Multiple roles of DDX17 in Human Immunodeficiency Virus type 1 replication

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“If you cannot - in the long run - tell everyone what you have been doing, your doing has been worthless.”

Erwin Schrödinger (1887-1961) - Nobel Prize winner in physics, 1933.

Abstract

Human immunodeficiency virus type 1 (HIV-1) is a small retrovirus that highly depends on the host cell machinery to replicate by completing its lifecycle and producing new infectious viral particles. The complexity of HIV-1 life cycle regulation only reflects the diversity of the virus-host interactions. Cellular helicases are enzymes involved in every step of nucleic acid metabolism, through rearranging ribonucleoprotein complexes. The understanding and the importance of helicases in HIV-1 replication started to emerge a decade ago. Since then, many studies reported the promoting or inhibiting effect of this protein family on HIV-1. My thesis project was to investigate the role of helicases in HIV-1 replication and comprises two parts. First, we performed a shRNA screen in SupT1 cells to knockdown 130 helicases and monitor their effect on the production of HIV-1 particles. This work allowed us to identify cellular pathways that are important for HIV-1 replication, as well as 35 potential helicases that dramatically affect virus production. Second, we chose to further investigate the role of DDX17 in HIV-1 replication. In addition to showing for the first time that a helicase is required for HIV-1 frameshift, we found that DDX17 promotes viral RNA packaging. Considering the role of DDX17 as a cofactor of the zinc antiviral protein (ZAP) in exosome-mediated HIV-1 mRNA degradation, this emphasizes the fact that helicases are multifunctional proteins. Finally, this work identifies helicases that potentially strongly modulate HIV-1 production. Individual investigation for each candidate will be needed to unravel the mechanisms underlying their effect on HIV-1 replication.

Résumé

Le virus de l'immunodéficience humaine de type 1 (VIH-1) est un petit rétrovirus qui dépend fortement de la machinerie cellulaire afin de compléter son cycle de réplication et produire de nouvelles particules virales infectieuses. La complexité de la régulation du cycle de réplication du VIH-1 reflète la diversité des interactions hôte-virus. Les hélicases sont des enzymes impliquées dans toutes les étapes du métabolisme des acides nucléiques, en réarrangeant les complexes ribonucléprotéiques. La compréhension de l'importance des hélicases dans la réplication du VIH-1 a commencé il y a une dizaine d'années. Depuis, plusieurs études ont rapporté les effets stimulateurs ou inhibiteurs de cette famille de protéines sur le VIH-1. Mon projet de thèse était d'investiguer le rôle des hélicases dans la réplication du VIH-1 ; il comprenait deux parties. Premièrement, nous avons supprimé l'expression de 130 hélicases au moyen de shRNAs dans les cellules SupT1. Ce travail nous a permis d'identifier les voies cellulaires majoritairement impliquées dans la réplication du VIH-1, ainsi que 35 hélicases affectant de manière drastique la production virale. Dans un second temps, nous avons choisi de nous intéresser plus en détails au rôle de la protéine DDX17 dans la réplication du VIH-1. En plus d'identifier pour la première fois une hélicase étant requise pour le décalage du cadre de lecture (-1), nous montrons que DDX17 favorise l'encapsidation de l'ARN viral. Considérant que DDX17 agit également en tant que co-facteur de ZAP (protéine antivirale zinc) dans la dégradation des ARNs du VIH-1 par l'exosome, cela souligne le fait que les hélicases sont multifonctionnelles. Finallement, au cours de ce travail nous avons identifié un

certain nombre d'hélicase ayant le potentiel de fortement moduler la production du VIH-1. Des études individuelles seront nécessaires afin de mettre à jour les mécanismes responsables de l'effet de chacun des candidats sur la réplication du VIH-1.

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List of abbreviations

(-)ssDNA	Minus strand strong-stop DNA
(+)ssDNA	Plus strand strong-stop DNA
AIDS	Acquired immunodeficiency syndrome
APOBEC3	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like
ART	Antiretroviral therapy
BER	Base excision repair
BLV	Bovine leukemia virus
BST-2	Bone marrow stromal cell antigen 2
CARD	Caspase activation and recruitment domain
CDC	Centers for Disease Control
CH	Cystein- and Histidine-rich
CNS	Central nervous system
cpz	Chimpanzee
CRA	Chemokine receptor antagonist
CTD	C-terminal domain
CTE	Constitutive transport element
CypA	Cyclophilin A
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DIS	Dimerization initiation site
DSB	Double-stranded break repair

dsDNA	Double-stranded DNA
dsRBD	double-stranded RNA binding domain
EC	Elite controller
EJC	Exon junction complex
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
FDA	U.S. Food and Drug Administration
FeLV	Feline leukemia virus
FI	Fusion inhibitorg
gag	**"**group-specific antigen"
GALT	Gut-associated lymphoid tissue
gor	gorilla
gp	glycoprotein
GPI	Glycosylphosphatidylinositol
gRNA	genomic RNA
HAART	Highly active antiretroviral therapy
HFV	Human foamy virus
HIF	HIV-1 influencing factor
HIV-1	Human immunodeficiency virus-type 1
HMBA	Hexamethylene bisacetamide
hnRNP	Heterogenous nuclear ribonucleoprotein

HR	Homologous repair
HTLV-1	Human T-cell leukemia virus-type 1
IFN	Interferon
INI	Integrase inhibitor
IN	Integrase
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISE	Intronic splicing enhancer
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
ISS	Intronic splicing silencer
IST	Inducer of short transcripts
kb	kilobase
KS	Kaposi's Sarcoma
LEDGF	Lens epithelium-derived growth factor
LGP-2	Laboratory of genetics and physiology 2
LINE-1	Long interspersed element 1
LMIC	Low- and middle-income countries
LTR	Long terminal repeat
MDA-5	Melanoma differentiation associated protein-5
MLV	Murine leukemia virus
MNR	Mismatch repair
MoMLV	Moloney murine leukemia virus

MPMV	Mason-Pfizer monkey virus
mRNA	messenger RNA
NC	Nucleocapsid
NER	Nucleotide excision repair
NES	Nuclear export signal
NHEJ	Non-homologous DNA end joining
NIS	Nuclear diffusion inhibitory signal
NK	Natural killer cells
NLS	Nuclear localization signal
nm	nanometer
NMD	Nonsense-mediated decay
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NPC	Nuclear pore complex
NRTI	Nucleoside reverse transcriptase inhibitor
nt	nucleotide
OD	Oligomerization domain
PAMP	Pathogen associated molecular pattern
PBS	Primer binding site
PCAF	p300/CREB-binding protein-associated factor
PCE	post-transcriptional control element
pDC	Plasmacytoid dendritic cell
PI	Protease inhibitor
PIC	Preintegration complex

PPT	Polypurine tract
PR	Protease
PRR	Pattern-recognition receptor
PTEF-b	Positive transcription elongation factor b
REV-A	Reticuloendotheliosis virus strain A
RGG	Arginine- and Glycine-rich
RIG-I	Retinoic acid-inducible gene I
RISC	RNA-induced silencing complex
RNP	Ribonucleoprotein
RRE	Rev response element
rRNA	ribosomal RNA
RSE	RSV stability element
RSV	Rous sarcoma virus
RT	Reverse transcriptase
SA	Splice acceptor
SAHA	Suberoxylanilide hydroxamic acid
SAMHD1	Sterile alpha motif and histidine/aspartic acid domain-containing protein 1
SD	Splice donor
SF	Superfamily
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIV	Simian immunodeficiency virus
SL	Stem-loop

SLFN11 Schlafen11

SNV Spleen necrosis virus

snRNA small nuclear RNA

SP Spacer peptide

SSBR Single-stranded break repair

ssm sooty mangabey

TAR Transactivation response

TB Tuberculosis

TGN *Trans*-Golgi network

TIP47 Tail-interacting protein of 47 kDa

TMG Trimethylguanosine

TNFα Tumor necrosis factor alpha

TRAMP Trf4/Air2/Mtr4 polyadenylation

TRIM Tripartite motif-containing protein

tRNA transfer RNA

U2AF U2 associated factor

UTR Untranslated region

VLP Virus-like particle

WS Werner's Syndrome

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Contribution to knowledge

During the course of my PhD program, I studied the implication of helicases in HIV-1 replication, which is the review presented in Chapter 2. We performed a shRNA-based screening to specifically address the role of helicases in HIV-1 replication. We identified 35 helicases that strongly modulate viral production. This constitutes the manuscript presented in Chapter 3. Furthermore, we were the first to report that a helicase, DDX17 affects HIV-1 frameshift. We further demonstrated that this particular helicase promotes the packaging of viral RNA in the viral particles, therefore contributing to virus infectivity. This second manuscript is presented in Chapter 4.

I also contributed to other studies, including the role of BST-2 in HIV-1 infectivity. Details about authors' contribution are provided at the end of each section.

List of publications

Rong L, Zhang J, Lu J, Pan Q, **Lorgeoux RP**, Aloysius C, Guo F, Liu SL, Wainberg MA, Liang C: The transmembrane domain of BST-2 determines its sensitivity to down-modulation by human immunodeficiency virus type 1 Vpu. *J Virol* 2009, 83:7536-7546.

Lorgeoux RP, Guo F, Liang C: From promoting to inhibiting: diverse roles of helicases in HIV-1 Replication**.** *Retrovirology* 2012, 9:79.

Lorgeoux RP, Pan Q, Liang C: Screen for helicases modulating HIV-1 infection in SupT1 cells (in preparation)

Lorgeoux RP, Pan Q, Le Duff Y, Liang C : DDX17 promotes the production of infectious HIV-1 particles through modulating viral RNA packaging and translation frameshift (*Virology*, accepted)

Chapter 1 – Introduction

1.1 HIV History

1.1.1 Discovery

1.1.1.1 First Cases

In 1981, a rare form of Kaposi's sarcoma (KS) emerged, more severe than the usually known KS and affecting young homosexual men in New York and California (United States) [1, 2]. Within the following months, the Centers for Disease Control (CDC) reported that the emergence of this new type of KS appeared to be concomitant with the recrudescence of opportunistic diseases such as Pneumocystis pneumonia, correlating with an immune deficient state of the patients [3]. Rapidly, this infection was reported to spread in the United States and taken very seriously by health professionals and medias. A real marathon began in order to understand the cause of this disease that was no longer only seen within the American homosexual men community, but also in drug users [4] and in the United Kingdom [5].

A New and Deadly Virus

From 1981 to 1982, the scientific community faced the disturbing reality of an unidentified pathological agent that caused the death of infected people within less than twenty months [1]. Then began the need of naming this agent. In the early days, names were given upon the observation that patients were belonging to certain communities. Thus, names appeared such as "Gay compromise syndrome" [6] or the four 'H' disease (Haitians, Homosexuals, Hemophiliacs, Heroin users) [7, 8], starting to provide some room for discrimination. Finally, in September 1982, the CDC firstly described the disease as we call it today, AIDS, standing for Acquired Immunodeficiency Syndrome. However, the causing agent of this syndrome remained unidentified until 1983, when two independent teams claimed the discovery of a new retrovirus. Dr Montagnier's group, from the Pasteur Institute in Paris, France, reported that they had isolated the retrovirus causing AIDS, and named it lymphadenopathy virus (LAV) [9]. Simultaneously, Dr Gallo's group, from the United States, identified the human T cells lymphotropic virus type 3 (HTLV-III) as the causing agent for AIDS [10]. After arguing for the name and the discovery of the newly found virus, the International Committee on Taxonomy of Viruses finally came to the decision, in 1986, that the virus would be called human immunodeficiency virus (HIV) [11]. Although many consider that it is the combination of both the knowledge and the experience of the two research teams that led to the discovery of HIV as the ethiological agent causing AIDS, only Dr Françoise Barré-Sinoussi

and Dr Luc Montagnier were awarded the Nobel Prize in Physiology or Medicine in 2008 for its discovery.

1.1.2 HIV Epidemiology

1.1.2.1 The Origins

Viruses can cross species barriers and often cause diseases in the new host. This is how simian immunodeficiency virus (SIV) was transmitted into humans and became HIV. Among numbers of theories that emerged regarding how the virus was first transmitted from monkeys to humans, the "hunter theory" remains the most common one. According to that theory, African hunters were contaminated by SIV through cuts or injuries when chimpanzees (cpz) were being killed and bloods put in contact. SIV then evolved to adapt to its new host, becoming HIV. In 1999, a SIVcpz strain very close to HIV-1 allowed researchers to identify *Pan troglodytes troglodytes* as the original sub-group of chimpanzee that transmitted SIV to humans in Southern Cameroon [12].

In 1998, analysis of a plasma sample from an adult Bantu male from 1959 who lived in Leopoldville, Belgian Congo (now Kinshasa, Democratic Republic of Congo) revealed that SIV jumped to human not long before 1959 [13]. Thereafter, HIV traveled to the island of Haiti and North America. Blood industry as well as national and international travels then contributed to spread HIV worldwide in the late 1970s early 1980s, leading to the pandemic that we know today.

1.1.2.2 Classification

1.1.2.2.1 *Retroviridae* Family

Two schemes are commonly used to classify viruses: the International Committee on Taxonomy of Viruses, and the Baltimore classification. According to the Baltimore classification, viruses belong to seven distinct groups (I-VII), depending on the type of nucleic acid that they carry and their replication mode [14] (Figure 1).

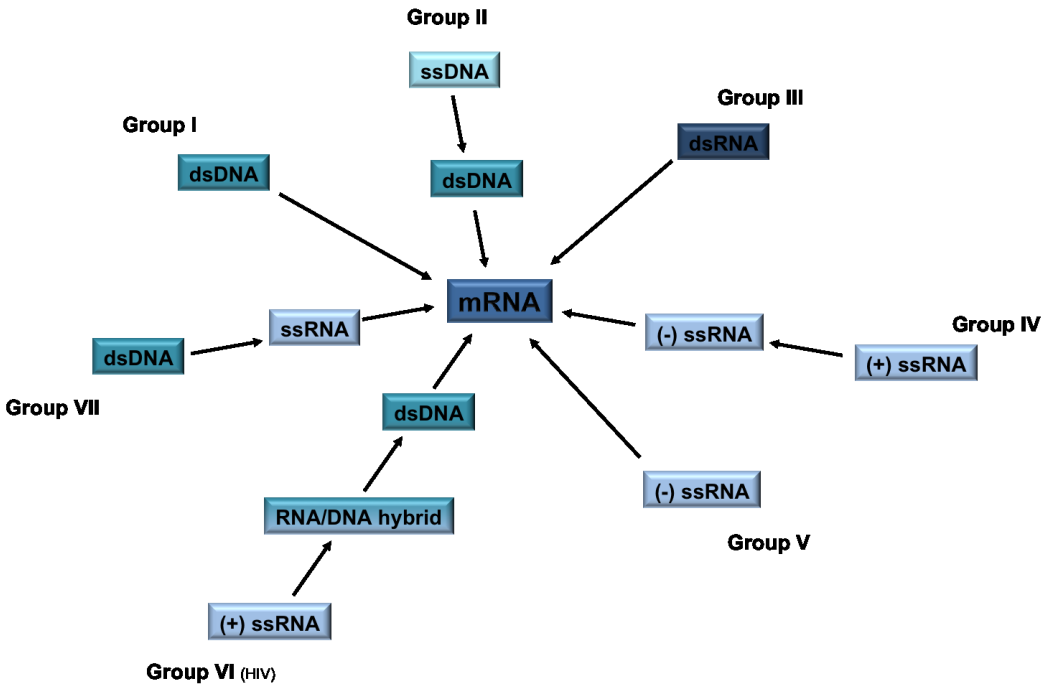


Figure 1 | Virus Classification
Viruses are classified into seven groups (I to VII), based on the type of nucleic acid that carries their genetic information and the pathway that viral mRNA is synthesized.

HIV belongs to the *Retroviridae* family that is part of Group VI viruses, according to the Baltimore classification. Retroviruses are further divided into *Orthoretrovirinae* and *Spumaretroviridae* subfamilies. *Orthoretrovirinae* comprises six *genera,* including *lentiviruses* (Table 1). Based on these morphological and biochemical features, it has been established that HIV belongs to the *lentivirus* (*lente*-, Latin for "slow") genus.

Table 1 | Retroviruses Family

Retroviruses family			
Subfamily	Genus	Host Type species	Host Species
Orthoretrovirinae	*Alpharetrovirus*		
	Betaretrovirus		
	Gammaretrovirus		
	Deltaretrovirus		
	Epsilonretrovirus		
	Lentivirus	*Bovine*	
		Equine	
		Feline	
		Ovine	
		Primate	*HIV*
			SIV
Spumaretrovirinae	*Spumavirus*		

A retrovirus is an enveloped ribonucleic acid (RNA) virus that replicates in a host cell using its enzyme reverse transcriptase (RT) to convert its single-stranded RNA genome into double-stranded DNA. The newly synthesized DNA is then integrated into the host genome by the viral integrase (IN) with the help of cellular co-factors, and becomes a provirus. This provirus undergoes the usual transcription to produce new viral RNA that is exported to the cytoplasm where it is translated, leading to the synthesis of all viral components needed to generate

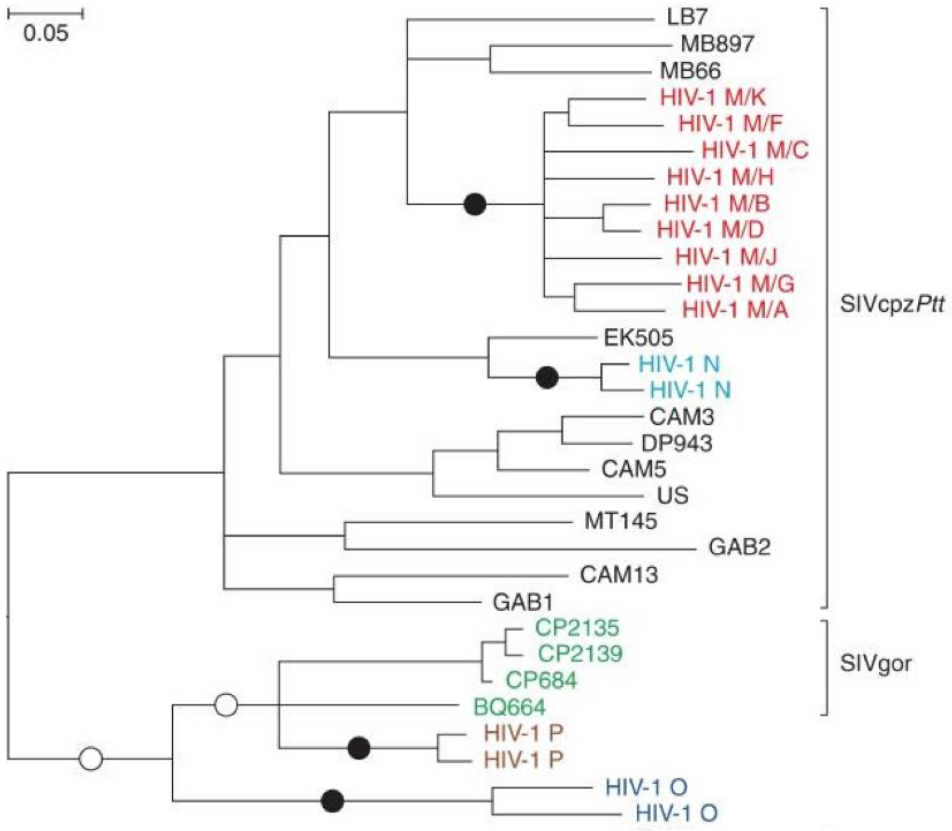
new viruses, including structural, regulatory and accessory proteins, enzymes and genomic RNA (gRNA).

Retroviruses normally infect somatic cells, but in rare cases, they are thought to infect germ cells, allowing the transmission of the virus genome on to the next generation. As opposed to previously mentioned exogenous retroviruses, these are called endogenous retroviruses, and are believed to play an important role in evolution.

1.1.2.2.2 HIVs

HIV encompasses two types, HIV-1 and HIV-2, depending on the original SIV strain that was transmitted to humans in West and Central Africa (Figure 2). HIV-1 is further divided into four groups: M (major), N (not-M, not-O), O (outlier), and P. M and N originated from SIVcpz, P is a new group that most likely arose from SIVgor (*Gorilla gorilla gorilla*) (Figure 2). Group M is the largest group and includes subtypes A, B, C, D, F, G, H, j and K (E and I have never been isolated as new strains but as recombinant viruses only) (Figure 2) [15]. HIV-2 was identified as a new strain in 1986, as its epitopes showed some discrepancy from those of HIV-1 [16]. HIV-2 is also further divided into subtypes, A to H.

A


0.05
LB7
MB897
MB66
HIV-1 M/K
HIV-1 M/F
HIV-1 M/C
HIV-1 M/H
HIV-1 M/B
HIV-1 M/D
HIV-1 M/J
HIV-1 M/G
HIV-1 M/A
SIVcpzPtt
EK505
HIV-1 N
HIV-1 N
CAM3
DP943
CAM5
US
MT145
GAB2
CAM13
GAB1
CP2135
CP2139
CP684
SIVgor
BQ664
HIV-1 P
HIV-1 P
HIV-1 O
HIV-1 O

B

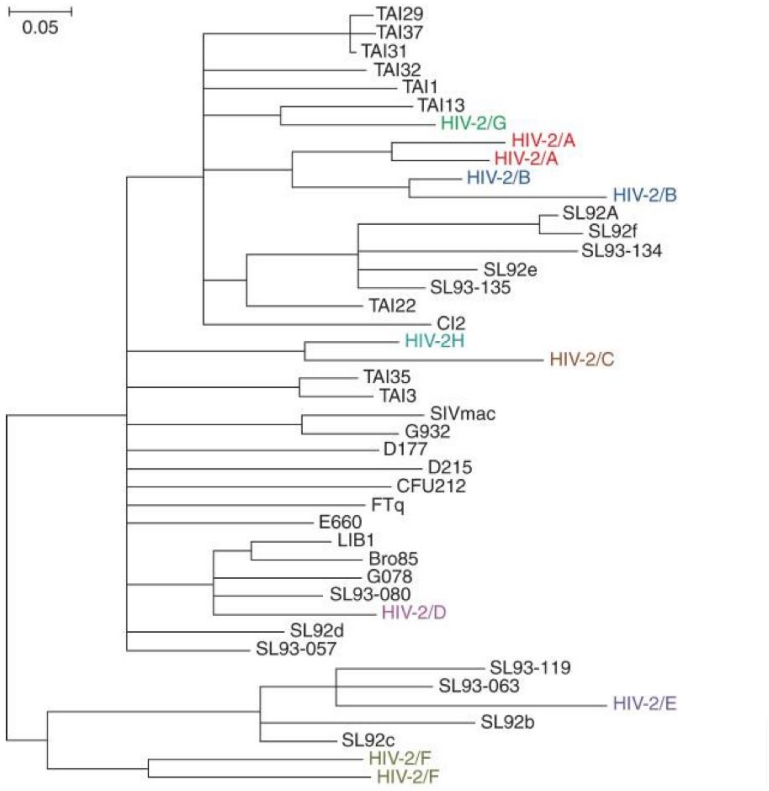


Figure 2 | Phylogeny of HIV-1
(**A**) Phylogenetic relationships between HIV subtypes and SIV viruses. *Pol* gene was used as a reference. Black circles indicate the four branches where cross-species transmission-to-humans has occurred. White circles indicate two possible alternative branches on which chimpanzee-to-gorilla transmission occurred. (**B**) HIV-2 origins. The phylogenetic relationships of representative SIVsmm and HIV-2 strains are shown for a region of the viral *gag* gene [15].

1.1.3 HIV Pandemic

To date, HIV/AIDS is one of the greatest challenges in global health with about 35 million people living with HIV [17] and more than 25 million people have died from the consequences of this infection [18] (Figure 3). With a

population of 0.9 billion, sub-Saharan Africa accounts for about 70% of worldwide-infected people, which includes 22.5 million individuals living with HIV and 1.2 million new infection per year [19]. As a result of lots of efforts, the number of newly infected people keeps declining. Nevertheless, lots of progress remains to be made in the fields of prevention, treatments and vaccines to stabilize the number of persons living with HIV and maybe reach the "zero new infection". Although both HIV types cause the same AIDS symptoms, they appear to behave and spread differently (Figure 3) [20].

