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Interaction du domaine nucléocapside de la polyprotéine Gag du VIH-1 avec la protéine cellulaire Unr: implication sur la traduction IRES-dépendante du virus

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I dedicate this thesis:

To my beloved parents

To my brothers Ahmad and Rawad

To the memory of my grandpa

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Table of Contents

ABBREVIATIONS

Aa	Amino Acid
AIDS	Acquired immune deficiency syndrome
ALIX	Apoptosis-Linked gene 2 Interacting protein X
Apaf-1	Apoptotic protease-activating factor 1
APCs	Antigen Presenting Cells
APOBEC3G/F	Apolipoprotein B mRNA-Editing enzyme-Catalytic polypeptide-like 3G/F
ARE	AU-rich instability element
AUF1	AU-rich binding factor 1
AZT	Azidothymidine
BAF	Barrier-to-autointegration
BMH	Branched Multiple Hairpin
CA	Capsid
CBC	Cap Binding Complex
CBP	p300/CREB Binding Protein
CDC	Center for Disease Control and Prevention
CPSF6	Cleavage and Polyadenylation Specific Factor 6
CRFs	Circulating Recombinant Forms
cPPT	Central PolyPurine Tract
Crm1	Chromosome maintenance 1
CrPV	Cricket Paralysis Virus
CSDs	Cold Shock Domains
CSPs	Cold Shock Proteins

CTD	C-Terminal Domain
CTS	Central Termination Sequence
CypA	Cyclophillin A
DIS	Dimer Initiation Site
DCC	Dosage Compensation Complex
EMCV	EncephaloMyoCarditis Virus
ER	Endoplasmic Reticulum
ESCRT-1	Endosomal Sorting Complex Required for Transport-1
ESE	Exonic Splicing Enhancers
ESS/ISS	Exonic/Intronic Splicing Silencers
FDA	Federal Drug Administration
FMDV	Foot-and-Mouth Disease Virus
Gag	Group specific antigen
GDP	Guanosine DiPhosphate
GTP	Guanosine TriPhosphate
GRID	Gay Related Immune Deficiency
gRNA	Genomic RNA
HAART	Highly Active Anti-Retroviral Therapy
HAT	Histone Acetyl Transferase
HAV	Hepatitis A Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
hnRNPA1	Heterogenous Nuclear Ribonucleoprotein A1

HRV	Human Rhinovirus
HuR	Human antigen R
ICTV	International Committee on Taxonomy of Viruses
IDUs	Injection Drug Users
INSTIs	Integrase Strand Transfer Inhibitors
IN	Integrase
IRES	Internal Ribosome Entry Site
ITAF	IRES-Trans-Acting Factors
La	Lupus autoantigen
(L) domains	Late domains
LDI	Long Distance Interaction
LEDGF/p75	Lens Epithelium-derived Growth Factor -75kDa
LTRs	Long Terminal Repeats
MA	Matrix
mCRD	Major Coding Region Determinant of instability
MHR	Major Homology Region
mRNP	Messenger RiboNucleoProtein complex
Msl-2	Male-Sex lethal 2
MSM	Men who have Sex with Men
Myr	Myristyl
Myr (e)	Myristyl-exposed
Myr (s)	Myristyl-sequestered
NA	Nucleic Acid

NCp7	Nucleocapsid protein
Nef	Negative Regulatory Factor
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside/Nucleotide analogue Reverse Transcriptase Inhibitors
NPC	Nuclear Pore Complex
nPTB	Neuronal isoform of PTB
NTD	N-terminal Domain
NUPs	NucleoPorins
ORFs	Open Reading Frames
PABP	Poly(A)-Binding Protein
PBS	Primer Binding Site
PAIP-1	PABP Interacting Protein
PCAF	P300/CBP-Associated Factor
PDI	Protein Disulfide Isomerase
PIC	Pre-Integration Complex
PIs	Protease Inhibitors
PPT	PolyPurine Tract
PR	Protease
PTB	Polypurine Tract Binding protein
PTH	ParaThyroid Hormone
PtdIns(4,5)P2	Phosphatidylinositol-4,5-biphosphate

R	Redundant
RanGTP	Ran bound to Guanosine TriPhosphate
Rev	Regulatory of Virion expression
RRE	Rev Response Element
RT	Reverse Transcriptase
SD	Splicing Domain
SIV	Simian Immune deficiency Virus
SU	Surface glycoprotein
TAR	Transactivation response Region
TRBP	TAR RNA Binding Protein
Tat	Transactivator of transcription
TM	Transmembrane protein
TNPO3	Transportin 3
Unr	Upstream of N-ras
UNG2	Uracil-DNA glycosylase 2
UTR	UnTranslated Region
U3	Unique in 3’
U5	Unique in 5’
VCC	Virus Containing Compartement
vDNA	Viral DNA
Vif	Viral infectivity factor
VLPs	Virus Like Particles
Vpr	Viral protein R

Vpu	Viral protein U
WHO	World Health Organization

Introduction

Chapter I-Human Immunodeficiency Virus type 1 (HIV1)

1.1 HIV Classification

Human Immunodeficiency Virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS) (Barré-Sinoussi et al. 1983). Human Immunodeficiency Virus is a member of the *Lentivirus* genus in the subfamily *Orthoretrovirinae* of the family *Retroviridae.* Retroviruses are part of a large diverse family of enveloped RNA viruses sharing common structures, compositions, and replicative properties. The family name *Retroviridae* is derived from the fact that its members reverse transcribe their genomic RNA (gRNA) into a linear double stranded DNA which is integrated into the host genome (Coffin et al. 1997). *Lentivirus* (Latin: Lenti meaning slow) genus includes non-oncogenic retroviruses that produce multi-

organ diseases characterized by long incubation periods, long-term illnesses, and persistent infections with latency periods (Campbell & Robinson 1998). Lentiviruses are also distinguished from the oncogenic retroviruses by their ability to infect non dividing cells such as macrophages and monocytes (Vodicka 2001). According to the recent classification of the International Committee on Taxonomy of Viruses (ICTV), the *Lentivirus* genus comprises nine species (Figure 1): six non-primate and three primate viruses which are Human Immunodeficiency Virus type 1 and 2 (HIV-1, HIV-2) and Simian Immunodeficiency Virus type (SIV).

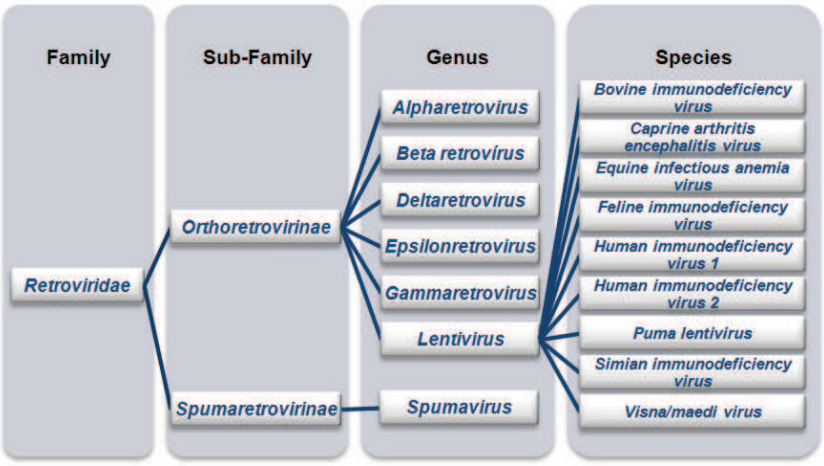

Figure 1: HIV taxonomy as classified by the International Committee on Taxonomy of Viruses (ICTV) 2013).

1.2 History of HIV infection

In 1981, the first clinical case of AIDS was observed in the United States of America after outbreaks of *Pneumocystis carinii* pneumonia (Gottlieb et al. 1981) and Kaposi's sarcoma (Friedman-Kein 1982) among young homosexual men. At that time, the disease did not have a name and it was thought to be restricted to the gay community so it was given different names including Gay Related Immune Deficiency (GRID) or gay cancer since it was often accompanied by cancer. Afterwards, it was found that the disease is not restricted to homosexuals and was thus defined properly by the Center for Disease Control and Prevention (CDC) as AIDS (CDC 1982, current trend updates). However, at that time, the causing agent of the disease was still unknown.

Later in 1983, the team of Professor Luc Montagnier at the Pasteur Institute in Paris isolated the virus from the lymph nodes of a patient with generalized lymphadenopathy corresponding to a prodromal phase of HIV infection (Barré-Sinoussi et al. 1983; Montagnier et al. 1984). Françoise Barré-Sinoussi and Luc Montagnier were awarded the Nobel Prize for Medicine in 2008 for the discovery of HIV (Lever & Berkhout 2008).

Few years after HIV discovery, a new lentivirus was isolated from West African AIDS patients in 1986. This new virus was related to the Simian Immunodeficiency Virus and in general associated with a lower viral load, a slower decrease of CD4 and clinical progression than HIV-1 (Marlink et al. 1994; Clavel et al. 1986). Since it was shown to cause AIDS, the virus was named HIV-2 (Le Guenno et al. 1987).

1.3 HIV Origin and Phylogeny

Genome sequence alignment of HIV-1 and HIV-2 isolates indicated that they share only 40% sequence homology (Guyader et al. 1987). Phylogenetic studies have showed that HIV-1 is closest to Simian Immunodeficiency Virus of chimpanzee (SIVcpz) while HIV-2 is closer to the Simian Immunodeficiency Virus of mangabeys (SIVsm) (

Figure 2). Thus, it was suggested that HIV-1 was derived from the SIVcpz by intraspecies transmission in West Africa (Gao et al. 1999). This could have occurred in West Equatorial Africa as a result of exposure to animal blood while hunting, butchering, or consumption of raw meat (Buonaguro et al. 2007). On the other hand, HIV-2 originated as zoonosis from the sooty mangabey (Reeves & Doms 2002).

Figure 2: HIV origin (A) HIV-1 originates from a single subspecies of the chimpanzee, Pan troglodytes troglodytes **(B)** HIV-2 originates from the sooty mangabey, Cercocebus atys **(C)** the radial phenogram demonstrates the close relationship between HIV and SIV env sequences (distances indicates the relative degree of evolutionary relatedness) (Stebbing et al. 2004).

Due to differences in transmission rates and virulence, HIV-1 is pandemic with a worldwide distribution, while HIV-2 is more endemic, with stable prevalence rates in most countries (Remy 1998; Coffin et al. 1997). For instance, even if HIV-2 is transmitted by the same routes as HIV-1, it is at a lower rate, probably because of a very low virus load in many asymptomatic individuals (Berry et al. 1998; Kanki et al. 1994).

Globally circulating HIV-1 strains are classified into four phylogenic groups: M, N, O, and P. The three groups M, N, and O are the result of independent cross species transmission events from SIV strains naturally infecting African primates to humans (Sharp & Hahn 2011).

Group M (Major) includes the pandemic strains of HIV and is globally distributed in almost every country. Since group M is globally distributed, this led to the predominance of different M subtypes in different areas. Group M is divided into nine subtypes (A-D, F-H, J, K) in addition to more than 40 different circulating recombinant forms (CRFs) generated when a population is co-infected with multiple subtypes (Sharp & Hahn 2011) (Figure 3). Group O (Outlier) regroups viruses isolated from individuals living in Gabon, Cameroon and Equatorial Guinea and represents less than 1% of HIV-1 infections (Peeters et al. 1997;

Mauclère et al. 1997). The group has a 30-50% sequence divergence from group M depending on the compared gene (Yamaguchi et al. 2003). A new HIV strain, distinct from group M and Group O, was isolated from Cameroon in 1998 and called Group N (New or nonM/nonO). To date, only 14 cases have been documented (Simon et al. 1998; Vallari et al. 2010; Delaugerre et al. 2011). Recently, in 2009, group P was discovered in a Cameroonian woman living in France (Plantier et al. 2009). Group P has been identified so far only in one other person also in Cameroon (Vallari et al. 2010; Mourez et al. 2013).

HIV-2 is divided into seven subtypes (A- G) with subtype A accounting for the majority of HIV-2 infections (Reeves & Doms 2002).

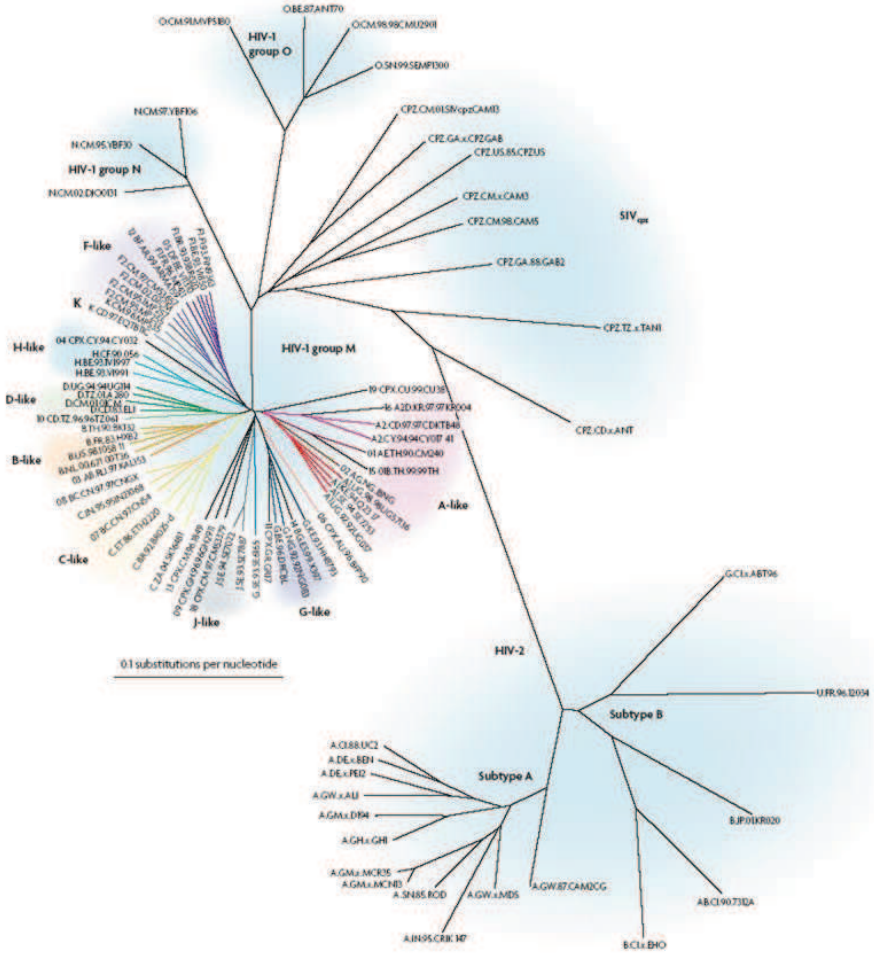

Figure 3: A comparison of the genetic similarity between different HIV and SIV strains by aligning the full genome sequences of 87 human and simian lentiviruses. HIV-2 and HIV-1 share around 50–60% sequence identity. The origins of the HIV-1 groups indicate two probable jumps from chimpanzee (groups M and N) and gorilla (group O) species. HIV-1 M subtypes probably evolved from a single introduction into the human population before diverging into different subtypes (Ariën et al. 2007).

1.4 Epidemiology of HIV infection

According to the World Health Organization (WHO), an estimated 35 million people were living with HIV in 2013 of which 3.2 million were children below the age of 15 (Figure 4).The increase of the number of people living with AIDS from previous years is due to the fact that more people are receiving the life-saving antiretroviral therapy. There were 2.1 million new HIV infections globally showing a 38% decline in the number of new infections from 3.4 million in 2001. In parallel, the number of AIDS deaths is also declining with 1.5 million AIDS deaths in 2013, down from 2.3 million in 2005. The highest mortality rate is in Sub-Saharan Africa where there is the highest HIV prevalence (Figure 5).

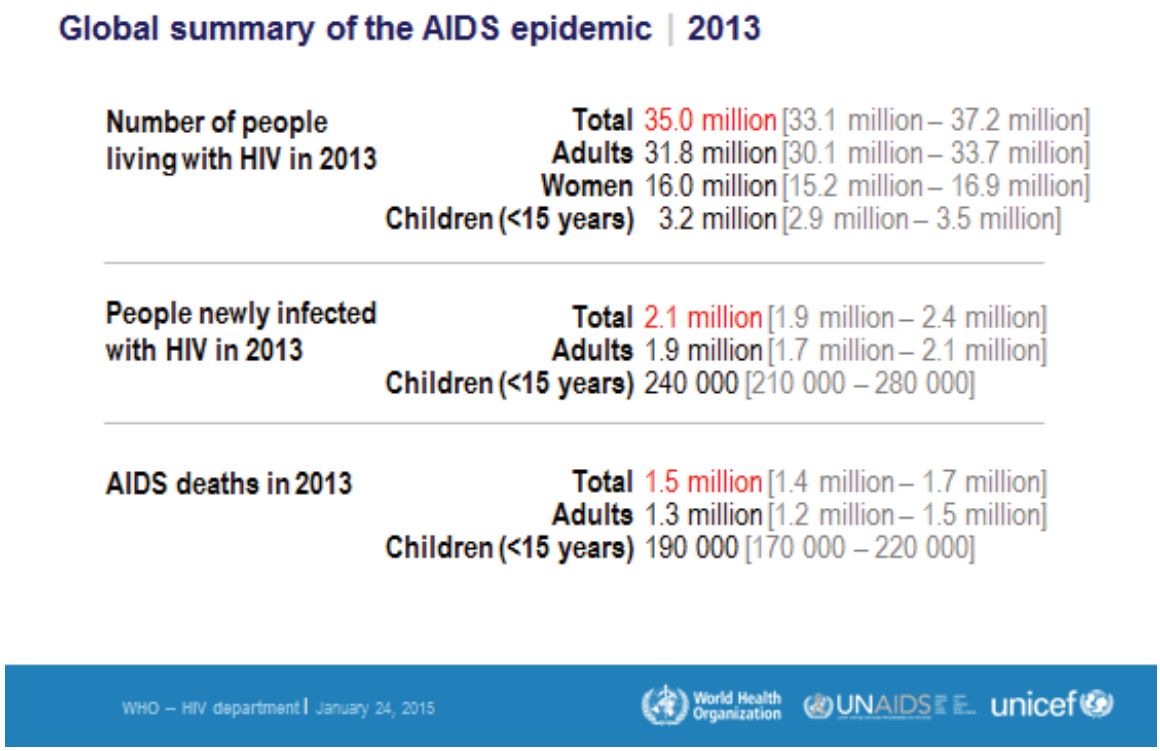

Figure 4: WHO 2013 statistics on AIDS

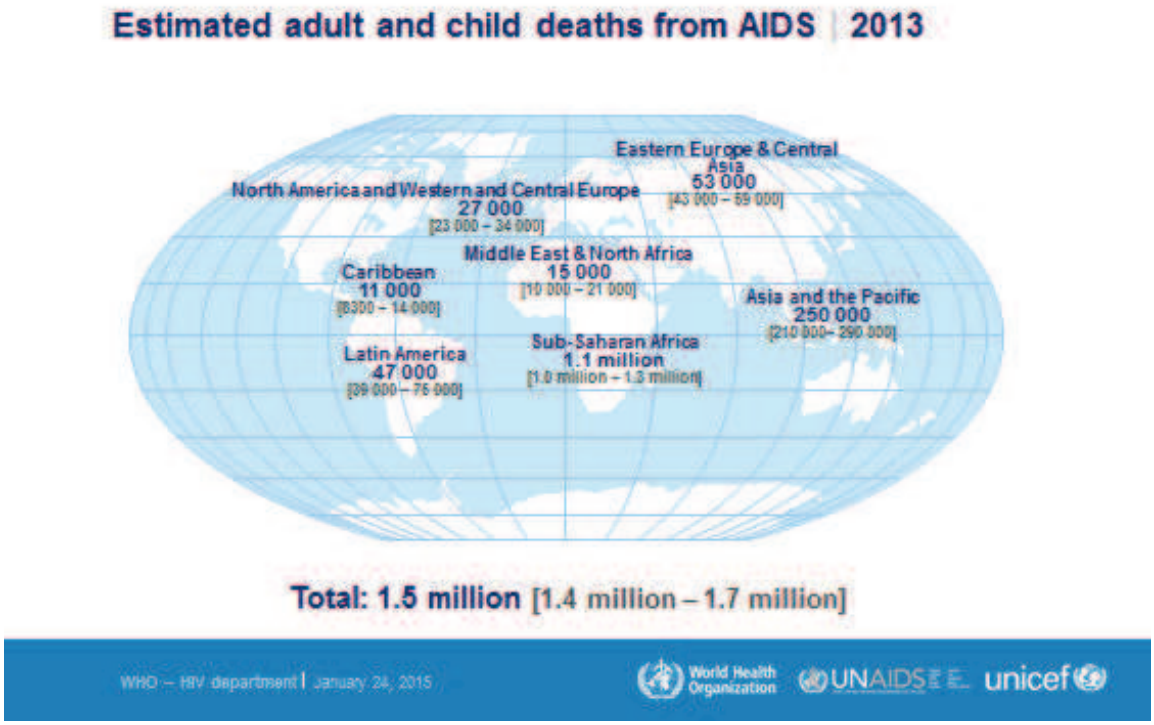

Figure 5: Geographic distribution of adult and children deaths due to AIDS in 2013 as estimated by WHO. The highest mortality rate is in Sub-Saharan Africa where there is the highest HIV prevalence

Molecular epidemiology studies showed that there is a specific geographic distribution pattern of HIV-1 subtypes. Globally, the most prevalent genetic subtypes are A, B, and C with subtype C accounting for more than half of all HIV-1 infections. Subtype C viruses are prevalent in the countries accounting for more than 80% of the global HIV-1 infections. Subtype A viruses are predominant in central and eastern Africa as well as Eastern Europe. Subtype B is dominant in Western and Central Europe, America, Australia, Northern Africa, and Middle East. In addition, CRFs are gaining more importance in the HIV pandemic constituting around 18% of infections (Buonaguro et al. 2007).

Subtype distribution was highly associated with demographic parameters suggesting highly compartmentalized epidemics, determined by social and behavioral characteristics of the patients (Abecasis et al. 2013). In France, viral diversity has increased in all risk groups over the last 15 years and although subtype B is the major subtype in France, the number of non-B virus infected patients is increasing (Chaix et al. 2013; Brand et al. 2014).

1.5 HIV transmission

HIV transmission occurs via direct sexual contact, vertical transmission from mother to child, or exposure to contaminated blood, or blood products (Sleasman & Goodenow 2003). The CDC reported that more than half of the new infections occur among men who have sex with men (MSMs). However, heterosexuals and injection drug users (IDUs) are also significantly affected by HIV (Figure 6). The mother to child transmission rate during pregnancy, labor, delivery, or breast feeding is estimated by the WHO to range from 15 to 45% in the absence of intervention.

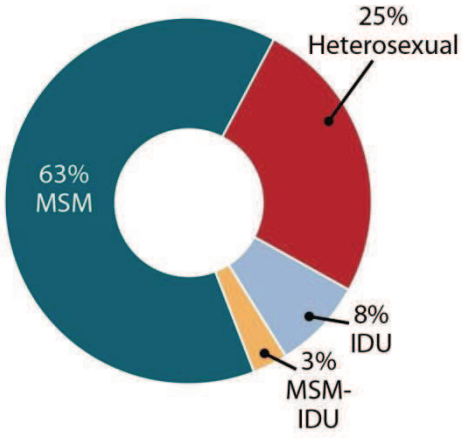

Figure 6: Estimated new HIV infection by route of transmission. Men who have sex with Men (MSM) remain the group most heavily affected by HIV in the United States accounting for more than 63% of new infections in 2010 (CDC 2010).

1.6 Progression of HIV infection

Although the course of HIV infection may vary among different patients, a common pattern is recognized. HIV-1 infection progresses in three phases: acute infection, chronic infection, and AIDS (Figure 7).

Acute infection

The early stage of HIV infection is the acute or primary infection. It corresponds to the period between the infection and the moment when HIV antibodies are detected in blood. This usually occurs 4 to 6 weeks after exposure although in some cases it can take up to 12 weeks.

Shortly after a person becomes infected with HIV, the virus replicates causing a huge rise of viremia (concentration of virus in blood) and rapid loss of CD4+ cells. This phase of intense viral replication promotes the dissemination of the virus throughout the body. This is accompanied by 'mononucleosis like' symptoms such as fever, sore throat, and cervical adenopathy. Rash and fever are the most characteristic symptoms of primary HIV infection. Eventually, the immune system develops a response against the infection bringing back the virus down to a steady-state level which varies depending on each individual.

Since HIV antibodies are not present in blood yet, diagnosis cannot be performed with standard serological tests. However, diagnosis is done based on the detection of viral RNA or p24 (HIV capsid protein) antigens in the plasma of infected individuals. Seroconversion, the onset of anti HIV-antibodies in blood, is the hallmark of transition from this phase to chronic infection (Pantaleo et al. 1993; Kahn & Walker 1998).

Chronic (latent) HIV infection is also referred to as the asymptomatic phase since no clinical symptoms are usually observed. During this phase, the infection is latent through a balance between CD4+ cell production and virus production. In some cases, the virus may be inactive in "reservoir cells" such as macrophages or CD4+ memory cells. However, the term 'latency' is misleading since in this period all patients have a gradual deterioration of the immune system manifested by the depletion of CD4+ cells. The inevitable outcome of this

progressive depletion of CD4 and immune system deterioration is the AIDS phase (Ho et al. 1995; Pantaleo et al. 1993).

AIDS

AIDS refers to Acquired Immunodeficiency syndrome. It is also called symptomatic phase. Progression to AIDS occurs when CD4+ T cells count falls below 200 cells per mm^3. At this point, the immune system failure leads to the emergence of opportunistic infections (Cytomegalovirus, toxoplasmosis...) and tumors (Kaposi's sarcoma, lymphoma...) which would lead to death (Pantaleo et al. 1993).

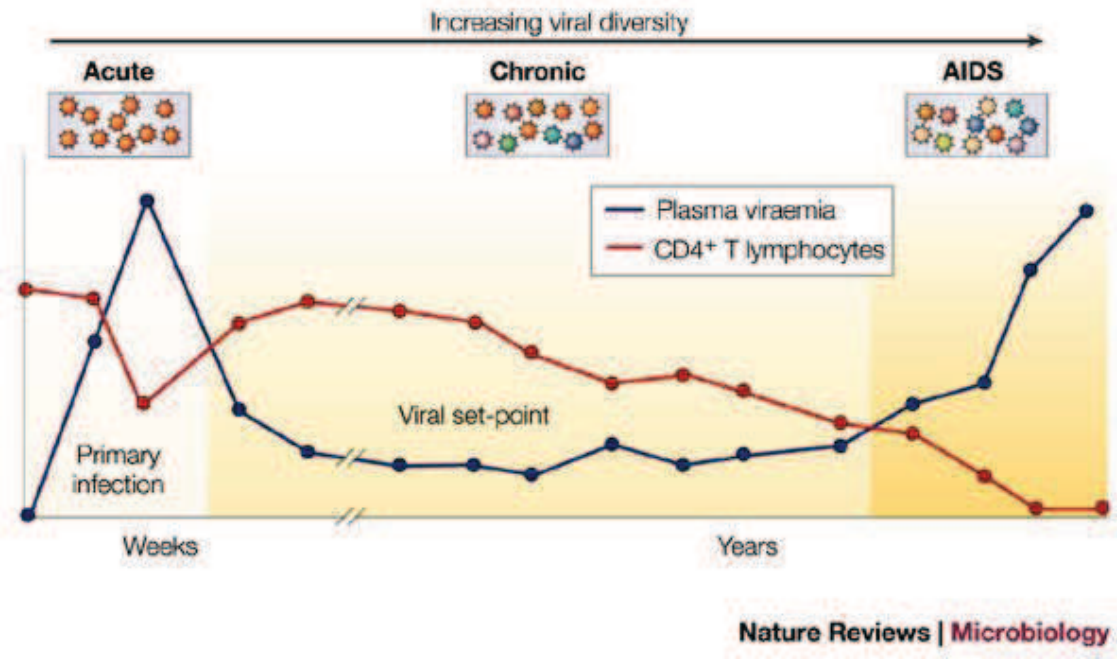

Figure 7: Schematic representation of the course of HIV infection defined by the level of viral replication. Plasma viremia is represented by the blue line whereas CD4+ T cells level is represented by the red line. Patterns of CD4 decline and virus load increase over the average course of untreated HIV infections (Simon & Ho 2003).

1.7 HIV treatment

Up to this moment, there is no drug that can eradicate HIV infection. However, antiretroviral treatment allows viral suppression and reduction of morbidity and mortality (Reviewed in Arts & Hazuda 2012; Este & Cihlar 2010). The Food and Drug Administration (FDA) approved till today 34 single commercial drugs to treat HIV infection. Those drugs are classified into six classes:

- Nucleoside/nucleotide analogue reverse transcriptase inhibitors (NRTIs): This was the first class of drugs approved by FDA in 1987 when the therapeutic activity of azidothymidine (AZT) was discovered. These analogues compete with the natural nucleotides for incorporation into the newly synthesized DNA. Since they lack a 3' hydroxyl group at the deoxyribose moiety, the formation of a phosphodiester bond is thus inhibited resulting in termination of the growing viral DNA chain.

- Protease inhibitors (PIs), first introduced in 1995, inactivate the viral protease (PR) preventing the cleavage of the viral polyproteins Gag and Pol and hence interfering in virion maturation and infectivity.

- Non nucleoside reverse transcriptase inhibitors (NNRTIs): In 1996, the first NNRTI drug nevirapine was approved. This drug class binds to a hydrophobic pocket near the reverse transcriptase enzyme active sites thus modifying the active site conformation and reducing polymerase activity.

- Fusion inhibitors: In 2003, the first peptide fusion inhibitor (enfuvirtide) was approved by the FDA, it targets the viral gp41 preventing the fusion of the viral and cellular membranes.

-CCR5 antagonists: are allosteric inhibitors which block the interaction of the virus with one of its receptors (CCR5) on the cell surface membrane. The first CCR5 antagonist (maraviroc) was approved in 2007.

-integrase inhibitors also known as integrase strand transfer inhibitors (INSTIs) are the most recent antiretroviral drugs approved by the FDA. The leading drug of this class, Raltegravir, rapidly became a powerful tool in the anti-HIV strategy.

After the first HIV-1 drugs were given as monotherapy, HIV care evolved to include a "cocktail" of antiretroviral agents. This was referred to as highly active retroviral therapy (HAART). Combinational therapy was found to dramatically suppress viral replication and reduce the plasma HIV-1 viral load. HAART regimes combine three drugs belonging to at least two classes of the commercially available drugs. Currently, there is three FDA approved drug cocktails (Arts & Hazuda 2012; de Béthune & Béthune 2010; Este & Cihlar 2010).

Despite the efficiency of HAART and the reduction in morbidity and mortality; HIV continues to spread in many parts of the world. This is because, even with extended antiretroviral treatment, the HIV reservoir is not eradicated because treatment does not affect the pool of latent proviruses in "reservoir cells" such as macrophages and CD4 T lymphocytes (Ho et al. 2013). In addition, the genetic variability of HIV strains confers drug resistance by the apparition of escaping mutants. Thus, a safe and effective vaccine would be a promising tool to stop the spread of HIV.

Despite all the attempts, development of such a candidate vaccine remains elusive (Wang et al. 2015; Ensoli et al. 2014). Trials using recombinant forms of the HIV envelope, the target of neutralizing antibodies in HIV infected persons, were unsuccessful due to the high variability of HIV antigens and also due to trimerization, glycosylation, or conformational changes. Attempts to induce T cell mediated immune response also failed since latently infected cells survive cytotoxic T lymphocyte surveillance. When those latent cells are activated, they have the ability to infect new cells before being cleared (Johnston & Fauci 2007). Virus like particles (VLPs), non-infectious particles capable of assembly, have been demonstrated to be potent and efficient stimulators of both humor and cell mediated immune response. Therefore, VLPs are considered as possible HIV vaccines (Yao et al. 2003; Jaffray et al. 2004).

Chapter II- Organization of the HIV-1 Particle

2.1 Structure of the Viral Particle

The structure of mature HIV-1 virions was determined by electron microscopy in 1987. HIV forms spherical, membrane enveloped virions with a diameter around 100-120 nm (Gelderblom et al. 1987). The viral envelope is composed of a lipid bilayer. From the HIV-1 envelope around 10 protein projections called spikes can be observed. Spikes are trimers of dimers of the envelope surface (SU, gp120) and transmembrane (TM, gp41) glycoproteins (Aloia et al. 1993; Checkley et al. 2011). During the virus budding a large array of cellular proteins are also incorporated inside the virus including actin, major histocompatibility complex proteins MHC I and MHC II, or intracellular adhesion molecules as ICAM1, CD28,… (Arthur et al. 1992; Ott 2008; Linde et al. 2013). Some of them like cyclophilinA were shown to play a role in the HIV infection process (Thali et al. 1994).

Directly, below the envelope, a matrix shell formed by the oligomerization of ~ 2000 copies of the matrix protein (MA, p17) lines the inner surface of the envelope (Figure 8 for a schematic representation of a mature virion). The conical shaped capsid assembled from the homo-oligomerization of ~ 2000 copies of the capsid protein (CA, p24) is located in the core of the viral particle. The capsid encloses two copies of the viral RNA genome covered by nucleocapsid proteins (NCp7) forming a ribonucleoprotein complex which also contains the viral enzymes protease, reverse transcriptase, and integrase (Frankel & Young 1998; Turner & Summers 1999). Additionally the viral accessory proteins Vpr (around 270 copies per virion (Müller et al. 2000), Vif, and Nef (10 copies/virion, (Welker et al. 1998) are enclosed within the virion in addition to a number of molecules of cellular origin implicated in the virus infection cycle. Among them are found: the reverse transcription primer $tRNA^{Lys3}$ (Kleiman et al. 2004), Cyclophillin A (Braaten et al. 1996), actin (Wilk et al. 1999), tRNA synthetase (Cen et al. 2001), and APOBEC3G (Zennou et al. 2004).

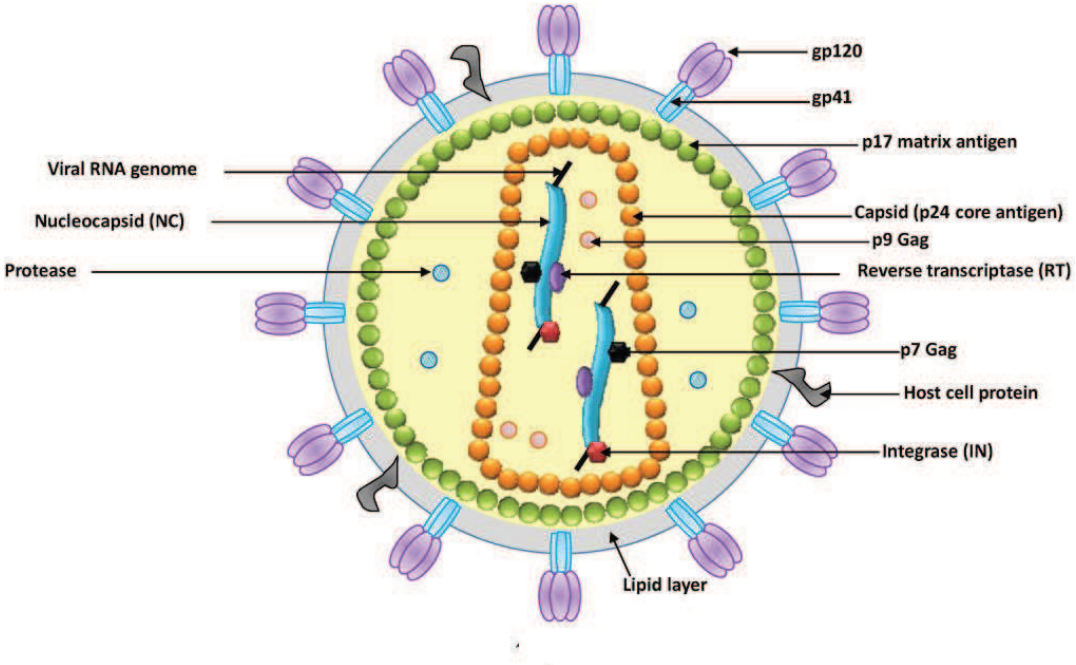

Figure 8: Schematic structure of the HIV-1 mature viral particle. The HIV virion is represented as a sphere containing two copies of the RNA genome associated to the nucleocapsid (NC) protein. The ribonucleocapsid is enclosed within a core made of capsid (p24) protein, which is surrounded by a shell of matrix (p17) proteins that associates to the virion envelope. The external glycoprotein is formed of a dimer of the subunits SU (gp120) and TM (gp41). The virus enzymes integrase (IN) and reverse transcriptase (RT), and protease are also indicated. Adapted from (Sherman & Greene 2002).

2.2 Genomic Organization of HIV

2.2.1 Proviral DNA

In order to replicate its genome, HIV needs to integrate a DNA copy (also called proviral DNA or vDNA) of its genome into the host chromosome. This genomic DNA comprises a central region coding for the structural, enzymatic, and accessory proteins flanked on both extremities by non-coding regions responsible for the control of integration, encapsidation and expression of the genome.

2.2.1.1 Coding Region

The viral genome codes for fifteen mature proteins. Nine of them, called the enzymatic and structural proteins, are obtained from the cleavage of the three polyproteins Gag, Gag-pol, and Env (Figure 9). The other ones are the regulatory (Tat, Rev) and auxiliary proteins (Nef, Vif, Vpr, Vpu) (Freed 2001; Frankel & Young 1998). HIV proteins and their functions will be further developed within this chapter.

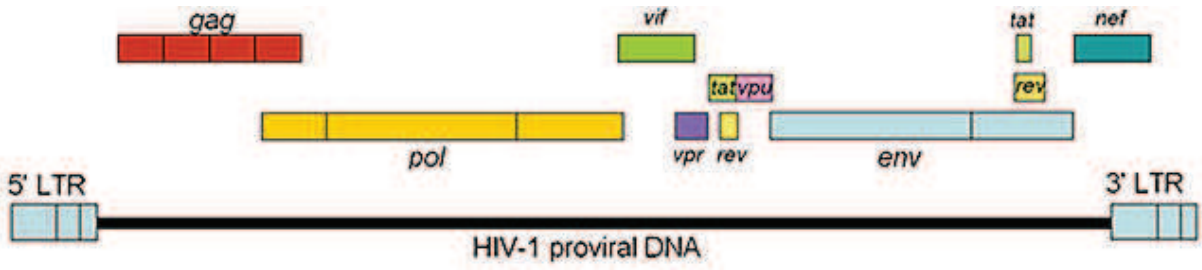

Figure 9: HIV-1 DNA genome scheme. All structural, accessory and enzymatic genes are indicated in addition to the two LTRs as present in an integrated provirus.

2.2.1.2 Non coding regions

All retroviruses possess at both ends sequences of nucleotides called Long Terminal Repeats (LTRs) derived from the 5' and 3' Untranslated Region (UTR) at the extremities of the viral genomic RNA (gRNA) (Temin 1982; Piekna-Przybylska et al. 2011). These sequences are important for the integration of the proviral DNA into the host genome, its transcription into viral RNA, as well as the reverse transcription of the gRNA into viral DNA (vDNA) (Pereira et al. 2000; Reicin et al. 1995).

Each LTR is divided into three regions: U3 (Unique in 3'), R (Redundant), and U5 (Unique in 5').

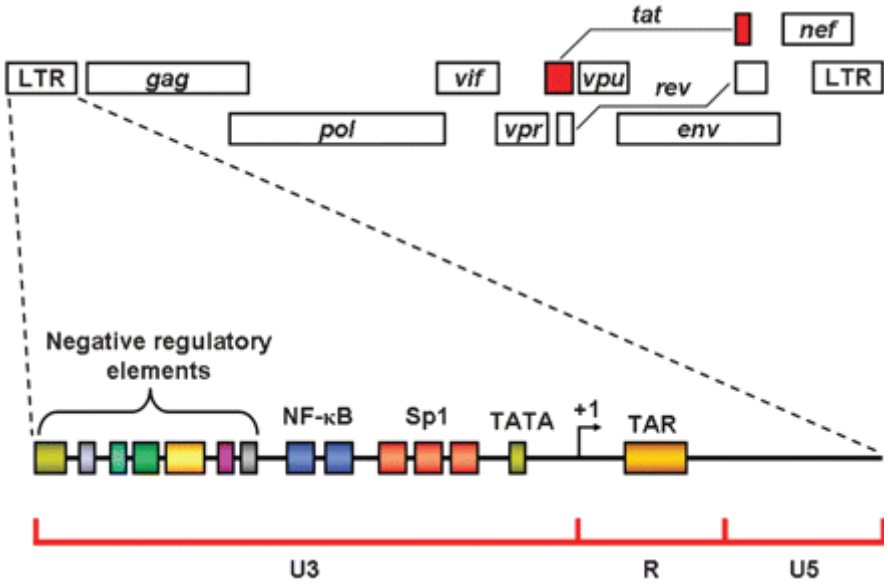

Figure 10: Detailed structure of the LTR. The LTR is divided into three regions U3, R, and U5 (Romani et al. 2010). It contains many binding sites for cellular factors implicated in transcription. Some of these sites such as the negative regulatory elements down regulate HIV-1 transcription when bound to regulatory proteins. The HIV-1 LTR contains a TATA box, two repeats of the transcription factor NF-Kb binding site, and three repeats of Sp1 binding site. The start of transcription occurs at the beginning of the R region indicated by (+1).

The U3 region (positions -454 to 0 in HXb2 strain vDNA) is a G- rich proviral DNA sequence which behaves as a promoter for the viral transcription of the integrated viral DNA by the cellular RNA polymerase II. The U3 region is further divided into three region: a core promoter harboring the TATA box and three binding sites for the transcription factor Sp1, an activator domain with two NF-κB binding sites, and a modulator domain which binds several cellular and transcriptional factors (Gaynor 1992).

Although both LTRs are identical in sequence, they are different in terms of function. The 5'-LTR directs the initiation of transcription whereas the 3' end is involved in the cleavage and polyadenylation of viral transcripts (Klaver & Berkhout 1994; Brown et al. 1991). Transcription of the HIV-1 provirus starts by the binding of cellular factors, including NF-κB, Sp1, the TATA box binding protein, and RNA polymerase II, to the promoter region in the 5′-LTR. Transcription starts at the beginning of the R region in the 5'-LTR and it ends in the R region of the 3'-LTR (Romani et al. 2010) resulting in the so-called 5'-and 3'-UTR in the gRNA (Figure 11).

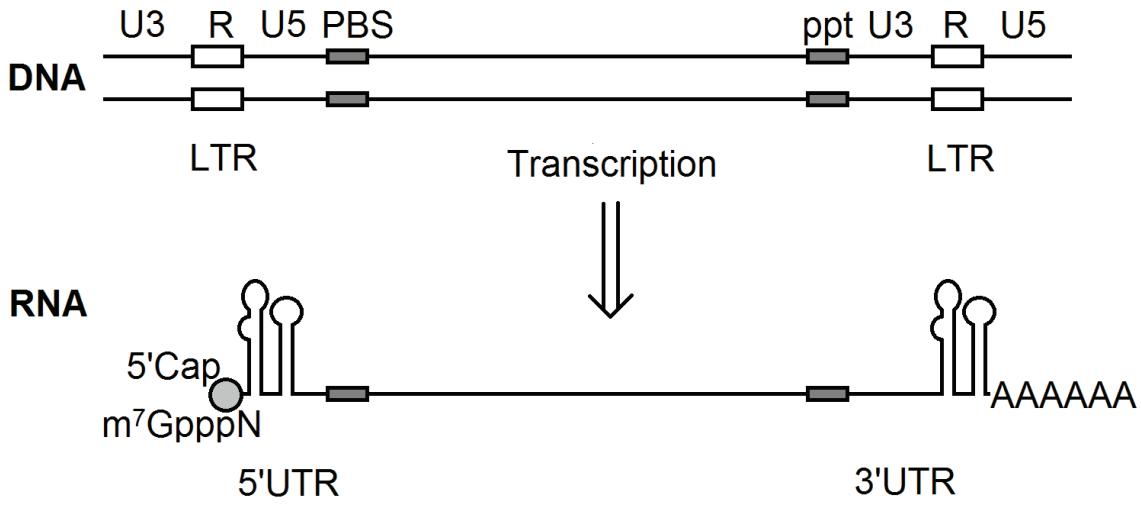

Figure 11: HIV proviral DNA (vDNA) and RNA (gRNA) transcription product. The vDNA is flanked by long terminal repeats (LTR) containing U3, R and U5 regions in addition to the reverse transcription signals: the primer binding site (PBS) and polypurine tract (PPT). Transcription of HIV DNA produces RNA capped at 5' end and polyadenylated at 3' end (Das et al. 1998).

2.2.2 Viral RNA (gRNA)

The HIV-1 genome consists of a dimer of two (+) identical RNA strands of 9.2 kb held together noncovalently with interactions involving a limited number of base pairs in the DIS loop of the LTR (Paillart, Shehu-Xhilaga, et al. 2004). These RNA molecules are capped at their 5'-end and polyadenylated at their 3'-end (Figure 11). Each strand of the viral genome has three major coding regions corresponding to the polyproteins Gag, Pol, and Env. The genome also encodes for the regulator (Tat, Rev) and the auxiliary proteins (Nef, Rev, Vif, Vpr, Vpu, and Tat). The coding regions are flanked by noncoding regions or Untranslated regions (UTRs) at both ends of the genome: 5'-UTR and 3'-UTR (Coffin et al. 1997). The Unique sequence in 5' (U5) is present only in the 5'-UTR whereas the unique sequence in 3' (U3) is present only in the 3'-UTR.

2.2.2.1 5’ -UTR

The highly structured 5’-UTR region is the most conserved part of the HIV genome and is involved in several steps of the viral life cycle including reverse transcription, RNA transcription, genomic packaging, translation, and dimerization (Berkhout 1996; Brasey et al. 2003).

The 5’-UTR of the full length gRNA contains in this order (Figure 12): the R region, the U5 region, the primer binding site (PBS), and the packaging signal ψ.

The repetitive sequence R: The 5’-UTR starts with the redundant R region (positions +1 to 95 in the HXb2 strain vDNA), a 96 nucleotide sequence, present at both ends of the viral genome. The +1 site corresponds to TAR beginning at the 5’ end where transcription starts and to the cap site. This R region plays an important role in reverse transcription and is divided into two hairpins with distinct functions: TAR and poly(A).

The TAR (Transactivation Responsive Region) hairpin in the R region corresponds to the first 58 nucleotides of the viral RNA (positions +1 to +58 in the HXb2 strain vDNA). TAR plays a significant role in the Tat-mediated transcription activation of the viral genome from the promoter localized in the 5’-LTR of the proviral genome. When the viral protein Tat is bound to TAR, it promotes the binding of a transcription elongation factor P-TEFb, composed of cyclin T1 and cdk9. Interaction of Tat with TAR and P-TEFb causes the hypophosphorylation of the C-terminal domain of RNA polymerase II hence increasing transcription (Karn & Stoltzfus 2012). Additionally, virus harboring mutations in the TAR region show a reduced ability in reverse transcription and packaging thus reflecting a role of TAR in both processes. However, TAR requirement is conformation dependent (Harrich et al. 2000). Additionally, TAR is important in the dimerization of the RNA genome since opening of the TAR hairpin leads to aberrant RNA dimerization and packaging (Das et al. 2012).

Moreover, the TAR stem-loop has been shown to inhibit translation directed by the 5’-UTR. However, the La autoantigen protein, an RNA binding protein implicated in cap-independent translation initiation of poliovirus RNA (Meerovitch et al. 1989), was shown to bind to the TAR stem-loop and to alleviate the translation repression of the 5’-UTR (Svitkin et al. 1994).

The poly(A) hairpin is localized downstream of TAR in the R region (positions +72 to +77 in the HXb2 strain vDNA); it contains the polyadenylation signal (AAUAAA) responsible for the addition of the poly(A) tail at the 3'-end of the mRNA. Despite the presence of this sequence on both extremities of the HIV RNA genome; it is functional only at the 3'-end while it is repressed at the 5'-end (Brown et al. 1991).

The Unique in 5' (U5), an 84 nucleotide sequence (positions +96 to +179 in the HXb2 strain vDNA), is the first sequence to be transcribed during reverse transcription. It is involved in the formation of the LTR required for the integration of the proviral DNA. The U5 region is also implicated in a long-distance base-pairing interaction with a region encompassing the start codon of the Gag open reading frame (Abbink & Berkhout 2003) suggested to regulate the translation of Gag. This structure is seen in one of the HIV-1 leader conformations thought to be implicated in the riboswitch which regulates multiple processes like gRNA dimerization and packaging (Abbink et al. 2005; Ooms et al. 2004).

The Primer Binding site (PBS) region is an 18 nucleotide sequence in 3' of U5 (+181 to +198 in HXb2 strain vDNA). It plays an important role in reverse transcription by serving as a primer binding site for the human primer of reverse transcription: $tRNA^{Lys3}$ (Kleiman 2002; Mak & Kleiman 1997).

The leader region or psi region: It is the region located between the PBS and the gag initiation codon (+236 to +336 HXb2 strain vDNA) and its secondary structure is composed of four hairpins called SL1 to SL4 connected by short linkers (Clever & Parslow 1997; Harrison & Lever 1992). SL1 contains the DIS sequence (Dimer Initiation site, +256 to +261), a 6 nucleotide stem loop, involved in the dimerization of the viral genome through formation of a kissing dimer intermediate with the DIS of another RNA molecule (Skripkin et al. 1994). SL2 contains the Splicing Domain (SD, +281 to +299) with a major splice donor site where all HIV-1 transcripts, with the exception of the full length mRNA, are cleaved at this site generating different viral mRNAs (O'Reilly et al. 1995). SL3 is the major packaging site harboring the ψ hairpin and is of particular interest since its sequence is highly conserved among different strains of HIV-1 (Hayashi & Iwakura 1993). SL4 harbors the gag start codon for the initiation of Gag translation.

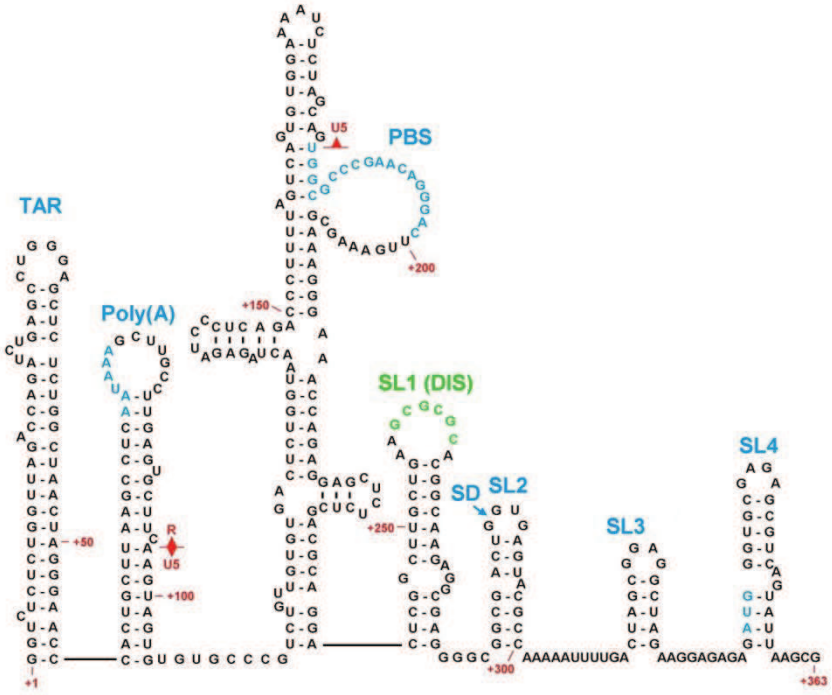

Figure 12: HIV-1 5' RNA Structural Elements. These are the TAR element, the poly(A) hairpin, the U5-PBS complex, and stem-loops 1–4. Nucleotides and numbering correspond to the HIV-1 HXB2 sequence (Russell et al. 2004).

2.2.3 Other Non-coding sequences

Other non-coding sequences exist within the viral genome outside the LTRs.

Polypurine tract (PPT, +8614 to +8638) **and central polypurine tract** (cPPT, +4318 to +4350) are purine rich sequences resistant to the RNase H activity of the reverse transcriptase. PPT is at the 3' end of the viral genome upstream of the U3 region, whereas cPPT is at the center of the genome in the open reading frame of the pol gene. During reverse transcription, the genomic viral RNA is degraded with the exception of these two sequences which serve as primers for the synthesis of the (+) DNA strand (Charneau & Clavel 1991; Charneau et al. 1992). In addition to this property, the main role of PPT and cPPT may be to protect the viral genome from DNA editing by the cytidine deaminase of the APOBEC family and other

factors (Hu et al. 2010). Both PPTs may counteract these enzymes, which are incorporated into the virions and act as natural defense barriers against HIV infection by causing lethal mutations within the viral genome (Wurtzer et al. 2006). cPPT is responsible during reverse transcription (RT) of the formation of a triple stranded DNA structure called DNA flap. A number of papers underlined the role of cPPT in the nuclear import and uncoating of the pre-integration complex (PIC) since HIV mutants lacking the central DNA flap were found defective in nuclear import which could be restored by re-insertion of the DNA flap sequence (Zennou et al. 2000; Sirven et al. 2000; Rivière et al. 2010; Arhel et al. 2007). However, other works showed that the cPPT is not absolutely necessary for nuclear import (Dvorin et al. 2002; Limón et al. 2002; Marsden & Zack 2007).

The Rev-Responsive Element (RRE, +7710 to +8061): is a 352 nucleotide highly structured RNA element located in the env gene. Being present on all partially spliced and unspliced viral RNA transcripts, it serves as a docking site for the viral protein Rev associated to nuclear export cell factors (Crm1; RanGTP…) resulting in the nuclear export of these transcripts (Fernandes et al. 2012; Malim et al. 1989; Farjot et al. 1999; Taura et al. 1998).

2.3 Viral Proteins

The viral genome contains three main open reading frames (ORFs) gag, gag-pol, and env which enable the synthesis of the structural proteins, viral enzymes, and envelope proteins respectively. The genome also encodes for six regulatory and auxiliary proteins Nef, Rev, Vif, Vpr, Vpu, and Tat (Freed 2001; Frankel & Young 1998).

The unspliced gRNA can be translated to provide a 55 kDa myristoylated Gag precursor protein (Pr55Gag) which is cleaved according to a highly ordered and controlled mechanism by the viral protease (PR) during the maturation step to yield the matrix p17 (MA), capsid p24 (CA), nucleocapsid p7 (NC) and p6 proteins. In addition, two peptides, SP1 and SP2, are also generated from sequences on both sides of the NCp7 coding region (Bell & Lever 2013).

Alternatively, a (-1) ribosomal frameshift can occur at the end of Pr55Gag translation resulting in the extension of the gag ORF and continued translation into the pol ORF generating Pr160 (Gag-Pol), a Gag-Pol fusion protein. This protein is cleaved concomitantly or right after the virus release (Kohl et al. 1988) leading to the viral enzymes protease (PRp12), reverse transcriptase (RTp66/p51), and integrase (INp32) in addition to the Gag cleavage products (Waheed & Freed 2012; Ganser-Pornillos et al. 2008), as a result of a highly complex and controlled mechanism (Pettit et al. 2005; Könnyű et al. 2013).

The Env, auxiliary and regulatory proteins are the translation products of mono-spliced (Env, Vpu, Vpr, Vif) and multi-spliced mRNA (Tat, Rev, Nef). The env ORF encodes for the glycosylated gp160 Env polyprotein precursor which is cleaved later to yield the surface glycoprotein (SU) gp120 and transmembrane protein (TM) gp41 (Decroly et al. 1994; Moulard & Decroly 2000).

The auxiliary and regulatory proteins are generated as a result of alternative splicing of the viral mRNA due to the presence of several donor and acceptor splicing sites. Regulatory proteins Tat and Rev are essential for virus propagation. In contrast, the auxiliary proteins Vif, Vpr, Vpu , and Nef are not necessary for virus propagation in cell cultures, but they seem to play a key role in viral pathogenesis *in vivo* (Freed 2001; Frankel & Young 1998).

2.3.1 Envelope proteins

The HIV *env* gene is expressed from a singly spliced mRNA coding also for Vpu (Hunter & Swanstrom 1990; Freed & Martin 1995). Translation of *env* to produce envelope proteins takes place on endoplasmic reticulum-bound ribosomes. Concomitantly to its translation on the rough endoplasmic reticulum, the envelope glycoprotein precursor (gp160) is N- and O-glycosylated with oligosaccharide side chains before its oligomerization (mainly as trimers) and trafficking to the Golgi apparatus (Schawaller et al. 1989; Bernstein et al. 1994; Leonard et al. 1989). In the Golgi, cellular furin and furin-like proteases cleave gp160 to yield the mature SU glycoprotein (gp120) and the TM glycoprotein (gp41) (Hallenberger et al. 1992). After cleavage, the two envelope proteins gp120 and gp41 stay associated by non-covalent

interactions to form a heterotrimeric complex composed of three copies of gp120 and gp41. The complexes of gp120/gp41 are trafficked to the plasma membrane where they are incorporated as trimeric spikes projecting from the viral envelope (Zhu et al. 2003). The surface glycoprotein gp120 is highly glycosylated (20-35 N-glycosylation sites) and is responsible for virion attachment to the cell surface by binding to CD4 receptors on the surface of infected cells. The transmembrane protein gp41 is less glycosylated (3-5 glycosylation sites) and mediates the anchoring of the envelope protein into the host plasma membrane after receptor binding (Checkley et al. 2011; Hunter & Swanstrom 1990; Freed & Martin 1995).

2.3.2 Gag and its Cleavage Products

Gag (group specific antigen) is a 55 kDa myristoylated multidomain protein. As it was mentioned earlier, during virion maturation, Gag is cleaved by the viral protease (Figure 13) into the matrix protein p17 (MA), capsid protein p24 (CA), nucleocapsid protein p7 (NC) and p6 protein in addition to the spacer peptides SP1 and SP2 (Ganser-Pornillos et al. 2008; Waheed & Freed 2012). The Gag protein is the central actor in particle formation. The role of Gag in assembly will be elucidated within the chapter 3.2.4.

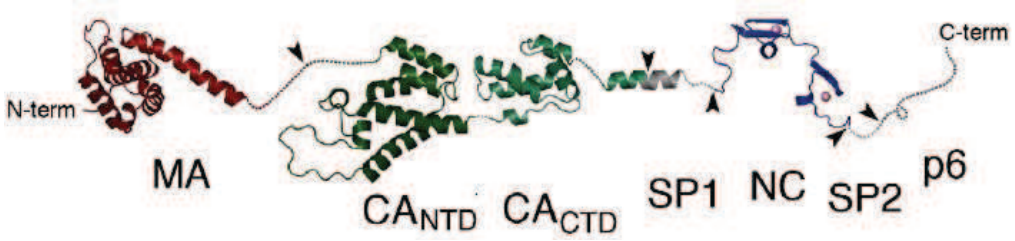

Figure 13: Model of the HIV-1 Gag polyprotein showing MA, CA with its N-terminal and C-terminal domains (NTD/CTD) and NC domain. Protease cleavage sites are indicated by arrow heads (Ganser-Pornillos et al. 2008).

2.3.2.1 Matrix (MA)

The Matrix protein is the N-terminal component of the Gag and Gag-Pol polyproteins. It is a multifunctional protein with several functions in both the early and late phases of viral replication. In a mature virus particle, the 132 amino acids MA protein lines the inner surface of the viral envelope (Frankel & Young 1998).

The MA domain targets Gag to its assembly site at the plasma membrane. A myristyl (myr) group cotranslationally attached to the N-terminus of the protein (Bryant & Ratner 1990) is responsible for anchoring Gag to the inner leaflet by its insertion into the plasma membrane. However, myristoylation alone is not sufficient for stable membrane binding (Yu et al. 1992). The myr group synergizes with a highly basic patch of amino acid residues to facilitate Gag anchoring to the plasma membrane through electrostatic interactions with the acidic phospholipids in the plasma membrane (Saad et al. 2006). Mutations blocking myristoylation or affecting the basic patch lead to inefficient Gag targeting to the plasma membrane (Ghanam et al. 2012; Bukrinskaya 2004).

Gag membrane binding is thought to be regulated by a "myristyl switch mechanism" where the myristate shifts between two conformations (Figure 14): the myristyl-exposed (myr e) and myristyl- sequestered (myr s) states (Spearman et al. 1997). The exposure of this myr is dependent on protein trimerization and factors causing protein self-association such as increasing Gag concentration or RNA binding. Thus, a MA monomer is in the myr (s) state. On the other hand, MA trimerization triggers the exposure of the myristyl group (Zhou & Resh 1996; Tang et al. 2004). Therefore, HIV-1 Gag multimerization is coupled to efficient membrane binding through mediating the exposure of the myristyl group (Resh 2004; Ono et al. 2005; Dalton et al. 2005). Moreover, it was shown that MA binds preferentially to phosphatidylinositol-4,5-biphosphate (Ptdlns(4,5)P2), a phospholipid enriched in the inner leaflet in the plasma membrane (Ono et al. 2004). In fact, depletion of Ptdlns (4,5)P2 from the plasma membrane leads to the accumulation of Gag at the late endosomes rather than at the plasma membrane. Moreover, this interaction also triggers the exposure of the myristyl switch suggesting a possible mechanism for Gag targeting to lipid domains where Ptdlns (4,5)P2 is enriched (Saad et al. 2006).

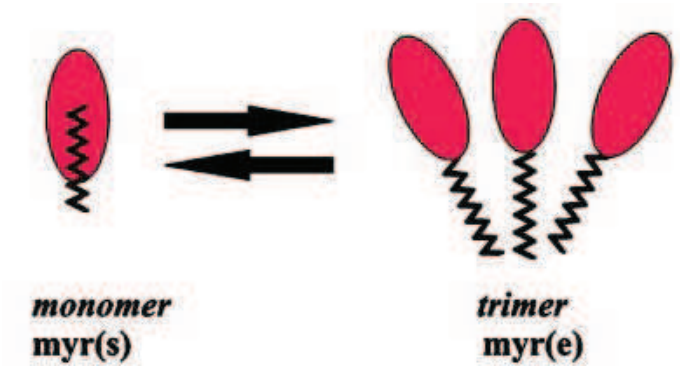

Figure 14: Myristyl switch as proposed by Tang et al. In monomeric Gag, the myristyl is sequestered inside the matrix domain of Gag; however, upon trimerization the myristyl group is exposed (Resh 2004).

Additionally, in most but not all cell lines, the MA domain of Gag is required for the incorporation of Env proteins into the viral particles. This cell dependent requirement of MA for gp41 incorporation suggests that host factors are involved in the interaction between MA and gp41 cytoplasmic tail and thus, required for Env incorporation into the virus (Dorfman et al. 1994; Freed & Martin 1996a; Murakami & Freed 2000).

Although not clearly understood, MA as a domain of the polyprotein Gag seems also to play a role in viral gRNA packaging. This is attributed to its basic RNA binding domain (aa 26-30) able in the absence of the NC domain, to drive Gag binding to viral and cellular RNAs (Burniston et al. 1999; Ott et al. 2005). The MA domain of Gag serves thus as one of the scaffolds that bring together Gag, RNAs, and Env to the plasma membrane.

As a part of the preintegration complex, the mature MA protein is also involved in the early steps of the life cycle. Since MA bears two nuclear localization signals (NLS) (Bukrinsky, Haggerty, et al. 1993; Haffar et al. 2000), it may contribute to the import of the preintegration complex (Gallay et al. 1996).

2.3.2.2 Capsid (CA)

Capsid is the second domain of the Gag polyprotein (mentioned CA as a domain of Gag or CAp24 as the mature form). Around 2000 CAp24 molecules arranged in hexamers form the

viral capsid, the virion core. CAp24 is a 24 kDa protein structurally divided into two independently folded domains: the N-terminal domain (NTD) and the C- terminal domain (CTD) connected by a short flexible linker. The capsid plays roles both in the early and late steps of the viral life cycle (Frankel & Young 1998; Waheed & Freed 2012).

A loop in the NTD interacts with the cellular protein cyclophilin A (CypA) leading to incorporation of the latter into HIV virions (Thali et al. 1994; Luban et al. 1993; Franke et al. 1994). Knocking down CypA or inhibiting its incorporation into virions caused a decrease in infection indicating that CypA is important in the early infection steps (Braaten et al. 1996; Braaten & Luban 2001). It is hypothesized that CypA could stabilize the trans conformation of the G89-P90 bond in CA and thus, affect the viral core stability (Bosco et al. 2002). It is also believed that CypA, at high CypA:CA ratio, could modulate CA-CA interactions within the lattice of the core, thereby causing its destabilization (Grättinger et al. 1999; Gamble et al. 1996). CAp24 is also a target of the cellular restriction factor TRIM5α which binds to and targets the core to the proteasome early in the infection (Malim & Bieniasz 2012).

The CTD of CA bears a stretch of 20 amino acids labeled as the Major Homology Region (MHR) due to its conservation across unrelated viruses (Wills & Craven 1991). The CTD plays a role mainly in assembly and is significant for capsid dimerization and Gag oligomerization (Lingappa et al. 2014).

Furthermore, CA plays also a positive role in the viral genome nuclear import. Several genome-wide siRNA studies identified Nup358, Nup 153, and the nuclear transport factor (TNPO3) as required for HIV-1 infection (Brass et al. 2008; König et al. 2008). This requirement was linked to CA where a specific mutation in CA determined the HIV-1 ability to use Nup 153, TNPO3, and to some extent Nup 358 (Lee et al. 2010). It was proposed by Price et al. that CA binding to the cellular protein cleavage and polyadenylation specific factor 6 (CPSF6) facilitates its use of nuclear import factors such as TNPO3 and nucleoporins. Inaddition, supporting evidence indicates a role of CA in integration efficiency, and chromatin targeting (Zhou et al. 2011; Valle-Casuso et al. 2012; Lee et al. 2010; Schaller et al. 2011).

2.3.2.3 Nucleocapsid (NC)

NC is a 55 amino acid basic protein characterized by two highly conserved zinc finger motifs connected by a basic sequence (Thomas & Gorelick 2008; B. P. Roques et al. 1997). NC exists in the cell and the virus as a domain of the Gag polyprotein (NC) and as a mature protein (NCp7). By binding to nucleic acids, NC and NCp7 are involved in many processes including reverse transcription, genomic RNA encapsidation and assembly of the viral particle by promoting Gag oligomerization. It coats the viral RNA protecting it from nucleases (Darlix 1995, Berkowitz 1993, Dorfmann 1993, Gorelick 1988). NC/ NCp7 will be discussed in more details in Chapter IV.

2.3.2.4 P6

The p6 domain comprises the 51 C-terminal amino acids of Gag and is important for the incorporation of the viral protein Vpr into the virion. It harbors two late (L) domains PTAP and YPXnL (Meng & Lever 2013), whose roles will be described in the next chapter.

2.3.2.5 Spacer peptides SP1 and SP2

SP1 and SP2 are two cleavage maturation products of Gag. SP1 (also called p2) is located between the CA and NC domains whereas SP2 (also called p1) separates the NC region from p6. The precise role of these spacers is still not clarified; however, they seem to regulate the protease cleavage rate (Pettit et al. 1994; Lee et al. 2012). Furthermore, SP1 has been shown to be essential for Gag polymerization and infectivity (Kräusslich et al. 1995) since any mutation in the first residues of SP1 destroys Gag ability to assemble. In link with these two properties, this spacer is supposed to stabilize the immature-like hexamer and its proteolysis is presumed to destabilize the Gag lattice leading to the formation of conical shaped mature capsid lattice (Gross et al. 2000; Datta et al. 2011; Ganser-Pornillos et al. 2008).

2.3.3 Viral Enzymes

2.3.3.1 Protease (PR)

The HIV-1 protease belongs to the family of aspartyl proteases with an active site formed at the interface of the two subunits of the active homo dimer (Navia et al. 1989; Wlodawer et al. 1989). It is released from the Gag-pol precursor by an autocatalytic mechanism. As the virus buds from the plasma membrane, immature particles are noninfectious. Gag and Gag-pol must be cleaved by the viral PR leading to conformational rearrangements within the particles producing mature infectious viruses. The action of PR is detailed in the maturation section of this manuscript (chapter 3.2.5).

Because PR activity regulation is crucial for virus infectivity, PR has been a prime target for the development of anti-HIV drugs. Nowadays several drugs are commercialized and included into HAART. However, drug resistance has emerged due to the genetic variability of the virus leading to selection of strains which can replicate in the presence of those drugs (Sayer et al. 2010; Broder 2010; Doherty et al. 2011).

