successfully developed an innovative strategy based in RNAi technology to identify newly cellular factors essential for HIV-1. We built up a feasible and reproducible shRNA screen in Jurkat T cells against cellular proteins that could contribute for HIV-1 replication, using a shRNA library covering all human "kinome" and "phosphatome". With this technique we were able to identify 14 newly proteins essential for HIV-1 replication but dispensable for cell viability. In this group of proteins we identified 2 phosphatases, 5 kinases, 1 hypothetical kinase-binding-protein, 2 phosphatase-binding-proteins and 4 other proteins with various functions (Chapter 2).

The construction of shRNA libraries that target mammallian genes (Paddison *et al.*, 2004) triggered the development of shRNA screens in various areas facilitating the identification and characterization of cellular genes (e.g. Tsuji *et al.*, 2005; Ngo *et al.*, 2006). Therefore, simultaneously with our study, other groups have also reported screens to identify cellular

proteins important for HIV-1 replication. In 2008 the first genome-wide screening was published by Brass et al., followed by other groups (Konig *et al.*, 2008; Zhou *et al.*, 2008; Yeung *et al.*, 2009) and ours. From these screens, hundreds of cellular proteins needed for HIV-1 to replicate were uncovered. However, a reduced overlap between the different studies was observed. These data highlights the complexity and wide connections between host cell and viruses (Goff, 2008; Kok *et al.*, 2009; Hirsch, 2010). Despite the different screens that have been developed, our strategy stands out due to its original platform with successful cut-off criteria which resulted in a very interesting output. The use of a T cell line to better mimic the natural host cell, together with the use of a restricted shRNA library against human kinases and phosphatases stood up our screen from the previous ones. Our screen was developed in a way that can be easily reproduced with different libraries and different host cells to study other cellular proteins that could have a role in HIV-1 replication.

Although, our major aim was the identification of kinases and phosphatases, we have also pulled out other proteins with different functions. Even though these proteins were not kinases or phosphatases, their putative connection to these proteins and/or their involvement in some signal pathways lead us to include them in the subsequent studies.

From all fourteen proteins identified in our screen, thirteen were validated as essential for HIV-1 replication with no significant effect in cell viability (chapter 2). These proteins were then characterized to understand their function in the HIV-1 life cycle. Crucial steps of the HIV-1 life cycle were analysed in cells deficient for the studied proteins and gathered data indicated that all protein were important during the early steps of HIV-1 infection, before integration, showing no effect in HIV-1 integration. We have also demonstrated that PRKD1, MAP3K2, MAPK9, RAD23B, EZH2, PPFIA2, PPFIBP1, WT1 and STK24 proteins have influence during HIV-1 LTR driven transcription. These results can lead to two hypotheses: these proteins are multifunctional, acting on different replication steps of HIV-1 replication; or the effect observed in HIV-1 entry could be an indirect effect of these proteins on the HIV-1 LTR promoter and other cellular promoters (chapter 3).

Derived from our screen and from the identification of CIB2, we decided to study the role of DNA-PKcs in HIV-1 replication. Due to the homology of CIB2 with CIB1, a DNA-PKcs interacting protein, we evaluated the role of DNA-PKcs during different steps of HIV-1 life cycle. Our results corroborate with previous studies that demonstrated the essential role of DNA-PKcs

for HIV-1 replication (Daniel *et al.*, 1999; Baekelandt *et al.*, 2000; Daniel *et al.*, 2004; Ariumi *et al.*, 2005). Nevertheless our data suggest a role for DNA-PKcs that has not yet been demonstrated. We discovered that DNA-PKcs has a crucial role during HIV-1-LTR driven transcription, and specifically in Tat transcription (elongated transcripts). Our results together with observations in *Chun et al.*, lead to a hypothetical function of DNA-PKcs in HIV-1 transcription through regulation of Sp1 transcription factor. Furthermore due to the dissimilarities between the CIB2 function and DNA-PKcs function in HIV-1 context, these two proteins do not seem to function as partners during HIV-1 replication (chapter 4).