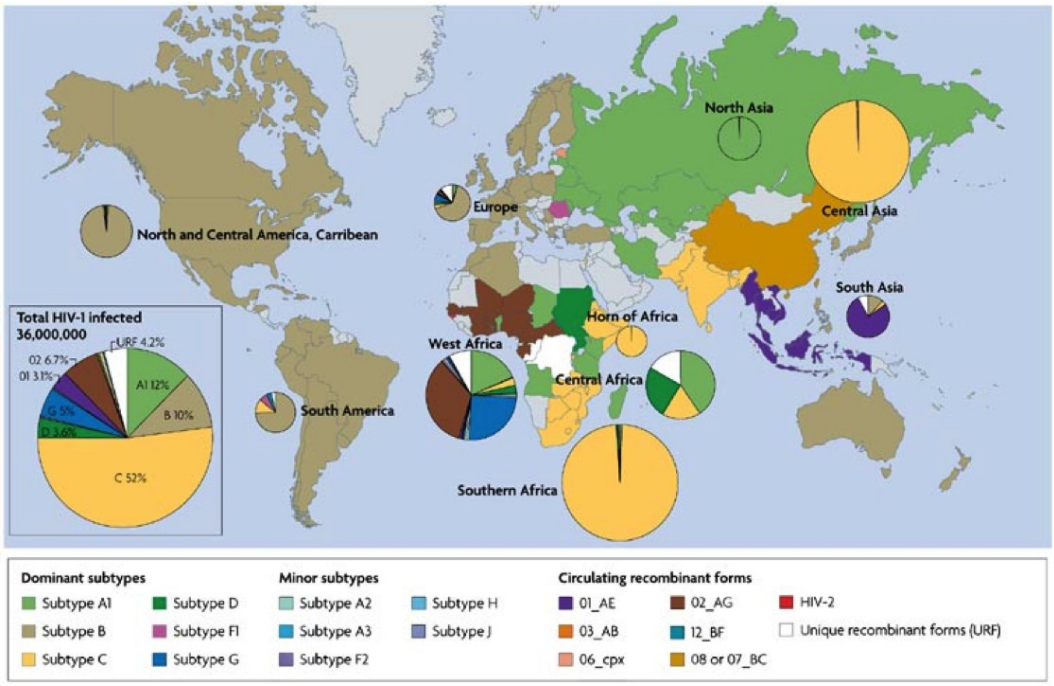


Figure 3 | HIV-1 Subtypes Distribution Map (2007)
Worldwide diversity and distribution of HIV-1 subtypes and recombinants [20].

While HIV-1 spreads worldwide, HIV-2 is less pathogenic, has a slower rate of disease progression and is confined in Central Africa (Figure 3) [17, 20, 21]. HIV-

1 group M is the most pathogenic group, responsible for the pandemic, with a majority of infections caused by subtype C in Africa and subtype B in Europe and America (Figure 3).

1.2 HIV-1 Replication

1.2.1 Viral Genome

Depending on the complexity of their genome, retroviruses fall into two categories: simple and complex [22]. Like all retroviruses, HIV-1 carries genes coding for *gag*, *pol* and *env*, leading to the expression of structural (MA, CA, NC), enzymatic (PR, RT, IN) and envelope (gp120, gp41) proteins, respectively (Figure 4). *Lentiviruses* and *Spumaviruses* are complex viruses. In addition to these fundamental genes common to all retroviruses, HIV-1 also encodes for regulatory (Tat, Rev, Nef, Vpr) and accessory (Vpu, Vif) proteins, rendering its nine kilobase (kb) genome more complex and providing it with powerful tools allowing tight regulation of the replication cycle and escape mechanisms to the restrictions imposed by the host cell (Figure 4). Along with the help of these regulatory proteins, non-coding HIV-1 sequences rearrange into specific RNA secondary structures that play key roles in protein expression. Indeed, HIV-1 genome is folded into numerous stem-loop structures that act as docking sites for both viral and cellular components and allow proper viral replication. The implication of such RNA regulatory *cis*-elements has been reported to modulate

various steps, including transcription (TAR), splicing (ESE, ESS, ISE, ISS), export (RRE), translation (PCE, IRES) and packaging (DIS, SL3). In the end and with the help of cellular factors, the tight regulation of HIV-1 genome expression allows nine genes to code for fifteen different proteins leading to the production of new virus particles.

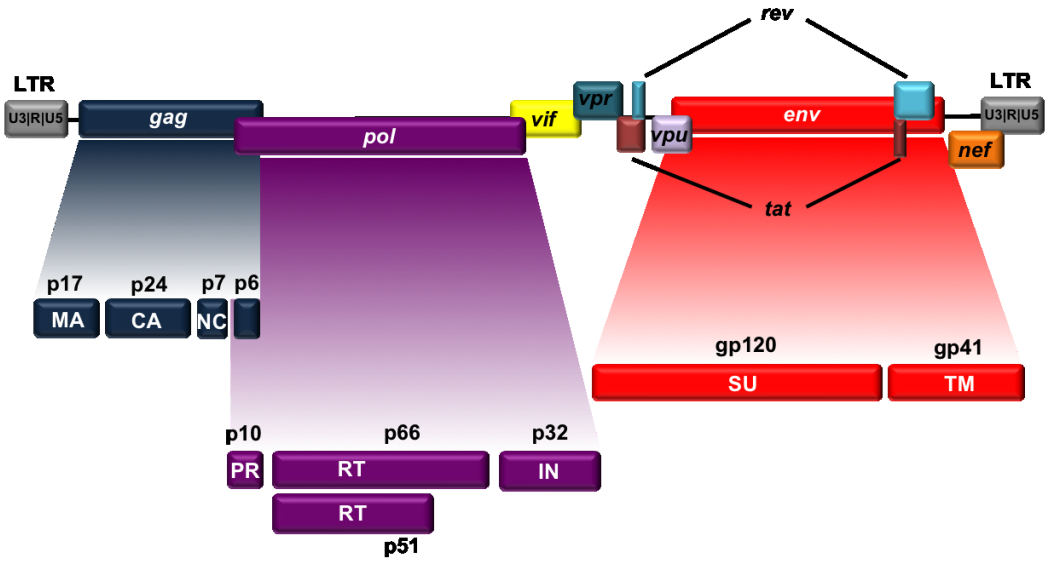


Figure 4 | HIV-1 Genome Organization
Representation of the nine open reading frames of HIV-1 genome. Gene products corresponding to the expression of the *Gag, Pol* and *Env* viral genes are indicated in the lower panel. MA = matrix; CA = capsid; NC = nucleocapsid; PR = protease; RT = reverse transcriptase; IN = integrase; Vif = viral infectivity factor; Vpr = viral protein R; Vpu = viral protein U; Nef = negative factor; SU = surface domain; TM = transmembrane domain; LTR = long terminal repeat.

1.2.2 Virus Particle

HIV-1 particle is an enveloped particle of a 100 nm diameter (Figure 5). Its envelope is made of the cellular lipid bilayer and includes the viral Env

glycoproteins SU (surface subunit) and TM (transmembrane subunit), cleavage products of the $gp160^{Env}$ precursor into gp120 and gp41, respectively, by the cellular protease furin [23]. Under the envelope, the viral matrix (MA) forms a layer under which is the core made of the viral capsid (CA). Inside this core, two molecules of HIV-1 gRNA are associated with both cellular and viral components. Viral components include the Pol enzymes (RT, IN and PR), the accessory protein Vpr, as well as the chaperone NC protein that coats the two strands of viral RNA (Figure 5). Cellular components are also incorporated, including $tRNA^{Lys3}$, helicases and many are yet to be characterized.

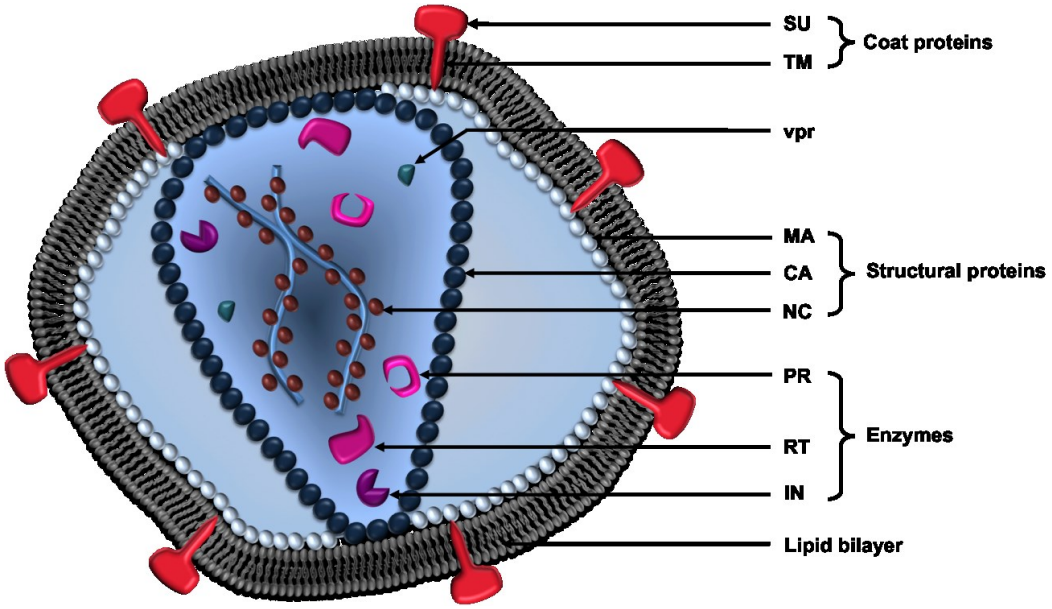


Figure 5 | HIV-1 mature particle
Structural representation of an HIV-1 mature particle. Two molecules of genomic RNA (gRNA, in blue) are coated with NC. Viral enzymes, Vpr, gRNA and NC are packaged into the core of the particles, made of HIV-1 CA. HIV-1 matrix MA forms a layer underneath the lipid bilayer in which the viral envelope protein are incorporated. MA = matrix; CA = capsid; NC = nucleocapsid; PR = protease; RT = reverse transcriptase; IN = integrase; SU = surface domain; TM = transmembrane domain.

1.2.3 Virus Replication Cycle

HIV-1 replication cycle can be divided in two majors stages: early and late. The early stage comprises all the steps prior to the irreversible integration of the viral genome into the host DNA and includes attachment, fusion, entry, reverse transcription, nuclear import and integration (Figure 6). The late stage includes transcription, splicing, export, translation, assembly, packaging, release

and maturation (Figure 6). The virus life cycle can also pause after integration and become dormant, which allows the establishment of reservoirs. This phenomenon is called latency.

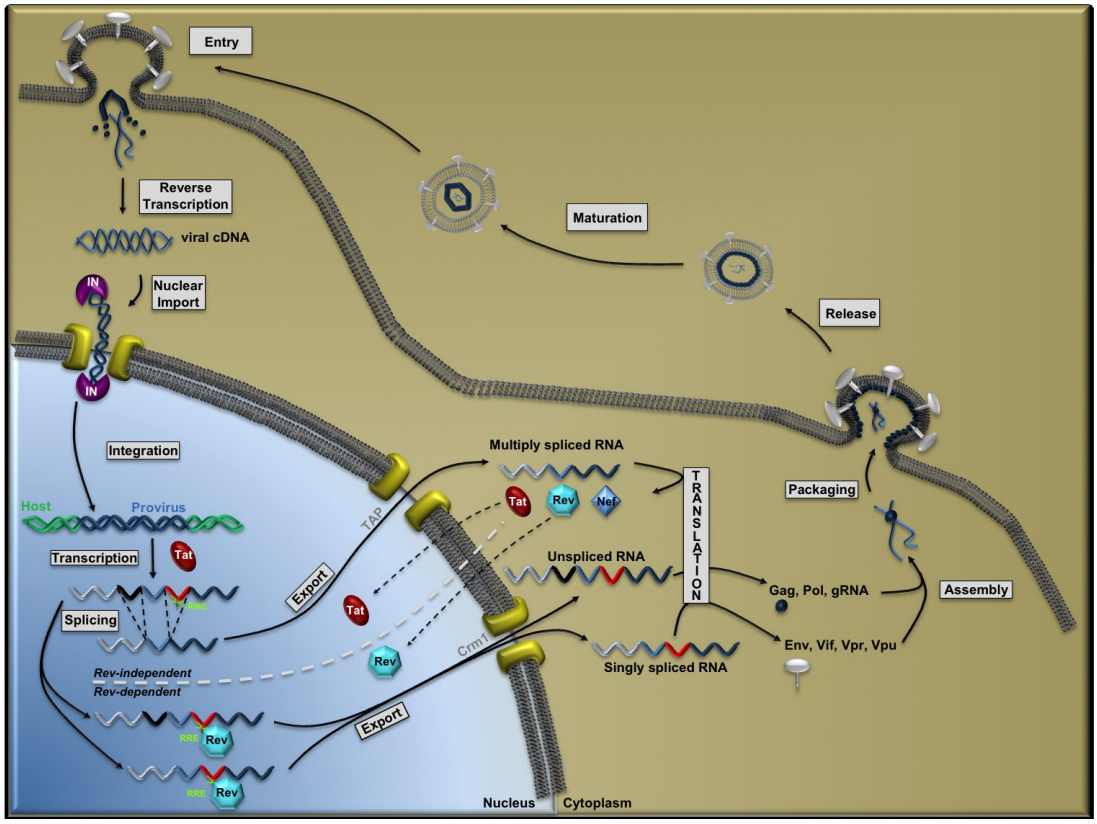


Figure 6 | HIV-1 Replication Cycle

1.2.3.1 Early Stages

1.2.3.1.1 Attachment, fusion and entry

As an enveloped virus, HIV-1 particle needs to fuse with the membrane of the target cell to release its genetic material and follow its replication cycle. Since the beginning of the pandemic, it was observed that HIV-1 infection correlated

with a decrease in the CD4 positive T-cells population. Very soon, CD4 antigen was identified as the receptor required by HIV-1 to enter the cells [24, 25]. However, although CD4 was shown to be necessary for the entry process, the fact that some HIV-1 strains were infecting T lymphocytes (T-tropic) while others were displaying specificity for macrophages (M-tropic) brought to attention that other receptors than CD4 were involved in the entry process. It took nearly a decade for many groups to discover, in 1996, that the seven transmembrane domains chemokine receptors CXCR4 (also called fusin) and CCR5 were the co-receptors that HIV-1 needs to successfully infect a target cell, responsible for T-tropism and M-tropism, respectively [26-31]. Polymorphism in the variable region V3 of gp120 confers specificity to these co-receptors, and HIV-1 viruses display X4 or R5 tropism depending on their requirement for either CXCR4 or CCR5 to complete the entry step.

Originally, HIV-1 envelope proteins are synthesized in the cytoplasm of an infected cell as a glycoprotein precursor, $gp160^{Env}$. Upon $gp160^{Env}$ proteolysis in the *trans*-Gogli network, gp120 and gp41 form non-covalent heterodimers that further associate into trimers within the envelope of the virus, forming spikes. These trimers are next directed to the lipid rafts in the plasma membrane, where they are incorporated in the envelope of the budding particle (less than 15 per virion [32]), subsequently allowing docking and fusion of the newly made virus with the target cell. Entry is a sequential process involving the formation of a trimolecular complex between gp120, CD4 and a co-receptor (CXCR4 or CCR5), causing a series of protein conformational changes, ultimately leading to the fusion of both viral and cellular membranes. First, gp120 binds to CD4, inducing

a conformational change in gp120 that exposes its co-receptor binding domain [33]. The even stronger interaction between gp120 and the co-receptor triggers a new structural change that allows the insertion of the hydrophobic N-terminal domain of gp41 (fusion peptide) in the plasma membrane (reviewed in [34]). The resulting six-helix structure brings the two membranes together, leading to fusion of the membranes and release of the viral core into the cytoplasm (Figure 7). Recently, HIV entry process has been reconsidered as a group showed that it was also occurring through a clathrin- and dynamin-dependent endocytosis mechanism, allowing efficient fusion of the viral and cellular membranes in the endosome, leading to infection [35].

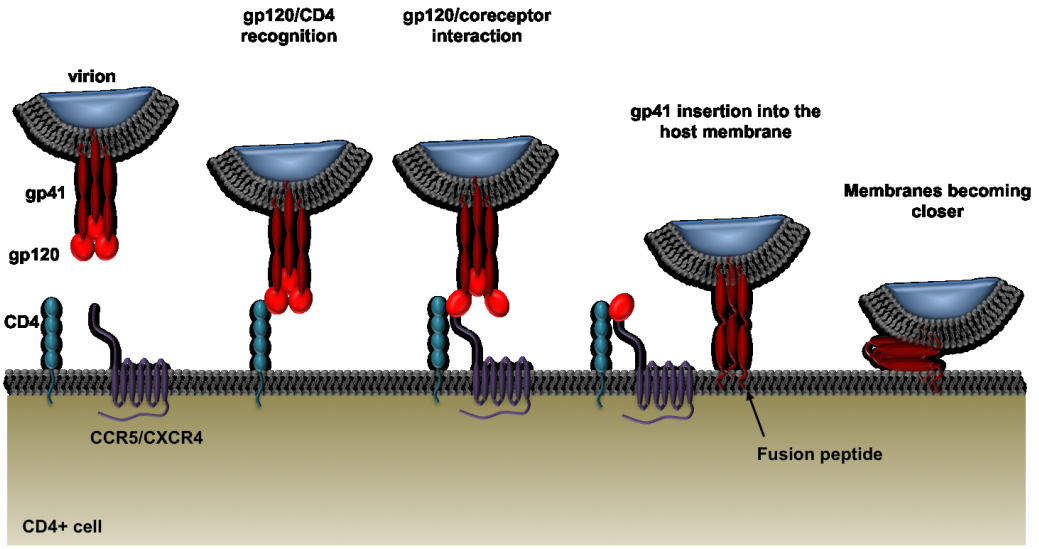


Figure 7 | HIV-1 Entry
Recognition of gp120 by CD4 allows virus docking to the plasma membrane of the target cell. Subsequent interaction between gp120 and the coreceptor (CXCR4 or CCR5) triggers a conformational change leading to the exposure of the fusion peptide and its insertion into the membrane of the target cell. Conformational réarrangement of gp41 induced the viral and cellular membrane to become closer, eventually leading to membrane fusion.

1.2.3.1.2 Uncoating and Reverse Trancription

Following entry, the viral core is released in the cytoplasm of the newly infected cell. Uncoating is a poorly defined process that leads to the core decapsidation and allows the transition from the reverse transcription complex (RTC) to the pre-integration complex (PIC). Although it has long been thought that the uncoating process occurrs immediately following the entry step, recent studies tend to demonstrate that it actually is a more progressive step where the capsid actively plays a role in the migration of the RTC towards the nuclear pore through interaction of viral CA with the cellular cytoskeleton [36].

The RTC subviral particle contains both viral and cellular components. Indeed, along viral components (two molecules of gRNA, Vpr, Vif, Nef, RT and IN), cellular factors are also essential for the cascade of reactions that lead to productive infection. For example, cyclophilin A (CypA) protects the viral core *via* its interaction with CA [37, 38], and $tRNA^{Lys3}$ initiates reverse transcription through its association with the primer binding site (PBS) on the viral RNA [39, 40].

RT is a multifunctional enzyme that carries DNA polymerase and RNase H activities. The DNA polymerase activity is both DNA- and RNA-dependent and the RNase H domain is responsible for the digestion of RNA from the RNA/DNA duplexes that are formed during the reverse transcription process. Reverse transcription is known to be an error-prone mechanism, leading to high virus mutation rates, responsible for immune escape and drug resistance. RT exists as a heterodimer, which is constituted by two subunits: p51 and p66. While p66 is the

catalytic subunit, p51 is obtained by the C-terminal cleavage of p66 and plays a regulatory function. Reverse transcription is a multistep cascade that occurs within the RTC and leads to the conversion of gRNA to dsDNA [41]. The reverse transcription process can be decomposed as follows: (Figure 8)

- Reverse transcription initiates with the hybridization of the 3' 18-nucleotide segment of the $tRNA^{Lys3}$ primer to the PBS region of the gRNA. This primer is elongated until the 5' end of the gRNA, resulting in the synthesis of the minus strand strong-stop DNA ((-)ssDNA). Simultaneously, the gRNA that is part of the DNA/RNA hybrid is degraded by the RNase H activity of RT;
- (-)ssDNA contains the repeat sequence R that is present in both the 5'LTR and the 3'LTR of the gRNA. This sequence allows the (-)ssDNA to transfer from the gRNA 5'R to the gRNA 3'R. This is the first strand transfer. Following this "jump", the viral DNA is synthesized towards the 3' end of the gRNA until the PBS region, and the gRNA, which serves as a template, is degraded *via* the RNase H activity of the RT enzyme, except for the two polypurin tracts (PPTs);
- The synthesis of the positive sense DNA strand initiates at the PPT sequences, leading to the formation of U+ (from the 3' PPT) and D+ (from the central PPT) DNAs. This step is named the plus strand strong-stop DNA, or (+)ssDNA. Following polymerization of the $tRNA^{Lys3}$, which generates a new PBS, the RNase H digests the primer and the PPTs;
- During the second strand transfer, the complementary sequences of the two PBS (one from the (-)ssDNA and the other from the (+)ssDNA) hybridize,

allowing the completion of DNA synthesis with the formation of the two LTRs. Furthermore, the formation of a DNA Flap during the elongation of the viral positive DNA strand is essential for the import of the PIC into the nucleus [42, 43].