2.3.3.2 Reverse Transcriptase (RT)

The name of the HIV-1 virus family is due to this enzyme since these viruses need to integrate their genome into the host chromosome and thus to reverse transcribe their gRNA into double DNA. RT is a heterodimer composed of the subunits p66 (560 aa) and p51 (450 aa), both derived from Gag-pol. p66 and p51 share the same amino terminus sequence. The larger subunit, p66 contains the active sites for both the DNA polymerization and RNase H activities. The p51 subunit is considered to mainly play a structural role (Wang et al. 1994). Reverse transcriptase is a multifunctional enzyme which presents RNA-dependent and DNA-dependent DNA-polymerase activities. It presents also a polymerase dependent and independent ribonuclease H activity that degrades the RNA strand from the RNA:DNA heteroduplex during and after reverse transcription, respectively (Peliska & Benkovic 1992; Gopalakrishnan et al. 1992; Peliska et al. 1994; Goff 1990). Reverse transcription is presented in details in part 3.1.2 of this manuscript.

RT has been the first target for anti-HIV drug design. Two major classes of drugs target RT: nucleoside/nucleotide analogue reverse transcriptase inhibitors (NRTIS) such as AZT and non-nucleoside reverse transcriptase inhibitors (NNRTIs) such as nevirapine (Frankel & Young 1998). At least two molecules from these classes are combined to a third antiviral drug targeting another HIV protein in the classical anti-HIV cocktails.

2.3.3.3 Integrase (IN)

HIV integrase is a 32 kDa protein (288 residues) encoded by Gag-pol, like the other viral enzymes. IN is active probably as a tetramer composed of two IN dimers bound to DNA (Ellison et al. 1995). IN is composed of three structural and functional domains: an N-terminal zinc binding domain (HHCC type) that facilitates oligomerization (Bushman et al. 1993), a catalytic central domain which adopts an RNase H fold and a C-terminal DNA binding domain involved in IN and DNA contact (Chiu & Davies 2004). IN is essential for the incorporation of vDNA into the chromosomal DNA of the target cell. The mode of action of integrase in presented in the integration in chapter 3.1.3 of this manuscript.

In addition to integration, IN was reported to be implicated in reverse transcription (Wu et al. 1999; Zhu et al. 2004) where it increases the processivity of RT and suppresses the formation of pause products (Dobard et al. 2007). It also enhances reverse transcription via its interaction with cellular proteins such as SIP2/Gemin 2 and cellular dynein light chain (Jayappa et al. 2015). IN may play also a role in processes nuclear import of the PIC to the nucleus of the host cell, and chromatin targeting (Ao et al. 2010; Hendrix et al. 2011). As the other enzymes, IN is also a target for antiretroviral drugs with several molecules approved by the FDA, as for example raltegravir in 2007.

2.3.4 Regulatory and auxillary proteins

2.3.4.1 Nef (Negative Regulatory Factor)

Nef gene encodes a 27 kDa (206 residues) N-terminally myristoylated protein associated with the cytoplasmic face of cellular membranes (Niederman et al. 1993). Nef is abundantly produced early in the infection when its mRNA represents three quarter of the total viral load mRNA of the cell (Klotman et al. 1991). Despite its name as negative factor, Nef is defined as

a pathogenic factor since a deletion in *nef* gene greatly reduced the severity of infection in humans or rhesus macaques infected by HIV-1 or SIV (Kestler et al. 1991; Kirchhoff et al. 1995). Nef plays multiple roles in the viral life cycle:

A) Nef downregulates CD4 receptors at the surface of infected cells by three pathways. First it redirects some CD4 from the trans-Golgi network to the endosomal compartment, second it triggers the endocytosis of CD4 which are at the membrane and third, it targets CD4 from the endosome to the lysosome for degradation (Kim et al. 1999; Piguet et al. 1998; Mangasarian et al. 1997). CD4 down regulation by Nef i) counteracts the inhibitory effect induced by high CD4 expression level in HIV-1 producer cells (Ross et al. 1999), ii) liberates Lck protein kinase bound to CD4 altering T-cell ability to respond to antigens and cytokines (Chrobak et al. 2010) and iii) prevent potentially lethal superinfection (Michel et al. 2005).
B) Nef downregulates MHCI molecules on the cell surface by a still uncertain mechanism, therefore blunting cytotoxic T cell (CTL) recognition of infected cells and escaping the immune response (Collins et al. 1998).
C) Nef delays the endocytosis and recycling of the T cell receptor (TCR-CD3). TCR is an important component of the immunological synapse formed between antigen presenting cells (APCs) and T cells for antigen recognition. Retarding endocytosis of TCR-CD3 results in less efficient synapse formation (Iafrate et al. 1997).
D) Nef enhances HIV infectivity by facilitating the penetration of the viral core into the cell cortical actin network known as a barrier for intracellular parasitic organisms (Campbell et al. 2004).

2.3.4.2 Vpr (Viral Protein R)

Vpr is a 14 kDa (96 residues) multitask protein packaged into mature virions through the interaction with the p6 domain of Gag. Despite its small size, many functions have been attributed to Vpr (Figure 15), including cell cycle arrest, apoptosis, nuclear import of the PIC, and accuracy of reverse transcription (Romani & Engelbrecht 2009; Guenzel et al. 2014). The HIV-1 genome encodes Vpr whereas the HIV-2 genome encodes both Vpr/Vpx proteins. Both

Vpr and Vpx are homologous proteins with functional and structural similarities. Vpx seems to be originated by gene duplication from Vpr (Strebel 2013).

A) Vpr does not harbor a classical nuclear localization site but it bears two N-terminal α-helices, and as part of the PIC, it facilitates the infection of non-dividing cells such as macrophages by shuttling the PIC to the nucleus (Miller et al. 1997; Vodicka et al. 1998).
B) Vpr blocks the proliferation of infected cells by inducing a G2/M cell cycle arrest which could explain the modest Vpr enhancement of LTR driven transcription, but it is not clear how this cell cycle block contributes to viral replication (Cohen et al. 1990; Jowett et al. 1995).
C) Vpr induces cell apoptosis during HIV-1 infection by permeabilizing the mitochondrial membrane and inducing the release of pro-apoptotic proteins. Additionally, Vpr induced G2/M cell cycle may also contribute to apoptosis (Guenzel et al. 2014).
D) Vpr interacts with several cellular proteins including UNG2 (uracil –DNA glycosylase 2) which is found in virions (R. Chen et al. 2002; Chen et al. 2004). UNG2 is a DNA-repair enzyme that specifically removes the RNA base enzyme uracil from DNA. The inclusion of Uracil in DNA can occur by mis-incorporation of dUTP or by cytosine deamination thus causing mutations in the HIV-1 genome (Guenzel et al. 2014). These mutations are responsible for the emergence of drug-resistant HIV-1 strains and for rendering the genome non-functional if they are too frequent. Vpr thus modulates the mutation rate of HIV (Mansky 1996).

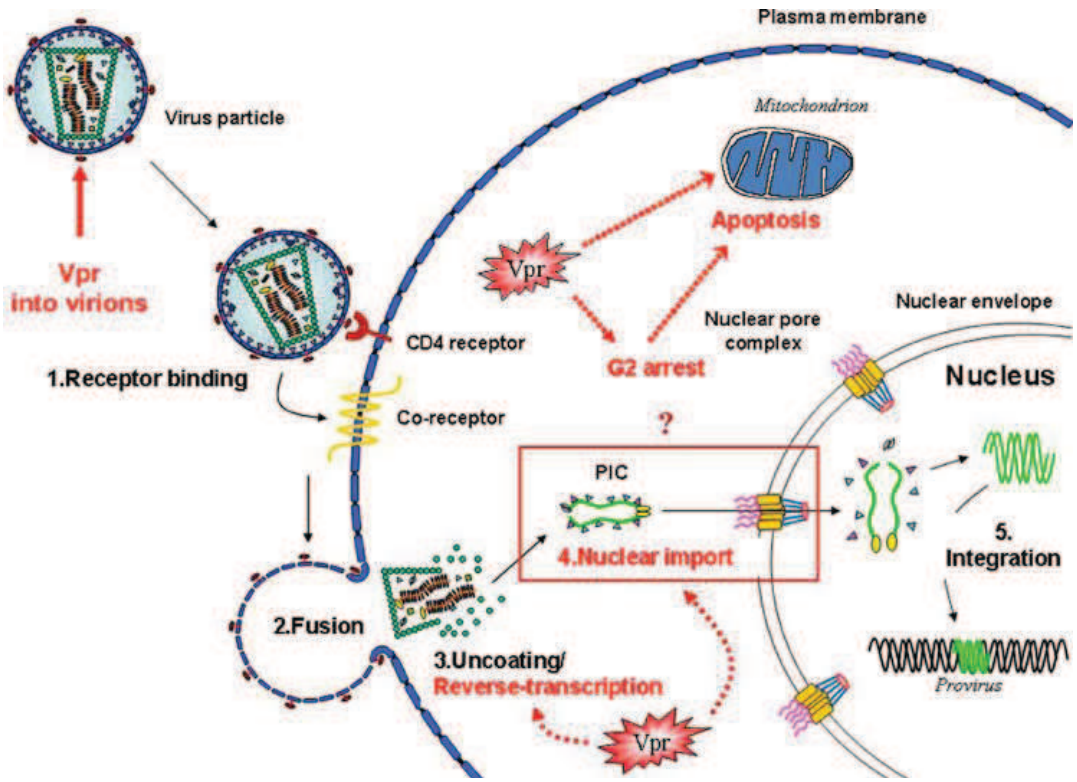

Figure 15: Schematic representation of the early steps of HIV infection. The scheme highlights the events where Vpr is involved. Vpr plays multiple roles during the life cycle including cell cycle arrest, apoptosis, nuclear import of the PIC, and accuracy of reverse transcription (Guenzel et al. 2014).

2.3.4.3 Vpu (Viral protein U)

Vpu is a 16 kDa (81 residues) type I integral membrane protein unique to HIV-1 and most SIV strains but absent in HIV-2. Vpu plays two main roles in infectivity which depend on two separate domains (Bour et al. 1995).

First, Vpu stimulates, in a cell type dependent manner, the release of viral particles from the surface of infected cells. In the absence of Vpu, viral particles cannot be released from the cells and thus accumulate at the plasma membrane, before their subsequent transport to endosomes for degradation (Miyakawa et al. 2009). The restriction factor, tetherin, a glycosylated type II integral membrane protein localized in cholesterol enriched cell plasma membrane domains, directly crosslinks the virions to the plasma or endosomal membranes (Neil et al. 2008; Van Damme et al. 2008; Perez-Caballero et al. 2009). Tetherin is a type I interferon induced protein which acts on a broad range of enveloped viruses (Yang et al.

2010) and is found associated to HIV virions (Fitzpatrick et al. 2010). Vpu antagonizes tetherin by downregulating its surface expression through several mechanisms. These mechanisms include its proteasomal or endo-lysosomal degradation and/or alteration of its trafficking towards the cell surface (Dubé et al. 2010). In HIV-2, host restriction by tetherin is counteracted by the Env protein (Malim & Emerman 2008; Dubé et al. 2010).

Second, Vpu plays a role in the degradation of newly synthesized CD4 receptors. By interacting with the newly-synthesized CD4 in the endoplasmic reticulum, Vpu facilitates their entry into the endoplasmic-reticulum-associated degradation pathway leading to a dramatic reduction of CD4 expression in infected cells (Lindwasser et al. 2007). CD4 degradation induces Env release from the ER and transport of gp120 and gp140 to the surface of the plasma membrane (Tiganos et al. 1997; Bour et al. 1995; Dubé et al. 2010).

2.3.4.4 Vif (Viral Infectivity Factor)

Vif, a 23 kDa (192 residues) basic protein produced during the late phase of the viral life cycle, interacts with the viral RNA (for which it has a chaperone activity) (Henriet et al. 2007; Khan et al. 2001; Bernacchi et al. 2007; Henriet et al. 2005), Gag and Gag-Pol (Huvent et al. 1998), and is found encapsidated in the virion. Vif mutant viruses show markedly reduced levels of viral DNA synthesis and produce highly unstable replication intermediates suggesting that Vif functions before or during DNA synthesis (Simon & Malim 1996). It was called as Viral Infectivity factor since Vif deficient viruses exhibit reduction or complete loss of infectivity in non-permissive cells as monocytes, macrophages, and primary lymphocytes. On the other hand, viruses produced from permissive cell lines (HeLa, 293T, Cos7, Jurkat...) are fully infectious. This difference is due to the expression of two natural cellular inhibitors of HIV replication APOBEC3G (hA3G) and APOBEC3F (hA3F) by non-permissive cells. APOBEC3G/F (Apolipoprotein B mRNA-editing enzyme catalytic polypeptide –like 3G/F) are cellular cytosine deaminases that catalyze cytosine deamination during (-) strand DNA synthesis resulting in i) degradation of viral DNA and ii) lethal hypermutagenesis. Most, but not all of these actions, necessitate hA3G and hA3F virion incorporation. Vif counteracts the effect of APOBEC3G/F by different mechanisms (Figure 16) including the inhibition of their virion packaging (Mariani et al. 2003; Li et al. 2004), their targeting for degradation by the

26S proteasome (Marin et al. 2003; Yu et al. 2003; Sheehy et al. 2003), and the inhibition of their translation (Mariani et al. 2003; Stopak et al. 2003; Mercenne et al. 2010).

In addition, Vif is also implicated in the control of viral reverse transcription (Dettenhofer et al. 2000; Goncalves et al. 1996), RNA dimerization, and viral assembly (Henriet et al. 2007).

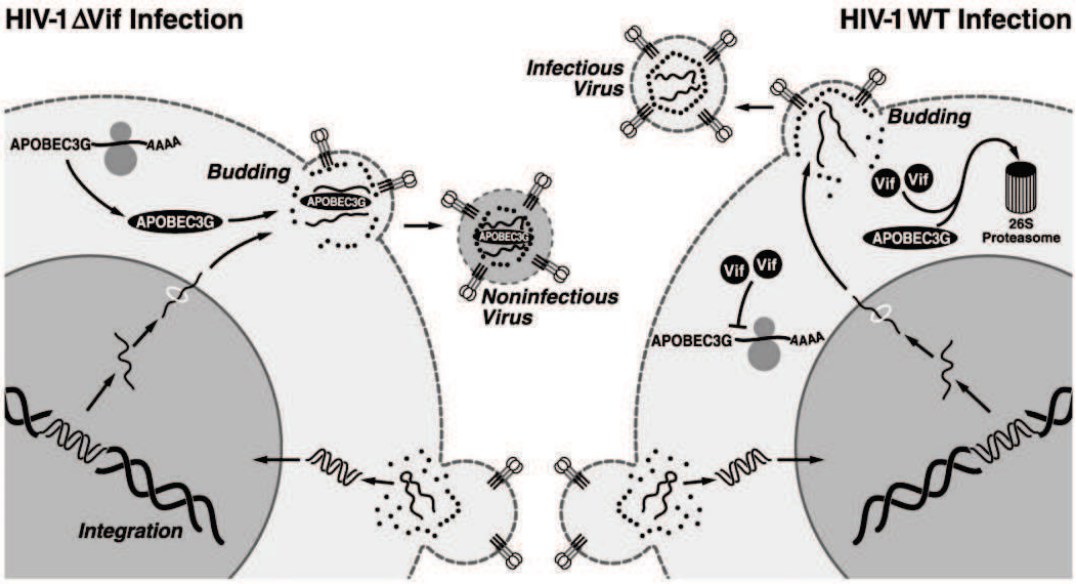

Figure 16: Vif counteracts the antiviral activity of cellular APOBEC3G by impairing APOBEC3G mRNA translation and by targeting APOBEC3G for proteasomal degradation. These mechanisms prevent APOBEC3G incorporation into virions. In the absence of Vif, APOBEC3G is incorporated into virions mutating the reverse transcribed viral DNA within infected cells. This renders APOBEC3G containing virions noninfectious (Stopak et al. 2003).

2.3.4.5 Rev (Regulatory of virion expression)

Rev is a 13 kDa (116 residues) transcriptional activator protein bearing a nuclear export signal (NES) (Fischer et al. 1995) and a nuclear localization signal (NLS) allowing its shuttling between the nucleus and the cytoplasm (Meyer & Malim 1994; Hope 1999). By Rev cooperative binding to RRE, the unspliced or partially spliced RNAs (≈ 9kb and ≈ 4kb HIV-1 mRNA which encodes Gag, Pol and Env) could be shuttled to the cytoplasm via the nuclear pore complex and thus avoid nuclear degradation (Malim et al. 1989; Daugherty et al. 2008). In the absence of Rev, the RNA remains in the nucleus and the splicing machinery quickly splices the RNA so only Tat, Nef, and Rev can be produced (Hope 1997; Fischer et al. 1994).

2.3.4.6 Tat (Transactivator of transcription)

The *tat* gene allows the expression of two small basic nuclear/nucleolar forms of the Tat protein with the minor form composed of 72 residues and the major form of 86 residues. When Tat binds to the nascent TAR RNA element during the LTR driven transcription, it provides a strong activation of the HIV-1 promoter by enhancing the processivity of the transcribing polymerases via an interaction with a cellular transcription factor called pTEFb (Dingwall et al. 1989; Dingwall et al. 1990).

Several other functions of Tat have been reported. Tat was shown to upregulate the expression levels of a number of cytokines, the HIV-1 co-receptor CCR5, and the interleukin-2 (IL-2) receptor (CD25) in HIV-1-infected cells. On the other hand, Tat downregulates several genes such as the MHCI. This regulation consequently induces immune suppression and apoptosis (Romani et al. 2010; D. Chen et al. 2002).

Even if Tat is not described as incorporated into the HIV-1 virion, it was described to have an influence on several steps, including the early steps, of the virus life cycle. Tat was shown to stimulate reverse transcription (Harrich et al. 1997; Apolloni et al. 2013; Kameoka et al. 2002; Chiu et al. 2002). Although data are still controversial. Tat's implication in late steps of viral replication were also mentioned, as for instance in HIV-1 RNA splicing, capping, and translation (Jablonski et al. 2010; Chiu et al. 2002; Braddock et al. 1993). In addition, Tat exhibits a chaperone activity, which may explain its ability to recruit tRNA and stimulate reverse transcription as efficiently as NCp7 (Kuciak et al. 2008; Boudier et al. 2014; Boudier et al. 2010; Apolloni et al. 2007; Harrich et al. 1997; Ulich et al. 1999; Lin et al. 2015).

Chapter III- HIV-1 Life Cycle

The HIV-1 life cycle can mainly be divided into two phases (Figure 17):

- an early phase which starts with the recognition of the host cell, viral entry, reverse transcription, uncoating, nuclear import and ends with the integration of the proviral DNA in the host genome.

- a late phase corresponding to the viral DNA transcription in the nucleus into RNAs, which are transported to the cytoplasm to be translated by the cellular machinery leading to virus protein production, viral particle assembly, budding, and maturation.

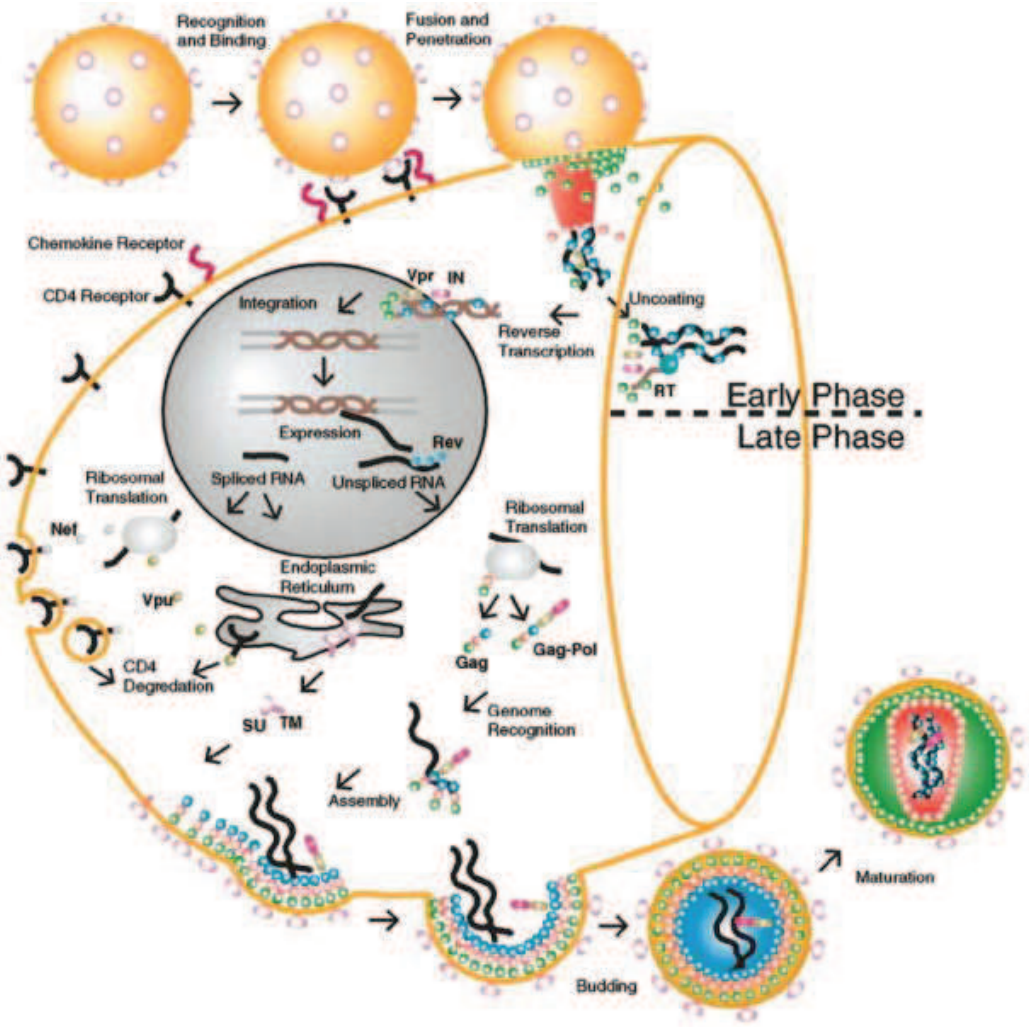

Figure 17: General features of the HIV-1 replication cycle (Turner & Summers 1999).

3.1 The early phase or the pre-integrative phase

3.1.1 Virus Entry

The entry of the viral capsid into the cell requires a fusion between the membranes of the HIV virion and the one of the host cell (Figure 18). The entry process is preceded by a binding or attachment phase during which the virus is adsorbed to the cell surface via specific or unspecific interactions with various types of cell surface molecules, such as the lectins DC-SIGN or heparin sulfate proteoglycans (Mondor et al. 1998; Geijtenbeek et al. 2000). The following entry process is triggered by sequential interactions between the HIV gp proteins and the cellular receptor (CD4) and coreceptors, CCR5 or CXCR4. The HIV-1 virus infects cells harboring the immunoglobulin superfamily member CD4 at their surface, such as T lymphocytes, monocytes, macrophages, follicular dendritic cells, Langerhans cells, and microglial cells (Coffin et al. 1997; Clapham & McKnight 2001; Nisole & Saïb 2004). Binding is initiated by the heterotrimeric complexes composed of the two Env glycoprotein products: gp120 and gp41 (Checkley et al. 2011).

The viral gp120 protein specifically recognizes and binds to CD4 receptors at the cell surface (Figure 18) (Dalgleish et al. 1984; Maddon et al. 1986). This interaction induces a gp120 conformational change that exposes a highly variable co-receptor binding site (Kwong et al. 1998). As a result, gp120 binds to one of the chemokine co-receptors CXCR4 or CCR5 (Feng et al. 1996; Deng et al. 1996; Dragic et al. 1996; Alkhatib et al. 1996; Berger et al. 1999). The co-receptor/gp120 binding induces then a PDI (protein disulfide isomerase)-mediated conformational change in gp120, resulting in the exposure of the gp41 fusion peptide which inserts itself in the plasma membrane of the host leading to fusion of virion and host membranes and delivery of the viral core in the cytoplasm (Barbouche et al. 2003; Gallo et al. 2003; Wilen et al. 2012).

The preferential use of one of the two co-receptors determines the tropism of a viral isolate. HIV-1 strains that use CCR5 receptor for viral entry are designated as R5 viruses. These isolates can infect macrophages and primary CD4+ T cells but not T cell lines. On the other hand, X4 viruses utilize CXCR4 receptor and can infect T cell lines as well as primary T cells.

R5X4 are “dual tropic strains” capable of utilizing either CCR5 or CXCR4 receptors (Berger et al. 1999; Clapham & McKnight 2001).

In parallel to this CD4-dependent entry, HIV-1 virions can enter the cell through clathrin mediated endocytosis. However, the fact that this entry is a productive entry leading to an infection is still subject to debate (Permanyer et al. 2010; Daecke et al. 2005; Miyauchi et al. 2009). It is however possible that endocytosis could be implicated in cell to cell viral transmission via cell-cell contacts known as virological synapses (Jolly & Sattentau 2004; Mothes et al. 2010; Hübner et al. 2009).

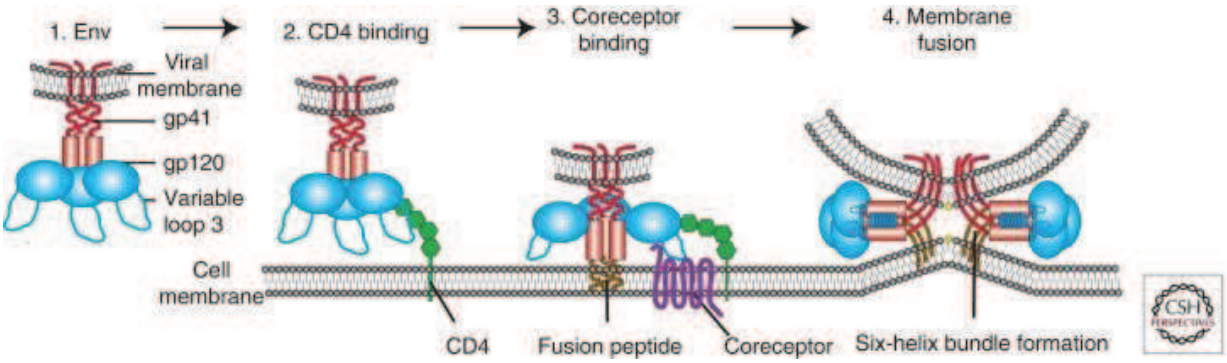

Figure 18: Overview of HIV entry. To deliver the viral capsid into cells, HIV Env, comprised of gp120 and gp41 subunits **(1)**, first attaches to the host cell, binding CD4 **(2)**. This induces conformational changes in Env, allowing coreceptor binding, which is mediated in part by the V3 loop of Env **(3)**. This initiates the membrane fusion process as the fusion peptide of gp41 inserts into the target membrane, followed by six-helix bundle formation and complete membrane fusion **(4)** (Wilen et al. 2012).

As virus entry is a key step for the viral infection, it is a target for pharmaceutical drugs in an attempt to inhibit HIV-1 replication. The first approved viral entry inhibitor was a 36-residue-long peptide called enfuvirtide whose structure is based on the one of gp41 fusion peptide. Its low oral bioavailability and its high production costs are important limiting factors for its use (Kilby et al. 1998). CCR5 is also an excellent target for therapy as individuals homozygous for a 32 base pair deletion in CCR5 (CCR5Δ32) resulting in a stop codon in CCR5 exhibit a profound resistance to infection by R5 HIV-1 virus (Samson et al. 1996; Dean et al. 1996; Huang et al. 1996). Maraviroc, a CCR5 receptor antagonist approved by FDA in 2007, binds

directly to CCR5 inhibiting gp120 association (Dragic et al. 2000). However, it is not active against viral isolates using CXCR4. Additionally, resistance to maraviroc is acquired by virus switching to use CXCR4 co-receptors as well as by acquiring mutations allowing gp120 to use drug-bound CCR5 receptors (Westby et al. 2007; Pugach et al. 2007). CCR4 agonists are as well potential therapeutic drugs against HIV-1 infection (Wu et al. 2015).

3.1.2 Reverse Transcription and uncoating

Retroviruses are defined by their ability to integrate a copy of their genome in the host chromatin. Therefore, the RNA genome should be reverse transcribed by the virion-packaged RT into double stranded DNA before integration of the viral genome in the host cell genome in the nucleus. Most retroviruses depend on mitosis to gain access to the nucleus; however, lentiviruses such as HIV-1 are able to infect non dividing cells by crossing the nuclear membrane most likely via the nuclear pore complex (NPC). The diameter of the nuclear pore is approximately 30 nm whereas the width of the core of the virus is 50-60nm; thus, uncoating of the viral core should occur prior to crossing the nuclear membrane. Uncoating is the disassembly of the protective conical core surrounding the viral genome. The core consists of a lattice of capsid protein hexamers containing two copies of the single stranded RNA genome, several viral proteins (NC, RT, IN, Vpr and perhaps, PR and Nef) and cellular proteins such as CypA in the outer part of the core and APOBEC3G (Ambrose & Aiken 2014; Hilditch & Towers 2014; Welker et al. 2000).

Uncoating occurs after fusion-dependent core delivery into the cytoplasm and before nuclear import; however, the timing and the location of this event is still debated (for reviews see Ambrose & Aiken 2014; Arhel 2010).The first model based on early works suggests that uncoating occurs close to the plasma membrane directly after fusion. This is based on the absence of significant amount of CAp24 within intracellular HIV-1 complexes early after infection (Karageorgos et al. 1993; Iordanskiy et al. 2006a; Bukrinsky, Sharova, et al. 1993; Heinzinger et al. 1994; Fassati & Goff 2001; Grewe et al. 1990). However, this model is based on biochemical studies of cell purified cores which are fragile and susceptible to lose part of their CAp24 content and structure during the isolation procedures. A second model proposes a gradual uncoating during reverse transcription and transport to the nuclear membrane. In this model, mainly based on microscopy studies, reverse transcription and

uncoating are coupled and may regulate reach other (Forshey et al. 2002; Arfi et al. 2009; Hulme et al. 2011; McDonald et al. 2002). Two lines of evidence demonstrate that quick uncoating impairs reverse transcription. First, TRIM5α, a restriction factor that blocks HIV-1 replication by targeting the capsid core for proteasomal degradation early after infection, causes premature uncoating and aborted reverse transcription (Malim & Bieniasz 2012; Stremlau et al. 2004). Additionally, Forshey et al. demonstrated that mutated capsids with increased or decreased core stability impair reverse transcription (Forshey et al. 2002). The third model suggests that uncoating occurs at the plasma membrane upon successful completion of reverse transcription. This model is supported by the observation that inhibition of reverse transcription by Nevirapine led to the accumulation of capsid cores at the nuclear membrane at late time points after infection (Arhel et al. 2007).

Multiple viral and cellular factors were found to play a role in the uncoating process including IN (Briones et al. 2010), Nef (Malim & Emerman 2008), prolyl isomerases Pin1 (Misumi et al. 2010), TRIM5α (Sebastian & Luban 2005), dynein and kynesin 1 (Lukic et al. 2014), and CypA (Luban et al. 1993). However, their implication is still unclear as none of them is absolutely necessary as revealed by the uncoating observed *in vitro* upon synthesis of full-length viral DNA by endogenous reverse transcription (Arhel et al. 2007).

The first viral nucleic acid complexes found inside the cell after entry are called reverse transcription complexes (RTC) as they undergo reverse transcription converting the single stranded RNA genome into double-stranded proviral DNA. Once the RNA is transcribed to DNA, the ribonucleoprotein complex is named as pre-integration complex (PIC). RTC and PIC exact composition is not fully known, but both of them contain the cellular and viral proteins necessary for reverse transcription, cytoplasmic transport, nuclear entry, integration, and expression.

Reverse transcription steps

Reverse transcription proceeds in series of steps (Figure 19) and requires the presence of reverse transcriptase, a multifunctional enzyme harboring RNA-dependent-DNA-polymerase, DNA-dependent-DNA-polymerase, and Ribonuclease H (RNase H) activities (For reviews see (Herschhorn & Hizi 2010; Isel et al. 2010; Mougel et al. 2009).

A) Reverse Transcription is initiated by the hybridization of the virion-packaged cellular $tRNA^{Lys3}$ primer to the PBS sequence located at the 5'-end of the viral genome.

B) The reverse transcriptase extends the 3'-end of the primer thereby synthetizing the (-) ssDNA containing copies of the U5 and R sequences.

C) As the primer is extended, the RNA template associated in a DNA/RNA heteroduplex with the newly synthesized DNA template is hydrolyzed by the RNase H activity of the RT. In order to elongate the minus strand, the (-) ssDNA is transferred to the 3' end of the genome where it anneals to the R region. This is referred to as the first strand transfer.

D) As the synthesis of the (-) DNA strand proceeds towards the PBS sequence in 5' of the gRNA, the RNAse H activity degrades the RNA template except for two purine rich sequences PPT and cPPT. Thus, the PPT and cPPT serve as primers for the (+) strand DNA which lead to the copy of U3, R, U5, and PBS regions from the 5'-end of the (-) strand forming the (+) ssDNA.

E) This synthesis part ends when the RT synthesizes the PBS sequences at both ends of the DNA strands. After tRNA and RNA primers degradation by RNAse H, the (+) ssDNA undergoes a second strand transfer facilitated by the annealing of the PBS sequences of both (+) and (-) DNA strands. This is followed by circularization of the DNA.

F) DNA synthesis continues leading to the formation of the proviral double stranded linear DNA bearing LTR (U3-R-U5) at each end (Freed 2001; Coffin et al. 1997; Arhel 2010). The final product of reverse transcription is a linear double stranded DNA with a central DNA flap corresponding to an overlap of about 100 nucleotides localized in the center of the genome called the central termination sequence (CTS).

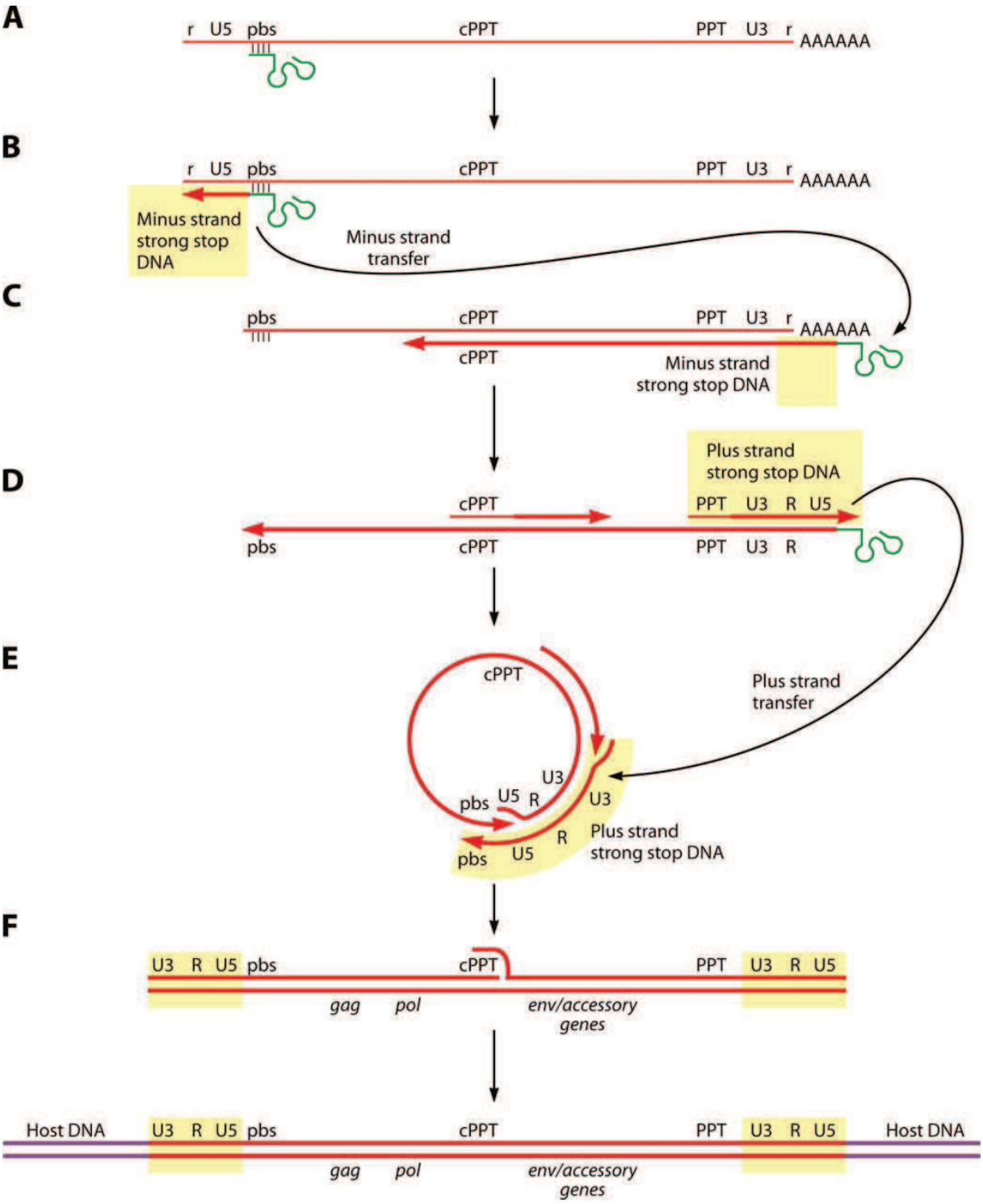

Figure 19: Schematic diagram of events in reverse transcription. Thin red lines represent gRNA whereas thick red lines represent viral DNA. tRNA primer is depicted in green. The letters correspond to the ones in the text above (Onafuwa-Nuga & Telesnitsky 2009).

The Reverse transcription process is highly regulated by cellular and viral proteins, with among them, NCp7, whose role will be described in chapter IV of this manuscript. The

proteins IN, Vpr, Nef and Tat were all found to play a role in regulating fidelity and efficiency of the reverse transcription even if their exact role is still unclear (Hu & Hughes 2012). As discussed in Chapter II, Vif is particularly interesting as it prevents APOBEC3G/F incorporation into virions and mutations during DNA synthesis (Henriet et al. 2009). Several cellular proteins are also implicated in RT regulation as for example, the DNA topoisomerase I (Top1), the kinase anchor protein I (AKAP1), and the human antigen R (HuR) (Warren et al. 2009).

3.1.3 Nuclear Import and DNA integration

Upon completion of reverse transcription, the viral complex becomes a pre-integration complex (PIC) competent for import of viral DNA into the nucleus and its integration in the host chromosomes. The double stranded viral DNA and IN protein form the heart of the PIC (Farnet & Haseltine 1991). The precise identification of the other viral or cellular components is not trivial (Matreyek & Engelman 2013). While MA, Vpr, and RT (Miller et al. 1997; Bukrinsky, Sharova, et al. 1993; Iordanskiy et al. 2006) were repeatedly identified as components of the PIC, the presence of NC and PR is still debated (Farnet & Haseltine 1991; Gallay et al. 1995; Karageorgos et al. 1993). In addition to viral proteins, cellular factors as BAF (Barrier-to-autointegration) (Chen & Engelman 1998), HMG1 (Y) (High Mobility Group Protein) (Farnet & Bushman 1997), the non-homologous DNA end joining pathway protein Ku (Li et al. 2001), and the transcription co-activator LEDGF/ p75 (Lens Epithelium-Derived Growth Factor -75kDa) (Llano et al. 2004) were also found within the PIC.

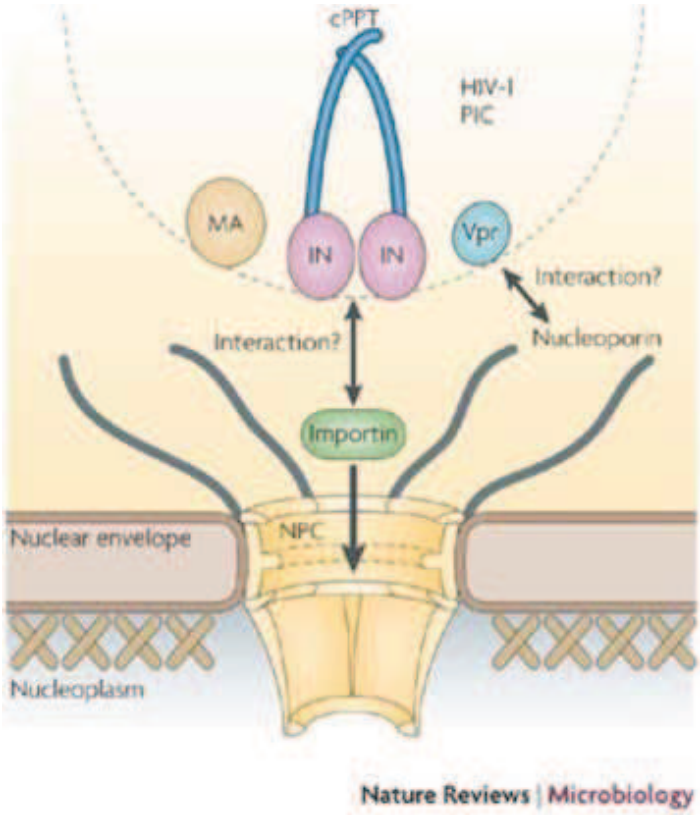

Figure 20: The viral factors MA, Vpr, IN, and cPPT have been proposed to facilitate HIV-1 PICs transport through the NPC. Although MA, Vpr and IN proteins have been reported to interact with importin and nucleoporins, a major component of NPCs, the contribution of these interactions to HIV-1 replication in non-dividing cells is controversial (Suzuki & Craigie 2007).

As the size of the PIC is too large for passive diffusion, nucleoporins (NUPs) are used to enter the nucleus (Sherman & Greene 2002; Suzuki & Craigie 2007). Although the precise mechanism is still debated, the transport of the PIC to the nucleus is thought to be facilitated by various proteins of the PIC even if none of them appears absolutely necessary or sufficient (Figure 20) (Rivière et al. 2010; Piller et al. 2003; Bukrinsky 2004; Fassati 2006). The "central DNA flap" has been implicated in nuclear import of the PIC (Zennou et al. 2000; Ao et al. 2004), as well as the karyophilic viral proteins IN (Gallay et al. 1997; Bouyac-Bertoia et al. 2001), MA (Haffar et al. 2000), and Vpr (Popov et al. 1998; Le Rouzic et al. 2002). Their roles are tightly linked to cellular nuclear transport proteins, such as proteins of the importin α pathway, Transportin 3, nucleoproteins Nup153 and Nup358 or a S/R family protein, CPSF6 (reviewed in Matreyek & Engelman 2013; Fassati 2012). Recent evidence highlighted the importance of the capsid in the PIC nuclear entry. The fact that CAp24 molecules were still found attached to the PIC (Arhel et al. 2007; Dismuke & Aiken 2006) and the recent

identification of cellular proteins implicated in the nuclear import/export pathway as CAp24 partners start to decipher this role (Price et al. 2012; Matreyek & Engelman 2013).

Following nuclear import, the proviral DNA is integrated into the host genome (reviewed by Craigie & Bushman 2012). Integration is catalyzed by the viral IN (Figure 21) which specifically recognizes the two LTRs at the ends of the viral DNA (Coffin et al. 1997). First, IN initiates the 3'-end processing by clipping off two nucleotides from the 3' termini of both blunt-ended strands leaving in all cases the conserved CA-3' sequence (3' processing). Next, IN cleaves the cellular target genome DNA. Then, the 3' recessed ends of the viral DNA are joined to the ends of the cleaved target cellular DNA (strand transfer). The single-strand gaps and the two-nucleotide overhang at the 5' ends of the viral DNA are filled by cellular repair enzyme even if the exact DNA pathway implicated is not unknown (Engelman et al. 1991; Vink, van Gent, et al. 1991; Vink, Yeheskiely, et al. 1991). The whole process results in a five base-pair duplication of the target genome.

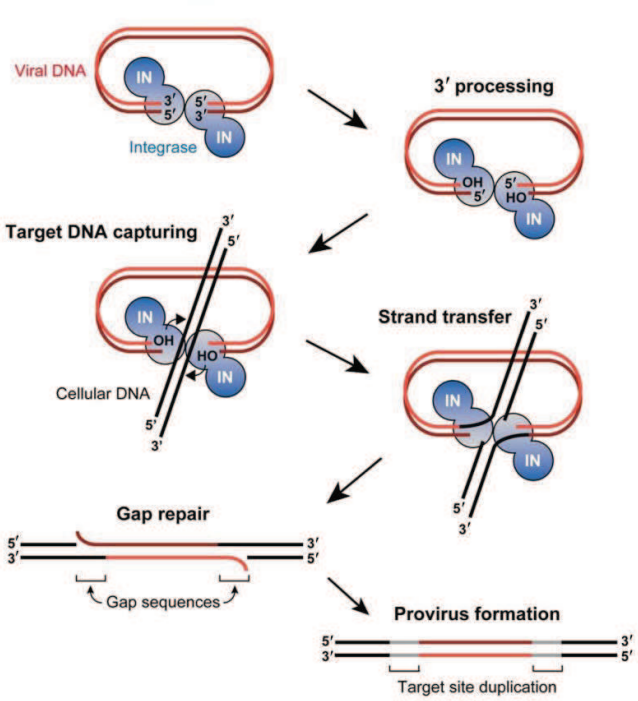

Figure 21: Integration scheme. Integration is initiated by IN recognition of both ends of viral DNA and subsequent removal of two (or three) nucleotides from each of the 3′ ends (3′ processing). The target DNA (i.e., chromosomal DNA) captured by IN is cleaved in a staggered fashion via the exposed hydroxy (OH) groups on the viral DNA ends. Then, the 3′ end of the viral DNA and the 5′ end of the target DNA are simultaneously linked (strand transfer). The excision of the mispaired 5′ viral DNA ends and filling in the single-strand gaps are carried out by cellular enzymes (Suzuki et al. 2012).

The integration of the provirus is not random and occurs at preferential sites of integration called “hot spots” which have been shown to be active transcription units allowing an efficient HIV transcription post integration (Schröder et al. 2002; Lewinski et al. 2006; Jordan et al. 2001). There are multiple integration sites of the provirus and most infected cells contain more than one integrated provirus (Jung et al. 2002). In addition to IN, multiple proteins were shown to enhance DNA integration *in vitro* (Reviewed in Suzuki & Craigie 2007; Bushman et al. 2005; Gabriel et al. 2012). These proteins include HMG-I (Y) a non-histone chromosomal protein important for transcriptional control and chromosomal architecture (Farnet & Bushman 1997), INI1/hSNF5 a core component of the SWI/SNF ATP-dependent chromatin-remodeling complex (Kalpana et al. 1994), Barrier to autointegration protein (BAF) (Chen & Engelman 1998), and the viral NC protein whose stimulatory role is described in chapter V of this manuscript (Gao et al. 2003; Poljak, Susan M Batson, et al. 2003).

A host protein of particular importance, the transcriptional mediator lens epithelium-derived growth factor (LEDGF/p75), was shown to boost both the efficiency of integration and targeting to active transcription units (Figure 22). Knockout or knockdown of this protein caused a substantial reduction of infectivity and much of genome integration targeting to transcription units was lost (Marshall et al. 2007; De Rijck et al. 2010). Data suggest a tethering model where LEDGF/p75 binds simultaneously IN and chromatin at active transcription sites thus directing integration to those sites (reviewed in Ciuffi & Bushman 2006; Craigie & Bushman 2012).

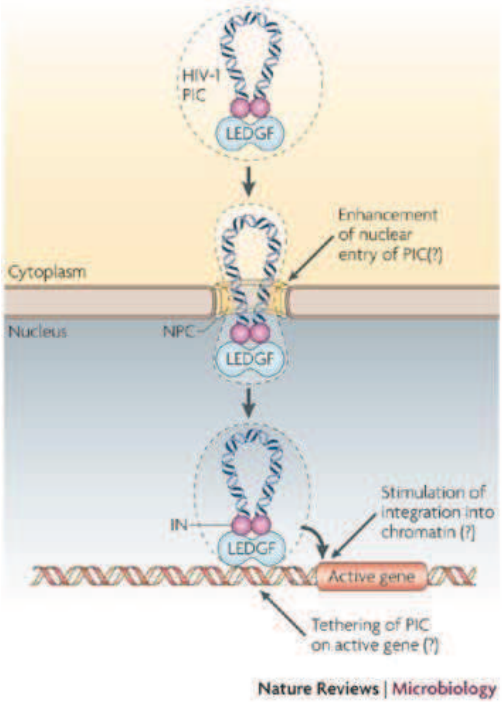

Figure 22: Proposed roles for lens-epithelium-derived growth factor (LEDGF/p75) in HIV-1 DNA integration. LEDGF/p75 might regulate HIV-1 replication through the tethering of IN and chromatin (Suzuki & Craigie 2007).

3.2 The Late Phase

3.2.1 Proviral cDNA expression

The integrated provirus acts as a transcription template that is regulated at the epigenetic, transcriptional, and postranscriptional level (reviewed in Karn & Stoltzfus 2012; Nekhai & Jeang 2006). Once integrated into the cellular chromatin, the proviral DNA is folded into nucleosomes bound to histones (Verdin et al. 1993). This structure prevents the access of cellular transcription factors to the proviral DNA thus repressing its expression and resulting in a state of viral latency. Nucleosome unfolding is the pre-requisite to any transcription initiation and this job is accomplished by the activation of chromatin- remodeling complexes which remodel histones by modifying histone-DNA interactions or by promoting post-translational acetylation of histones (Marcello 2006; Gatignol & Jeang 2000). These complexes are activated by cytokines, chemokines, cellular events, or recruited by the viral protein Tat after its production.

The transcription of the viral genes requires the host transcriptional machinery and is modulated by several cellular and viral proteins. The HIV-1 5'-LTR behaves as a powerful and highly optimized promoter for viral transcription of the integrated vDNA by the cellular RNA polymerase II. It possesses binding sites for numerous transcription factors including Sp1, NFκB, AP-1, and NF-AT (Pereira et al. 2000; Garcia et al. 1992; Kawakami et al. 1988; Leonard et al. 1989; Nabel & Baltimore 1987; Li et al. 1991; Ross et al. 1991). However, in the absence of Tat, the basal transcriptional activity of the HIV-1 LTR is low and results in production of abortive short viral transcripts reflecting a block in the transcription elongation phase (Kao et al. 1987; Toohey & Jones 1989; Romani et al. 2010). This activity is increased in the presence of Tat which is produced early after infection from rare multiply spliced small mRNA transcripts generated despite the elongation defect. These transcripts lead to the synthesis of a few Tat molecules sufficient to stimulate HIV transcription elongation, leading to the production of additional Tat transcripts and proteins. Tat can also be translated from tat mRNA by an Internal Ribosome Entry Site (IRES) dependent mechanism (Charnay et al. 2009).

By binding to the U-rich bulge within TAR stem loop in the R region, Tat recruits a positive transcription elongation factor, P-TEFb, composed of a kinase component called CDK9 and a cyclin component cyclin T1 (Figure 23) (Dingwall et al. 1989; Dingwall et al. 1990; Zhou et al. 2003). This stable complex first phosphorylates the TAR-bound negative elongation factor NELF which releases paused transcription elongation complexes (Yamaguchi et al. 1999; Zhang et al. 2007; Fujinaga et al. 2004) and phosphorylates also serine residues in the CTD of RNA polymerase II during elongation. This increases the RNA polymerase II processivity leading to full length HIV-1 transcripts (Kim & Jang 2002).

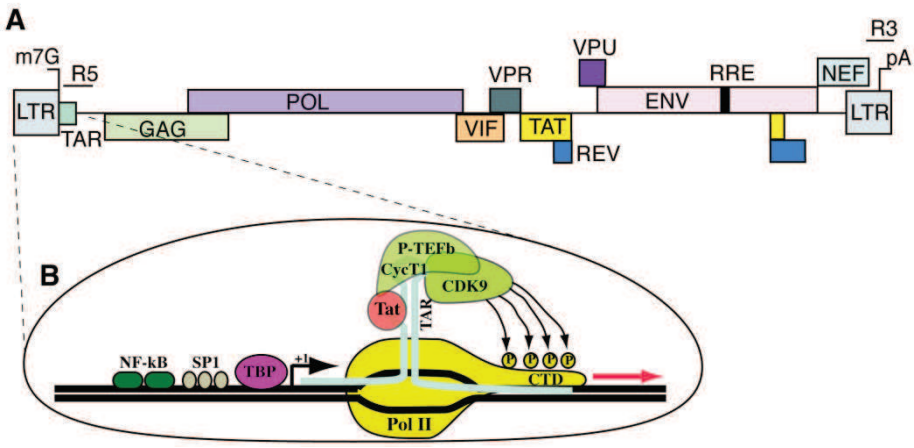

Figure 23: Tat-promoted transcription of the viral genes. (A) Map showing the genomic organization of the HIV-1 provirus. **(B)** Schematic representation of viral transcription. A number of cellular transcription factors assemble onto the LTR promoter. Binding of Tat onto the TAR sequence promotes assembly of the components of P-TEFb (CycT1 and CDK9). The kinase activity of CDK9 phosphorylates the RNA polymerase II CTD to facilitate elongation (Caputi 2011).

In parallel, Tat recruits enzymes with histone acetyl transferase (HAT) activity including the p300/CREB binding protein (CBP), P300/CBP-associated factor (PCAF), hGCN5, and TIP 60. These interactions result in Tat acetylation and HIV-1 LTR transcription enhancement (Weissman et al. 1998; Benkirane et al. 1998; Col et al. 2001; Kamine et al. 1996; Marzio et al. 1998; Caputi 2011).

In parallel, the phosphorylation of P-TEFb stimulates the human capping enzyme to cap HIV-1 mRNAs (Zhu et al. 1997; Herrmann & Rice 1995; Zhou et al. 2003).

3.2.2 Splicing and nuclear export of viral mRNAs

Although the transcription of the proviral DNA generates a single RNA, more than 40 HIV transcripts are produced due to alternative splicing of the pre-mRNA containing multiple splicing sites (Figure 24) (Reviewed in Stoltzfus & Madsen 2006; Tazi et al. 2010). This is a crucial step to ensure the production of the full set of mRNAs to encode for all the viral proteins. Most HIV-1 strains use four different splice donor or 5'-splice sites (5'ss) and eight different acceptor or 3'-splice sites (3'ss) to generate nearly 40 different transcripts encoding a total of nine proteins (Schwartz et al. 1990; Karn & Stoltzfus 2012). HIV RNAs can be singly spliced (encoding Env, Vif, Vpr, and Vpu), multiply-spliced (encoding Tat, Rev, and

Nef), or unspliced (encoding Gag and Gag-Pol) (Purcell & Martin 1993; Schwartz et al. 1990; Karn & Stoltzfus 2012).

Splicing is controlled by cellular factors which bind to short cis- acting sequences flanking HIV-1 splice sites: exonic splicing enhancers (ESE) and exonic/intronic splicing enhancers (ESS/ ISS) (Stoltzfus & Madsen 2006; Nekhai & Jeang 2006).

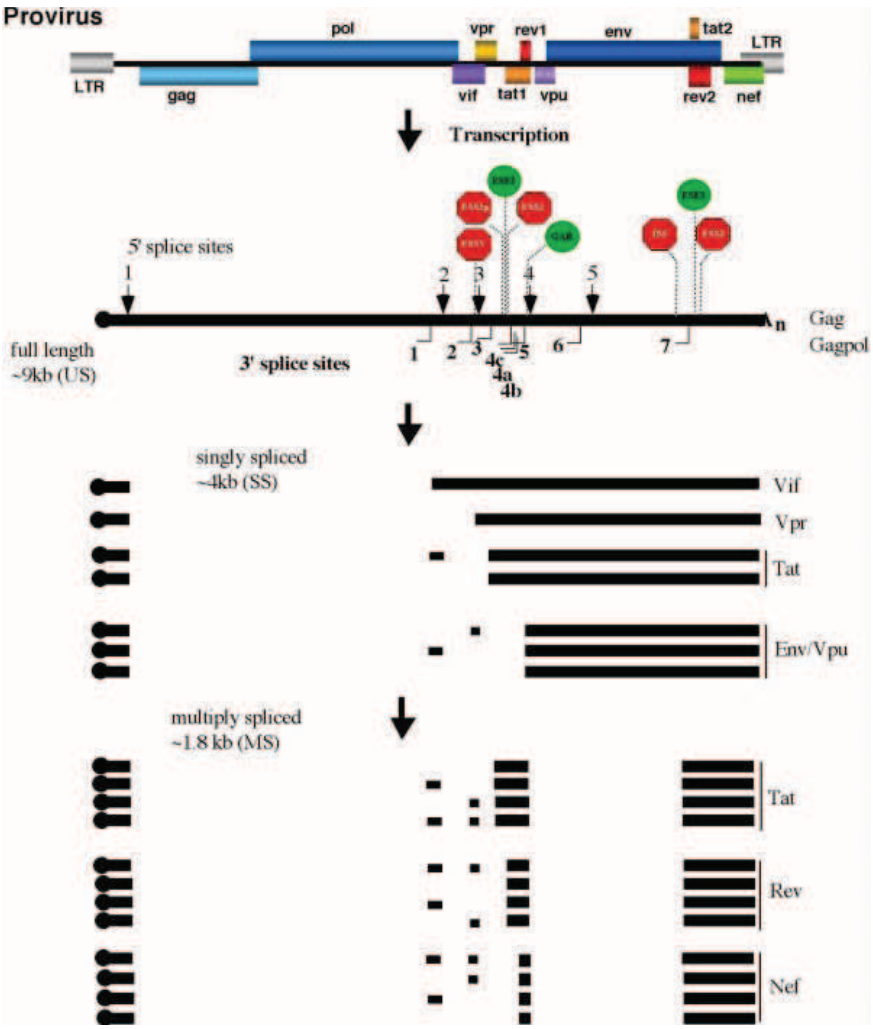

Figure 24: Processing of HIV-1 RNA. Outlined in the figure are the cis-acting components of the HIV-1 RNA which control its processing. Indicated are the positions of the 5' splice sites (arrows above the unspliced RNA), 3' splice sites (brackets below the unspliced RNA), and the various ESS and ESE elements that modulate splice site use. At top is an outline of the viral genome and on the bottom, the exons which comprise the major spliced forms (4.0 kb singly spliced and 1.8 kb multiply spliced) of the genomic RNA are indicated by black boxes. Multiple spliced RNAs combining or excluding various exons encode each of the viral accessory proteins (Cochrane et al. 2006a).

In an infected cell, the first transcribed RNAs are fully spliced by the cellular splicing machinery. This is the early phase of gene expression in which Tat, Rev, and Nef proteins are synthesized. Tat functions as a trans-activator of viral transcription as discussed above. Nef indirectly enhances transcription by positively affecting transcription factors such as NFAT, NFκB, and AP-1. Rev protein is responsible for the conversion from early HIV gene expression to late gene expression by mediating the transport of the mono spliced and non-spliced mRNA out of the nucleus into the cytoplasm (Wu & Marsh 2003; Freed 2001).

Rev recognizes RRE on unspliced (9 Kb) and mono spliced mRNAs (4-5 Kb) (Malim et al. 1989; Mann et al. 1994) and, via its NES signal, it recruits the cellular export factor Crm1 (Chromosme maintenance Region 1) and the nuclear export factor Ran bound to guanosine triphosphate (RanGTP). The complex is then transported through the nuclear pore via interactions with the NPC (Figure 25). Once in the cytoplasm, GTP hydrolysis to GDP destabilizes the Rev complex releasing the RNA and then Rev re-enters the nucleus via interaction with importin β (Neville et al. 1997; Fukuda et al. 1997; Fornerod et al. 1997; Askjaer et al. 1998; Dayton 2004; Henderson & Percipalle 1997). Cellular factors such as DDX1 and DDX3 are also associated with the Rev-RRE-Crm1 complex (Yedavalli et al. 2004; Fang et al. 2005).

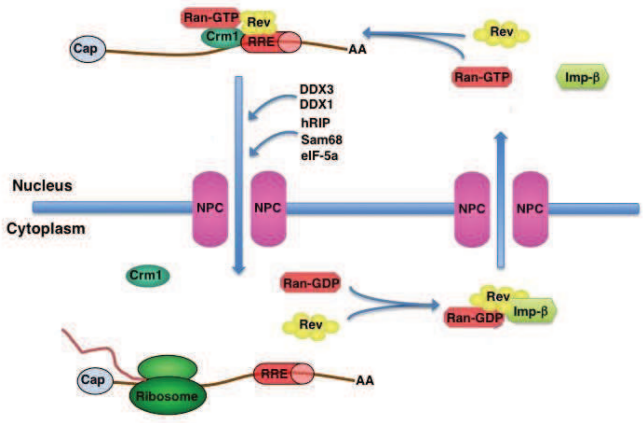

Figure 25: HIV-1 RNA export to the cytoplasm. 9 and 4 kb viral RNAs are exported to the cytoplasm upon interaction of the Rev/Crm1/RanGTP complex with the RRE sequence within the RNA. The DDX1 and DDX3 helicases facilitate translocation of the Rev-RNA complex to the cytoplasm through the nuclear pore complex (NPC). Other host cell factors have positive (hRIP, SAM68, eIF-5a) effects on the Rev-dependent export of the viral RNA but their role is less understood. Importin-b and RanGDP are required for import of Rev into the nucleus (Caputi 2011).

On the other hand, the export of multi-spliced RNAs relies on the endogenous nuclear pathway which involves a range of host cell factors including Tap-Nxt1 and the RNA helicase Dbp5 (Cullen 2005; Schmitt et al. 1999).

3.2.3 Translation of Viral Proteins

In the cytoplasm, the cellular machinery of the host cell is hijacked by the virus in order to synthesize the viral proteins (Bolinger & Boris-Lawrie 2009). The translation of Gag and Gag –Pol polyproteins to give the Gag precursor can be initiated by either a classical cap dependent mechanism or by Internal Ribosome Entry Site (IRES). Cap and IRES dependent gag translation will be discussed in Chapter V.

The synthesis of Gag-Pol precursor is obtained by a ribosomal frameshift at the end of the translation of the Gag precursor. The presence of a sliding sequence associated with a stem-loop allows a shift of one nucleotide to the 5' of the reading frame. This frameshift occurs ~ 5% of the time and results in the production of Gag-Pol/Gag ratio of ~1/20 ratio (Jacks et al. 1988; Bidou et al. 1997; Brierley & Dos Ramos 2006). Frameshifting necessitates two essential factors on the RNA: a slippery heptanucleotide sequence (UUUUUUA) where frameshift occurs, and a downstream RNA element called frameshift stimulatory signal (FSS) that controls the shift efficiency (Giedroc & Cornish 2009; Staple & Butcher 2005).

The Env and Vpu protein synthesis is obtained from a bicistronic mRNA which contains two overlapping ORFs: vpu and env. The translation of env protein depends on a leaky scanning mechanism (Schwartz et al. 1990; Schwartz et al. 1992).

3.2.4 Viral Assembly, Budding, and Maturation

Although Gag on its own is capable of self-assembly to form VLPs (viral-like particles) in the absence of other viral proteins or even viral RNA, these VLPs are defective and thus unable to enter into any cell (Gheysen et al. 1989; Mergener et al. 1992; Campbell & Rein 1999). The production of mature virions requires the encapsidation of the genomic RNA, the incorporation of the gp120/gp41 glycoprotein complex, and the Gag-Pol precursor. In most cells including CD4(+) lymphocytes, assembly takes place at the plasma membrane where

budding occurs. However, in primary human macrophages, the other main HIV target cells, assembly and budding take place in large intracytoplasmic vacuoles (Orenstein et al. 1988) called Virus-Containing Compartment (VCC). Newly formed immature particles accumulate with time in the lumen of these VCC (Gaudin et al. 2013) which in contrast to what was first supposed are not late endosomes or multivesicular bodies (Raposo et al. 2002; Pelchen-Matthews et al. 2003) but are nowadays described as specialized, intracellular sequestered portions of plasma membrane with dynamic connections to the extracellular milieu as they are accessible to extracellular molecules (Bennett et al. 2009; Mlcochova et al. 2013). It is worth noting that macrophages remain infectious for a long time and resist to the cytopathic effect of HIV (Koppensteiner et al. 2012; Sharova et al. 2005).

Virus assembly results in the formation of viral particles composed of viral proteins and two copies of the gRNA but also an important proportion of cellular RNAs (reviewed in Adamson & Freed 2007; Lingappa et al. 2014; Cimarelli & Darlix 2002). Gag is the central player in HIV-1 particle formation as it orchestrates assembly (reviewed in Kutluay & Bieniasz 2010; Sundquist & Kräusslich 2012) by initially undergoing oligomerization upon binding to the gRNA which acts as a scaffold. Gag oligomers were recently observed and described in the cytoplasm, in the form of compact oligomers which progressively assemble during their trafficking to the plasma membrane (El Meshri et al. 2015). Furthermore, according to *in vitro* data, Gag may also traffics to the plasma membrane under a compacted form also named the U-form (Figure 26) (Munro et al. 2014; Datta et al. 2007).

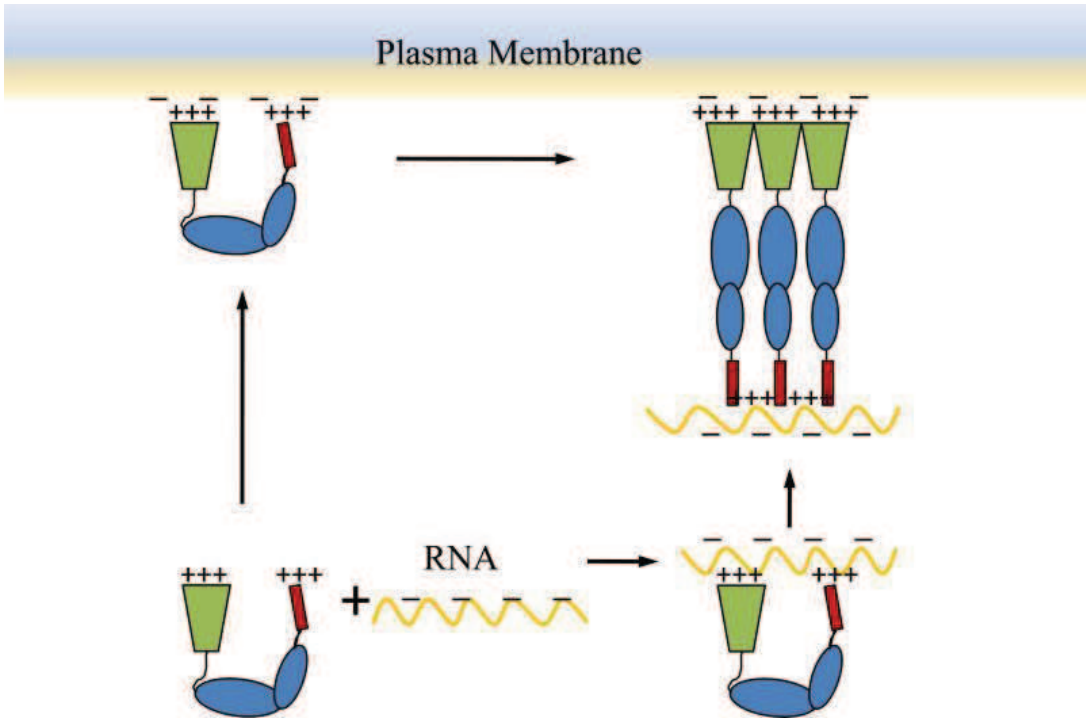

Figure 26: Model of HIV-1 assembly. In this model, Gag is thought to assume a compact U-shape conformation with both ends near each other. Both the N-terminal MA domain (green) and the C- terminal NC domain (red) are positively charged, but the MA domain has a much higher affinity for negatively charged phospholipids than for RNA. The NC domain exhibits the inverse pattern (Rein et al. 2011).

In fact, Gag multimerization is primarily mediated by CA-CA interaction that starts in the cytoplasm where Gag is first found as oligomers and ends at the plasma membrane where higher order multimers are finally obtained (Nermut et al. 2003; Kutluay & Bieniasz 2010; El Meshri et al. 2015; Larson et al. 2003). SP1, MA, and NC stabilize Gag multimerization and assembly (Burniston et al. 1999; Ott et al. 2005; Datta et al. 2011; de Rocquigny et al. 2014; Alfadhli et al. 2011). MA and NC act via their RNA binding capacities but also in case of MA by its myr moiety and its basic domain (O'Carroll et al. 2012; Li et al. 2007; Ono et al. 2004; Chukkapalli et al. 2008). The cell plasma or endosomal membranes are used as a second assembly platform for Gag/RNA multimerization (Hogue et al. 2009; Alfadhli et al. 2011; Ott et al. 2005; Rein et al. 2011; Pelchen-Matthews et al. 2003).