The work presented in this thesis disclosed the function of several cellular proteins during HIV-1 replication, giving a significant contribution to the understanding of complex interplay

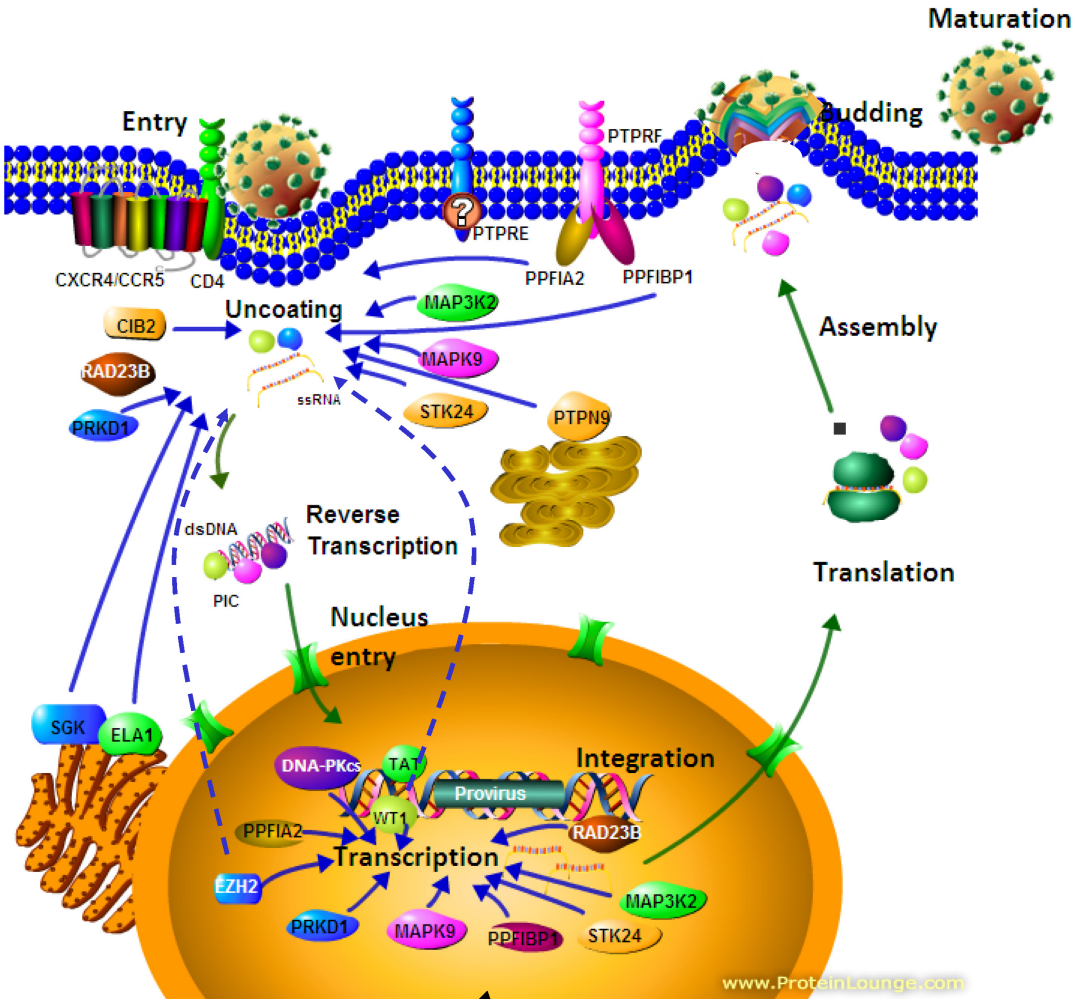

Figure 5.1. Schematic representation of the studied proteins and their function in the HIV-1 life cycle. Green arrows indicate the different steps of HIV-1 life cycle; blue arrows point out where in the HIV-1 life cycle the studied proteins have a positive role. Dash arrows indicate a possible indirect effect in HIV-1 entry mediated by HIV-1 LTR transcription. This figure was designed through the Protein lounge software: Copyright www.proteinlounge.com, 2010

between HIV-1 and its host. Our major conclusions are schematized in figure 5.1.

With the identification and characterization of new cellular proteins with an important role in the HIV-1 life cycle, we also open new perspectives for studying the signalling pathways involved in the interaction between the virus and the host. Moreover, the core analysis performed for the 14 identified proteins revealed that these proteins could be hypothetically connected through common signal pathways in two distinct networks. In network 1, ERK1, Jnk 1 and NFκB were the convergent proteins (figure 2.4 in chapter 2) and in network 2 was cyclin A (figure 2.5 in chapter 2). Future studies could be performed to evaluate the importance of the studied proteins in signal pathways such as ERK1/2, Jnk 1 and NFκb signaling cascades, and corroborate the importance of these signal cascades with the essential role of the identified proteins in the HIV-1 life cycle.

In conclusion the results presented in this thesis have contributed to a better understanding of the complex and intrinsic interplay between cellular factors and HIV-1 and have provided a powerful tool for the identification of new viral co-factors based in RNAi screen analysis. The identification of cellular proteins, specially kinases and phosphatases, essential for HIV-1 replication, but not important for cell survival, furnish promising targets for the development of new therapeutic strategies against HIV-1.

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