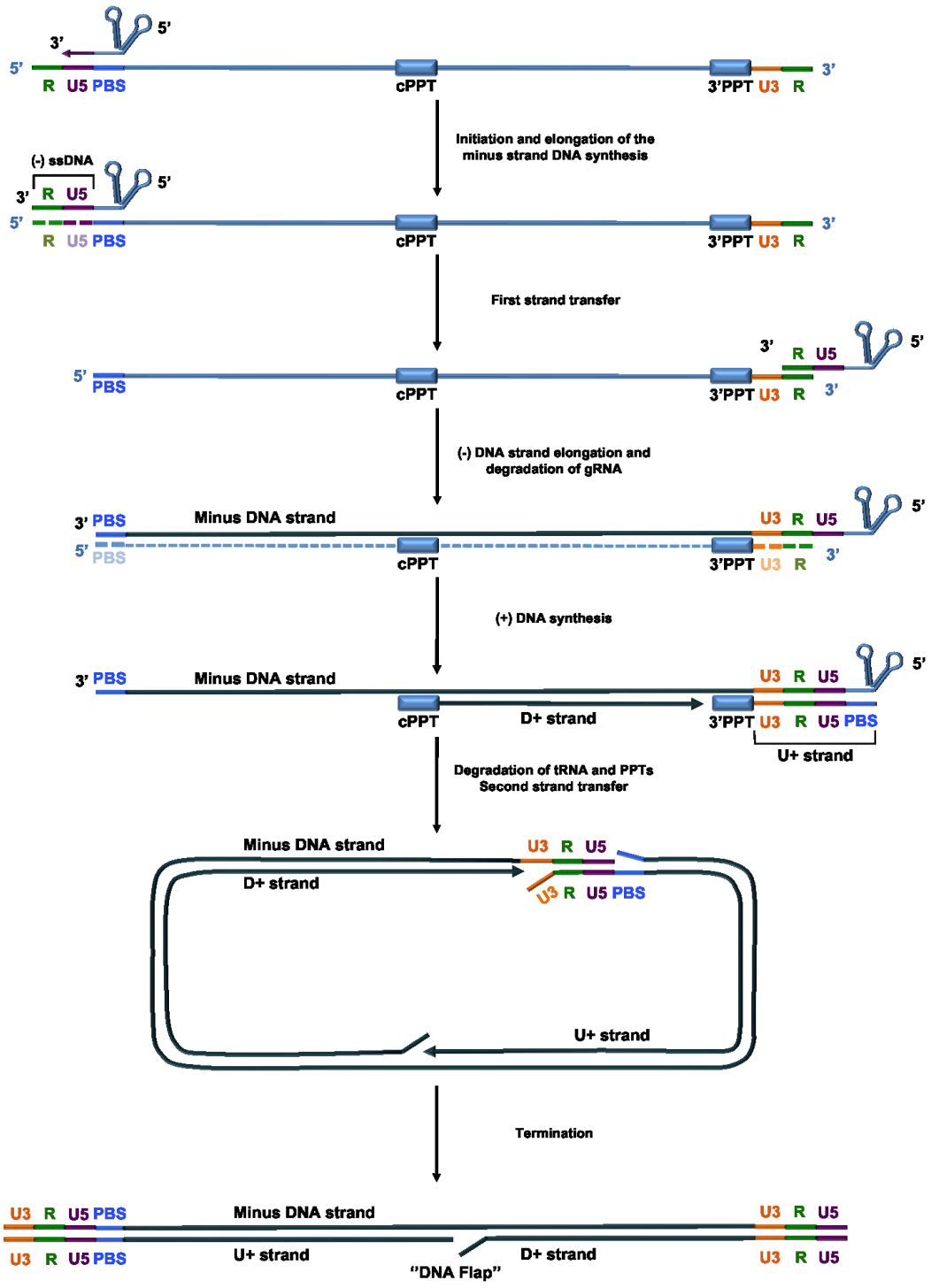


Figure 8 | Reverse Transcription of HIV-1 gRNA

1.2.3.1.3 Nuclear import of the Preintegration Complex

Similarly to other lentiviruses, HIV-1 has the ability to infect non-dividing cells and therefore pass through the intact nuclear envelope. Following reverse transcription, the newly synthesized viral DNA, which is part of the RTC, uses the microtubule network to travels towards the nucleus [44]. During this migration the composition of the ribonucleoprotein complex changes, and the viral CA, along with the DNA Flap, drives the maturation of the RTC into PIC [45]. This complex recruits the cellular machinery that leads to its efficient translocation through the nuclear pore complex. The size of the PIC being about 30 nm [46], HIV-1 needs a dynamic process to deliver its genome through the nuclear pore, which size is comprised in a 16-20 nm range [47, 48]. The PIC is composed of at least three viral proteins including MA, Vpr and IN and cellular proteins such as LEDGF (Lens Epithelium-Derived Growth Factor), TNPO3 (Transportin 3) and other importins [49]. The cellular components that are included in the PIC can vary depending on the cell type, to recruit specific factors allowing efficient nuclear translocation [49].

- MA was the first viral component to be identified as a player in HIV-1 nuclear import [50]. MA contains two nuclear localization signals (NLSs), and interacts with cellular importin α, contributing to PIC translocation to the nucleus. However, some studies reported that MA was not essential and that viruses mutated within its NLSs were still able to replicate [49, 51, 52];

- Considering the fact that Vpr does not carry any NLS motif, it was suggested that it accumulates in the nucleus through another mechanism involving other nuclear import pathways [53]. Vpr has been shown to interact with importin α [54], and to stimulate HIV-1 replication, especially in macrophages [49]. Like MA, Vpr is not essential, but its loss seriously impedes HIV-1 replication [49]
- IN carries a NLS that makes it highly karyophilic [55]. In addition to its natural nuclear localization, HIV-1 IN is recognized by importin α [56]. However, this interaction does not seem to be involved in nuclear import [57], and it would rather be through the interaction with a combination of importins (α1, α3, 7), TNPO3/transportin-SR2 and LEDGF/p75 that IN would participate in the nuclear import of HIV-1 DNA [49];
- The capsid protein CA was recently reported to be involved in the regulation of HIV-1 nuclear import by remodeling the composition of the PIC in importins and nucleoporins. However, no interaction has yet been found between CA and such importins or nucleoporins, suggesting that CA could affect the import step by regulating the maturation of the RTC into PIC, therefore influencing the composition of the latter [49].
- The DNA Flap structure, which is formed during the reverse transcription, also seems to regulate HIV-1 DNA nuclear import. However, the mechanism whereby this motif promotes nuclear import of the PIC remains unclear. More studies will be needed to elucidate whether the DNA Flap facilitates the

uncoating process or if it is involved in structural conformation changes allowing the recruitment of cellular factors [49].

1.2.3.1.4 Integration

Upon import of the PIC to the nucleus, the viral DNA is covalently integrated into the host genome. This critical step is the irreversible event catalyzed by the tetrameric viral IN that leads to the establishment of a permanent infection and forms the basis of latency, allowing the creation of virus reservoirs. The integration process can be decomposed in two distinct enzymatic reactions: the initiation, occurring in the cytoplasm and the insertion, taking place in the nucleus. In the cytoplasm, HIV-1 IN dimer targets the two viral LTRs and triggers a nucleophilic attack that leads to the cleavage of a conserved CA dinucleotide, yielding to the exposure of free CA^{OH} 3'-hydroxyl groups, essential for the second step (Figure 9) [58]. In the nucleus, HIV-1 IN performs a second nucleophilic attack resulting in a nick in the host DNA, therefore creating free 5'-phosphate extremities in the cellular chromatin. Finally, IN tetramers join the viral DNA ends to the 5'-ends of the host genome and the cellular DNA repair machinery completes the integration process by filling up the gaps and covalently linking the two DNA strands, thus leading to successful DNA strand transfer (Figure 9) [58].

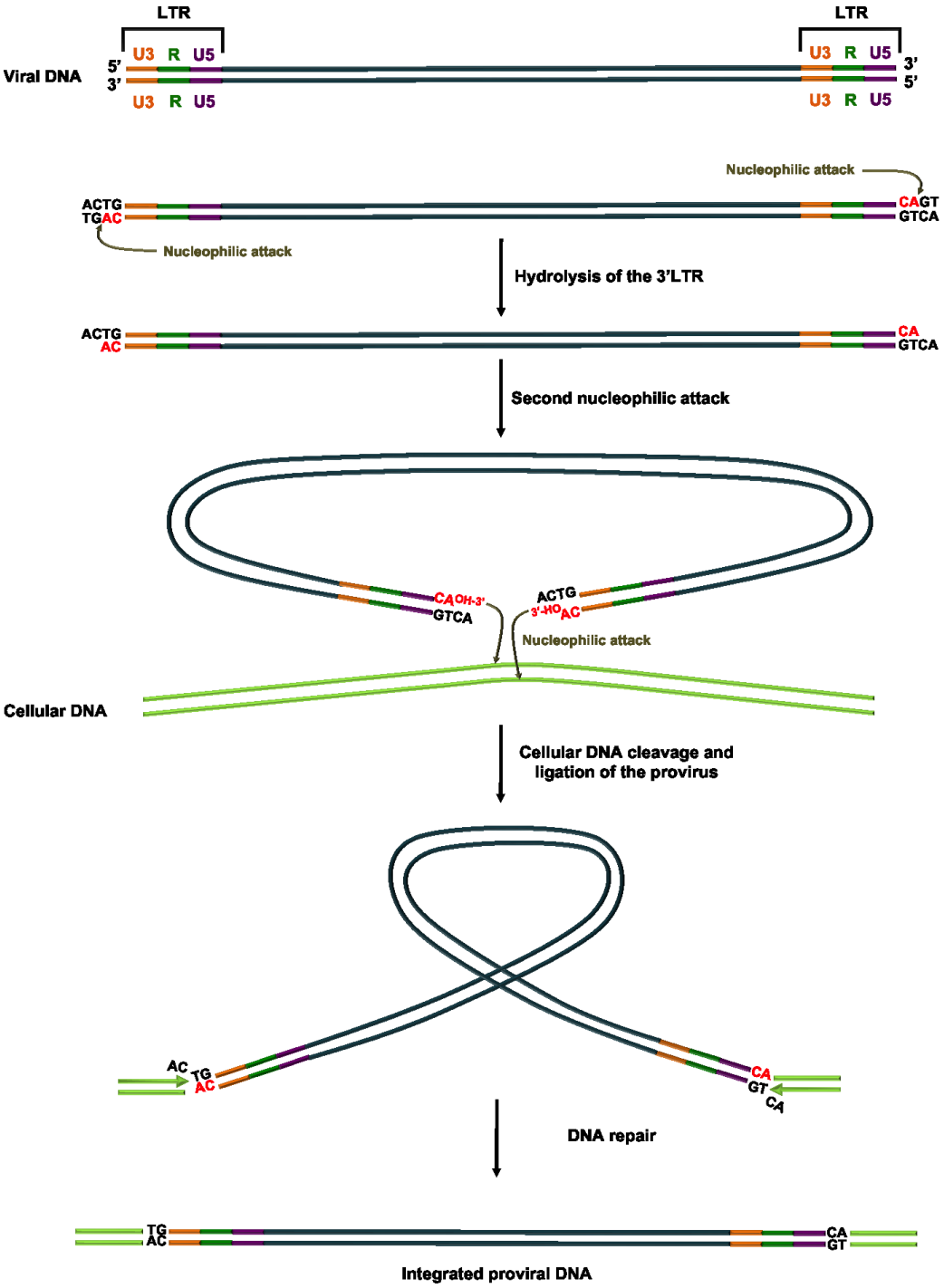


Figure 9 | Integration of HIV-1 DNA into the cellular DNA

Several viral and host factors can modulate the efficiency of the integration mechanism, including LEDGF/p75, HIV-1 NC and HIV-1 Rev [58, 59].

LEDGF/p75 promotes the integration process through bridging the chromatin to the C-terminal domain of HIV-1 IN [60]. HIV-1 NC facilitates the joining of the two viral DNA ends, leading to successful insertion of the viral genome [61]. Recently, Rev was reported to interact with HIV-1 IN and LEDGF/p75, preventing the integration process to occur, presumably in the attempt to reduce superinfection and death of the host cell [61].

1.2.3.2 Late Stages

While early stages of HIV-1 replication mainly depend on the viral enzymes that are initially incorporated into the viral core, late stages require many types of cellular machinery and different virus-host interactions to produce viral RNAs and proteins that are necessary for the assembly of new particles.

1.2.3.2.1 Transcription

1.2.3.2.1.1 Tat-independent Transcription

Once integrated in the host genome, HIV-1 provirus behaves like endogenous DNA and is transcribed into RNA by the cellular RNA polymerase II. HIV-1 DNA transcription can be decomposed in two steps: Tat-independent (basal transcription) and Tat-dependent. Basal HIV-1 DNA transcription is a low-

efficient process that is stimulated by a viral DNA motif known as inducer of short transcripts (IST) [62]. This IST promotes the formation of transcription complexes that are incapable of efficient elongation, resulting in non-polyadenylated 50-nucleotides viral RNAs [62]. Upon viral infection, macrophages release cytokines that activate infected cells, allowing translocation of NF-κB from the cytoplasm to the nucleus. NF-κB binds to the promoter activation region and induces basal transcription as a result of chromatin decondensation within the LTR [63]. Furthermore, Vpr and IN also display stimulatory effects on viral basal transcription by rearranging chromatin structure [64, 65], allowing SP-1 factor to interact with the promoter region and initiate elongation [66]. This process leads to the production of low amounts of full length viral RNAs that are constitutively spliced by the cellular machinery.

1.2.3.2.1.2 Tat-mediated Transcription

Tat protein acts a *trans*-activator of viral transcription and plays a key role in viral genome expression. Through binding to the TAR hairpin within the 5' UTR of the newly synthesized viral RNA, Tat contributes to the recruitment of various cellular factors, including p-TEFb and PCAF, that will enhance elongation and transcriptional activity by more than 100-fold [67]. First, p-TEFb, which is a heterodimer constituted of CycT1 and CDK9, is recruited to the loop of the TAR structure [68]. This complex triggers the phosphorylation of the CTD of RNA pol II, resulting in an increase of its activity and higher amounts of viral

RNA produced. Following transcription, these RNAs are subjected to alternative splicing and exported *via* the Rev-independent or Rev-dependent pathway.

1.2.3.2.2 Splicing

Carrying only 9 genes, HIV-1 has evolved ingenious, complex and highly regulated splicing mechanisms allowing the production of the 15 viral proteins that are necessary to generate new infectious particles. Therefore, HIV-1 transcripts can be constitutively or alternatively spliced, leading to the production of ~30 RNA variants that are grouped under three main species: 2kb (multiply spliced), 4kb (singly spliced) and 9kb (full length).

1.2.3.2.2.1 Constitutive Splicing

Constitutive RNA splicing consists in removing non-coding sequences (introns) allowing the juxtaposition of coding sequences (exons). The cellular complex responsible for the splicing events is called spliceosome. The spliceosome machinery is composed of five core snRNPs (U1, U2, U5 and U4/U6) and includes more than 300 other proteins that regulate the splicing process [69]. Splicing is initiated by the recognition of the 5' splice site (5'SS) by U1 and by the binding of splicing factor 1 (SF1) to the branch point (BP). The heterodimer U2AF (U2AF65/U2AF35) recognizes and binds to the polypyrimidine tract within the intron, upstream of the splicing acceptor (SA) site,

and induces the recruitment of U2 that replaces SF1 at the splicing donor (SD) site. The interaction between U1-pre-mRNA-U2 and U4-U5-U6 triplex then triggers conformational rearrangements in the mRNA. These structural rearrangements allow two successive transesterification reactions, resulting in the excision of the intron and the ligation of the two exons side by side [69] (Figure 10A). For HIV-1, constitutive splicing leads to the production of the 2kb RNA species, responsible for the expression of the regulatory protein Tat and Rev and the accessory protein Nef (Figure 11).

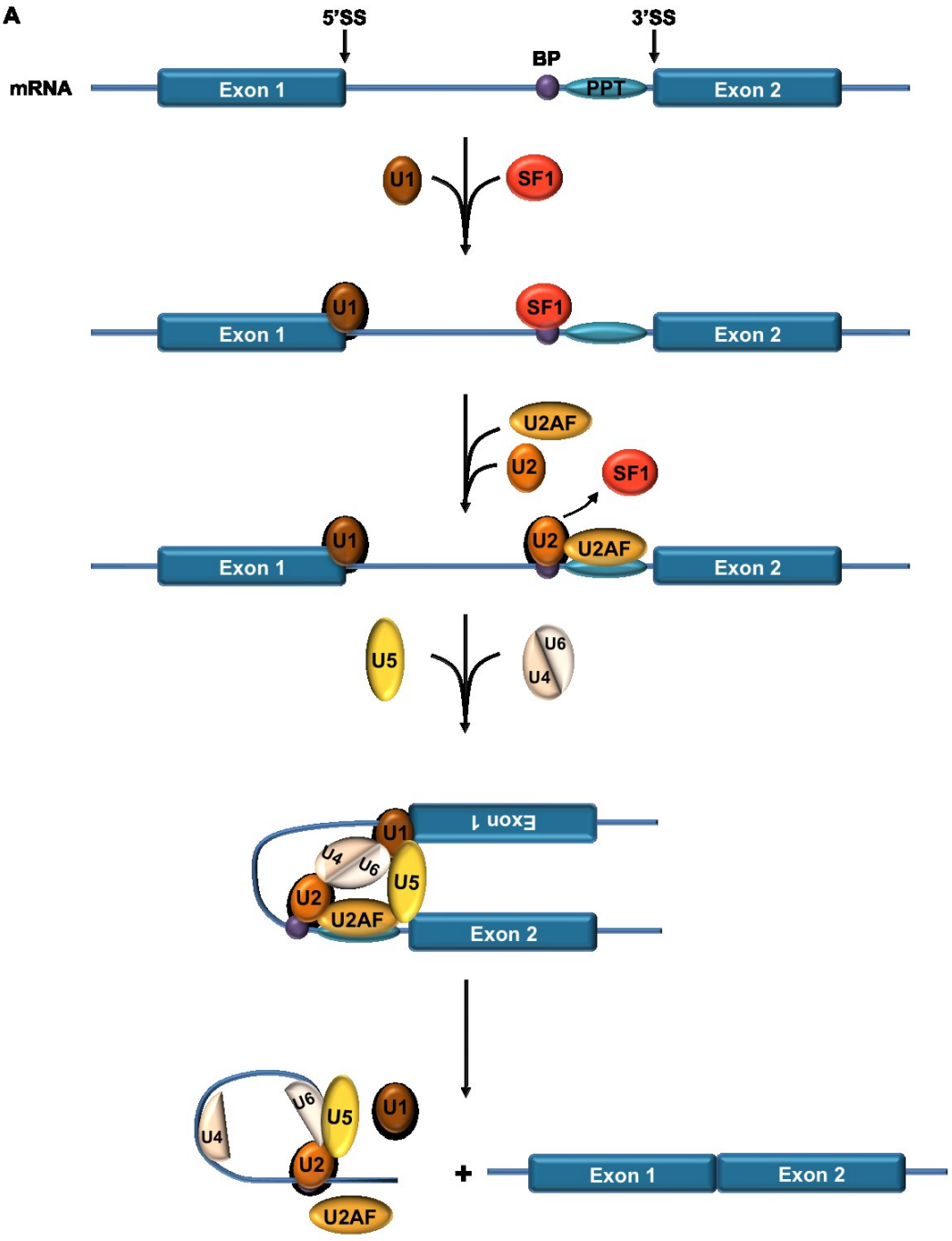

A
5'SS
3'SS
BP
mRNA
Exon 1
PPT
Exon 2
U1
SF1
U2AF
U2
U5
U6
U4
B
hnRNP
SR
SR
hnRNP
ESS
Exon 3
ESE
ISE
ISS

Figure 10 | Basic mechanism of splicing
(**A)** The four conserved motifs that enable recognition of RNA by the spliceosome are: the exon–intron junctions at the 5′ and 3′ ends of introns (the 5′ splice site (5′ SS) and 3′ SS), the branch point (BP) sequence located upstream of the 3′ SS and the polypyrimidine tract (PPT) located between the 3′ SS and the BP. U1 and splicing factor 1 (SF1) are recruited to the 5'SS and the BP, respectively. U2AF binds to the PPT and recruits U2 that replaces SF1. U4/U6 and U5 associate into a triplex and trigger a conformational change of the RNA, allowing the formation of a catalytic complex and the two exons to become closer, leading to the excision of the intron. (**B**) Exonic and intronic splicing modulatory sequences and regulatory proteins. Exon 1 and Exon 2 are constitutive; Exon 3 represents an alternatively spliced exon (adapted from [70]).

1.2.3.2.2.2 Alternative splicing

Alternative splicing of HIV-1 RNA allows the production of 4kb and 9kb RNA species, responsible for the synthesis of structural, envelope and accessory proteins (Figure 11). This highly regulated process is one of the mechanisms that HIV-1 has evolved to control the equilibrium among viral protein amounts that is necessary for virus production. To be able to produce such various RNA species, HIV-1 uses the combination of 5 splicing donor sites (D1, D1A, D2, D3 and D4) and 9 splicing acceptor sites (A1, A1A, A2, A3, A4c,a,b, A5 and A7) [71, 72]. Alternative splicing process requires signals that promote or inhibit exon definition, resulting in specific intron excision and synthesis of unique viral transcripts. Viral RNA secondary structures play essential roles in the regulation of alternative splicing events. Particular rearrangements into stem loop structures within introns and exons of the viral RNA act as *cis*-regulatory elements that allow the recruitment of specific spliceosome components, leading to distinct outcomes regarding exon definition. While exonic and intronic splicing enhancers

(ESE and ISE) facilitate exon definition, exonic and intronic splicing silencers (ESS and ISS) repress it [73]. Thus, ISS and ESS recruit hnRNP A/B and hnRNP H [74, 75], blocking access to the adjacent SA site, while ISE and ESE recruit SR proteins such as SRp75, facilitating exon definition (Figure 10B) [76]. Once specific SA and SD sites are selected, the spliceosome complex is recruited and triggers the excision of the intron, as described above.

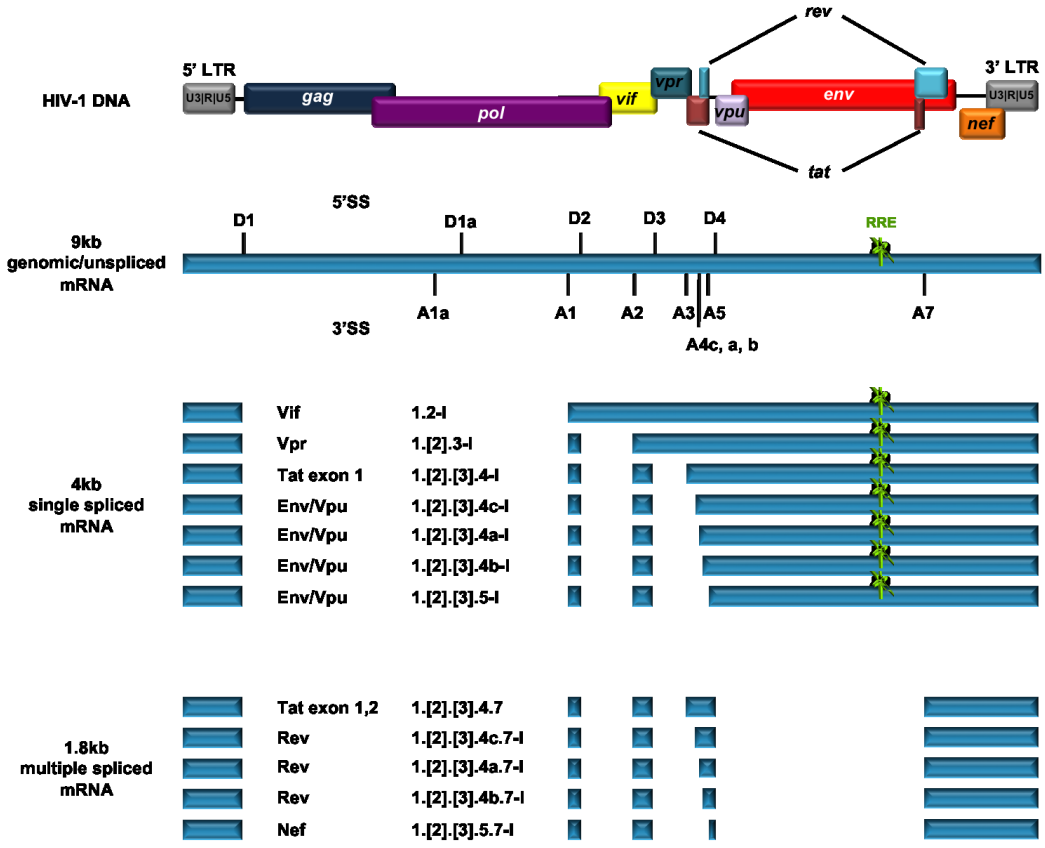


Figure 11 | HIV-1 mRNA splicing profiles
Upper panel: representation of the genomic organization of HIV-1 DNA. Lower panel: transcription allows the synthesis of pre-mRNA that contains many 5' and 3' splice sites (5'SS and 3'SS, respectively). Alternative splicing leads to the production of single spliced (4kb) and multiple spliced (1.8kb) HIV-1 mRNA. Only major spliced species are represented. « I » indicates incompletely spliced mRNAs. LTR, long terminal repeat; D, donor; A, acceptor; RRE, Rev response element (adapted from [72]).

1.2.3.2.3 Nuclear Export

1.2.3.2.3.1 Rev-independent Nuclear Export: TAP pathway

Similarly to transcriptional events, two mechanisms responsible for HIV-1 RNA export exist: Rev-independent and Rev-dependent. Following transcription, RNAs need to cross the nuclear pore complex (NPC). This export process requires transporters that recognize different RNA species and deliver them into the cytoplasm [77]. Non-coding RNA molecules are recognized by exportins that leave the nucleus in a Ran-dependent mechanism. These include transfer RNA (tRNA), which is recognized by exportin-t, micro RNA (miRNA) by exportin-5, small nuclear RNA (snRNA) and rRNA by Crm1 (also named exportin-1). Conversely, export of mRNA is independent of the Ran pathway. Along with transcription, the nascent RNA molecules become associated with cellular proteins including CBC (cap-binding complex), ALY, Yral, TAP/NXF1 and p15/Mtr2 that together dock the mRNP complex to the NPC and transport the mRNP across the nuclear membrane [77]. The multiply spliced HIV-1 RNA molecules follow this latter route to leave the nucleus and allow the new production of the first viral proteins: Tat, Rev and Nef. The fact that Tat and Rev carry a NLS in their sequence then allows them to be translocated to the nucleus where they will stimulate transcription and export of incompletely spliced viral RNAs, respectively (Figure 12).