As already discussed in Chapter II, the MA domain of Gag serves as a scaffold that brings together Gag, RNAs and Env to the plasma membrane (Dorfman et al. 1994; Alfadhli et al. 2011; Ott et al. 2005; Freed & Martin 1996b). The role of NCp7 in assembly and encapsidation of the viral genome will be elucidated in chapter IV.

The mechanism of Gag transport to the plasma membrane is not fully understood but it is evident that Gag must interact with a large amount of host cell machineries including microtubules networks, motor proteins and vacuolar complexes. In fact, Gag interacts with the kinesin superfamily member KIF4 whose downregulation reduces particle production and increases intracellular Gag degradation (Hirokawa & Noda 2008). However, this idea is still debated and some studies suggest that Gag is transported to components of the endocytic pathway before membrane transport (Dong et al. 2005; Perlman & Resh 2006; Resh 2005). During their oligomerization and transport to the plasma membrane, Gag proteins are accompanied with a cytoplasmic host complex containing P bodies, and host proteins such as Staufen, ABCE1, and DDX6 which facilitate assembly (Zimmerman et al. 2002; Reed et al. 2012; Cochrane et al. 2006b).

3.2.5 Budding and Maturation

The final step in the virus assembly is budding where the virus particle is pinched off from the host cell membrane. For this purpose, HIV-1 is capable of hijacking the cellular machinery to allow the budding of the immature viral particles via the endosomal sorting complexes required for transport (ESCRT) (reviewed in Votteler & Sundquist 2013; Demirov & Freed 2004; Sundquist & Kräusslich 2012; Meng & Lever 2013). Through its p6 late domain, the Gag precursor recruits the cellular ESCRT machinery necessary for the virion release. p6 includes two important domains for the recruitment of the ESCRT machinery: the PTAP domain that specifically binds to the TSG101 subunit of the ESCRT-I complex (Garrus et al. 2001; Martin-Serrano et al. 2001; VerPlank et al. 2001) on one hand, and the YPXL domain (Tyr-Pro-Lys-X, where X is any residue) that specifically binds to the apoptosis-linked gene 2-interacting protein ALIX (Strack et al. 2003; Usami et al. 2009; Zhai et al. 2008; Wollert et al. 2009) on the other hand. The TSG101/ESCRT-I protein and ALIX will allow the recruitment of ESCRT-III complex proteins (specifically CHMP-2, CHMP-4) and VPS4 which will mediate membrane fission and release of the budding virion (Morita et al. 2011; Jouvenet et al. 2011).

The budding virions are non-infectious and must undergo a final maturation step corresponding to a finely tuned series of viral precursor protein cleavages by the viral protease (For a review Bukrinskaya 2004; Sundquist & Kräusslich 2012). Viral maturation

begins during or immediately after budding. The interaction between Gag-Pol precursors during assembly permits the dimerization of the PR domains forming an active homodimer. The mature viral PR is thus liberated by an autocatalytic mechanism (Debouck et al. 1987). Then, PR will sequentially cleave Gag and Gag-pol releasing the structural proteins (MA, CA, NC, p6) and viral enzymes (PR, IN, RT) (Wiegers et al. 1998). As the immature virion matures, MA remains associated with the membrane, NC condenses with the dimeric gRNA, CA reassembles to form a closed conical capsid shell encompassing the NC-RNA complex (Figure 27). These structural rearrangements result in a mature infectious virion capable of infecting new cells (Vogt 1996; Balasubramaniam & Freed 2011a). It is important to add that the viral particle during assembly and budding will incorporate, specifically or not, an important amount of cellular molecules including RNAs and proteins (Chertova et al. 2006; Tremblay et al. 1998; Ott 2008; Santos et al. 2012; Linde et al. 2013).

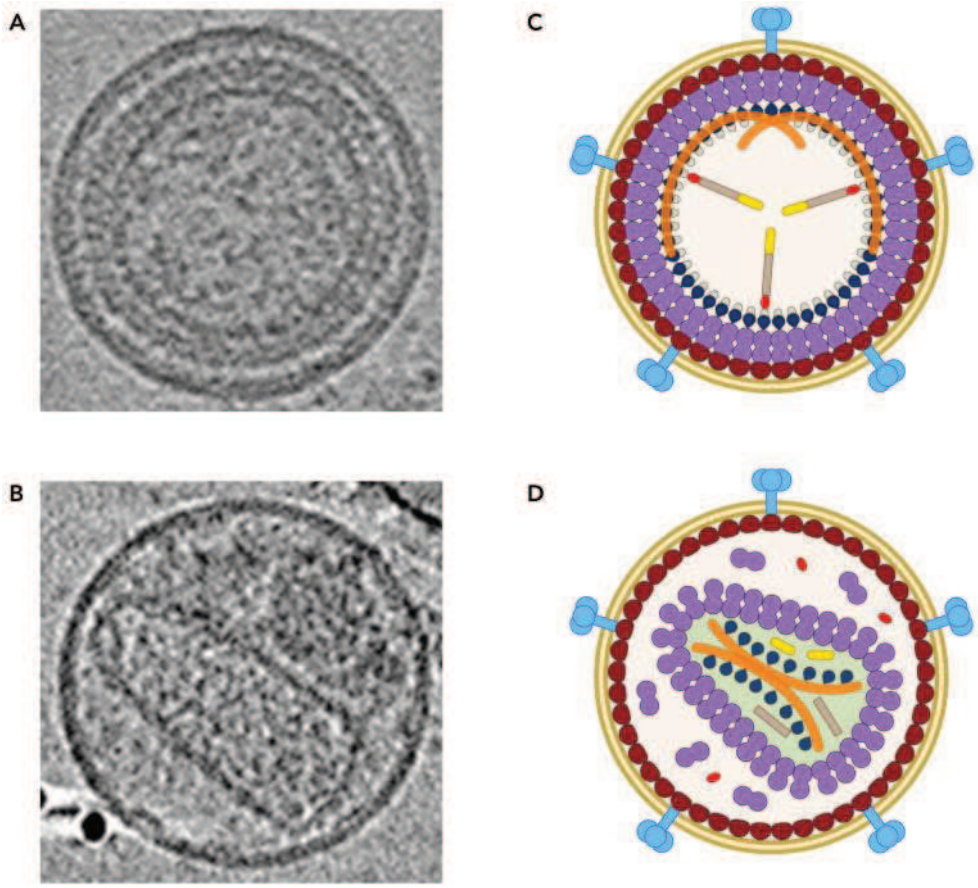

Figure 27: HIV-1 virion. Central slices through cryo-EM tomograms of immature ***(A)*** and mature **(*B*)** virus particles that are ~130 nm in diameter. Schematic representation of immature **(*C*)** and mature **(*D*)** HIV-1 particles (Balasubramaniam & Freed 2011).

Chapter IV- The HIV-1 Nucleocapsid protein

4.1 NCp7: a cleavage product of Gag

Retroviral Nucleocapsid proteins are small highly basic nucleic acid binding proteins bearing one (Henderson et al. 1981) or two copies of a highly conserved CCHC zinc binding domain called zinc fingers (Berg 1986; South et al. 1990; Covey 1986) flanked by basic residues. Noticeably, among the Spumaretroviruses (Linial 1999) and the gypsy retrovirus of the fruit fly *Drosophila melanogaster* (Darlix et al. 1995), NCs which are only present as a domain of the unprocessed protein Gag, do not harbor any zinc finger.

NC is usually generated by the cleavage of the Gag structural polyprotein by the viral protease. In the case of HIV-1, NCp7 is released after a set of five HIV-1 Pr55Gag protease cleavages (Figure 28) which occur at different rates (Pettit et al. 1994). The first cleavage occurs at the SP1/NC junction and results in the formation of NCp15 comprising NCp7-SP2-p6 domains on one hand and MA-CA-SP1 domains on the other hand (Shehu-Xhilaga et al. 2001). The second cleavage requires that the NCp15 binds to RNA in order for the reaction to proceed (Mirambeau et al. 2007; Sheng et al. 1997), and it produces NCp9 (which is basically NCp7 attached to the SP2 peptide). The final proteolytic cleavage generates the predominant 55 amino acid NCp7 after NCp9 cleavage liberating SP2 (Coren et al. 2007; Henderson et al. 1992; Tanchou et al. 1998). In parallel, the MA-CA-SP1 domain is first cleaved between MA and CA liberating MA and CA-SP1 latter cleaved to give CA and SP1. However, the proteolytic cleavage is not 100% efficient since some NCp9 and NCp15 forms were detected by western blot of HIV-1 virions (Gorelick, Gagliardi, et al. 1999a; Tanchou et al. 1998).

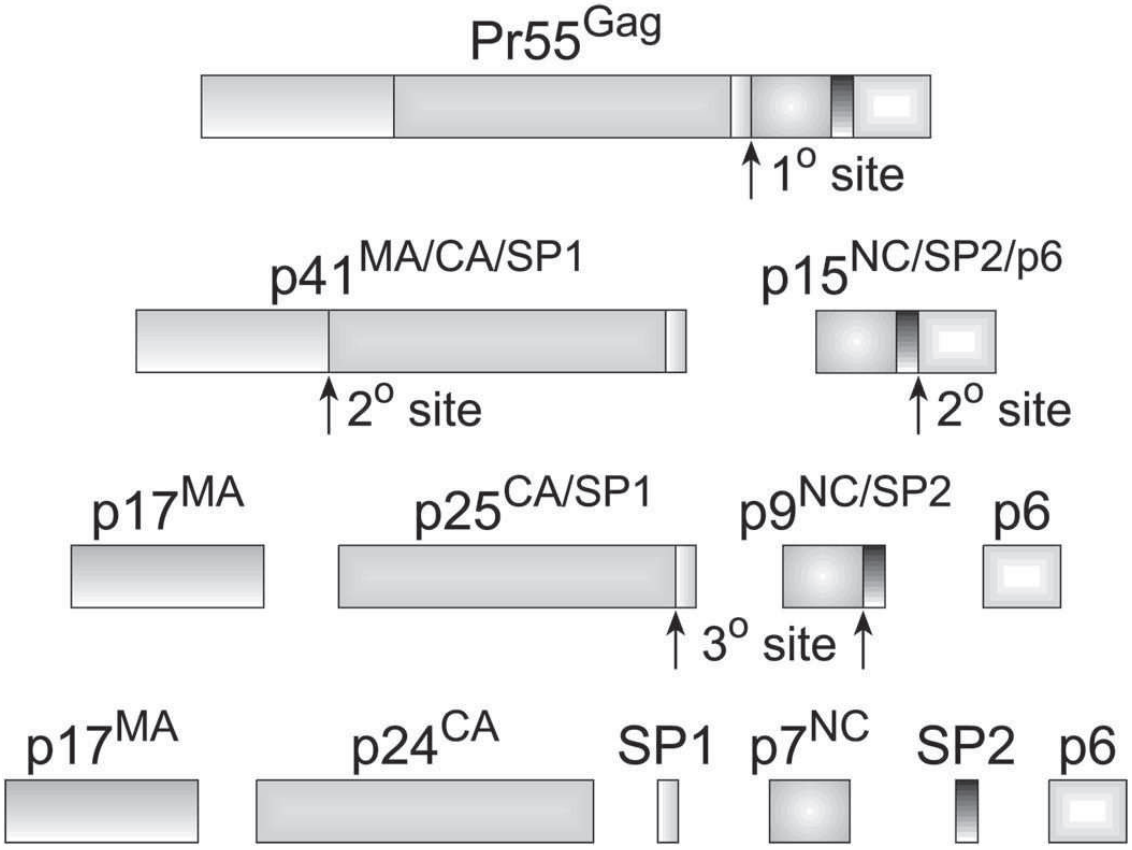

Figure 28: First, second, and third PR cleavage sites are ordered according to their processivity rates (Thomas & Gorelick 2008).

4.2 Nucleocapsid Structure

The HIV-1 nucleocapsid (NCp7) is a 55 amino acid basic protein bearing two copies of a highly conserved zinc finger motif of the CCHC type (Cys-X2-Cys-X4-His-X4-Cys) bridged by a short basic and 6-7 residues flexible linker and flanked by N- and C-termini rich in basic residues (Figure 29). The proximity of the two zinc fingers is due to the conformation of the linker between the two CCHC motifs (Morellet et al. 1992; Summers et al. 1992), and notably to the proline residue in the linker which favors the formation of a bend between the two zinc fingers. This orientation is also stabilized by hydrophobic and hydrogen interactions between the zinc fingers implicating mainly the amino acids Phe16, Asn17, Gly19, and Trp37 (Déméné, Jullian, et al. 1994; De Guzman et al. 1998).

The zinc fingers are capable of chelating zinc with a very high affinity (in the order of 10^{-13} M) (Bombarda et al. 2007; Bombarda et al. 2001; Mély et al. 1996). Zinc binding acts as the main driving force for the NCp7 folding as in the absence of Zn^{2+}, NCp7 is mainly an unstructured peptide. Coordination of Zn^{2+} is central for the protein activity. Furthermore, NCp7 sequence is highly conserved across all HIV-1 strains. The conservation of the CCHC motifs among different retroviral NCp7s reflects the biological importance of the zinc fingers. Mutations affecting the zinc fingers or the linker have a negative impact on the retroviral cycle (Déméné, Dong, et al. 1994; Dorfman et al. 1993; Gorelick, Gagliardi, et al. 1999b). Although the N-terminus sequence is variable, the basic character of its amino acids is always conserved (Beltz et al. 2005; Bourbigot, Ramalanjaona, Boudier, G. F J Salgado, et al. 2008; Egelé et al. 2004).

The CCHC motifs together with the basic linker form the central globular NCp7 domain with the flanking N- and C-terminal regions being disordered and independent from the zinc fingers. The flexibility of NCp7 structure is a characteristic of almost all RNA chaperones (Cristofari & Darlix 2002). The main goal of the folded zinc fingers is to properly present amino acids critical for nucleic acid recognition and genomic RNA packaging.

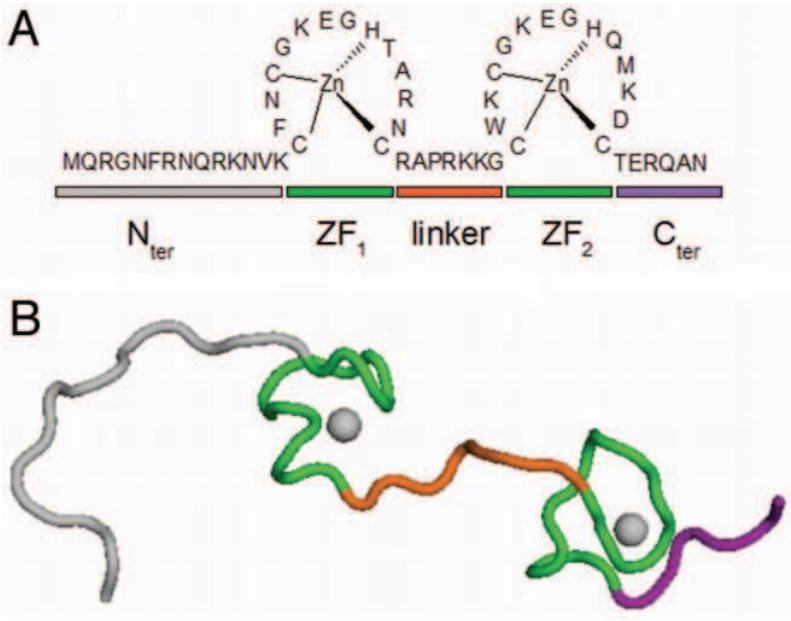

Figure 29: 3D structure and sequence of the HIV-1 NCp7. (A) NCp7 sequence. The two zinc fingers (ZF) are in green, the basic linker in orange, and the N and C terminals are in grey and violet respectively. **(B)** Scheme of NCp7 structure (Godet et al. 2012; Godet & Mély 2010).

4.3 Nucleic Acid Binding

NCp7 is capable of binding specifically or non-specifically nearly any nucleic acid (NA) sequence. NCp7 binding is highly dependent on the ionic strength (Athavale et al. 2010; R J Fisher et al. 1998; Mély et al. 1995; Urbaneja et al. 1999). The fifteen basic residues of NCp7 are responsible for non-specific interactions where NCp7 binds with electrostatic interactions to any sequence of around 6 nucleotides (Lapadat-Tapolsky et al. 1995; Mély et al. 1995). This binding mode is presumably the most common in the virions in which the NCp7 concentration can reach the millimolar range and thus induce an extensive gRNA coating by 1500-2000 NCp7 copies protecting it from nucleases (Chen et al. 2009; Lapadat-Tapolsky et al. 1993). On the other hand, a number of specific NCp7/NA interactions were reported in the literature (C. Vuilleumier et al. 1999; Berkowitz et al. 1996; Urbaneja et al. 2002; Wu et al. 1997; Amarasinghe et al. 2000; De Guzman et al. 1998; Bourbigot, Ramalanjaona, Boudier, Gilmar F J Salgado, et al. 2008). NCp7 binds with high affinity to UG or TG rich single stranded sequences. The hydrophobic platform formed at the top of the two folded zinc fingers is responsible for these specific interactions. More specifically, the residue Trp37 (ZF2) plays a key role (C. Vuilleumier et al. 1999; Morellet et al. 1994; Bombarda et al. 1999; Lam et al. 1994; Mély et al. 1993) through stacking interactions with exposed guanidine residues, more specifically found in the following sequences: TG, TXG, or GXG where X could be any base (C. Vuilleumier et al. 1999; R J Fisher et al. 1998; Avilov et al. 2009; Berglund et al. 1997; Avilov et al. 2008). Specific interactions play an important role in the recognition of the Psi encapsidation signal of the gRNA enabling its selection by Gag among a large excess of cellular RNA during virus assembly (Lever et al. 1989; Muriaux & Darlix 2010).

The structure of NCp7 bound to small nucleic acid sequences was determined in a 1:1 complex with either DNA (Morellet et al. 1998; B P Roques et al. 1997; Schüler et al. 1999) or RNA (Amarasinghe et al. 2000; Amarasinghe et al. 2001; De Guzman et al. 1998) sequences. The zinc fingers direct the recognition of the nucleic acid whereas the N- and C-terminal regions stabilize the nucleoprotein complex. Upon nucleotide binding, the proximity of the zinc fingers is reinforced.

4.4 RNA chaperone

NAs adopt thousands of different conformations; however, most of them are biologically inactive. Therefore, to acquire a biologically active conformation, NAs must reach the most thermodynamically stable conformation (Draper 1992; Treiber & Williamson 2001). NAs are helped during that process by proteins called nucleic acid chaperones (Cristofari & Darlix 2002). They lower the energy barrier allowing breakage and re-formation of base pairs (Herschlag 1995). RNA chaperones are present in all living organisms including viruses where they play vital functions particularly in terms of NA replication (Cristofari & Darlix 2002; Schroeder et al. 2004; Zúñiga et al. 2009).

The first highlighted viral RNA chaperones were the nucleocapsid proteins of the avian and murine retroviruses viruses: MuLV NCp10 and RSV NCp12 (Prats et al. 1988). Later, a chaperone like activity was demonstrated for the HIV-1 NCp7 (Barat et al. 1993; Darlix et al. 1990; De Rocquigny et al. 1992; Dib-Hajj et al. 1993). Of all NC proteins studied to date, HIV-1 NCp7 possesses the strongest NA chaperone activity (Levin et al. 2005). The NC chaperone activity relies on three properties, i/ its ability to aggregate NAs upon binding into ribonucleoprotein complexes, ii/ its capacity to partially destabilize NA structures in an ATP-independent manner, and iii/ a rapid on-and-off NA binding kinetics (Darlix et al. 2011). All NC forms from Gag to NCp7 exhibit various levels of chaperone activity, depending on their degree of processing. As an example, the binding of NCp7 to NA is non-cooperative while those of NCp9 and NCp15 are cooperative (Cruceanu, Gorelick, et al. 2006; Khan & Giedroc 1994). Furthermore, Gag binding to NAs is stronger than the one of NCp7 (Rein 2010).

The NCp7 chaperoning activity varies depending on the NCp7 to NA molar ratio (Figure 30) which determines the level of NA occupancy. Three levels of NA occupancy and thus, three discrete modes of NCp7 activity and function exist (Ivanyi-Nagy et al. 2005; Cristofari & Darlix 2002; Auxilien et al. 1999; Guo et al. 1997; Stoylov et al. 1997).

1) The binding mode occurs when NCp7 concentration is limited (1:100nt). Thus, NCp7 binds with high affinity to specific viral sequences but does not promote aggregation or chaperoning.

2) The chaperoning mode occurs at a high degree of RNA occupancy (1:15 to 1:7 nt). In this case, NA molecules can undergo structural rearrangements.
3) The saturation mode occurs at saturating levels of NCp7 (1:5 or 1:1 nt). Thus, the NA molecules are entirely coated by NCp7.

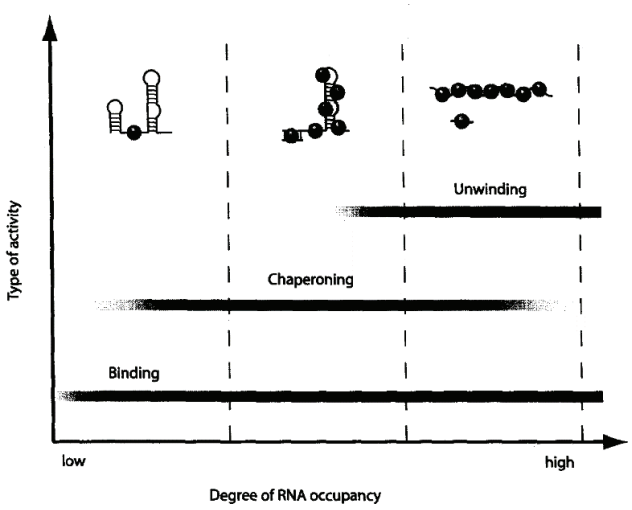

Figure 30: The scheme presents the connection between the degree of RNA occupancy by NCp7 molecules (gray circles) and its activity. This scheme illustrates the different types of activity associated with RNA chaperone proteins according to the degree of RNA occupancy. The three modes depicted in the scheme can be classified as noncrowded, crowded, and overcrowded (Cristofari & Darlix 2002).

4.5 Roles of NC in the viral life cycle

The chaperone activity of NCp7 explains to a large extent the critical roles played by NCp7 in both early and late phases of the viral life cycle.

4.5.1 NCp7 roles in the early phase

The reverse transcription is the step in the viral life cycle where the chaperone roles of NCp7 are clearly required. The ability of NCp7 to destabilize NAs and its ability to anneal NAs are important in facilitating several reverse transcription steps.

4.5.1.1 Initiation of Reverse Transcription

Annealing of the cellular primer $tRNA^{Lys\ 3}$ to gRNA is a basic requirement for the initiation of reverse transcription. In order for this to occur, the tRNA structure should be disrupted allowing its 3' nucleotides to base pair with nucleotides in the PBS region of the gRNA. This unfolding and thus the annealing can be forced *in vitro* using heat (Beerens & Berkhout 2000). However, under physiological conditions, NCp7 and NC in Gag strongly enhance the annealing rate (Barat et al. 1993; Hargittai et al. 2001; Hargittai et al. 2004; Huang et al. 1998; Khan & Giedroc 1992; Tisne 2005). Prats et al. (1988) showed that a mutation within the NCp7 coding region prevented placement of tRNA on PBS (Prats et al. 1988).

4.5.1.2 Efficiency of Reverse Transcription

During reverse transcription, the DNA product is frequently incomplete due to the pausing of RT at specific sites on the RNA template (Messer et al. 1985; Dudding et al. 1991; DeStefano et al. 1992; Klarmann et al. 1993; Arts et al. 1995; Wu et al. 1996). Addition of NCp7 greatly reduces RT pausing (Figure 31), increasing the production efficiency of full length DNA (Wu et al. 1996; Tanchou et al. 1995; Ji et al. 1996). This is explained by the chaperone properties of NCp7, which can break base pairs and thus eliminate the secondary structures hindering the reverse transcription process (Rein et al. 1998).

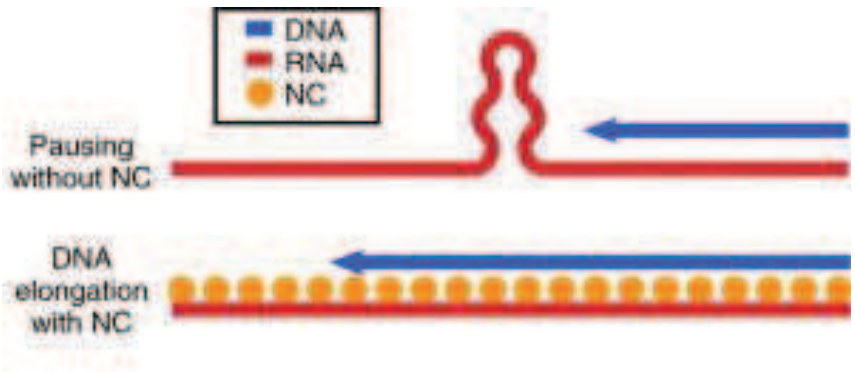

Figure 31: Effect of NCp7 protein on DNA elongation during reverse transcription. Addition of NC reduces the pausing of reverse transcriptase by transiently destabilizing secondary structure regions in the template RNA (Rein et al. 1998).

4.5.1.3 First Strand transfer

The first strand transfer is an obligatory step for the formation of the complete (-)DNA complementary to the gRNA (Refer to Chapter III of the manuscript where reverse transcription is discussed). The newly synthesized (-)ssDNA is transferred from the 5' to the 3' extremity of the gRNA in a reaction involving the pairing of bases complementary to the R regions of both NAs (Gilboa et al. 1979). The chaperone role of NCp7 is particularly important for hybridization of TAR and cTAR motifs included in this R regions of the gRNA and the (-) DNA respectively (Figure 32). Using an *in vitro* reverse transcription system, it was shown that minus strand transfer is inefficient due to the stability of the TAR stem loop that prevents hybridization with its complementary cTAR sequence in (-)ssDNA under physiological conditions in absence of NCp7 (Guo et al. 1997; Lapadat-Tapolsky et al. 1997; Lapadat-Tapolsky et al. 1995; Baudin et al. 1993; You & McHenry 1994). However, the addition of NCp7 induces a destabilization of the secondary structure of both TAR and cTAR (Bernacchi et al. 2002; Azoulay et al. 2003) thus accelerating the annealing and first strand transfer (Guo et al. 1997; Darlix et al. 1993; Allain et al. 1994; Peliska et al. 1994; Kim et al. 1997; You & McHenry 1994). NCp7 destabilization and self-priming inhibition properties were shown to rely on the two zinc fingers and the basic linker (Beltz et al. 2005). Furthermore, NCp7 stimulates RT RNase H activity on RNA/DNA duplexes (Roda et al. 2003).

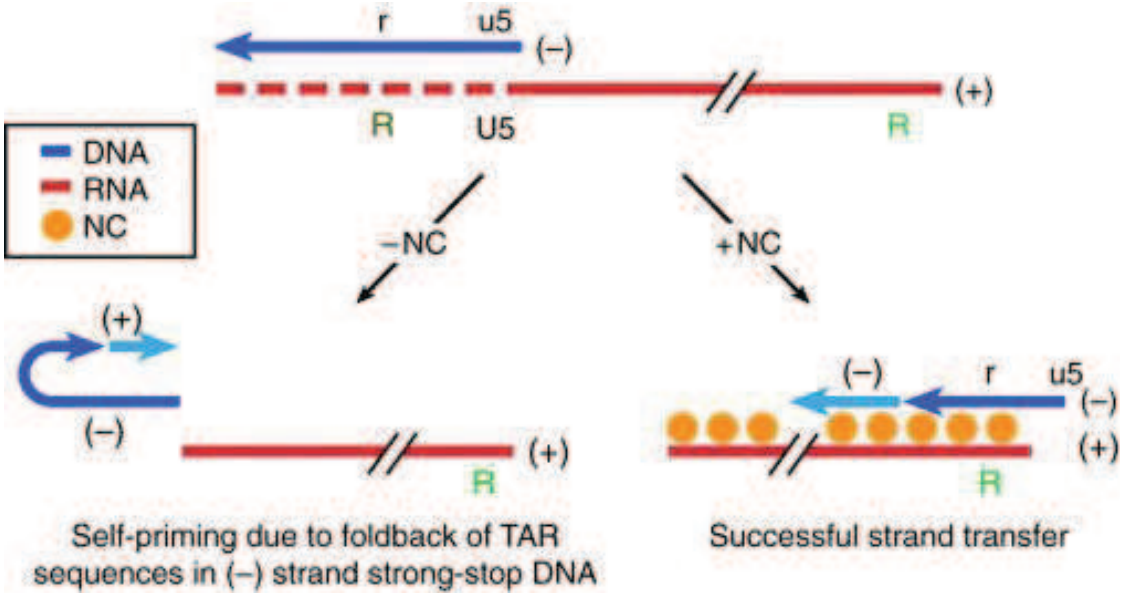

Figure 32: Effect of NCp7 on strand transfer during reverse transcription. Synthesis of a complete (-) strand DNA copy relies on the annealing of the 'r' sequence in the (-) strand DNA to 'R' sequences at the 3' end of the template RNA. Moreover, the (-) strand DNA folds back on itself which results in extension of (-) strand with (+) strand sequences. Addition of NC prevents this reaction increasing the synthesis of full length (-) strand DNA (Rein et al. 1998).

4.5.1.4 Second Strand Transfer

The second strand transfer during reverse transcription requires the annealing of the DNA (+) and (-) copies of PBS to form a circular intermediate. NCp7 binds PBS sequences with good affinity and chaperones the annealing reaction between the complementary PBS sequences. In the absence of NCp7, (+) PBS was reported to spontaneously anneal to (-) PBS mainly via nucleation mediated by their single stranded overhangs. Although the PBS stem is only slightly destabilized by NCp7 (Bourbigot, Ramalanjaona, Boudier, Gilmar F J Salgado, et al. 2008), it is sufficient for NCp7 to increase the annealing kinetics and switch the reaction pathway to a loop-loop kissing mechanism that does not occur in the absence of NCp7 (Egelé et al. 2004; Egelé et al. 2005; Ramalanjaona et al. 2007). Godet et al. (2011) demonstrated that NCp7 can 'freeze' PBS conformations competent for annealing. In addition, this mechanism is strictly dependent on the integrity of both zinc fingers (Godet et al. 2011).

4.5.1.5 Viral DNA integration

As the reverse transcriptase product is transported to the nucleus, the viral DNA is a target for cellular nucleases. However, NCp7 coats the viral genome protecting it against nucleases (Krishnamoorthy 2003; Lapadat-Tapolsky et al. 1993; Tanchou et al. 1995). Furthermore, the inverted repeat sequences at the end of the LTR were shown to be protected by NCp7 and IN binding (Bampi et al. 2004). NCp7 was also found to strongly stimulate DNA integration by IN under physiological conditions (Buckman et al. 2003; Carteau et al. 1997; Poljak, Susan M Batson, et al. 2003; Thomas et al. 2006). This was shown *in vitro* (Carteau et al. 1997; Carteau et al. 1999; Gao et al. 2003; Poljak, Susan M Batson, et al. 2003) and *in vivo* in cell-culture based system. Specifically, some NCp7 mutants as NC_{H23C} were shown to be highly defective in integration and by consequence profoundly replicative defective (Gorelick, Fu, et al. 1999; Thomas et al. 2006). The mechanism by which NCp7 influences the integration *in vivo* is not well understood. It could be, as *in vitro*, by directly enhancing the enzymatic steps of integration and also by assisting the formation of a complex functional for integration. It is important to keep in mind that NCp7 localization in the PIC is still a subject of debate.

4.5.2 NC roles in the late phase

As in late phase, NC functions as a domain of Gag, I will refer to it as Gag(NC).

4.5.2.1 Role of NC in the selection and encapsidation of the viral genome

The encapsidation of the viral genome by Gag(NC) is an obligatory step for the formation of infectious virions. The genomic RNA is specifically encapsidated due to its selective discrimination from the large excess of cellular RNA (Aldovini & Young 1990; Cimarelli & Darlix 2002). This specific encapsidation is a result of the interaction of the NC domain within Gag with the encapsidation signal ψ in the 5'-UTR of the gRNA (Clever et al. 1995; Lochrie 1997; D'Souza & Summers 2004; Jewell & Mansky 2000).

The ψ signal (shown in Figure 33) is a 120 nucleotides stretch presenting a stable secondary structure composed of 4 stem loops SL1 to SL4 (Baudin et al. 1993; Clever et al. 1995; Harrison & Lever 1992). Mutations affecting elements in these sequences significantly

reduced the encapsidation of the viral genome (Clever & Parslow 1997; Aldovini & Young 1990). Genetic studies demonstrated that all the four SLs play independent roles in RNA packaging (Clever & Parslow 1997; Harrison et al. 1998; McBride & Panganiban 1996; McBride et al. 1997; Hayashi et al. 1992). SL2 and SL3 have the strongest affinity for Gag(NC) while SL4 stabilizes the Psi structure via RNA-RNA interactions (Amarasinghe et al. 2001). *In vitro*, NC binds with high affinity to SL1, SL2 and SL3 recognizing a GXG motif (Robert J. Fisher et al. 1998; C Vuilleumier et al. 1999). Since SL1 contains the DIS sequence responsible for promoting dimerization of the genomic RNA and since dimerization was suggested to be linked to packaging (Russell et al. 2004; Laughrea et al. 1997), Gag(NC) binding to SLs is thought to chaperone the gRNA dimerization. It was shown that the integrity of both Zn fingers is required for the encapsidation (Dorfman et al. 1993; Gorelick et al. 1990; Aldovini & Young 1990; Zhang & Barklis 1995). However, the two Zn fingers of HIV-1 are not functionally equivalent and the first Zn finger is more prominent in RNA selection and packaging (Gorelick et al. 1993; Schwartz et al. 1997). Although the Zn fingers are necessary for encapsidation, they are not sufficient and the flanking basic residues are also implicated (Dannull et al. 1994; Cimarelli et al. 2000; De Guzman et al. 1998; Poon et al. 1996).

Furthermore, Gag(NC) exhibited a 10-fold higher affinity to these sequences than mature NCp7 (Cruceanu, Urbaneja, et al. 2006).

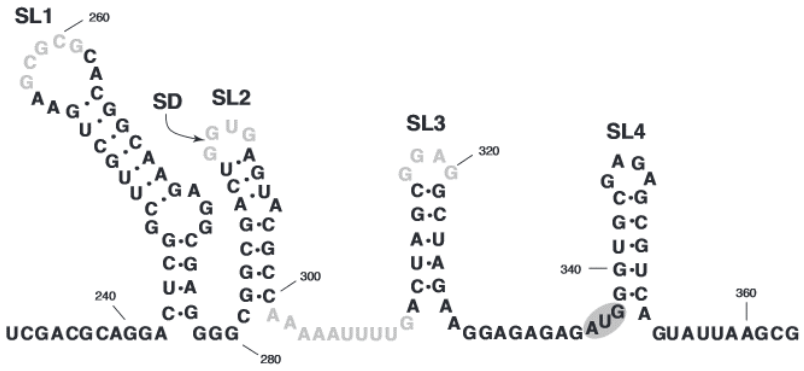

Figure 33: ψ signal of HIV-1 gRNA composed of four stem loops (SL). High affinity binding sites are in gray. The gag translation initiation codon is indicated. The major splice donor site (SD) is indicated by the arrow (Darlix et al. 2002).

4.5.2.2 Role of NC in the dimerization of the viral genome

The DIS hairpin sequence is sufficient to induce HIV genome dimerization in vitro and is believed to promote the dimerization of the viral genome through formation of a kissing dimer intermediate with the DIS of another RNA molecule. Its conservation among different HIV strains reflects the significance of this motif (Paillart et al. 1994; Paillart, Shehu-Xhilaga, et al. 2004; Haddrick et al. 1996; Laughrea & Jetté 1994; Muriaux et al. 1995; Skripkin et al. 1994). This process can be divided in two steps. The first step corresponds to the engagement in a kissing loop base pairing interaction of the palindromic sequences (GCGCGC for HIV-1 group B) of two SL1 motifs (Figure 34) thus forming a loose RNA dimer (Dardel 1998; Ennifar et al. 2001; Ennifar et al. 1999). The subsequent step is the transformation of this complex into a more stable extended dimer (Ennifar et al. 1999; Girard et al. 1999). This is accomplished via the opening of the hairpin stem by incubation either with NC protein (D. Muriaux et al. 1996) or at high temperature (Laughrea & Jetté 1996; D Muriaux et al. 1996). Thus, NC by chaperoning the most stable structure appears to promote the formation of the extended duplex at physiological temperatures (Feng et al. 1999; D. Muriaux et al. 1996; Rist & Marino 2002).

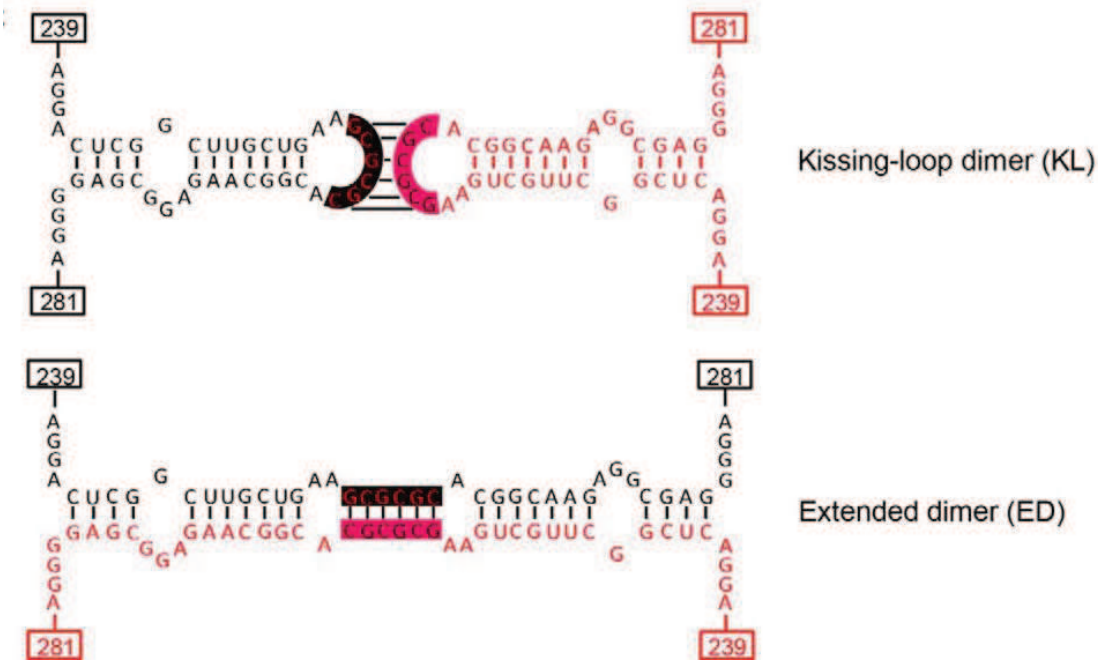

Figure 34: Proposed scheme of DIS sequence dimerization. Schematic representation of the NC-induced structural conversion of the DIS sequence from the kissing complex to the extended complex (van Bel et al. 2014).

Deletions or mutations in the SL1/DIS sequence lead to reduction in genomic RNA dimerization and packaging in addition to diminishing virus production (Berkhout & van Wamel 1996; Laughrea et al. 1997; Paillart et al. 1994; Paillart et al. 1996).

Structure Models for the 5'-UTR (discussed in Chapter V)

Since the dimerization signal of retroviruses is located in a region that is crucial for retroviral replication, the 5' region contains in addition to the DIS several functional elements like TAR, primer binding site, splice donor site, and the packaging signal. The secondary structure is crucial for the activity of these domains. Two alternative secondary structures were proposed for the HIV 5'-UTR (Figure 35) in which the functional elements are either accessible or silenced. The LDI (Long Distance Interaction) is believed to direct the gRNA towards translation whereas the BMH (Branched Multiple Hairpin) is believed to allow the dimerization and the encapsidation of the gRNA (Abbink & Berkhout 2003; Berkhout et al. 2002; Huthoff & Berkhout 2001a; Huthoff & Berkhout 2001b). It has been hypothesized that NC induces this conformational switch which would lead to dimerization and encapsidation or translation of the gRNA (Huthoff & Berkhout 2001b).

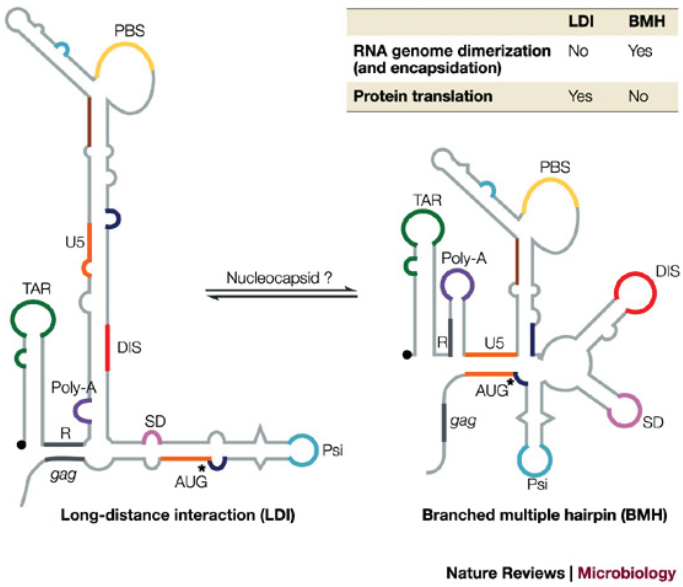

Figure 35: Model showing the putative conformational switch proposed to regulate translation and packaging of the HIV-1 genomic RNA (Paillart, Shehu-Xhilaga, et al. 2004).

4.5.2.3 Role of NC in the assembly of the viral genome

In parallel to encapsidation and dimerization, the genome is trafficked to the plasma membrane and used as a platform for Gag multimerization. These two processes are highly dependent on one another. The NC domain of Gag plays a key role in Gag assembly since the deletion of NC domain or its two zinc fingers resulted in a deficiency of Gag oligomerization (Ott et al. 2009; Johnson et al. 2002; Crist et al. 2008; El Meshri et al. 2015; de Rocquigny et al. 2014; Muriaux & Darlix 2010) and accumulation at the plasma membrane (Ono & Freed 2001; Ono et al. 2005; Li et al. 2007; El Meshri et al. 2015).

The role of Gag(NC) in the assembly is strongly related to its ability to form with NAs a scaffold for Gag oligomerization (Cimarelli & Darlix 2002; Muriaux et al. 2001; Jouvenet et al. 2009). This initial step of NC-RNA recognition is a key determinant for the initiation of Gag packing and routing to the plasma membrane (Ott et al. 2009; Alfadhli et al. 2005). The initiation of particle production is delayed by an NC-deleted Gag in comparison to the wild type Gag (Ott et al. 2009). This NC RNA interaction is thought to be non-specific (Campbell and Rein 1999) and mainly promoted by the basic residues of NC rather than the Zn fingers (Cimarelli et al. 2000; Burniston et al. 1999; Wang et al. 2004; Ono et al. 2005; Ott et al. 2009).

Although many studies did not find any role of Gag(NC) in Gag trafficking to the plasma membrane (Burniston et al. 1999; Muriaux et al. 2004; Grigorov et al. 2007), other studies reported that Gag(NC) mutants are deficient in reaching endosomes and membrane binding (Grigorov et al. 2007; Hogue et al. 2009).

A recent study done in our lab by Elmeshri et al (2015) demonstrated a role of Gag(NC) in the rapid trafficking of Gag oligomers to the plasma membrane. This is probably achieved via the interaction of NC with cytoskeleton proteins (actin, tubulin) in addition to cellular motor proteins such as KIF4 (Liu et al. 1999b; Jolly et al. 2007; Martinez et al. 2008). Additionally, Gag (NC) interacts electrostatically with the negatively charged phospholipids upon initial binding of Gag to the plasma membrane (Kempf et al. 2015). A loss of this interaction upon Gag(NC) deletion could explain the poor binding of a Gag(NC) deletion mutant to the plasma membrane (El Meshri et al. 2015).

Furthermore, Gag(NC) cooperates with p6 and SP2 in particle budding via its association with the ESCRT proteins Tsg101 and ALIX required for viral budding. The interaction between the NC and the Bro-1 domain of ALIX helps to recruit the budding machinery, ESCRTIII and VPS4, promoting viral release (Popov et al. 2009; Popova et al. 2010).

Gag(NC) Interaction with host factors promoting assembly and budding

Gag(NC) interacts with several host proteins especially during the late phase of the viral lifecycle (Mirambeau et al. 2010; Friedrich et al. 2011; Muriaux & Darlix 2010; Ott 2002). Several cellular proteins (Staufen1/ABCE1/Bro1/actin/ nucleolin/ APOBEC3G, etc.) were described to interact with the Gag NC domain (Figure 36) with some of them playing a role in particle assembly or budding (Popov et al. 2009; Chatel-Chaix et al. 2008; Bacharach et al. 2000; Lingappa et al. 2006; Wilk et al. 1999; Luo et al. 2004).

Actin is one of the first cellular proteins shown to be an HIV-1 NC ligand. It interacts with the N-terminal domain (Krogstad et al. 2002) of both mature NCp7 and NC as a domain of Gag (Wilk et al. 1999; Liu et al. 1999a). This interaction is shown to affect viral budding and actin was shown to be incorporated into the virus (Gladnikoff et al. 2009; Chen et al. 2007; Chertova et al. 2006; Ott 2008). However, further studies are needed to analyze this interaction and its consequences.

Nucleolin is an RNA binding chaperone protein usually present in the dense granular region of the nucleolus; however, it was found at the plasma membrane of HIV-1 infected cells (Ueno et al. 2004). It could possibly play a role in the nucleocytoplasmic transport of gRNA as it is able to bind to gRNA (Cochrane et al. 2006). Nucleolin interacts with the NC domain of Gag and Psi region of gRNA thus promoting assembly, budding, and release of viral particles (Bacharach et al. 2000).

ALIX is a protein of the ESCRT family playing a role in enhancing virus budding. On one hand; ALIX interacts with the p6 domain of Gag to recruit the ESCRT machinery. On the other hand, the Bro 1 domain of ALIX interacts with the NC domain of Gag cooperating in the ESCRT recruitment (Popov et al. 2009; Dussupt et al. 2009; Popova et al. 2010).

Staufen1 is a protein found in early ribonucleoprotein assembly complexes of HIV-1. It plays a role in RNA trafficking and metabolism. Staufen1 is recruited by the Gag(NC), more particularly by the zinc fingers (Chatel-Chaix et al. 2008), and it favors gRNA encapsidation. Gag and Staufen are thought to actively recruit cellular proteins involved in assembly. Furthermore, Staufen1 is incorporated into the virus (Mouland et al. 2000; Chatel-Chaix et al. 2004).

The cellular ABCE1 protein is a member of the ATP-binding cassette (ABC) transporters superfamily which transports molecules across cellular membranes. It directly binds Gag after its translation assisting the early stages of viral assembly (Zimmerman et al. 2002). ABCE1/ Gag binding is mediated through the basic residues of NC (Lingappa et al. 2006).

In addition, Gag(NC) likely interacts with restriction factors such as the Prion Protein (PrP) and human Discs Large (hDlg1) protein. PrP is RNA chaperone protein that affects negatively virion production probably through the interaction of Gag(NC) and gRNA (Leblanc et al. 2004; Gabus et al. 2001). Human hDlg1 is a scaffold protein recruited beneath the plasma membrane. Its interaction with the Gag(NC) modulates the distribution of Gag in the plasma membrane and was shown not to affect particle assembly and release but to affect their infectivity (Perugi et al. 2009).

Finally, genetic screens have identified more than 1000 host proteins that may interact with the viral NC protein; however, these interactions have to be validated and their potential roles have to be disclosed (Brass et al. 2008; Jäger et al. 2011; König et al. 2008; Honglin Zhou et al. 2008).

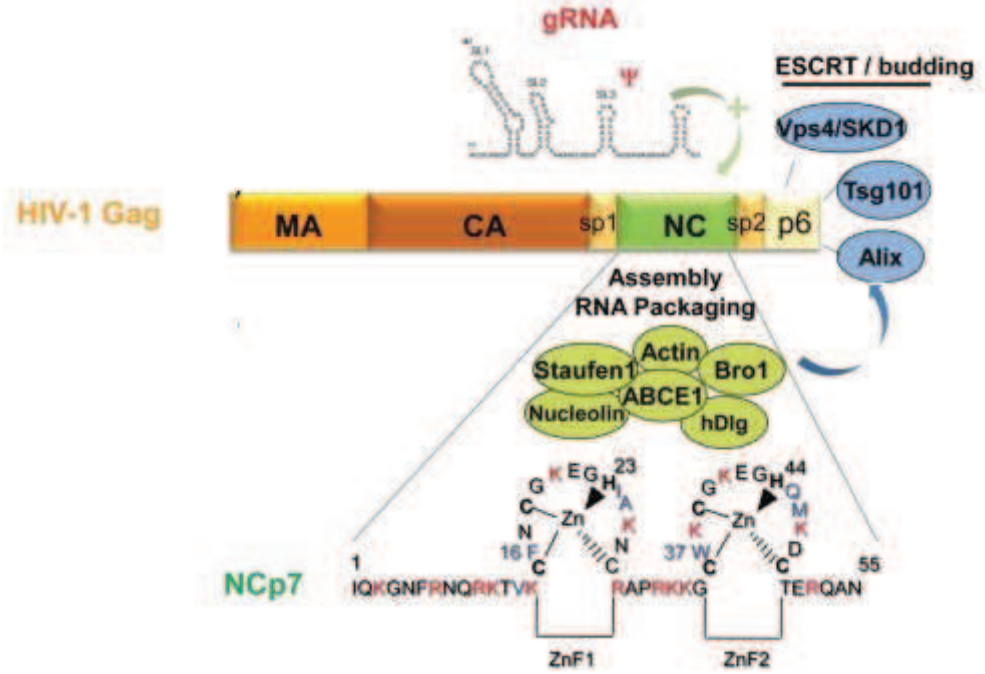

Figure 36: Cellular factors associated to Gag polyprotein precursor and its NC domain. The NC domain of Gag is necessary for gRNA recruitment via its binding to the Psi packaging signal. The NC domain is a key factor for Gag multimerization on the gRNA. NC interacts with cellular factors such as actin, Staufen1, the Bro1 domain of ALIX and ABCE1. NC interacts with the Bro-1 domain of Alix participating with p6 in the recruitment of the budding machinery. The 55 amino acids of mature NC are shown. NCp7 is a highly basic (in red) protein containing two highly conserved zinc fingers and a plateau of hydrophobic amino-acids (in blue) involved in RNA-NC interactions, RNA chaperone activities and particle assembly (adapted from Muriaux and Darlix 2010).

Chapter V- HIV-1 Translation

5.1 Overview of Eukaryotic Translation

Translation is the final stage in the gene expression pathway where proteins are synthesized by the ribosome from the mRNA according to the information coded by the latter. This is a complex process involving interactions between three classes of ribonucleic acids (rRNA, tRNA, and mRNA) and proteins. mRNA is a ribonucleotide sequence harboring two post transcriptional modifications: the 5'cap(m^7G), a methyl guanosine attached via a 5' to 5' triphosphate bridge to the RNA 5'-end, and a poly(A) tail at its 3' end corresponding to long stretch of adenylate residues (range 67 to 92 in mammalian cells, Subtelny et al 2014). The translation of the mRNA is initiated at the start codon (AUG encoding a Met) and ends by one of the three stop codons (UAG, UGA, UAA). The region between the start codon and the stop codon is the coding sequence or the open reading frame (ORF) translated by the ribosome. The nucleotides are read in triplicates where each triplicate is called a codon and codes for a specific amino acid and each codon is recognized by a specific tRNA. tRNA is a small RNA of 75-95 nucleotides folded into a three dimensional structure with three hairpin loops. One of these hairpins contains a sequence called anti-codon, a sequence of three nucleotides complementary to the codon on the mRNA. In another hairpin, the tRNA carries the amino acid corresponding for that codon. When the tRNA recognizes and binds to its corresponding codon in the ribosome, the tRNA transfers the appropriate amino acid to the end of the growing amino acid chain. Then the tRNAs and ribosome continue to decode the mRNA molecule until the entire sequence is translated into a protein (Rodnina & Wintermeyer 2009; Ramakrishnan 2011). It is important to note that translation is a cyclical process, and ribosomal subunits that participate in initiation derive from the recycling of post-termination ribosomal complexes (post-TC).

The translation process is divided into three main steps: initiation, elongation, and termination. The most important and most regulated one is the initiation step which will be the focus of this chapter. The initiation step corresponds to the assembly of the elongation-competent 80S ribosome on the mRNA, in which the initiation codon is base-paired with the anticodon loop of initiator tRNA in the ribosomal P-site. During the elongation step, the

mRNA is decoded by the 80S ribosome thus forming the polypeptide chain. Finally, the termination of the translation occurs with the release of the nascent polypeptide when the ribosome encounters a stop codon (Groppo & Richter 2009; de Breyne et al. 2013).

Initiation of Translation

The initiation of translation is in any case the rate-limiting step in translation; it mainly corresponds to the recruitment of the 40S ribosome to the mRNA. Two different mechanisms have been described for initiation of translation: the canonical-cap dependent one also mentioned as the scanning mechanism and the less common one, the internal ribosome entry site (IRES)-dependent.

5.1.1 Cap-Dependent Translation Initiation

This mechanism of translation initiation requires at least 12 initiation factors (eiFs) (Jackson et al. 2010) and starts by the formation of the 43S preinitiation complex composed of the 40S ribosomal subunit, the eIF2-GTP-Met-tRNA^{Met} ternary complex, eIF3 and other initiation factors (Figure 37, steps 2 and 3). In parallel, another complex of eIFs binds the mRNA 5'cap. It consists of eIF4E which physically binds the cap, eIF4A an RNA helicase which unwinds the RNA secondary structures and the scaffolding protein eIF4G. The mRNA is circularized by the eIF4G binding to the poly(A) binding protein (PABP) positioned on the poly(A) tail at the 3'end of the mRNA (Figure 37, step 4).

The 43S preinitiation complex recognizes the mRNA by binding of eIF3 to eIF4G (Figure 36, step 5). The 43S pre-initiation complex scans the mRNA in a 5' to 3' direction until it encounters the start codon and recognizes it by base pair formation with the tRNA (Figure 37, step 6). Binding of the pre-initiation complex to the start codon leads to the formation of the 48S initiation complex (Figure 37, step 7). Subsequently, the eIF5B-dependent joining of the 60S subunits to the 48S complex is concomitant with hydrolysis of eIF2-GTP and release of most of the initiation factors leading to the 80S initiation complex (Figure 37, step 8). Finally, another step of GTP hydrolysis of eIF5B occurs before the 80S complex is rendered competent for elongation (Figure 37, step 9) (Balvay et al. 2007; Groppo & Richter 2009;

Gebauer & Hentze 2004; Jackson et al. 2010).

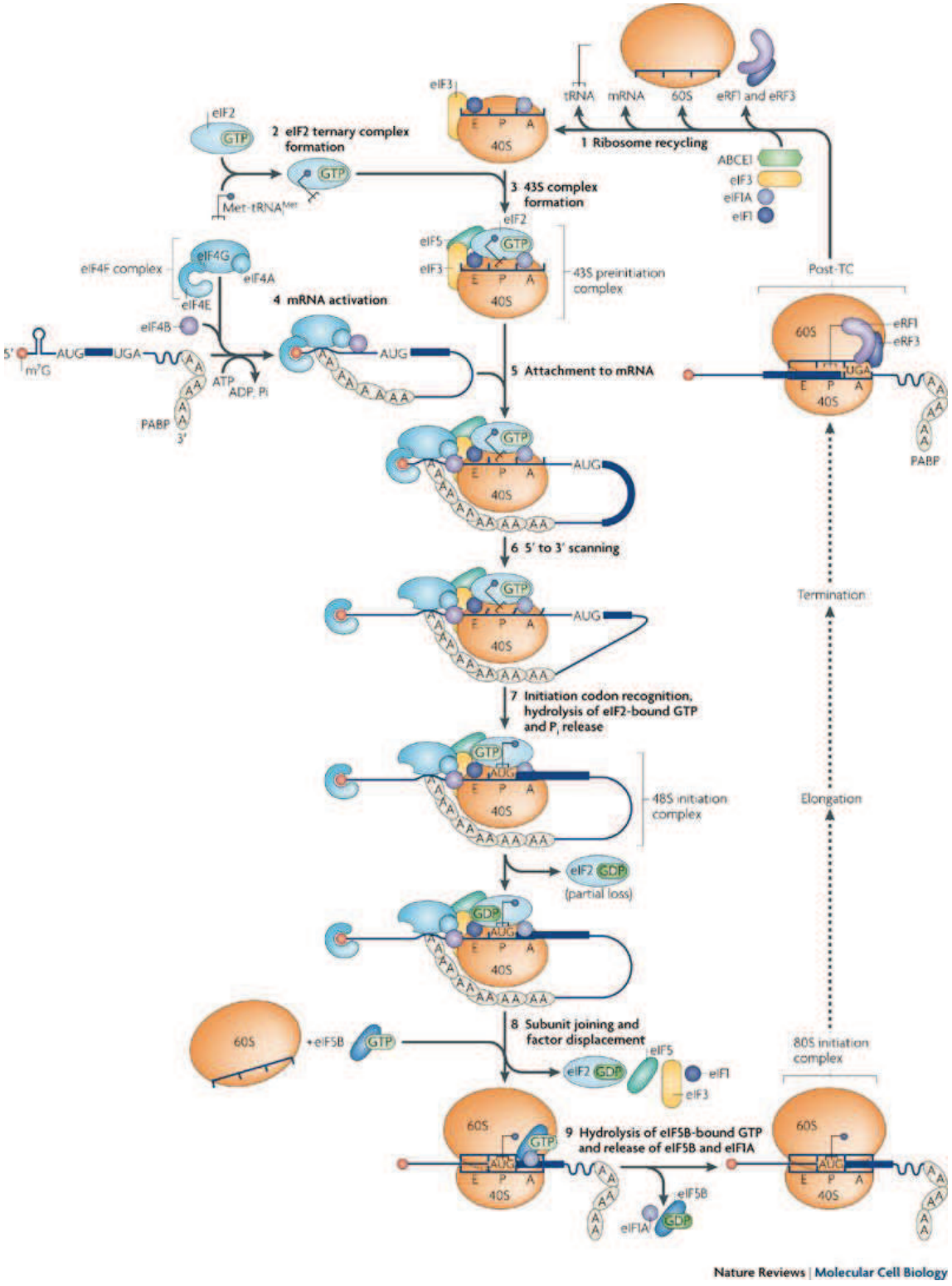

Figure 37: Canonical pathway of eukaryotic translation initiation (Jackson et al. 2010).

5.1.2 Internal Ribosome Entry Site (IRES) Dependent Translation Initiation

Internal Initiation of translation was first demonstrated for the picornavirus family followed by the discovery of other types of IRES in other RNA viruses (Jang et al. 1988). Later, the first reports on cellular IRESs started to appear and the list of cellular mRNA harboring IRESs is increasing since then (Macejak & Sarnow 1991; Baird et al. 2006; Komar & Hatzoglou 2005). *Picornaviridae* is a family of single stranded positive RNA viruses represented by poliovirus (PV) and encephalomyocarditis virus (EMCV). The genomic RNA of these viruses is not capped and its 5'-UTR genomic RNA is long and highly structured, thus making it difficult for the classical ribosomal scanning to occur. In addition, picornavirus proteases cleave eIF4G and PABP needed, as seen earlier, for cap-dependent translation. Despite all those factors, picornavirus protein synthesis still occurs at an important level during infection, suggesting another mechanism of translation initiation (Martínez-Salas & Fernández-Miragall 2004; Balvay et al. 2007; Roberts et al. 2009).

This alternative mechanism which does not require the cap or the cap binding protein eIF4E initiates translation by direct ribosome binding to the 5'-UTR. The IRES dependent translation allows RNAs to bypass the canonical cap-dependent translation control mechanisms thus allowing protein synthesis to occur under cellular stress conditions including hypoxia, apoptosis, angiogenesis, heat shock, irradiation, G2/M cell cycle arrest and viral infection (Stein et al. 1998; Lang et al. 2002; Holcik et al. 2000; Stoneley et al. 2000; Akiri et al. 1998; Fernandez et al. 2001; Kim & Jang 2002; Pyronnet et al. 2001). All IRESs have in common highly structured GC rich RNA regions (Balvay et al. 2007; Balvay et al. 2009). Despite their common structure, each IRES needs a different limited set of the canonical initiation factors to be functional.

5.1.2.1 IRES classification

According to their structure and sequence; most of the viral IRESs have been classified into four types (Jackson et al. 2010). Cellular IRESs are however much more variable (Figure 38) even if they are often compared to picornaviruses IRES.

Type I and type II include the picornaviruses IRESs with type I including the Polio enterovirus (PV) and human rhinovirus (HRV) and type II containing the cardiovirus EMCV (encephalomyocarditis virus) and the aphtovirus IDU
(Foot-and-mouth disease virus). For these two types of IRES, most of the canonical initiation factors are necessary with the exception of eIF4E and the N-terminal part of eIF4G. The cleavage of the latter by viral proteases renders it nonfunctional for cap-dependent translation but still active for the virus RNA translation thus allowing the virus to by-pass the cellular protein synthesis shut-down (Ohlmann et al. 1996; Pestova et al. 1998; Prévôt et al. 2003).

Type III includes the flaviviruses such as hepatitis C (HCV) IRES. Initiation of translation can occur without the need of eIF1A, 4A, 4B and 4F as these IRESs are able to bind the 40S ribosomal subunit directly.

Type IV canonical IRES is the one of the dicistrovirus CrPV (cricket paralysis virus). These IRESs initiate translation without the need of any initiation factor, initiator tRNA, or a proper AUG start codon (Deniz et al. 2009; Wilson et al. 2000; de Breyne et al. 2013). In this type of viruses, translation can occur even under extreme stress conditions.

Besides these classified IRESs, a number of unconventional IRESs is also found. Among them is the one of the lentivirus. As described below, the presence of a functional IRES in the 5'-UTR of the lentivirus genome was a debate for a long time as they were described to have on one hand type III IRES properties capable of binding directly to the ribosome 40S subunit (Locker et al. 2011), and on the other hand, they require all the eIF's, with the exception of eIF4E as type I and II IRESs do. They are described later in this manuscript. They are part of a larger group of IRESs localized on 5'-capped and polyadenylated mRNAs and can undergo both kinds of translation initiation mechanisms according to the cell's physiological conditions (de Breyne et al. 2013).

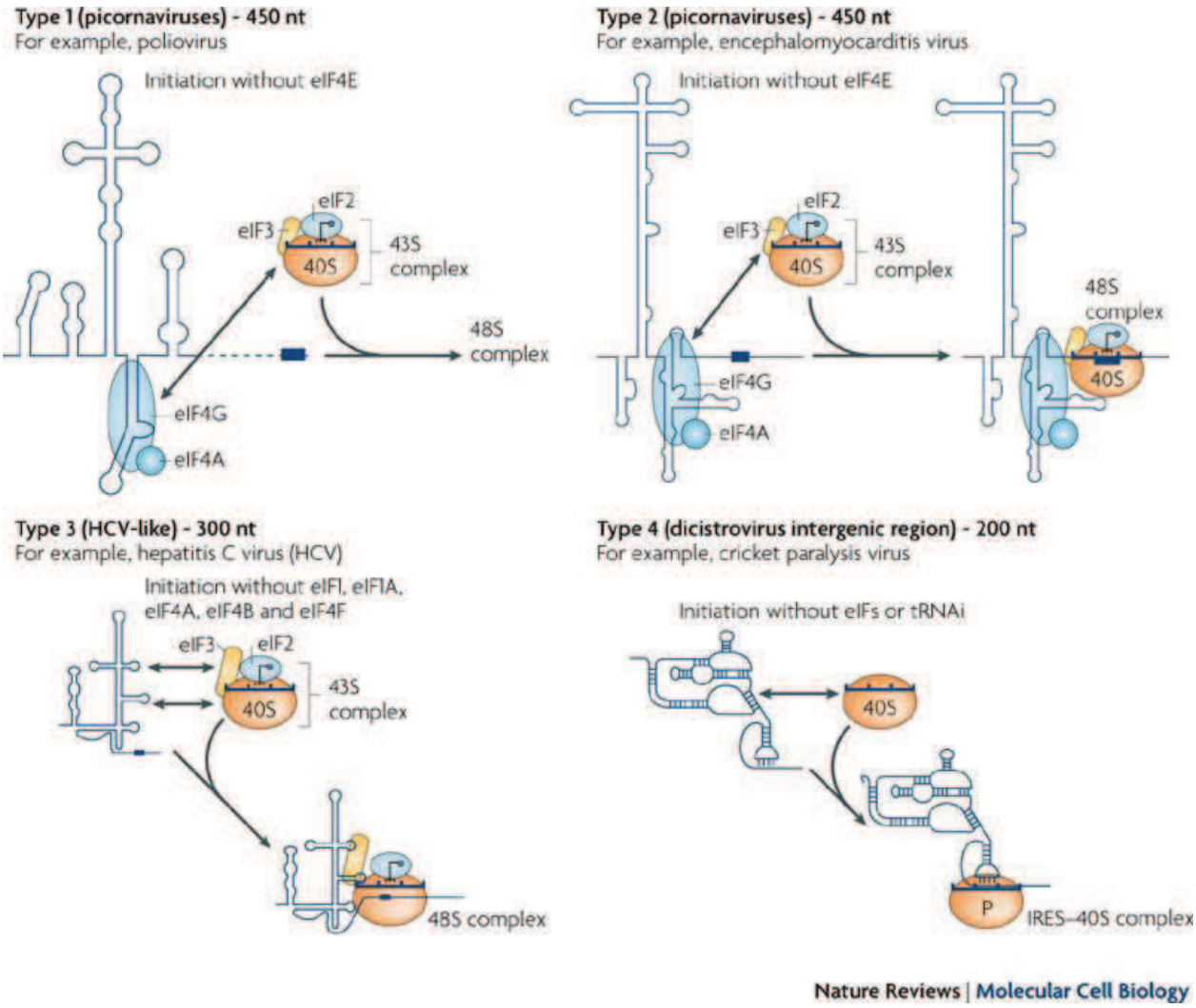

Figure 38: Different IRES groups and their needs in initiation factors (Jackson et al. 2010).

5.1.2.2 IRES Trans-Acting Factors

IRES-dependent translation initiation requires in addition to certain initiation factors the presence of a subset of RNA binding proteins called IRES Transacting Factors (ITAFs). The requirements differ quite markedly for different IRESs even between closely related IRESs (Table 1). Among these ITAFS are the La autoantigen, Polypyrimidine Tract binding protein (PTB), Heterogenous Nuclear Ribonucleoproteins A1 (hnRNP A1), Heterogenous Nuclear Ribonucleoproteins C1 and C2 (hnRNPC1/C2), and Up-stream of N-ras (Unr) which will be focused upon in the next chapter (King et al. 2010; Komar & Hatzoglou 2011; Stoneley & Willis 2004; Balvay et al. 2007; de Breyne et al. 2013). It is worth noting that all the IRESs are not stimulated at the same level by a given ITAF. For example, even if FMDV and HRV

IRESs have a very strong requirement for PTB (Hunt & Jackson 1999a; Pilipenko et al. 2000), the EMCV IRES is only slightly stimulated by PTB (Pestova et al. 1996; Kaminski & Jackson 1998) whereas the PV and HAV IRESs occupy an intermediate position between the two extremes FMDV and EMCV (Hunt & Jackson 1999a; Yu et al. 2011). All type I IRESs require PCBP2 (Walter et al. 1999) which seems to act in conjunction with SRp20 at least for PV IRES stimulation (Bedard et al. 2007). Additionally, type I HRV IRES has a strong requirement for the Unr protein which does not seem to affect PV IRES *in vitro* (Hunt et al. 1999) but was found highly stimulatory after cell transfection (Boussadia et al. 2003).

As for the mechanism, there is no evidence that any ITAF recruits directly the 43S ribosomal subunit; it more probable that ITAF binding remodels the RNA secondary structure into conformations capable of recruiting the initiation complex. One result supporting this affirmation is the fact that ITAF binding to some IRESs not only protects the RNA from degradation but also exposes some residues to chemicals (Pilipenko et al. 2000).

Virus family	IRES	IRES type	ITAF
Picornavirus	PV	I	PTB, PCBP2, PCBP1, La, Unr, SRp20
	FMDV	II	PTB, ITAF45, La
	HRV	I	PTB, PCBP2, La, Unr, hnRNPA1
	HAV		PTB, PCBP2
	EMCV	II	PTB, La
Flavivirus	HCV	III	PTB, PCBP2, La, hnRNP D, hnRNP L
Dicistrovirus	CrPV	IV	
Retrovirus	HTLV-1		PTB, La
	HIV-1		La
	SIV		
	MoMuLV		
Cellular	Apaf-1		PTB, nPTB, Unr
	Unr		PTB, Unr

Table 1: ITAF requirements for different IRESs. Data compiled from (Fitzgerald & Semler 2009; Jackson 2013; King et al. 2010).

5.2 HIV-1 Translation

As it was mentioned before, the translation of the full length HIV-1 mRNA produces mainly the precursors Pr55Gag and Pr160Gag-Pol. Pr55Gag is translated by the conventional translation elongation whereas Pr160Gag-Pol is produced by a -1 programmed ribosomal frame shift. Both cap-dependent and IRES-dependent translation initiation have been proposed to account for HIV-1 translation initiation (Figure 39). In parallel, another shorter Gag isoform is synthetized during HIV-1 infection (Pr40Gag) which corresponds to translation initiation at an alternative AUG start codon. This isoform was shown to be important for HIV-1 infection in cultured cells (Buck et al. 2001).

Several mechanisms of translation initiation were shown to occur for HIV-1. In fact, the identification of an IRES in the Gag coding region was rapidly commonly accepted and described as responsible for Pr40Gag synthesis. The presence of a functional second IRES localized in the HIV-1 5'-UTR was however much more debated as it was not identified in HIV-2 (de Breyne et al. 2013) in contrary to the one in the Gag coding region found in both HIVs (Herbreteau et al. 2005). Nowadays, these three kinds of initiation acting in parallel are accepted but still a lot of work has to be done to determine precisely when and for what purpose each of them is used during the time course of an infection. In fact, Monette et al showed that 70% of Gag synthesis is due to the IRES-dependent translation initiation (Monette et al. 2013). However, Amorim et al showed that HIV-1 protein synthesis is highly dependent on the cap-dependent translation initiation during the first 24-48 hours of infection while IRES-dependent translation is important later for particle production (Amorim et al. 2014). Most of the debate is linked to the fact that IRES activity experiments were in different *in vitro* systems which is problematic considering the HIV-1 IRES dependency on cellular factors.

In my PhD I focused on the HIV-1 5'-UTR IRES named the HIV-1 IRES in this manuscript for simplification. The HIV-1 cap- and 5'-UTR dependent translation initiation will be presented in the next paragraphs.

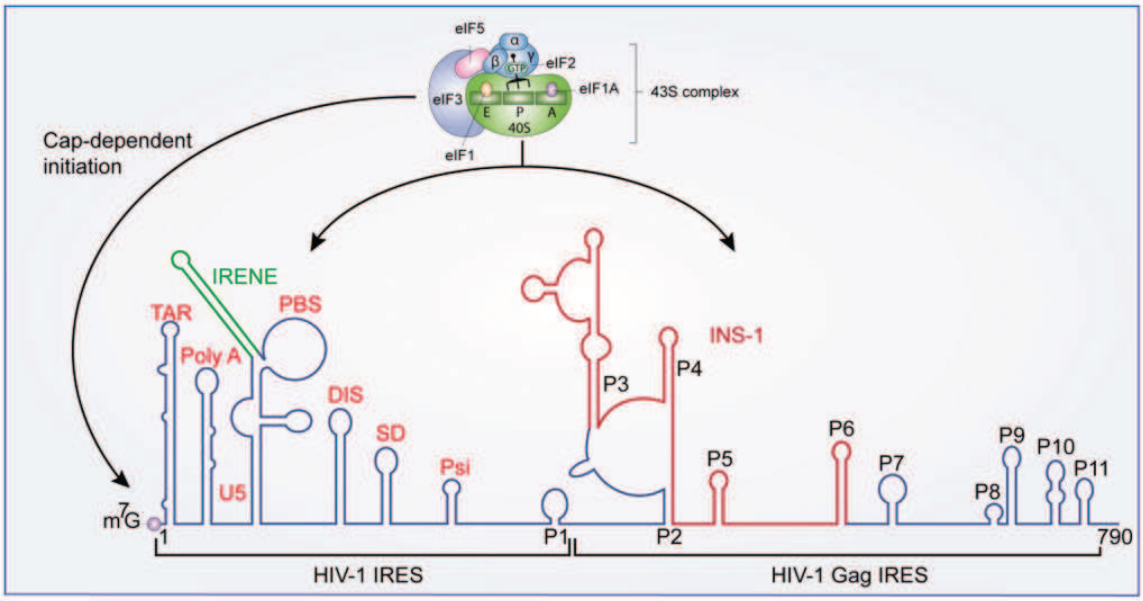

Figure 39: HIV-1 IRESs and HIV-1 Gag IRESs. The 43S preinitiation complex can mediate both cap-dependent and IRES-mediated translation initiation (Guerrero et al. 2015).

5.2.1 HIV-1 Cap-dependent Translation

HIV viral mRNAs, as all RNA polymerase II products, are capped at their 5'-end and polyadenylated at their 3'-end, thus, they undergo cap-dependent translation initiation in a manner similar to cellular mRNA. As it was discussed before, the cap-dependent translation initiation depends on the recognition of the cap by the eIF4F complex for the recruitment of the 43S pre-initiation complex.

Cap-dependent translation of HIV-1 mRNA has been demonstrated both *ex vivo* (Chamond et al. 2010; Guerrero et al. 2015; Miele et al. 1996) and *in vitro* (Ricci et al. 2008; Chamond et al. 2010; Guerrero et al. 2015) although the high degree of RNA structures in the HIV 5'-UTR was shown, in rabbit reticulocyte, to limit the unwinding step of the ribosomal scanning (Miele et al. 1996). Interestingly, the HIV-1 5'-UTR was unable to promote translation when cloned into a dicistronic construct with downstream reporter gene, so that Miele et al. proposed that HIV-1 translation occurs exclusively by a cap-dependent mechanism (Chamond et al. 2010; Guerrero et al. 2015; de Breyne et al. 2013).

In another study, Ricci et al. (2008) performed competition experiments *in vitro* in rabbit reticulocyte lysates (RRL) using either a cap analogue that competes with the cap mRNA for the recruitment of eIF4F or L-protease from the foot and mouth disease virus (FMDV) that cleaves eIF4G and impairs the cap dependent translation. This resulted in a diminished HIV-1 translation but not a total extinction; so that it was proposed that HIV-1 Pr55Gag synthesis relies both on cap- and IRES-dependent translation initiations.

This latter affirmation was supported by a recent study showing a possible ribosomal scanning of the HIV-1 5'-UTR as the insertion of upstream AUG codons within the 5'-UTR caused a decrease in translation from the native HIV-1 AUG codon (Berkhout et al. 2011).

Ribosomal Scanning Barriers

Among all the RNA structures within the 5'-UTR, the TAR sequence is the major obstacle for ribosomal scanning as it was shown to inhibit translation alone or in the context of the 5'-UTR. In order to overcome this hurdle, HIV takes advantage of the host translation machinery. The translation of the HIV-1 5'-UTR in RRL increases upon the addition of HeLa cell extracts suggesting that cellular factors in the extracts are responsible for overcoming the translational inhibition mediated by the 5'-UTR (Soto-Rifo, Limousin, et al. 2012; Vallejos et al. 2011). A variety of cellular proteins have been demonstrated to bind TAR element and promote cap-dependent viral translation.

Soto Rifo et al. (2012b) demonstrated that the RNA helicase DDX3 promotes translation initiation of HIV-1 genomic RNA via binding to the HIV-1 5'-UTR and interacting at the same time with eIF4G and PAPB. This interaction could promote the unwinding of the double stranded RNA structures allowing the assembly of the pre-initiation complex in an ATP dependent manner even in the absence of eIF4E. Down regulation of DDX3 results in a specific inhibition of the HIV-1 5'-UTR driven expression and this was confirmed by showing that DDX3 is important for HIV-1 gRNA translation in the context of viral ureplication (Soto-Rifo, Rubilar, et al. 2012). The specific recruitment of DDX3 to the HIV-1 RNA is mediated by DDX3 interaction to the TAR-binding viral protein Tat (Lai et al. 2013). Additionally, it was demonstrated in RRL that the viral protein Rev, at low concentration, binds specifically to DDX3 thus stimulating HIV-1 translation. In the contrary, high Rev

concentrations block nonspecifically all ribosomal scanning via an unknown mechanism (Groom et al. 2009).

Other TAR binding proteins include the Lupus autoantigen (La) whose binding to TAR element relieves the translational repression. This is probably achieved by La's dsRNA unwinding activity (Svitkin et al. 1994; Hühn et al. 1997). Staufen and TAR RNA binding protein (TRBP) were also reported to bind TAR and facilitate RNA translation (Dugré-Brisson et al. 2005; Dorin et al. 2003). The RNA helicase A (RHA) as well interacts with the HIV-1 5'-UTR enhancing LTR directed gene expression (Bolinger et al. 2010).

5.2.2 HIV-1 IRES-Dependent Translation

A strong argument for the relevance of the HIV-1 IRES is the fact that it was described as functional in several HIV-1 strains including the proviral wild-type pNL4.3 HIV-1 clone (Brasey et al. 2003) as well as in CXCR4 (X4)-tropic primary isolate HIV-LAI (Gendron et al. 2011). Moreover, Vallejos et al. demonstrated that not only laboratory HIV-1 strains exhibited IRES activity, but also natural variants of HIV-1 exhibited this activity (Vallejos et al. 2012).

5.2.2.1 HIV-1 IRES activity is cell type and state dependent

Many studies demonstrated this IRES activity in cellular states where the cap dependent translation initiation was inhibited (Guerrero et al. 2015; de Breyne et al. 2013). The HIV-1 IRES activity was first discovered by Brasey et al in 2003 by cloning the 5'-UTR of PNL4.3 HIV-1 proviral clone into dual luciferase vector containing the *Renilla* luciferase gene (Rluc) upstream and the firefly luciferase gene downstream (Fluc). To ensure that the two cistrons are independently translated, a defective EMCV IRES (ΔEMCV) known to inhibit ribosome initiation and read- through was inserted upstream of the HIV-1 5'-UTR. The translational activity of the HIV-1 5'-UTR was monitored using Fluc as the readout whereas Rluc served as a translational control. The IRES exhibited poor activity in an *in vitro* rabbit reticulocyte lysates (RRL); however, it was active when supplemented with HeLa cell extracts and even more stimulated with the addition of G2/M arrested HeLa cell extracts. Then, they performed

a serious of deletions at the 5' and 3' region of the 5' UTR and IRES activity was mapped to the region with the nucleotides 104-336 (Brasey et al. 2003).

This suggested the need of cellular factors over-regulated in G2/M to act as chaperones or ITAFs rendering the IRES active or factors down-regulated in G2/M which would be IRES repressors. These results were in agreement with other studies (Miele et al. 1996; Vallejos et al. 2011). Vallejos et al. found that the HIV-1 IRES is more effective in RRL supplemented with G2/M cellular extracts than when supplemented with G1 or non-synchronized cells. They also demonstrated that some portions of the 5'-UTR are protected from chemical attack specifically in the presence of G2/M cellular extracts. Thus, they concluded that G2/M cellular extracts contain proteins that stimulate HIV-1 translation by binding to RNA regions critical for HIV-1 IRES activity (Vallejos et al. 2011). The fact that this activity is cell dependent is in agreement with the data of Plank et al showing that it is four times more active in Jurkat cells than in HeLa cells and even more active in other T cell lines, the HIV-1 natural targets (Plank et al. 2013).

5.2.2.2 HIV-1 IRES important structural features

The minimal active HIV-1 IRES was restricted to the region spanning nucleotides 104 to 336 in pNL4-3 strain which also contains several RNA motifs important for HIV-1 life cycle (PBS-DIS-SD-Psi) (Brasey et al. 2003). Even if the HIV-1 IRES seems to be only weakly affected by mutations or deletions, (Vallejos et al. 2011; Vallejos et al. 2012; Plank et al. 2013) mutating the region at the base of the DIS stem-loop and surrounding the four way junction had great negative effects suggesting that these nucleotides are critical for IRES activity (Plank et al. 2013). These results are in agreement with the data provided by Gendron et al (2011) where they reported a sensitivity of IRES activity to mutations in this apical DIS loop area. Hence, this RNA region is suggested to be a binding site for putative proteins which modulate HIV-1 IRES activity (Gendron et al. 2011). This hypothesis is supported by the study of Vallejos et al. (2011) which identified changes in the chemical probing profile of the IRES in this region upon the addition of cellular extracts (Vallejos et al. 2011).

Through a mutational analysis, Gendron et al. defined regions of HIV-1 IRES that are important for its activity. Three positive determinants increased IRES activity (nt 202-217, nt

240-242, and nt 302-305) whereas one negative determinant called IRENE in the stem loop preceding the PBS (nt 134-178) decreased IRES activity. Deletions or mutations in the positive determinants could either remove the binding site of an unknown cellular factor or result in an IRES conformational change (Gendron et al. 2011).

5.2.2.3 Factors modulating HIV-1 IRES activity

Several cellular proteins were reported as factors modulating HIV-1 IRES activity (Guerrero et al. 2015; de Breyne et al. 2013). Monette et al. showed that the heterogenous nuclear ribonucleoprotein A1 (hnRNP A1) acts as an IRES transacting factor. This IRES activity was coupled to increased expression and cytoplasmic retention of hnRNP A1 upon viral infection (Monette et al. 2009). eIF5A and the Rev co factors DDX3 and hRIP (Human Rev Interacting Protein) were also found to increase HIV-1 IRES activity. Their overexpression after 293T cells transient transfection along with a bicistronic dual luciferase reporter construct increased both cap-and IRES-dependent translation with a further increase of the latter. The inverse results were obtained after the knock down of their expression (Liu et al. 2011).

On the contrary, the human embryonic lethal abnormal vision (ELAV)-like protein (HuR) functions as a repressor of HIV-1 IRES activity by impacting post-integration steps of the HIV-1 replication cycle. This repression does not require a direct binding between HuR and the viral RNA but most likely via protein-protein interaction according to an unknown mechanism (Rivas-Aravena et al. 2009).

Additionally, the HIV-1 IRES activity was found to be inhibited by the instability element (INS-1), a cis acting element present in the gag ORF. This inhibition is partially rescued by Rev or hnRNPA1 overexpression (Valiente-Echeverría et al. 2013). Brasey et al. also defined the *gag* ORF to negatively influence the IRES-dependent translation initiation in the context of bicistronic mRNA (Brasey et al. 2003). The addition of the *gag* ORF could favor a U5-AUG duplex formation thus inhibiting translation.

Apart from hnRNP A1, the proteins mentioned here are not the classical viral ITAFs. As far as the latter are concerned, it is important to mention that the La autoantigen was found to interact with HIV-1 and 2 5'-UTR especially with the TAR stem (Chang et al. 1994;

Waysbort et al. 2001) even if it was not functionally shown that this interaction has a specific impact on the IRES activity (Svitkin et al. 1994).

Another study from Vallejos et al (2011) identified several proteins from G2/M HeLa cell extracts to be HIV-1 5'-UTR binding partners, among them hnRNP L known to be an HCV ITAF but not PTB, PCBP1/2 or Unr. No functional data concerning HIV-1 IRES stimulation were available (Vallejos et al. 2011).

5.2.2.4 Why IRES dependent translation is significant?

After having a look at the two mechanisms of translation initiation of HIV-1 RNA and their regulation, one can wonder how this is related to the cellular modifications induced during an HIV-1 infection.

During the course of viral infections, an increase of reactive oxygen intermediates is often reported. This is also the case for an HIV-1 infection (Deshmane et al. 2009). This oxidative stress impairs cap-dependent translation initiation and stimulates IRES-dependent translation initiation as it was shown for HCV and HIV-1 (MacCallum et al. 2006; Gendron et al. 2011).

During the course of an HIV-1 infection, cap-dependent translation is also impaired by the viral PR which cleaves the initiation factors eIF4G and PABP required for cap-dependent translation (Ventoso et al. 2001; Castelló et al. 2009). This viral strategy is already well known for PV but Ohlmann et al. (2002) reported that eIF4G1 but not eIF4G2 is cleaved by the viral HIV-1 PR both in rabbit reticulocyte and cell lysates. However, unlike PV protease which cleaves in one site, they showed that the cleavage occurs at three sites and affects both cap-dependent as well as IRES-dependent translation, an event which is most likely to occur late in the infection time course (Ohlmann et al. 2002).

Moreover, the expression of the viral protein Vpr promotes a cell cycle arrest in the G2/M phase (Zhao et al. 2011). This is associated with phosphorylation of eIF4E and disruption of the eIF4F complex (Sharma et al. 2012). These events render the cap dependent translation initiation inactive. Despite the global decrease of the cap-dependent translation, the translation of the HIV-1 structural proteins is conserved. One hypothesis is the flexibility of HIV-1 to

undergo cap independent translation using a different set of initiation or modulating factors overexpressed in G2/M as discussed in the above section (Monette et al. 2013; Amorim et al. 2014; de Breyne et al. 2012). The HIV-1 mRNA expression seems to be even more complex. Recently, Sharma et al. showed that the full length unspliced mRNA as well as singly spliced HIV-1 mRNAs were associated with cap binding complex (CBC) even after their viral encapsidation. It is in contrast to multi-spliced cellular mRNAs which use this complex only for nuclear export and the first round of their translation which is important for the quality control of the transcript but is replaced by the eIF4F complex for the latter translation initiation (Moore 2005; Maquat et al. 2010; Sharma et al. 2012). The HIV-1 mRNA could thus initiate translation more than once even in the absence of a functional eIF4F complex.

5.2.2.5 Translation and encapsidation

In addition to the IRES, several important RNA structural elements are located in the 5'-UTR including the PBS, DIS, and ψ important for RNA packaging in new virions. Thus, an overlap occurs between translation signals and packaging signals. In fact, the full length retroviral RNA plays a role both as a messenger RNA and as genomic RNA meaning that the same HIV-1 RNA can be either translated or packaged into new retroviral particles. It was shown that an HIV-1 RNA does not need to be translated to be packaged and that by consequence the packaging mostly happens in *trans* ie by Gag synthesized from any RNA (Butsch & Boris-Lawrie 2000; Nikolaitchik et al. 2006). It was suggested that a transition between translation and packaging occurs through RNA riboswitch which plays a significant role in the viral life cycle.

Several structural models were proposed for the 5'-UTR after the analysis of secondary and tertiary structures of the different parts of the HIV-1 leader region. Huthoff and Berkhout analyzed the folding of the 5'-UTR in RRL by chemical probing experiments and computer assisted RNA folding analysis based on thermodynamic parameters (Huthoff & Berkhout 2001b). They showed that it can fold into two alternative conformations: the Branched Multiple Hairpin (BMH) and the Long Distance Interactions (LDI). Both conformations are mutually exclusive and they do not migrate with the same speed on non-denaturing gel electrophoresis. The BMH would be the dimerization competent conformation which favors viral encapsidation and replication by exposing the dimerization and packaging signals. On

the other hand, the LDI, the most stable conformation, would be the dimerization incompetent conformation where the DIS region is in long distance interaction with the poly(A) region resulting in the destruction of the poly(A), SD, SD and Psi stem-loops. The switch between the two conformations may occur during virus infection as it is favored by RNA, NCp7, and magnesium (Huthoff & Berkhout 2001b; Lu, Heng, Garyu, et al. 2011; Abbink et al. 2005; Berkhout & van Wamel 2000). Studying various HIV-1 leader mutants indicated a correlation between LDI-BMH equilibrium, dimerization, and packaging of viral RNA (Abbink et al. 2005; Ooms et al. 2004). However, these two conformations observed *in vitro* were not seen *in vivo*. Paillart et al. showed that the RNA structure in infected cells and virions are highly similar and did not observe any long distance interaction arguing against the LDI conformation for the RNA *in vivo* (Paillart, Dettenhofer, et al. 2004). Weeks at el. used a SHAPE analysis to determine the secondary structure of the HIV-1 genome. They observed that the 5'-UTR maintains the same conformation whether it is inside or outside the viral particle. This conformation, called the Week's conformation, is highly similar to the BMH conformation proposed by Huthoff and Berkhout (Wilkinson et al. 2008; Watts et al. 2009). Different BMH-like RNA secondary structures have been proposed (Heng et al. 2012; Lu, Heng & Summers 2011). Additionally, an alternative LDI conformation where the loop of the DIS base pairs with U5 was proposed (Heng et al. 2012; Lu, Heng, Garyu, et al. 2011). A study done by Lu et al. also supported a translation/packaging RNA structural switch mechanism (Figure 40) where the DIS bound to the U5 is displaced by AUG upon U5:AUG formation (Lu, Heng, Garyu, et al. 2011).

Though it is appealing to hypothesize that changes in conformations could explain the molecular switch between translation and packaging; this does not seem to be the case since viral protein synthesis was driven in both BMH and LDI structures in addition to intermediates (Abbink et al. 2005; Vallejos et al. 2011).

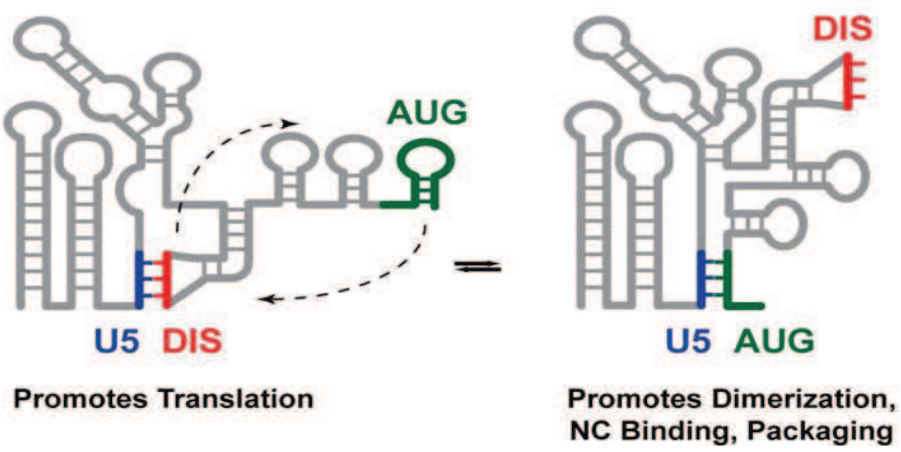

Figure 40: Schematic representation of the translation/packing RNA structural switch mechanism where the DIS bound to the U5 is displaced by AUG upon U5: AUG formation. The conformational change associated with U5: AUG base pairing sequesters the *gag* codon inhibiting translation. On the other hand, it exposes the DIS and the NC binding sites, thus promoting genome packaging (Lu, Heng, Garyu, et al. 2011).

IRES Regulation and Packaging

However, as the activity of the IRES is concerned, several groups hypothesized that the various viral and cellular factors would influence it by switching the RNA from one structure to another as it is the case for Gag. In fact, Gag expression inhibits its own synthesis while it favors the BMH structure (Anderson et al. 2007; Lu, Heng, Garyu, et al. 2011). In fact, Gag exerts a concentration dependent effect on its own production where it is increased at low Gag concentrations and inhibited by high Gag concentrations. Thus, the presence of Gag on the viral mRNA may impede ribosomal scanning along the 5'-UTR. Thus, high concentrations of Gag would probably inhibit translation favoring packaging of newly synthesized viral particles (Anderson et al. 2007). In this model, in the early steps of viral infection, the canonical cap dependent mechanism is used in order to ensure a rapid stock of structural proteins and enzymes. Then, Gag production will stimulate its own translation as discussed above. As the viral life cycle progresses, Gag molecules bind the genomic RNA blocking ribosomal scanning thus causing the switch to IRES-dependent translation. Moreover, as Vpr expression blocks the cycle in G2/M phase, which is not favorable for cap dependent translation, viral protein production is pursued by the IRES-dependent translation which is active in the G2/M phase. Accumulation of Gag and Gag-pol leads to protease maturation which cleaves initiation factors thus inhibiting cellular and viral translation and triggering assembly (Balvay et al. 2007).

Chapter VI- Unr (Up-stream of the N-ras)

6.1 *Unr* gene: Discovery and Evolutionary Considerations

The *unr* gene was identified as a transcription unit localized immediately upstream of the *N-ras* gene in the genome of several mammalian species (Doniger & DiPaolo 1988; Jeffers et al. 1990; Nicolaiew et al. 1991). Its homozygous knock-out is lethal in mice reflecting its essential role (Dormoy-Raclet et al. 2005).

The *N-ras* and *unr* locus is constituted of a tandem of closely linked genes transcribed in the same orientation found in humans, rats, mouse, and guinea pig genomes. With respect to the large size of the mammalian genome, the small, highly conserved 150bp intergenic region between *unr* and *N-ras* in addition to the ubiquitous expression of both genes is unusual (Doniger et al. 1992; Jeffers et al. 1990; Nicolaiew et al. 1991; H Jacquemin-Sablon & Dautry 1992). This raised the question of a possible transcriptional interference between the two genes where the expression of the downstream gene is negatively regulated by the expression of the upstream gene. In order to answer this question, Boussadia et al. evaluated the impact of *unr* transcription on *N-ras* expression by deleting the *unr* promoter. They found that the expression of *N-ras* is mainly controlled by its own promoter with *unr* transcription being a modulator of this expression rather than a major determinant (Boussadia et al. 1997).

Unr gene was detected in all tested vertebrates including fish, mammals, birds, and reptiles suggesting that *Unr* was present at the beginning of the vertebrate evolution (fish) and was maintained throughout (reptiles, birds, mammals) (Ferrer et al. 1999). Additionally, *Unr* was found in *Drosophila Melanogaster* (Abaza et al. 2006). The encoded Unr proteins exhibit a high degree of conservation across these species as illustrated by the 99% sequence identity between rat and human Unr proteins (G Triqueneaux et al. 1999).

6.2 Unr expression

Northern blot analysis showed that *unr* expresses three transcripts of different sizes (4.2, 3.8, and 3.2 kb) as a result of the use of three different polyadenylation signals present in the 3'-non translated region. These three transcripts were detected in most mammalian tissues and Drosophila embryos with an additional fourth transcript found only in the testis (Jeffers et al. 1990; Abaza et al. 2006). Overall expression of Unr protein is ubiquitous in all tissues with a higher expression in the skeletal muscles, heart, and testis with an additional developmental regulation in the latter (Jeffers et al. 1990; Nicolaiew et al. 1991; López-Fernández et al. 1995; H Jacquemin-Sablon & Dautry 1992). On the other hand, *unr* expression is practically absent in the small intestine of adults (López-Fernández et al. 1995).

In humans, two protein isoforms are detected as a result of alternative splicing of exon 5. The major and minor isoforms of the protein are approximately 85 (767 aa) and 88 kDa (798 aa) respectively (Boussadia et al. 1993; Hélène Jacquemin-Sablon & Dautry 1992).

Several transcriptional (Hélène Jacquemin-Sablon & Dautry 1992) and post-transcriptional mechanisms were proposed to regulate Unr expression. The level of Unr expression was shown to correlate with the proliferation state of different cell types (lymphocytes, synovial fibroblasts…), Unr being highly expressed in proliferative and undifferentiated precursor cells (Masuda et al. 2002; Nicolaiew et al. 1991). Furthermore, the 3'-UTR of *unr* mRNA was shown to specifically destabilize *unr* mRNA *in vitro* and *in vivo* (Dormoy-Raclet et al. 2005). Mammalian Unr expression is also regulated at the translational level via the presence of an IRES localized in the highly conserved *unr* mRNA 5'-UTR. The unr IRES activity exhibit a cell-type specificity being more efficient in HeLa and HuH7 cells than in embryonic stem (ES) cells. Furthermore the IRES activity is downregulated by polyprymidine tract-binding protein (PTB) and by Unr itself (Dormoy-Raclet et al. 2005; Cornelis, Sandrine A Tinton, et al. 2005). PTB and Unr being part of the IRES Trans-Acting Factors (ITAFs) family known to regulate diverse cellular and viral IRESs are described later within this chapter (Tinton et al. 2005; Hunt & Jackson 1999b; Mitchell et al. 2003).

6.3 Unr cellular localization

The intracellular localization of Unr was investigated by immunofluorescence as well as by cell fractionation experiments which indicated that Unr is mainly a cytoplasmic protein. Additional cellular fractionation of the cytoplasm showed that it is partly soluble and partly associated with the Endoplasmic Reticulum. This distribution is consistent with the function of Unr in translation regulation (discussed below). A small fraction of Unr was however detected at a low level in the nucleus (Jacquemin-Sablon et al. 1994; Ferrer et al. 1999).

6.4 Unr Domain Structure

The initial sequence analysis of the Unr proteins did not show any link to any other protein found within the database (Jeffers et al. 1990; Nicolaiew et al. 1991). However, after looking for more distant relations such as conserved motifs or secondary structures, Doniger et al. (1992) demonstrated the presence of five cold shock domains (CSDs) within the Unr protein (Figure 41) (Doniger et al. 1992). These evolutionary conserved domains of about 70 aa are found in both prokaryotes and eukaryotes in which they mediate binding to single stranded nucleic acids. Unr is however particular in the cold-shock protein family as it is the only one to contain five CSDs which all share a common sequence signature not found in the other family proteins (Alexander K Goroncy et al. 2010; Hunt et al. 1999).

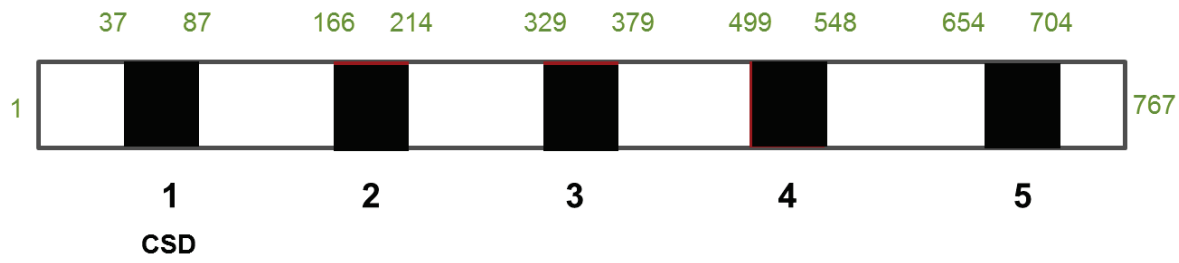

Figure 41: Unr protein: an RNA binding protein with five cold shock domains.

Cold-Shock Domains

Cold-shock domains belong to the oligosaccharide/oligonucleotide fold (OB). OB folds are composed of a β barrel with a five-stranded antiparallel β barrel. Many of them contain two conserved aromatic and basic motifs RNP1 and RNP2 located in strands β2 and β3, respectively both implicated in single stranded RNA/DNA binding (Arcus 2002; Theobald et al. 2003; Landsman 1992).

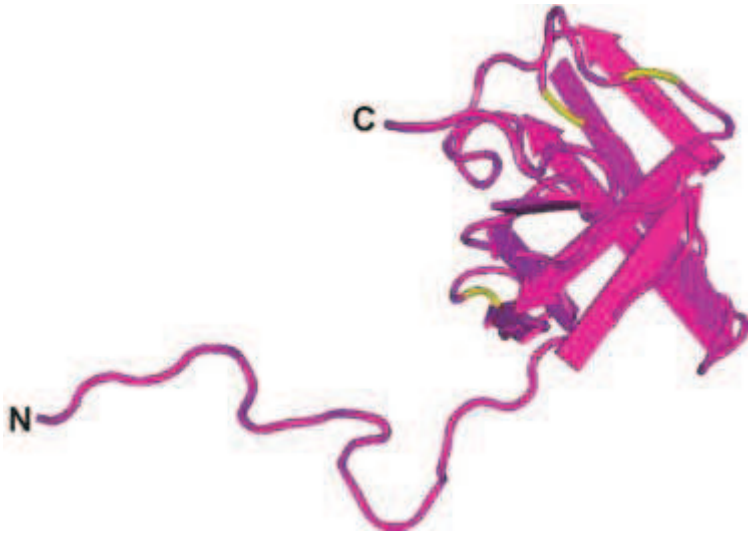

Figure 42: Structure of the CSD1 of human UNR (Mihailovich et al. 2010).

CSDs were initially identified in bacterial cold shock proteins (CSPs). The latter are small (7.4 kDa) mostly acidic proteins essentially composed of one CSD (Figure 42). They were named cold shock proteins since their expression is strongly induced in response to a decrease in temperature allowing cell adaptation to low temperatures. However, most of the CSPs are implicated in physiological processes during normal cellular conditions. Bacterial CSPs bind single stranded nucleic acids with a Kd value in the micro or nano molar range. CSPs play physiological roles in transcription and translation regulation and RNA turnover, primarily linked to their RNA chaperone activity (Ermolenko & Makhatadze 2002; Horn et al. 2007; Graumann & Marahiel 1998). For example, CspA, the major cold-shock protein of *Escherichia coli*, was found to promote single strandness in mRNA molecules at low temperatures to facilitate their translation.

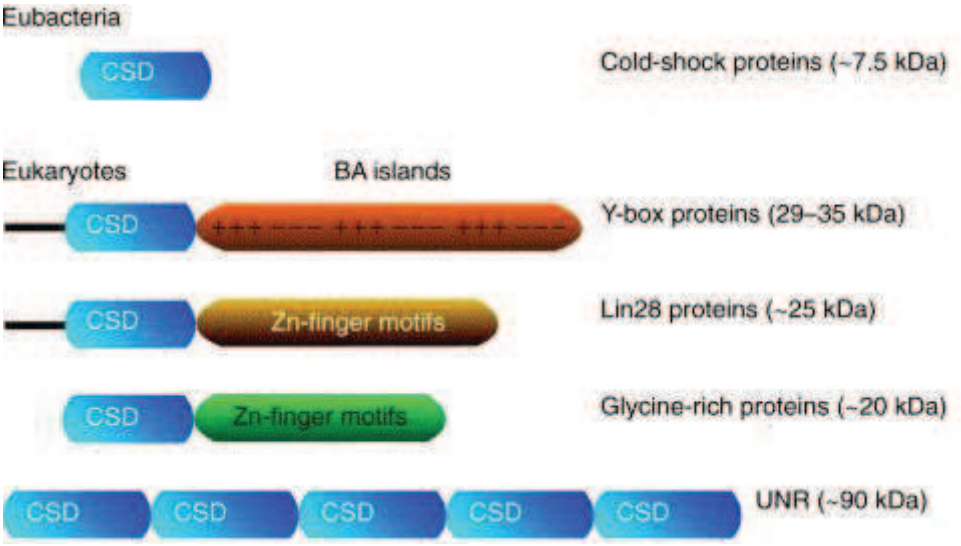

Figure 43: Proteins that contain CSD domains. Bacterial cold-shock proteins are only composed of one CSD whereas eukaryotic cold shock proteins harbor an auxiliary variable domain at the CSD C-terminus. Examples of auxiliary domains are basic/aromatic (BA) islands as found in the Y-box proteins, Zn-finger motif in Lin28 proteins and glycine rich motif in plant proteins. The Unr protein is an exception with five cold-shock domains (Graumann & Marahiel 1998).

In contrast to bacterial CSPs, eukaryotic counterparts contain auxiliary domains in addition to the CSD and are referred to CSD proteins (Figure 43). Eukaryotic CSD proteins display multiple functions and are characterized by a variable N-terminal sequence, a conserved CSD domain, and diverse auxiliary C-terminal domains (Mihailovich et al. 2010; Sommerville 1999; Kohno et al. 2003).

The Y-box protein family is among the most widely studied eukaryotic CSD proteins. They were named as Y proteins for their ability to bind the Y-box sequence of MHC II promoters via their CSD domains (Didier et al. 1988). The Y-box CSD-domain is thought to be implicated in the capacity of the Y-box proteins to regulate mRNA translation by specifically sequestering certain mRNAs in messenger ribonucleoprotein complex (mRNP). The specificity is given by the sequence-specific RNA binding property of the CSD domain which is associated to an RNA binding C-terminal domain composed of basic/aromatic (BA) islands (Mihailovich et al. 2010; Sommerville 1999). This property was shown for both human YB-1 (Nekrasov et al. 2003; Pisarev et al. 2002) and the *Xenopus* FRGY2 proteins (Murray et al. 1992; Matsumoto et al. 2003; Bouvet et al. 1995; Ranjan et al. 1993).

A second class of eukaryotic cold shock domains bears C-terminal retroviral zinc fingers. This class includes the highly conserved Lin-28 protein initially discovered in *Caenorhabditis elegans* and a family of glycine-rich plant proteins (Moss et al. 1997; Karlson & Imai 2003). Lin-28 is involved in the *let-7* miRNA family posttranscriptional biogenesis. Let-7 miRNA family is implicated in the control of cell differentiation and acts as tumor suppressor (Nam et al. 2011; Piskounova et al. 2011; Thornton & Gregory 2012). Most plant cold shock proteins act as RNA chaperones in response to a decrease in temperature, but they also induce stress tolerance and regulate development (Sasaki & Imai 2011).

The third class of eukaryotic shock domains bears RGG motifs of closely spaced Arg-Gly-Gly repeats and is found in invertebrates (Skehel & Bartsch 1994; Franco et al. 1997; Thieringer et al. 1997; Salvetti et al. 1998; Hayman & Read 1999). However, the function of these invertebrate cold shock domains is still not identified (Burd & Dreyfuss 1994).

The last member of the cold-shock protein family is our protein of interest, the mammalian Unr protein, which is structurally unusual as it is the only one to be composed of a succession of five cold shock domains. Unr structural organization is discussed in the next section.

6.5 RNA binding properties of Unr

Since Unr is composed of five cold shock domains, it was expected to bind nucleic acids as other cold shock proteins do. In their first characterization of the human Unr protein in 1994, Jacquemin-Sablon et al. tested its ability to bind DNA and RNA *in vitro*. They found that Unr exhibited a high affinity (Kd in the nanomolar range) for both single stranded DNA and RNA. However, Unr demonstrated a low affinity (Kd around 100nM) for double stranded DNA and RNA and structured RNA (tRNA) thus differentiating itself from Y box factors which bind double stranded NA as strongly as single stranded ones (Jacquemin-Sablon et al. 1994). An *in vitro* selection amplification approach (SELEX) identified two consensus motifs for Unr binding: 5’(A/g)5AAGUAA/g-3’ and 5’-(A/g)7A/gAACG/a(A/gG/a)-3’. These sequences are characterized by a central conserved motif harboring pyrimidine preceded by a purine tract. Both parts of the consensus motif seem to be necessary for Unr binding. Additionally, stable secondary structures do not exist for the consensus motif thus suggesting that Unr differentiates RNA based on their primary structure rather than their secondary structure.

Redundancy was observed among the CSD's of Unr for RNA binding. Although the N-terminal CSD seems to be the major contributor of binding energy as alone it presents only a 5 times decrease in affinity for the selected RNA sequence, a Unr protein lacking the CSD1 is able to bind to this RNA with the same affinity as the full length Unr (G Triqueneaux et al. 1999). In contrast, this redundancy was not observed with the Unr natural substrates. For example, mutation of any of the CSDs was found to severely impair the Unr binding to the HRV IRES (Brown & Jackson 2004) whereas only CSD1 binds to msl-2 RNA (Abaza & Gebauer 2008). This could highlight Unr's flexibility in target recognition even if Unr is characterized as a purine-rich RNA sequence binding protein due to the high concentration of purines in its target sequences. At least 9 natural Unr targets (shown in the table below) were described so far (Mihailovich et al. 2010).

mRNA	Binding site	Sequence
PITSRLE kinase	IRES (nt 380 to 1)	Purine rich sequences with AAGUA core motif
Apaf-1	IRES (nt -233 to 1)	Purine rich region within a stem-loop structure
c-fos	ORF (nt -397 to 484)	Purine rich sequences
HRV-2	IRES (nt -509 to -435 and nt -172 to 1)	ND
c-Myc	IRES (nt -233 to 1)	ND
PABP	5'-UTR (nt -432 to -372)	Four Oligo(A)s separated by pyrimidine stretches of 3-6 nt
PTH	5'-UTR (nt -432 to -372)	ND
Unr	IRES (nt -447 to - 186)	ND
msl-2	3'-UTR (nt -3231 to 3276)	Two Oligo(U)s embedded in a purine rich sequence
SELEX	NA	AAGUA/G or AACG motifs downstream of a purine rich stretch

Table 2: Identified natural targets of Unr with their binding site and sequence. Adapted from (Mihailovich et al. 2010).

The higher affinity of CSD1 (20 -fold higher in comparison to CSD5) observed by Triqueneaux et al. was explained after the 3D structure determination by NMR of each independent human Unr CSD. The orientation of several amino acids both in RNP-1 and

RNP-2 binding sites of CSD-1 was found to be different from the one of their counterparts in the other CSDs (Alexander K. Goroncy et al. 2010).

6.6 Functions of Unr

Unr has been found to play diverse biological roles by binding to a variety of RNAs and regulation factors via its multiple CSDs. Unr behaves as an ITAF regulating the activity of several viral and cellular IRESs in addition to its own IRES. Furthermore, Unr is involved in the regulation of cap-dependent translation (Duncan et al. 2006; Abaza et al. 2006) and mRNA stability (T. C. Chang et al. 2004). Additionally, Unr expression is regulated along the cell cycle implying a role in the control of cell proliferation and death. Furthermore, in *Drosophila*, Unr was shown to play a role in the nucleus by regulating the binding of the Dosage Compensation Complex to the male X-chromosome to increase its translation (Patalano et al. 2009a). My primary focus will be on the role of Unr in translation and especially, in IRES-mediated translation.

6.6.1 Unr Regulates IRES-dependent translation

Most of the work done on Unr demonstrated that it behaves as an ITAF regulating the activity of several viral and cellular IRESs in addition to its own IRES. As it was discussed earlier, a number of cellular and viral mRNAs are translated by an unconventional mechanism of direct internal ribosome entry. This unusual mechanism was first discovered in picornavirus RNA (Jang et al. 1988).

The translation of Rhinovirus and poliovirus IRESs is inefficient in RRL unless they are supplemented with HeLa cell extracts (Borman et al. 1993; Brown & Ehrenfeld 1979). Hunt el al. demonstrated that two separable activities purified from HeLa cells were required for proper HRV IRES translation. The first one was identified as the polyprimidine-tract binding protein (PTB), whereas the second one was Unr found in association with another protein named Unrip for Unr interacting protein (Hunt & Jackson 1999b; Hunt et al. 1999). It is worth noting that the functional implication of Unrip on IRES activity regulation is not known so far (Evans et al. 2003; Hunt et al. 1999). Both PTB and Unr work synergistically to stimulate

HRV IRES dependent translation and Unr binding is suggested to be required for efficient PTB binding (Anderson et al. 2007).

Although recombinant purified Unr was incapable of activating PV IRES, even in the presence of PTB, it was later implicated in the stimulation of PV IRES. In fact, after HeLa cell lysate fractionation, a PV IRES stimulatory activity was identified one fraction which after further analysis was attributed to Unr (Hunt et al. 1999). These observations were confirmed by the group of Jacquemin-Sablon when they showed only a weak activity of the poliovirus IRES in ES with Unr knockout. This activity was recovered after Unr cell overexpression (Boussadia et al. 2003). The 10 fold lower affinity of Unr for PV IRES compared to HRV IRES explained why purified Unr was non-functional (Hunt et al. 1999).

	ITAF implicated	**Synergy with Unr**	**References**
HRV	PTB (R) PCBP2 (R)	Yes ?	(Boussadia et al. 2003; Hunt & Jackson 1999a; Anderson et al. 2007; Brown & Jackson 2004; Anderson & Lever 2006)
PV	PTB (R) PCBP2 (R)	Yes No	(Boussadia et al. 2003)
EMCV	PTB hnRNPD		(Boussadia et al. 2003)
FMDV	PTB ITAF45		(Boussadia et al. 2003)
Apaf-1	PTB Unr (I)	Yes	(Mitchell et al. 2001;Mitchell et al. 2003)
EV1 human	PTB PCBP1	?	(Lin et al. 2009)
Unr	PTB (D) hnRNP C1/C2 (I)		(Dormoy-Raclet et al. 2005; Cornelis, Sandrine A Tinton, et al. 2005; Schepens et al. 2007)
Cmyc	PCBP1 (I) PCBP2 (I)	Yes Yes	(Evans et al. 2003)
PITSLRE	Unr (I)		(Schepens et al. 2007; Tinton et al. 2005)

Table 3: List of IRESs where Unr acts as an ITAF. Other implicated ITAFs are also listed. (R) means that the ITAF is required for IRES activity. (I) and (D) refer to an increase and decrease in IRES activity respectively. (B) means that the ITAF was shown to bind to the IRES RNA. Synergy with Unr is also indicated.

This Unr dependency is specific to some IRESs including the one of HRV and poliovirus, as it is not observed for the IRESs from the EMCV or FMDV (Boussadia et al. 2003). The binding of Unr to HRV-2 IRES was mapped to two distinct stem-loop domains in which specific A-rich nucleotides sequences located in single strand regions of the stem loops are critical for this binding (Anderson et al. 2007). As mentioned earlier, this binding and the resulting IRES activation requires the presence of all the five CSDs (Brown & Jackson 2004).

Unr also regulates the activity of a variety of cellular IRESs including the proto-oncogene c-Myc, the apoptotic protease–activating factor Apaf-1, the cell cycle PTSRLE kinase, and its own IRES (Dormoy-Raclet et al. 2005; Mitchell et al. 2003; Evans et al. 2003; Tinton et al. 2005). In a similar mechanism to HRV IRES, Unr exhibits its effect on Apaf-1 IRES in cooperation with the neuronal isoform of PTB (nPTB). The binding of Unr to the Apaf-1 IRES mediates a change in conformation allowing nPTB binding and hence ribosome landing (Figure 44). For this activity, Unr has been proposed to function as a RNA chaperone, as suggested for ITAF in general (Mitchell et al. 2001; Mitchell et al. 2003).

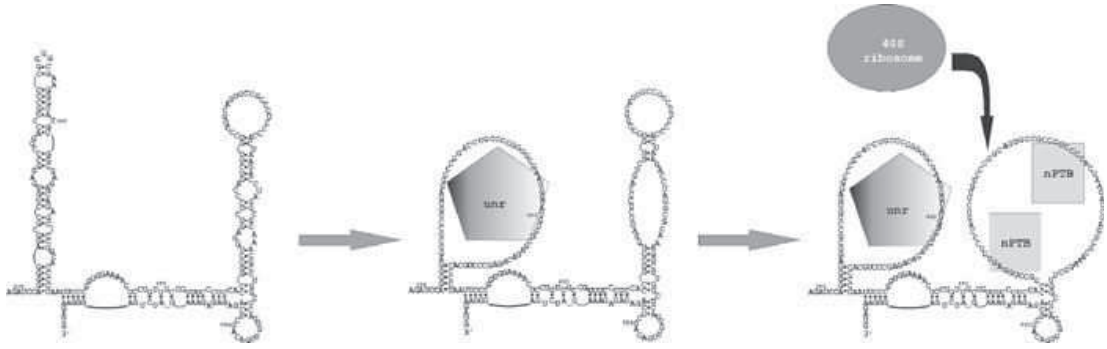

Figure 44: Binding model of Unr and nPTB to the Apaf-1 IRES. Unr modifies the Apaf-1 IRES structure, so that the stem-loop structure is further opened allowing PTB binding and ribosome landing (Mitchell et al. 2003).

Finally, Unr translation is driven by an IRES that is down regulated by PTB (Cornelis, Sandrine A. Tinton, et al. 2005) and Unr itself as Unr IRES activity is higher in Unr deficient cells showing that Unr regulates its own IRES through a negative autoregulatory loop that contributes to fine tune Unr levels in the cells (Dormoy-Raclet et al. 2005). Unr IRES activity is in contrast stimulated by hnRNP C1/C2, a nuclear protein that translocates to the cytoplasm during mitosis and binds to IRES RNA thus disrupting PTB and Unr binding (Schepens et al. 2007).

6.6.2 Unr regulates Cap-Dependent Translation

In addition to its role as an ITAF, Unr is implicated in the regulation of the cap-dependent translation of specific mRNA as the one of male-sex lethal 2 (*msl-2*) in *Drosophila*. The inhibition of *msl-2* RNA translation is an essential regulatory step for X-chromosome dosage compensation in *Drosophila melanogaster* (Abaza et al. 2006; Duncan et al. 2006).

In different organisms, sex is determined by regulatory mechanisms that cause males (XO or XY) and females (XX) to differ in the dose of X chromosome. This unequal distribution of X chromosomes leads to developmental defects or death; thus, animals have evolved a process which ensures equal levels of X-linked gene products in males and females. This process is known as dosage compensation. Different organisms have developed different dosage compensation mechanisms; however, exclusive to X chromosomes (Figure 45). Female mammals inactivate one X chromosome, hermaphrodite worms keep both their X chromosomes active but half express both X chromosomes, while *Drosophlila* females keep both X chromosomes active. In this case, *Drosophila* males increase their X chromosome transcription two folds in order to compensate for the difference in gene dosage (Mendjan & Akhtar 2007; Meyer 2005).

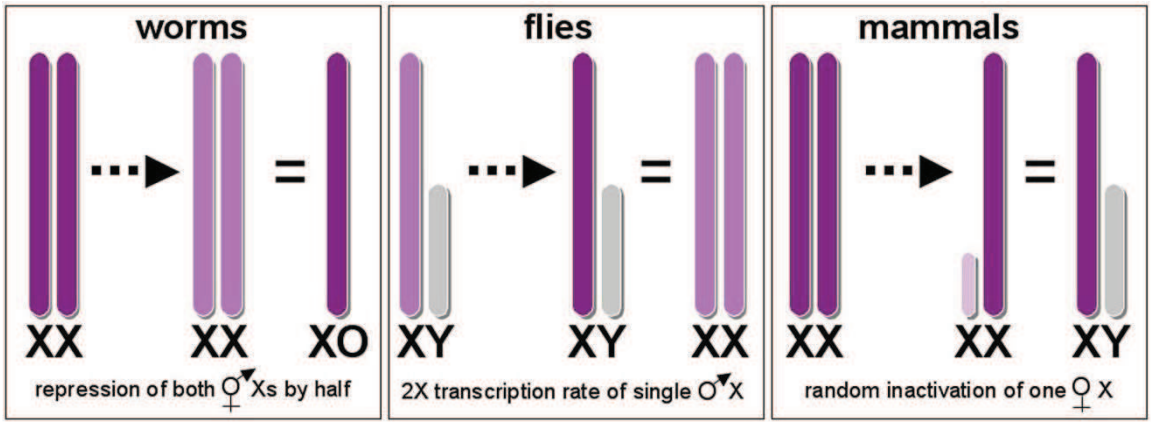

Figure 45: Diverse strategies for dosage compensation in worms, flies, and mammals (Meyer 2005).

For this compensation activity in males, the dosage compensation complex (DCC) or male – specific lethal (MSL) complex is assembled and binds to hundreds of sites along the X chromosome. However, in females the MSL complex assembly is limited due to the lack of one of its nucleating protein msl-2. The female-specific RNA-binding protein sex lethal

(SXL) prevents msl-2 synthesis by binding to both UTR regions of *msl2* pre-mRNA. Although Unr is expressed in both males and females, it was identified to be a regulator of dosage compensation only in female *Drosophila* via coordinating with SXL in *msl-2* translational control (Figure 46).

To do so, in female cells, SXL binds first to Uridine rich sequences in the 3'-UTR of *msl-2* mRNA and recruits Unr which also binds to *msl-2* mRNA leading to the inhibition of the 43S cap dependent pre-initiation complex recruitment (Abaza et al. 2006; Duncan et al. 2006). Crystal structure, NMR, small angle-X-ray, and neutron scattering data of the SXL-UNR-*msl2* ribonucleoprotein complex showed an intertwined network of ternary interactions resembling a 'triple zipper'. The cooperative complex formation leads to a 1000-fold increase in RNA binding affinity to Unr (Hennig et al. 2014). Although CSD1 of Unr is required for the binding to *msl2* mRNA and SXL complex, it is not sufficient for translation repression suggesting the contribution of other Unr domains through their interactions with other relevant factors (Abaza & Gebauer 2008). One of these factors was reported to be the poly(A)-binding protein (PABP) (Duncan et al. 2009). By another unknown mechanism, Unr was also described to promote the msl binding to male X-chromosome, a role which implies that Unr functions in the nucleus (Patalano et al. 2009).

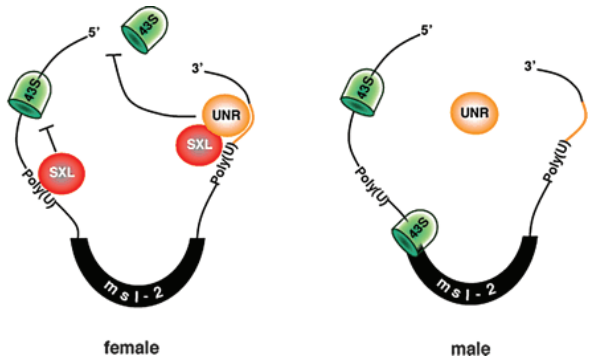

Figure 46: Model for Unr's role in translation repression of *msl-2* mRNA in *Drosophila*. In females flies, SXL protein binds to poly(U) stretches at both the 5'- and 3'-UTRs of the *msl-2* mRNA. Then, SXL bound to the 3'-UTR recruits Unr to nearby purine-rich sequences, thus inhibiting the recruitment of the 43S ribosomal complex at the 3' end of the mRNA. The scanning of ribosomes which managed to escape this, will be inhibited by SXL bound at the 5'-UTR. In males, in the absence of SXL protein, Unr does not bind to *msl-2* mRNA and does not affect translation(Abaza et al. 2006).

6.6.3 Unr regulates the stability and translation of mRNA

Studies have showed that Unr is an essential player in regulating the stability of mRNA by binding to purine rich sequences. Unr has notably been shown to be involved in regulating the stability of the mRNAs coding for PTH (parathyroid hormone) and c-Fos.

The post transcriptional regulation of PTH gene expression is mediated by binding of regulatory factors to an instability element in the 3'-UTR of PTH mRNA. AU-rich binding factor 1 (AUF1) was described as a member of the protein-binding complex that protects PTH mRNA from degradation (Sela-Brown et al. 2000). By affinity chromatography, Dinur at al. (2006) showed that recombinant Unr also binds PTH 3'-UTR transcript. Since protein binding is usually associated with an increased stability of the mRNA, the effect of Unr overexpression and knockdown on PTH mRNA stability was investigated. Unr depletion was shown to decrease PTH gene expression. In parallel, Unr overexpression increased PTH mRNA levels for a full length PTH mRNA but not for an mRNA lacking the 3'-UTR instability element. This suggests that Unr binding stabilizes PTH mRNA probably in a manner similar to AUF1 and, thus, that Unr, together with other proteins in the RNA binding complex, determine PTH mRNA stability (Dinur et al. 2006).

In contrast to PTH mRNA stabilizing effect, Unr plays a central role in destabilizing c-Fos mRNA. The *c-fos* gene is a proto-oncogene transiently expressed in the cell in response to extra cellular stimuli. However, c-fos mRNA is highly unstable as it is rapidly degraded via two different pathways. The first one is mediated by an AU-rich instability element (ARE) found within the 3'-UTR (Chen et al. 1995), whereas the second is due to a destabilizing purine rich motif in the mCRD (major coding region determinant of instability) in the c-fos ORF (Chen et al. 1992). Studies of c-fos mCRD-mediated decay have shown that it occurs through a deadenylation process tightly coupled to translation. Five proteins, including Unr, an heterogenous nuclear ribonucleoprotein (NSAP1), PABP, the PABP interacting partner 1 (PAIP-1), and the AU-rich element binding protein (hnRNP D) were identified to play a role in this process. Unr plays an essential role in this multi-protein complex by binding to both the purine rich sequence in the mCRD and to the protein PABP, creating thus a physical link between the mCRD and the poly(A) tail in the absence of mRNA translation. The role of the other proteins in the complex is not clearly understood. They probably interact with PABP via

PAIP-1. Prior to translation, this complex protects the poly(A) from an attack by the poly(A) nuclease CCR4. However, during translation, the movement of the ribosome disrupts the complex thus allowing the CCR4 nuclease to access the poly(A) tail. As a consequence, mRNA is deadenylated and degraded (Grosset et al. 2000; T.-C. Chang et al. 2004).

6.6.4 Unr regulates cell differentiation, proliferation, and death

Unr regulates cell differentiation, proliferation, and death in a cell specific manner. As it was already mentioned, Unr is essential for dosage compensation of X chromosome in *Drosophila* females. Unr performs dual opposing functions in regulation of dosage compensation in males and females: repression of DCC formation in female flies, and promotion of DCC assembly on male X chromosome (Patalano et al. 2009). This specificity applies as well to proliferation as for Unr deficient homozygous mice for which late embryonic lethality was discovered at mid-gestation (Boussadia and Jacquemin-Sablon unpublished observations). However, Unr-deficient ES stem cells grew normally suggesting that Unr affects cell proliferation in a cell-type specific manner (Boussadia et al. 1997). The differentiation of embryonic stem cells into primitive endoderm is inhibited by Unr whereas its knockout leads to differentiation. Moreover, Unr acts both as a positive and negative regulator of apoptosis depending on the cell type and this activity was described to be linked to Unr RNA binding (Elatmani et al. 2011; Dormoy-Raclet et al. 2007). Additionally, the erythroid blast proliferation and differentiation was severely affected when Unr expression was reduced. Cells with reduced Unr expression became pycnotic and failed to mature to anucleated.

Aim

The HIV-1 nucleocapsid plays important roles in the early and late phases of the HIV-1 infection cycle. The crucial role of NC is mainly due to its chaperone properties mediating nucleic acid rearrangements into their most thermodynamically stable conformation. In the early phase of the viral life cycle, the mature form of NC (NCp7) is thus undoubtedly implicated in the reverse transcription process. NCp7 is also thought to promote the integration of the viral genome. Furthermore, during the late phase of the life cycle, as a domain of the polyprotein Gag, NC selects and packages the genomic RNA forming a ribonucleoprotein (RNP) platform for assembly of new virions. Finally, in the infected cells and the virions, both forms of NC coat the viral RNA protecting it from nuclease attack.

Apart from its well- known roles in chaperoning RNA rearrangements and reverse transcription, a lot of questions about NC are still not answered or debated. This is particularly the case for its involvement in the steps of the early infection phase happening after the reverse transcription of the viral genome into double stranded DNA. Although NCp7 was found in the nucleus shortly after infection, there is an ongoing debate about its presence in the integration competent RNP called pre-integration complex (PIC). A number of questions regarding the cellular distribution of NCp7 after the release of the viral capsid in the cytoplasm have to be answered. For example, does NCp7 enter the nucleus on its own or associated to cellular RNAs or as part of the PIC?

In the late phase of the infection, as NC participates to gRNA selection and coating, what is its influence on the switch between assembly and translation? How the NC domain of Gag influences these two processes which are also highly related to gRNA transport and targeting to the plasma membrane and Gag multimerization?

In fact, the proviral DNA integrated into the host genome is transcribed into genomic RNA (gRNA) by RNA polymerase II and then exported to the cytoplasm without splicing. Then, this RNA is either packaged into new virions or translated to give the viral proteins. In contrast to HIV-2, where RNA has to be translated before its packaging (cis packaging), HIV-1 RNA can be packaged without prior translation. Thus, the same pool of RNA acts as a

messenger RNA or genomic RNA. The balance between translation and packaging may occur through an RNA riboswitch that targets RNA for either translation or encapsidation. The current model suggests that NCp7 and Gag are implicated in this ribosomal switch. However, this switch is still debated. In parallel, a temporal regulation of HIV-1 gRNA translation has to be performed. It was shown that HIV-1 gRNA can drive both cap-dependent and IRES-mediated translation with the latter being a convenient way for the virus to sustain an efficient translation even after the block of the cell in the G2/M phase or the cleavage of cell factors necessary for cap-dependent translation during the course of the infection. Both of these mechanisms are likely influenced by NC binding to the gRNA through an unknown mechanism.

During the course of my PhD, I first focused my work on the identification of NC or NCp7 cellular protein partners which could help addressing some of these questions. Several genome wide studies identified cellular proteins which are partners of viral proteins and thus, potentially important for viral infection. Among these proteins, I carefully selected proteins which may influence the viral cycle, interact with NC or NCp7 and are involved in the cellular processes mentioned earlier. The selected proteins were tested by confocal microscopy for their co-localization with NCp7 or Gag. Their interaction with NC or Gag(NC) was tested by FRET (Fluorescence Resonance Energy Transfer), visualized by FLIM (Fluorescence Lifetime Imaging Microscopy) and/or co-immunoprecipitation. Even if several of them were positive, I decided to focus my attention on the cold shock protein Upstream of N-ras (Unr). This choice was justified by several facts: i / Unr is an IRES transacting factor modulating translation driven by several viral and cellular IRESs; ii / it is overexpressed in G2/M phase; iii / the NCp7 protein preferentially recognizes, on the gRNA, sites located in regions carrying the HIV-1 5'-IRES.

In this context, the objective of my thesis work was to characterize i) the interaction of Unr with NCp7 as a mature form or as a domain of the polyprotein Gag , ii) the effect of Unr on the HIV-1 5'-UTR IRES activity and its potential modulation by NCp7 or Gag, and iii) the influence of Unr in HIV-1 infection.

Materials and Methods

I-Material

A- Cell lines

HeLa cells (ATCC CCL2) is an adherent immortal cell line of human origin. They have the morphology of epithelial cells, as they were taken from an adenocarcinoma of the cervix in a patient named Henrietta Lacks in 1951. They are transformed by human papillomavirus type 18.

293T cells (ATCC CRL-3216) cells are derived from human embryonic kidney (HEK) transformed with adenovirus 5 expressing SV40 large T-antigen, allowing episomal replication of plasmids carrying the SV40 origin of replication.

B- DNA constructs

Name	Promoter	Insert	Tag Position	Resistance	Vector backbone	Source
Unr constructs						
pEF-ctrl	EF-1a	None	None	Ampicillin	pEF	J-Sablon
pEF-Flag-Unr	EF-1a	Unr	Flag N-terminal	Ampicillin	pEF	J-Sablon
pmCherry-Unr	CMV	Unr	mCherry N-terminal	Kanamycin	pmCherry-C1	Institut Pasteur
pUnr-HXGWA	T7	Unr	6 X His N-terminal	Ampicillin	pHXGWA	IGBMC
Gag constructs						
pGag-mCherry	CMV	Gag	mCherry between aa 369 and 370 of Gag	Ampicillin	pCDNA3.1	
pGag-TC	CMV	Gag	Tetra-cysteine C-terminal	Ampicillin	pCDNA3.1	
pGag- ΔNC-TC	CMV	Gag	Tetra-Cysteine C-terminal	Ampicillin	pCDNA3.1	
pGag-G2A	CMV	Gag mut G2A	None	Ampicillin	pCDNA3.1	
pGag-	CMV		None	Ampicillin	pCDNA3.1	

LeucineZipper						
pGagM368A	CMV	Gag mut M368A	None	Ampicillin	pCDNA3.1	
pGag-eGFP	CMV	Gag with eGFP between aa 369 and 370 of Gag	eGFP internal	Ampicillin	pCDNA3.1	
pGagG2A-mCherry	CMV	Gag with mCherry between aa 369 and 370 of Gag	eGFP internal	Ampicillin	pCDNA3.1	
NCp7 constructs						
pNCp7	CMV					
pFlag-NCp7	CMV	NCp7	3XFlag N-terminal	Ampicillin	pcIneo	Institut Pasteur
pNCp7-eGFP	CMV	Ncp7	eGFP C-terminal	Ampicillin	pCDNA3.1	
pNCp7-mCherry	CMV	Ncp7	mCherry C-terminal	Ampicillin	pCDNA3.1	
IRES constructs						
dlHIV-1 IRES	CMV	RLuc-HIV-1 pNL4.3 IRESwt-FLuc	None	Ampicillin	pcDNA3	Vallejos et al. 2012
dlVar2	CMV	RLuc-HIV-1 pNL4.3 IRESwt-FLuc	None	Ampicillin	pcDNA3	Vallejos et al. 2012
dlHIV1-211	CMV	RLuc-HIV-1 pNL4.3 IRESmutA211G-FLuc	None	Ampicillin	pcDNA3	
dlHIV1-212	CMV	RLuc-HIV-1 pNL4.3 IRESmutG212C-FLuc	None	Ampicillin	pcDNA3	
dlHIV1-213	CMV	RLuc-HIV-1 pNL4.3 IRESmutU213A-FLuc	None	Ampicillin	pcDNA3	
dlHIV1-214	CMV	RLuc-HIV-1 pNL4.3 IRES mutA214G-FLuc	None	Ampicillin	pcDNA3	
dlIRES 205CCU207	CMV	RLuc-HIV-1 pNL4.3 IRES mutAGC205-207CCU-FLuc	None	Ampicillin	pcDNA3	
dlIRES 210UUCAU214	CMV	RLuc-HIV-1 pNL4.3 IRES mutAAGUA210-214UUCAU-FLuc	None	Ampicillin	pcDNA3	
dlIRES 210CCAGC214	CMV	RLuc-HIV-1 pNL4.3 IRES mut AAGUA210-214CCAGC-FLuc	None	Ampicillin	pcDNA3	
pRAF Apaf dual lucefirase pcDNA3.1	SV40/T7 CMV	RLluc-5'UTR(1-578)/Apaf1-FLluc	None	Ampicillin	pBluescript	Mitchell et al. 2001

pIRES HIV-1 wt	CMV/T7	HIV-1 pNL4.3 IRESwt (1-336)	None	Ampicillin	pcDNA3.1 (+)Zeo	
pIRES Apaf-1 wt	CMV/T7	Apaf-1 pNL4.3 IRES (1-581)	None	Ampicillin	pcDNA3.1 (+)Zeo	
Lentivirus constructs						
pSiCoR-luciferase	CMV	SIN-LTR-Psi-cPPT-LoxP-CMV-Luciferase	None	Ampicillin	pSicoR	Adapted from pSicoR (Ventura et al. 2004)
pMD2.G	CMV	VSV-G	None	Ampicillin		Trono lab Addgene
pCMV-dR8.91	CMV	Gag-Pol-cPPT-RRE pNL4.3 strain	None	Ampicillin		Trono lab Addgene
Control plasmids						
pCI-neo	CMV		None	Ampicillin		
pcDNA3.1	CMV		None	Ampicillin	pCDNA3.1	
peGFP	CMV			Kanamycin		
pFlag stop	CMV		3XFlag	Ampicillin	pcIneo	
dlΔEMCV IRES	CMV	RLuc-ΔEMCV-IRES-FLuc	None	Ampicillin	pcDNA3	Vallejos et al. 2012
pRF	SV40/T7	RLuc-FLuc		Ampicillin	pBluescript	Mitchell et al. 2001

C- Primary Antibodies

Name	Organism	Source	Reference	Type	Dilution (WB)
Anti-Flag M2 Clone M2	Mouse	Sigma	F1804	IgG1, Monoclonal	1/ 10 000
Anti-Gag (p24 Gag #24-4)	Mouse	NIH AIDS reagent	6521	Mono	1/ 10 000
AntiUNR;CSDE1	Rabbit	Proteintech	13319-1-AP	IgG Polyclonal	1/1000
Anti-eGFP	Mouse	Proteintech	66002.1.Ig	IgG2a Monoclonal	1/ 10 000
Anti-GAPDH	Mouse	Millipore	MAB374	IgG1, Monoclonal	1/ 5 000
Anti-Phospho-Histone H3 (Ser10)	Rabbit	Cell Signaling	3377	IgG Monoclonal	1/ 1000

D- Secondary Antibodies

Name	Organism	Source	Reference	Label	Dilution
Anti-mouse	Goat	Promega	W402B	HRP	1/ 10 000
Anti-mouse (Kappa light chain)	Rat	Abcam	AB99632	HRP	1/5 000
Anti-rabbit	Goat	Promega	W401B	HRP	1/ 10 000
Anti-goat	Rabbit	Southern Bioth	S6160-05	HRP	1/ 8 000
Protein A		Invitrogen	101023	HRP	1/ 10 000

E- Primers

Name	Sequence
qPCR	
qPCR lucif Roche fwd	5'-TGAGTACTTCGAAATGTCCGTTC-3'
qPCR lucif Roche rev	5'GTATTCAGCCCATATCGTTTCAT-3'
qPCR 18S Fwd	5'TGTGGTGTTGAGGAAAGCAG-3'
qPCR 18S Rev	5'TCCAGACCATTGGCTAGGAC-3'
Mutations	
mut IRES FW UUCAU	(Phos)GGGACACTTGAAAGCGATTCATAAGCCAGAGGAG
mut IRES rev	(Phos)TGTTCGGGCGCCACTGCTAGAG
mut IRES FW CCAGC	(Phos)GGGACTTGAAAGCGACCAGCAAGCCAGAGGAG
mut IRES 211AG FW	(Phos)GGGACTTGAAAGCGAAGGTAAAGCCAGAGGAG
mut IRES 212 GC FW	(Phos)GGGACTTGAAAGCGAAACTAAAGCCAGAGGAG
mut IRES 213 UA FW	(Phos)GGGACTTGAAAGCGAAAGAAAAGCCAGAGGAG
mut IRES 214 AG FW	(Phos)GGGACTTGAAAGCGAAAGTGAAGCCAGAGGAG
mut IRES 205-207 CCT FW	(Phos)GAACAGGGACTTGAACCTGAAAGTAAAGCCAGAG
mut IRES 205-207 CCT REV	(Phos)GGGCGCCACTGCTAGAGATTTTCC
Cloning	
AttB1-CSDE1-no Koz	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAGCTTTGATCCAAA CCTTCTC
AttB2-CSDE1-stop	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTCAATGACACCAGCTT GAC
HIV IRES EcoR FW	CGGAATTCGGTCTCTCTGGTAAGACCAG
HIV IRES Xba Rev	CGCTCTAGATCTCTCTCCTTCTAGCCTCC
Apaf IRES EcoR FW	CGGAATTCAAGAAGAGGTAGCGAG
Apaf IRES Xba Rev	CGCTCTAGACATCTTCCCTCAGATCTTTCTCTC

II-Methods

A- Cell Biology

Cell Culture

HeLa and 293T cells were cultured in DMEM - Dulbecco's Modified Eagle Medium (Gibco® Life Technologies ref 21885) -supplemented with 10% fetal bovine serum (FBS, Gibco) and a mixture of penicillin (100 U/mL) and streptomycin (10 U/mL) antibiotics (Lonza DE17-602E). Cells were cultured in 75cm2 flasks and placed into a thermostatically controlled CO_2 incubator at 37°C (95% H_20, 5% CO_2). Subcultures were made every 3 or 4 days by trypsinization until the cells reached twenty passages. Cells were washed once with 5mL 1×PBS, diluted from 10×PBS (Phosphate Buffer Saline, Biowhitakker®, BE17-517Q; 1.4g/L KH_2PO_4, 90g/L NaCl, 7.95g/L Na_2HPO_4; pH 7.4) and detached by the action of 2mL of trypsin/EDTA/PBS (Lonza BE02-007E) for 2 min at 37°C. The action of trypsin was stopped by adding 8mL complete DMEM. Then, cells were centrifuged for 5min at 1100rpm and resuspended in 10mL of complete DMEM. Cells were seeded into new 75cm2 flasks containing 20mL complete DMEM.