1.2.3.2.3.2 Rev-dependent Nuclear Export: CRM1 Pathway

In addition to the multiply spliced RNA, full length and singly spliced HIV-1 RNAs must also be exported to the cytoplasm. Since mRNAs do not normally leave the nucleus before complete splicing, HIV-1 requires a specific mechanism to export incompletely spliced transcripts. All 4kb and 9kb viral mRNAs possess an intron within the *env* gene that contains a complex stem-loop structure, the Rev responsive element (RRE) motif. This RRE recruits the viral protein Rev along with other cellular factors, including DDX1 and DDX3, to promote the export of intron-containing viral transcripts *via* the CRM-1/Ran-GTP pathway (mechanism further described in Chapter 2) (Figure 12).

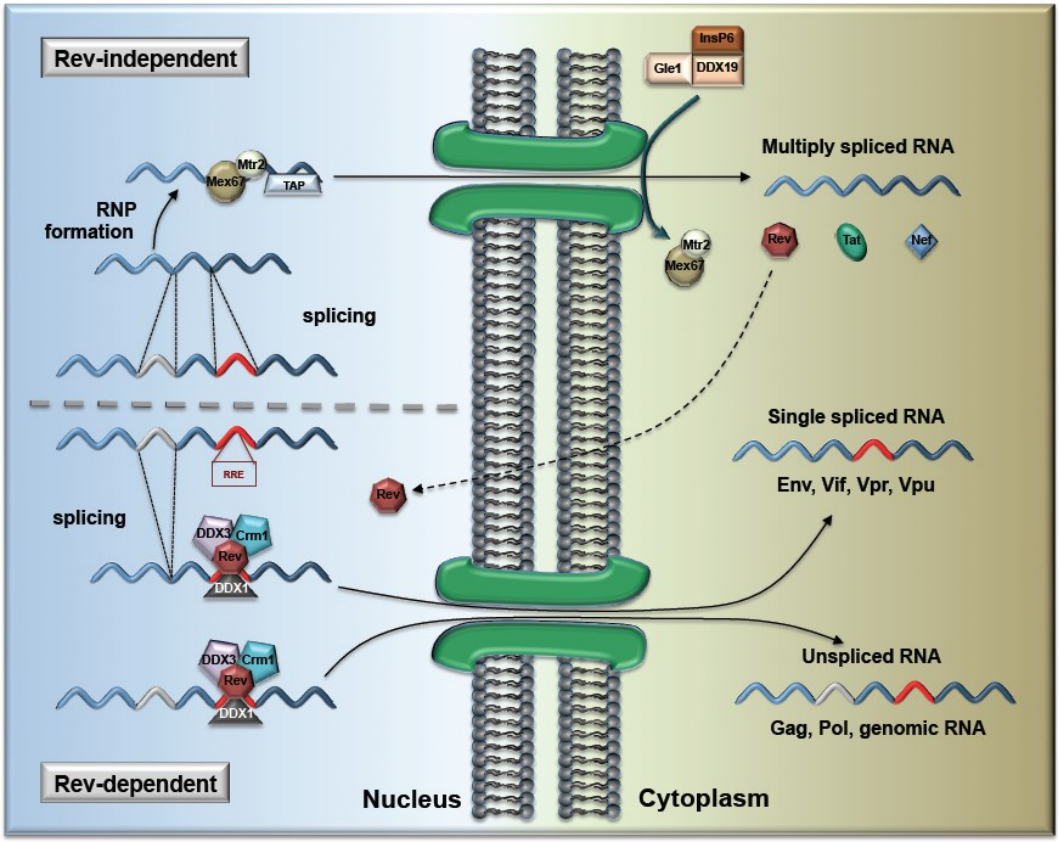


Figure 12 | HIV-1 RNA export pathways

Constitutively spliced HIV-1 RNA associates with Mex67, Mtr2 and TAP before being exported to the cytoplasm. Gle1 and InsP6 are present at the cytoplasmic face of the NPC, Dbp5 (human DDX19 homolog) remodels the mRNP and removes RNA transporters Mex67 (human TAP homolog) and Mtr2 (human NXT1 (nuclear transport factor 2-like export factor 1) homolog) [78, 79]. Uncompletely spliced HIV-1 RNAs associates with Rev, DDX1, DDX3 and Crm1 and are exported to the cytoplasm to be translated into structural proteins, envelop proteins and accessory proteins.

1.2.3.2.4 Translation

Upon the export of HIV-1 transcripts to the cytoplasm, the cellular translational machinery is used to produce all viral proteins:

- Multiply spliced mRNA is translated into Tat, Rev and Nef;
- Singly spliced mRNA is translated into $gp160^{Env}$, Vif, Vpr and Vpu;
- Full length mRNA is translated into $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$.

To synthesize the diversity of proteins that the virus needs to produce new particles, HIV-1 has evolved in generating bi-cistronic RNAs: *env/vpu* and *gag/gag-pol*, that require additional regulatory mechanisms.

- *vpu* and *env* coding sequences overlap and Vpu initiation AUG codon is located upstream of the Env initiation codon. Weak recognition of Vpu initiation codon results in a leaky scanning of the ribosomal complex on the viral RNA, allowing Env synthesis [80].

- *gag* and *pol* coding sequences are supported by the full length viral RNA but do not share the same reading frame. To overcome this restriction and to be able to express *pol* gene, HIV-1 uses a slippery sequence “AAUUUUUUAG”, called (-1) frameshift. In ~5% of cases, this frameshift results in the loss of recognition of the

gag stop codon and the ribosomal scanning of *pol* sequence, allowing the synthesis of $Pr160^{Gag\text{-}Pol}$ precursor and expression of optimal ratio of viral proteins [81, 82].

Several Gag isoforms also exist, as a result of cap-independent translation, due to the presence of an internal ribosome entry site (IRES) within the viral 5' UTR. This IRES-dependent translation mode has been proposed to stimulate protein synthesis during the Vpr-induced G2/M cell cycle arrest, when the cap-dependent translation is inactivated [83-85]. Recently, the viral protein Rev was identified as an additional factor regulating HIV-1 translation, through binding to the viral 5' UTR. While high levels of Rev non-specifically inhibit translation, low concentrations tend to have a stimulatory effect [86].

1.2.3.2.5 Assembly and Packaging

Virus particles are enveloped particles made of a phospholipid bilayer, which protects the viral core composed of the gRNA, $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ precursors. $Pr55^{Gag}$ supplies the structural proteins (MA, CA and NC) as well as three peptides (p2, p1 and p6, also called "spacer peptides" SP2, SP1 and SP6). In addition, $Pr160^{Gag\text{-}Pol}$ provides the viral enzymes (PR, RT and IN) (Figure 13A). Gag plays a key role in the assembly process, as it encodes all the information necessary for the production of virus-like particles (VLPs) [87, 88]. Indeed, distinct functional domains of $Pr55^{Gag}$ allow for the recruitment and encapsidation

of gRNA dimer, Gag assembly and cohesion, Gag targeting to the plasma membrane and binding to the envelop proteins [88] (Figure 13B).

- HIV-1 NC is a multifunctional protein that facilitates viral replication at many levels, including late stage. Its two zinc fingers confer NC high affinity for nucleic acids, and NC-RNA interaction through the genomic packaging signal Ψ induces the packaging of viral gRNA dimer. The two RNA strands are non-covalently dimerized in their 5′ UTR through formation of a "kissing-loop" structure mediated by the dimer initiation site (DIS) [89]. Within mature particles, NC coats the viral genome, acting as a chaperone and participating in further steps of the virus replication cycle. Furthermore, through binding to HIV-1 RNA, NC allows the formation of Gag/Gag-Pol complexes and Gag multimerization [90, 91];
- HIV-1 MA is responsible for targeting Gag to the assembly sites of the plasma membrane, the lipid rafts [88]. Upon Gag multimerization, HIV-1 MA adopts a new conformation resulting in the exposure of its N-terminal myristoylated domain. This domain interacts with the cellular phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P(2)), which triggers the anchorage of Gag in the plasma membrane [92]. In addition to promoting Gag assembly, HIV-1 NC also recruits and incorporates Env into the plasma membrane [88]. Following translation in the endoplasmic reticulum (ER) as a $gp160^{Env}$ precursor, Env polyprotein is directed to the *trans*-Golgi network (TGN) where it undergoes extensive glycosylation. The cellular protease called furin cleaves $gp160^{Env}$ precursor into non-covalently linked gp41 and

gp120 subunits that assemble into spikes at the plasma membrane [23]. Acting as an adaptor, the cellular TIP47 (tail-interacting protein of 47 kDa) binds to the cytoplasmic tail of gp41 and the CTD of MA, thus bridging Gag to Env and mediating Env spikes incorporation in the viral envelope [93];

- HIV-1 CA plays a mechanistic role in the assembly process, maintaining the cohesion between the 1500 CA molecules forming the viral core [88].

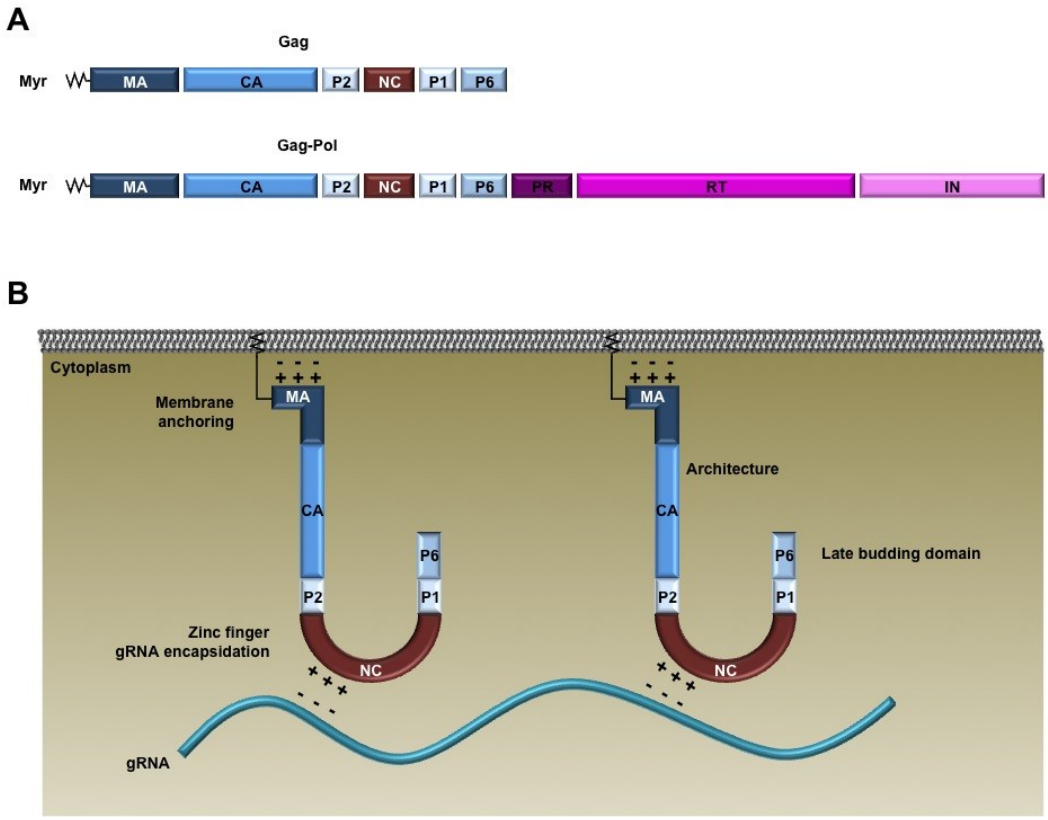


Figure 13 | HIV-1 assembly
(**A**) Schematic organization of Gag and Gag-Pol precursors. (**B**) Association of Gag with the plasma membrane and the viral genomic RNA (gRNA) (adapted from [94]). CA = capsid; MA = matrix; gRNA = genomic RNA.

1.2.3.2.6 Budding

Budding of HIV-1 particles was first described in macrophages within structures very similar to late endosomes [95, 96]. However, a controversy emerged regarding the specific location of virus budding. Although the composition of HIV-1 envelope is very similar to the one of late endosomes, it is also rich in phosphatidylserine, sphyngomyelin, saturated phosphatidylcholine and cholesterol, characteristic of the lipid rafts within the plasma membrane [97]. It is now accepted that the budding process can occur at both sites, and that it happens mainly in late endosomes for macrophages [95, 98, 99] and at the plasma membrane for lymphocytes [100]. In both cases, the virus recruits the cellular ESCRT (endosomal sorting complex required for transport) machinery to release particles.

$Pr55^{Gag}$ uses its p6 domain, or "late" domain, to recruit all the necessary cellular elements that are required for the release of viral particles, which includes the ESCRT system. This system is composed of four complexes: ESCRT-0, I, II and III, with 0 and I being indispensable for HIV-1 release [101, 102]. The late domain p6 contains two motifs, PTAP and YPXnL (where X can represent any amino acid and $n = 1 \sim 3$). To promote budding of new viral particles, p6 interacts with the N-terminal domain of TSG101 (ESCRT-I subunit) through its PTAP motif [103], and with the cellular protein ALIX through its YPXnL motif [104]. TGS101 and ALIX can also interact directly through the PTAP motif carried by ALIX [104].

1.2.3.2.7 Maturation

The first details regarding the maturation process arose from pictures obtained from electronic microscopy studies showing that two types of viral particles exist (Figure 14). While immature virions display a dense layer right underneath the envelope, mature ones contain a structurally organized cone-shaped core. The maturation process allows the rearrangement of $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ components within the particle, which transforms the non-infectious viral particles into mature virions capable of infecting new target cells.

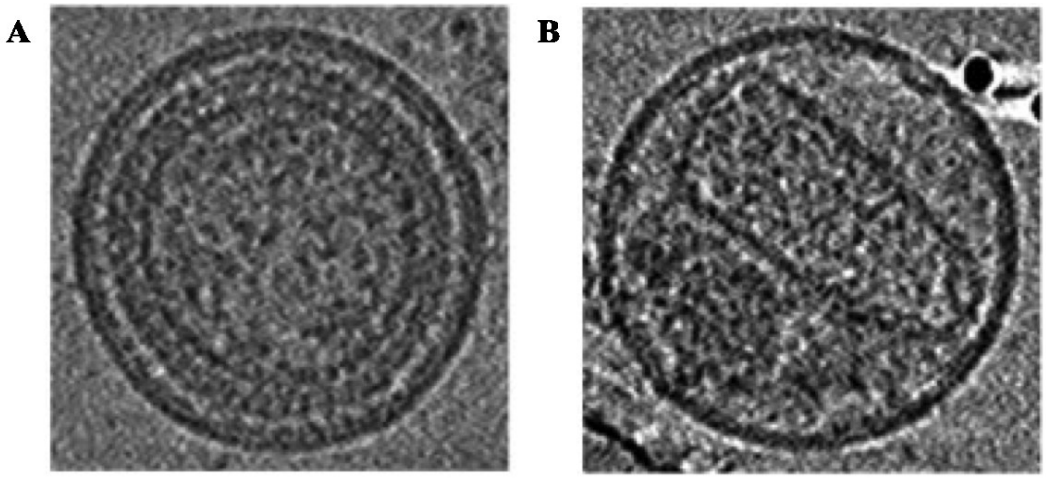


Figure 14 | HIV-1 particles
Electronic microscopy of immature (**A**) and mature (**B**) HIV-1 viral particles [105].

During the assembly process, Gag and Gag-Pol multimerize, which brings together the PR domains, causing their dimerization and activation [88, 106]. This self-activation triggers an autocatalytic cleavage that ultimately releases the PR enzyme. In a series of five sequential and ordered proteolytic reactions, HIV-1 PR

releases all the maturation intermediates, including SP1, NC, MA, CA and SP2 [88, 107-109]. The first cleavage occurs at the C-terminus of SP1, which separates MA-CA-SP1 from NC-SP2-p6. A second cleavage separates MA from CA-SP1 and NC-SP2 from p6. SP1 is then cleaved from CA, which further aggregates to form the cone-shaped viral core [107].

1.2.3.3 Latency

1.2.3.3.1 Rationale

Since 1996, highly active antiretroviral therapy (HAART) successfully managed to reduce viral replication to undetectable levels, as being measured per number of viral RNA copies in the blood. However, HAART is only effective in cells that actively transcribe the viral genome, and inefficient in latently infected cells. Latency is defined as a reversibly non-productive state of infection of individual cells [110]. Therefore, these cells remain able to produce new infectious viruses upon reactivation of the viral genome transcription. This latency phenomenon results in the early establishment of extremely stable reservoirs where HIV-1 provirus remains in a dormant state.

These reservoirs have now been identified and are found in many distinct tissues, including the lymphoid tissue (CD4+ resting memory T cells), the brain (central nervous system, CNS), the gut-associated lymphoid tissue (GALT), and the bone marrow (hematopoietic progenitor cells) [110, 111].

Statistically, the half-life of a latently infected cell can reach 44 months [112], meaning that it would take up to 60 years to eradicate the HIV-1 reservoirs in patients taking HAART treatment [113]. Moreover, a few weeks interruption of HAART treatment results in the restoration of the reservoirs. As a consequence, today HAART remains a life-long therapy that is also associated with compliance, resistance, toxicity and cost issues [111]. Therefore, reservoirs are considered as the major barrier to HIV-1 eradication, and it is of crucial interest to understand latency mechanisms in order to, one day, cure HIV-1.

1.2.3.3.2 Mechanisms

The observation that latent HIV-1 is found in resting memory CD4+ T cells, but not in naive CD4+ T cells, brought to attention that the cells become infected during the transition from activated effectors to resting memory cells [110]. Indeed, no specific cellular repressors have been found to force the provirus to enter a latent state [114]. In fact, latency is rather caused by a combination of factors that results in the inactivation of viral transcription.

- The transcriptional environment is not suitable for HIV-1 gene expression. The transcription of the provirus is highly regulated by the viral protein Tat. In the context of latency, it was shown that less Tat is available, leading to lower amounts of viral genes expressed. This phenomenon is also called “molecular switch” [114]. Furthermore, host cellular transcription factors,

including pTEFb, NF-κB, NFAT are sequestered in the cytoplasm, which results in the non-activation of the elongation process [110, 114, 115];

- Normally, HIV-1 provirus preferentially integrates into an active region of the host chromosome, which provides a favorable environment for transcription [116]. In some cases, the provirus integrates in "gene deserts" where the DNA is compacted into heterochromatin. Therefore, chromatin condensation renders the viral genome less subject to transcription [117];
- Finally, epigenetic regulation also restricts transcriptional activity, through post-translational modifications of histones, including phosphorylation, acetylation, methylation and ubiquitination [117].

1.2.3.3.3 Approaches

The latency state highly correlates with a loss of Tat activity. Consistently, the expression of Tat in *trans* from an ectopic promoter greatly unable the cells to enter latency [118]. The resulting strategy to purge HIV reservoirs is to reactivate the transcription of the provirus, eventually inducing cell death, a strategy also called "shock and kill" [119]. To date, several approaches are being envisaged to eliminate latency, including:

- Treatment with SAHA (suberoxylanilide hydroxamic acid), a HDAC inhibitor that reactivates HIV-1 provirus transcription [115];
- Treatment with HMBA (hexamethylene bisacetamide), an inducer of transcriptional elongation [119];

- Activators of PKC (Protein kinase C), a cellular protein involved in signal transduction pathways activated upon adaptive immune response [119]. One approach is to develop PKC activators [115];
- Activators of IL-7, a cytokine that can stimulate transcription through activation of the JAK/STAT pathway [120]. However this approach is controversial since IL-7 was also reported to be involved in the maintenance of reservoirs [121];
- Methylation inhibitors. HIV-1 CpG islands are methylated, which contributes to the establishment of latency through inhibiting transcription [115];
- Gene therapy. Introducing a pool of CD4+ CCR5Δ32/Δ32 cells, resistant to HIV-1, through bone marrow transplant. Although this has been done once in human, the "Berlin Patient" [122], this technique would be limited to patients with lymphomas or leukemias [111] and remains at high risks.

Finally, a consensus is that a combination of immune priming of CD8+ T cells with HIV-1 Gag along with reactivation of transcription activity would be necessary to escape from latency [110]. Moreover, limitations arise from the difficulty to measure and target HIV reservoirs, which renders the design of models for eradication and trials very challenging.

1.3 Evolution of HIV Disease

1.3.1 Stages of HIV-1 Infection and Disease Progression

In non-treated patients, HIV-1 results in a chronic infection of the immune system, leading to the progressive depletion of the central players in the defense mechanisms, the CD4+ T cells. CD4 is the main receptor that HIV-1 uses to enter the cells, located at the surface of several cell types, including monocytes/ macrophages, dendritic cells, and cells from the lymphoid tissues [24, 25]. Due to their localization in the mucosa, dendritic cells are the first to be exposed to the virus. Upon their migration to the lymph nodes, they transmit the virus to monocytes, macrophages and CD4+ T cells [123], allowing further dissemination of viruses in the blood circulation. HIV-1 infection can be decomposed in three major phases: primary infection (acute), asymptomatic (chronic), and symptomatic (AIDS) (Figure 15).

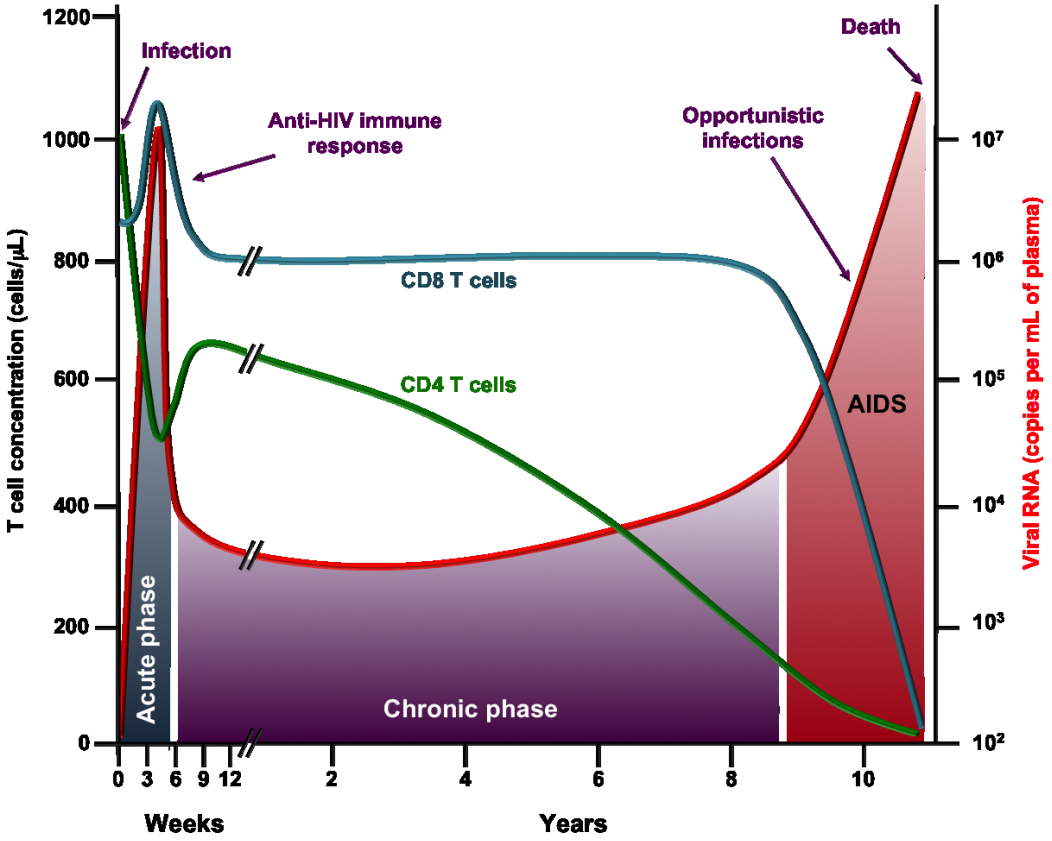


Figure 15 | Evolution of HIV-1 infection in non-treated patients
Natural history of HIV-1 infection. Representative progression of plasma viral load (Viral RNA) (red) CD8 T cell counts (blue) and CD4 T cell counts (green) in a normal progressor. AIDS occurs when CD4 T cell counts drop to <200 cells/µL. Time post-infection is indicated in weeks and then in years, with the acute phase of infection being indicated by weeks 1–5. The asymptomatic phase of infection can last up to 8-9 years post infection. (Adapted from [124, 125]).