Cell Transfection

Transfection is the introduction of foreign DNA into eukaryotic cells. All transfections carried out during the experiments mentioned here are transient, that is to say, the introduced genetic material does not integrate into the genome of the host cell. Cells were transfected by PEI (polyethylenimine) using the JetPEI® kit (Polyplus TransfectionTM; 101-10N). The latter is a cationic polymer which compacts DNA into positively charged complexes capable of interacting with the anionic peptidoglycans present on the cell surface. The formed complexes are then internalized by endocytosis and released into the cytoplasm after rupture of the endosomes. PEI also protects the DNA in endosomes by buffering the pH.

For each test sample, two Eppendorf tubes are prepared: one containing the plasmid DNA resuspended in 150mM NaCl solution, the other containing PEI (7.5mM stock solution) also resuspended in 150mM NaCl solution at a ratio of 2µl of PEI per µg of transfected plasmid

DNA. The PEI solution is added to the DNA and the mixture is then vortexed and left at room temperature for 20 minutes to allow the formation of DNA-PEI complexes. The content is finally deposited drop by drop in the cells supernatant (ie complete DMEM). For all the transfections mentioned in this manuscript, the final amount of transfected DNA was normalized (if necessary) by adding an empty plasmid to the plasmids of interest.

Cell Cycle Analysis

Hela cells are plated in 6-wells plate at a density of 3.10^5 cells/well. The next day, cells are treated with 100ng/ml of nocodazole and incubated for 16 hours at 37°C. Live cells are harvested by trypsinization and then centrifuged together at 1200 rpm for 5 min. Pellets are then washed with PBS and fixed with 500µl of 70% ice-cold ethanol for at least 4 hours. Ethanol is removed by centrifugation at 1200rpm and cells are resuspended with 500µl of PBS containing 100µg/ml RNase A and 25µg/mL propidium iodide. The DNA fluorescence is then measured by flow cytometry using a FACScalibur provided by Becton Dickinson (USA). Cell cycle analysis is performed using ModFiLT software and the percentages of cells distributed, according to their DNA content, is calculated.

B- Biochemistry

Whole cell protein extraction

48h post-transfection, 293T cells are trypsinized and resuspended in 2 mL of complete DMEM medium. Cells are centrifuged for 5min at 1500 rpm and pellets are resuspended in 500µL of lysis buffer (Tris-HCl 10mM pH=7.5, NaCl 150mM, EDTA 1mM, and 1% NP40, complete MINI, EDTA free protease inhibitor Cocktail Tablets, Roche). Lysates are kept for 20 min on ice before centrifugation at 14,000rpm at 4°C for 20min (centrifuge Megafuge 40R, ThermoScientific) to remove cell debris.

Protein Quantification by Bradford Assay

The Bradford assay is a colorimetric method to determine the quantity of proteins present in a sample. It is based on the change of color of Coomassie blue upon interaction with the basic and aromatic amino acids of the proteins. We used the Bio-Rad protein Assay reagent (ref: 500-0006). 1mL of "Bio- Rad Protein Assay" (BIO RAD) solution is diluted in 4 ml of water to obtain the working reagent. The reference range is performed with increasing amounts of BSA (Bovine Serum Albumin) diluted to one-tenth in a TRIS buffer.

Volumes (µL)	**1**	**2**	**3**	**4**	**5**
Bradford	200	195	190	185	180
BSA (1.44 mg/mL)	0	5	10	15	20

10 µL of each test sample are added to 190µL of reagent. Assays are performed in duplicates. The plate is stirred for 15min before measuring the absorbance of each well at 525nm by a spectrophotometer Safas FLX- Xenius. Absorbance measurements are made with respect to the "blank" protein-free control and calibration line Absmoy = f(concentration of BSA) is used to calculate the concentration of each sample.

Co-Immunoprecipitation

HeLa cells are cotransfected with the plasmids expressing proteins of interest and 48h post-transfection, cells are washed in 1× PBS and lysed in buffer (Tris-HCl 10mM pH=7.5, NaCl 150mM, EDTA 1mM, and 1% NP40, complete MINI, EDTA free protease inhibitor Cocktail Tablets, Roche). After centrifugation, the protein concentration is assessed by a Bradford assay as described above. A lysate aliquot corresponding to 90 micrograms of protein is taken to make a deposit "input" to verify that the proteins have been overexpressed. We add DTT (dithiothreitol) 50mM to reduce disulfide bridges, 4× Laemmli Blue (277.8mM Tris-HCl, 44.4% glycerol, 4.4% SDS and bromophenol blue 0.02%), SDS provides a uniform negative charges load of proteins (negatively charged) in order to separate them based on their

molecular weight, and water to bring the volume to 90 µl. The tubes "input" are then denatured for 5min at 95°C, then stored at -20°C.

A 1.5mg protein equivalent of lysates is incubated 2h at 4°C with 1µg of anti-Flag M2 antibody (Sigma, F1804) on a rotating wheel. After equilibration, 50µL Dynabeads protein A (Life technologies, 10002D) is added and the mixture is incubated for 1h at 4°C. Beads are washed 3 times with cold 1× lysis buffer, resuspended in Laemmli sample buffer (Laemmli, Bio-Rad, 161-0747), boiled for 5 min, and analyzed by SDS-PAGE and Western blotting.

SDS-PAGE and Western Blotting

All input samples and immunoprecipitates are separated by acrylamide gel SDS-PAGE (10%, 2 h at 120Volts, minigel system BioRad, running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.8) then transferred to a polyvinylidene fluoride membrane (PVDF), previously activated in ethanol, for 2 hours at 100 Volts in a semi- liquid system (transfer buffer (25mM Tris, 192mM glycine, 30% ethanol, pH 6.8).
Membranes are blocked with 10mL of a 3% casein solution in TBS-T (Tris buffered saline-Tween20 0.2%; Sigma T9039) for one hour at room temperature and then probed with primary antibody diluted in 3% casein TBS-T for 1h at room temperature. After incubation the membrane is washed 3 times for 10min TBS-T and finally incubated with a horseradish peroxidase HRP-conjugated secondary antibody diluted 1/10000 in 3% casein TBS-T for 1h at room temperature. The membrane is washed again 3 times and the membrane is revealed using a chemiluminescent ECL system (Amersham) on a LAS 4000 apparatus. Sometimes, a home-made substrate solution for the HRP enzyme (250 mM luminol; coumaric acid 90 mM; 30% hydrogen peroxide; 1M Tris pH 8.8) was used.

In vitro RNA transcription and Biotinylation

Linearized plasmids (25-30µg) were incubated for 3 hours at 37°C in a final volume of 300µL containing 30µL of 10× T7 buffer, 4mM each of dNTPs, 5mM DTT, 1mM spermidine, 0.055 % (v: v) Triton X100, 0.05 mg/mL BSA, 1U/µL RNasin, and 3µL (1/100 of Vfinal) bacteriophage T7 RNA polymerase of bacteriophage T7. After the transcription reaction is over, the mixture is treated by DNase for 1 hour at 37°C, RNA is extracted by phenol-

chloroform, and loaded on a column for size exclusion chromatography (Tosch Bioscience, 06729). Collected RNA fractions are precipitated overnight by 100% ethanol and then washed with 70% ethanol and resuspended in water.

RNA is biotinylated using the 5'-end labeling kits (Vector Labs) according to manufacturer's instructions.

C- Cell Assay

Dual luciferase Assays

HeLa cells are seeded in 12 well plates at a density of 8.10^4 cells/well. After twenty four hours, cells are transfected with 200ng of the dual luciferase IRES construct and 250ng of each plasmid coding protein of interest. Total plasmid DNA is completed by an empty vector to 1µg. Twenty-four hours post transfection, cells are lysed in 1× lysis buffer (Promega) (250µL/well) for 15min at room temperature. Firefly and *Renilla* luciferase activities are measured with the Dual-Luciferase Reporter Assay System (Promega, E1910) according to the manufacturer's protocol, using a luminometer (Berthold, TriStar LB941). Briefly, 20µl of sample is introduced in each well of a 96-well, flat-bottomed, white plate (Greiner Bio-One) and first, 70µl of luciferase assay reagent II (LARII) is added and the luminescent signal is read just after substrate addition (accumulation acquisition time 10 sec). After firefly luciferase quantification, the reaction signal is quenched, and the *Renilla* luciferase simultaneously activated, by adding 70µl Stop&Glo® Reagent to each sample. *Renilla* luciferase luminescence is quantified just after reagent addition for 10 seconds.

D- Virus production, infection, quantification

Viral Production and Quantification

Stocks of VSV-G pseudotyped HIV-1 pseudoparticles is prepared by transfecting 3µg of pMD2.G, 12 µg of pSiCoR-luciferase, and 6 µg of pCMV-dR8.91 vectors into 293T cells using the standard Jet transfection protocol (PolyPlus, France). At 48 hour post transfection, cell culture supernatants are collected, clarified on 0.45µm PVDF filter (Millipore

SLHV033RS), and concentrated in Vivaspin20 (Sartorius, VS2031). Supernatants containing pseudoparticles are stored in 500 µL aliquots at -80°C. In parallel, viral stocks are titrated by anti-p24 enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Innotest® HIV Antigen mAb).

Viral infection after siRNA transfection

To infect HeLa cells, cells are seeded in 6 well plates at a concentration of 5 x 10^4 cells/well. The next day, cells are either transfected with 50nM a control non target siRNA or Unr siRNA (Dharmacon LU-015834-00-0002 and D-001810-10-20), in order to knock down the protein of interest using Jet (PolyPlus transfection, France). 48 hours post transfection, cells are infected by adding an amount of viral supernatant equivalent to 360ng of p24/well. Polybrene was added to the viral supernatant and the medium in a final concentration of 1 µg/ml. Viral particles were mixed with 1µM of AZT (zidovudine) is used as a positive control whereas non infected cells are used as negative control. After 8 hours, the medium is changed and replaced by a virus-free medium and the cells are kept for 48 hours after infection before lysis and RNA extraction.

RNA extraction

RNA is extracted with RNeasy Plus Mini kit (Qiagen) according to manufacturer's instructions extraction. Briefly, cells are lysed directly in the well with 350µl of Buffer RLT PLUS supplied with the Qiagen kit. Lysates are centrifuged and the supernatant is transferred into a gDNA Eliminator spin column. One volume of 70% ethanol is added to the flow-through and the mix is loaded into RNeasy spin column. The column is washed by 700µl of RW1 buffer and 500µl of RPE buffer respectively. Then, RNA is eluted by adding 50µl of RNase free water to the RNeasy spin column.

Quantitative PCR

After quantification, RNA is reverse transcribed into cDNA using iScript ^{TM} Select cDNA Synthesis Kit (Bio-Rad). For each condition, 1µg RNA is mixed with 4µl of the 5 x iScript reaction mix, 1µl iScript Reverse transcriptase, and the final volume is completed to 20 µl by

nuclease free water. The mix is incubated for 5 minutes at 25^{o} C, 30 minutes at 42 ^{o} C, and then 5 minutes at 85 ^{o} C in a Biorad T100 thermal cycler. A non-enzymatic control (NEC) which contains all the reaction mixture except reverse transcriptase is run in parallel to the samples. This NEC will be used in qPCR to test for contaminating DNA.

Each reaction mix contains 300nM of each of forward and reverse primers, 50ng reverse transcribed cDNA template, and 10µL of 2x Fast SYBR® Green Master Mix (Applied Biosystems, 4385612) in a total reaction volume of 20µl. Mixes are prepared in a MicroAmp® Optical 96-well Reaction Plate (Applied Biosystems, 4346907). Two controls are run in parallel to the samples: a non-template control in which cDNA is replaced by water, and a non-enzymatic control described in the above paragraph.

Amplification of cDNA is carried out by real-time quantitative PCR as follows: 95^{o}C for 20sec and 40 cycles (95^{o}C for 3sec, 60^{o}C for 3sec) and detected using an ABI 7000 Sequence Detection System (Applied Biosystems). Gene expression is normalized to housekeeping gene (18S). Plates are sealed with an Optical Adhesive Cover (Applied Biosystems).

Cycle thresholds (C_T) are determined per transcript in triplicate with the following probes:

Luciferase Forward: 5'-TGAGTACTTCGAAATGTCCGTTC-3'

Luciferase Reverse: 5'GTATTCAGCCCATATCGTTTCAT-3'

18S Forward: 5'TGTGGTGTTGAGGAAAGCAG-3'

18S Reverse: 5'TCCAGACCATTGGCTAGGAC-3'

Relative levels of mRNA gene expression are calculated using the $2^{-\Delta\Delta Ct}$ method

E- Imaging Protocols

Cell fixation

Cells are washed once with 1×PBS and fixed with 4% Paraformaldehyde (PFA) in 1× PBS for 15 minutes at room temperature. After fixation cells are washed three times with 1× PBS.

Fluorescence and Immunofluorescence (IF)

Twenty four hours prior to transfection, HeLa cells are grown on previously ethanol sterilized glass coverslips in 12-well plates at a concentration of 8.10^4 cells per well. Twenty four hours post-transfection, cells are washed twice with 1× PBS and then fixed with 1mL of 4% PFA for 15 min at room temperature, and permeabilized with 1mL of a 1% solution of Triton-×100 (detergent, resulting in the formation of pores in the cell, 100% stock solution) in 1× PBS for 10 min at room temperature. The nonspecific sites are then blocked with a BSA solution (bovine serum albumin, Euromedex, ref 04-100-811-C) 1% in 1× PBS for 60 min. The slides are placed in contact with a volume of 50 µl of anti-Flag M2 antibody diluted at 1/250 in 1% BSA, previously deposited on the parafilm. After a series of 3 washes with 1× PBS, the cells are placed in contact for 40 minutes at room temperature with 50 µl of anti-mouse antibody coupled to Alexa Fluor 568 diluted at 1/1000 or 1/2000 in 1%. BSA. The coverslips are then mounted on slides with a drop of Prolong® Gold antifade reagent (Invitrogen ref 36930) and stored at 4 ° C protected from light until use.

Confocal Microscopy

Laser scanning confocal microscopy represents one of the most significant advances in optical microscopy used for biological applications. Confocal microscopy technique is based on the illumination of the sample by a laser beam where the fluorescence which does not originate from the focal plane of the lense used is removed using a diaphragm (pinhole) placed in front of the detector. In this way, only the photons originating from the focal plane cross the diaphragm and participate in the formation of the image. Thus, it is possible to obtain a sharp and thin optical section of the focal plane.

The cellular localization of the proteins of interest coupled to fluorophores (eGFP, mCherry) is visualized by confocal microscopy with a Leica SPE equipped with a 63× 1.4NA oil immersion objective (HXC PL APO 63×/1.40 OIL CS). The eGFP images are obtained by scanning the cells with a 488-nm laser line and using a 500- to 555-nm band-pass filter for emission. For the mCherry images, a 561-nm laser line is used with a 570- to 625-nm band-pass filter. Images are analyzed by Image J software.

FRET (Förster/fluorescence Resonance Energy Transfer)

FRET is a non-radiative process by which energy from an excited fluorescent donor (eGFP) is transferred to a nearby fluorescent acceptor (mCherry). FRET is only possible when the donor emission spectrum overlaps with the acceptor absorption spectrum. It also requires that the donor and acceptor have a favorable orientation and that the distance between the two is less than 10nm (Förster distance). If the two fluorophores (eGFP and mCherry) fused to the proteins of interest are at a distance less than 10nm, there is an energy transfer between the donor (eGFP) and the acceptor (mCherry), which results in a decrease in the fluorescence lifetime (τD) of the donor (Figure 47). The eGFP emission spectrum is then moved to lower life times. There are three methods of FRET measurements: intensity fluorescence measurements, acceptor photobleaching, and fluorescence lifetime measurements.

In our studies, we used FLIM (Fluorescence-lifetime imaging microscopy) technique to visualize FRET. FLIM is a technique that allows acquiring an image according to the lifetime of the fluorophore observed (here the donor). Visualization by FLIM has the advantage of overcoming the level of fluorescence intensity of both the fluorophores and thus be independent of the level of expression of the proteins studied.

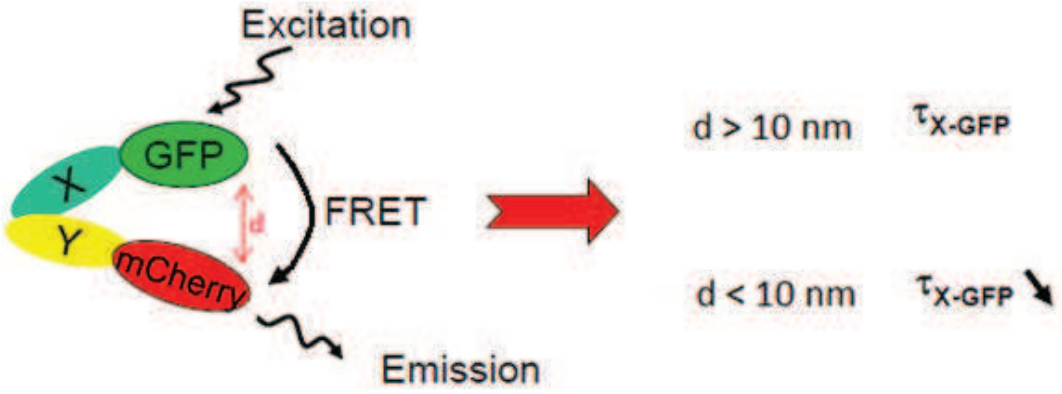

Figure 47: Fluorescence Resonance Energy Transfer. When the two proteins of interest are within a distance of less than 10nm, an energy transfer will occur from the donor (eGFP) to the acceptor (mCherry) thus decreasing the lifetime of the donor.

Time-correlated single-photon counting FLIM measurements are performed on a homemade two-photon excitation scanning microscope based on an Olympus IX70 inverted microscope with an Olympus 60× 1.2NA water immersion objective operating in the descanned fluorescence collection mode (Clamme et al. 2003; Azoulay et al. 2003). Two-photon excitation at 900nm is provided by a mode-locked titanium-sapphire laser (Tsunami; SpectraPhysics) or an Insight DeepSee (Spectra Physics) laser. Photons are collected using a set of two filters: a shortpass filter with a cutoff wavelength of 680nm (F75-680; AHF, Germany) and a band-pass filter of 520 ± 17 nm (F37-520; AHF, Germany). The fluorescence is directed to a fiber-coupled APD (SPCM-AQR-14-FC; PerkinElmer), which is connected to a time-correlated single photon counting module (SPC830; Becker & Hickl, Germany). Typically, samples are continuously scanned for about 60s to achieve the appropriate photon statistics in order to investigate the fluorescence decays. Moreover, to reach the Nyquist–Shannon sampling criteria, we carried out FLIM measurements using20 µm × 20 µm scale and 256 pixels × 256 pixels. Data are analyzed using a commercial software package (SPCImage v2.8; Becker & Hickl, Germany). For FRET experiments with eGFP, the FRET efficiency (E) is calculated according to:

$$E=1-(\tau DA/\tau D)=1/1+(R/R0)^6$$

where τDA is the lifetime of the donor in the presence of the acceptor and τD is the lifetime of the donor in the absence of the acceptor, R is the distance between donor and acceptor and R_0 is the Förster distance for which E = 50%.

This report is used to determine whether two proteins interact or not: the threshold for E is set at 5%, and the higher the FRET efficiency, the closest the interactors.

F- Molecular Biology

Transformation of competent bacteria and plasmid amplification

Each plasmid is transformed by heat shock in 50µL *E. coli* DH5α chemically competent bacteria. For this, competent bacteria are thawed on ice and incubated with the transforming plasmid on ice for 30 minutes before being heat shocked for 30 seconds at 42°C and replaced

for 2 minutes on ice. The bacteria are resuspended in 1mL of LB medium (tryptone 10g/L, yeast extract 5g/L, NaCl 10g/L, pH 7) without antibiotics and incubated 1h at 37°C with shaking. Transformed bacteria are selected on LB agar plates supplemented with suitable sterile antibiotic (ampicillin 100 µg/mL, kanamycin 50µg/mL). Plasmid amplification is performed clonally. Plasmids are purified on anion exchange column (Nucleobond Xtra Midi Kit Plus; Macherey-Nagel) following the manufacturer's protocol.

Site Directed Mutagenesis

In order to generate IRES mutants, we used a Phusion site-directed mutagenesis kit from Thermo scientific (F-541) according to the manufacturer's instructions which consist of three steps.

- amplification of the full IRES plasmid to be mutated with phosphorylated primers harboring the desired mutations. Amplication is carried using a Biorad T100 thermal cycler. Primers sequences are listed in the primer section.

-ligation of the PCR product using Quick T4 DNA ligase

-transformation of DNA with ultra-competent cells (NEB) and verification of the mutant sequence

	Volume (µL)	**Final Concentration**
H2O	Up to 50 µL final volume	
5X Phusion HF Buffer	10	1x
10Mm dNTPs	1	200µM each
Fwd Primer	2.5	10 µM
Reverse Primer	2.5	10 µM
DNA		1ng
DNA polymerase Phusion hot start (2U/µl)	0.5	0.02U/ µL

Gateway Cloning

To clone Unr into different constructs (eGFP, mCherry, PHXGWA), we used the Gateway cloning technology which is a fast and highly effective universal cloning method for transferring DNA fragments in multiple vectors. This technology is based on the specific recombination system used by the lambda phage to integrate its DNA in the *E. coli* chromosome. This integration process (lysogeny) is catalyzed by two enzymes: the phage λ encoded protein Int (integrase) and the *E.coli* protein IHF encoded protein (Integration host factor). Upon integration, the recombination between *att*B and *att*P sites generate *att*L and *att*R sites that flank the integrated phage λ DNA. This is a reversible process and the excision reaction is catalyzed by the Int and IHF in combination with the phage λ DNA.

This gateway cloning system, an *in vitro* version of the integration and excision reactions, facilitates the transfer, between vectors, of heterologous DNA sequences flanked by altered att sites. The system is based on two recombination reactions. First, the BP reaction permits recombination of substrate flanked by attB sequences (PCR product flanked by attB sequences) with a donor vector flanked attP sequences, which generates entry clone flanked by attL sites (entry vector). This reaction is catalyzed by the enzyme BP Clonase ™ mix (consisting of integrase Int and IHF integration factor). A fraction of this reaction is directly used for transforming bacteria DH5α. The selection is made by two mechanisms: the ccdB gene originally localized in the donor vector (whose product is toxic to the bacterium DH5α) allows negative selection of the non-modified donor vectors while the antibiotic gene resistance allows the positive selection. Positive entry clones, verified by sequencing, are used for the second recombination reaction: LR reaction. It allows recombination of a substrate flanked by attL sequences (entry vector) with a destination vector flanked by the attR sequences. The reaction catalyzed by LR Clonase^{TM} mix (consisting of integrase Int, Xis excisionase of IHF and integration factor). The main advantage of this technique is that a DNA sequence can be cloned in multiple vectors suitable for such diverse systems as bacteria, cells mammalian or plant cells, bypassing digestion steps – ligation.

The attB flanking PCR products were realized using Phusion polymerase and the primers attB1-CSDE1-noKoz and AttB2-CSDE1-stop given in the primer section.

All the construction realized with this technique were sequenced by the GATC Biotech AG service.

IRES cloning into T7 vectors

The HIV-1, 211 HIV-1 mutant, and the Apaf-1 IRESs were amplified by PCR using the primers HIV IRES EcoR FW and HIV IRES Xba Rev or Apaf-1 IRES EcoR FW and Apaf-1 IRES Xba rev given in the primers section. After purification of the PCR product on NucleoSpin® Gel and PCR Clean-up Columns (Macherey Nagel), they are digested with EcoRI and XbaI enzymes and ligated into a XbaI/EcoRI digested and SAP (Shrimp alkaline phosphatase, Biolabs) dephosphorylated pcDNA3.1(+)/Zeo vector. Ligations are transformed in DH5α bacteria and plated on ampicillin resistant plates. Plasmids are extracted from grown colonies and their plasmid DNA content extracted using nucleospin columns (Macherey Nagel) and send to GATC Biotech AG for sequencing.

Results

Part I: The IRES transacting factor Upstream of N-Ras interacts with HIV-1 Gag/NCp7: functional implication on the IRES driven translation and infection.

Due to its nucleic acid (NA) chaperoning ability, NCp7 is a major player in the early and late stages of the viral cycle. In the early phase, it chaperones NA during reverse transcription and influences integration of the viral genome. In the late phase, as a domain of Gag, it binds to the packaging signal (ψ) in the 5'UTR participating in the dimerization and the selective encapsidation of the genomic RNA (gRNA) into the viral particles.

In retroviruses and HIV-1 in particular, the full-length viral RNA acts both as a messenger and gRNA. The balance between these two key roles must be maintained to allow an efficient virus replication. The processes of gRNA translation and encapsidation/packaging are determined by several viral 5' leader region elements including the 5'-cap structure and the Internal Ribosome Entry Segment (IRES) for the first, and the dimer initiation site (DIS), and the packaging signal (ψ) for the latter.

HIV-1 translation proceeds by either a cap-dependent or an IRES-dependent mechanism. Recent data showed that the two mechanisms of translation initiation are active at different levels according to the cellular state and infection progress. An IRES directly recruits the 40S ribosome independently of the 5'cap and without the need of some initiation factors required for the cap-dependent translation. The molecular mechanism by which the host translation apparatus recognizes the IRES is unknown, but for other viral or even cellular IRESs, some canonical initiation factors as well as specific cellular proteins, named IRES transacting factors (ITAFs), participate in the recognition process. HIV-1 bears two IRESs, one in the Gag open reading frame responsible for the synthesis of the 40kDa Gag isoform as well as the full length Gag, and the other, the HIV-1 IRES, within the 5' leader region found in all HIV-1 mRNA. Knowing that 40S ribosome recruitment by IRESs depends on RNA secondary and tertiary structures, the IRES activity is possibly regulated by structural changes within the IRES.

The HIV-1 5'-UTR was suggested to fold into two alternative conformations where, according to the conformation, either the dimer initiation signal (DIS) or the Gag start codon is exposed. As an RNA chaperone, NC binds to the 5'-UTR in proximity to the IRES region which overlaps the packaging (ψ) signal in the 5'-UTR. This binding is thus suggested to destabilize one of the conformers thus exposing the DIS and promoting switch towards dimerization and gRNA packaging.

In order to better understand the role of viral proteins and their interaction with host factors, Jager et al. identified by affinity purification coupled to mass spectroscopy analysis, potential cellular protein partners of NC and Gag. One of the proteins is Upstream of N-ras (Unr). Unr is a cytoplasmic protein with five cold shock domains described as an ITAF for viral and cellular IRESs including the one of poliovirus (PV), the human Rhinovirus (HRV), and the apoptotic protease –activating factor Apaf-1. Since Unr as a potential HIV-1 ITAF would bind to the HIV-1 IRES region in close proximity to the NC binding site, it is interesting to confirm the NC/Unr interaction and to analyze the role of Unr on HIV-1 IRES activity.

During the course of my PhD, I started with dual luciferase experiments to look for the effect of Unr on the HIV-1 IRES activity, which led me to identify Unr as an ITAF for the HIV-1 IRES. This effect was studied in the presence of NCp7 and Gag which both modulated the HIV-1 IRES activity. Furthermore, I looked for the effect of IRES mutations that possibly interfere with Unr binding on the HIV-1 IRES activity. I was able to identify regions where mutations greatly decrease the IRES activity. Using infectivity assays, I investigated the role of Unr in early infection by using a VSV-pseudotyped lentivirus model where I showed that Unr knock down decreased the infection. Most of this work is presented in the following article manuscript that will be submitted soon.

Protein purification, RNA synthesis, and electrophoretic mobility shift assay (EMSA) are in progress in order to further show Unr binding to the HIV-1 IRES. This part is presented under the form of supplementary data where I also present the path I took before I started working on Unr. I include as well some extra negative results that are worth knowing.

Manuscript 1

The IRES transacting factor Upstream of N-Ras interacts with HIV-1 Gag/NCp7: functional implication on the IRES driven translation and infection.

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The Human Immunodeficiency virus type 1 (HIV-1) gRNA is used both for packaging into new virions and translation of the viral proteins. The HIV-1 5'-UTR has been shown to drive cap-dependent translation initiation under normal physiological conditions, and IRES-dependent protein synthesis during the cell cycle arrest induced by the viral infection. The HIV-1 genomic mRNA harbors two IRES elements: the first one lies within the 5'-UTR while the second one lies within the gag coding region. However, the HIV-1 5'-UTR IRES is not functional in an *in vitro* translation system unless supplemented by cellular extracts from cells blocked in G2/M. Upstream of N-ras (Unr) protein is an ITAF (IRES-transacting factor), overexpressed in G2/M, which regulates Rhinovirus and Poliovirus IRESs as well as its own IRES-driven translation. Interestingly, Unr has been recently found as a partner of the HIV-1 nucleocapsid protein, which as a mature protein (NCp7) or a domain of Gag, plays several important roles in the viral life cycle. Using a dual luciferase assay, Unr overexpression was shown to act as an ITAF increasing HIV-1 IRES dependent translation. Point mutations in a putative Unr binding site in the HIV-1 IRES were found to alter both the IRES activity and its activation by Unr, suggesting a strong dependency of the IRES on Unr. Unr stimulation effect is furthermore counteracted by NCp7, but not by Gag overexpression, which increases the IRES activity in an additive manner to Unr, suggesting a differential Unr effect on the early and late phases of the infection. An RNA dependent interaction of Unr with both Gag and NCp7 was identified by FRET-FLIM and immunoprecipitation experiments. Finally, knockdown of Unr in HeLa cells leads to a decrease in the infection by a non-replicative lentivector, proving its functional implication in the early phase.

Introduction

The human Immunodeficiency virus type-1 (HIV-1) belongs to the *Lentivirus* genus of the *Retroviridae* family. Its positive-stranded RNA genome (gRNA) resulting from the polymerase II-mediated transcription of the integrated viral genome (vDNA) is capped and polyadenylated. This gRNA is thus used both for packaging into new virions and translation of the viral precursor proteins. Interestingly, the highly structured 5' non-coding region (5'-UTR) of HIV-1 has first been shown to block ribosomal scanning *in vitro* (Miele et al, 1996) but it was later shown that it is not the case in cultured cells thus showing that the cap-dependent translation is dependent on cellular factors and physiological conditions (Berkhout et al, 2011; Ricci et al, 2008). However, the cap-dependent translation is not the sole mechanism described for HIV-1 translation. IRES-dependent translation has been described for HIV-1 especially under conditions where cap-dependent translation is impaired. In fact, the highly structured 5' extremity of the HIV-1 gRNA harbors two Internal Ribosome Entry Site (IRESs). IRESs were first described among picornaviruses before their identification in other viral and cellular RNAs. IRESs are highly structured RNA domains found most often in the 5' non-coding region of messenger RNAs which allow the 40S ribosomal subunit recruitment without the need of the 5'-cap structure or cap-dependent translation mandatory initiation factors. For HIV-1, the first functional IRES described is localized within the gag ORF (Buck et al, 2001). It drives the expression of both the full length and a shorter isoform of the Gag polyprotein (Gagp40) from an alternative AUG start codon. This IRES unconventionally drives translation from an AUG localized at its 5' extremity and is also found functional in others retroviruses including HIV-2 (Herbreteau et al, 2005; Locker et al, 2011; Weill et al, 2010). A second, more conventional IRES, is found in the 5'-UTR of the viral gRNA and thus in all HIV-1 mRNA more precisely between nucleotides 1-336, with nucleotides 104-336 representing the minimal functional sequence (Brasey et al, 2003). This genome region includes several secondary structures implicated in genome transcription, encapsidation, dimerization and reverse transcription including : TAR, poly(A), PBS, DIS and Psi (Brasey et al, 2003). Since this IRES is not functional in HIV-2, its functionality in HIV-1 was a subject of a long debate since its first description in 1996 by Brasey et al. The contradictory results observed for this IRES are in part explained by the fact that it is not functional in a rabbit reticulocyte lysate (RRL) *in vitro* system (Miele et al, 1996). However,

it becomes functional upon RRL supplementation by cellular extracts especially from HeLa cells blocked in G2/M (Rivas-Aravena et al, 2009; Vallejos et al, 2011). Additionally, it also becomes functional when used *in vitro* with HeLa cells based translation extracts (Brasey et al, 2003), in cell-based assays (Brasey et al, 2003; Gendron et al, 2011; Liu et al, 2011; Monette et al, 2009; Vallejos et al, 2012) or in *Xenopus laevis* oocytes (Rivas-Aravena et al, 2009; Vallejos et al, 2012; Vallejos et al, 2011). This underlines its dependence on cellular factors, as it was already shown for Polio- or Rhinoviruses IRESs (Borman et al, 1993; Meerovitch et al, 1993) or for the cellular IRES from the Apaf1 mRNA (Mitchell et al, 2001).

Recent studies supported the HIV-1 IRES requirement for IRES transacting factors (ITAFs) as cap- and IRES-dependent mechanisms of translation initiation were shown to be active at different levels according to the cellular state and the progress of the infection (Amorim et al, 2014; Monette et al, 2013). The IRES is particularly active when the cell is blocked in G2/M as it is the case during HIV-1 infection (Amorim et al, 2014; Gendron et al, 2011; Monette et al, 2013; Rogel et al, 1995).

So far, the number of ITAFs identified for viral or cellular IRESs is around fifteen. Although their modes of action are poorly known, they are suggested to act as RNA chaperones rearranging the secondary structures of the IRES or as adaptor proteins bridging the RNA to the proteins of the 40S ribosome subunit (King et al, 2010). In any case, their ability to regulate the activity of IRESs and their variable level of expression in different cell types is one of the leading explanation for the difference of activity observed for a given IRES across various cell types (Balvay et al, 2009; Filbin & Kieft, 2009; Fitzgerald & Semler, 2009). One of these ITAFs, the cytoplasmic protein Unr (Upstream of N-Ras) contains 5 Cold Shock Domains (CSDs) which bind NAs as well as proteins (Ferrer et al, 1999; Jacquemin-Sablon et al, 1994). The IRES regulating action of Unr has been demonstrated for the Polio- and Rhinoviruses IRESs, in addition to cellular IRESs including Apaf1 and its own IRES (Boussadia et al, 2003; Brown & Jackson, 2004; Mitchell et al, 2003; Tinton et al, 2005). As an ITAF of the HIV-1 IRES and being overexpressed in cells blocked in G2/M, Unr could explain the sustained synthesis of HIV-1 proteins during the course of an HIV-1 infection despite the cell block in G2/M or the cleavage by HIV-1 protease of the canonical cap-dependent initiation factor eIF4G.

The HIV-1 nucleocapsid protein is present in two forms in the virus particle and the infected cell, as a domain of the polyprotein Gag (NC-Gag) and as a product of cleavage of Gag (mature form, NCp7). Because of its ability to bind to nucleic acids (NA) (Clever et al, 1995; Clever et al, 2002; Dannull et al, 1994), NC/NCp7 is a major player in both the early and late stages of the viral cycle (Darlix et al, 2007; Darlix et al, 1995; Thomas & Gorelick, 2008). In the early phase, it protects the incoming gRNA from cellular nucleases, chaperones NAs during reverse transcription and probably promotes the integration of the provirus (Darlix et al, 2011). In the late phase, it participates in the selective encapsidation and dimerization of the gRNA into the viral particles (Berkowitz et al, 1996). The encapsidation results in the formation of high order Gag multimers on the gRNA and the concomitant trafficking of this ribonucleoprotein to the cell plasma membrane (Berkowitz et al, 1996; Cimarelli et al, 2000; de Rocquigny et al, 2014; El Meshri et al, 2015). The central role of NC/NCp7 in infection is emphasized by the drastic loss of infectivity observed after single point mutations in either of its two zinc fingers (Dorfman et al, 1993).

Interestingly, Unr has been recently found as a partner of the NCp7 protein in affinity purification analysis coupled to mass spectroscopy (Jager et al, 2012). In the present work, we found by biochemical and microscopy approaches that Unr acts as an ITAF for the HIV-1 IRES and that this activity is regulated by NC-Gag, but not by NCp7. We further showed that Unr interacts with NCp7 and the NC domain of Gag. Finally, a knockdown of Unr by siRNA was found to decrease HeLa cells infection by pseudotyped HIV-1 pseudoparticles.

Materials and Methods

Mammalian Cell Culture

HeLa cells and 293T cells were maintained in Dulbecco's modified Eagle medium (Gibco® Life Technologies ref 21885) supplemented with 10% fetal calf serum (Invitrogen Corporation, France) and a mixture of penicillin (100 U/mL) and streptomycin (10 U/mL) antibiotics (Lonza DE17-602E) at 37°C in a 5% CO_2.

Plasmids and proteins

The dlHIV-1, dlVAR2 and dl-Apaf (pRAF) were generous gifts from M. Vallejos and A. Willis and were described earlier (Vallejos et al 2012 and Mitchell et al 2001). The plasmid constructs for VSV-G pseudotyped HIV-1 particles, namely pMD2.G and pCMV-dR8.91 were obtained from Addgene. pSicoR-luciferase was obtained by the replacement of the eGFP cDNA in pSicoR-eGFP by the firefly luciferase cDNA between the NheI and EcoRI cloning sites. In PEF-Flag-Unr (generous gift from J. Sablon), the Unr gene is cloned with its N-terminus fused to a Flag sequence and expressed under the control of an EF-1a promoter. peGFP-Unr and pmCherry-Unr were cloned by Gateway® cloning from PEF-Flag-Unr, using the following primers (AttB1-CSDE1 : 5'-ggggaccactttgtacaagaaagctgggtttagtcaatgacaccagcttgac-3' and AttB2-CSDE1-Stop : 5'-ggggaccactttgtacaagaaagctgggtttagtcaatgacaccagcttgac-3') into the destination vector peGFP-C1-GW. IRES mutants were generated by site directed mutagenesis using the Phusion site-directed mutagenesis kit (Thermoscientific, F1504) according to the manufacturer's instructions with the following primers purchased from Sigma.

IRES mut rev	(Phos)TGTTCGGGCGCCACTGCTAGAG
IRES mut U fwd	(Phos)GGGACACTTGAAAGCGATTCATAAGCCAGAGGAG
IRES mut C fwd	(Phos)GGGACTTGAAAGCGACCAGCAAGCCAGAGGAG
IRES mut 211 fwd	(Phos)GGGACTTGAAAGCGAAGGTAAAGCCAGAGGAG
IRES mut 212 fwd	(Phos)GGGACTTGAAAGCGAAACTAAAGCCAGAGGAG
IRES mut 213 fwd	(Phos)GGGACTTGAAAGCGAAAGAAAAGCCAGAGGAG
IRES mut 214 fwd	(Phos)GGGACTTGAAAGCGAAAGTGAAGCCAGAGGAG

NCp7 was synthesized by solid phase peptide synthesis using the FMOC (Fluorenylmethyloxycarbonyl chloride) technology. At the end of the synthesis, two equivalents of biotin-NHS (Biotin N-hydroxysuccinimide) were added before the deprotection of the peptide.

Dual luciferase Assays

HeLa cells were seeded in 12 well plates with a density of 8×10^4 cells/ well. Twenty four hours post seeding, cells were transfected, using jetPEI™ Polyplus Transfection™ according to the manufacturer's protocol, with 200 ng of the dual luciferase IRES construct and 250 ng of each construct coding for the protein of interest. Total plasmid DNA was completed by an empty vector to 700 ng. Twenty four hours post transfection, firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol, using a luminometer (Berthold, TriStar LB941). Briefly, cells were lysed in 1×lysis buffer (Promega) (250 µL/well) for 15 min at room temperature and 20 µL of sample was introduced in each well of a 96-well, flat-bottomed, white plate (Greiner Bio-One). 70 µL of luciferase assay reagent II (LARII) was injected into each well and the luminescent signal was accumulated for 10 s. Then, the firefly luciferase reaction signal was quenched by adding 70 µL Stop & Glo® Reagent to each well and the *Renilla* luciferase activity signal was measured for 10s. Statistics were done using the student t- test.

Co-Immunoprecipitation

293T cells were seeded in a 6 well plate at a concentration of 60×10^4cells/well 24h before cotransfection with 2 µg of the each of the plasmids coding for the proteins of interest. 48 h post-transfection, cells were washed in 1×PBS and lysed in RIPA buffer (Tris-HCl 10 mM pH=7.5, NaCl 150 mM, EDTA 1 mM, and 1% NP40, complete Mini-EDTA free protease inhibitor Cocktail Tablets (Roche)). After centrifugation to remove cell debris, the protein concentration was assessed by a Bradford assay. An input fraction (30 µg) was kept to check the protein expression level, and the equivalent of 1 mg of lysate was incubated 2 h at 4°C with 1 µg of anti-Flag antibody (Sigma, F1804) on a rotating wheel. After equilibration, 50 µL of Dynabeads protein A (Life technologies, 10002D) were added and the mixture was incubated for 1h at 4°C. Beads were washed 3 times with cold 1×lysis buffer, resuspended in Laemmli sample buffer (Bio-Rad, 161-0747), boiled for 5 min, and analyzed by Western blotting.

Western Blots

Protein samples were electrophoresed on a 12% SDS-PAGE gel. Subsequently, proteins were transferred onto a polyvinylidene difluoride membrane (Amersham, RPN303F) and blots were probed with mouse monoclonal antibody anti-CAp24 Gag (AIDS Reagent Program, 6521 #24-4; Simon et al 1997; Fouchier et al 1997), anti-eGFP (Proteintech, 66002-1), anti-Unr (Proteintech, 13319-1-AP), or anti-GAPDH (Millipore, MAB374). After several three PBS washes, secondary anti-mouse or anti-rabbit antibodies (Promega WB401B and W402B) conjugated to the horseradish peroxidase were added to the membrane and proteins were visualized by an homemade chemioluminescent ECL system on an Image Quant LAS 4000 apparatus (GE healthcare).

Confocal microscopy

HeLa cells were seeded on a glass coverslip in 12 well plates with a density of 8×10^4 cells/ well, 24 hours before being transfected with the appropriate plasmids as described above. Twenty four hours post transfection, cells were washed with PBS and fixed with 4% Paraformaldehyde (PFA) in 1×PBS for 15 minutes at room temperature. After fixation, cells were washed three times with 1×PBS and mounted on slides using Prolong Gold Antifade Reagent (Invitrogen Reference 36930). The cellular localization of the proteins of interest coupled to eGFP or mCherry was visualized by confocal microscopy with a Leica SPE equipped with a 63× 1.4 NA oil immersion objective (HXC PL APO 63×/1.40 OIL CS). The eGFP images were obtained by scanning the cells with a 488-nm laser line and using a 500- to 555-nm band-pass for emission. For the mCherry images, a 561-nm laser line was used with a 570- to 625-nm band-pass. Images were analyzed by the Image J software.

Fluorescence lifetime imaging microscopy (FLIM)

FLIM measurements were performed using the time-correlated single photon counting approach, on a homemade two-photon excitation scanning microscope based on an Olympus IX70 inverted microscope with an Olympus 60× 1.2NA water immersion objective operating in the descanned fluorescence collection mode (Azoulay et al, 2003; Clamme et al, 2003). Two-photon excitation at 900 nm was provided by a mode-locked titanium-sapphire laser

(Tsunami, Spectra Physics) or an Insight DeepSee (Spectra Physics) laser. Photons were collected using a set of two filters: a short-pass filter with a cutoff wavelength of 680 nm (F75-680; AHF, Germany) and a band-pass filter of 520 ± 17 nm (F37-520; AHF, Germany). The fluorescence was directed to a fiber-coupled APD (SPCM-AQR-14-FC; PerkinElmer), which was connected to a time-correlated single photon counting module (SPC830, Becker & Hickl, Germany). Typically, the samples were continuously scanned for about 60 s to achieve the appropriate photon statistics in order to reliably analyze the fluorescence decays. Moreover, to reach the Nyquist–Shannon sampling criteria, we carried out FLIM measurements using 20 μm × 20 μm scale and 256 pixels × 256 pixels. Data were analyzed using a commercial software package (SPCImage v2.8; Becker & Hickl, Germany). For Förster resonance energy transfer (FRET) experiments, the FRET efficiency (E) was calculated according to: $E=1-(\tau_{DA}/\tau_{D})$, where τ_{DA} is the lifetime of the donor (eGFP) in the presence of the acceptor (mCherry) and τ_{D} is the lifetime of the donor in the absence of the acceptor.

Viral Production and Quantification

Stocks of VSV-G pseudotyped HIV-1 pseudoparticles were prepared by transfecting 3 μg of pMD2.G, 12 μg of pSiCoR-luciferase, and 6 μg of pCMV-dR8.91 (Addgene) vectors into 293T cells using the standard JetPEI transfection protocol (PolyPlus). At 48 hours post transfection, cell culture supernatants were collected, clarified on a 0.45 μm PVDF filter (Millipore SLHV033RS), and concentrated twice by a Vivaspin20 centrifugal concentrator (Sartorius, VS2031). Supernatants containing pseudoparticles were stored in 500 μL aliquots at -80°C. In parallel, viral stocks were titrated by anti-p24 enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Innotest® HIV Antigen mAb).

Viral infection

HeLa cells were seeded in 6 well plates at a concentration of 5×10^{4} cells/well. The next day, cells were either transfected with 50 nM of control siRNA or Unr siRNA in order to knock down Unr (Dharmacon LU-015834-00-0002 and D-001810-10-20), using the Jet Prime transfection protocol (PolyPlus transfection, France). 48 hours post transfection, cells were infected with the viral pseudoparticle supernatant equivalent to 360 ng of p24/well. 1 μM of

AZT (zidovudine) was used as a positive control, whereas non infected cells were used as negative control. After 8 hours, the medium was changed and replaced by a virus-free medium and the cells were kept for 48 hours after infection, before lysis and RNA extraction.

Quantitative PCR

RNA was extracted with RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions and was transcribed into cDNA using the iScript ^{TM} Select cDNA Synthesis Kit (Bio-Rad). qPCR master mixes were prepared in a MicroAmp® Optical 96-well Reaction Plate (Applied Biosystems) with 2× Fast SYBR® Green Master Mix (Applied Biosystems, 4385612) according to the manufacturer's recommendations. Plates were sealed with an Optical Adhesive Cover. qPCR reactions contained 300 nM forward and reverse primers and 50 ng/ µL reverse transcribed cDNA template in a total reaction volume of 20 µL. Amplification of cDNA was carried out by real-time quantitative PCR and detected using an ABI 7000 Sequence Detection System (Applied Biosystems). Gene expression was normalized to housekeeping genes and samples were run alongside RT-negative cDNA (produced without reverse transcriptase) and H_2O controls.

Cycle thresholds (C_T) were determined per transcript in triplicate with the following probes:

Luciferase Forward: 5'-TGAGTACTTCGAAATGTCCGTTC-3'

Luciferase Reverse: 5'GTATTCAGCCCATATCGTTTCAT-3'

18S Forward: 5'TGTGGTGTTGAGGAAAGCAG-3'

18S Reverse: 5'TCCAGACCATTGGCTAGGAC-3'

Relative levels of mRNA gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Results

HIV-1 IRES activity is stimulated by Unr

To provide further information in the debate on HIV-1 IRES activity and functional relevance (Amorim et al, 2014; Berkhout et al, 2011; Brasey et al, 2003; Gendron et al, 2011; Monette et al, 2009; Monette et al, 2013; Ricci et al, 2008; Valiente-Echeverria et al, 2013; Vallejos et al, 2012; Vallejos et al, 2011), we investigated the putative role of upstream of N-ras (Unr), as an ITAF of the HIV-1 IRES. We selected Unr because i) it is an ITAF for polio- and rhinoviruses as well as for cellular IRESs whose mRNA are capped and polyadenylated similarly to the one of HIV-1, ii) its expression is cell cycle regulated, showing an overexpression in G2/M and iii) it potentially interacts with both Gag and NCp7 (Jager et al, 2012).

The HIV-1 IRES functionality was tested in a well-established dual luciferase system (Vallejos et al, 2012), similar to the one used by Brasey and Gendron (Brasey et al, 2003; Gendron et al, 2011). In this system, the first 336 nucleotides of the pNL4.3 HIV-1 clone are inserted into a dual luciferase construct (dl) between an upstream *Renilla* luciferase gene (Rluc) and a downstream firefly luciferase gene (Fluc). A defective encephalomyocarditis virus (ΔEMCV) IRES known to inhibit ribosome reinitiation and read-through is also present upstream of the tested IRES (Figure 1A). After transient transfection and CMV promoter-driven transcription in HeLa cells, the resulting bicistronic mRNA is translated via a cap-dependent mechanism for the Rluc gene and an IRES-dependent one for the Fluc gene. The ratio of the Fluc/Rluc activities reflects the IRES activity normalized to the cap dependent one. These bicistronic mRNAs offer the advantage to allow the IRES to be studied, in the absence of virus infection, thus eliminating potential interferences from the viral replication cycle or from other regions of the gRNA which could influence the HIV-1 IRES (Berkhout, 1996; Brasey et al, 2003; Valiente-Echeverria et al, 2013).

The activity of the HIV-1 IRES was assessed in this system, in comparison to two other IRESs, namely the IRES of a clinical isolate (VAR2), previously described as being 4 times more active than the HIV-1 IRES in HeLa cells (Vallejos et al, 2012) and the cellular IRES of Apaf-1 mRNA which is also active in HeLa cells (Coldwell et al, 2000; Mitchell et al, 2001).

Using HeLa cells transiently transfected by the dl constructs, we found that all IRESs are active (Figure 1B), with the HIV-1 IRES being 1.7 times more active than the one of Apaf-1, but 2.5 times less active than the one of the clinical variant VAR2. We next investigated the effect of Unr overexpression on these different IRESs. Cells were thus co-transfected with the corresponding dl constructs and pEF-Flag-Unr and, luciferase activity was quantified 24 h post transfection. All IRESs presented an increase of their activity of around 40% (Figure 2).

In the case of Apaf-1, this Unr-mediated increase was expected, since Unr was shown to be a stimulating ITAF of the Apaf1 IRES (Mitchell et al, 2003). Similar to the Apaf-1 IRES, Unr increases the translation activity of HIV-1 IRES, suggesting that Unr acts as an ITAF of this IRES.

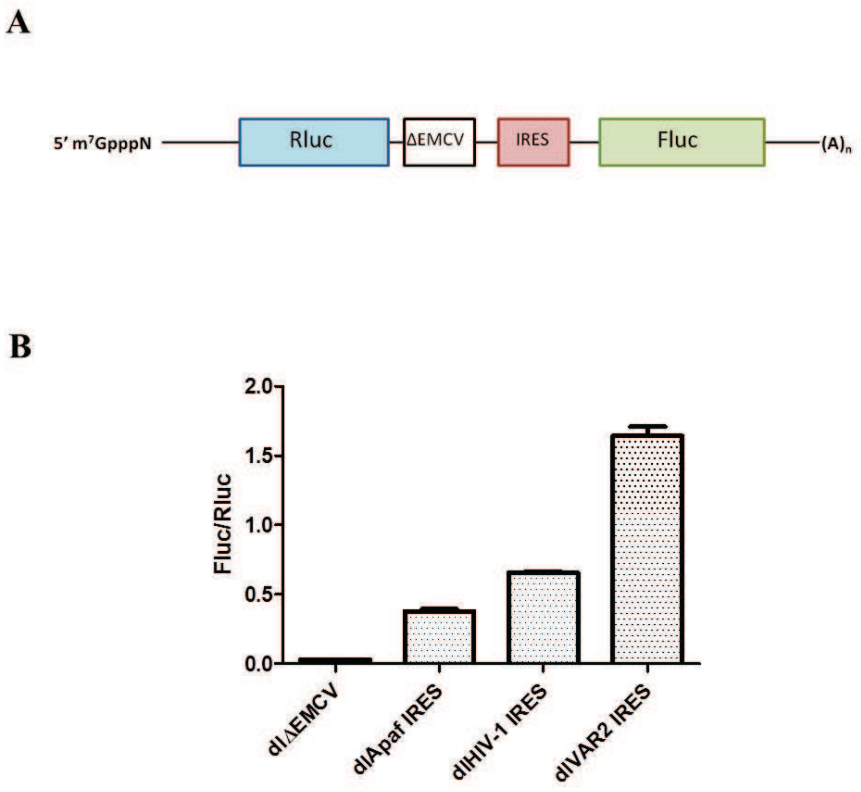


Figure 1: IRES activity measured by a dual luciferase assay. (A) Scheme of the construct used to perform the dual luciferase test. The IRES is inserted into a dual luciferase contruct (dl) between an upstream Renilla luciferase gene (Rluc) and a downstream firefly luciferase gene (Fluc). **(B)** Activity test in HeLa cells of dlHIV-1 IRES, dlVAR2 IRES and dlApaf-1 IRES. The dlΔEMCV IRES was used as a negative control. The IRES activity is monitored by the ratio of Fluc/Rluc luciferase activities. Measurements were performed in HeLa cells 24 hours post transfection. Histograms represent the mean ± SEM for at least 3 independent experiments done in triplicate.

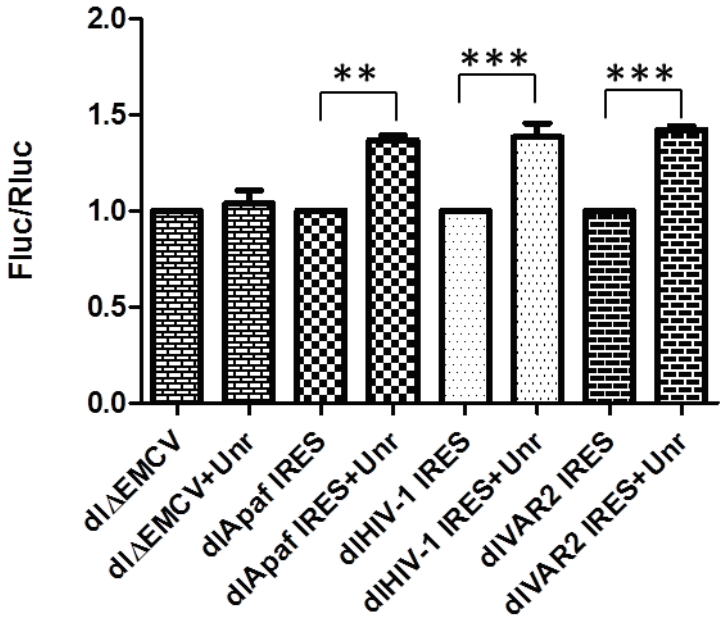


Figure 2: Effect of Unr overexpression on the activity of HIV-1 IRES. The IRES activity is monitored by the ratio of Fluc/Rluc luciferase activities in the dual luciferase assay normalized for each IRES to the one obtained in the absence of Unr overexpression. Two variants of HIV-1 IRES (HIV-1 and VAR2) are tested. The Apaf-1 and ΔEMCV IRESs are given as positive and negative controls respectively. Measurements were performed in HeLa cells 24 hours post transfection. Histograms represent the mean ± SEM of at least 3 independent experiments done in triplicate **, $p < 0.01$; ***, $p < 0.001$.

Identification of Unr binding site in the HIV-1 IRES

An *in vitro* selection approach (SELEX) identified two purine rich unstructured consensus sequences $(Pu)_5AAGUA(Pu)$ or $(Pu)_8AAC(Pu)_3$, as preferential binding sites for Unr (Triqueneaux et al, 1999). With the aim of mapping the Unr binding site on the HIV-1 IRES, we searched for the presence of such sequences in regions predicted to be single stranded within the minimal active pNL4.3 HIV-1 IRES sequence localized between nucleotides 104-336. We identified two sequences of this type, namely the $(Pu)_2(Py)(Pu)_2AAGUA(Pu)$ sequence at the level of nucleotides 205-215 and the $(Pu)_8AACA(Pu)_3$ sequence at the level of nucleotides 183-197.

Based on the SELEX analysis mentioned before, we designed two IRES mutants of nucleotides in positions 210-214 in order to reduce the binding affinity to Unr. To do so, we

selected for each position nucleotides which were never found in the RNAs selected as Unr partner in the SELEX selection i.e. C and U at positions 210, 211 and 214 or A and C at position 212 and finally G and A at position 213 (Triqueneaux et al, 1999). The mutations were inserted into the HIV-1 IRES cloned in the dual luciferase construct to give the mutU and mutC constructs (Table 1).

Clone	Sequence
Wild type sequence	210 AAGUA 214
mutU	210 **UUCAU** 214
mutC	210 **CCAGC** 214
mut211	210 A**G**GUA 214
mut212	210 AA**C**UA 214
mut213	210 AAG**A**A 214
mut214	210 AAGU**G** 214

Table 1: Mutations in the HIV-1 IRES sequence to identify Unr binding site

The two IRES mutants, mutU and mutC, tested in the dual luciferase assay showed a dramatic activity decrease of respectively, 75 and 84% in respect with the WT level, in the absence of Unr overexpression (Figure 3A). When Unr was overexpressed, both mutants became insensitive to Unr, while a 40% increase was observed for the WT IRES. This loss of sensitivity for the two IRES mutants to Unr overexpression suggests that the mutated 210-214 sequence may be involved in the Unr binding site. To exclude the possibility that the observed effect may be due to the destabilization of the IRES structure by the simultaneous mutation of residues 210-214, we generated a set of single point mutated IRESs referred to as mut211, mut212, mut213, and mut214 (Table 1). Such single point mutations were shown to not significantly destabilize the IRES structure (Gendron et al, 2011; Plank et al, 2013; Vallejos et al, 2012; Vallejos et al, 2011).

The single point mutants showed different levels of IRES activity and response to Unr. Mut211 exhibited a strong reduction (73%) in IRES activity and was not responsive to Unr overexpression (Figure 3B). A less important reduction in the IRES activity was observed for mut213 and mut214 with respectively, 52 and 75% of the WT activity. They also respond at

different levels to an Unr overexpression with an increase of 27 and 60%, respectively. Mut213 is thus even more responsive than the WT IRES to Unr. Interestingly, mut212 activity is stronger than the one of the WT IRES (44% increase in absence of Unr overexpresssion), while its response to Unr overexpression is close to the WT one (48% for mut212, as compared to 40% for the WT; Figure 3B).

Taken together, our data indicate that the 210-214 sequence plays an important role in the HIV-1 IRES activity and its regulation by Unr. It strongly suggests that this sequence corresponds to an Unr binding site and that Unr can modulate the HIV-1 IRES activity.

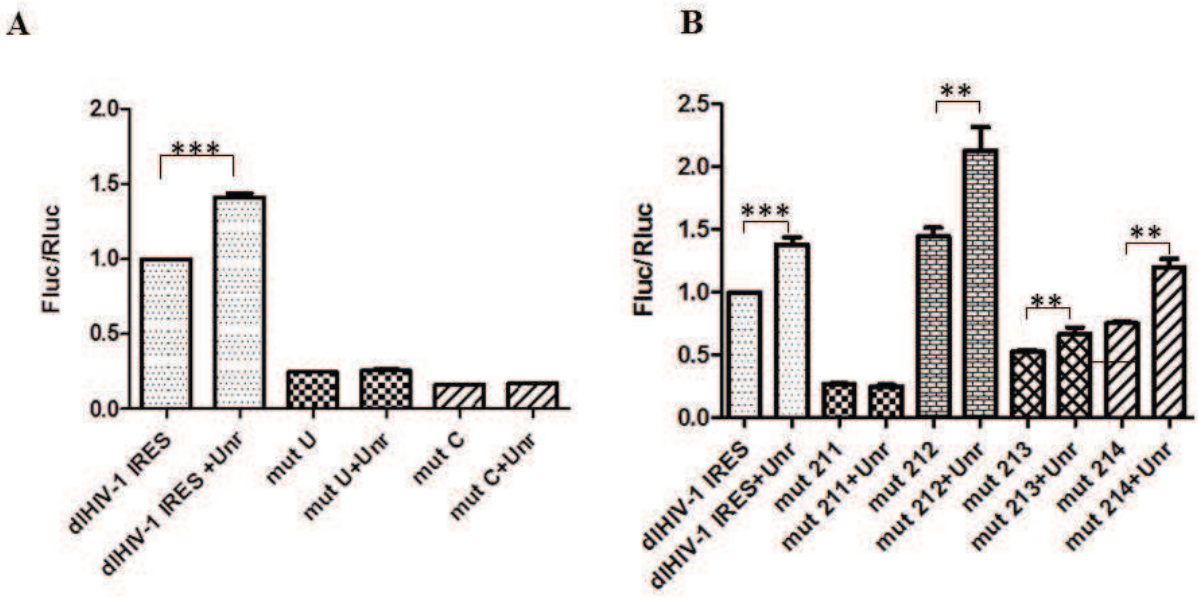


Figure 3: Determination the activities of mutated HIV-1 IRES with/ without Unr overexpression. (A) Activities of mutU and mutC IRES activities with/without Unr overexpression. **(B)** Activities of mut211, mut212, mut213, and mut214 with/ without Unr overexpression. The IRES activity is expressed by the ratio of Fluc/Rluc luciferase activities in the dual luciferase assay. Measurements were performed in HeLa cells, 24 hours post transfection. The ratios are normalized to the one of the WT HIV-1 IRES without Unr overexpression. Histograms represent the mean ± SEM of at least 3 independent experiments done in triplicate **, $p < 0.01$; ***, $p < 0.001$.

Opposite effects of Gag and NCp7 on Unr-promoted HIV-1 IRES activity.

As the HIV-1 5'-UTR harbors several functional domains including the primary gRNA packaging signal (Abd El-Wahab et al, 2014) and the primer binding site (PBS) (Wilkinson et al, 2008) which are targets of the NC domain of Gag and NCp7, we wondered whether the

HIV-1 IRES activity can be modulated by NCp7 or Gag. Therefore, we investigated the effect of NCp7 and Gag overexpression on the HIV-1 IRES activity with or without co-expression of Unr using our dual luciferase system. While NCp7 overexpression does not affect HIV-1 IRES activity, it counteracts the stimulating effect of Unr overexpression on this activity (Figure 4A). In contrast to NCp7, Gag overexpression increases the IRES activity by about 30%, close to the level of Unr stimulation. When both Gag and Unr are overexpressed, their effects on IRES activity are clearly additive, leading to a 60% increase with respect to the control without any overexpression (Figure 4B). As the main contributor of the nucleic acid binding activity of Gag is the NC domain, which is endowed with a potent nucleic acid chaperone activity, it could be hypothesized that the stimulatory effect of Gag on the IRES activity is driven by NC. We thus overexpressed a Gag mutant where the NC domain is deleted (GagΔNC) with the dual luciferase construct. In line with our hypothesis, this mutant only increased by 11% the HIV-1 IRES activity. The overexpression of Unr and GagΔNC gave the same HIV-1 IRES activity as in the presence of Unr alone, which is in agreement with the need of the NC domain for Gag stimulation effect.

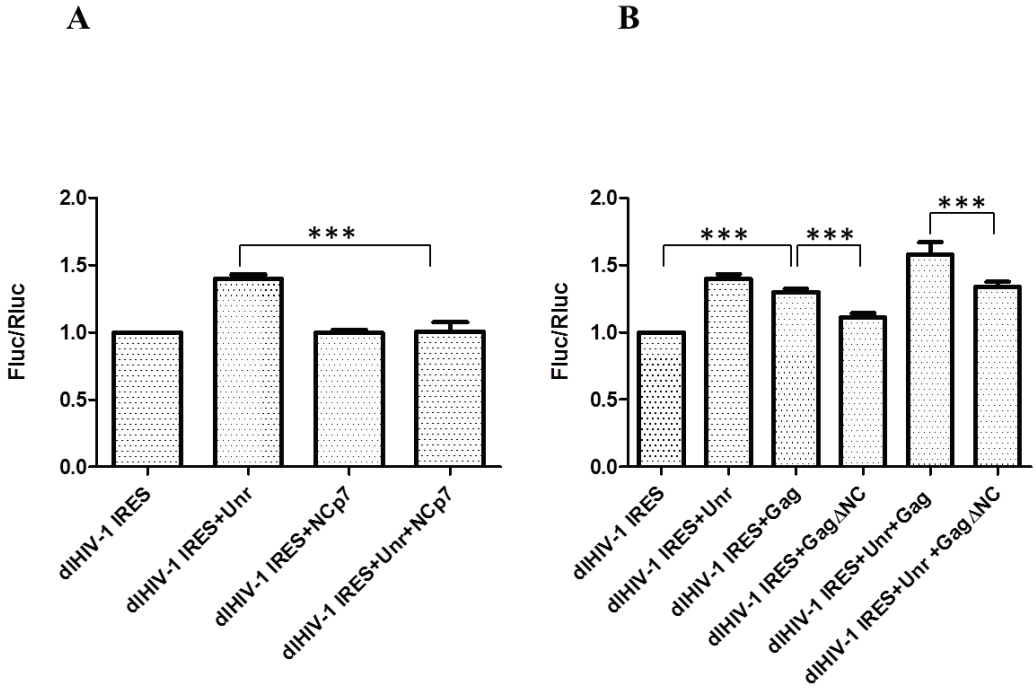


Figure 4 : Effect of NCp7 (A) and Gag or GagΔNC (B) on HIV-1 IRES activity with or without Unr overexpression. The IRES activity is expressed by the ratio of luciferase activities Fluc/Rluc in the dual luciferase assay. The measurements were performed in HeLa cells 24 hours post transfection. Histograms represent the mean ± SEM of at least 3 independent experiments done in triplicate **, $p < 0.01$; ***, $p < 0.001$.

NCp7/ Gag and Unr co-localize in the cytoplasm

Since we have demonstrated that Gag and NCp7 can modulate the Unr effect on HIV-1 IRES, and since Jäger et al. identified, by mass spectrometry, Unr as being part of affinity-purified protein complexes bound to strep-tagged NCp7 and Gag overexpressed in HEK cells (Jager et al, 2012), our next objective was to confirm the interaction between Unr and NCp7 and/or Gag. In order to observe the possible co-localization of NCp7/Gag and Unr, we transiently expressed mCherry-Unr with NCp7-eGFP or eGFP-Unr with Gag-mCherry in HeLa cells. The cellular distributions of the fusion proteins are in accordance with the known distributions of both proteins, with Unr being mainly cytoplasmic (Figure 5C) and NCp7 being found in all cell compartments with a preferential localization in the cytoplasm and the nucleoli (Figure 5A). Interestingly, a strong co-localization of Unr with NCp7 was observed (Figure 5-D3). In contrast, no strong colocalization was observed for Gag, which is rapidly transported to the membrane after its translation (Figure 5-E3). No change in localization of any of the proteins was detected upon expression (Figure 5).

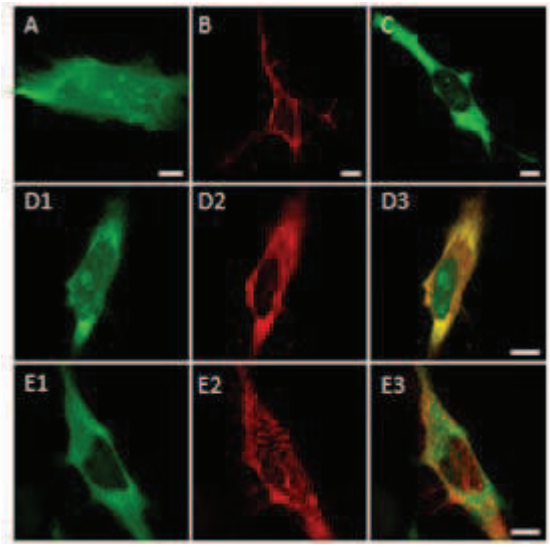


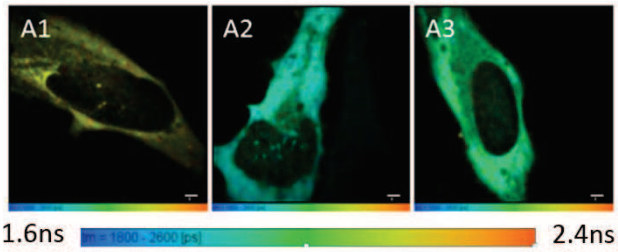
Figure 5: Colocalization of NCp7 and Gag with Unr. Intracellular distribution of NCp7-eGFP (A), Gag-mCherry (B), eGFP-Unr (C) in HeLa cells fixed with paraformaldehyde. Cells were observed by confocal microscopy 24 hours post transfection with constructs coding for each protein. D1 and D2 show NCp7-eGFP and Unr-mCherry respectively. E1 and E2 show eGFP-Unr and Gag-mCherry, respectively. D3 and E3 show the composite images of D1-D2 and E1-E2, respectively. The bar scale represents a distance of 10µm.

NCp7 interaction with Unr visualized by FRET-FLIM

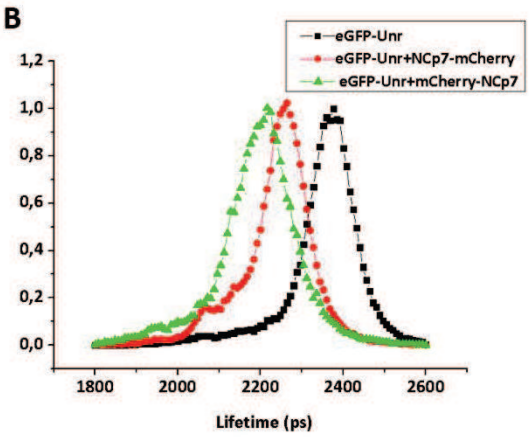
Since mCherry-Unr was seen to co-localize with NCp7-eGFP in fixed HeLa cells, we wondered if they could interact. To this end, we performed FRET-FLIM (Förster resonance energy transfer- Fluorescence Lifetime Imaging Microscopy) experiments on live cells co-expressing the two proteins of interest fused to eGFP and mCherry as fluorescence donor and acceptor, respectively. FRET between eGFP- and mCherry-labeled proteins only occurs when the two fluorophores are less than 8 nm apart, a distance corresponding to intermolecular protein–protein interactions (Bastiaens & Squire, 1999; Day et al, 2001; Voss et al, 2005). FRET can be unambiguously quantified from the fluorescence lifetimes of the eGFP-labeled proteins, measured at each pixel of the cell image, using the FLIM technique. Indeed, FRET resulted in a decrease in the eGFP lifetime that does not depend on the instrumentation or the concentration of the fluorophores, thus clearly demonstrating an interaction between the two proteins. As a control, cells expressing eGFP-Unr in the absence of NCp7 or Gag were imaged (Fig. 6A1). The fluorescence lifetime (τ) of eGFP-Unr, as indicated through a color code in figure 6A1, was found very homogeneous over the cell, with a value of 2.37 ± 0.02 ns, Figure 6B) which is very similar to that of free eGFP (2.39 ± 0.08 ns; Salah et al, 2015). Co-expression of eGFP-Unr with NCp7 labeled by mCherry at its N-terminus (mCherry-NCp7) or C-terminus (NCp7-mCherry) resulted in a significant decrease of the fluorescence lifetime, as shown by the color change in respect to the control (Fig. 6A2 and A3). This is even more visible when the eGFP-Unr lifetime distribution of all the measured pixels in 20 imaged cells is plotted (Fig. 6B), showing a significantly shifted distribution towards lower lifetime in the presence of mCherry labeled NCp7. The average lifetime values in the presence of NCp7-mCherry or mCherry-NCp7 are 2.25 ± 0.05 ns and 2.21 ± 0.04 ns respectively (Fig. 6C) giving average FRET values of 5.1 and 6.8 %. These percentages of FRET are above the 5% threshold, required to define a bona fide interaction between two partners (Voss TC et al, 2005), but are nevertheless rather low. One possible explanation to these low FRET percentages is that a significant amount of eGFP-Unr may not interact with NCp7 and exhibit thus a 2.4 ns lifetime that artificially increases the mean lifetime that is recovered using a mono-exponential model. To take into account the coexistence of bound and free eGFP-Unr populations, we analyzed the fluorescence decays with a two components model: $F(t)=\alpha_1 e^{-t/\tau 1}+\alpha_2 e^{-t/\tau 2}$ where the long-lived lifetime τ_2 was fixed at the lifetime of

eGFP-Unr expressed alone (2.37 ns), while the short component τ_1 and the populations (α_1 and α_2) associated with the two lifetimes were allowed to float. Using this bi-exponential fluorescence decay model, it appeared that 21 to 26 % of the eGFP-Unr proteins was able to FRET with NCp7-mCherry and mCherry-NCp7, respectively with a high percentage of FRET above 35%. This underlines that a limited fraction of Unr can interact with NCp7, bringing the two fluorophores close to each other.

A



B



C

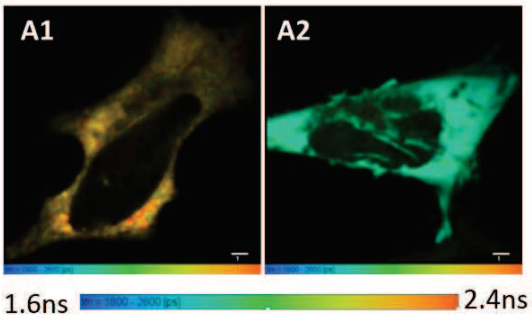
	eGFP-Unr	eGFP-Unr+ + NCp7-mCherry		eGFP-Unr + mCherry-NCp7	
Average lifetime (τ, ns)	2.37±0.02	One population[1]	Two populations[2]	One population	Two populations
		2.25 ± 0.05	1.46 ± 0.09	2.21 ± 0.04	1.52 ± 0.05
Amplitude (%)	----------	----------	21 ± 6	----------	26 ± 4
FRET Efficiency (%)	----------	5.1	38.5	6.8	35.7

Figure 6: Unr and NCp7 interaction monitored by FRET-FLIM. HeLa cells were transfected with DNA constructs coding eGFP-Unr **(A1)** with NCp7-mCherry **(A2)** or mCherry-NCp7 **(A3)**. Live cells were imaged 24h post-transfection. The eGFP fluorescence lifetime was determined using a single-exponential model and converted into a color code ranging from blue (1.6 ns) to red (2.4 ns). **(B)** Distributions of τ values expressed in ps for cells expressing eGFP-Unr alone (black); eGFP-Unr with NCp7-mCherry (red) or eGFP-Unr with mCherry-NCp7 (green). **(C)** Fluorescence lifetimes of eGFP-Unr, in the presence of NCp7-mCherry or mCherry-NCp7, analyzed by one or two populations analysis. The lifetimes are expressed, as mean ± SEM for about 20 cells. For the two population analysis, the long lifetime was fixed to 2.37 ns and the shorter lifetime as well as its amplitude were determined and expressed as mean ± SEM for about 20 cells. The lifetime of eGFP-Unr expressed alone was obtained through a one population analysis.

Gag interaction with Unr visualized by FRET-FLIM

Given that Gag and Unr partially colocalized in the cytoplasm, we conducted the same kind of FRET/FLIM experiments on live HeLa cells co-expressing the two proteins eGFP-Unr and GagG2A-mCherry as fluorescence donor and acceptor respectively. Because Gag is rapidly transported to the membrane shortly after its translation in the cytoplasm, we used to evidence the interaction with Unr, a previously published Gag-G2A (Glycine at position 2 mutated to Alanine) mutant lacking the N-terminus myristoylation motif responsible for the localization of Gag to the plasma membrane. This mutant shows a predominantly cytoplasmic localization associated to a low multimerization degree even if it preserved its full RNA binding capability. As before, the fluorescence lifetime of eGFP-Unr was monitored in HeLa cells without (Figure 7A1) or with GagG2A-mCherry co-expression (figure 7A2). A significant decrease in the fluorescence lifetime of Unr-eGFP was observed (figure 7B), when it was co-expressed with GagG2A-mCherry from 2.23 ±0.07 ns to 1.31 ± 0.04 ns with a single population analysis corresponding to a FRET efficiency of 7.5%. As for NCp7, a two populations analysis revealed that around 25% of the total Unr-eGFP population was implicated in an interaction with GagG2A corresponding to a strong FRET efficiency (46 %).

A



B

	eGFP-Unr	eGFP-Unr + Gag G2A-mCherry	
Average lifetime (τ, ns)	2.41±0.02	One population[1]	Two populations[2]
		2.23 ± 0.07	1.31 ± 0.04
Amplitude (%)	----------	----------	25.5 ± 4.5
FRET Efficiency (%)	----------	7.5	46.3

Figure 7: Unr and GagG2A interaction monitored by FRET-FLIM. HeLa cells were transfected with DNA constructs coding for eGFP-Unr **(A1)** with GagG2A-mCherry **(A2)**. Live cells were imaged 24h post-transfection. The eGFP fluorescence lifetimes were determined using a single-exponential model and converted into a color code ranging from blue (1.6 ns) to red (2.4 ns). **(B)** Fluorescence lifetimes of eGFP-Unr, in the presence of NCp7-mCherry or mCherry-NCp7, analyzed by one or two populations analysis. The lifetimes are expressed, as mean ± SEM for about 20 cells. For the two population analysis, the long lifetime was fixed to 2.41 ns and the shorter lifetime as well as its amplitude were determined and expressed as mean ± SEM for about 20 cells.

NCp7 and Gag interaction with Unr monitored by co-immunoprecipitation

To confirm the interaction between Unr and Gag/NCp7, we performed co-immunoprecipitations (co-IP) on 293T cells co-transfected with constructs coding for Flag-Unr and NCp7-eGFP. The immunoprecipitates were analyzed by western blot. All the fusion proteins were well overexpressed as observed in the input lanes 1 to 5 in figure 8A. As Flag-Unr is specifically immunoprecipitated by the anti-Flag antibody (in figure 8A compare lanes 8 and 9 with lane 10); the presence of NCp7-eGFP in the co-IP complex with Flag-Unr (compare lane 9 with lane 8) confirms that Flag-Unr and NCp7-eGFP interact specifically. The interaction between NCp7 and Unr was further confirmed by the co-IP of eGFP-Unr with Flag-NCp7 using an anti-eGFP antibody (data not shown). Moreover, we also used a synthetic NCp7 biotinylated at its N-terminus (Biotin-NCp7) and fixed it on streptavidin beads before addition of 293T cell lysates overexpressing Flag-Unr protein (Fig 8B). A western blot using an anti-Flag antibody confirmed the good expression of Flag-Unr (Fig 8B lanes 2 and 4) and the specific presence of Unr only in the precipitates with streptavidin beads covered by Biotin-NCp7. The RNA dependent character of the NCp7 interaction was also shown by co-IP experiments (data not shown).

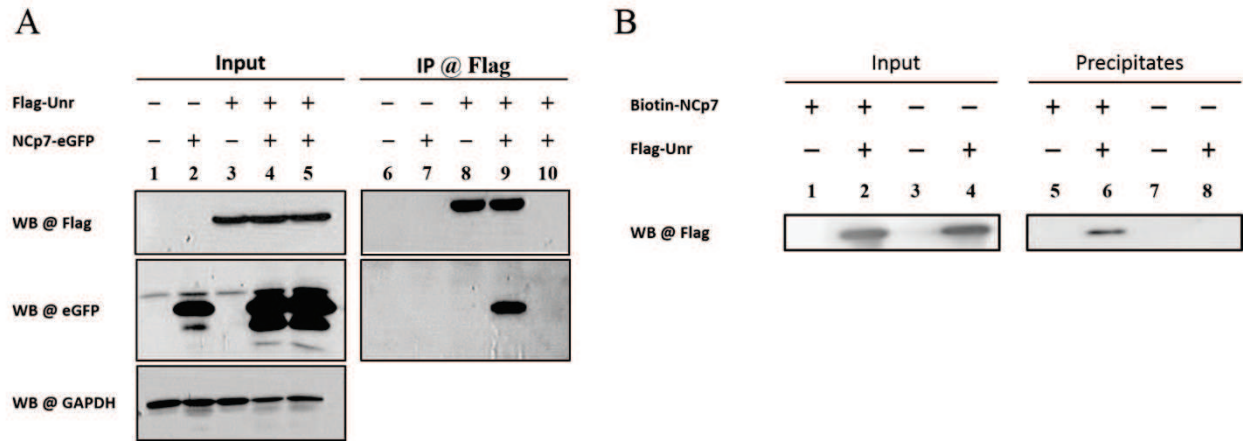


Figure 8: Co-immunoprecipitation of NCp7 and Unr. (A) Co-immunoprecipitation of NC-eGFP with Flag-Unr. 293T cell lysates expressing diverse combinations of proteins of interest after transient transfection were subjected to immunoprecipitation with protein A beads linked to an anti-Flag antibody. The immunoprecipitates were analyzed by acrylamide gel SDS-PAGE and revealed by Western blotting, using anti-eGFP and anti-Flag antibodies diluted to 1/10000 and 1/4000 respectively. Homogenous loadings were checked using a western blot against GAPDH (antibody diluted to 1/5000) **(B)** Co-precipitation of Flag-Unr with biotinylated NCp7 bound to streptavidin beads. 293T cells were transfected with an empty plasmid or a plasmid coding for Flag-Unr. 48 hours post transfection, cell lysates were added to streptavidin beads alone or streptavidin beads conjugated with biotinylated NC.

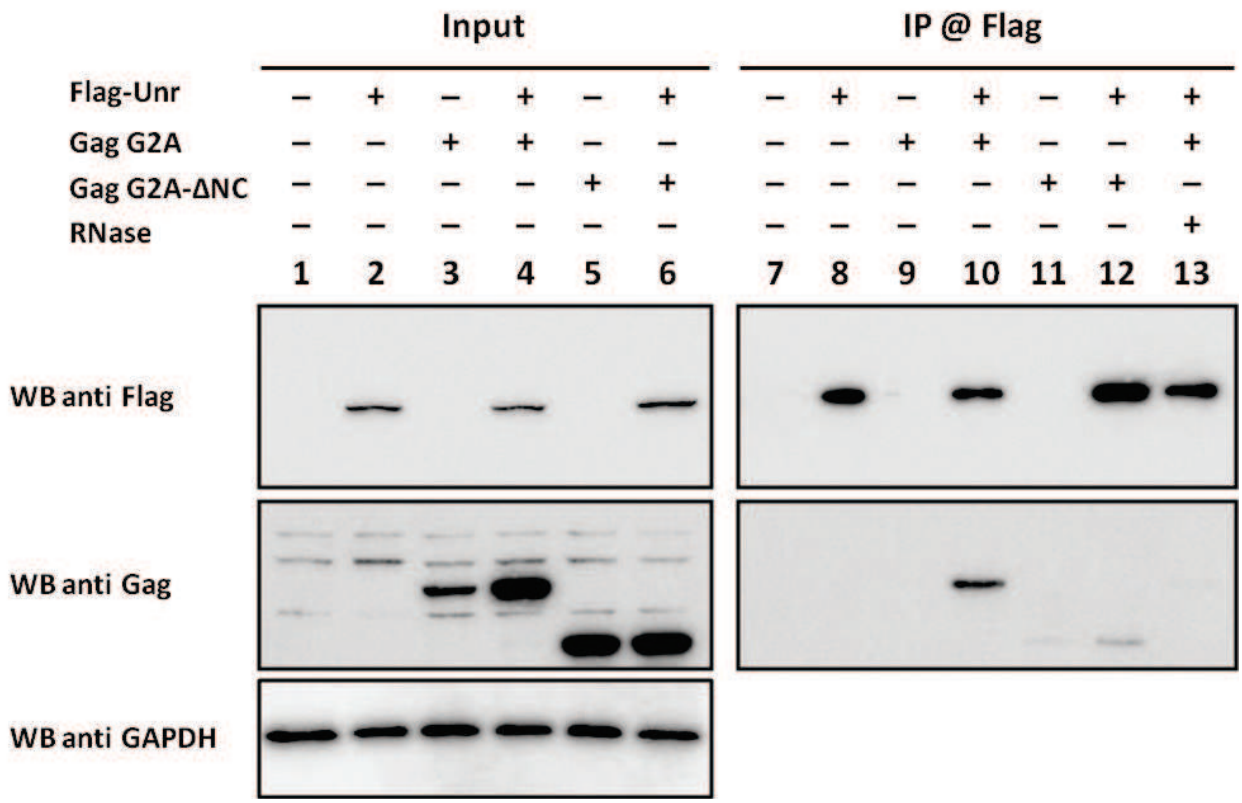


Figure 9: Co-immunoprecipitation of GagG2A with Flag-Unr. 293T cell lysates expressing proteins of interest after transient transfection were subjected to immunoprecipitation with anti-Flag antibody (diluted to 1/4000) conjugated to protein A beads. The immunoprecipitates were analyzed by acrylamide gel SDS-PAGE and revealed by Western blotting, using anti-Gag antibody (diluted to 1/10000). Homogenous loadings were checked using a western blot against GAPDH (diluted to 1/5000).

As we measured an interaction by FRET/FLIM between GagG2A and Unr, we next validated this interaction by co-IP experiments. HeLa cells were transfected with different combinations of constructs coding for Flag-Unr, GagG2A or a GagG2A mutant with a deleted NC domain (GagG2A-ΔNC). All the proteins were correctly expressed as revealed by western blot on input cell lysates (Figure 9 lanes 1 to 6). The lysates were subjected to an immunoprecipitation with an anti-Flag antibody able to imunoprecipitate specifically Flag-Unr (Figure 9 lanes 8, 10 12 and 13). GagG2A specifically co-immunoprecipitated only when Flag-Unr was co-expressed (compare figure 9 lanes 10 with lane 9). In contrast, only a weak co-IP was observed when the NC domain of GagG2A was deleted (Figure 9 lane 12), while no co-IP was observed when a lysate expressing GagG2A and Flag-Unr was treated with RNase before the co-IP (Figure 9 lane 13).

Altogether, these results confirm the interaction of both NCp7 and Gag with Unr. The Gag/Unr interaction is RNA dependent and to a large extent mediated by the NC domain.

Unr knockdown reduces infection by an HIV-1 lentivector.

To analyze the effect of Unr protein on HIV-1 viral infection, we used, as a model of the early stages of infection (post-entry to the integration), a non-replicative lentivirus pseudotyped with the VSV (Vesicular Stomatitis Virus) glycoprotein. In this lentivector genome, the viral genes were replaced by a CMV promoter driving the synthesis of firefly luciferase mRNA, the level of cells infection could be monitored by measuring the enzymatic Fluc activity or the quantity of mRNA coding Fluc in the infected cells. As Unr is part of the cellular translation machinery and the production of firefly luciferase protein could be influenced by Unr knockdown, we quantified the Fluc mRNA by RT-qPCR and normalized it to that of the 18S housekeeping gene mRNA. HeLa cells were infected 48 hours after transfection with either a control siRNA or siRNA against Unr. The reverse-transcriptase inhibitor AZT (zidovudine) was used as a positive control. In comparison to non-treated cells or cells treated with control siRNA, we observed a 30% decrease in the infection after treatment with a siRNA against Unr. This decrease has to be compared with the 70% decrease observed when AZT was used (Figure 10A). The proper Unr knockdown was verified by western blot in comparison to GAPDH (Figure 10B). Since this model only mimics the early phase of infection, the decrease on infection upon Unr knockdown suggests that Unr is important in the early phase of cell infection.

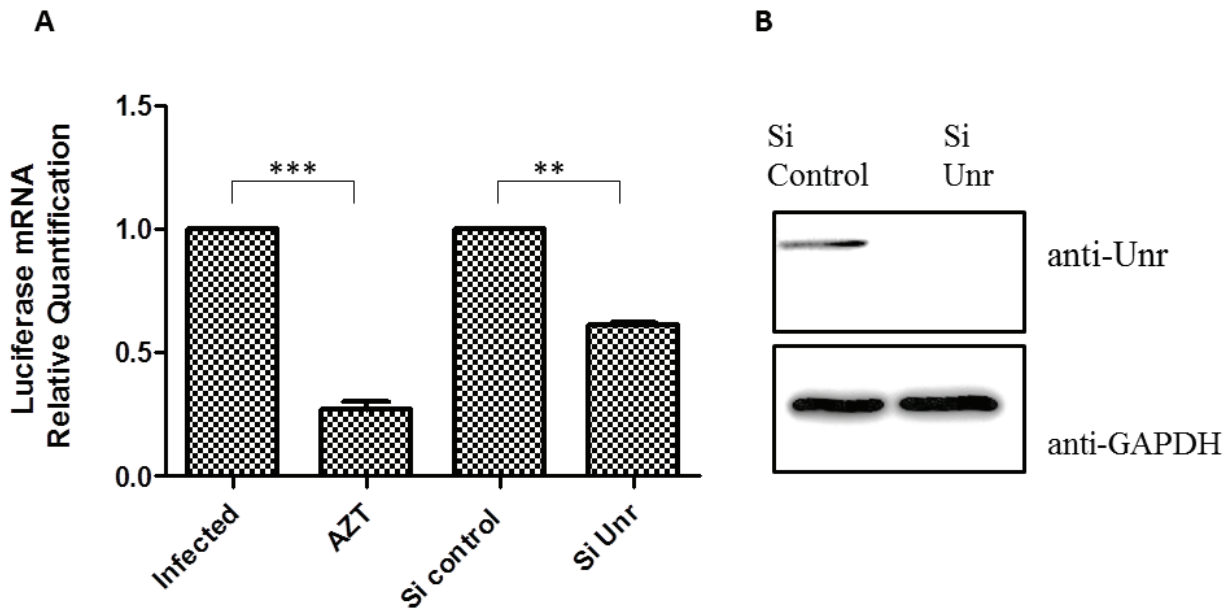


Figure 10: Unr knockdown inhibition of infection. (A) Firefly luciferase mRNA quantification by RT-qPCR after infection of HeLa cells with firefly-coding HIV-1 pseudoparticles. HeLa cells were non treated, treated with AZT, control siRNA, or siRNA against Unr before infection. The graph shows the quantification of the luciferase mRNA relative to the mRNA of the housekeeping gene 18S. The quantification was realized using the delta Ct quantification method. Histograms represent the mean ± SEM of at least 3 independent experiments done in triplicate **, $p < 0.01$; ***, $p < 0.001$. **(B)** Control of Unr knockdown after siRNA treatment. Infections were done 48 hours after siRNA transfection using either a control siRNA or a siRNA against Unr. Cell lysates were subjected to a SDS-PAGE and a western blot against Unr (antibody diluted to 1/1000) or GAPDH (antibody diluted to 1/5000) as a loading control, 48 hours after infection.

Discussion

Since Unr has been implicated in the IRES-mediated translation of Poliovirus and Rhinovirus in addition to cellular IRESs (Boussadia et al, 2003; Brown & Jackson, 2004; Mitchell et al, 2003; Tinton et al, 2005), we assumed in this paper that Unr could also be implicated in the HIV-1 IRES dependent translation. In fact, the HIV-1 5'-UTR bears two IRESs, one in the 5'-UTR found in the gRNA and all viral mRNAs and a second one in the Gag coding region which drives the synthesis of Gagp55 and a 40 kDa truncated form of Gag. Since the HIV-1 IRES was shown to be not functional in RRL unless supplemented with cellular extracts especially from G2/M blocked cells, it was assumed to be regulated by cellular factors. Among the factors reported to increase the HIV-1 IRES activity are the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), eIF5A and the Rev co factors DDX3 and hRIP (Liu et al, 2011; Monette et al, 2009). However, the regulatory mechanism is likely to differ among those factors.

In this study, we used a dual luciferase system based on cell transfection with a bicistronic construct in which the firefly luciferase and the renilla luciferase expressions are under the control of the cap-dependent and the HIV-1 IRES translation respectively. We showed for the first time that Unr is an ITAF having a stimulatory action on the activity of the HIV-1 IRES. Moreover, we identified a single stranded region between nucleotides 205-215 of the 5'-UTR, as a binding site of HIV-1 IRES. This region is located in the bottom of the PBS loop and close to the NC binding sites (Levin et al, 2010; Wilkinson et al, 2008). Mutations in this sequence greatly affected the IRES activity. This decrease is most likely not linked to a loss of the proper folding of the HIV-1 IRES as this IRES was shown to be highly resistant to mutations and deletions (Gendron et al, 2011; Plank et al, 2013; Vallejos et al, 2012; Vallejos et al, 2011). Three out of the four point mutations (mut 211, 213 and 214) presented a decrease in the IRES activity and response to Unr, with mut211 being totally inactive and non-responsive to Unr overexpression. On the other hand, mut212 was more active than the WT and showed a more sensitive response to Unr overexpression. The functional implication of this sequence is in line with a previous study (Gendron et al, 2011), which identified the sequence 202-217, as being able to increase the IRES activity. Furthermore, the SHAPE reactivity of nucleotides 211-213 was shown to be modified upon aldrithiol-2 (AT-2) treatment, compromising the NC-RNA interaction. Therefore, this region is not only implicated in IRES activity but is also structurally modified by NC binding. This is in line with the fact that NCp7, in our dual-luciferase assay, does not present any stimulatory effect and even counteracts the Unr action. In this context, it would be highly interesting to characterize the structures of both the wild-type and the mutant IRES structures by SHAPE and determine the Unr binding affinity to these mutants in comparison to the WT IRES.

Interestingly, Gag was observed to behave differently to NCp7 in our assay, showing a stimulatory effect on the HIV-1 IRES, which is additive to that of Unr. This effect of Gag is in line with the ability of this protein to stimulate its own translation when expressed at low concentration (Anderson et al., 2006). To our knowledge, the contribution of the IRES-dependent translation on the effect of Gag on its own translation was not deciphered. However, this stimulatory effect was attributed to the matrix domain rather than the NC domain (Anderson & Lever, 2006). The IRES stimulation observed with Gag in contrary to NCp7 as well as the stimulatory effect of Gag on its own translation could be tentatively

explained by the differences in the nucleic acid chaperone properties of the two proteins, especially at low protein concentrations (Jones et al, 2011; Roldan et al, 2005; Wu et al, 2010). Using FRET-FLIM and co-immunoprecipitation experiments, we clearly evidenced the interaction of both mature NCp7 and Gag with Unr when overexpressed, confirming the physical interaction between these proteins predicted by Jäger et al. (Jager et al, 2012) . We further showed that this interaction is RNA dependent and that, in the case of Gag, the interaction is mainly mediated by its NC domain. The RNA dependence of the interaction is consistent with the fact that Unr (Hunt et al, 1999; Triqueneaux et al, 1999) as well as NCp7 and Gag (Athavale et al, 2010; Fisher et al, 1998) are RNA binding proteins and that other NC interactions with host proteins were reported to be RNA dependent (Burnett & Spearman, 2007).

The functional implication of Unr in HIV-1 infection was tested using an infection model based on nonreplicative lentiviral VSV- pseudotyped particles which mimic the early phase of the infection (entry to integration). Unr knockdown induced a 30% decrease of the infection suggesting that Unr is implicated in the early phase of the life cycle, most probably in the decapsidation, reverse transcription, or integration step. This mechanism is unknown; however, one possibility is that Unr associates with the nucleocapsid protein or the RNA to facilitate, via its potential chaperone activity, the reverse transcription in the cytoplasm.

In our model, in the early phase, when vRNA translation is not necessary, NCp7 protects the gRNA from Unr IRES stimulation effect preserving the gRNA for reverse transcription. In this phase however, Unr is necessary for an optimal infection. This role could be linked to the chaperone activity of Unr as well as to the proximity of the Unr binding sites to the PBS binding site on the HIV-1 5'UTR. In the late phase, since HIV-1 depends on both cap dependent and IRES dependent translation to translate viral proteins during infection as evidenced by Amorim et al. (2014), it could be possible that in the early hours of infection, translation proceeds by cap dependent translation allowing synthesis of sufficient Gag protein to activate the IRES dependent translation when the cap dependent translation is shut off by a G2/M block. This block has the consequence of increasing Unr translation which could thus, with the help of Gag stimulate the translation of most of the viral messenger RNA which all harbor HIV-1 5'UTR IRES.

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Supplementary Data

In this chapter I will present the extra work that is not included into an article because the results were not pursued further or were not finalized at the time of writing of this manuscript.

1-Proteins tested for interaction with NCp7

When I arrived to the laboratory, I started to work on BAP-1, an ubiquitin hydrolase protein that was identified as a cellular partner of Gag and NCp7, based on a yeast two-hybrid screen of a human cDNA library obtained from mRNA expressed in a CEM lymphocyte cell line (Richard Benarous). Indeed, in this two-hybrid screen, 10 independent cDNA clones coding BAP-1 were obtained when NCp7 was used as bait whereas one clone was obtained when Gag was used as bait. Thus, during my first year of PhD, I concentrated my work on this potential hit and cloned BAP-1 in different constructs in an attempt to validate its interaction with NCp7 and Gag. Both with co-immunoprecipitation and FRET-FLIM experiments, I failed to validate it.

Thus, I turned my focus on other recently published studies which performed genome-wide RNA interference based screening or affinity tagging coupled to mass spectrometry in order to identify cellular proteins influencing HIV-1 infection or interacting with viral proteins, respectively (Jäger et al. 2011; König et al. 2008; H Zhou et al. 2008; Brass et al. 2008). From these lists, we carefully selected proteins which may interact with a high probability with NCp7 (high MiST score in Jäger study). As selection criteria for these proteins, we used i/ their presence in the lists of NCp7 partners; ii/ their identification as proteins influencing HIV-1 infection in the siRNA studies; iii/ their presence in the virion (Ott 2008; Chertova et al. 2006); iv/ their cellular localization looking for a possible co-localization with NCp7 and v/ the functional cellular pathway in which they are implicated. We selected 16 proteins (Table S1), based on these criteria.

From the different constructs we received from specialists of these different proteins, we cloned the targeted cDNA into different vectors in order to obtain several fusion constructs (eGFP, Flag, mCherry) to be used in microscopy and biochemical studies. After testing these

proteins by confocal microscopy for their co-localization with NCp7, I looked for an interaction with NCp7 using FRET-FLIM and/or co-immunoprecipitation. It is worth to note that we focused on NCp7 more than on Gag since we were particularly interested in the early phase of the infection and on the roles of NCp7 as a mature protein. The tested proteins and the results of these experiments are listed in the table below:

Protein		**Colocalization with NCp7**	**Co-IP detected**
SAP 49	Splicing factor 3b, Subunit 4, 49Kd	No	ND
STRAP	Human protein threonine kinase receptor associated protein	**Yes**	ND
Unr	Upstream of N-ras	**Yes** (Cytoplasm)	**Yes**
Nol 12	Human Nucleolar protein 12	**Yes** (Nucleolus)	**Yes**
PRMT1	Protein arginine methyl transferase 1	**Yes** (Cytoplasm)	ND
Sam 68	GAP-associated tyrosine phosphoprotein p62 (Sam68)	No	ND
RNH1	Ribonuclease/Angiongenin Inhibitor 1	**Yes** (Cytoplasm)	ND
MCM3	Minichromosome Maintenance Complex Component 3	No	ND
EXOSC10	Exosome component 10	**Yes** (Nucleolus)	NT
Ap4	Adaptor protein-4 complex	No	ND
NuSAP	Nucleolar and spindle associated protein 1	**Yes** (Nucleolus)	**Yes**
SHP	Small heterodimer partner	No	ND
RanBP8	Ran binding protein 8	**Yes** (Cytoplasm)	ND
Nup 62	Nucleoporin protein 62	No	ND
Nup 153	Nucleoporin protein 153	No	ND
Nup 214	Nucleoporin protein 214	No	ND

Table S1: List of tested proteins. Results of co-localization and co-immunoprecipitation experiments with NCp7 are indicated. ND: not detected NT: not tested

Among the tested proteins, six co-localized with NCp7. These proteins were RNH1, NuSAP. Nol12, PRMT1, Strap, and RanBP8. The localization of these proteins and their co-localization with NCp7 is shown in Figure S1. Nol12 and NuSAP colocalized with NCp7 in the nucleolus (Figure S1-C2 and C3) whereas RNH1, PRMT1, Strap, and RanBP8 colocalized with NCp7 in the cytoplasm (Figure S1-C1, C4, C5, and C6).

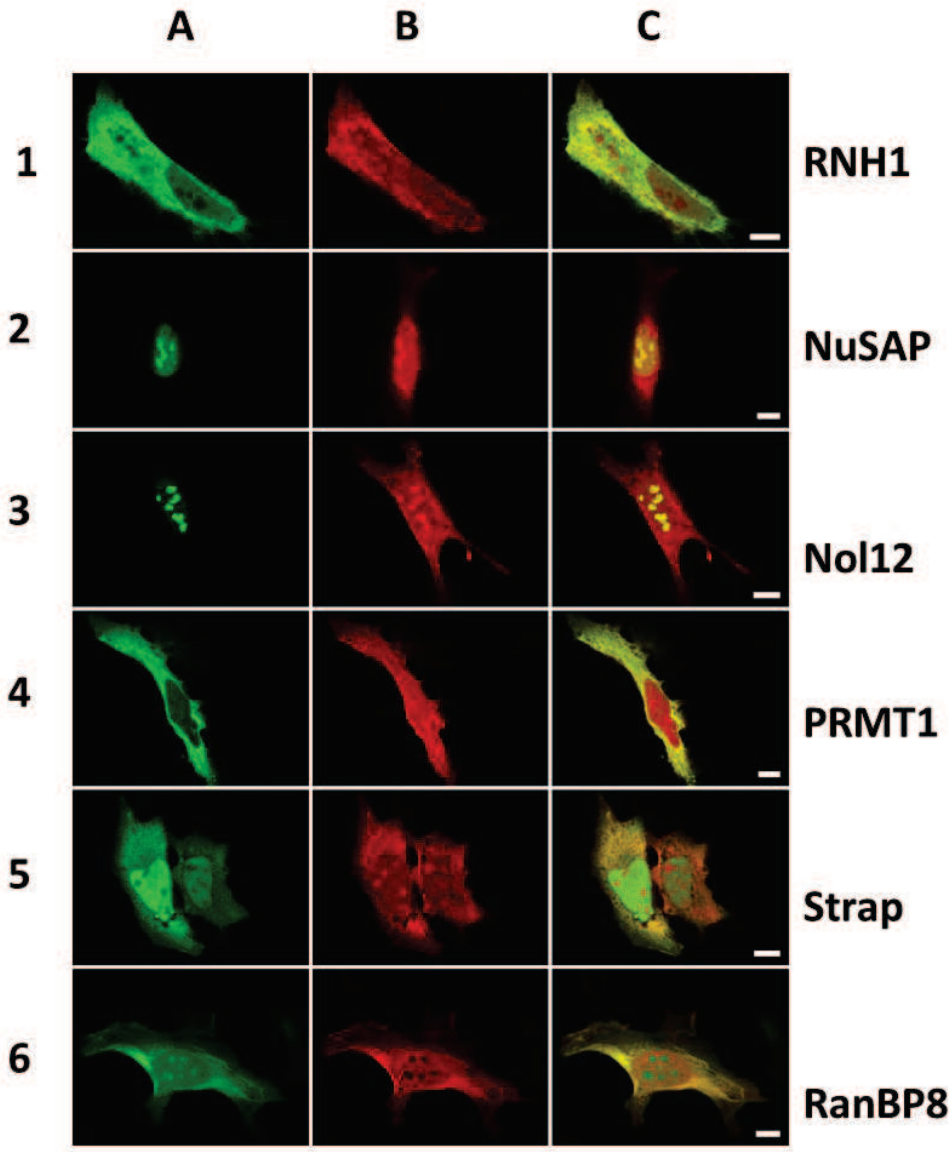

Figure S1: **Co-localization of NCp7 with selected proteins.** HeLa cells were transfected with constructs coding for the mentioned proteins fused to eGFP or to mCherry, fixed by PFA, and imaged by confocal microscopy 24 hours post-transfection. **A1-A5** show the localization of RNH1, Nol 12, NuSAP, PRMT1, and Strap proteins fused to eGFP. **B1-B5** show the localization of mCherry-NCp7. **C1-C5** show the composite images of their corresponding A and B images. **A6** shows the localization of NCp7-eGFP, **B6** shows the localization of RanBP8-mCherry. **C6** is the composite image of A6 and B6. The scale bar corresponds to 10µm.

Among all the tested proteins, I was able to validate by co-immunoprecipitation the interaction of three proteins with NCp7, namely i) the Upstream of N-ras (Unr), ii) the Nucleolar protein 12 (Nol 12), and iii) the Nucleolar and spindle associated protein-1 (NuSAP). My colleague Sarwat Zgheib is currently investigating the interaction of NCp7 with Nol12 in her thesis project. The co-immunoprecipitation of NuSAP and Nol 12 with NCp7 is shown in the Figure below.

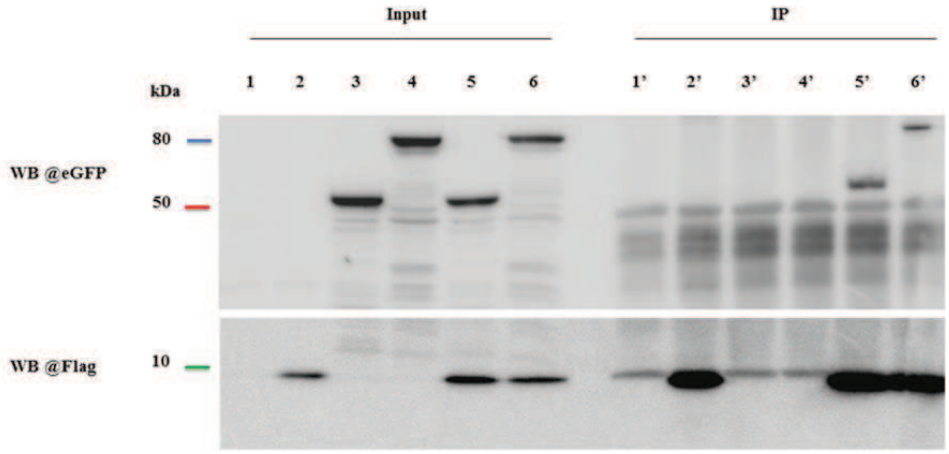

Figure S2: Co-immmunoprecipitation of eGFP-Nol12 and eGFP-NuSAP with Flag-NCp7. 293T cell lysates expressing proteins of interest after transient transfection for 24 hours were subjected to immunoprecipitation. The immunoprecipitates were analyzed by acrylamide gel SDS-PAGE and revealed by Western blotting. Input lanes correspond to the following cell lysates: lane 1: non transfected, 2: Flag-NCp7, 3: eGFP-Nol12, 4:eGFP-NuSAP, 5: eGFP-Nol12 + Flag-NCp7, 6: eGFP-NuSAP + Flag-NCp7. Lanes 1' to 6' correspond to the immunoprecipitates of the input samples (1 to 6 respectively) with anti-Flag coupled to protein A sepharose beads. Lanes 5' and 6' indicate the coimmunoprecipitation of eGFP-Nol12 and eGFP-NuSAP respectively with Flag-NCp7

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2-Complementary results on Unr and NCp7/Gag interaction

2.1-NCp7 interaction with endogenous Unr

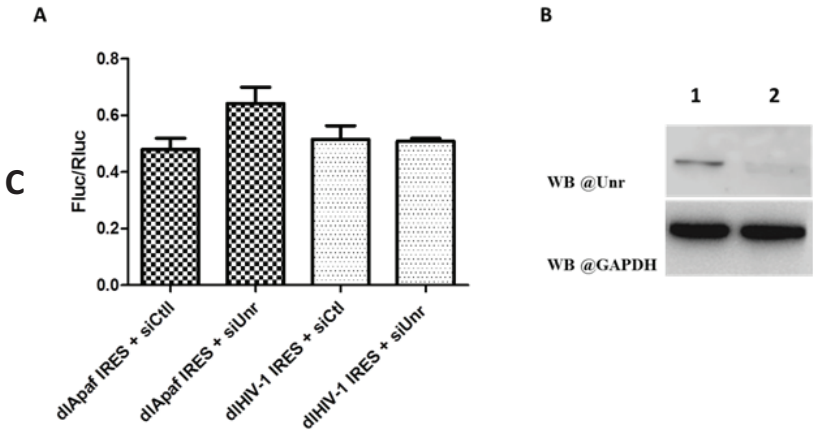
As I presented earlier in the previous article, I validated by co-immunoprecipitation and FRET FLIM, the interaction between Unr and NCp7 or Gag after transient overexpression in cells. It would have been interesting to further validate this interaction by showing the interaction between NCp7 or Gag with the endogenous Unr. I attempted to perform this by immunoprecipitating tagged NCp7 and looking for the co-immunoprecipitated endogenous Unr by western blot. However, I was not able to detect any Unr in the co-immunoprecipitated complex. This is certainly due to the low affinity of the available antibodies. In fact, Unr was only weakly detected by western blot in the input and thus, even if there is a co-immunoprecipitation, the amount of co-immunoprecipitated protein could be below the antibody detection level. In an attempt to overcome this obstacle, I tried to immunoprecipitate endogenous Unr and look for NCp7, but the antibody was not capable of immunoprecipitating endogenous Unr. Furthermore, considering that the interaction could be weak, I tried to crosslink endogenous Unr with NCp7 in cell lysates using dithiobis(succinimidyl propionate) with no success. Additionally, as Unr is known to be overexpressed in G2/M, I tried to block the cells in G2/M by overexpressing the viral protein Vpr or by a nocodazole treatment. Even if cells were blocked in G2/M as checked by FACS using propidium iodide, the level of endogenous Unr detected was not increased and no coimmunoprecipitation was detected.

2.2-Effect of Unr knockdown on IRES activity

As we discovered that Unr increases IRES activity, we wondered whether Unr knockdown could influence or diminish IRES activity in the cell. Therefore, we knocked down Unr in HeLa cells by transfecting siRNA against Unr and looked for HIV-1 IRES activity in the dual luciferase assay. When we checked for siRNA efficiency, we were not able to detect any Unr after siRNA transfection (Figure S3-B), but as the antibody against Unr is weak, it is possible that the Unr siRNA-transfected cells still contain a sufficient amount of Unr to induce IRES activity. This residual amount of Unr is quite likely, because HeLa cells are known to express Unr at an important level. In this context, the Fluc/Rluc ratio was not affected but there was a decrease in both Firefly and Renilla activities (Figure S3-A and C). This shows an effect of

the Unr knockdown on both the cap- and the IRES-dependent translation or a general toxicity of the knockdown. It is also possible that other cellular factors can substitute for Unr, in the condition of its knockdown, on IRES.

A problem linked to a weak knockdown or a cellular toxicity induced by Unr knockdown is supported by the observation of this knockdown on the Apaf-1 IRES activity. Since Unr is known to be an ITAF for the Apaf-1 IRES, we also attempted to silence Unr expression and looked at the Apaf-1 IRES activity in HeLa cells. Similar to HIV-1 IRES, Unr knockdown did not affect Apaf-1 IRES activity but decreased both Firefly and Renilla luciferase activities (Figure S3-A and C). Even if a weak increase could be seen, this is not statistically significant (Figure S3-A). Similar to HIV IRES, Unr knockdown decreased both Firefly and Renilla luciferase activities with Apaf-1 IRES (Figure S3-C).



C

IRES	siRNA	Firefly luciferase RLU	Renilla Luciferase RLU
HIV-1 IRES	Ctl	36380	69430
HIV-1 IRES	Unr	9713	19137
Apaf IRES	Ctl	30583	62719
Apaf IRES	Unr	10368	16859

Figure S3: **Unr knockdown effect on HIV-1 IRES activity. A**. Ratio of FLuc/RLuc activities obtained in the dual luciferase assay for Apaf-1 and HIV-1 IRESs with Unr knockdown by siRNA. Twenty four hours after siUnr or siCtl transfection in HeLa cells, cells were transfected with dlHIV-1 IRES or dlApaf IRES. Luciferase activities measurements were performed 24 hours post transfection. **B**. Unr detection in HeLa cell lysates after Unr knockdown. HeLa cell lysates were subjected to an SDS-PAGE analysis coupled to an anti-Unr or GAPDH western blot 24 hours after transfection with an siRNA control (lane 1) or an SiRNA against Unr (lane 2) **C**. Firefly and Renilla luciferase activities obtained in the dual luciferase assay realised as in A. The values are expressed as Relative Luminescence Unit (RLU).

2.3-Unrip, PTB, and La effect on HIV-1 IRES

When Unr was identified as a stimulating ITAF of the poliovirus IRES, it was identified in a HeLA cell lysate fraction. In this fraction, another protein was purified with Unr: Unrip. However, up till today, no data showed that Unrip is implicated in the IRES mediated translation regulation. We seeked to study Unrip's influence on the HIV-1 IRES activity. Our data show that Unrip also increases the IRES activity and that overexpression of Unr and Unrip increases IRES activity in an additive manner (Figure S4). This was not put in the article as this result needs further validation.

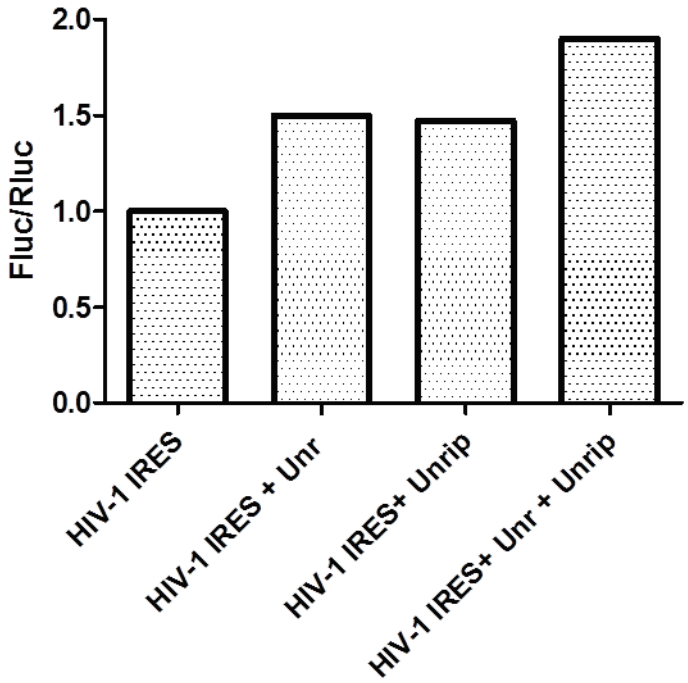


Figure S4: Effect of Unrip overexpression on the activity of dlHIV-1 IRES. The IRES activity is expressed by the ratio of Fluc/Rluc luciferase activities in the dual luciferase assay. Measurements were performed in HeLa cells 24 hours post transfection with the dual construct and constructs coding Unrip or Unr.

As PTB and La are among the classical ITAFs known to function on different cellular and viral IRESs, and Unr was reported to function in synergy with PTB for some of them, we tried to look for any effect of PTB or La on HIV-1 IRES activity. Neither La nor PTB seem to

affect HIV-1 IRES activity when overexpressed alone (Figure S5); however, it would be interesting to look at their effect when overexpressed with Unr.

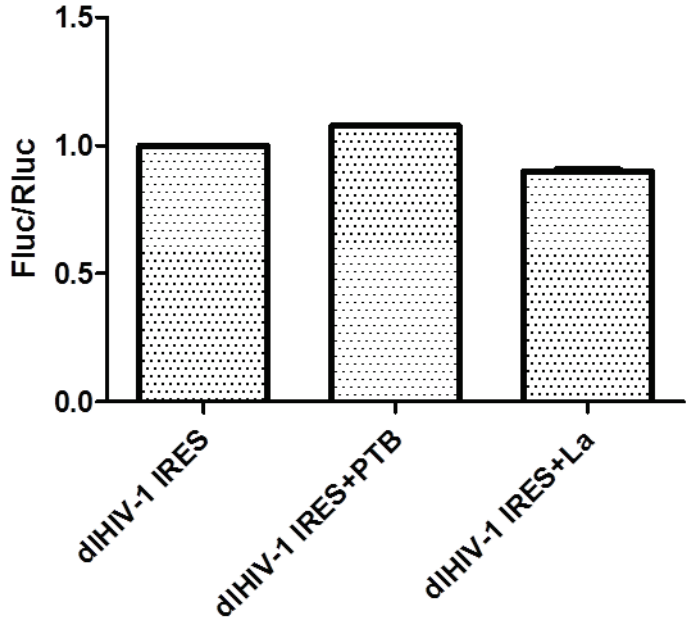


Figure S5: Effect of PTB and La overexpression on the activity of dlHIV-1 IRES. The IRES activity is expressed by the ratio of Fluc/Rluc of luciferase activities in the dual luciferase assay. Measurements were performed in HeLa cells 24 hours post transfection with the dual luciferase construct and the constructs coding the mentioned proteins.

2.4-Unr binding to the HIV-1 IRES RNA

Since Unr is an ITAF for HIV-1 IRES, Unr is expected to bind the HIV-1 5'-UTR region where the IRES is located. We expected that Unr could bind to the wild type HIV-1 IRES but not the 211 mutant which dramatically decreases the IRES activity (see article). To show this, we performed an electrophoretic mobility shift assay using Unr protein purified from bacterial cell lysates and IRES RNAs obtained after *in vitro* transcription (HIV-1, mut211, and Apaf IRES as a positive control).

Protein purification

Unr was cloned into a His- tag vector. The His-tagged Unr protein was expressed in *E.coli* BL21. Different protocols were tried for Unr purification including Nickel affinity batch and column purifications. We tried to denature and re-fold the protein by Urea. In all our attempts, the protein was eluted; however, with a numberof contaminants. With one step purification protocols, we were never able to get a sufficiently pure protein for our assays. We tried different second step purifications in our lab including ion exchange chromatography and size exclusion chromatography but it was not successful because the protein was either lost due to aggregation or purified at a very low quantity.

Then, we seeked help from the platform at IGBMC specialized in protein purification. The protein was purified using nickel column and eluted by an imidazole gradient. Even after extensive washes, the eluted protein was not pure. Thus, gel filtration was performed as a second step. The eluted protein was pure, but the protein concentration was very low. Due to its aggregation propensity, the protein was difficult to concentrate to a working concentration. Optimization of the protein purification protocol is still needed to get a good concentration of a pure non aggregated protein.

RNA synthesis

The dlHIV-1 IRES, mut 211 IRES, and the dlApaf-1 IRESs were cloned into pcDNA3.1 vector bearing T7 promoter. *In vitro* RNA transcription and purification was done at the Institut de Biologie Moleculaire et Cellulaire in Strasbourg with the help of Dr. Serena Bernacchi. RNA was then biotinylated with a 5'-end biotinylation kit (Vector Lab) according to the manufacturer's instructions. RNA was stored ready for use in electrophoretic shift binding assay (EMSA) using the Chemiluminescent RNA EMSA Kit.

EMSA

The Chemiluminescent RNA EMSA Kit optimization with the control supplied with the kit was used. The kit works in our hands nicely with the supplied controls. The kit will be used in a close future to check Unr binding to the HIV-1 IRES RNA.

Part II: Investigating the Cellular Distribution and Interactions of the HIV-1 Nucleocapsid Protein by Quantitative Fluorescence Microscopy

As mentioned earlier, although a lot is known about the role of NCp7 in the early and late phase of the viral life cycle especially in reverse transcription where it chaperones the initiation of reverse transcription and the two obligatory strand transfers, little is known about the fate of NCp7 after the completion of viral DNA synthesis. Based on the lower affinity of NCp7 to dsDNA in comparison to ssRNA, it is suggested that NCp7 is released after the reverse transcription of the viral DNA. The fate of the released NCp7 is totally unknown. It has been reported that NCp7 is found in the nucleus shortly after infection (Zhang & Crumpacker 2002), but the way it enters the nucleus is debated. Indeed, NCp7 may enter the nucleus on its own or as part of the PIC.

Therefore, my second axis of research was focused on the distribution and the fate of NCp7 in the cell, with a special focus on the identification of its potential intracellular ligands. To address this question, we used a model based on the transient overexpression of eGFP-labeled NCp7 in HeLa cells, to investigate by a combination of fluorescence microscopy techniques the intracellular distribution and dynamics of labeled NCp7. My focus was mainly on studying the interaction with cellular DNA and RNA. By FRET-FLIM between NCp7-eGFP and nucleic acids labeled by Sytox Orange, I found that cellular RNAs and notably ribosomal RNAs are the major binding partners of NCp7 in the cytoplasm and nucleolus.

Manuscript 2

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RESEARCH ARTICLE

Investigating the Cellular Distribution and Interactions of HIV-1 Nucleocapsid Protein by Quantitative Fluorescence Microscopy

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Abstract

The nucleocapsid protein (NCp7) of the Human immunodeficiency virus type 1 (HIV-1) is a small basic protein containing two zinc fingers. About 2000 NCp7 molecules coat the genomic RNA in the HIV-1 virion. After infection of a target cell, the viral core enters into the cytoplasm, where NCp7 chaperones the reverse transcription of the genomic RNA into the proviral DNA. As a consequence of their much lower affinity for double-stranded DNA as compared to single-stranded RNAs, NCp7 molecules are thought to be released in the cytoplasm and the nucleus of infected cells in the late steps of reverse transcription. Yet, little is known on the cellular distribution of the released NCp7 molecules and on their possible interactions with cell components. Hence, the aim of this study was to identify potential cellular partners of NCp7 and to monitor its intracellular distribution and dynamics by means of confocal fluorescence microscopy, fluorescence lifetime imaging microscopy, fluorescence recovery after photobleaching, fluorescence correlation and cross-correlation spectroscopy, and raster imaging correlation spectroscopy. HeLa cells transfected with eGFP-labeled NCp7 were used as a model system. We found that NCp7-eGFP localizes mainly in the cytoplasm and the nucleoli, where it binds to cellular RNAs, and notably to ribosomal RNAs which are the most abundant. The binding of NCp7 to ribosomes was further substantiated by the intracellular co-diffusion of NCp7 with the ribosomal protein 26, a component of the large ribosomal subunit. Finally, gradient centrifugation experiments demonstrate a direct association of NCp7 with purified 80S ribosomes. Thus, our data suggest that NCp7 molecules released in newly infected cells may primarily bind to ribosomes, where they may exert a new potential role in HIV-1 infection.

Introduction

The nucleocapsid protein (NCp7) of the human immunodeficiency virus type 1 (HIV-1) is a small basic protein resulting from the cleavage of the Gag polyprotein precursor by the viral

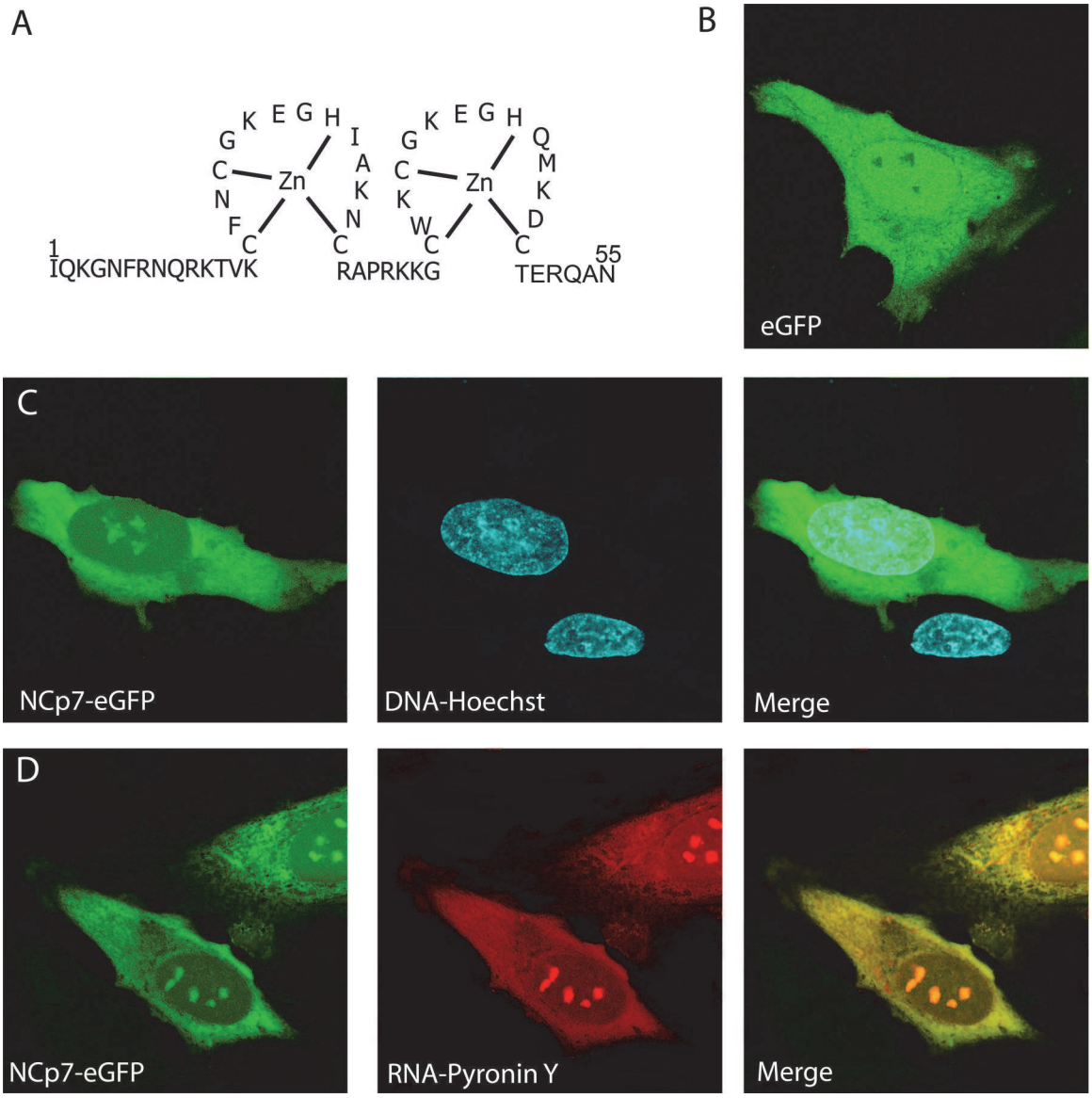

Fig 1. Intracellular distribution of NCp7-eGFP. (A) Amino acid sequence of NCp7. Confocal images of HeLa cells expressing transiently eGFP (B) or NCp7-eGFP (C, D). Comparison with the localization of DNA labeled by 1.6 μM Hoechst 33342, (C) and RNA labeled by 1 μM Pyronin Y. The cyan color of the merge panel in (C) indicates colocalization of NCp7 with DNA in the nucleus. The nearly uniform yellow color of the merge panel in (D) indicates a strong colocalization of NCp7 with RNA all over the cell.

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protease. The mature form of NCp7 contains 55 amino acids forming two highly conserved CCHC zinc-finger motifs connected by a short flexible linker and flanked by unfolded N- and C-terminal basic domains (Fig. 1A) [1,2]. In their zinc bound form, the two zinc fingers exhibit similar folding and are in close proximity [2–4].

NCp7 is endowed with key functions in the early and late phases of HIV-1 replication [5–12]. These functions rely on its chaperone properties, which direct the rearrangement of nucleic acids (NAs) into their most thermodynamically stable conformation [13–16]. Within the reverse transcription complex (RTC), NCs are thought to be required for the initiation and the two obligatory strand transfer reactions of viral DNA synthesis by the RT enzyme [8,17–24]. In the nucleus, NCp7 may assist the integrase-mediated integration of the viral DNA into the host

cell DNA [25,26]. Later, during virus assembly, the nucleocapsid domain of the Gag polyprotein is responsible for the selection and packaging of the genomic RNA into assembling virions [7,27–29]. Last, in the mature virus the genomic RNA dimer undergoes condensation and is coated and protected by about 2000 molecules of NCp7 in the nucleocapsid substructure [30–33].

Though the mechanism of the chaperoning functions of NCp7 has been widely studied, its trafficking and interactions with cellular components during the early stages of infection are unclear. Once viral DNA synthesis is completed, the RTC becomes the so-called preintegration complex (PIC), which is the integration-competent HIV-1 complex that drives integration of the viral DNA into the host cell DNA. However, the exact composition of the PIC and notably its content in NCp7 as well as the timing of the viral capsid uncoating are debated (for review, see [34]). Based on the much lower affinity of NCp7 for double-stranded DNA than for single-stranded RNAs [17,35,36], it was hypothesized that NCp7 molecules are progressively released during viral DNA synthesis [35,37]. In support of this hypothesis, immunolabeling of NCp7 in H9 cells acutely infected with HIV-1, revealed a diffuse cytoplasmic localization of the protein 4 hours post infection as well as a nuclear localization 18 hours post infection, suggesting that the released NCp7 molecules may diffuse in the cytoplasm and the nucleus [38]. Moreover, NCp7 was also detected in the nucleus 8 hours post infection in infected P4 cells [39]. Due to the large number of NCp7 molecules initially present in the infecting viruses, a significant fraction might be released in the cytoplasm and in the nucleus of the infected cells. It remains that the fate and possible role of these released NCp7 molecules are still unknown.

In this context, in order to characterize the possible intracellular distribution of these released NCp7 molecules and to identify their potential interactants, we used HeLa cells transfected with eGFP-labeled NCp7 as a model. Using a combination of advanced quantitative fluorescence microscopy techniques, we found that NCp7 was distributed in the cytoplasm and the nucleoli where it mainly binds to ribosomal RNAs. Moreover, direct monitoring and mapping of the dynamics of NCp7 and ribosomal proteins further confirmed that NCp7 can bind to ribosomes. Finally, the binding of NCp7 to purified 80S ribosomes was directly demonstrated by gradient centrifugation experiments.

Materials and Methods

1. Constructs

The NC domain of Gag was PCR amplified from the Gag-TC plasmid kindly provided by D. Ott [40]. The PCR product was purified using a PCR purification kit (Macherey Nagel GmbH, Germany), digested with BamH1 and then ligated in pEGFP-N1 to produce NC-eGFP. The integrity of all constructs was confirmed by DNA sequencing (GATC Biotech, Germany).

2. Cell Culture and DNA transfections

HeLa cells were cultured on 35 mm glass coverslips (μ-Dish IBIDI, Germany) in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Invitrogen Corporation, France) at 37°C in a 5% CO_2 atmosphere. Transfection was performed with jetPEI (PolyPlus transfection, France) according to supplier's recommendations. All experiments were done between 16–24 hours post DNA transfection.

The Hoechst 33342, Pyronin Y, Sytox Orange and RNase were purchased at Sigma Aldrich. Staining of nucleic acids was performed by incubating live HeLa cells for 30 minutes with either Hoechst 33342 (1.6 μM) or Pyronin Y (1 μM). For the RICS experiments, the Pyronin Y concentration was 10 nM and the incubation time was reduced to 5 minutes. Due to the high concentrations of RNAs in cells and the rather high binding affinity of Pyronin Y for RNAs [41],

only marginal amounts of Pyronin Y molecules are expected to diffuse in their free form in the cells. For Sytox Orange staining, cells were fixed for 15 minutes with 4% PFA solution, then permeabilized using 1% saponin and incubated for 30 minutes with 2.5 μM Sytox Orange. To digest the cellular RNAs, cells were treated for 30 minutes at 37°C by a mixture of RNaseA and RNase T1 (Ambion Rnase cocktail, Life Technologies, France) added at 25 U/mL and 100 U/mL, respectively.

3. Linear sucrose density gradient analysis

Human 80S ribosomes were purified from HeLa cells as previously reported [42] and incubated with NCp7 for 30 minutes at 4°C. The mixture was loaded on 15–30% sucrose density gradients prepared in buffer A (20 mM Tris-HCl pH 7.5, 2 mM $Mg(OAc)_2$, 150 mM KCl) and centrifuged at 25 000 rpm for 10.5 hours using a SW41 rotor. The gradients were fractionated and fractions corresponding to the peak of 80S ribosomes were precipitated with cold acetone. Proteins in the fractions were separated by SDS-PAGE and probed by western blotting using specific antibodies against NCp7 (polyclonal antibody kindly provided by R. Gorelick), RPS7 (abcam ab57637) and RPL26 (abcam ab59567), and peroxidase-labeled secondary antibody. NCp7, RPS7 and RPL26-specific bands were visualized by luminescence.

4. Confocal microscopy and Raster Image Correlation Spectroscopy

Confocal microscopy and Raster Image Correlation Spectroscopy (RICS) experiments were performed on a Leica SP2 microscope equipped with a 63× oil immersion objective (NA = 1.2). eGFP fluorescence was excited with a 488 nm argon laser line and the emitted fluorescence (500–550 nm) was detected by a PMT detector. The RICS analysis was performed using the SimFCS software developed by the Laboratory for Fluorescence Dynamics (http://www.lfd.uci.edu). For each cell, a stack of 50 images (256×256 pixels with a pixel size of 50 nm) was acquired at 400 Hz (2.5 ms between the lines) with a pixel dwell time of 4.8 μs. Moving average subtraction was performed to remove the contribution of slow moving structures and cellular displacements [43]. The average spatial correlation was fitted by a three dimensional diffusion model. A 50 nM solution of eGFP in water was used for calibrating the focal volume, assuming that its diffusion constant D is 90 $\mu m^2/s$ [43].

5. Fluorescence correlation spectroscopy

FCS measurements were performed on a home-built multiphoton microscope based on an inverted microscope (IX70, Olympus, Japan) [44,45]. The excitation light at 900 nm was provided by a mode-locked Ti:Sapphire laser (Tsunami, Spectra Physics, CA) or a broad band Insight Deep See laser (Spectra Physics, CA) delivering femtosecond pulses at 80 MHz frequency. The laser beam was focused by a 60× (NA = 1.2) water immersion objective (Olympus, Japan). Emitted fluorescence was filtered using a short-pass filter with a cutoff wavelength of 680 nm (F75–680; AHF, Germany) and a band pass filter of 520/17 nm (F37–520; AHF, Germany). The fluorescence was directed to a fiber-coupled APD (SPCM-AQR-14-FC; Perkin Elmer, CA), and the normalized autocorrelation function was calculated on-line with a hardware correlator (ALV5000, ALV GmbH, Germany).

The focal volume was measured prior to experiments using a 50 nM solution of tetramethylrhodamine (TMR) with a known diffusion coefficient (D = 592 $\mu m^2/s$ at 37°C) [46]. For each condition, measurements were performed in the cytoplasm and the nucleus of 16 different cells. For each measurement, 50 acquisitions of 5 seconds duration were realized and the autocorrelation curves were calculated. Five averages of ten autocorrelation curves were calculated and analyzed independently by the Quickfit software [47], using the anomalous diffusion

model:

$$G(\tau) = \frac{1}{V_{eff}\langle C\rangle}\frac{1}{\left(1+\left(\frac{\tau}{\tau_D}\right)^{\alpha}\right)}\frac{1}{\sqrt{1+\left(\frac{r_0}{z_z}\right)^2\left(\frac{\tau}{\tau_D}\right)^{\alpha}}} \quad (1)$$

where *Veff* and $<C>$ represent the effective focal volume and the average concentration of fluorescent molecules, respectively while r_0 and *zz* correspond to the lateral and axial dimensions of the focal volume, respectively. τ_D corresponds to the transit time and α is a coefficient related to the anomalous diffusion. The size of the beam waist in the focal plane was determined by a calibration measurement and the diffusion coefficients were calculated from the transit time values:

$$D = \frac{\omega_{XY}^2}{4\tau_D} \quad (2)$$

Noticeably, Equation 2 only rigorously applies in the case of free diffusion [48]. Nevertheless, for the sake of comparison, apparent diffusion constants were calculated with the same equation in case of anomalous diffusion [49].