1.3.1.1 Acute Phase

The acute phase only lasts for a few weeks (Figure 15) and is characterized by an extremely high viremia, causing the rapid dissemination of viruses to the whole body. The viral load can thus reach a hundred million particles per milliliter of plasma [124]. During this phase, most patients do not show any

symptoms. However, ~30% of patients get similar symptoms to these of mononucleosis (fever, rash, swollen lymph nodes) [124], reflecting the activation of the immune system that recognizes viral antigens and starts producing antibodies against the viral capsid. This phenomenon is called seroconversion, and the detection of antibodies by ELISA allows diagnosis of HIV-1 infection. Within a few weeks, the level of CD4+ T cells drops while CD8+ T cell population increases. Although, CD4+ and CD8+ T cell populations rapidly come back to normal, effects of early CD4+ T cell depletion in the alimentary tract cause local immunodeficiency that persists over the whole course of the disease. At the end of this phase, the viremia decreases as a result of the immune response (Figure 15).

1.3.1.2 Chronic Phase

The asymptomatic, or chronic, phase starts immediately after the acute phase and can last for several years (Figure 15). During this phase, the immune system remains activated and a fragile equilibrium takes place between the production of viruses and their elimination by the immune system. This constant activation has two beneficial outcomes for HIV-1. First, the continuous production of CD4+ T cells provides new targets for the virus. Second, it allows the switch from naive T cells to memory T cells, thus establishing latency through providing the perfect support. This results in a constantly low viremia and high levels of CD8+ cytotoxic T cells. Over the years, this equilibrium fails and the

CD4+ T cell population collapses to levels as low as 200-300 cells/mm^3 of plasma, allowing the viremia to rise progressively (Figure 15). Furthermore, constant immune activation induces chronic inflammation, leading to the destruction of the mucosa and the lymph node tissues, favoring translocation of bacteria and microbial infections [126].

1.3.1.3 Acquired Immunodeficiency Syndrome (AIDS) Phase

This final phase is declared when the production of new lymphocytes no longer compensate their destruction by the virus, leading to a complete depletion of the CD4+ and CD8+ T cell populations (Figure 15). Therefore, million of viral particles are produced everyday and the viremia rises tremendously, saturating the already weakened immune system. In terms, opportunistic diseases such as tuberculosis and pneumocytosis emerge, latent viruses such as herpesvirus start replicating, causing cancers and eventually leading to the death of the patient.

1.3.1.4 Elite Controllers

1.3.1.4.1 Characteristics

As described in the previous sections, HIV-1 disease progression in non-treated patients is normally characterized by a decline in CD4+ T cells eventually

leading to an increase in viremia and death of the individual. However, 2% to 15% of the people who are infected with HIV-1 belong to the category of "long term non-progressors", that have variable and detectable viral loads and for whom the disease progression evolves less rapidly to AIDS [127]. These patients have stable CD4 counts for at least five years, although they are not under antiretroviral therapy.

An even more restricted population of untreated patients, about 1%, called "elite controllers" (ECs), appears to be able to control viral replication by keeping the viremia to undetectable levels (< 50 RNA copies/mL of blood using common techniques for detection) and maintaining high levels of CD4 counts [128] (Figure 16). However, although these patients do not show any clinical sign of disease progression, their specific anti-HIV-1 CD8 immune response is highly activated, and ultrasensitive techniques were able to detect a low but existing residual viral replication [127]. The study of ECs is therefore becoming a priority and is expected to give us a better understanding of the immune response mechanisms, in order to adopt new strategies for the development of treatments and vaccines.

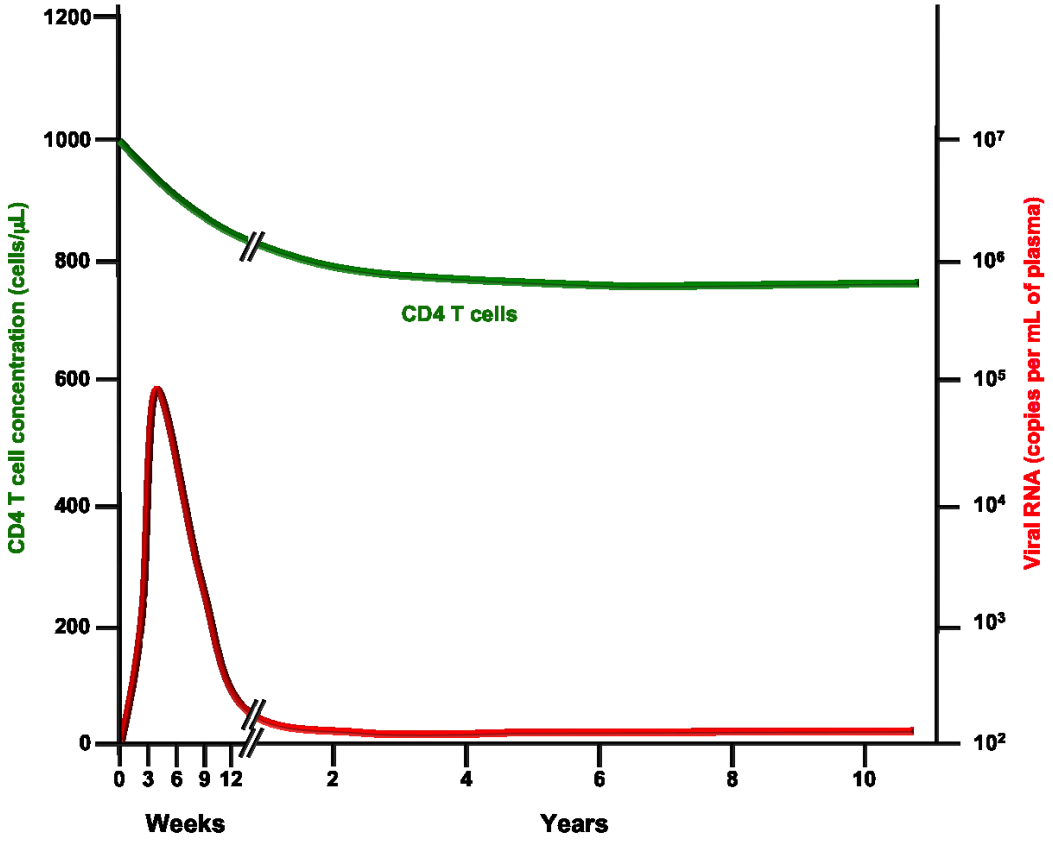


Figure 16 | Evolution of HIV-1 infection in Elite Controllers
Theoretical plasma viral load measurements (red) and CD4 T cell counts (green) for a representative Elite Controller. Viral load (red) was determined to be approximately 10^5 copies/mL in the acute phase of infection and reaches <50 copies/mL during the asymptomatic phase of infection. CD4 T cell counts remain relatively steady throughout infection, but may undergo a slight decline, as indicated here. (Adapted from [125])

1.3.1.4.2 Mechanisms

The mechanisms underlying the ability for ECs to naturally block the viral replication remain unclear. It was first hypothesized that attenuated viruses could be the cause of such unusual, early and immediate stop in disease progression. However, various studies showed that elite controllers were infected with fully

replication-competent viruses [129, 130]. Second, due to the slower disease progression observed in individuals that are heterozygous CCR5+/CCR5Δ32 [131], a genetic explanation for the ability of ECs to control HIV-1 was suspected. Again, this hypothesis was ruled out by genotyping analysis, which revealed that CCR5 genes from ECs have generally two wild type alleles [132]. Moreover, the proportion of ECs that are homozygous for the mutation Δ32 in the CCR5 gene is comparable to the one from the general population [133]. Third, considering the moderate efficacy of neutralizing antibodies in normal progressors, it was proposed that adaptive immunity, and more precisely the B-lymphocytes response, was involved. However, ECs patients actually have slightly lower levels of neutralizing antibodies [134], probably due to the fact that such adaptive immune response is related to the amount of circulating viruses, and that ECs have low viremia [127]. Furthermore, although natural killer (NK) cells appear to have a distinctive activation profile [135], they are not found to play a critical role in the control of HIV-1 in ECs [136].

Finally, numbers of studies focusing on the implication of adaptive immunity tend to point out the importance of HIV-1-specific CD8+ T cell response [132]. More precisely, ECs overexpress HLA class I alleles HLA-B*57 and HLA-B*27, which seems to correlate with low viremia [137]. Moreover, CD8+ T cells from ECs secrete multiple cytokines, including IL-2, interferon (IFN) and tumor necrosis factor alpha (TNFα), while CD8+ T cells from normal-progressors only secrete IFN-γ [138]. In addition, CD8+ T cells from ECs are much more efficient at loading lytic granules and delivering granzyme B to the CD4+ infected target

cells [139]. Altogether, these studies put CD8+ T cell response on a pedestal for unraveling the mechanisms underlying the control of HIV-1 disease progression.

1.3.2 HIV Management

1.3.2.1 *Ante* 1996

At the beginning of the pandemic in the early 1980s, there were no treatments available for HIV-1 infected patients, but interferon. The first antiviral drug to be approved by the U.S. Food and Drug Administration (FDA) was zidovudin (AZT), a nucleoside reverse transcriptase inhibitor (NRTI), in 1987 [140]. For the first time, we were able to prevent HIV replication [141]. Until 1991, AZT was the only drug available to treat infected patients. From 1991 to 1995, the FDA approved four new NRTIs and the first protease inhibitor (PI), widening the collection of anti-HIV drugs (Table 2). However, these drugs were used in the context of monotherapy, and patients started developing resistance to treatment, due to random mutations during the viral replication. Rapidly, monotherapy became ineffective in preventing HIV replication, and the scientific community decided to combine multiple drugs in order to observe a lasting effect of the treatment.

1.3.2.2 Highly Active Anti-Retroviral Therapy (HAART)

HAART has first been introduced at the 11th International Conference on AIDS in Vancouver (Canada) by Drs. Ho and Shaw, in July 1996. Rapidly, HAART has proved that the combination of one PI with two other drugs (usually NRTIs) could stably reduce the viremia, increase the number of CD4+ T cells and improve the health of HIV infected patients. Today, 33 drugs have been approved by the FDA and 31 are still being commercialized (Table 2) [140].

Basic science continuously improves our understanding of the complex mechanisms underlying HIV-1 replication. Along with research in pharmacology, this knowledge led to the development of anti-retroviral drugs that are grouped into six classes: chemokine receptor antagonist (CRA), fusion inhibitors (FIs), NRTIs, NNRTIs, integrase inhibitors (INIs), and PIs (Table 2) [142].

- CRA. To date, Maraviroc is the only chemokine receptor antagonist. It reversibly binds to CCR5 and blocks the interaction of Env gp120 with its co-receptor CCR5;
- Fusion inhibitors act extracellularly to prevent the fusion between HIV and the target cell through impeding Env gp41 conformational changes;
- NRTIs are the largest family of anti-HIV drugs. They block the viral replication at the reverse transcription step by competing with cellular nucleosides. Thus, the viral RT incorporates NRTIs in the newly synthesized

DNA, resulting in the early termination of the elongation of the proviral genome;

- NNRTIs bind to the p66 subunit of the viral RT heterodimer, inducing a conformational change that alters the active site of the enzyme;
- INIs block the viral integration step by binding metallic ions in the active site of HIV-1 IN, resulting in the inhibition of the viral strand transfer;
- PIs act as competitive inhibitors though binding to the viral PR and preventing the cleavage of viral substrates.

Different classes of anti-HIV drugs are combined into multidrug regimens that prevent the emergence of resistance (Table 2). More anti-HIV drugs are currently under investigation, including the new class of maturation inhibitors.

Table 2 | FDA approved antiretroviral drugs used for HIV-1 treatment as of December 2012, adapted from [140].

Class	Brand name	Generic Name	Manufacturer	FDA approval
CRA	Selzentry	Maraviroc	Pfizer	06-Aug-07
FI	Fuzeon	Enfuvirtide (T-20)	Hoffman-La Roche	13-Mar-03
NRTI	Retrovir	Zidovudine (ZDV), or Azidothymidine (AZT)	GlaxoSmithKline	19-Mar-87
	Videx	Dideoxyinosine (ddI)	Bristol Myers-Squibb	09-Oct-91
	Zerit	Stavudine (d4T)	Bristol Myers-Squibb	24-Jun-94
	Epivir	Lamivudine (3TC)	GlaxoSmithKline	17-Nov-95
	Combivir	Lamivudine + zidovudine	GlaxoSmithKline	27-Sep-97
	Ziagen	Abacavir sulfate (ABC)	GlaxoSmithKline	17-Dec-98
	Trizivir	Abacavir, AZT and 3TC	GlaxoSmithKline	14-Nov-00
	Videx EC	Enteric coated didanosine (ddI EC)	Bristol Myers-Squibb	31-Oct-00
	Viread	Tenofovir disoproxil fumarate (TDF)	Gilead	26-Oct-01
	Emtriva	Emtricitabine (FTC)	Gilead Sciences	02-Jul-03
	Epzicom	Abacavir and Lamivudine	GlaxoSmithKline	02-Aug-04
	Truvada	TDF and Emtricitabine	Gilead Sciences, Inc.	02-Aug-04
NNRTI	Viramune	Nevirapine (NVP)	Boehringer Ingelheim	21-Jun-96
	Rescriptor	Delavirdine (DLV)	Pfizer	04-Apr-97
	Sustiva	Efavirenz (EFV)	Bristol Myers-Squibb	17-Sep-98
	Intelence	Etravirine	Tibotec Therapeutics	18-Jan-08
	Viramune XR	Nevirapine (NVP)	Boehringer Ingelheim	25-Mar-11
	Edurant	Rilpirivine	Tibotec Therapeutics	20-May-11
INI	Isentress	Raltegravir	Merck & Co., Inc.	12-Oct-07
PI	Invirase	Saquinavir mesylate (SQV)	Hoffman-La Roche	06-Dec-95
	Norvir	Ritonavir (RTV)	Abbott Laboratories	01-Mar-96
	Crixivan	Indinavir (IDV)	Merck	13-Mar-96
	Viracept	Nelfinavir mesylate (NFV)	Agouron Pharmaceuticals	14-Mar-97
	Agenerase	Amprenavir (APV)	GlaxoSmithKline	15-Apr-99
	Kaletra	Lopinavir and Ritonavir (LPV/RTV)	Abbott Laboratories	15-Sep-00
	Reyataz	Atazanavir sulfate (ATV)	Bristol Myers-Squibb	20-Jun-03
	Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03
	Aptivus	Tipranavir (TPV)	Boehringer Ingelheim	22-Jun-05
	Prezista	Duranavir (DRV)	Tibotec, Inc.	23-Jun-06
Multi-class products	**Atripla**	**EFV + FTC + TDF**	**Bristol Myers-Squibb and Gilead Sciences**	**12-Jul-06**
	Complera	**FTC + Rilpivirin + TDF**	**Gilead Sciences**	**10-Aug-11**
	Stribild	**Eviltegravir + Cobicistat + FTC + TDF**	**Gilead Sciences**	**27-Aug-12**

1.3.2.3 Today's Challenges

Although lots of progress have been made in drug discovery, HIV infection still cannot be cured with current therapies and patients must undergo life-long treatment [143]. Today, several challenges must be seriously addressed in order to be able to completely manage HIV infection and eventually eradicate the virus. Among them, the emergence of multidrug resistance, transmission of drug-resistant HIV strains, simplification and accessibility to treatments and development of new drugs and vaccines are the key questions that remain at the heart of HIV management.

1.3.2.3.1 Increase Accessibility to anti-HIV Treatments

In 2010, less than half of HIV infected people were having access to antiretroviral therapy (ART) when needed in low- and middle-income countries (LMICs) [144]. Furthermore, the budget allocated to fight HIV in LMICs decreased in 2010, increasing the gap between the needs and the resources that are made available [144]. However, while only 10% of the people who needed ART in North Africa was actually initiating treatment, other regions and countries have higher access to anti-HIV drugs, like Botswana where up to 93% of HIV infected population has access to treatment [144]. This raises the fact that national and international policies must be improved to better distribute resources in order to provide access to anti-HIV drugs equally well in LMICs.

1.3.2.3.2 Decrease Drug Resistance and Increase Adherence to Treatment

Due to the lack of proofreading activity of the viral RT enzyme, HIV has high mutation rates that allow it to escape from monotherapeutic treatments. With the combination of multi-class drugs, HAART keeps a constant pressure on the virus through inhibiting distinct steps of viral replication, therefore rendering the emergence of resistance quasi impossible [141]. However, the lack of adherence to treatments allows the virus to quickly start replicating again, leading to the emergence of multi-drugs resistance and treatment failures [143, 145]. Lots of progress has been made to facilitate adherence, including the combination of several drugs in a single pill, which radically changed the perception of HIV treatment compare to the ~25 pills a day that were needed in the early days. Pharmacists, chemists and biochemists play a key role in the development of new technologies and drug-pharmacokinetics enhancers. These progresses will allow the next generation drugs to be more potent and more rapidly active upon delivery [143, 146].

1.3.2.3.3 Reduce Adverse Effects

Although HAART has radically changed the course of HIV/AIDS progression by transforming a fatal disease into a chronic infection, anti-HIV drugs yet go along with a series of side effects in a variable proportion of patients, usually not life-threatening. In some cases, patients experience short-term adverse

effects such as toxicities, rash, hypersensitivity, anemia and jaundice. However, they can also be subjected to long-term side effects, including lipodystrophy, distal sensory periphery neuropathy and cardiovascular, hepatic and renal complications [147]. Such consequences can render adherence more complicated since living conditions of the patient are deteriorated and, in some cases, physical signs encourage social discrimination (e.g. lipodystrophy). It is therefore of high interest and priority to limit adverse effects in order to improve the quality of life and the adherence to treatment of the patient.

1.3.2.3.4 Development of Vaccines

Although HAART is considered a major triumph in the management of HIV/AIDS, the emergence of resistance forces the scientific community to continue investigating means to create a suitable vaccine. Indeed, developing a vaccine that could provide complete immune protection against HIV remains the ultimate goal that the scientific community wants to reach in order to eradicate HIV. Several attempts have been made in the field of vaccine development, but none has been successful enough to satisfy the expectation of a complete protection against HIV. Several approaches are envisaged, including:

- the development of broadly neutralizing antibodies [148];
- the study of the virus interactome to identify cellular factors involved in HIV-1 replication as potential targets [149];

- the investigation of the role of CD4(+) T cells and their stimulation in developing durable functional antibody responses [150];
- the development of animal models to design new vaccine studies [151].

Considering all the efforts that are put into the development of vaccines against HIV and the knowledge that has been accumulated over the past 30 years studying the virus, we must realize that we still need to better understand the diversity of the mechanisms that HIV uses to replicate and to escape the host immune system. Further comprehension of the immune response will allow us to develop vaccines that can provide both mucosal and systemic immunity to protect individuals against sexual and blood transmission modes [143].

1.4 Innate Immune Response to HIV-1 Infection

The immune system protects the organism from infections caused by pathogenic agents, including bacteria and viruses. Vertebrates have evolved two types of immune responses. The first line of defense is constituted by the innate immune response, which is fast and non-specific. The specific adaptive immune response is the second line of defense, progressively established upon activation of the innate immune response. This section will be focusing on the several levels of activation of the innate immune response and how HIV-1 hijacks this cellular pathway.

1.4.1 Innate Sensors

Cells possess mechanisms that enable them to instantly detect the presence of pathogens, including viruses. Until recently, innate sensors involved in HIV-1 recognition were poorly understood. However, recent studies start bringing to light the diversity of the cellular sensors that activate the innate immune response. Upon HIV-1 infection, the cellular pattern-recognition receptors (PRRs) sense the viral pathogen associated molecular pattern (PAMP) that triggers the activation of the first layer of the innate immune response. Among the PRRs, we find the Toll-like receptors (TLRs) and the retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) [152].

In human, the TLR family comprises ten members [153] that are present either at the cell surface (TLR1, 2, 4, 5, 6) or within the endosomes where they sense viral nucleic acids [153, 154]. While TLR7 and TLR9 are constitutively expressed in plasmacytoid dendritic cells (pDCs) and recognize ssRNA and dsDNA respectively, TLR3 is preferentially expressed in DCs and senses dsRNA [155]. HIV-1 nucleic acid sensing through these three TLRs promotes the transcription of multiple cytokine and chemokine genes, including type I IFN [155]. DCs also express TLR8, which recognizes ssRNA [156]. Interestingly, TLR8 rather has a promoting effect on HIV-1 transcription through the recruitment of cyclin-dependent kinase-7 and the cooperation with DC-SIGN that recruits pTEFb, resulting in the activation of RNA pol II [152].

Along with the TLRs, the RLRs are also positioned at the frontline of the innate antiviral response and are involved in RNA recognition. RLRs are a small family that comprises three members: RIG-I, MDA-5 and LGP-2. RIG-I and MDA-5 possess two N-terminal caspase activation and recruitment domains (CARDs) as well as a C-terminal DExD/H box RNA helicase motif [157]. The helicase domain recognizes the viral RNA, inducing a conformational change of RIG-I, resulting in its dimerization. Subsequent activation of the NF-κB pathway leads to the production of type I IFN [157].

Both TLR and RLR pathways recruit transcription factors NF-κB, IFN regulatory factor (IRF)-3 and -7 that lead to the production of type I and III IFNs [158, 159].

1.4.2 IFN-Induced Signaling Pathways

1.4.2.1 Description of IFN Family

Interferons are cytokines that are secreted by host cells in response to the challenge by pathogenic agents or tumor cells. Their function is to stimulate the immune system by activating immune cells such as macrophages and NK cells, resulting in pulling an alarm that warns the host of the presence of a pathogen and prepares it to fight against the invasion. They are divided in three major classes: type I IFN, type II IFN and type III IFN.

- Type I IFN is a superfamily that comprises five members in human: IFN-α, IFN-β (beta), IFN-ω (omega), IFN-κ (kappa) and IFN-ε (epsilon) [158]. Type I interferon usually refers to IFN-α, which has 13 subtypes and is secreted by leukocytes, and IFN-β, which is produced by fibroblasts [158].
- Type II IFN is limited to only one member, IFN-γ, which is secreted by immune cells such as activated T cells and NK cells [158].
- Type III IFN contains three members IFN-λ1, IFN-λ2 and IFN-λ3 that are considered as type I-like IFNs [160, 161].

1.4.2.2 Stimulation of the IFN Pathway

Once produced, type I IFNs (mainly IFN-α and IFN-β), as well as type III IFNs, can activate the IFN signaling pathway in both autocrine and paracrine manners. The IFN receptor IFNAR (IFN-α/β receptor) is constituted of two subunits IFNAR1 and IFNAR2, which are associated with the tyrosine kinase 2 (Tyk2) and the Janus tyrosine kinase 1 (JAK1), respectively. Upon binding to INFAR, IFN-α/β trigger the phosphorylation of JAK1 and Tyk2 that further promote the phosphorylation and dimerization of STAT1 and STAT2 [158, 162]. Subsequently, the phosphorylated heterodimer STAT1/STAT2 recruits the cellular factor IRF-9 to form the IFN stimulated gene (ISG) factor 3 (ISGF3) complex [158]. Following its translocation to the nucleus, ISGF3 recognizes a DNA motif named IFN-stimulated response element (ISRE), thus activating the

transcription of ISGs [158, 163]. Transcription of ISGs accounts for the expression of several hundreds of genes that lead to the synthesis of a proteomic arsenal that constitutes the early antiviral response.

1.4.2.3 How HIV-1 Turns the IFN Pathway into its Own Benefit

HIV-1 has evolved several mechanisms to escape the innate immune response. As a lentivirus, HIV-1 genome is integrated into the host DNA, which is not without reminding us that a significant amount of our genome, the endogenous retroviruses (ERVs), originates from the colonization from other viruses. If the host were provided an antiviral mechanism to eliminate integrated retroviral DNA, it would lead to the complete destruction of the whole genome. As a consequence, the cell has evolved to tolerate such "foreign" DNA [152]. Therefore, HIV-1 found a way to remain discrete, which allows it to be "safe" as long as its proviral DNA is integrated. Moreover, it is important to note that monkeys that are naturally infected with SIV avoid disease progression due to the fact that their immune system "ignores" the virus. On the contrary, disease progression in human is correlated with a constant activation of the innate immune response [157]. This observation forces us to consider that the IFN pathway must somehow be beneficial to the virus. Indeed, among the many regulatory sequences that are carried by the HIV-1 promoter, one is specifically related to the activation of the innate immune response. This region, downstream the HIV-1 LTR, spanning nt +200 to +217, is homologous to the ISRE of the ISG

promoter, and was reported to be essential for the production of HIV-1 RNA and proteins [164].

1.4.2.4 Restriction Factors

Among the several hundreds of ISGs, which expression is stimulated upon viral components recognition, a special class, the restriction factors, emerged a decade ago and seems to specifically block retroviral replication. Although they are not constitutively expressed in all cell types and they are not always conserved among different host species, these host restriction factors have been shown to restrict viral replication at diverse stages of the virus life cycle, including reverse transcription, genome replication and release. These factors include tripartite motif-containing protein (TRIM5α), apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC3), bone marrow stromal antigen 2 (BST-2) and sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1 (SAMHD1) [157, 165]. As a countermeasure, HIV has evolved to overcome these blocks by producing viral proteins that bind to the host restriction factors and target them to degradation.

1.4.2.4.1 TRIM5α

HIV-1 can successfully enter primate cells, including human and old monkey cells. However, HIV-1 infection of old monkey cells does not lead to the

production of new particles, indicating the presence of a restriction mechanism that is missing in human cells. TRIM5α has been identified as the restriction factor responsible for the resistance of old monkey cells to HIV-1 [166]. Through direct interaction with the viral CA protein, TRIM5α mediates the early disruption of HIV-1 capsid, resulting in non-productive infection [167, 168]. Interestingly, the human homolog of TRIM5α has a low affinity for HIV-1 CA, which results in the loss of restriction [168]. In human cells, CypA binds to HIV-1 CA, which confers protection against TRIM5α [169, 170]. The discovery of TRIMCyp as a fusion protein accounts for this divergence in the restriction of HIV-1 between old monkey and human cells. In Old World primates, long-interpersed element 1 (LINE-1) replication resulted in the insertion of the *CypA* sequence into the *trim5α* gene, thus creating a new fusion protein TRIMCyp [171] that bridges the viral capsid to the proteasome-dependent degradation [172].

1.4.2.4.2 APOBEC3G

Among the seven members that compose the APOBEC3 family, APOBEC3G is the one that exerts the stronger anti-HIV-1 effect [157]. The observation that some cell types were permissive to Vif-defective HIV-1 virus replication while other cell types were non-permissive led to the discovery of the restriction factor APOBEC3G [173]. APOBEC3G is a DNA deaminase that can inhibit viral replication by inducing G to A mutations within the retroviral cDNA during reverse transcription [174]. HIV-1 Vif specifically binds to APOBEC3G

and links it to the ubiquitin-mediated proteasome degradation machinery. As a consequence, HIV-1 Vif prevents APOBEC3G from being incorporated into viral particles, thus overcoming the host restriction [175].

1.4.2.4.3 BST-2

Similarly to the discovery of ABOBEC3G as the restriction factor antagonized by HIV-1 Vif, the observation that Vpu-defective HIV-1 infection was cell type-dependent led to the identification of BST-2, also known as tetherin, as a new restriction factor [176, 177]. In non-permissive cells, BST-2 tethers the virus particles to the plasma membrane of infected cells, thus inhibiting viral release [178, 179]. BST-2 has unique structural features that include a N-terminal cytoplasmic tail, a single helical transmembrane domain, a coiled-coil ectodomain, and a C-terminal glycosylphosphatidylinositol (GPI) anchor [180, 181]. The two coiled-coil domains facilitate dimerization through juxtaposing two BST-2 molecules, preferentially in the same orientation [181, 182]. Although the exact mechanism whereby the virus particles are held at the cell surface remains unclear, it is known that BST-2 inserts its GPI anchor into the viral envelope layer or the plasma membrane, thereby inhibiting the release of the virus [183, 184]. Subsequently, these virus particles are internalized and degraded in the endosomes [178].

To overcome the restriction imposed by BST-2, HIV-1 encodes the Vpu protein, which directly interacts with the transmembrane domain of BST-2 and removes it

from the cell surface [185, 186]. However, it remains unclear whether Vpu prevents BST-2 trafficking and/or if it subverts BST-2 recycling to the plasma membrane [187, 188].

1.4.2.4.4 SAMHD1

It has long been known that macrophages and DCs are more resistant to HIV-1 infection than CD4+ T cells, and that Vpx protein, found in HIV-2 or SIV but absent in HIV-1, could increase the susceptibility of these cell types to be infected by HIV-1 [189, 190], suggesting that Vpx can overcome the innate restriction displayed by myeloid cells. Recently, SAMHD1 was identified as the cellular factor responsible for such resistance [191, 192]. Through depleting the cellular pool of dNTPs, SAMHD1 blocks viral reverse transcription, thus preventing HIV-1 cDNA synthesis [193, 194]. To overcome this restriction, Vpx acts as a scaffold through binding to the C-terminal domain of SAMHD1 and recruiting the E3 ubiquitin ligase complex, resulting in SAMHD1 polyubiquitination and degradation [191, 192, 195].

1.5 Research Projects

Several research axes are being investigated in Dr. Liang's laboratory, including the mechanism of LINE-1 retrotransposition, the study of IFITMs and MX proteins as well as the role of helicases in HIV-1 replication. My project consists in studying the role of helicases in HIV-1 replication and find new members of this family that can affect viral replication. To reach that objective we performed a screen to identify candidates for further investigation.

First, I will present the role of helicases in HIV-1 replication. This will be the focus of Chapter 2.

Second, I used a shRNA approach to stably knockdown cellular helicases in SupT1 cells and monitored HIV-1 replication in these cell lines with the aim to identify new helicases that are able to either promote or inhibit HIV-1 replication. This will be the focus of Chapter 3.

Third, among the candidates isolated from the first step, I further investigated the role of DDX17 in HIV-1 replication. The study of DDX17 revealed that this helicase affects HIV-1 replication at many stages of the virus life cycle, including viral RNA packaging and Gag-Pol frameshift. This work will be presented in Chapter 4.

Chapter 2 - Role of Helicases in HIV-1 Replication (Review)

Adapted from [196].

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From promoting to inhibiting: diverse roles of helicases in HIV-1 Replication

Rene-Pierre Lorgeoux[1,3], Fei Guo[4] and Chen Liang[1,2,3*]

2.1 Introduction

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus. Replication of HIV-1 RNA genome involves reverse transcription by viral reverse transcriptase, integration into cellular DNA by viral integrase, and transcription by cellular RNA polymerase II. HIV-1 RNA is subject to the regulation by viral proteins including Tat, Rev, and Gag that recognize specific viral RNA structures. Tat binds to the TAR (transactivation response) RNA that is located at the very 5' end of viral genome, and further recruits cellular factors including the P-TEFb (positive transcription elongation factor b) complex to HIV-1 promoter and

enhances transcription [197]. Rev recognizes the RRE structure that is located within the envelope protein-coding region and promotes the nuclear export of unspliced and partially spliced viral RNA through recruiting the CRM1 (chromosome region maintenance 1, also named exportin 1) nuclear export machinery [197]. Gag recognizes the viral RNA packaging signals located at the 5' untranslated region (5'UTR) and recruits two copies of full-length HIV-1 RNA into each virus particle [198]. Recent studies begin to reveal that a group of cellular proteins named helicases modulate HIV-1 replication through interacting with Tat, Rev and Gag proteins.

The importance of helicases in HIV-1 replication began to receive much attention when DDX3 was identified as an essential factor of the Rev/CRM1/RRE RNA export complex in 2004 [199]. The role of helicases in HIV-1 replication was further highlighted in a 2006 review where the authors stated that the story of HIV-1 and helicase would continue to unfold [200]. Indeed, in the subsequent years, more helicases were discovered that not only promote but also, in some cases, restrict HIV-1 replication. This review is aimed at providing an up-to-date account of the HIV-1 and helicase story with a focus on helicases that exert specific association with Tat, Rev, Gag or viral RNA. We also briefly discuss the possible involvement of helicases in HIV-1 RNA packaging and viral DNA integration, as well as how HIV-1 evades the recognition by the RNA helicase RIG-I. An overview is provided in Figure 17 to illustrate the helicases that are known to modulate a distinct step of HIV-1 RNA replication. The general role of helicases in viral infection is discussed in two recent excellent reviews [201, 202].

Methods to study the activity of RNA helicases in the context of viral replication are described in [203].

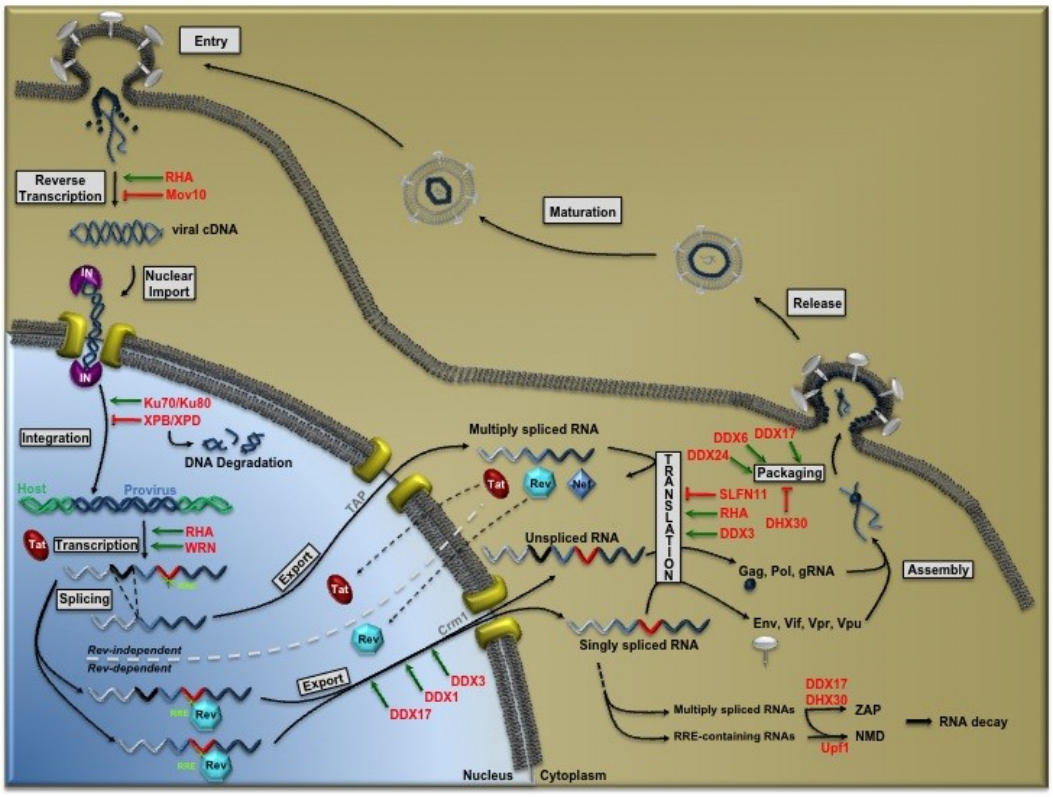

Figure 17 | Putative Roles of helicases in HIV-1 life cycle
HIV-1 infection starts with the entry step and ends with production of mature and infectious virus particles. The flow of the virus life cycle is narrated with arrows. Helicase involvement at distinct steps of HIV-1 life cycle is illustrated. Names of helicases are highlighted in red letters. Green arrows indicate a stimulating effect of a specific helicase on HIV-1 replication, the red lines denote inhibition.

2.2 Helicases Share Conserved Core Structures and Have Diversified Functions

Helicases are enzymes that hydrolyze NTPs and use the energy to unwind nucleic acid duplex or translocate along nucleic acid strand [204]. They are ubiquitously expressed and participate in every cellular event involving nucleic acids. Helicases are characterized by two RecA-like domains that exhibit ATP-binding as well as nucleic acid-binding properties. Among the conserved motifs that helicases carry are the Walker A and B boxes that bind and hydrolyze NTPs, as well as the "arginine fingers" that couple NTP hydrolysis with nucleic acid unwinding or translocating activities. On the basis of their conserved motifs and enzymatic properties, helicases are classified into six superfamilies (SF) [204]. SFI and SFII have the most members, they function in a monomeric or a dimeric form. Many SF1 and SFII helicases contain the DExD/H motif [205]. Members of SFIII to SFVI are of viral or bacterial origin and often form hexamers [206, 207]. Depending on whether unwinding RNA or DNA duplexes, helicases are also grouped as RNA helicases and DNA helicases. But this definition can become ambiguous for some helicases such as RNA helicase A (RHA) that are able to unwind both RNA and DNA [208]. An RNA helicase database is now available at http://www.rnahelicase.org [209].

The biochemical properties of helicases can be defined with four measurable parameters. These include, 1) translocation rate which represents the number of bases translocated per second, 2) directionality of action either from 5' to 3' or from 3' to 5', 3) processivity which is characterized by the number of rounds of catalysis before a helicase falls off the substrate, 4) step size which represents the number of base pairs translocated during each NTP hydrolysis event [204]. Helicases differ considerably in their biochemical properties. For example, hexameric helicases often translocate long distance on DNA before falling off, whereas RNA helicases of the DEAD-box family unwind a short stretch of double-stranded RNA of no more than two helical turns. Even among DEAD-box helicases, they exhibit a wide array of activities [205]. For example, the DEAD-box protein eIF4AIII, upon binding to ATP, shows RNA clamping activity and serves to recruit the core components of exon junction complex (EJC) [210]. DDX21 (also named RH-II/GuA) acts as a strand annealer in an ATP-independent manner [211]. These biochemical properties often determine the biological functions of helicases.

Helicases are also regulated by co-factors. For example, the translation initiation factor eIF4A alone exhibits low ATP-dependent helicase activity [212]. Binding to eIF4B and eIF4H greatly enhances the ability of eIF4A to unwind RNA [213]. In addition, a local activation of the yeast helicase Dbp5 by inositol hexakisphosphate 6 (InsP6) and Gle1 leads to the removal of mRNA export factor Mex67 when mRNA arrives at the cytoplasmic side of the NPC [78, 79]. Therefore, despite the conserved motifs and the similar folding that all helicase

core domains share, each helicase is highly specific in its way to modify nucleic acid structures and regulate nucleic acid functions.

2.3 Helicases as the Co-factors of HIV-1 Tat

HIV-1 Tat protein activates viral RNA synthesis [197]. Tat binds to the TAR RNA and recruits transcription factors to stimulate both transcription initiation and elongation. These transcription factors include p300/CREB-binding protein-associated factor (PCAF) and P-TEFb. P-TEFb contains cyclin T1 (CycT1) and cyclin-dependent kinase 9 (CDK9). CDK9 hyperphosphorylates the CTD of RNA polymerase II and activates transcription elongation (Figure 18) [197]. In addition to these transcription factors, two helicases, the Werner syndrome (WRN) helicase and RHA, were reported to act as co-factors of Tat and enhance HIV-1 gene expression [214, 215].

WRN is a member of the RECQ helicase family that also consists of RECQL (RECQ protein-like), BLM (bloom syndrome), RECQ4/RTS (Rothmund-Thomson syndrome) and RECQ5. RECQ helicases harbor the DEAH motif and belong to SFII. They are capable of resolving complex DNA structures that often block DNA replication fork progression [216]. In addition to its role in DNA recombination, WRN also promotes RNA polymerase II-dependent transcription, which is partially attributable to its ability to stimulate the DNA-unwinding activity of DNA topoisomerase I [217]. In line with its role in transcription, WRN

was recently shown to interact with HIV-1 Tat and promote HIV-1 LTR transactivation (Figure 18) [214]. WRN and Tat are co-localized within the nuclei of HIV-1 infected cells. The purified recombinant GST-Tat is able to pull down the endogenous WRN. WRN appears to enhance HIV-1 gene expression by facilitating the recruitment of PCAF and P-TEFb to HIV-1 LTR [214]. In support of this role of WRN, ectopic expression of wild type WRN in human lymphocytes increases HIV-1 p24(Gag) production and viral replication.

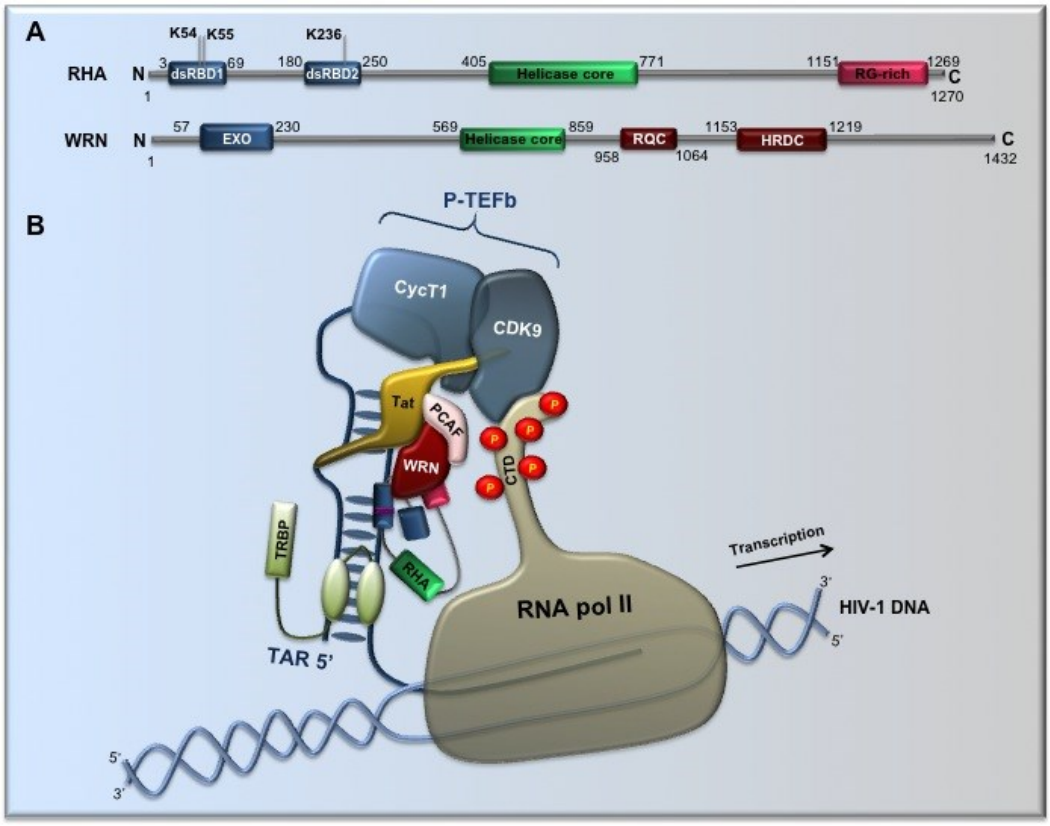

Figure 18 | Interaction of WRN and RHA with the TAR/Tat complex
(**A**) Domain structures of RHA and WRN. The amino acid positions of each illustrated domain are indicated. dsRBD, double-stranded RNA-binding domain; RG-rich, arginine (R) and glycine (G)-rich region; EXO, exonuclease domain; RQC, RecQ Conserved domain; HRDC, helicase RNase D C-terminus domain. (**B**) WRN interacts with Tat and helps the recruitment of the P-TEFb complex (consisting of CycT1 and CDK9) to the HIV-1 promoter. RHA binds to the stem

of TAR RNA via its dsRBD and also interacts with WRN. TRBP, TAR RNA binding protein.

In addition to the WRN helicase, RHA has also been shown to promote TAR-dependent HIV-1 gene expression [215]. RHA contains the DEIH Walker B motif, is a DEXH helicase. In addition to the helicase core domain, RHA has two double-stranded RNA-binding domains (dsRBDs) at its N-terminal region and the arginine- and glycine-rich (RGG) repeats at its C-terminal region (Figure 18) [218]. These latter domains target RHA to its RNA substrates. HIV-1 TAR RNA has been shown binding to the N-terminal dsRBDs of RHA [215, 219]. This interaction allows RHA to affect a few steps of HIV-1 replication including transcription. Similar to WRN, RHA increases both basal activity from HIV-1 LTR and Tat transactivation (Figure 18) [215]. It is unclear whether RHA directly interacts with Tat as WRN does. Interestingly, the dsRBD II and the RGG repeats of RHA directly interact with the N-terminal exonuclease domain of WRN, and stimulate its exonuclease activity [220]. With such an interaction, RHA promotes the WRN-mediated degradation of D-loop DNA as well as the unwinding of Okazaki fragment-like hybrids [220, 221]. It is thus conceivable that these two helicases may act together to promote HIV-1 RNA synthesis (Figure 18).

2.4 The Essential Role of Helicases in Rev-dependent RNA Export

The intron-containing cellular RNA cannot leave the nucleus before they are completely spliced. HIV-1 needs to evade this form of cellular surveillance in order to export its full-length and partially spliced RNA into the cytoplasm and produce viral structural proteins and accessory proteins. This viral evasion relies on the Rev protein that binds to the HIV-1 RNA sequence RRE and communicates the intron-containing HIV-1 RNA to the CRM1 nuclear export pathway for export [222]. Crossing NPC is not a trivial task for the RNP complex. Remodeling is required so that the RNP is able to thread through the NPC channel. Snay-Hodge and colleagues first reported in 1998 that in yeast, the Dbp5 RNA helicase (human DDX19 homolog) associates with the NPC and is essential for mRNA export [223]. It was later shown that following activation by Gle1 and InsP6 at the cytoplasmic face of the NPC, Dbp5 remodels mRNP and removes RNA transporters Mex67 (human TAP homolog) and Mtr2 (human NXT1 (nuclear transport factor 2-like export factor 1) homolog) [78, 79]. It remained unknown whether a similar role of helicase is required for Rev/CRM1/RRE-mediated export of intron-containing HIV-1 RNA until Yedavalli and colleagues reported the essential role of DDX3 in this export event (Figure 19) [199]. It is noted that, in addition to helicases, other cellular factors have also been shown to promote Rev/RRE-mediated RNA export. One such example is Mtr3 that binds to the Rev/RRE complex and facilitates the export of HIV-1 RNA [224, 225].

DDX3 is a DEAD-box protein (Figure 3A). Although DDX3 has been shown to interact with RNA transport factors TAP/NXF1 and REF/Aly, it does not appear to play a role in bulk mRNA export [226-228]. It is interesting to note that Ded1

(yeast DDX3 homolog) modulates translation by controlling the conformation of eIF4F-mRNA complex [229], suggesting a role of Ded1/DDX3 in translation. The function of DDX3 in RNA export was not recognized until DDX3 was found to participate in the Rev-dependent export of unspliced and partially spliced HIV-1 RNAs [199]. Rev is co-immunoprecipitated with DDX3, but a direct interaction between the two proteins has not been experimentally demonstrated. Rather, the purified GST-CRM1 is able to pull down the *in vitro* translated DDX3. This direct interaction depends on the DDX3 fragment at amino acid positions 260 to 517 that does not include the NES sequence, and is Ran-GTP independent (Figure 19A, 19C), which suggests that instead of a cargo, DDX3 acts as an effector in the CRM1-mediated nuclear export pathway.