Fluorescence cross-correlation spectroscopy (FCCS) measurements in live cells were performed on the same setup, according to the recommendations of Bacia et al [50]. An excitation of 1020 nm was provided by a femtosecond laser (Insight, Spectra Physics, CA) source. Fluorescence emission was collected and separated from excitation by a first dichroic (SP 720 nm, Semrock, NY). A second dichroic (LP 585, Chroma, VT) was used to split the emission in green (BP 525–39, Semrock, NY) and red (BP 660–52, Semrock, NY) channels. The filters were selected to minimize the bleed-through of eGFP and mCherry emission between the two channels. The bleed-through was quantified with cells expressing eGFP or mCherry alone. Power intensity was adjusted to optimize the count rate and minimize the noise and saturation effects for cells transfected with eGFP alone, mCherry alone, eGFP-mCherry fusion protein and with eGFP and mCherry, simultaneously. The calibration of the focal volume at 1020 nm was performed prior to experiments using a 50 nM solution of eGFP preliminary characterized at 930 nm by FCS measurements. FCS and FCCS correlation curves were obtained by averaging 60 measurements of 5 s for five different cells. Curves were calculated and analyzed independently by the Quickfit software [47], using the anomalous diffusion model (1). Values of the FCCS parameters were extracted with the Global FCCS Fit plugin of the Quickfit software.

6. Fluorescence lifetime imaging microscopy (FLIM)

Time-correlated single-photon counting FLIM was performed on the same two-photon microscope as described for FCS, using an excitation wavelength of 900 nm. Imaging was carried out with a laser scanning system using two fast galvo mirrors (Model 6210, Cambridge technology, MA), operating in the descanned fluorescence collection mode. The fluorescence was directed to a fiber coupled APD (SPCM-AQR-14-FC, Perkin Elmer, CA), which was connected to a time-correlated single photon counting (TCSPC) module (SPC830, Becker & Hickl, Germany), operating in the reversed start-stop mode. Typically, the samples were scanned continuously for about 80 s to achieve appropriate photon statistics to determine the fluorescence decays. Data were analyzed using a software (SPCImage V2.8, Becker & Hickl, Germany), which uses an iterative reconvolution method to recover the lifetimes from the fluorescence decays. The FRET efficiency reflecting the distance between the two chromophores was calculated

according to:

$$E = \frac{R_0^6}{R_0^6 + r^6} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (3)$$

where R_0 is the Förster radius, r the distance between donor and acceptor, τ_{DA} is the lifetime of the donor in the presence of the acceptor, and τ_D is the lifetime of the donor in the absence of the acceptor.

7. Fluorescence Recovery After Photobleaching (FRAP)

FRAP experiments were performed on an iMIC microscope (Till Photonics, Germany) equipped with a Cobolt Dual Calypso Laser 491/532 nm (Sweden) and a 60× TIRFM (1.45 NA) objective (Olympus, Japan). Fluorescence images were acquired on an EMCCD camera (Andor Tech, Ireland). To avoid possible artifacts of overexpression, only cells expressing low but detectable amounts of protein were chosen for analysis. Briefly, five images were acquired in an epifluorescence configuration before bleach. Photobleaching was done on a selected area in confocal mode by a 488 nm (30% of intensity) laser scanning 30 lines during 30 ms. Recovery was followed in epi configuration at 50 frames per second acquisition rate during 5–10 seconds. For each time point, the intensity of the bleached region was corrected for the bleaching due to the illumination during imaging and the fluorescence intensity was normalized to the pre-bleached intensity. FRAP recovery curves were generated and fitted using the Offline Analysis (Till Photonics, Germany). The normalized curves were fitted by a double exponential rise model:

$$F(t) = A_1(1 - e^{-t/\tau_1}) + A_2(1 - e^{-t/\tau_2}) \quad (4)$$

where A_1 and A_2 represent the amplitudes and τ_1 and τ_2 the decay times of the two components. The bleached surface S and the values of the recovery halftimes $t_{1/2}$ were used to calculate the diffusion coefficients:

$$D = \frac{S}{4t_{1/2}} \quad (5)$$

For all measurements, cells were maintained at 37°C.

Results

1. Intracellular localization and binding partners of NCp7-eGFP

Fig. 1 shows typical HeLa cells expressing either eGFP (Fig. 1B) or NCp7-eGFP (Fig. 1C, D). Unlike eGFP which is homogenously distributed all over the intracellular volume, NCp7-eGFP is preferentially localized in the cytoplasm and the nucleoli of the transfected cells. NCp7-eGFP is also observed in the nucleoplasm, but at a smaller intensity than in the other compartments. The distribution differences between NCp7-eGFP and eGFP clearly indicate that the localization of the former is mainly driven by NCp7. A nucleolar localization of NC proteins has been reported for HIV-1 and Rous Sarcoma virus in chicken, mouse and human cells and seems to be a common feature among retroviruses [51]. NC proteins may be retained in the nucleoli through their binding to cellular proteins or RNAs [51]. Moreover, the accumulation of NCp7-eGFP in the nucleoli suggests that it can cross the nuclear envelope, either by diffusion or by an active transport. In the cytoplasm, the distribution is diffuse, indicating that NCp7 does not bind to any specific cytosolic structure or compartment.

Since the biological role of NCp7 is mainly related to its interactions with nucleic acids, we compared its intracellular distribution with that of cellular DNAs and RNAs. Fig. 1C reports cells expressing transiently NCp7-eGFP labeled with Hoechst 33342, a dye that binds specifically to the minor groove of DNA molecules. As expected, we observed a strong signal in the nucleus, due to the high concentration of cellular DNA in this compartment. This pattern does not correspond to the NCp7-eGFP cellular distribution, though from the merge figure, it can be seen that NCp7-eGFP and the Hoechst dye colocalize in the nucleus. Using the RNA specific dye Pyronin Y [52,53], we observed a nearly perfect match of the intracellular distribution of this label with NCp7-eGFP (Fig. 1D) that was confirmed by the uniform yellow color of the cell in the merge panel. This colocalization of NCp7-eGFP with RNA all over the cell suggests that cellular RNAs are the major targets of NCp7-eGFP expressed in HeLa cells. DNA molecules in the nucleoplasm may also be binding partners for NCp7-eGFP, but both the lower accumulation of NCp7-eGFP in the nucleoplasm as compared to the nucleoli and cytoplasm, and the only partial colocalization of NCp7-eGFP with Hoechst suggest that DNA represent a less favored target as compared to RNA. This was expected, since NCp7 exhibits preferential binding to RNAs [17,36], so that binding to cellular RNAs in the nucleoli and cytoplasm is more likely than binding to DNA in the nucleoplasm.

In order to further evidence the interactions of NCp7 with cellular RNAs and DNAs, Fluorescence Lifetime Imaging Microscopy (FLIM) was performed to monitor the Förster resonance energy transfer (FRET) between NCp7-eGFP and Sytox Orange, a specific marker of nucleic acids [54]. FRET corresponds to a non radiative energy transfer between a fluorescent donor and an acceptor when they are less than 8 nm apart. This very short distance implies that the labeled molecules interact together, so that FRET can be used to evidence intermolecular interactions [55–57]. In cells, FRET can be followed ideally by the FLIM technique that measures the fluorescent decay at each pixel of the cell image. From these decays, the fluorescence lifetimes (τ) of the FRET donor could be extracted. These lifetimes are absolute parameters that do not depend on the instrumentation or the local concentration of the fluorophores, so they will be only sensitive to FRET. Therefore, the shortening of the fluorescence lifetime of the donor by FRET provides a direct evidence for a physical interaction between NCp7-eGFP and labelled NAs (reviewed in [58]). Sytox Orange was used as a FRET acceptor because its absorption spectrum overlaps the emission of eGFP. However, as this dye does not diffuse across the plasma membrane, FLIM measurements were performed on fixed and permeabilized cells. Noticeably, the permeabilization by saponin caused a slight decrease of the eGFP fluorescence lifetime from 2.43 ns to 2.2–2.3 ns in both eGFP and NCp7-eGFP expressing cells (S1 Table). Staining of eGFP-expressing cells with Sytox Orange caused a less than 5% decrease of the eGFP lifetime in all cell compartments, with the exception of nucleoli, where a 9% decrease was observed (Fig. 2A and 2B). This decrease likely resulted from the high concentration of Sytox Orange molecules bound to the densely packed RNA in the nucleoli, so that the probability to find Sytox Orange molecules close to eGFP molecules gets no more negligible [59]. However, this decrease in the lifetime is small in comparison with the up to 25% decrease in the NCp7-eGFP lifetime observed in the presence of Sytox Orange (Fig. 2C and 2D). Since the autofluorescence of the cells was found to represent less than 1% of the emission of NCp7-eGFP, this large decrease in the lifetime can unambiguously be attributed to FRET, confirming a direct interaction between NCp7-eGFP and labelled NAs. Interestingly, the FRET efficiencies observed in the nucleoli and the cytoplasm (20–25%, Fig. 2D and Table 1) were markedly higher than the FRET efficiency (13%) in the nucleoplasm, confirming that NCp7-eGFP proteins bind less efficiently to DNA as compared to RNA.

To further evidence the NCp7/ RNA interactions, cells were subjected to an RNase treatment to solely remove RNAs (Fig. 2E and Table 1). Results show that the FRET efficiencies

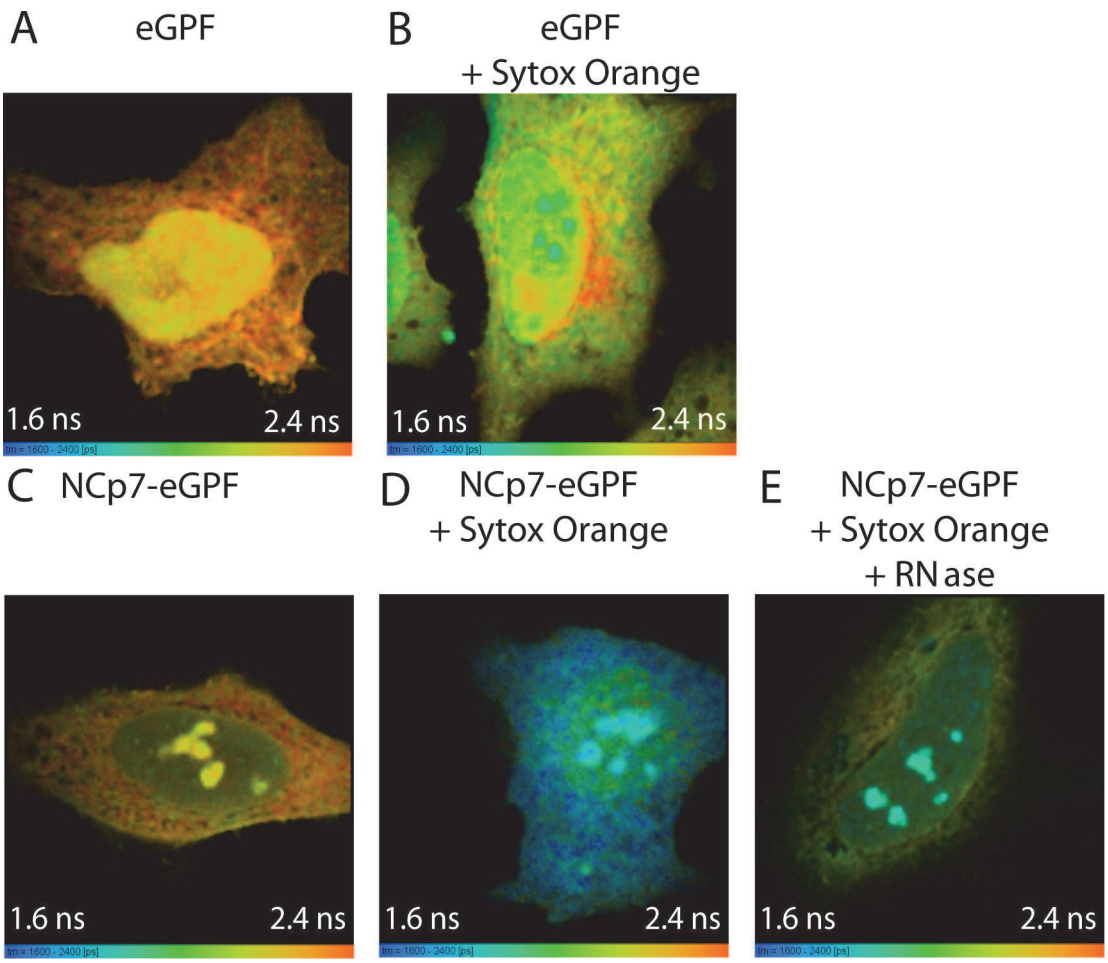

Fig 2. Interaction of NCp7-eGFP with nucleic acids monitored by two-photon FLIM. Fluorescence lifetime color-coded images of eGFP (A, B) and NCp7-eGFP (C, D, E) in the absence (A, C) or in the presence (B, D, E) of 2.5 μM Sytox Orange. In panel E, a mixture of RNaseA and RNase T1 was added at 25 U/mL and 100 U/mL, respectively. The time-resolved decays were fitted with a mono-exponential function. Excitation wavelength was 900 nm. Emission of eGFP was selectively collected through a 515/10 nm filter to remove any contribution from Sytox Orange emission.

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Table 1. Fluorescence lifetimes and FRET efficiencies for cells expressing eGFP or NCp7-eGFP in the presence of Sytox Orange.

	eGFP	eGFP + Sytox Orange	NCp7-eGFP	NCp7-eGFP + Sytox Orange	% FRET	NCp7-eGFP + Sytox Orange + RNAse	% FRET
	τ (ns)	τ (ns)	τ (ns)	τ (ns)		τ (ns)	
Whole cell	2.26±0.03	2.16±0.01	2.20±0.06	1.75±0.03	**20**	1.99±0.04	**10**
Cytoplasm	2.30±0.02	2.20 ± 0.02	2.24±0.07	1.69±0.04	**25**	1.97±0.03	**12**
Nucleus	2.20±0.02	2.11± 0.04	2.11±0.05	1.84±0.04	**13**	1.93±0.05	**8.5**
Nucleoli	2.20±0.02	2.0 ± 0.02	2.13±0.07	1.70±0.05	**20**	1.86±0.05	**13**

The lifetime values were extracted from FLIM color coded images such as in Fig. 2, and given as mean +/- SD for 10–13 cells. FRET efficiencies were calculated by comparison with the lifetimes of NCp7-eGFP in the corresponding cell compartments. The SD for the FRET values is about 2%. Cells were fixed with 4% PFA and permeabilized with 1% saponin, as described in Materials and Methods.

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dramatically decreased both in the cytoplasm (from 25% to 12%) and the nucleoli (from 20% to 13%). Both the high FRET values and their strong decrease upon RNase treatment confirm that NCp7-eGFP interacts with cellular RNAs in the cytoplasm and the nucleoli. The residual FRET observed after RNase treatment is probably due to complexes of NCp7 bound to incompletely digested RNA fragments or to DNAs. A small decrease in the FRET efficiency was also observed in the nucleoplasm (from 13% to 8.5%). Since DNA should not be affected by the RNase treatment, this FRET decrease may reflect the digestion of nuclear RNAs, such as mRNAs. Noticeably, the presence of incompletely digested RNAs in the nucleus after RNase treatment suggested by the significant signal observed in this compartment (Fig. 2E) could be explained by the rather soft cell permeabilization treatment based on saponin. As previously reported [60], this detergent destabilizes cholesterol containing membranes and is thus not very efficient for nuclear membrane permeabilization. As a consequence, the RNase molecules may not efficiently enter into the nucleus and may thus incompletely digest the nuclear and nucleolar RNAs.

Taken together, the data of Fig. 1 and Fig. 2 indicate that cellular RNAs are likely the major binding partners of NCp7 in the cytoplasm and nucleoli. This conclusion is consistent with the observation that HIV-1 particles contain in addition to the genomic RNA, representing about half of the total RNA, an heterodisperse assortment of cellular RNA species, which are believed to be encapsidated by the NC domain of Gag, in proportion to their cellular level [61].

2. Intracellular dynamics of NCp7-eGFP

To further characterize NCp7-eGFP in cells, we performed a series of quantitative fluorescence microscopy experiments in order to explore its intracellular dynamics. In a first step, FRAP measurements were performed to determine the fraction of NCp7-eGFP molecules that are mobile in the intracellular environment and to obtain a first estimation of the NCp7-eGFP diffusion rates in comparison to eGFP alone [62].

Approximately 5 μm^2 areas in the cytoplasm and in the nucleus of eGFP and NCp7-eGFP expressing cells were photobleached by a 488 nm laser (Fig. 3A). A time lapse sequence of 5–10 seconds was acquired just after the bleaching in order to monitor the fluorescence recovery. Representative experimental curves for eGFP and NCp7-eGFP are shown in Fig. 3B and 3C, respectively. As shown by the distribution of the residuals, a double exponential fit (Equation 4 in Materials and Methods section) was required to adequately fit the recovery curve. This biphasic shape of FRAP curves in the cellular environment is usually interpreted as a consequence of molecular crowding and presence of obstacles [63–65]. The recovery curves show that the diffusion of eGFP is much faster than that of NCp7-eGFP. The halftime of fluorescence recovery $t_{1/2}$ corresponds to 0.07s and 0.32 s for eGFP and NCp7-eGFP, respectively.

The values of $t_{1/2}$ were used to estimate the diffusion coefficients according to Equation 5 in Materials and Methods section. It should be noted that it represents only a rough estimation, due to the experimental conditions (bleaching in confocal mode, observation in epi illumination) and the oversimplified 2D diffusion model used for fitting. In spite of these limitations, the intracellular D value ($\sim$20 $\mu m^2/s$, Fig. 4A) determined for eGFP was in good agreement with the values reported in the literature [66,67]. The diffusion rate constant of NCp7-eGFP was found to be $\sim$4 times slower than that of eGFP. As NCp7 is a relatively small protein (Fig. 1A), its fusion to eGFP causes only a small increase of the hydrodynamic radius from 2.77 nm for eGFP alone [68] to 3.04 nm for NCp7-eGFP. This increase represents less than 10%, so that the 4-fold difference in the diffusion kinetics of these two proteins likely results from the interaction of NCp7 with cellular partners.

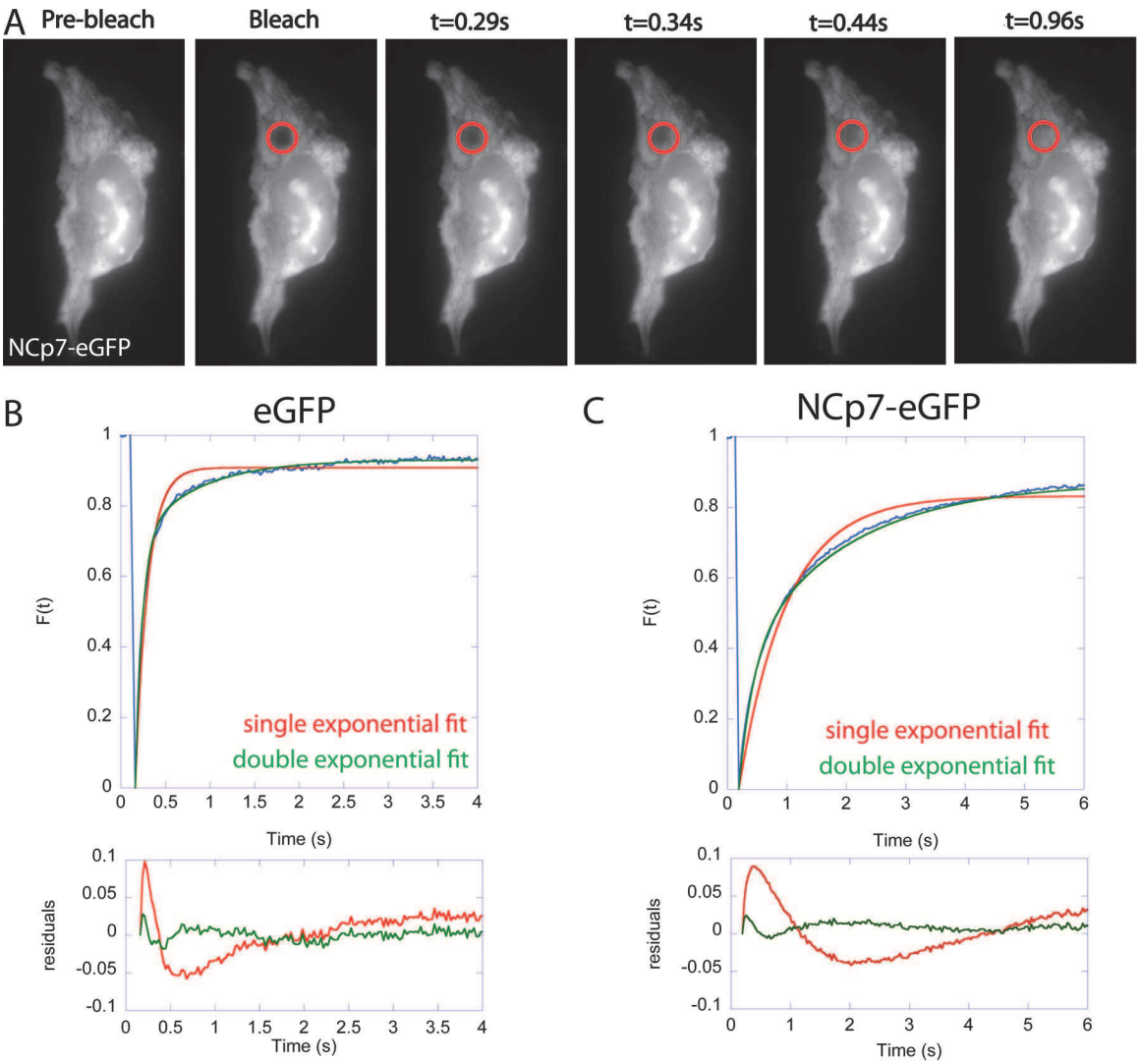

Fig 3. FRAP experiments in eGFP and NCp7-eGFP expressing cells. (A) Time lapse sequence of typical FRAP measurements in the cytoplasm of an NCp7-eGFP expressing cell. The bleached region is highlighted by the red circle. Normalized fluorescence recovery curves of (B) eGFP and (C) NCp7-eGFP in the cytoplasm. For both eGFP and NCp7-eGFP, the distribution of residuals indicated a much better fit of the recovery curves with a double exponential as compared to a single exponential fit.

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Another key information provided by FRAP measurements is the mobile fraction (MF) of NCp7-eGFP molecules that can be deduced from the fluorescence level reached at equilibrium after recovery. MF represents the percentage of fluorescent molecules that diffuse and thus exchange with the photobleached ones. On the opposite, the proportion of NCp7-eGFP molecules bound to static or slowly moving cellular structures can be deduced from the immobile fraction (1-MF). As shown in Fig. 4B, the MF of eGFP (representing freely diffusing control molecule) is about 95% in the cytosol and 87% in the nucleus. Very similar percentages of MF were obtained for NCp7-eGFP in both the cytoplasmic (87%) and nuclear compartments (88%), indicating that NCp7-eGFP molecules form mobile complexes with their cellular partners and notably RNAs, and that only a small fraction (if any) of the NCp7-eGFP molecules are bound to immobile structures.

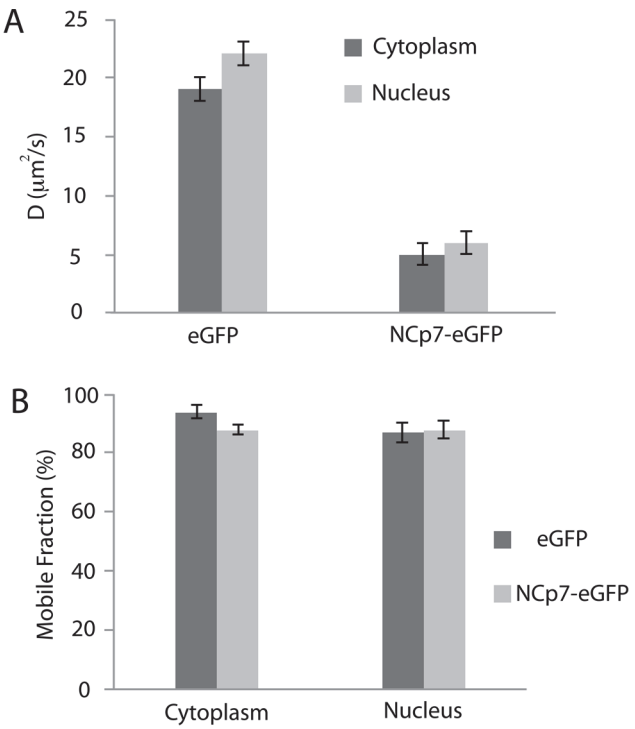

Fig 4. FRAP-based estimation of diffusion coefficient values (A) and mobile fraction (B) of eGFP and NCp7-eGFP in the cytoplasm and in the nucleus.

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To more accurately characterize the intracellular dynamics of NCp7-eGFP, we performed FCS [69–72] and RICS [43,66,73] measurements. Both methods characterize the translational diffusion of fluorescent molecules (or molecular complexes). They are based on the analysis of fluorescence fluctuations during the diffusion of fluorescent molecules through the focal volume of the microscope. FCS provides information about the diffusion time and mode, the local concentration, and about the molecular brightness of the diffusing fluorescent species. The intensity profile of the fluorescence signal at different locations in cells expressing eGFP or NCp7-eGFP was recorded over time and was analyzed by an autocorrelation function (Fig. 5). The autocorrelation curves were adequately fitted with a 3D anomalous diffusion model with one population (Equation 1 in Materials and Methods section). The fit was significantly better than with a free 3D diffusion model with one component, as shown by the residuals (Fig. 5A). Alternatively, a good fit was also obtained with a free 3D diffusion model with two populations, corresponding to free and bound NCp7-eGFP molecules (data not shown). In this case, the fraction of free NCp7-eGFP molecules did not exceed 5%, suggesting that the vast majority of NCp7-eGFP molecules are bound to cellular components, Moreover, the diffusion constant of the slow component was found to be very similar to the diffusion coefficient obtained with the anomalous diffusion model (data not shown).

The autocorrelation curves of NCp7-eGFP were then compared with those of the freely diffusing eGFP protein (Fig. 5B). For eGFP, the ratio of its diffusion coefficient (D) in the intracellular environment to the one in water (D_0) was found to be 0.306 for the cytosol and 0.280 for the nucleus, in good agreement with previously reported values in HeLa cells, swiss 3T3 fibroblasts and MCKD cells [74–76]. For NCp7-eGFP, its D value in the cytoplasm (4.5 ± 1 $\mu m^2/s$, Table 2) was found to be 8 times slower than the eGFP one (34 ± 3 $\mu m^2/s$). Similarly, a 5.5-fold difference was observed between the corresponding D values in the nucleus. These large

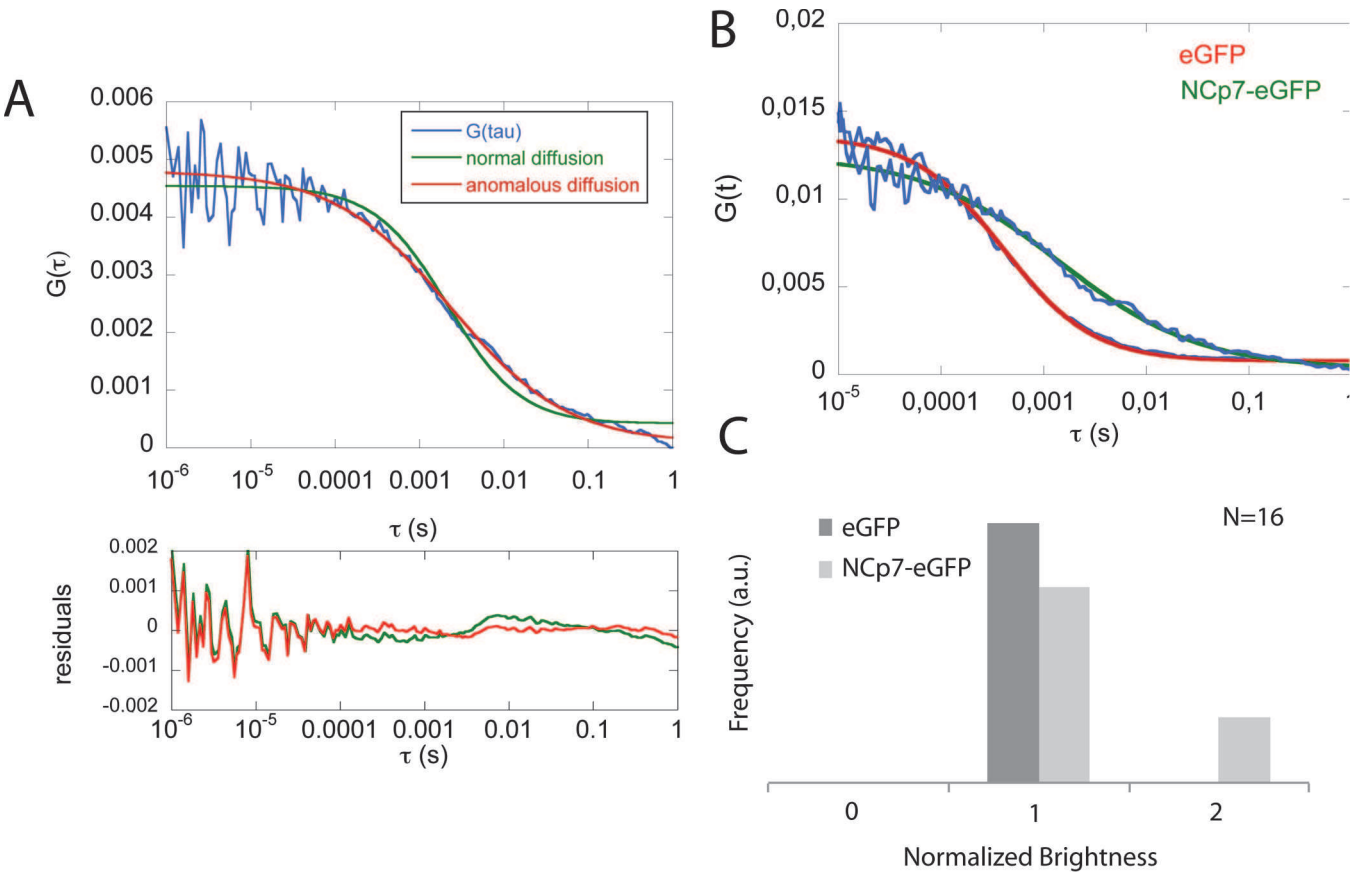

Fig 5. FCS measurements in eGFP and NCp7-eGFP expressing HeLa cells. (A) Experimental autocorrelation function (blue) of NCp7-eGFP in HeLa cells fitted with a model for free (green) and anomalous (red) 3D diffusion. The residuals indicate that a better fit was obtained with the anomalous diffusion model. (B) Comparison of autocorrelation curves for eGFP and NCp7-eGFP diffusion in the cytoplasm of HeLa cells. Fits (solid lines) were performed with the anomalous diffusion model. (C) Histogram of the brightness analysis for eGFP and NCp7-eGFP (N = 16).

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differences between NCp7-eGFP and eGFP clearly confirmed that NCp7-eGFP molecules diffuse in the cell in the form of complexes of rather large molecular weight as compared to eGFP molecule. Moreover, the anomalous diffusion coefficient α was found to be close to 1 for eGFP, which indicates that eGFP molecules diffuse in a regime close to free diffusion both in the cytoplasm and the nucleus. For NCp7-eGFP, the average α value is 0.65 ± 0.03 in the cytosol and

Table 2. Diffusion coefficients (D) and anomalous coefficients (α) inferred from FCS and RICS measurements of eGFP and NCp7-eGFP expressing cells.

		Cytoplasm		Nucleus	
		D (μm²/s)	α	D (μm²/s)	α
FCS	eGFP	34 ± 3	0.92 ± 0.08	31 ± 1	0.95 ± 0.09
	NCp7-eGFP	4.5 ± 1	0.65 ± 0.03	7 ± 3	0.60 ± 0.09
RICS	eGFP	28 ± 3		25.5 ± 2	
	NCp7-eGFP	3 ± 1		5 ± 2	

The D and α values are given as means +/- SD for 800 correlation curves in 16 cells (FCS) and 40 measurements in 10 cells (RICS).

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0.60 ± 0.09 in the nucleus, indicating that the motion of the NCp7-containing complexes is substantially slowed down either by the obstructed environment [77] or by transient binding events to cytoplasmic or nuclear components [78].

Moreover, FCS curves allowed us to further determine the brightness of these complexes, which is obtained by dividing the average number of photons emitted per second by the average number of fluorescent species diffusing through the focal volume. Comparing this value with the brightness of the eGFP molecules (considered as monomers) measured in the same conditions, helped us to determine the number of NCp7-eGFP molecules in the diffusing complexes. Brightness analysis (Fig. 5C) showed that the complexes do not contain more than two NCp7-eGFP molecules, clearly excluding that the complexes correspond to high molecular weight NCp7-eGFP aggregates.

Taken together, our data suggest that NCp7-eGFP molecules diffuse in both the nuclear and the cytoplasmic compartments in the form of large complexes that contain only one or two NCp7-eGFP molecules.

In order to map the spatial diffusion of NCp7-eGFP, FCS measurements were completed by RICS measurements. RICS analyses the fluorescence intensity fluctuations between neighboring pixels by spatially autocorrelating the image in x and y directions [66]. The fluorescence signal is acquired while the laser beam scans a region of the cell (Fig. 6A), generating a stack of 50–100 images. Afterwards, an average spatial correlation surface of these images is calculated (Fig. 6B) and fitted with a 3D diffusion model (Fig. 6C) in order to obtain information about the diffusion and the concentration of fluorescent molecules. Because the pixel dwell time ($\sim$µs) is much shorter than the time period between two lines ($\sim$ms), the amplitude of the spatial correlation surface (SCS) in the x direction reflects fast diffusion processes. Corresponding amplitudes appear in the y direction, when slowly diffusing molecules are present. As expected, the SCS of the rapidly diffusing eGFP molecules has significant amplitude only in the x direction, while for NCp7-eGFP, it broadens in the y direction, confirming the slower motion of the NCp7-containing complexes (Fig. 6B). The main advantage of RICS compared to FCS for cellular measurements is its lower sensitivity to photobleaching. Since the laser beam is scanning during the acquisition, the illumination time of the excited molecules is much shorter compared to FCS (µs versus ms). The average values of diffusion coefficients measured for eGFP and for NCp7-eGFP by RICS are summarized in Table 2 and are in full agreement with the FCS data, confirming that NCp7-eGFP molecules bind to its cellular partners within large complexes.

Another advantage of the RICS approach is the possibility to choose the size of the analyzed area. The lower size limit of this area is given by the distance travelled by the protein during the scan and corresponds to 2 μm^2 (40x40 pixels) for the fast moving eGFP. Consequently, in a stack of images of 1024x1024 pixels, we calculated the D values in windows of 64x64 pixels to draw the diffusion maps of eGFP and NCp7-eGFP in HeLa cells (Fig. 7). For eGFP, the diffusion coefficients are in the range of 20–40 $\mu m^2/s$, with a large majority of values being comprised between 25 and 35 $\mu m^2/s$, in line with previous reports [67]. The distribution of D values was rather homogeneous all over the cell, with no obvious deviation for any compartment. For NCp7-eGFP, the overall D values were much lower and differences in the D values between cell compartments appeared. In the cytoplasm, the diffusion D values were homogeneously distributed between 1.5 and 4 $\mu m^2/s$ all over the cytoplasm, thus no specific region of lower or higher mobility could be evidenced. The diffusion in the nucleus was found to be faster and more heterogeneous than in the cytoplasm, being comprised between 3.5 and 6.5 $\mu m^2/s$. Preliminary tracking experiments suggested that these rather high D values in the nucleoplasm may be related to a directed transport of the NCp7-containg complexes in this compartment (data not shown). Further studies are in due course to clarify the mechanism of

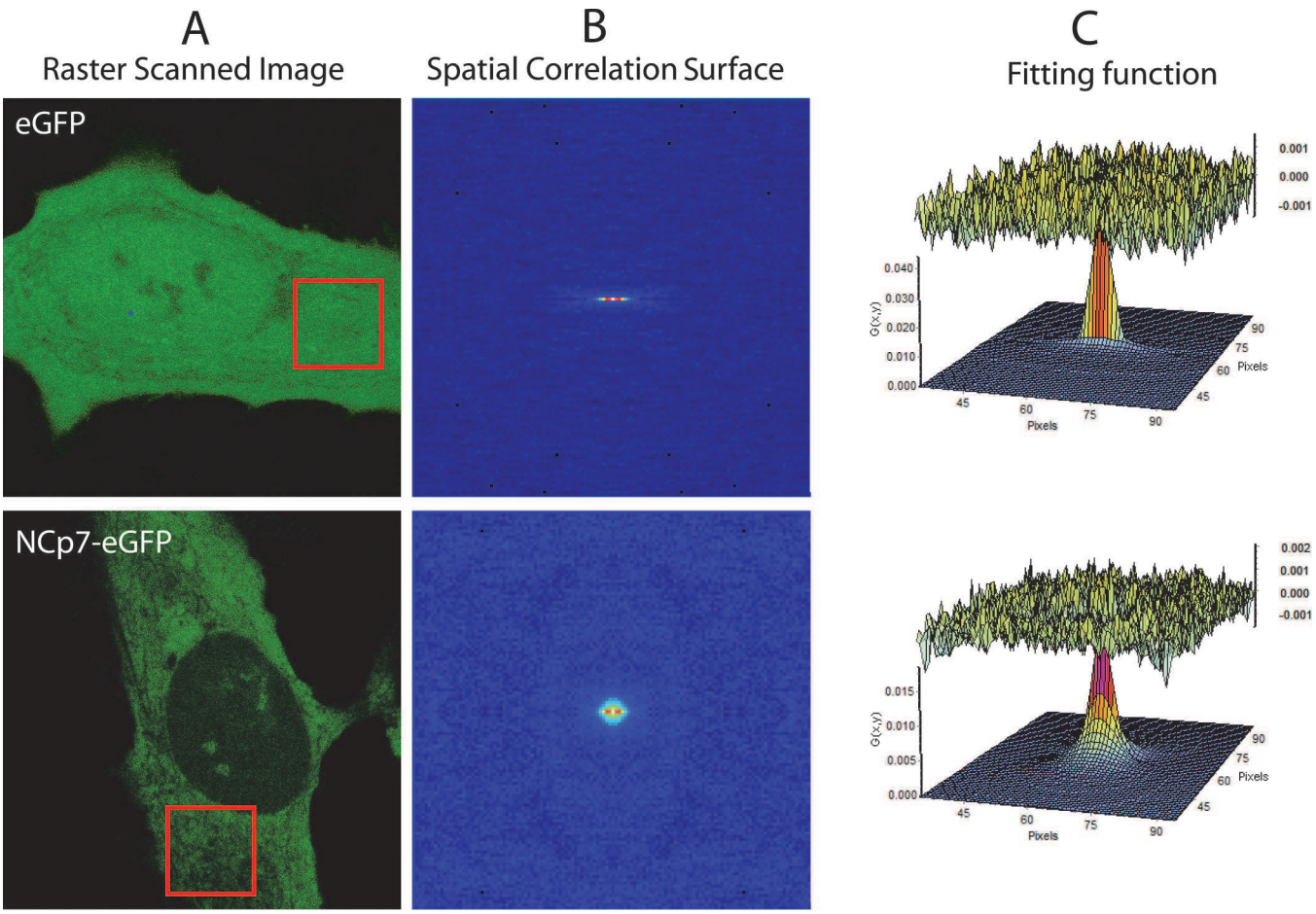

Fig 6. NCp7-eGFP dynamics in HeLa cells monitored by RICS. (A) A series of confocal images of eGFP and NCp7-eGFP expressing cells was acquired. A 128x128 pixel region was analyzed by calculating the two-dimensional spatial autocorrelation function represented as a spatial correlation surface (B) that was fitted by a 3D diffusion model (C), revealing the values of the diffusion coefficients and the number of diffusing molecules.

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this directed motion. In contrast, the D value drops to ~0.5 μm^2/s in the nucleoli, indicating that the NCp7-eGFP molecules probably form larger complexes with cell partners in the nucleoli than in the nucleoplasm or that the NCp7-containing complexes are further slowed down by the dense and compact environment of the nucleoli.

As NCp7 molecules were found to be largely associated with RNAs in the cytoplasm (Figs. 1 and 2), we next compared the cytoplasmic diffusion of NCp7-eGFP with the diffusion of cellular RNAs labeled with Pyronin Y. The average diffusion coefficient of cytoplasmic RNAs (5.8 +/- 1 μm^2/s) is significantly higher than the NCp7-eGFP one (3.2 +/- 0.9 μm^2/s), suggesting that NCp7-eGFP does not bind randomly to all cellular RNAs but interacts preferentially with large RNA molecules and/or with RNA molecules within ribonucleoprotein complexes. Since ribosomal RNAs represent about 80% of the total RNA in HeLa cells [79], it was tempting to speculate that NCp7 binds rRNAs in ribosomes. To further test this hypothesis, we measured the diffusion constant of the ribosomal protein L26 (RpL26), taken as an example. L26 is located at the surface of the large ribosomal 60S subunit. Interestingly, the D values (4.0 +/- 1.5 μm^2/s) of RpL26-eGFP were close to those of NCp7-eGFP, strengthening the hypothesis that NCp7-eGFP binds RNAs in ribosomes.

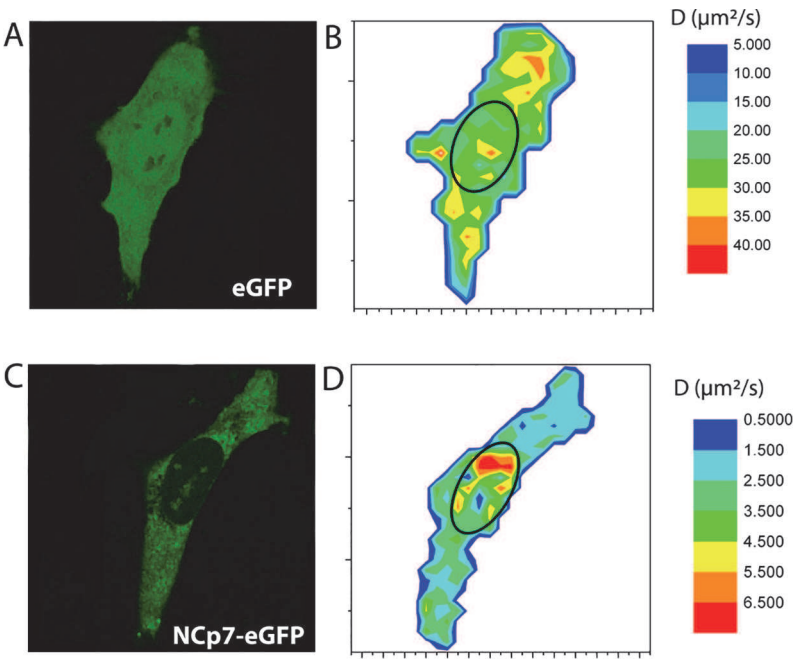

Fig 7. Confocal images (A, C) and RICS-based diffusion maps (B, D) of eGFP and NCp7-eGFP in HeLa cells. The color coded images (B, D) represent the values of the diffusion coefficients measured in the cell. The blue colors at the cell borders are artifacts, due to averaging with the exterior of the cells. Confocal images for the same cells (A, C) are given to identify the cell compartments and contour.

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To further confirm the interaction between NCp7 and ribosomes, FCCS experiences were performed on HeLa cells co-transfected with RpL26-eGFP and NCp7-mCherry. The FCCS technique correlates the temporal fluorescence fluctuations coming from two differently labeled molecules diffusing through the small sample volume defined in our case by a two-photon excitation. In the absence of spectral bleed-through, the cross-correlation function is different from zero only if both labeled proteins diffuse together, which implies that they belong to the same complex. A typical FCCS curve recorded on cells co-expressing RpL26-eGFP and NCp7-mCherry is shown in Fig. 8 (black trace), showing that the two proteins diffuse together and thus, belong to the same complex. It should be noted that these results show only that the two proteins are parts of the same ribonucleoprotein complex but not that they directly interact together. Quantitative analysis of FCCS curves revealed that the complexes containing both labeled proteins diffuse with a diffusion constant of 4 (+/- 3) $\mu m^2/s$, fully consistent with the diffusion constants of the NCp7-eGFP and RpL26-eGFP proteins measured by RICS. Furthermore, comparative analysis of the FCCS curve (Fig. 8, black curve) with the autocorrelation curves of NCp7-mCherry (Fig. 8, red curve) and RpL26-eGFP (Fig. 8, green curve) shows that between 40 to 70% of NCp7-mCherry molecules cross-correlate with RpL26-eGFP. Thus, the FCCS data clearly indicate that NCp7 and RpL26 diffuse together in the cytoplasm within the same ribosomal complex.

Finally, we performed gradient centrifugation experiments to directly assess whether NCp7 associates with purified 80S ribosome (Fig. 9). Purified human ribosomes were incubated in the absence or in the presence of purified NCp7 and submitted to fractionation experiments on a linear 15–30% sucrose gradient. The central peak fractions (Fig. 9A) were collected and analyzed by western blot. Antibodies directed against RpS7 and RpL26 proteins, present in the small and large ribosomal subunit, respectively were used to detect the ribosomes, while NCp7

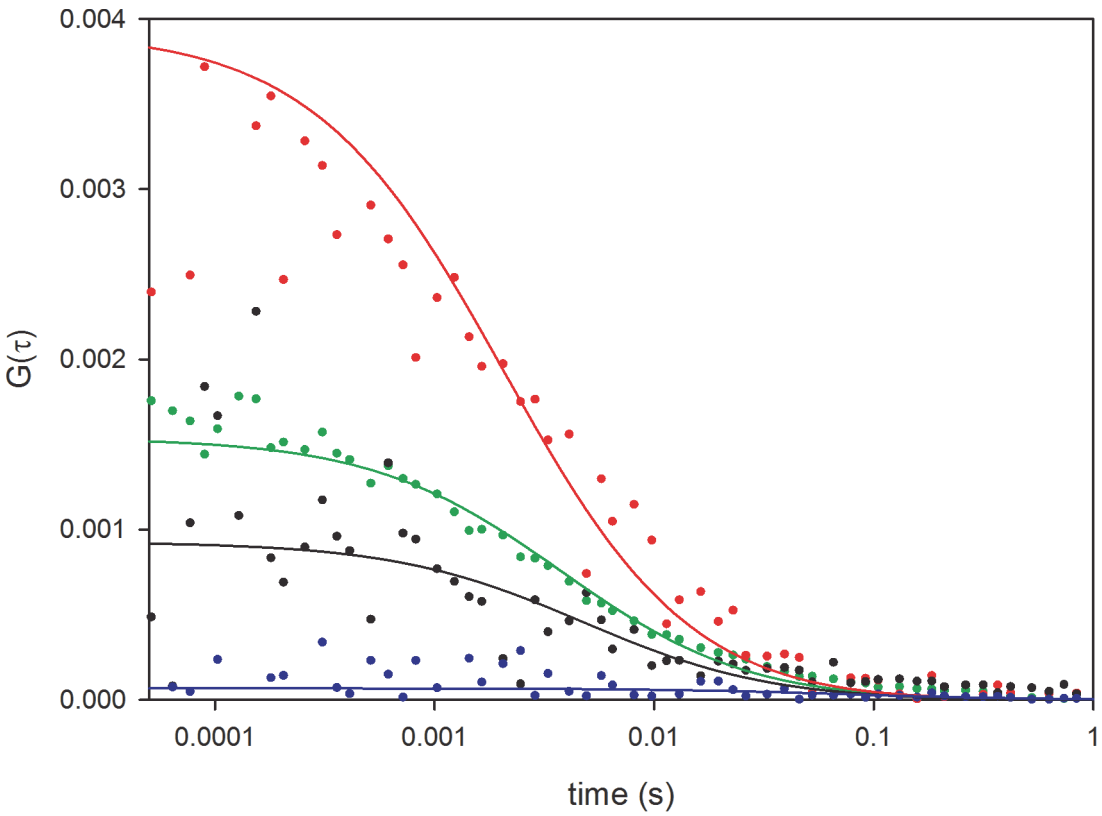

Fig 8. FCCS measurements on HeLa cells expressing RpL26-eGFP and NCp7-mCherry. The green, red and black curves denote the autocorrelation curve of RpL26-eGFP in the green channel, the autocorrelation curve of NCp7-mCherry in the red channel, and the cross-correlation curve between the two channels, respectively. As a negative control, the blue curve corresponds to the cross-correlation between eGFP and mCherry proteins co-expressed in HeLa cells. The close to zero value of the FCCS curve of the negative control not only shows that the two fluorescent proteins do not diffuse together, but also that there is marginal spectral bleed-through between the green and red channels. The solid lines correspond to the fit of the curves to the anomalous 3 D diffusion model. Diffusion constants of 5.6 (+/- 0.7) $\mu m^2/s$, 6 (+/- 3) $\mu m^2/s$ and 4 (+/- 3) $\mu m^2/s$ were obtained for RpL26-eGFP (green), NCp7-mCherry (red) and the RPL26-eGFP/NCp7-mCherry complex (black), respectively.

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was detected with a polyclonal antibody. A strong cosedimentation of NCp7 and ribosomal proteins (Fig. 9B, compare lanes 3 to lanes 1 and 2) was observed when NCp7 was incubated with the ribosomes prior to centrifugation, demonstrating a clear association of NCp7 with the 80S ribosome.

Discussion

NCp7 is a nucleic acid chaperone protein with key functions in the early and late phases of HIV-1 replication. Its implications in the reverse transcription process are extensively documented (for reviews, see [12,17,22,80]), but only little is known on NC's fate following the completion of viral DNA synthesis. A model was recently proposed in which due to its lower affinity for dsDNA as compared to ssDNA and vRNA, a large fraction of NCp7 is believed to be released in the cytoplasm during viral DNA synthesis by RT, so that only a small fraction of NCp7 molecules remains associated with the PIC to activate viral DNA integration by the IN enzyme [35,37]. This model is supported by the fact that NCp7 is present at a low level in the nucleus of newly infected cells and barely detectable in the PIC by biochemical techniques [25,38,39,81]. The intracellular distribution and biological role of NCp7 molecules released from the incoming viral particles in newly infected cells are yet totally unknown. In an attempt

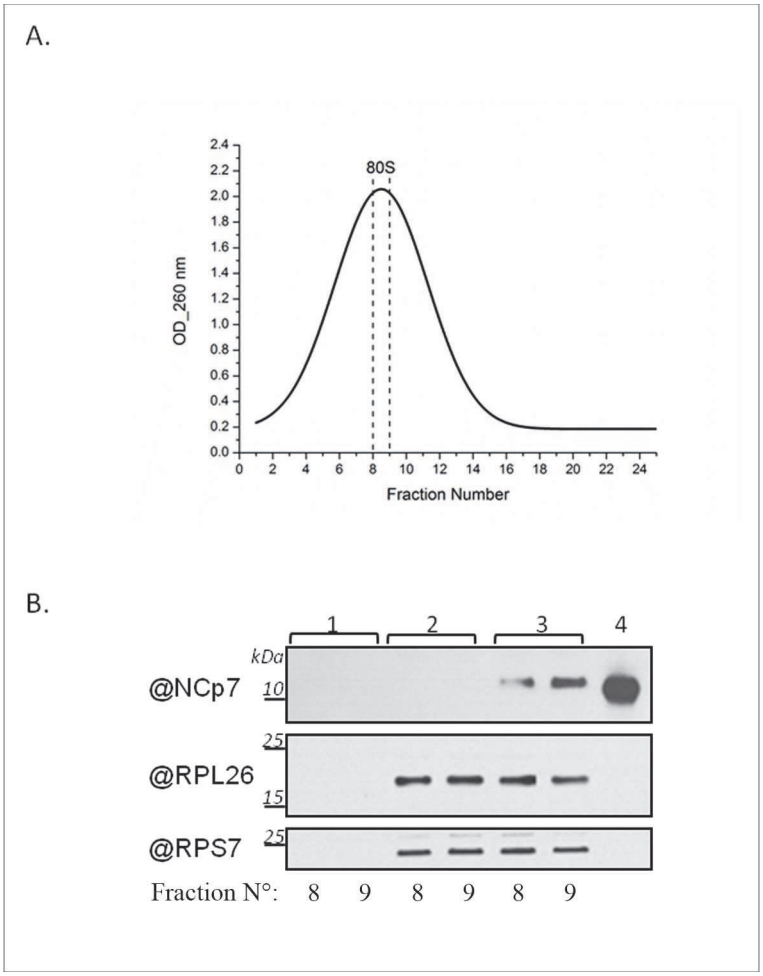

Fig 9. NCp7 cosediments with 80S ribosomes. (A) Sucrose gradient fractionation profile of purified 80S ribosomes (0.9 μM) incubated with NCp7 (13.3μM). Ribosome/NCp7 ratio was about 1/15. The peak fractions (8–9) were precipitated and further analyzed by western blot. (B) Western blot of fractions 8–9 from sucrose gradient fractionations performed with only NCp7 peptide (lanes 1); only 80S ribosomes (lanes 2); and 80S ribosomes and NCp7, together (lanes 3). NCp7 and ribosomal proteins were detected with polyclonal NCp7 and RpS7 antibodies, and monoclonal RpL26 antibodies. As a control, 90 nM of NCp7 peptide was loaded (4).

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to explore the fate and possible roles of NCp7 dissociating from the incoming virions, we used a model system based on the expression of eGFP-labeled NCp7 in HeLa cells to study by a combination of fluorescence microscopy techniques the intracellular distribution and dynamics of labeled NCp7, in order to identify its possible intracellular ligands.

Confocal microscopy (Fig. 1) showed that eGFP-NCp7 mainly localizes in the cytoplasm and the nucleoli. The presence of NCp7 in the nucleoli and to a lower extent in the nucleoplasm, is in line with the nuclear localization of NCp7 after infection [38,39] and the possible chaperoning role of NCp7 in vDNA integration by the viral integrase [32,82–84]. In agreement with the data reported by Lochmann et al. [51], our results show also that NCp7 can enter into the nucleus independently of the PIC. Colocalization studies further revealed a nearly perfect match of the intracellular distribution of NCp7 and cellular RNAs. In addition, the high FRET efficiency observed by FLIM between Sytox Orange labeled nucleic acids and NCp7-eGFP together with the large drop of this FRET efficiency upon RNase treatment (Fig. 2) clearly

indicate that NCp7 directly interacts with cellular RNAs both in the cytoplasm and the nucleoli. Thus, cellular RNAs appear to be the major intracellular ligand of NCp7. This high tropism for cellular RNAs may well explain why about half of the total virion RNA consists of cellular RNA species, probably packaged via the NC domain of Gag [61]. The binding of NCp7 to cellular RNAs is likely poorly specific since analysis of the composition of the cell RNA species in the virions revealed that they were roughly in proportion to their level in the cell [61].

The significantly lower amount of NCp7 and lower FRET efficiency in the nucleoplasm, together with the lower sensitivity of the FRET efficiency upon RNase treatment further suggest that NCp7 can also bind to double-stranded DNAs in the nucleus, but less efficiently than to RNAs. This conclusion is in line with the significantly lower affinity for double-stranded DNAs in comparison with single stranded RNA and DNA sequences [17,36]. FRAP (Fig. 4) and FCS (Fig. 5, Table 2) experiments further showed that the intracellular concentration of free NCp7-eGFP was negligible and that nearly all NCp7-eGFP molecules diffused in the form of complexes of rather high molecular weight. Moreover, most of these complexes were found to be mobile, being able to diffuse, while only a limited fraction of these complexes were associated with slowly moving or immobile structures. Interestingly, only one or two molecules of NCp7 per complex were found. This clearly excludes any cooperative binding of NCp7 to its cellular partners and strengthens the hypothesis that NCp7 may bind rather uniformly to RNA molecules in the cell. This low amount of proteins in the complexes may be explained by the high concentration of RNAs in the cell and the fact that in all experiments, we used cells displaying low to moderate expression of NCp7-eGFP. Moreover, the absence of cooperative binding of NCp7 to RNAs is in agreement with *in vitro* measurements showing, at best, a moderate cooperative binding of NCp7 to model RNAs [85,86]. Finally, RICS (Figs. 6–7) and FCCS experiments (Fig. 8) together with sucrose gradient fractionation data (Fig. 9) indicated that NCp7 was associated, in the cytoplasm, with ribosomes, probably via a binding with rRNAs, as the latter represent about 80% of all RNA in HeLa cells [79]. Therefore, as a non specific RNA binding protein, NCp7 will have a strong probability to bind to rRNAs. In addition, a number of ribosomal proteins including RpL26 were identified as NCp7 potential cellular partners in screens based on affinity tagging purification combined with mass spectrometry [87], clearly indicating that they potentially constitute with rRNA, additional binding sites for NCp7 in the ribosomes. Thus, ribosomes appear to be a major target for NCp7. Along this line, the mobile and immobile fractions of complexes seen by FRAP in the cytoplasm may tentatively be attributed to free and membrane-bound ribosomes, while the high concentration of NCp7 in the nucleoli may be related to the high molecular weight precursors of ribosomes in this compartment. Moreover, the binding of NCp7 to these high molecular weight precursors in the crowded environment of the nucleoli may well explain the low diffusion coefficients observed in this compartment. Since in the ribosome biogenesis, the ribosome precursors need to shuttle through the nucleoplasm to reach the cytoplasm, the complexes of NCp7 with these ribosome precursors may belong to the RNase-sensitive population of complexes seen by FRET/FLIM in the nucleoplasm.

In conclusion, our data indicate that NCp7 expressed in HeLa cells shows a binding tropism for RNAs, notably for rRNAs in ribosomes. It can thus be proposed that NCp7 leaving the RTC during and/or after viral DNA synthesis by RT in newly infected cells will first bind to ribosomes. As a consequence, NCp7 may influence protein synthesis, thus contributing to the infection process. Experiments are in progress to further characterize the interactions of NCp7 with ribosomes and ribosomal proteins, in order to tackle the new potential role of NCp7 in HIV-1 infection and replication.

Supporting Information

S1 Table. Effect of fixation and cell permeabilization on eGFP and NCp7-eGFP lifetimes. In the absence of Sytox Orange labeling, the distribution and lifetime values of eGFP are homogeneous all over the nucleus, so that nucleoli cannot be identified. Therefore, only one value is represented for the whole nuclear compartment.
(DOC)

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Author Contributions

Conceived and designed the experiments: HA EB BK PR ER HdR YM. Performed the experiments: HA NT EB LR HK. Analyzed the data: HA EB LR HK. Wrote the paper: HA EB LR YM.

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General Conclusions and Future Perspectives

The nucleocapsid protein NCp7 has been extensively studied in recent years for its role as a nucleic acid chaperone. This chaperone activity is responsible for its well-known role in reverse transcription in the early phase of the viral life cycle. NC is also implicated as a domain of Gag Gag(NC) in the late phase of the viral life cycle, participating in RNA packaging, assembly, and viral particle budding. However, a lot of questions are still debated especially regarding the fate of NC after reverse transcription and its possible implication in regulating translation and packaging. Since NC is implicated in gRNA packaging via its binding to the ψ domain of the HIV-1 5'-UTR and since gRNA acts also as a messenger RNA, Gag(NC) is suggested to play a role in regulating translation and packaging (Anderson & Lever 2006).

During the course of my PhD, in an attempt to address the above questions, I worked on two different projects. While my first project focused on the identification of new NC cellular protein partners and the understanding of HIV-1 IRES dependent translation, the second project focused on the cellular distribution and fate of the incoming virus NCp7 after reverse transcription.

In my first project, with the aim of better understanding the role of NCp7, we looked for cellular partners of NC. Among the identified partners, we selected Unr, known to be implicated in regulation of translation driven by a number of viral and cellular IRESs. By using a dual luciferase assay in cells, we demonstrated for the first time that Unr acts as an ITAF increasing the HIV-1 IRES activity when overexpressed in cells. This is interesting as the HIV-1 IRES is known to be poorly active in rabbit reticulocyte lysates (RRL) unless supplemented with G2/M arrested HeLa cell extracts reflecting the need of cellular factors which are overexpressed in G2/M (Brasey et al. 2003). Interestingly, Unr is known to be overexpressed in the G2/M phase of the cell cycle (Tinton et al. 2005; Schepens et al. 2007). In this context, one could wonder if Unr is essential for the HIV-1 IRES-dependent translation as it is for other cellular and viral IRESs. As an example, in the case of HRV and PV, IRES-dependent translation was found to be ten-fold less efficient in unr -/- ES cells than unr +/+ or unr +/- cells (Boussadia et al. 2003). On the other hand, Unr exhibited an inhibitory effect on its own IRES as unr IRES activity was two-fold higher in Unr -/- ES than Unr +/+ cells (Dormoy-Raclet et al. 2005). Furthermore, the complementation of a RRL *in vitro* translation system with purified Unr protein stimulated Apaf-1 and PITSRLE IRESs activities (Mitchell

et al. 2001; Tinton et al. 2005). In order to answer the question of Unr being essential in HIV-1 IRES, we did several experiments during this PhD work. For example, we knocked down Unr by HeLa cell transfection with a siRNA against Unr in parallel to a non-target control siRNA. This did not induce any change in IRES activity of neither Unr nor Apaf-1 IRES even though Unr is well documented to be necessary for Apaf-1 IRES activity (Mitchell et al. 2001). This negative result could be explained by an incomplete Unr knockdown. In fact, Unr is highly abundant in HeLa cells but only weakly detected by western blot. It is thus possible that the available anti-Unr antibody used in this study is unable to detect the small remaining quantity of Unr that is sufficient to potentialize HIV-1 and Apaf-1 IRESs. We thus plan to use the rabbit reticulocyte lysates in an *in vitro* translation system which does not contain Unr and in which the HIV-1 IRES is not active in order to see if its complementation with purified Unr would rescue the IRES activity.

Although Unr has been reported by several teams to act as an ITAF for PV, HRV, Apaf-1, PITSRLE IRESs, and its own IRES; its mechanism still remains not well known. It is also the case for many other ITAFs. It has been proposed that the major mode of action of ITAFs is to act as RNA chaperones stabilizing the three dimensional structure required for efficient assembly of the 48S ribosomal subunit. In this line, it was shown that Unr binding to Apaf-1 IRES changes its structure to allow PTB binding and ribosome landing. A synergy between Unr and other ITAFs was also described for several others IRESs. In this context, it was thus interesting to determine whether such a synergy may also occur in the case of HIV-1. We found that Unrip, which was described as part of the HeLa cell lysates fraction increasing poliovirus activity, also affects the HIV-1 IRES activity in synergy with Unr. As these results are still preliminary, they need to be completed and validated. As far as PTB is concerned, no effect on HIV-1 IRES was observed but we cannot exclude that PTB needs Unr to influence HIV-1 IRES. This hypothesis could be tested both in RRL and with the dual luciferase assay in cells.

Another strategy employed to further investigate if Unr is essential to the HIV-1 IRES activity, was to mutate the potential Unr binding region found at the level of nucleotides 205-215 in HIV-1 5'UTR. This sequence is characterized by a purine rich sequence surrounding a Unr binding sequence identified by a SELEX approach (Gérard Triqueneaux et al. 1999). We measured the activity and Unr-response of these mutants in a dual luciferase assay in cell. The

mutants showed different degrees of activities and response to Unr overexpression ranging from a total loss of activity (mut U and C and 211) to an increase in IRES activity and Unr response (mut212). Surprisingly, point mutations were sufficient to modify the IRES activity. This is interesting as the HIV-1 IRES activity and structure were shown to be highly resistant to deletions and mutations (Vallejos et al. 2011; Vallejos et al. 2012; Plank et al. 2013; Gendron et al. 2011). Furthermore, most of the mutations which decreased the IRES activity were also less responsive to Unr overexpression suggesting that these mutants have a lower affinity to Unr. Altogether, those data imply that HIV-1 IRES activity is highly dependent on Unr.

Since NCp7 and Gag(NC) bind to the HIV-1 5'-UTR and act as RNA chaperones, we studied NCp7 and Gag(NC) influence on IRES activity showing that mature NCp7 overexpression does not affect IRES activity whereas Gag increases it. This Gag effect is dependent on the Gag NC domain as a GagΔNC mutant showed only a weak stimulation. This is in line with the study published by Anderson et al in 2006 which showed that Gag, at low concentration, could stimulate its own translation with a mechanism which depends on the presence of the MA domain of Gag, independently of Gag binding to the RNA via the NC domain. It would be interesting to determine whether the stimulation they observed was due to a specific IRES stimulation. Since this study also showed that high Gag concentrations inhibit Gag translation, it would be interesting to test these conditions in our assay (Anderson & Lever 2006).

Unr ITAF activity on the HIV-1 IRES was totally abolished when NCp7 was overexpressed in contrary to Gag overexpression whose effect on the IRES is additive to the one of Unr. The fact that Unr stimulation was diminished upon NCp7 overexpression can be explained by several hypotheses, among them the possibility that the binding site of Unr on the RNA is masked by NCp7. Another explanation could be linked to the interaction observed between Unr and NCp7 (Jäger et al. 2011). We confirmed the interaction by co-immunoprecipitation and FRET-FLIM and found that NC, as a mature protein or as a domain of Gag, binds Unr when overexpressed. We showed that this interaction is mediated by RNA. The differential influence of Gag or NCp7 on Unr ITAF activity could thus be linked to the fact that the interaction between NCp7 and Unr would impede the latter from binding to the HIV-1 5'UTR or recruiting translation initiation factors (another hypothesis for the mechanism of action of

ITAFs). But Gag(NC) interaction with Unr would not be able to do so, due to its presence at low density on RNA coverage in the beginning of the late phase or due to the participation of others domains of Gag in the mechanism. Answers to these questions could be obtained by *in vitro* binding studies (gel shift assay, fluorescence anisotropy…) between purified Unr, Gag, Gag mutants, and NCp7 proteins and *in vitro* transcribed RNA.

As NCp7 overexpression seems to counteract the Unr-driven HIV-1 IRES stimulation and because in the early phase of the infection the incoming viral RNA is covered by NCp7, one would have expected that Unr knockdown should have no effect on the early phase of the infection. However, using a non-replicative VSV-pseudotyped lenti-virus infection system, we found a significant decrease of the infection after Unr knockdown. As the infection level was measured independently of any cell dependent translation process, this decrease is linked to an Unr action on any viral process from entry to integration but most likely before nuclear import. One possibility is that Unr associates with the nucleocapsid protein or the RNA to facilitate, via its potential chaperone activity, the reverse transcription in the cytoplasm.

In our model, in the early phase, when vRNA translation is not necessary, NCp7 protects the gRNA from Unr IRES stimulation effect preserving the gRNA for reverse transcription. In this phase however, Unr is necessary for an optimal infection. This role could be linked to the chaperone activity of Unr as well as to the proximity of the Unr binding sites to the PBS binding site on the HIV-1 5'UTR. In the late phase, since HIV-1 depends on both cap dependent and IRES dependent translation to translate viral proteins during infection as evidenced by Amorim et al., it could be possible that in the early hours of infection, translation proceeds by cap dependent translation allowing synthesis of sufficient Gag protein to activate the IRES dependent translation when the cap dependent translation is shut off by a G2/M block. This block has the consequence of increasing Unr translation which could thus, with the help of other cellular (Unrip, …) or viral (Gag) factors, stimulate the translation of most of the viral messenger RNA which all harbor HIV-1 5'UTR IRES.

Several experiments should be done to complete and to pursue this work in order to:

1. confirm a direct binding between Unr and HIV-1 5'_IRES. We will test recombinant Unr binding to the HIV-1 5'-UTR and its mutants by electrophoretic mobility shift assay. The

specific loss of interaction between Unr and the mutants will be validated by the determination of the mutant's structure by SHAPE analysis. We will also map Unr binding site to the HIV-1 5'-UTR by footprinting analysis.

2- confirm Unr effect on HIV-1 IRES. We will work with an *in vitro* rabbit reticulocyte lysate system. Since it is well documented that the HIV-1 IRES is not active in rabbit reticulocyte lysates unless HeLa cell extracts are added, we will test the effect of RRL complementation with purified Unr on HIV-1 IRES activity. This system will also allow us to test combinations of Unr with several viral and cellular proteins such as NCp7, Gag (NC), Unrip….

3- validate the role of Unr on the HIV infection. We will collaborate with a team which works with WT virus in order to test the overexpression and knock down of Unr in both the early and late phases of the infection.

4- study the role of Unr in the early phase of the viral life cycle by looking for the effect of Unr and NCp7 in reverse transcription.