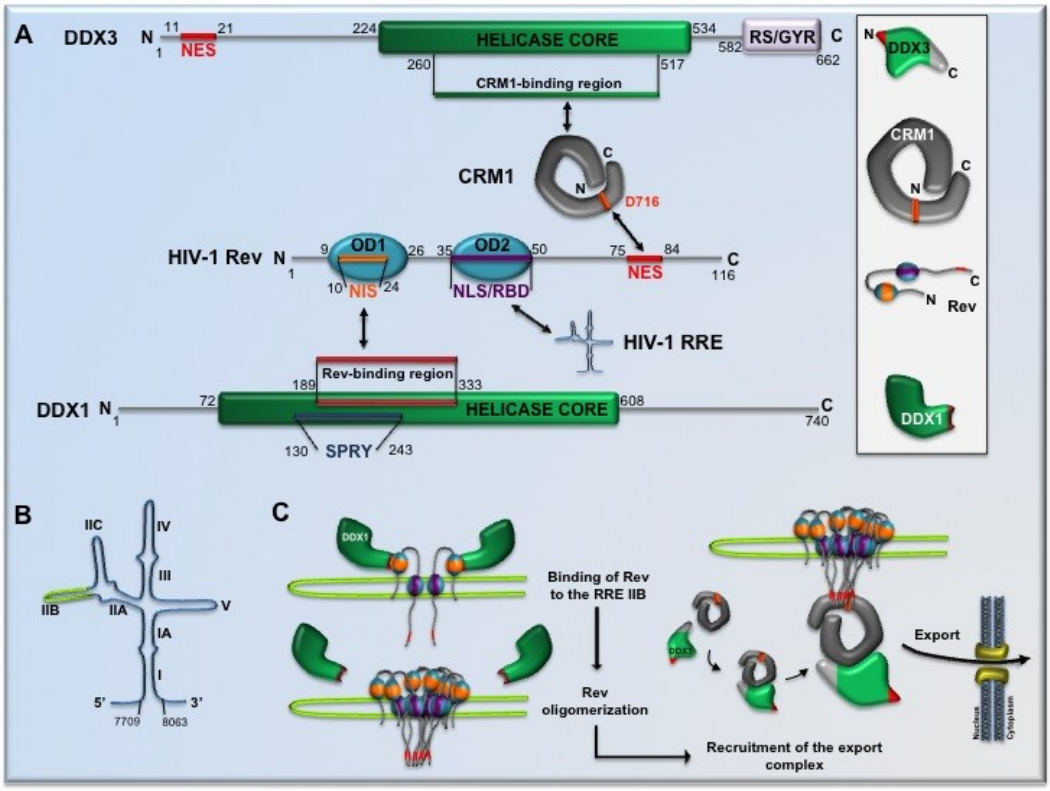

Figure 19 | DDX1 and DDX3 promote Rev-dependent RNA export
(**A**) The domain structures of DDX3, Rev and DDX1 are illustrated. CRM1 binds to the helicase core of DDX3. DDX1 directly interacts with Rev. The protein regions involved in these interactions are highlighted. NES, nuclear export signal; NLS, nuclear localization signal; OD1, oligomerization domain 1; RBD, RNA binding domain. (**B**) The secondary structures of the RRE RNA. The IIB stem-loop is highlighted in light green, which serves as the binding site of Rev. (**C**) A hypothetical model to illustrate the roles of DDX1 and DDX3 in Rev-mediated RNA export. DDX1 binds to Rev and promotes Rev oligomerization on the RRE IIB. Then the oligomerized Rev molecules recruit the CRM1/DDX3 binary complex (one or multiple copies) and together export viral RNA into the cytoplasm.

In support of the important role of DDX3 in Rev-dependent HIV-1 RNA export, knockdown of DDX3 or expression of the dominant negative mutant of DDX3 significantly diminishes HIV-1 replication [230]. Mutation of a unique fragment between the helicase motifs I and Ia diminishes the ability of DDX3 to bind to HIV-1 RNA and impairs HIV-1 replication [231]. Interestingly, a ligand of this unique region reduces HIV-1 infection of HeLaP4 cells, suggesting the possibility of targeting this domain to abrogate the function of DDX3 in HIV-1 replication. It remains to be tested whether DDX3 is involved in CRM1-mediated export of cellular RNAs such as snRNA and rRNA, and to elucidate the molecular details regarding how DDX3 promotes RNA export.

In addition to DDX3, the RNA helicase DDX1 has also been reported to associate with Rev and promote the export of RRE-containing viral RNA (Figure 19A) [232]. Purified DDX1 exhibits RNA-dependent ATPase activity. The DDX1 sequence from amino acids 189 to 333 directly interacts with the NIS at amino acids 10 to 24 in Rev. Through this interaction, DDX1 promotes Rev

oligomerization on the RRE RNA (Figure 19B, 19C) [233, 234]. This function of DDX1 is important because coordinate binding of multiple copies of Rev, rather than Rev monomer, to the RRE is required for initiating RNA export [235]. In support of its role as a co-factor of Rev, the low DDX1 level in astrocytes results in a predominant cytoplasmic location of Rev, which partially accounts for the poor susceptibility of this cell type to HIV-1 infection [236]. On the basis of these observations, we propose that DDX1 and DDX3 act sequentially in the Rev-dependent RNA export (Figure 19C). DDX1 first binds to Rev and promotes Rev oligomerization on the RRE RNA. Then the oligomerized Rev molecules, through presenting multiple copies of NES, recruit the CRM1/DDX3 complex that subsequently exports the RRE-containing HIV-1 RNA into the cytoplasm.

A recent proteomic study led to the finding of more helicases that associate with HIV-1 Rev [237]. In addition to DDX1 and DDX3, these include DDX5, DDX17, DDX21, DHX36, DDX47 and RHA. Silencing DDX5, DDX17 or DDX21 significantly modulates the production of HIV-1 particles, suggesting a functional role of these helicases in HIV-1 replication. It remains to be further investigated how each of these helicases affects the function of Rev and whether they play redundant roles or are involved in distinct steps of Rev-mediated RNA export. Interestingly, these helicases were not reported to associate with Rev in a separate protoemic study that employed the affinity tagging and purification mass spectrometry methods to identify cellular factors that interact with each of the 18 HIV-1 proteins [238]. This discrepancy may reflect the RNA-dependent nature of the Rev-helicase interaction.

2.5 Helicases in HIV-1 Particles

Gag makes HIV-1 particles [239]. In addition to viral RNA and viral proteins, a variety of cellular factors find their way into virus particles via direct or indirect interactions with Gag [240, 241]. Among these many cellular factors are two helicases, RHA and MOV10 (Moloney leukemia virus 10 homolog) [39, 242-244]. These two helicases both affect HIV-1 reverse transcription but with opposite outcomes.

RHA interacts with Gag in an RNA-dependent manner [39]. Knockdown of RHA in virus producer cells diminishes the infectivity of progeny HIV-1 particles, suggesting a functional role of the presence of RHA in the virions [39, 219]. This deficit in infectivity is caused at least in part by decreased viral reverse transcription [39, 219]. A further analysis of the viral RNA complex within the RHA-depleted virus particles reveals a reduced level of $tRNA^{Lys3}$ that is annealed onto the PBS [245]. This latter finding is verified by the *in vitro* study showing that the purified recombinant wild type RHA, but not its helicase-null mutant K417R, assists Gag/NC in promoting the formation of $tRNA^{Lys3}$/viral RNA binary complex [245]. Moreover, this binary viral RNA complex that is formed with the assistance of RHA exhibits higher efficiency in reverse transcription [245], which suggests that RHA not only promotes the annealing of $tRNA^{Lys3}$ onto viral RNA

but also helps the viral RNA complex to adopt conformations in favor of the action of viral reverse transcriptase.

In contrast with the stimulatory role of RHA in HIV-1 reverse transcription, MOV10 exerts an inhibitory effect [242-244]. MOV10 is a SFI RNA helicase, and has the DEAG Walker B motif. In addition to the helicase core domain, MOV10 has a long N-terminal region that bears a cysteine- and histidine-rich (CH) domain (Figure 20A). Detailed mutagenesis analysis showed that the MOV10 sequence at amino acid positions 261 to 305 interacts with the basic linker of the NC domain of Gag protein (Figure 20B) [246]. In addition, efficient viral incorporation of MOV10 also requires the helicase core domain downstream of this (261 to 305) region (Figure 20B). It remains controversial in terms of which step of viral reverse transcription is suppressed by MOV10. Burdick and colleagues reported a defect at the late stage of reverse transcription [244], whereas Wang et al and Furtak et al observed a reduction in the yield of early HIV-1 cDNA products (Figure 20C) [242, 243]. Testing purified MOV10 in cell-free HIV-1 reverse transcription assays is one way to elucidate the molecular details of its inhibition activity. The ability of MOV10 to dampen reverse transcription may enable it to have a role in controlling endogenous retroelements. Indeed, two recent studies reported that MOV10 inhibits the retrotransposition of both LTR and non-LTR endogenous retroelements including LINE-1, Alu and IAP [247, 248].

MOV10 may not act alone to inhibit HIV-1 reverse transcription. It is known that MOV10 binds to Ago2 and is a player in the microRNA pathway [249]. This function allows MOV10 to control local protein translation at synapses and modulate synaptic plasticity [250]. Ago2 was recently detected in HIV-1 particles [251]. This latter finding raises the possibility that MOV10 and Ago2 may get packaged into virus particles as one complex and, together, modulate the function of viral RNA.

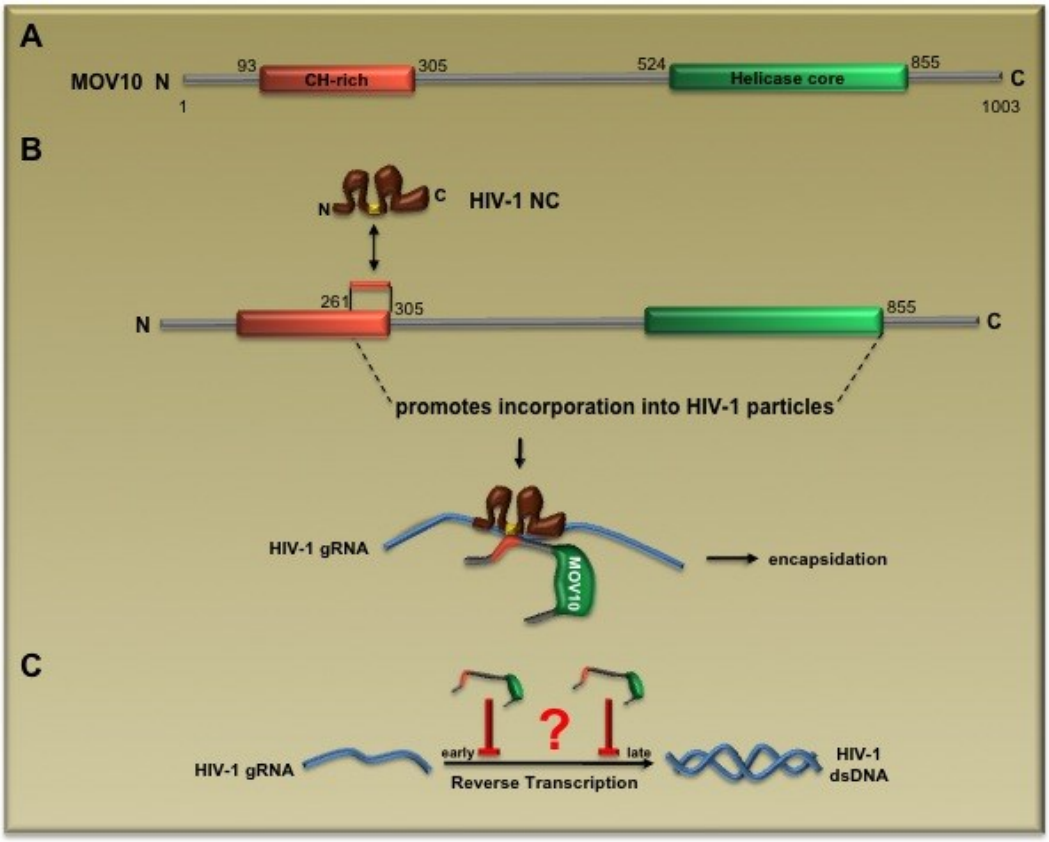

Figure 20 | MOV10 inhibits HIV-1 reverse transcription
(**A**) Domain structures of MOV10. MOV10 carries a CH-rich (cysteine/histidine-rich domain in the N-terminal region and a helicase core domain in the C-terminal region. (**B**) The basic linker of NC (highlighted in yellow) interacts with the (aa 261-305) region of MOV10, which contributes to the incorporation of MOV10 into HIV-1 particles. (**C**) MOV10 diminishes HIV-1 reverse transcription either at the early or the late phase of the reaction.

2.6 RHA and Schlafen11 in HIV-1 RNA Translation

The role of RHA in HIV-1 replication goes beyond enhancing viral transcription and reverse transcription. RHA also increases HIV-1 RNA translation (Figure 21) [219]. This function of RHA depends on its binding to the R/U5 sequence of HIV-1 RNA that has been named post-transcriptional control element (PCE). The PCE exists in the 5'UTRs of different retroviruses, including spleen necrosis virus (SNV), Mason-Pfizer monkey virus (MPMV), human foamy virus (HFV), reticuloendotheliosis virus strain A (REV-A), human T-cell leukemia virus type 1 (HTLV-1), feline leukemia virus (FeLV), and bovine leukemia virus (BLV) [252]. Studies show that RHA augments translation by promoting the association of PCE-containing RNA with polyribosomes [253]. This translation mechanism may have a cellular origin, since the translation of cellular junD mRNA is stimulated by RHA in a 5'UTR-dependent manner [254].

RHA is not the only helicase that promotes the translation of mRNA having structured 5'UTR. DHX29 has been reported to facilitate the formation of the 48S translation initiation complex on the AUG codon of mRNAs such as neutrophil cytosolic factor 2 (NCF2) and CDC25 that harbor secondary structures at their 5'UTRs [255, 256]. These findings suggest that in addition to the RNA helicase

eIF4A that functions as a conical translation initiation factor, the translation of specific mRNA may benefit from the action of other helicases (17).

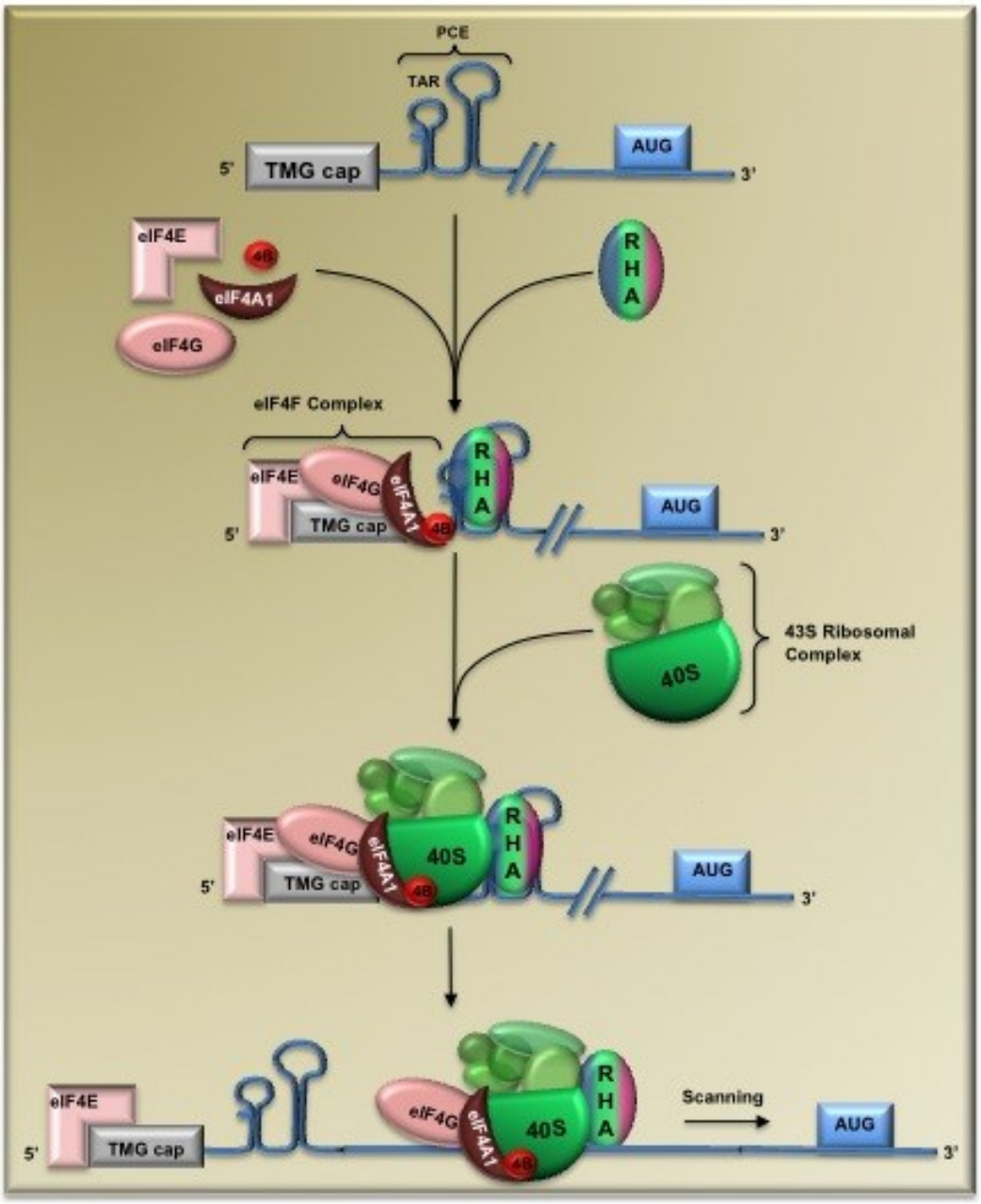

Figure 21 | RHA stimulates HIV-1 protein translation
Translation initiation factor eIF4E binds to the 5'cap structure of HIV-1 RNA together with eIF4G and eIF4A1 in the context of the eIF4F translation initiation complex, which further recruits the 43S ribosomal complex to the mRNA. RHA

binds to the R/U5 region of HIV-1 RNA and is proposed to assist the 43S ribosomal complex to scan the HIV-1 5'UTR and locate the translation start codon AUG.

RHA contributes to one of the several translation mechanisms that HIV-1 has harnessed to ensure efficient production of viral proteins. First, the activity of internal ribosome entry site (IRES) has been detected in the HIV-1 5'UTR and the Gag-coding region [257, 258], which allows translation to initiate in a cap-independent fashion. It is noted that HIV-1 PCE and IRES are mapped to different sequences of the 5'UTR [219, 258], indicating that they represent distinct translation mechanisms. Second, The Rev/RRE-exported viral RNAs have a trimethylguanosine (TMG) cap at their 5' ends as opposed to the 7-methylguanosine (m7G) at the 5' end of most cellular mRNA [259]. The TMG cap is synthesized by the PIMT enzyme (peroxisome proliferator-activated receptor-interacting protein with methyltransferase) that is recruited to HIV-1 RNA through binding to Rev. As a result, PIMT increases the translation of Rev-exported viral RNA. It is postulated that this mechanism ensures the production of optimal amounts of viral structural proteins at the late stage of HIV infection to produce virus particles. This finding also explains why Rev, besides its role in RNA export, also enhances translation [260, 261].

These different translation mechanisms contribute to HIV-1 protein production at different levels and under different conditions. RHA, through binding to R/U5 that is present on both spliced and unspliced HIV-1 RNAs, promotes the synthesis of all HIV-1 proteins, whereas the TMG cap, whose formation is Rev-dependent,

increases the translation of viral structural proteins. In regard to HIV-1 IRES, its activity is cell cycle-dependent and responds to oxidative stress [258, 262, 263].

It is well known that the genetic code is universal and that its redundancy allows distinct tRNAs to bring the same amino acid to the ribosome during the polypeptide chain synthesis. However, like other viruses, HIV-1 seems to have a species-specific codon bias that results in a diminution of the GC content and an increase of adenine as the third nucleotide of the codon [264-266]. Upon infection with HIV-1, the composition of the tRNA pool changes to favour the incorporation of amino acids corresponding to HIV-1-specific codons [267].

Recently, Schlafen11 (SLFN11) was identified as a new helicase/ISG that inhibits the translation of retroviruses [268]. Through direct binding to tRNAs, SLFN11 can regulate the composition of tRNA pools, therefore preventing the enrichment in "HIV-1-prefered tRNAs", resulting in the inhibition of viral translation [268]. To date, no HIV-1 component has been identified to counteract SLFN11.

2.7 Upf1 Associates With the 3'UTR of HIV-1 RNA

HIV-1 RNA has a long 3'UTR that represents one of the signals, in addition to the pre-mature termination codon (PTC) and the upstream open reading frame (uORF), that are recognized by the nonsense-mediated decay (NMD) machinery [269]. This long 3'UTR scenario is particularly true for the unspliced HIV-1 RNA in which the termination codon of the *gag-pol* gene is located approximately 4 kb from the 3' end of viral RNA. Although much is

known about PTC-triggered NMD, it remained unclear how sensing the length of 3'UTR is achieved by the NMD machinery until Hogg and Goff reported the association of Upf1 with HIV-1 3'UTR in an RNA length-dependent manner (Figure 22) [270].

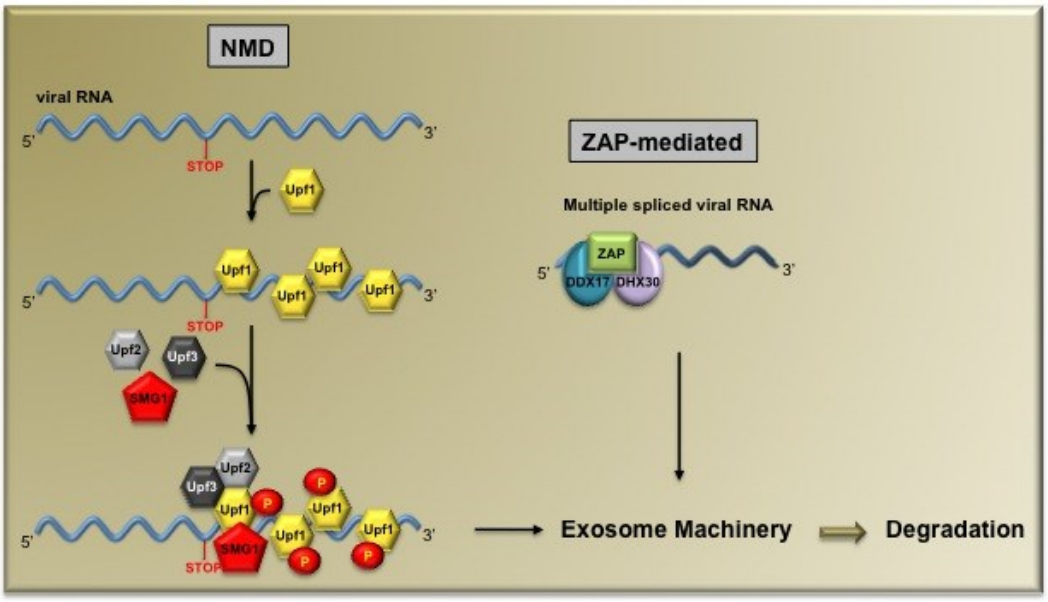

Figure 22 | Roles of Upf1 and ZAP in HIV-1 RNA degradation
Multiple copies of Upf1 first bind to the long 3'UTR and then recruit Upf2, Upf3 and SMG1 to assemble the NMD machinery. The exosomes are then recruited to degrade RNA. ZAP, together with DDX17 and DHX30, recognizes multiply spliced HIV-1 RNA and sends the RNA to exosomes for degradation.