In parallel, I was working on a second project focusing on the fate of NCp7 after its participation, especially through its chaperone role, to the reverse transcription process. In fact, due to the lower affinity of NCp7 for double stranded DNA (dsDNA) in comparison to single stranded DNA (ssDNA) and RNA, it is believed that a large fraction of NCp7 is released from the RTC in the cytoplasm during reverse transcription and only a small fraction remains associated to the PIC. The NCp7 that is still associated to the PIC could thus enter, as a part of the PIC, into the nucleus where it could, participate in the viral DNA integration mediated by IN (Buckman et al. 2003; Carteau et al. 1997; Carteau et al. 1999; Poljak, Susan M. Batson, et al. 2003; Thomas et al. 2006).

We studied, using a combination of advanced quantitative fluorescence microscopy techniques, the cellular distribution of NCp7 by using a model based on the overexpression of eGFP-labelled NCp7 in HeLa cells.

Taken together, our data support a model whereby NCp7 dissociates from the reverse transcription complex after the completion of viral DNA synthesis. After that, it diffuses in

the cytoplasm, binding to RNAs and ribosomes. Additionally, NCp7 enters the nucleolus by an unknown mechanism where it likely participates in the integration of the viral DNA. Although in HeLa cells, NCp7 is capable of entering the nucleus without the need of the pre-integration complex, it could be possible that, in the context of viral infection, small amounts of NCp7 stay associated with the PIC and hence enter the nucleolus.

Since our model was based on the overexpression of NCp7, it would be interesting to study NCp7 distribution in a context that mimics a viral infection. One idea is to track incoming viral particles labeled at the level of the nucleocapsid protein, by for example using the tetra-cysteine/ FlAsH approach (Pereira et al. 2011).

The association of NCp7 with the ribosome suggests a possible role of NCp7 in translation or ribosome biogenesis since it is associated with RNA in the nucleoli known to be rich in ribosomal RNA and to be the localization of ribosome assembly. Other studies in our lab have demonstrated the association of NCp7 and Gag(NC) with the ribosomal protein RPL7 (El Mekdad Hala, thesis manuscript). Studies are in progress to characterize the interaction between NCp7 and Gag(NC) and the ribosomal proteins and determine the role of Gag(NC) and/or NCp7 in the translation.

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French Summary

Humaine (VIH) et le Syndrome d'ImmunoDéficience Acquise (SIDA)

Le Virus de l'Immunodéficience Humaine, ou VIH, est un virus propre à l'Homme, responsable du Syndrome d'ImmunoDéficience Acquise. Le SIDA est en fait le dernier stade de l'infection par le virus, et donc le plus symptomatique, précédant le décès. Depuis sa découverte, il a été responsable de plus de 36 millions de morts à travers le monde (Chiffres OMS – octobre 2013). On parle de pandémie globale.

En 2012, environ 35 millions de personnes vivaient avec le VIH, sachant que le nombre de nouvelles infections annuelles était de 2,5 millions. En France, les politiques de prévention et de dépistage permettent de maintenir un nombre annuel de nouvelles infections stable à 7 000.

Cette maladie a été découverte aux Etats-Unis au début des années 1980, sans en connaître alors l'agent infectieux. Les symptômes étaient ceux retrouvés lors de maladies opportunistes telles que la toxoplasmose cérébrale, la pneumocystose, la tuberculose, le sarcome de Kaposi … Le fait que l'on retrouve, au début de l'épidémie, ces symptômes principalement chez les homosexuels a généré de nombreux préjugés, et notamment le fait que l'on qualifie cette maladie de « gay cancer ». En 1983, Françoise Barré-Sinoussi et Jean-Claude Chermann dans l'équipe du Pr Montagnier à l'Institut Pasteur de Paris identifient pour la première fois le virus. Les Pr Montagnier et Barré-Sinoussi recevront le prix Nobel de médecine en 2008 pour cette découverte.

Le VIH appartient à la famille des *Retroviridae* et au genre *Lentivirus*. Dans ce genre, on trouve le FIV - *Feline Immunodeficiency Virus* - et le SIV - *Simian Immunodeficiency Virus* -, homologues chez l'animal du VIH. On caractérisera plus tard la particule virale, mais il est important de savoir que celle-ci est capable de générer de très nombreuses mutations, à l'origine de son infectivité, mais surtout de sa résistance aux différents traitements existants. Dans ce manuscrit, nous nous intéresseront uniquement au VIH-1, car ce virus est le plus pathogène et de répartition mondiale ; en opposition au VIH-2 que l'on retrouve presque exclusivement en Afrique de l'Ouest.

Connaître l'agent infectieux à l'origine du SIDA a permis de comprendre son mode d'infection et son agressivité. Ce virus est très fragile, comme tous les virus enveloppés, il nécessite donc un contact rapproché avec l'individu pour qu'il y ait une nouvelle infection. L'infection peut avoir lieu lors d'un rapport sexuel non protégé, au contact direct des muqueuses ; lors d'une transfusion sanguine, notamment par les usagers de drogues par voie intraveineuse (UDI), si ces derniers s'échangent leurs seringues ; ou encore lors d'un accouchement par voie basse ou de l'allaitement maternel, si la mère est infectée. Aujourd'hui, il est connu que cette maladie n'est plus réservée aux homosexuels et peut toucher n'importe qu'elle personne ayant un rapport sexuel non protégé avec une personne séropositive pour le VIH. Ceci fait du préservatif le premier moyen de prévention contre l'infection par voie sexuelle pour l'ensemble de la population.

Le VIH cible le système immunitaire de l'hôte en infectant principalement les lymphocytes T CD4+ mais également les monocytes/macrophages, les cellules dendritiques…. Après une lente déplétion en cellules immunitaires, l'organisme n'est plus capable de se défendre contre les agressions extérieures. Cette immunodépression rend le patient séropositif vulnérable aux agents infectieux responsables de maladies opportunistes. Ce sont ces dernières qui causent le décès du patient, et pas le VIH directement. Cependant

l'évolution de la maladie n'est pas prédictible et les traitements actuels retardent l'apparition du stade SIDA.

En 2013, nous sommes, en partie, capables de maitriser l'infection en combinant au moins deux des quatre catégories d'antiviraux suivantes: inhibiteurs de la transcriptase inverse, de la protéase, de l'intégrase et de la fusion ; qui ciblent tous une étape différente du cycle de réplication viral.

Cependant, la grande capacité du virus à muter engendre des mutants d'échappement résistants ; ce qui nécessite de poursuivre la recherche de nouvelles cibles et de penser à de nouvelles stratégies pour élaborer un premier vaccin dirigé contre le VIH. En effet, trente ans après la découverte du virus, aucun vaccin n'est capable de prévenir l'infection et il n'existe pas de traitement curatif. Il est uniquement possible de retarder l'apparition des symptômes en limitant l'infection de nouvelles cellules immunitaires.

C'est la raison pour laquelle de nombreux laboratoires de recherche travaillent sur le cycle de réplication du VIH ainsi que sur les interactions du virus avec les protéines cellulaires.

C'est le cas du laboratoire de Biophotonique et Pharmacologie de l'Université de Pharmacie de Strasbourg où j'ai effectué ma thèse, au sein de l'équipe de biophotonique des interactions moléculaires et cellulaires, sous la direction du Pr Yves Mely.

6.7 La structure de la particule virale

Le VIH est un rétrovirus enveloppé, de forme sphérique de 120 nm de diamètre. Il possède deux copies d'ARN génomique simple brin, de polarité positive.

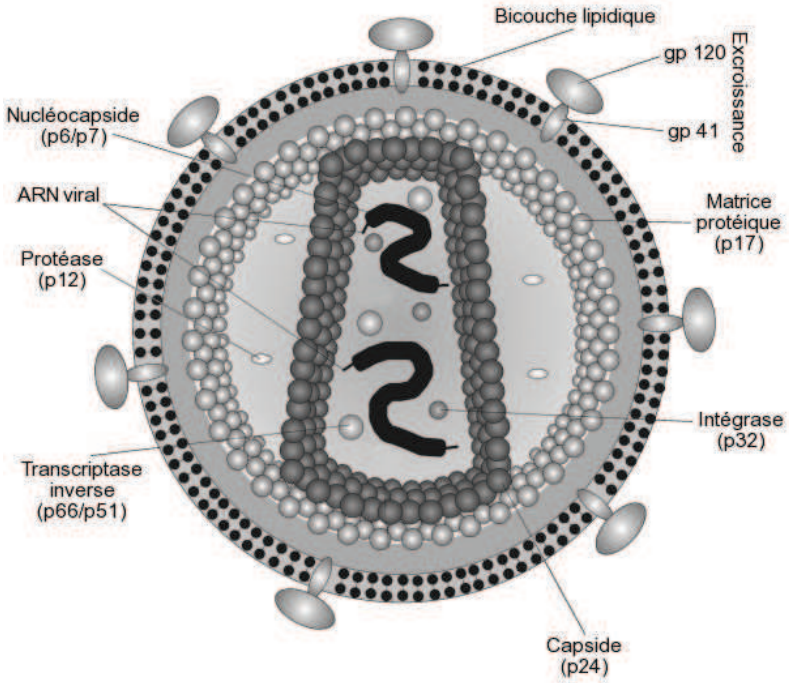


Figure 1 : Structure de la particule virale mature du VIH-1.

Le virion représenté correspond à la forme mature de la particule virale. La bicouche lipidique empruntée, à la cellule infectée, enveloppant le virus et dans laquelle sont enchâssées les spicules virales composées des deux glycoprotéines gp120 et gp41. Sous cette membrane se trouve la protéine de structure, la matrice. Au cœur de la particule, la capside de forme conique renferme une ribonucléoprotéine composée de deux copies de l'ARN génomique recouvertes de protéines de nucléocapside associées à des enzymes virales (intégrase, protéase).

De l'extérieur vers l'intérieur, on trouve **(Figure 1)** :

- L'enveloppe, composée d'une bicouche lipidique d'origine cellulaire, qui est le support des deux glycoprotéines responsables de la reconnaissance avec la cellule cible : gp120 et gp41. Ces glycoprotéines sont organisées sous forme de spicules, et vont permettre l'interaction entre la particule virale et les récepteurs et co-récepteurs cellulaires CD4, CCR5 et CXCR4 ;
- La matrice, composée de l'assemblage de protéines (MA, p17) ;
- La capside, de structure conique, propre au VIH-1, est constituée de protéines de capside (CA, p24) ;
- Deux copies d'ARN viral simple brin de polarité positive, contenant toutes les informations nécessaires à la production de nouvelles particules virales recouvertes de protéine de nucléocapside (NCp7).

La nucléocapside et l'ARN font partie du complexe nucléoprotéique qui contient également des enzymes virales essentielles telles que :

- La transcriptase inverse (RT), responsable de la rétrotranscription de l'ARN viral en ADN proviral,
- L'intégrase (IN), nécessaire pour l'intégration de l'ADN proviral à l'ADN génomique. La machinerie cellulaire effectuera la transcription de cet ADN intégré au génome cellulaire et la traduction des ARN messagers viraux (ARNm) pour permettre la production de nouveaux composants viraux,
- La protéase (PR), qui permet la maturation du virus.

Toutes ces protéines sont des cibles potentielles de recherche afin de mieux comprendre le mode d'infection du VIH-1 et de développer de nouveaux outils thérapeutiques.

6.8 Le génome viral

Le génome du VIH-1 est un ARN monocaténaire de 9kb de polarité positive. Au sein du virion, on retrouve deux copies du génome associées en dimère. Aux extrémités 5' et 3', on trouve des régions non codantes appelées UTR - *Untranslated Region* - sur l'ARN; ou LTR - *Long Terminal Repeat* - sur l'ADN proviral intégré au génome cellulaire. Ces régions LTR sont en fait des séquences synthétisées lors de la transcription inverse afin de contrôler et de diriger l'intégration au génome de la cellule hôte. Elles portent également des signaux de régulation de la transcription virale impliquant des protéines virales accessoires codées par ce même génome et des protéines cellulaires.

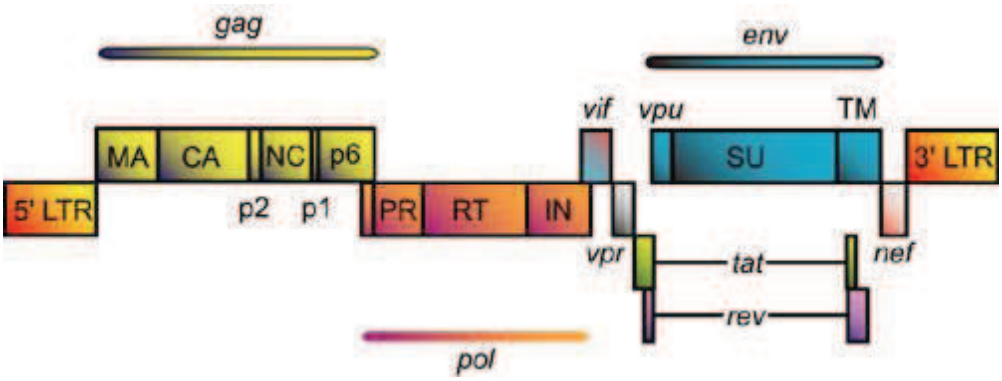


Figure 2*:* **Représentation schématique du génome du VIH-1**. Le génome porte aux extrémités deux régions non codantes. Les diverses régions codantes sont représentées par des rectangles. Les introns de Tat et Rev sont représentés par un trait plein. Les polyproteines Gag, Env et Pol sont également indiquées.

A partir de l'extrémité 5' du génome, les régions codantes sont les suivantes (**Figure 2**):

- Le gène *gag*, qui est un gène particulièrement important pour la suite de notre étude. Il code pour la polyprotéine Gag, qui sera ensuite clivée, au sein de la particule virale, par la protéase virale en protéines de structure du virus :
    - Protéine de matrice (MA),
    - Protéine de capside (CA),
    - Protéine de nucléocapside (NCp7),
- Le gène *pol*, code pour une polyprotéine clivée par la protéase virale afin de générer les enzymes virales suivantes :
    - Protéase (PR),
    - Rétrotranscriptase (RT),
    - Intégrase (IN)
- Le gène *env*, code pour une polyprotéine clivée cette fois-ci par une protéase cellulaire lors de son transport à la membrane plasmique, afin de donner les protéines suivantes :
    - Glycoprotéine gp120, protéine de surface (SU),
    - Glycoprotéine gp41, protéine transmembranaire (TM),
    - Protéine régulatrice Tat – transactivator of transcription – ayant de nombreux rôles dont celui de réguler la transcription virale *via* les séquences LTR,
    - Protéine régulatrice Rev – regulatory of virion expression – ayant un rôle dans l'export nucléaire des ARN viraux non totalement épissés.

Le génome possède aussi des gènes accessoires ou auxiliaires, codant pour les protéines Vif, Vpr, Vpu et Nef, non essentielles à la réplication dans les cellules T humaines, mais optimisant l'infectivité virale chez l'homme.

La protéine virale Gag

Gag est une polyprotéine virale de 55 kDa codée par le gène *gag*. A l'heure actuelle, le rôle de Gag est connu dans la phase tardive du cycle viral, lors des étapes d'assemblage et de bourgeonnement des particules virales (Freed, 1998).

Gag est d'abord synthétisée dans le cytoplasme où elle se lie à l'ARN génomique par ses domaines NCp7 et MA ce qui favorise sa multimérisation et sa localisation à la membrane plasmique (**Figure 3**). L'ancrage de Gag au feuillet interne de la membrane se fait *via* une myristoylation post-traductionnelle de Gag sur la Glycine en position 2. L'accumulation de protéines Gag sous la membrane plasmique sera responsable du bourgeonnement d'une particule virale immature non infectieuse. Lors de la maturation virale, Gag sera clivée par la PR afin de donner diverses protéines de structure. La majeure partie de la maturation de Gag aura lieu dans la particule virale après le bourgeonnement de cette dernière.

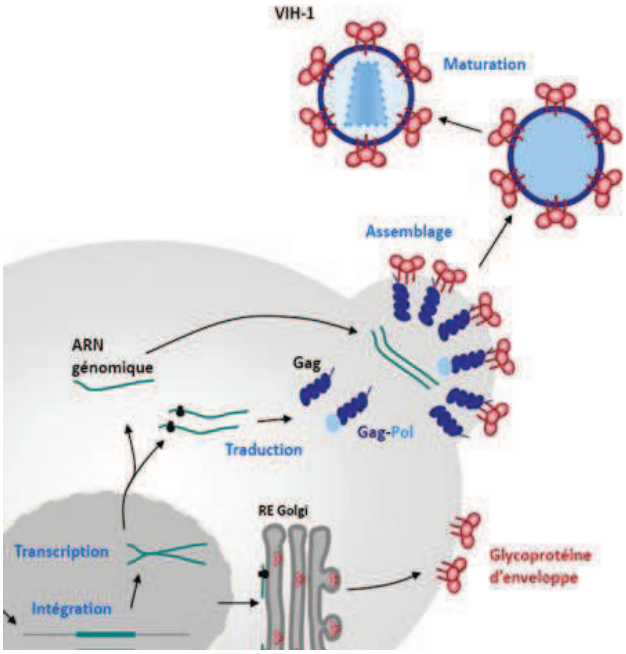
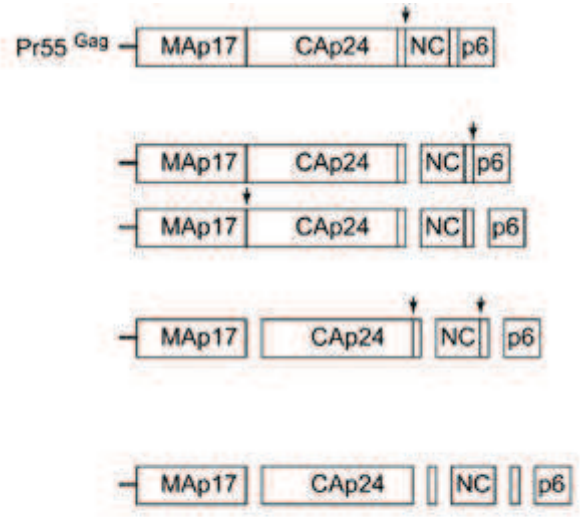


Figure 3 : Etapes tardives de la réplication de VIH-1

La polyprotéine Gag est synthétisée par des ribosomes cytoplasmiques et est retrouvée sous diverses formes dans la cellule infectée, en tant que protéine seule ou en tant que domaine dans la protéine Gag-Pol (obtenue par un décalage de phase lors de la traduction). Gag est responsable du bourgeonnement de la particule virale immature dans laquelle elle sera clivée lors de sa maturation en virion infectieux.

Au final, la maturation de Gag permet la production de plusieurs protéines de structure du virus (**Figure 4**).

- MAp17 est la protéine de matrice qui contient un domaine responsable du ciblage des radeaux lipidiques situés au niveau de la membrane plasmique cellulaire.
- La protéine de capside CAp24, compose la capside conique du VIH-1 au sein du virion
- La protéine NCp7, que l'on présentera en détail plus tard, chaperonne des acides nucléiques est impliquée dans de nombreuses étapes du cycle viral.



(Figure tirée de Ott D *et al.* (2003) J.Virol).

Figure 4 : Représentation du clivage du précurseur GagPr55.

Le clivage du précurseur GagPr55 par la PR virale a lieu dans un ordre finement régulé présenté ici, permettant d'obtenir outre les protéines MA, CA, NC et p6, deux peptides p2 et p1, et divers intérmédiaires de clivages. Figure tirée de (Ott et al, 2003).

Au sein d'une cellule infectée, il est possible de retrouver à un moment donné, Gag et ses divers produits de clivage. En effet, une cellule peut être infectée par plusieurs virus qui seront donc, à un moment donné, à des étapes différentes du cycle viral. La cellule infectée est

également une cellule productrice de nouveaux virions. Gag peut donc être retrouvée sous ses différentes formes au sein d'une cellule. Ceci est à prendre en compte lorsque l'on étudie le rôle des interactions de Gag avec des protéines cellulaires afin de ne pas se limiter à penser à un cycle viral indépendant.

La protéine virale de nucléocapside NCp7

La protéine NCp7 est retrouvée dans les cellules et le virus sous deux formes : soit sous sa forme mature (NCp7, 7 kDa), soit en tant que domaine au sein de la polyprotéine Gag (Gag-NC).

La protéine virale NCp7 est obtenue après clivage de la polyprotéine Gag par la protéase virale lors de sa maturation dans le virion. NCp7 est une protéine fortement basique, composée de 55 acides aminés, avec un poids moléculaire de 7 kDa. Elle est caractérisée par deux motifs en doigts de zinc (Zn), Cys-X2-Cys-X4-His-X4-Cys, ou CCHC, fortement conservés, qui se lient aux acides nucléiques avec une très forte affinité (Mely et al, 1994) formant alors un plateau hydrophobe à la surface de de la protéine (**Figure 5**). Ce dernier est indispensable à l'interaction avec les acides nucléiques et donc à son activité chaperonne de ces derniers. On sait aujourd'hui que toute mutation ponctuelle dans la structure du doigt de zinc affaiblit considérablement les activités de la NCp7 (Dorfman et al, 1993).

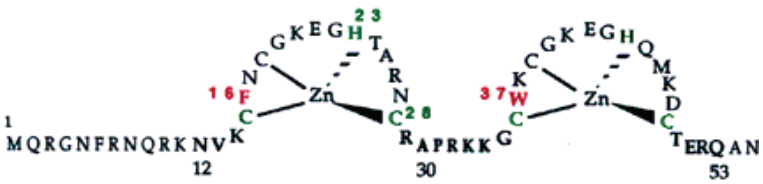


Figure 5 : Séquence protéique de la NCp7 du VIH-1.

Les résidus cystéines et histidines responsables de la liaison du zinc au sein des deux doigts de zinc de type CCHC sont représentés en vert. Les acides aminés responsables de la formation du plateau hydrophobe sont représentés en rouge.

Le cycle de réplication viral

Le cycle viral du VIH-1 est séparé en deux grandes phases : la phase précoce, allant de l'entrée du virus dans la cellule jusqu'à l'intégration du génome viral dans celui de la cellule hôte ; et la phase tardive, caractérisée par la transcription, la traduction, l'assemblage et le bourgeonnement.

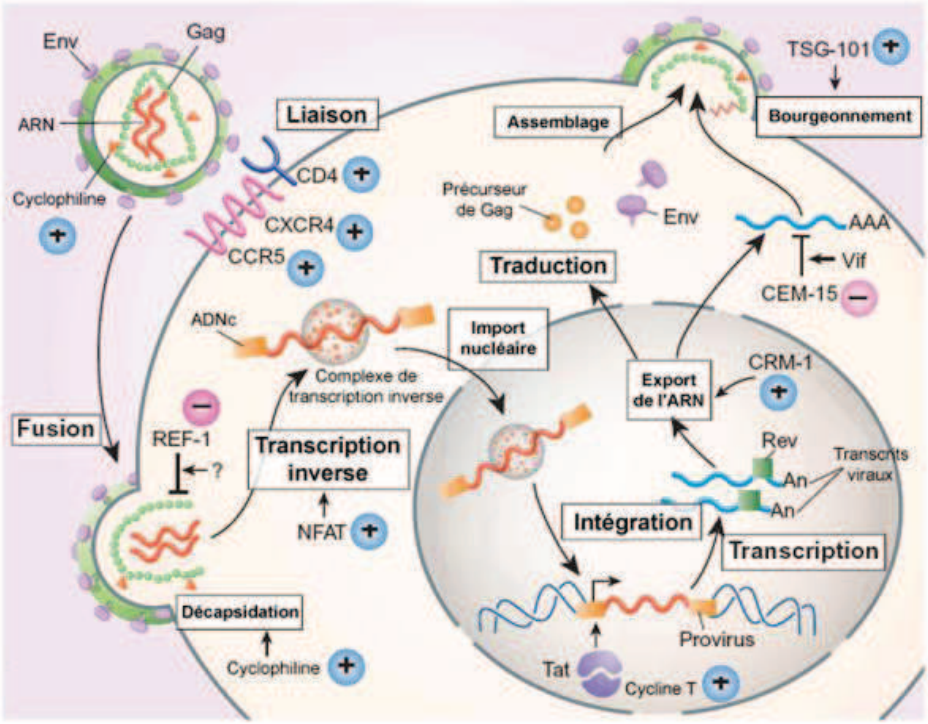


Figure 6 : Cycle de réplication de VIH-1. Le cycle du VIH-1 est divisé en phase précoce qui comporte la liaison du virus à la surface de la cellule, l'entrée du virus par fusion membranaire, la décapsidation permettant l'entrée du matériel génétique, la transcription inverse, l'import nucléaire et l'intégration. Vient ensuite la phase tardive qui consiste en la transcription des ARN messagers et génomiques viraux, leur export du noyau, leur traduction, l'assemblage des particules virales suivi du bourgeonnement de la particule et de sa maturation.

Le cycle de réplication du VIH commence par la reconnaissance de la cellule cible (**Figure 6**). En effet, les cellules du système immunitaire telles que les lymphocytes T, les cellules dendritiques et les monocytes/macrophages expriment un antigène à leur surface, le CD4. Ce dernier est reconnu par la protéine de surface virale, gp120. En plus de l'interaction gp120/CD4, il y a intervention de co-récepteurs CCR5 ou CXCR4 qui définissent le tropisme des différentes souches du virus ; car le CCR5 est préférentiellement exprimé par les lymphocytes T, les macrophages et les cellules dendritiques ; alors que CXCR4 est uniquement exprimé à la surface des lymphocytes T.

Après cette liaison, on assiste à un réarrangement des protéines de reconnaissance du virus et le peptide de fusion de la protéine transmembranaire gp41 est alors exposé, permettant la fusion de l'enveloppe virale et de la membrane plasmique. Le matériel génétique peut alors pénétrer la cellule sous forme de complexe nucléoprotéique appelé CTR, pour complexe de réverse transcription ou complexe de rétrotranscription. Ce complexe contient l'ARN génomique viral, l'$ARNt_3^{Lys}$ et différentes protéines cellulaires et virales dont MA, Nef, Vif, NCp7, IN, Tat, Vpr….

On assiste à une décapsidation progressive associée à la libération du CTR dans le cytoplasme de la cellule et à la reverse transcription de l'ARN génomique en ADN proviral. transformant alors le CTR en complexe de pré-intégration (CPI). Le CPI, outre l'ADN viral, comporte un certain nombre de protéines virales et cellulaires nécessaires à l'intégration et expréssion du génome viral.

Ce complexe est importé à l'intérieur du noyau de la cellule. Les *lentivirus* ont la particularité, contrairement aux autres rétrovirus, de voir leur materiel génétique entrer dans le noyau de cellules qui ne se divisent pas. L'ADN viral est intégré au génome cellulaire grâce à l'IN ; afin que la machinerie cellulaire permette la transcription du génome viral sous la régulation de la protéine virale Tat. Divers ARNs messagers sont produits, épissés et exportés pour la synthèse de protéines virales. Le nouvel ARN génomique est également synthétisés mais non épissé, il sera alors exporté du noyau à l'aide de la protéine virale Rev pour être soit traduit pour donner des protéines virales Gag et Gag-Pol; soit être encapsidé par Gag et transporté à la membrane pour constituer le matériel génétique de nouveaux virons.

L'assemblage nécessite la protéine Gag, qui s'auto-assemble et s'associe aux autres éléments constituant le virions : protéines virales et ARN. La protéine Gag dirige également le bourgeonnement de la particule virale qui emprunte sa membrane à la bicouche de la membrane plasmique cellulaire. La maturation concomitante ou après le bougeonnement est nécessaire afin d'obtenir des particules virales matures infectieuses. La maturation voit la morphologie du virion changer drastiquement et les protéines être clivées pour donner diverses protéines virales (MA, CA, NCp7, PR, RT, IN, …).

Rôle de la NCp7 dans le cycle viral

La forme mature est présente dans les étapes précoces du cycle viral (**Figure 7**). De par son activité chaperonne, NCp7 prend en charge les acides nucléiques afin de favoriser leur conformation biologiquement active la plus stable et ainsi réguler plusieurs étapes de la rétrotranscription (Godet et al, 2006). De plus, elle protège l'ARN viral des nucléases cellulaires et influencerait l'efficacité d'intégration de l'ADN proviral dans le génôme de la cellule hôte (Poljak et al, 2003) suggérant son association aux acides nucléiques viraux (ARN génomique, ADN proviral) tout au long de la phase précoce du cycle viral.

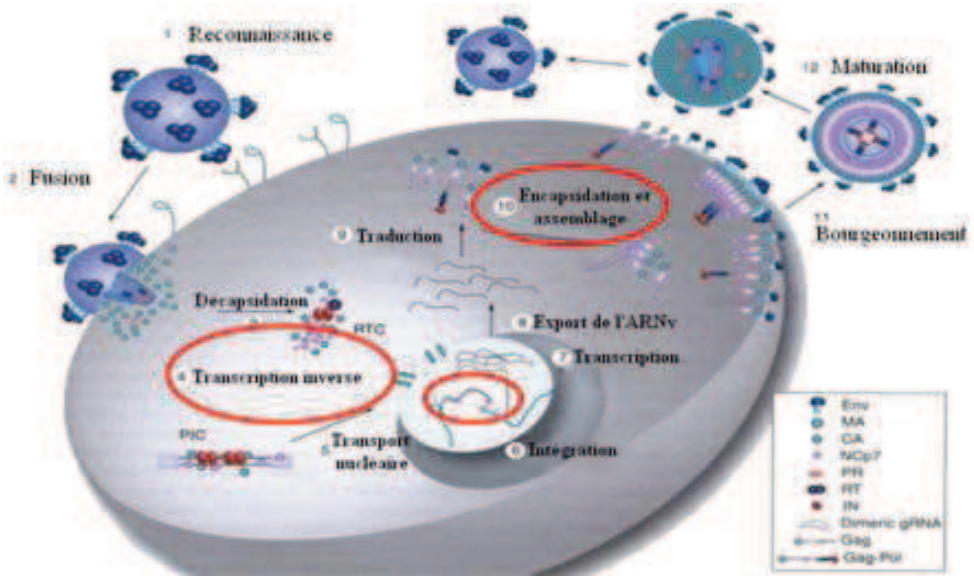


Figure 7 : Rôles de NCp7 au cours du cycle de réplication de VIH-1

La protéine NCp7 mature est présente lors des étapes précoces (1 à 6), ayant un rôle de chaperonne vis-à-vis des acides nucléiques lors de la rétrotranscrition (5) et de l'intégration (6) de l'ARN viral au génome cellulaire. Les étapes tardives (7 à 12) font intervenir NCp7 en tant que domaine de la protéine Gag.

Au sein de Gag ; NCp7 joue un rôle déterminant pour la sélection et la dimérisation du nouvel ARN viral formé. De plus, au cours de l'assemblage de Gag jusqu'à son ancrage dans la membrane plasmique, le domaine NCp7 participerait à la polymérisation des protéines Gag *via* des interactions NC/NC et la présence de l'ARN.

Cette protéine joue donc un rôle tout au long du cycle viral. Ses différents rôles majeurs ainsi que sa forte conservation de séquence au sein des différentes souches virales,

justifient le fort intérêt de la recherche d'antiviraux ciblant la NCp7. Il est important de rappeler que même s'il est facile d'associer un type d'activité à chacune des formes de NCp7 (mature ou Gag-NC), une cellule infectée contiendra de la NC sous ses deux formes en plus ou moins grande quantité au cours de l'infection.

Les IRES de VIH-1 et les ITAFs cellulaires

Chez les cellules eucaryotes, dans les conditions normales, la synthèse de nouvelles protéines se fait par une traduction des ARN messagers coiffe-dépendante. En effet, la grande majorité des ARN cellulaires portent une protéine de coiffe à leur extrémité 5' ajoutée après la transcription. Cette coiffe est reconnue par la protéine de coiffe eIF4E à laquelle s'associe un ensemble de facteurs d'initiation, les eIF - *Eukaryotic Initiation Factors* - qui recrutent le ribosome. Ce dernier va parcourir l'extrémité 5' non codante jusqu'au premier signal de début de traduction, un codon AUG, codant pour une méthionine. Il synthétise alors la protéine en incorporant les acides aminés dans l'ordre 5'-3' de la chaîne nucléotidique, jusqu'au codon de fin de traduction (**Figure 8**).

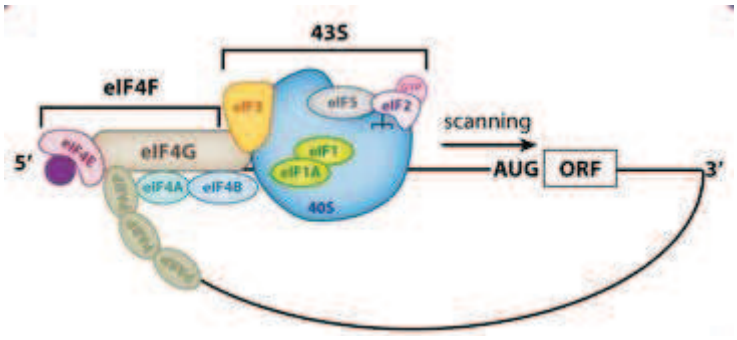


Figure 8: Représentation schématique de l'initiation coiffe-dépendante de la traduction

La traduction coiffe-dépendante débute en 5' grâce aux recrutements de facteurs d'initiation (eIF) et permet le recrutement du ribosome. La traduction commence au premier codon AUG et se termine au codon STOP. La circularisation de l'ARN par l'interaction eIF4G/PABP permet d'augmenter l'intensité de la traduction.

Il existe un second type de traduction qui permet de s'affranchir de tout ou partie des eIFs, on parle de traduction coiffe indépendante qui dépend de la présence d'IRES - *Internal Ribosome Entry Site*, ou site d'entrée interne des ribosomes. Le recrutement est rendu possible par la présence de nombreuses structures secondaires au sein des IRES permettant le recrutement du ribosome en s'affranchissant, suivant le type d'IRES, de tout ou partie des eIFs.

Les IRES ont été tout d'abord mis en évidence sur des ARNm viraux. Certains virus dont les ARNms ne sont pas coiffés, comme les picornavirus, utilisent exclusivement ce type de traduction tout en inhibant la traduction coiffe-dépendante en clivant certains facteurs d'initiation (eIF4G pour les picornavirus) indispensables pour la traduction coiffe-dépendante mais pas pour celle dirigée par leurs IRES. D'autres virus utilisent les deux types de traduction et peuvent également bloquer la cellule en phase G2/M (cas du VIH-1) ce qui inhibe la traduction coiffe dépendante. Toutes ces stratégies permettent de favoriser la traduction des ARNs viraux au détriment des ARNs cellulaires (Chamond et al, 2010).

Le virus du VIH-1 présente dans son génome deux IRES (**Figure 9**) : le premier IRES est situé dans la région 5' non codante (IRES 5' UTR), le second dans la région codante du gène de Gag (Vallejos et al, 2012; Vallejos et al, 2011).

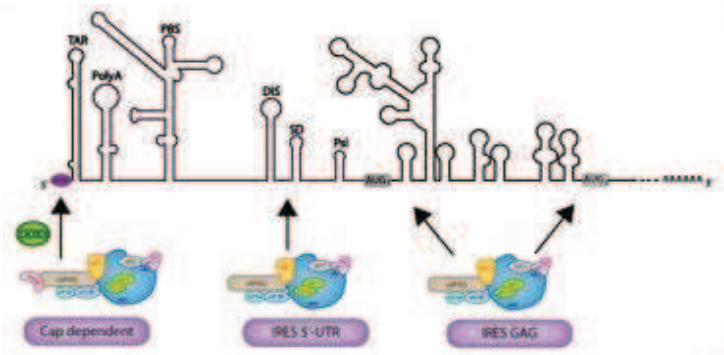


Figure 9 : Représentation schématique de l'extrémité 5' de l'ARN génomique de VIH-1

Le VIH-1 utilise les deux types de traduction : soit en recrutant les ribosomes au niveau de la coiffe située à l'extrémité 5' de l'ARN viral soit au niveau de deux structures internes appelées IRES (IRES 5'UTR ou IRES Gag). Les deux traductions ne faisant pas appel aux mêmes facteurs d'initiation.

Des IRES ont également été identifiés sur des ARN cellulaires, principalement sur des ARNm codant des protéines de stress ou exprimées lorsque la traduction coiffe dépendante est inhibée. Les deux types de traduction font intervenir de nombreux facteurs régulateurs. En plus de certains facteurs d'initiation également utilisés par la traduction coiffe-dépendante, les IRES utilisent des ITAFs - *IRES Transactivating Factors* – qui sont des protéines liant l'IRES et présentant une activité chaperonne pour ces ARNs, permettant de modifier leur conformation afin d'optimiser son interaction avec le ribosome. Chaque type d'IRES fait appel à un ou plusieurs ITAFs particuliers.

Dans ce projet, nous nous sommes intéressés à l'un de ces ITAF, la protéine cellulaire Unr, ou *Upstream of N-Ras*.

La protéine cellulaire Unr

Unr, ou *Upstream of N-ras* est une protéine cytoplasmique de 85 kDa, exprimée de façon ubiquitaire dans les cellules mammaliennes. Des homologues d'Unr sont retrouvés chez la drosophile, le ver *C. elegans* et le poisson zèbre. Elle se lie à l'ARN grâce à 5 domaines de liaisons, appelés Cold Shock Domain (Triqueneaux et al, 1999) répartis régulièrement tout au long de la séquence (**Figure 10**).

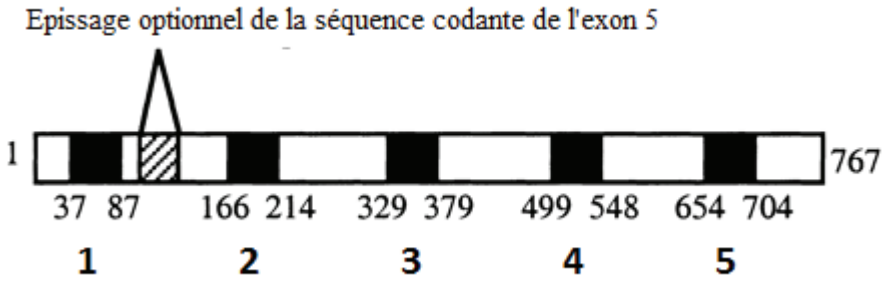


Figure 10 : Représentation schématique de la protéine Unr.

Les 5 régions Cold Shock Domain (CSD) sont représentées en noir et réparties régulièrement au sein de la protéine. Un site d'épissage alternatif est également représenté expliquant l'identification de deux isoformes de la protéine Unr chez la souris.

Il a été mis en évidence que la protéine Unr agit comme un ITAF pour les IRES du poliovirus et du rhinovirus (Hunt et al, 1999) stimulant leur activité. Unr est également capable de réguler la traduction de son ARNn en inhibant cette dernière via la présence d'un IRES dans la région 5' non codante de l'ARNm d'Unr. La présence de cet IRES lui permet d'être surexprimée en phase G2/M du cycle cellulaire.

D'autres rôles ont été attribués à Unr comme la régulation de la traduction coiffe dépendante mais aussi de la stabilité des ARN messagers *via* des mécanismes traduction dépendant (Chen & Shyu, 2003; Grosset et al, 2000).

Objectifs

La protéine de nucléocapside (NCp7) du virus de l'immunodéficience humaine de type 1 (VIH-1) est présente dans la cellule infectée et la particule virale soit en tant que domaine de la polyprotéine Gag (55kDa) soit sous sa forme mature (NCp7, 7kDa) après le clivage de Gag lors de la maturation virale. La protéine NCp7 joue un rôle important lors de plusieurs étapes du cycle viral dont la transcription inverse, l'intégration du génome viral, le recrutement des acides nucléiques (AN), l'assemblage et le bourgeonnement viral. Son implication fonctionnelle multiple ainsi que la grande conservation de sa séquence au sein des différentes souches virales font de la NCp7 une cible de choix pour le développement de nouveaux anti-rétroviraux. L'étude des interactions entre la NCp7 et des protéines cellulaires est essentielle pour la compréhension des mécanismes impliquant la NCp7 dans le cycle viral. Trois cribles pangénomiques basés sur des siRNA ont permis l'identification d'un ensemble de protéines cellulaires potentiellement importantes dans l'infection par le VIH-1. De plus, un crible basé sur l'immunoprécipitation de complexes protéiques analysés par spectrométrie de masse a permis d'identifier les protéines cellulaires associées à chaque protéine du VIH-1 surexprimée individuellement en cellule. A partir de ces données, nous avons sélectionné une série de protéines nucléaires et nucléolaires. Leur co-localisation avec la protéine NCp7 fut dans un premier temps vérifiée par microscopie confocale en sur-exprimant transitoirement les diverses protéines en fusion à des étiquettes fluorescentes (eGFP ou mCherry). Dans un second temps, l'interaction des protéines les plus intéressantes avec la NCp7 fut testée par co-immunoprécipitation ou par FRET (Fluorescence Resonance Energy Transfer) visualisée par FLIM (Fluorescence Lifetime Imaging Microscopy).

Ces analyses ont permis d'identifier la protéine ubiquitaire, cytoplasmique, « upstream of N-ras » (Unr, CSDE1) comme partenaire de NCp7. Les objectifs de cette thèse sont de valider et caractériser l'interaction NCp7/Unr et de déterminer le rôle fonctionnel d'Unr au cours de l'infection.

Résultats

Unr interagit avec NCp7

Nous avons dans un premier temps démontré une co-localisation entre NCp7 et Unr surexprimées en cellules HeLa en fusion à des étiquettes fluorescentes. La localisation de la protéine mCherry-Unr est cytoplasmique et correspond à celle décrite pour la protéine endogène Unr. Elle colocalise dans ce compartiment cellulaire avec la protéine NCp7-eGFP suggérant une possible interaction entre les deux protéines. Nous avons alors montré l'interaction entre NCp7 et Unr par des expériences de co-immunoprécipitation (co-IP). Dans un premier temps, nous avons montré que la protéine NCp7-eGFP co-immunoprécipite avec la protéine Flag-Unr (Figure 11). Cette interaction a été confirmée par la co-IP de Flag-Unr lors de l'immunoprécipitation de NCp7-eGFP. L'interaction NCp7/Unr est ARN dépendante comme le prouve des expériences de co-IP sur des lysats cellulaires traités à la RNase. Pour valider ces données, nous avons montré qu'une protéine NCp7 synthétique biotinylée et couplée à des billes de streptavidine était capable de précipiter la protéine Flag-Unr à partir de lysats de cellules HeLa surexprimant Flag-Unr. En utilisant les mêmes techniques, nous avons tenté de détecter une interaction entre NCp7-eGFP surexprimée en cellules ou la protéine synthétique biotine-NCp7 et la protéine endogène Unr sans succès pour l'instant. Ceci peut, en partie, être expliqué par le fait que les anticorps anti-Unr disponibles commercialement ne sont capables de détecter que très médiocrement Unr dans des lysats de cellules HeLa ou 293T.

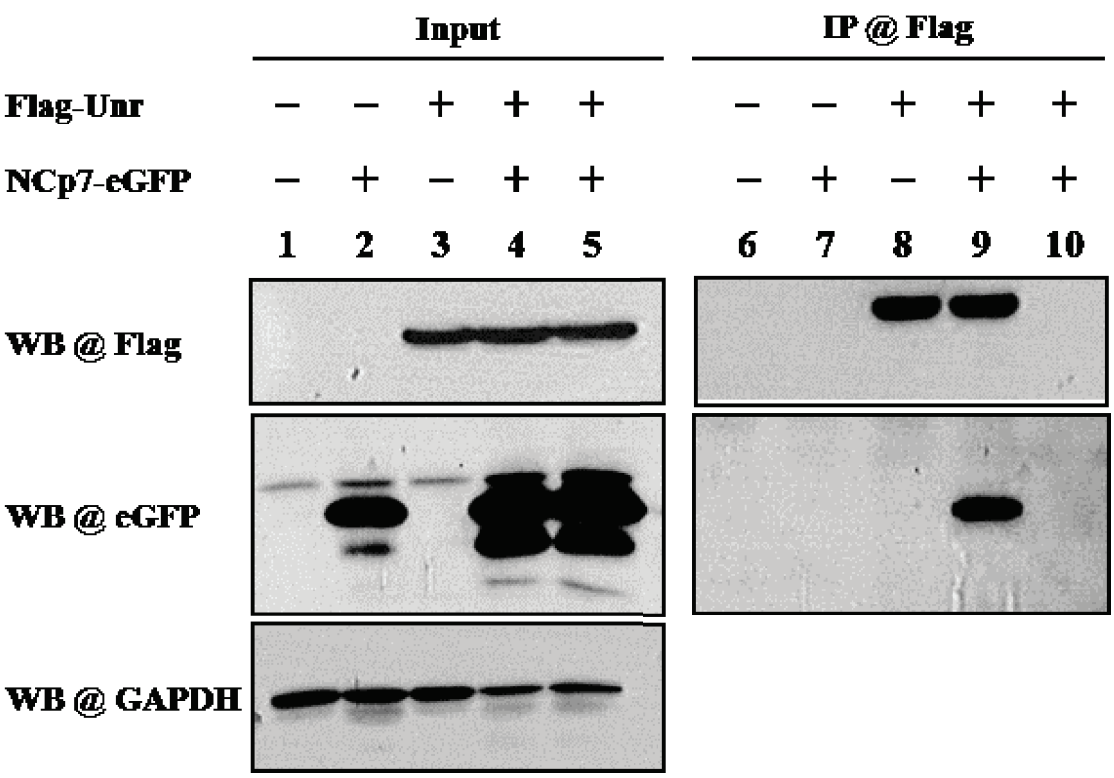


Figure 11: Co-immunoprecipitation de NCp7 et Unr. (A) Co-immunoprecipitation de NCp7-eGFP avec Flag-Unr. Des lysats de cellules 293T exprimant diverses combinaisons de protéines d'intérêt après transfection transitoire ont été soumis à des immunoprecipitation par des billes couplées à la protéine A liés à un anticorps anti-Flag. Les immunoprécipitats ont été analysés par gel d'acrylamide SDS-PAGE et révelés par Western blot à l'aide d'anticorps anti-eGFP et anti-Flag dilués au 1/10000 et 1/4000 respectivement. L'homogénéité des dépots a été vérifiée par un anticorps anti-GAPDH dilué au 1/5000.

Pour confirmer l'interaction observée par co-IP, nous avons réalisé des expériences de FRET sur des cellules vivantes co-exprimant Unr et NCp7 fusionnées à eGFP et mCherry comme donneur et accepteur de FRET, respectivement. Une interaction entre les deux protéines induit une diminution du temps de vie du donneur de fluorescence (eGFP) permettant la visualisation du FRET par FLIM. Nous avons observé une diminution significative du temps de vie moyen de fluorescence de la protéine eGFP-Unr en présence de NCp7-mCherry, indiquant l'existence de FRET entre les deux protéines marquées. Des résultats similaires ont été obtenus avec mCherry-NCp7 comme accepteur et eGFP-Unr

comme donneur. Ces résultats montrent l'existence d'une interaction directe entre les deux protéines.

Comme NCp7 est également présente sous forme de domaine au sein de la protéine Gag, nous avons étudié l'interaction Gag/Unr. Cette interaction est fortement probable puisque Gag est localisée dans le cytoplasme avant sa migration vers la membrane cytoplasmique où elle organise l'assemblage et au bourgeonnement du virus. Nous avons dans un premier temps observé par microscopie confocale la co-localisation de Gag et d'Unr dans le cytoplasme de cellules sur-exprimant les deux protéines. Une co-IP de Gag ou de Gag-G2A (mutant de myristoylation de Gag défectif pour une interaction stable de Gag avec la membrane plasmique cellulaire) a été observée lors de l'immunoprécipitation de Flag-Unr. Une proportion plus importante de Gag-G2A que de Gag est par ailleurs trouvée associée à Unr soulignant la localisation essentiellement cytoplasmique de cette interaction. L'observation de FRET entre eGFP-Unr et Gag-G2A a permis de valider cette interaction. Donc, à la fois NCp7 sous forme mature et la protéine Gag sont capables d'interagir avec la protéine Unr dans le cytoplasme.

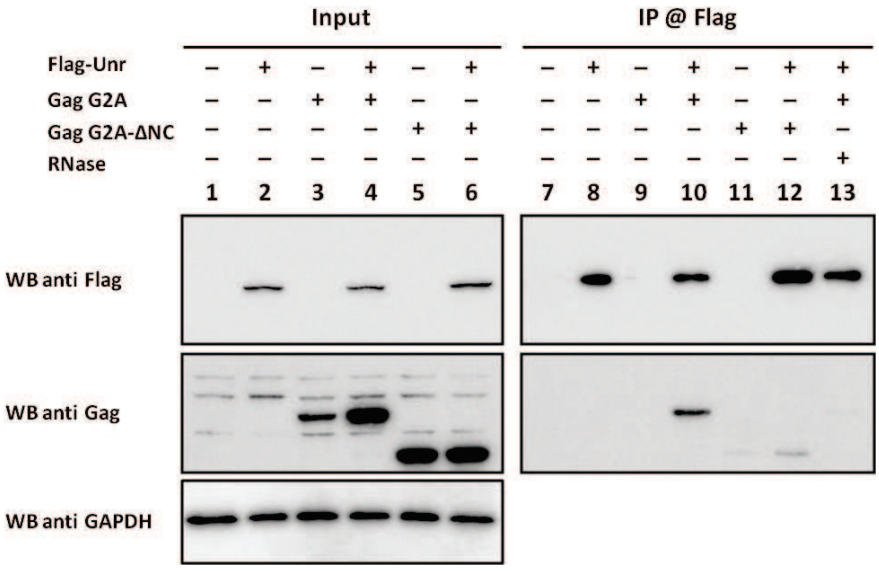


Figure 12: Co-immunoprecipitation de GagG2A avec Flag-Unr. Des lysats de cellules 293T exprimant diverses combinaisons de protéines d'intérêt après transfection transitoire ont été soumis à des immunoprecipitation par des billes couplées à la protéine A liés à un anticorps anti-Flag. Les immunoprécipitats ont été analysés par gel d'acrylamide SDS-PAGE et révelés par Western blot à l'aide d'anticorps anti-Gag dilués au 1/10000. L'homogénéité des dépots a été vérifiée par un anticorps anti-GAPDH dilué au 1/5000.

Afin d'analyser l'implication fonctionnelle de la protéine Unr dans l'infection par le VIH-1, nous avons utilisé un modèle de pseudoparticules lentivirales non replicatives pseudotypées par la glycoprotéine du virus de la stomatite vésiculeuse (VSV). Ce modèle permet d'étudier les phases précoces de l'infection post-entrée jusqu'à l'intégration. L'efficacité d'infection est mesurée par l'activité luciférase des cellules infectées résultant de l'expression du pseudo-génome viral, codant la luciférase, intégré au génome de la cellule. L'extinction de l'expression d'Unr par des siRNA montre une diminution significative de l'infection sur l'infection par le virus.

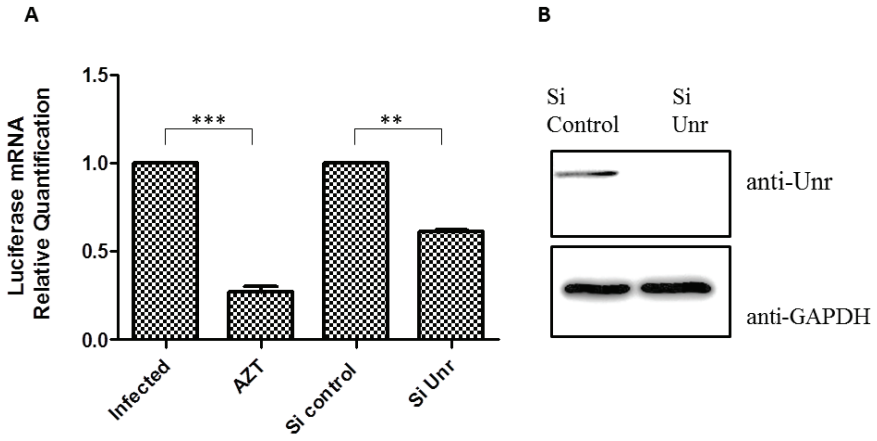


Figure 13: L'extinction d'Unr inhibe l'infection par un pseudovirus HIV-1. (A) Quantification par RT-qPCR de l'ARNm codant la Firefly luciférase (Fluc) après infection de cellules HeLa par un pseudovirus codant Fluc. Des cellules HeLa ont été soit : non traitées, traitées par de l'AZT, transfectées par un siARN contôle ou un siARN contre Unr avant infection. Les histogrammes représentent la quantification de l'ARNm codant la luciférase normalisée sur celle de l'ARNm du gène ménager 18S. La quantification a été réalisée par la méthode des delta Ct. Les histogrammes représentent la moyenne ± erreur type de la moyenne de 3 expériences indépendantes réalisées en triplicats **, $p < 0.01$; ***, $p < 0.001$. **(B)** Contrôle par western Blot de l'extinction d'Unr après traitement par siARN. Les infections ont été réalisées 48 heures après transfections des siARN. Les lysats cellulaires ont été soumis à un gel SDS-PAGE suivi d'un western blot contre Unr (anticorps dilué au 1/1000) ou GAPDH (anticorps dilué au 1/5000) pour le contrôle des dépots, 48 heures post-infection.

Afin d'étudier l'influence d'Unr sur l'activité de l'IRES 5'-UTR VIH-1, un test de « dual-luciférase » a été réalisé. Ce test est basé sur une construction plasmidique dans laquelle l'IRES 5'-UTR VIH-1 est placé entre le gène de la Renilla luciférase (Rluc) et celui de la Firefly luciférase (Fluc). Les activités luciférases mesurées dans ce test permettent

d'exprimer la fonctionnalité de l'IRES en calculant le ratio Fluc/Rluc, reflet de la traduction IRES-dépendante (Fluc) normalisée par l'activité de la traduction coiffe-dépendante (Rluc). Nous avons utilisé deux variants du de l'IRES du VIH-1 le premier correspondant à la souche de laboratoire pNL4.3 (dlVIH-1) et le second issu d'une souche isolée chez un patient infecté (VAR2). La sur-expression d'Unr en cellules HeLa a pour conséquence d'augmenter spécifiquement les traductions dépendantes des IRES dlVIH-1 et VAR2.

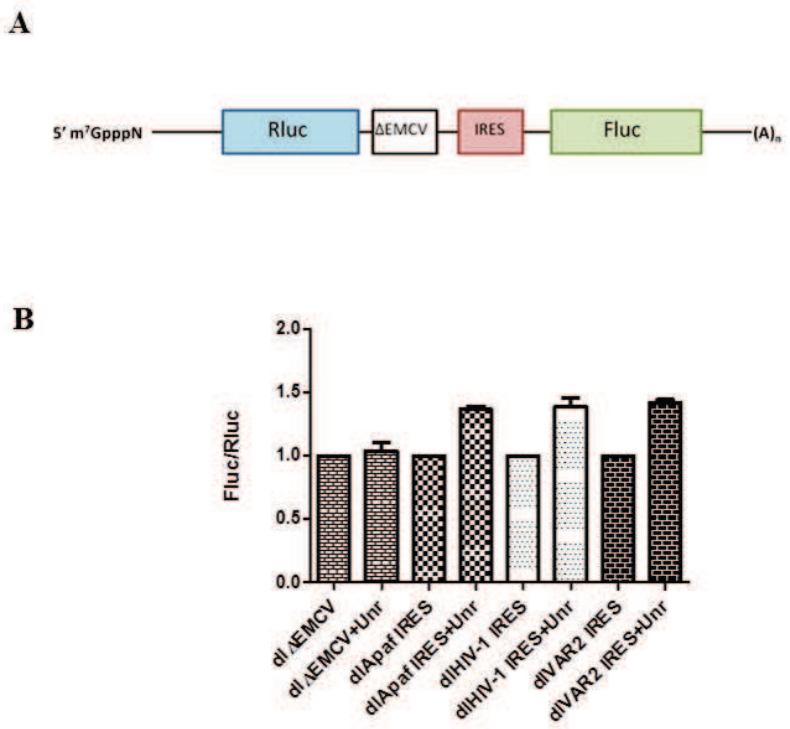


Figure 14: Ativité de l'IRES mesurée par le test de dual luciferase. (A) Schema de la construction utilisée pour réaliser le test de dual luciferase. L'IRES est inséré dans la construction de dual luciferase (dl) entre le gène codant la Renilla luciférase (Rluc) et celui codant la firefly luciférase (Fluc).

Cet effet est inhibé par la co-expression de NCp7 mais pas par celle de la protéine Gag. Cette inhibition pourrait être la conséquence d'une compétition entre NCp7 et Unr pour

la séquence IRES qui n'aurait pas lieu avec Gag. Par ailleurs, comme pour l'infection virale, l'extinction de l'expression d'Unr par siARN n'a pas d'effet sur l'activité de l'IRES. Ceci pouvant être expliqué par une imparfaite extinction d'Unr difficilement détectable par Western-Blot à cause de la mauvaise sensibilité des anticorps commerciaux disponibles.

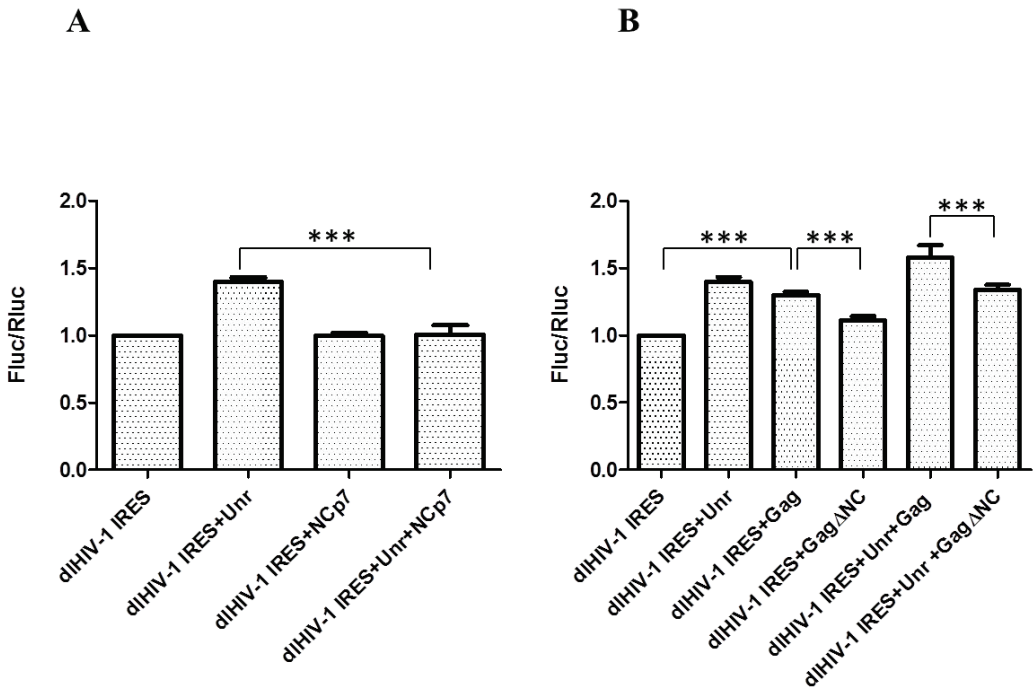


Figure 15 : Effet de la NCp7 (A) et de Gag ou de GagΔNC (B) sur l'activité de l'IRES du VIH-1 en présence ou en absence de la surexpression d'Unr. L'activité de l'IRES est exprimée par le ratio des activités des luciférases Fluc/Rluc mesurées dans le test de dual luciférase. Les mesures ont été réalisées sur des lystas de cellules HeLa 24 heures post transfection. Les histogrammes représentent la moyenne ± erreur type de la moyenne de 3 expériences indépendantes réalisées en triplicats ***, $p < 0.001$.

Nous avons identifié dans l'IRES 5'-UTR VIH-1 une séquence (nt 210-216 sur pNL4.3) correspondant au motif de liaison préférentielle d'Unr correspondant à des séquences polypurines sur des sections monobrins d'ARN entourant des séquences consensus de type AAGUA et AACG (Triqueneaux et al, 1999). Plusieurs mutations ponctuelles ont été

introduites dans cette séquence au sein de la construction « dual-luciférase » dlVIH-1 et l'activité des IRES obtenus a été testée. Toutes les mutations de ce site potentiel de liaison d'Unr diminuent la capacité de l'IRES à diriger une traduction coiffe-indépendante (Figure 16). Ces résultats sont en accord avec l'hypothèse de l'activité d'ITAF d'Unr pour l'IRES du virus VIH-1.

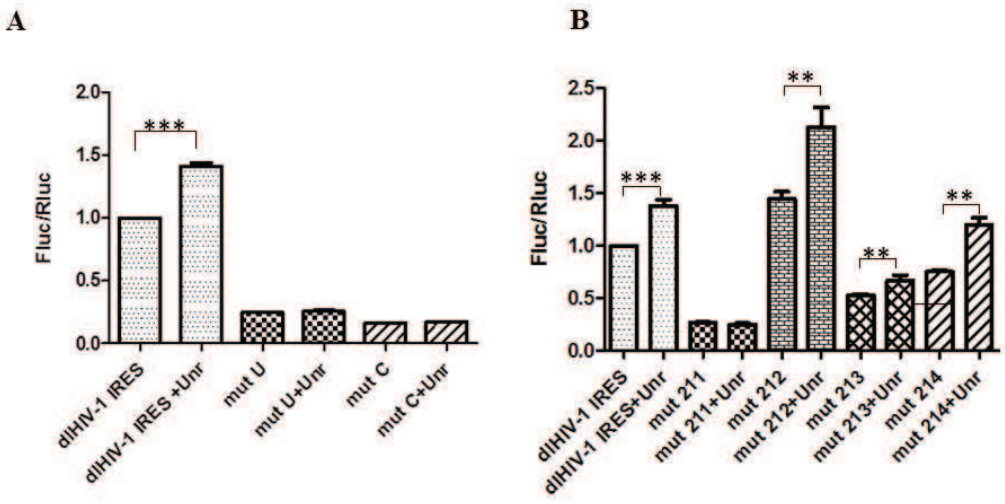


Figure 16: Determination des activités des mutants de l'IRES du VIH-1 avec ou sans surexpression d'Unr. **(A)** Activitées des mutants mutU et mutC de l'IRES avec ou sans surexpression d'Unr. **(B)** Activitées de mut211, mut212, mut213, et mut214 avec ou sans surexpression d'Unr. L'activité de l'IRES est exprimée par le ratio des activités Fluc/Rluc dans le test de dual luciferase. Les mesures ont été réalisées sur des cellules HeLa, 24 heures post transfection. Les ratios sont normalisés sur celui de l'IRES du virus VIH-1 sauvage sans surexpression d'Unr. Les histogrammes représentent la moyenne ± erreur type de la moyenne de 3 expériences indépendantes réalisées en triplicats **, $p < 0.01$; ***, $p < 0.001$.

Conclusion

En conclusion, nous avons montré une interaction entre la protéine de nucléocapside NCp7 mature et en tant que domaine au sein de la polyprotéine Gag du virus du VIH-1 et la protéine cellulaire Unr. Nous montrons également pour la première fois qu'Unr est, comme pour les polio- et rhinovirus, un ITAF de l'IRES du VIH-1. De plus, cette activité est spécifiquement inhibée par la co-expression de la protéine NCp7 mais pas par celle de la protéine Gag. Des mutations ponctuelles d'un site potentiel de liaison d'Unr sur l'IRES du VIH-1 diminuent fortement la capacité de traduction de cet IRES suggérant une dépendance

forte de l'IRES pour la présence d'Unr. Ces résultats suggèrent qu'Unr participerait à la régulation de l'équilibre entre traduction et encapsidation du génome viral au cours des différentes phases du cycle. Pour finir, Unr semble jouer un rôle significatif dans l'infection par le VIH-1 puisque son extinction par des siARN est capable de diminuer l'infection par un pseudovirus modèle des phases précoces de l'infection.

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Résumé

La protéine de nucléocapside (NC) du virus de l'immunodéficience humaine (VIH-1), sous sa forme mature NCp7 ou en tant que domaine de la polyprotéine Gag, joue de nombreux rôles dans les phases précoce et tardive de l'infection. La NC est une protéine à deux doigts de zinc, chaperonne des acides nucléiques. Afin de mieux comprendre son rôle dans l'infection, nous avons cherché de nouveaux partenaires cellulaires de la NCp7 et identifié une protéine de liaison aux ARNs, Upstream of N-ras (Unr), dont l'interaction avec Gag et NCp7 a été confirmée par co-immunoprécipitation et FRET-FLIM. L'interaction entre Gag et Unr est dépendante de l'ARN et médiée par le domaine NC. Unr est une ITAF (IRES transacting factor) régulant la traduction médiée par plusieurs IRESs cellulaires et viraux. L'ARN génomique du VIH-1 possède deux IRESs dont un localisé dans la région non traduite en 5' qui permet aux ARNm viraux de conserver un fort niveau de traduction lorsque la traduction coiffe-dépendante de la cellule est affaiblie par l'arrêt du cycle viral induit par l'infection. L'activité de cet IRES, appelé IRES VIH-1, dépend fortement de facteurs cellulaires mal identifiés à ce jour. En utilisant un système de dual luciférase, nous avons montré qu'Unr est une ITAF dont la surexpression stimule l'IRES VIH-1. Des mutations ponctuelles de cet IRES, dans un motif consensus de liaison à Unr, altèrent à la fois l'activité de l'IRES et sa réponse à Unr suggérant que l'activité IRES dépend fortement de Unr. L'effet d'Unr sur l'IRES est inhibé par la surexpression de NCp7 mais pas par celle de Gag dont l'effet stimulateur sur l'IRES est additif de celui d'Unr suggérant un rôle d'Unr différent dans les phases précoce et tardive de l'infection. Pour finir, le knockdown de l'expression d'Unr entraîne une diminution significative de l'infection par un pseudovirus non réplicatif soulignant l'implication fonctionnelle d'Unr dans la phase précoce. A partir de ces résultats, un modèle est finalement proposé et discuté. Dans un second projet, nous nous sommes intéressés au devenir de la protéine NCp7 libérée dans le cytoplasme après la transcription inverse. Pour ce faire, nous avons utilisé comme modèle des cellules HeLa où NCp7 est surexprimée et étudié par des techniques de microscopie de fluorescence avancées, la localisation et la dynamique cellulaire de NCp7. Nous avons pu montrer que NCp7 se lie préférentiellement aux ARNs ribosomaux dans le nucléole et le cytoplasme, suggérant un possible rôle de NCp7 dans la traduction.
Mots clés : VIH, nucléocapside, NCp7, Unr, Gag , IRES

Abstract

The Human Immunodeficiency Virus-1 (HIV-1) nucleocapsid protein (NC), as a mature protein (NCp7) or as a domain of the polyprotein Gag, plays several important roles in both the early and late phase of the infection. NC is a nucleic acid chaperone protein with two zinc fingers. In order to gain a better understanding of its roles in infection, we searched for new cellular protein partners of NCp7. We identified the RNA binding protein Unr, Upstream of N-ras, whose interaction with both Gag and NCp7 was confirmed by co-immunoprecipitation and FRET-FLIM. The Unr interaction with Gag is RNA dependent and mediated by its NC domain. Unr is an ITAF (IRES trans-acting factor) regulating the translation driven by several viral and cellular IRESs. The HIV-1 genomic mRNA harbors two IRESs elements: one of them found within the HIV-1 5'-Untranslated region drives HIV-1 mRNA translation when the cap-dependent translation is diminished due to the infection-induced cell cycle arrest. The activity of this IRES, called HIV-1 IRES, is highly dependent on cellular factors which are still not all identified. Using a dual luciferase assay, Unr was shown to act as an ITAF, increasing the HIV-1 IRES dependent translation. Point mutations of the HIV-1 IRES in a consensus Unr binding motif were found to alter both the IRES activity and its activation by Unr suggesting a strong dependency of the IRES on Unr. Unr stimulation effect is furthermore counteracted by NCp7, but not by Gag overexpression, which increases the IRES activity in an additive manner to Unr suggesting a differential Unr effect on the early and late phases of the infection. Finally, knockdown of Unr in HeLa cells leads to a decline in infection by a non-replicative lentivector proving its functional implication in the early phase. From those results, a functional model was proposed and discussed. In a second project, we focused on the fate of the NCp7 proteins that are released in the cytoplasm, after reverse transcription. To address this question, we used as a model, HeLa cells overexpressing NCp7 proteins and we investigated by advanced microscopy techniques, the distribution and dyamics of the NCp7 proteins. We found that NCp7 is mainly associated to ribosomal RNAs both in the nucleoli and the cytoplasm, suggesting a potential role of NCp7 in translation.
Key words : HIV, nucleocapsid, NCp7, Unr, Gag , IRES