Upf1 is a key component of the NMD core machinery [269]. As an SFI RNA helicase, Upf1 exhibits nucleic acid-dependent ATPase activity and the 5' to 3' RNA unwinding activity [271]. Using the RNA affinity purification technique and the mass spectrometry method, Hogg and Goff discovered that Upf1 associates with HIV-1 3'UTR and other model 3'UTRs [270]. When the abundance of Upf1 association with a 3'UTR exceeds a certain threshold, the RNA is marked as a

potential substrate for NMD. Frequent translation readthrough counters this mechanism by displacing Upf1 from the 3'UTR [270]. Interestingly, rare translation readthrough also rescues the RNA from NMD without affecting the association of Upf1 with 3'UTR, which suggests a two-step model for Upf1 to sense 3'UTR and to potentiate decay. Since HIV-1 and other retroviruses use the frameshift mechanism to read through the stop codon of Gag in order to produce the Gag-Pol polyprotein, this translation mechanism may protect HIV-1 RNA from Upf1-mediated RNA decay. In support of this possible counter measure, it has been reported that Rous sarcoma virus (RSV) has the RSV stability element (RSE) that contains a frameshift pseudoknot and prevents the Upf1-dependent degradation of unspliced RSV RNA [272]. However, the story of Upf1 and HIV-1 may be more complicated. One study shows that knockdown Upf1 in HeLa cells leads to decreased levels of both HIV-1 RNA and viral Gag protein and that this observation is independent of the role of Upf1 in NMD [273]. This study concludes that HIV-1 has evolved to use Upf1 to stabilize viral RNA. Further studies are warranted to define how Upf1 modulates HIV-1 replication in HIV-1 natural target cells such as primary CD4+ T cells.

In addition to Upf1, HIV-1 RNA was recently shown being subject to ZAP (zinc finger antiviral protein)-mediated degradation (Figure 22) [274]. ZAP was originally reported to inhibit murine leukemia virus (MLV) infection [275]. Interestingly, ZAP causes the degradation of multiply spliced HIV-1 RNA while spares the unspliced and singly spliced viral RNA [276, 277]. This degradation process can be initiated either by shortening the 3' polyadenylation tail or by

removing the 5' cap. Two RNA helicases, DDX17 and DHX30, were found as co-factors of ZAP [274, 278, 279], which may function by remodeling the viral RNP and assisting the recruitment RNA degradation machinery. A similar role of RNA helicase in cellular RNA degradation has been reported for Mtr4 in yeast that bridges the TRAMP (Trf4/Air2/Mtr4 polyadenylation) complex to exosomes and remodels substrate RNA molecules [280, 281].

2.8 DDX24 and DHX30 Modulate HIV-1 RNA Packaging

Each HIV-1 particle packages two copies of unspliced viral RNA that are non-covalently linked via the RNA stem-loop structure SL1 that is defined as the dimerization initiation site (DIS) [198, 282, 283]. The NC domain of Gag is primarily responsible for recognizing the RNA packaging signals that comprise the SL1, SL2 and SL3 RNA structures at the 5'UTR [284, 285]. The nuclear magnetic resonance (NMR) structures of the 712-nt HIV-1 5'-leader RNA reveal a structure-based coordination of HIV-1 RNA packaging, dimerization and translation [286]. In addition to these cis-acting viral RNA signals at the 5'UTR, the Rev/RRE system has also been shown to significantly augment HIV-1 RNA packaging [287]. A direct involvement of helicases in HIV-1 RNA packaging has not been documented, although it is known that bacteriophages use helicases as motors to "thread" phage DNA into their capsids [288]. Nonetheless, a couple of

helicases have been implicated in modulating the genome packaging of some retroviruses including HIV-1. For example, DDX6 was reported to affect the viral genome packaging of foamy virus, a spumaretrovirus [289]. Relocation of DDX6 from P bodies and stress granules to virus assembly sites at the perinuclear region was seen in cells infected with foamy virus. However, no interaction was detected between DDX6 and Gag, DDX6 was neither seen in the virus particles [289]. As opposed to the reported role of DDX6 in foamy virus assembly, DDX6 binds to HIV-1 Gag and promotes Gag assembly, independent of viral RNA packaging [290]. We previously observed that knockdown of the RNA helicase DDX24 diminishes HIV-1 RNA packaging [291]. This effect was seen only for Rev/RRE-exported, not for CTE (constitutive transport element)-exported viral RNA, which likely results from the interaction of DDX24 with Rev. With its predominant location within the nucleolus, DDX24 may gain access to HIV-1 RNA through association with Rev and participates in viral RNA remodeling. The effect may then extend to the viral RNA packaging event that takes place within the cytoplasm. In contrast to the stimulatory effect of DDX24, another RNA helicase, DHX30, inhibits HIV-1 RNA packaging [292], which may be attributable to its accessory role in ZAP-mediated HIV-1 RNA degradation [278].

2.9 The Putative Role of Helicases in HIV-1 DNA Integration

Integration of HIV-1 DNA into cellular DNA is catalyzed by viral integrase in the context of pre-integration complex (PIC) [293, 294]. The PIC consists of the full-length HIV-1 DNA, integrase, viral and cellular factors that assist viral DNA integration. In addition to a number of cellular proteins such as BAF (barrier-to-autointegration factor), Gemin2, EED (embryonic ectoderm development), integrase interactor 1, and LEDGF/p75 (lens epithelium-derived growth factor), the helicase DDX19A was recently shown to likely associate with the PIC [295]. Using the yeast two-hybrid method, Studamire and Goff screened for cellular proteins that interact with the integrase of Moloney murine leukemia virus (MoMLV) [296]. The candidates include several helicases such as Ku70/XRCC6, DDX5 and DDX18. It would be interesting to test whether these helicases also associate with HIV-1 integrase and whether they play a functional role in HIV-1 DNA integration. It should be noted that no helicase has ever been shown experimentally to interact with HIV-1 integrase, therefore a direct role of helicase in HIV-1 DNA integration remains to be established.

Despite not being components of the PIC, helicases in the DNA repair machinery may participate in HIV-1 DNA integration in an indirect manner. For example, unintegrated HIV-1 DNA has been reported to be the substrate of the non-

homologous DNA end joining (NHEJ) pathway [297]. Knockdown of the Ku80 DNA helicase, a key player in NHEJ, reduces HIV-1 DNA integration and diminishes viral replication in human CEM4fx cells [297]. In one study, 232 host DNA repair proteins were silenced using siRNA oligos and the effects on HIV-1 DNA integration were measured [298]. The targeted proteins are involved in base excision repair (BER), nucleotide excision repair (NER), NHEJ, single strand break repair (SSBR), double strand break repair (DSB), mismatch repair (MMR), and homologous recombination (HR). The results revealed an important role of the BER pathway in HIV-1 DNA integration [298, 299]. Notably, knockdown of a few DNA repair helicases including ERCC3 and RECQL4 diminishes HIV-1 infection, suggesting their role in viral DNA integration [298].

Instead of assisting HIV-1 DNA integration, certain DNA repair machineries exert inhibitory effects. For example, NER-deficient cells that are mutated in the helicases XPB and XPD are more susceptible to transduction by HIV-based retroviral vectors owing to an increase in the integrated viral DNA [300, 301]. This suggests a role of these two DNA helicases, and likely via the underlying NER pathway, in defending cells against retroviral integration. In conclusion, no helicase has been reported to specifically interact with HIV-1 integrase and hereby directly modulate viral DNA integration. Evidence does suggest that some helicases become involved in HIV-1 DNA integration in the context of DNA repair pathways.

2.10 RIG-I is Curtailed by Viral Protease for Sensing HIV-1 RNA

A small family of DExD/H helicases including RIG-I (retinoic acid-inducible gene I), MDA-5 (melanoma differentiation associated protein-5) and LGP2 (laboratory of genetics and physiology 2) recognize viral RNA and trigger interferon production [302]. Although transfecting the monomeric and dimeric HIV-1 RNA into cells triggers interferon production in a RIG-I-dependent manner, HIV-1 infection of monocyte-derived macrophages does not induce interferon response [303]. This suggests that HIV-1 has a mechanism to evade the recognition by RIG-I. Indeed, further studies showed that HIV-1 protease removes RIG-I from the cytosol to an insoluble fraction, therefore inhibiting RIG-I-mediated antiviral signaling [303].

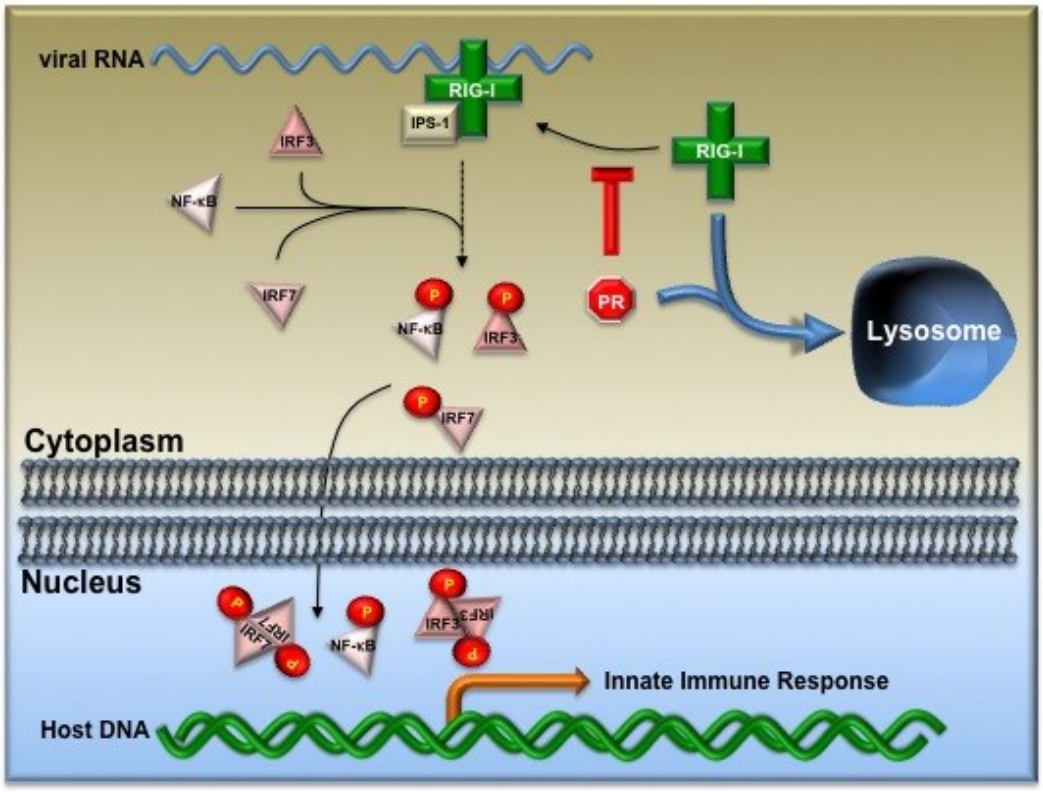

Figure 23 | Role of RIG-I in HIV-1 RNA sensing
HIV-1 RNA is recognized by RIG-I that recruits its adaptor IPS-1. Activation of the IPS-1 pathway results in the phosphorylation, dimerization and translocation of IRF-3 and IRF-7, leading to the transcriptional activation of ISGs. HIV-1 PR inhibits RIG-I signaling through targeting RIG-I to the lysosome for degradation.

2.11 Conclusion

HIV-1 engages helicases to facilitate viral replication at different steps. This engagement is achieved by interacting with helicases via either viral RNA or viral proteins. For example, RHA binds to the R/U5 region of HIV-1 RNA and promotes viral gene expression and viral reverse transcription [39, 215, 219, 245].

DDX1 and DDX3 are associated with the Rev/RRE/CRM1 complex and regulate viral RNA export [199, 232, 234]. Also, WRN interacts with Tat and elevates HIV-1 gene expression [214]. Recruiting these helicases to viral RNP complex at different stages of viral replication reflects the need of HIV-1 to harness cellular helicases to overcome certain rate-limiting steps of viral RNA replication or to accomplish an activity that rarely occurs to cellular RNA such as reverse transcription. Opening the door to cellular helicases also exposes the virus to helicases that are deleterious to HIV-1 replication. One such example is MOV10 that finds its way into HIV-1 particles and impairs viral reverse transcription [242-244].

More cellular helicases than described herein may associate with HIV-1 given that a dozen of helicases have been reported in several genome-wide functional screens that were aimed at identifying cellular proteins modulating HIV-1 infection. These include DDX10, DDX19, DDX33, DDX53, DDX50, DDX55, DDX60L, FBXO18, IGHMBP2, YTHDC2, HFM1, RECQL4, RUVBL2 [295, 304-306]. Furthermore, studies also show that HIV-1 infection alters the expression of a handful of cellular helicases [307, 308]. Last but not least, in a recent study aimed at comprehensively mapping the interactions between cellular factors and each of the HIV-1 18 proteins, DDX49 was reported to associate with Gag, DDX20 with Vpr, and RECQ1 with Pol [238]. An important task in the future is to characterize the interactions of these helicase candidates with HIV-1 and to decipher their functions in HIV-1 infection.

How many helicases does HIV-1 really need? How many of these helicases play redundant roles in HIV-1 replication? In addition to these questions, we also lack a detailed knowledge at the molecular and enzymatic levels regarding how a helicase promotes or impedes a specific step of HIV-1 replication. It will be a challenging task to determine experimentally when and where a specific helicase becomes associated with HIV-1 RNP. It is more challenging then to discern the structural and biochemical changes that a specific helicase introduces to the HIV-1 RNP complex. Knowing these biochemical and enzymatic details will not only help to further elucidate the role of a helicase in HIV-1 RNA metabolism, but will also aid the discovery of helicase inhibitors that may have the potential for treating HIV-1 infection [309].

Chapter 3 – Screen for Helicases Modulating HIV-1 Infection in SupT1 Cells (Research Article)

Cellular helicases are central players in the metabolism of nucleic acids. As a retrovirus without its own helicase, HIV-1 must exploit cellular helicases to successfully accomplish its lifecycle. In the past decade, several studies have reported the role of a few helicases in HIV-1 replication. In order to obtain a more comprehensive view regarding how cellular helicases modulate HIV-1 infection, we utilized the shRNA technique and knocked down 130 cellular helicases in SupT1 cells. By measuring HIV-1 production in the helicase knockdown and control SupT1 cells, we identified 35 helicases as potential key players in HIV-1 replication. These 35 helicases are distributed in eight cellular pathways among which DNA repair and gene expression machineries comprise the greatest number of candidates, highlighting the importance and the complexity of HIV-1 integration and viral gene expression regulation. We propose nine helicases as particularly promising candidates, including BTAF1, ERCC6L2, BLM, DDX48, TNFRSF6B, POLQ, RECQL, DDX17 and eIF4A1.

3.1 Introduction

Human immunodeficiency virus type 1 (HIV-1) is a small virus which genome codes for only fifteen proteins. Therefore, HIV-1 needs to recruit cell mechanisms through the recruitment of cellular factors to promote its replication, and to overcome the host restriction by expressing specific viral proteins. As a result of their essential functions in the virus lifecycle, viral enzymes have been the most exploited targets for the development of anti-HIV-1 drugs. However, the constant emergence of multiple drug resistance necessitates the quest for new targets and new therapies. To that extent, anti-HIV-1 drug targets are no longer limited to viral proteins, but also include cellular factors that have been proved to play critical roles in the replication of the virus. Such an example is the development of Maraviroc that blocks viral entry through inhibiting the interaction between HIV-1 gp120 and the CCR5 co-receptor [310]. Therefore, the identification of factors that are involved in viral-host interactions constitutes a crucial aspect in the understanding of which cellular pathways HIV-1 utilizes for its purpose. In 2001, two major breakthroughs revolutionized research: the sequencing of the human genome [311] and the discovery of RNA interference mechanisms in mammal cells [312]. Access to these new knowledge and technology have allowed for the development of new strategies to characterize virus-host interactions. Since 2007, a series of genome-wide studies have been performed with the aim to identify the host factors involved in HIV-1 replication [313-316]. Different approaches have been considered to knock down cellular

proteins and monitor HIV-1 replication, in order to screen for cellular factors affecting either the early or late stage of viral replication [304, 317-321]. Simultaneously, a second axis of AIDS research focused on the study of human genes polymorphism associated with distinct outcomes regarding disease evolution in HIV-1 infected patients [322-324]. The single-nucleotide polymorphisms (SNPs) were analyzed in genome-wide association studies with the attempt to identify genetic markers that are associated with the course of HIV/AIDS progression [149, 325-327]. Additional screening studies investigating the human-HIV-1 interactome also contribute to a better understanding of the cellular mechanisms that are hijacked by HIV-1 to replicate and the strategies that the cell develops to inhibit viral replication [238, 328].

RNA helicases are a large family of proteins that are involved in every step of RNA metabolism [329, 330]. They rearrange RNA structures and ribonucleic complexes (RNPs) using the energy derived from the hydrolysis of nucleotide triphosphates (NTPs) [331]. As a retrovirus, RNA is central to the replication of HIV-1. However, unlike some viruses that encode their own helicase, HIV-1 needs to exploit cellular ones to aid viral replication. To date, 17 cellular helicases have been reported to promote or inhibit HIV-1 replication at various stages, including reverse transcription, latency, transcription, export, translation, packaging and viral RNA stability [196, 268, 332, 333]. Interestingly, across all the genome-wide studies that have been performed, only 11 helicases were isolated as candidates for being HIV-1-influencing factors (HIFs). These include DDX3X [304, 317, 319], DDX5 [319], DDX6 [238], DHX8 [316], DHX9 (also known as RNA helicase A) [319], DHX15 [318], DDX20 [238], DDX40 [324],

DDX49 [238] and DICER1. Overall, as much as 14 helicases that are known to modulate HIV-1 replication were not identified as potential HIFs in the previous genome-wide screening studies (Figure 24). With the aim to gain a more comprehensive understanding of the role of cellular helicases in HIV-1 replication, we have performed a shRNA-based screening study and tested 130 cellular helicases for their possible effect on HIV-1 production in SupT1 cells. We have identified 35 helicases as potential HIFs, which include 41% (7 out of 17) of the helicases that have already been described in detailed studies.

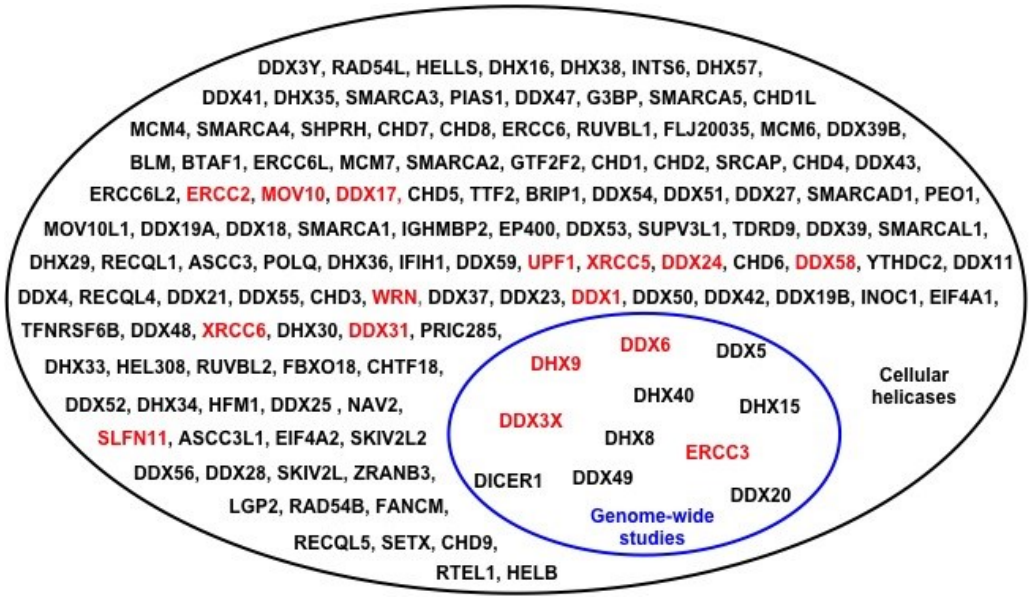

Figure 24 | Human helicases
The black circle represents human helicases. The blue circle represents human helicases that have been identified as candidates in genome-wide studies investigating for cellular factors that influence HIV-1 replication. Helicases in red have been individually studied for their effect on HIV-1.

3.2 Material and Methods

3.2.1 SupT1 Cell Lines

SupT1 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen). Mission short hairpin RNA (shRNA) transduction particles were purchased from Sigma (Cat. No. SH2621) to knock down 130 helicases in SupT1 cells. Each helicase was targeted on average by 3 to 5 shRNA clones. A control shRNA that had the scrambled RNA target sequence was also purchased from Sigma. HIV-1 stock was prepared by transfecting 293T cells with HIV-1 BH10 clone, together with a plasmid expressing the glycoprotein (G) of VSV. VSV G protein was used to enhance the infection efficiency of wild-type HIV-1 particles, and the VSV G pseudotyped HIV-1 BH10 particles were used in screening experiments. The level of viruses was titrated by infecting TZM-bl indicator cells. First, stable SupT1 cell lines were created to express each individual shRNA. SupT1 cells ($1.5x10^6$) were infected with 50 μL of the Mission shRNA transduction particles supplemented with Polybrene (5 μg/ml) in a 24-well plate, and spun at 1800 rpm for 45 minutes at room temperature. After 48 h, puromycin (2 μg/ml) was added to select for stably transduced cells. Cells were cultured with puromycin for at least 72h to ensure complete selection. Following puromycin selection, SupT1 cells (10^6) were challenged with VSV-G HIV-1 BH10. The next day, cells were spun at 1800 rpm

for 5 minutes to remove the supernatant containing VSV-G pseudotyped HIV-1 viruses and resuspended in 1 mL fresh RPMI. The supernatant was harvested 40h post infection for further analysis and the cells were lysed for western blot analysis.

3.2.2 Cell Viability Assay

To assess cell viability, we monitored cell proliferation using the CellTiter $^{96®}$ AQ_{eous} one solution cell proliferation assay kit (Promega). Briefly, this assay is a colorimetric method that determines the number of viable cells. The kit contains two chemicals: the MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfphenyl)-2H-tetrazolium) and the stability agent PES (phenazine ethosulfate). MTS is bioreduced into a colored formazan product by the cellular NADH or NADPH, which is produced by dehydrogenase enzymes in metabolically active cells. The MTS/PES mix is added to the cells and incubated for 4 hours at 37°C. Formazan product formation is measured by absorbance at 490nm using a spectrophotometer.

3.2.3 Lentiviral Particles

Bacterial stocks of shRNA-containing plasmids were purchased from Sigma. HEK293T cells (10^6) were transfected with 3µg shRNA, 3µg PLP1 (HIV-

1 Gag-Pol), 1μg PLP2 (HIV-1 Rev) and 0.25μg VSV-G, using lipofectamine2000 and according to the manufacturer protocol (Invitrogen). Lentiviral particles-containing supernatants were harvested 40 hours following transfection. SupT1 cell lines were generated as described in the previous section.

3.2.4 Virus Production Assay

The amount of infectious viruses in culture supernatants was determined by infecting the TZM-bl indicator cells. Cells were seeded in 24-well plate at a density of $4x10^4$ cells per well 1 day before infection. 50μL of supernatant from infected SupT1 cells were used to infect TZM-bl cells. Forty-eight hours after infection, cells were lysed in 1X passive lysis buffer (Promega), and levels of firefly luciferase activity in the cell lysates were measured using the Luciferase Assay kit and the Glomax luminometer (Promega).

3.3 Results

3.3.1 Design of the Helicases Screening

To identify cellular helicases that can modulate HIV-1 replication, we performed a two-step shRNA-based screen in SupT1 cells, using a shRNA library obtained from SIGMA (Figure 25). In the first step, we individually knocked down 130 cellular helicases (Table 3). Each shRNA sequence was inserted into the lentiviral pLKO.1 vector [334] and packaged into a VSV-G pseudotyped particle (obtained from SIGMA). After transduction with the VSV-G packaged shRNA, SupT1 cells were treated with puromycin, in order to select for stable knockdown cell lines. The use of puromycin selection allowed us to eliminate any helicase knockdown that would be lethal, since the cells would die and subsequently could not be selected. However, some cells could survive despite of the toxicity of some shRNAs, with a diminished metabolism. Therefore, to ensure that the cells were healthy, we analyzed the viability for every selected cell line, and excluded those exceeding 25% deviation compare to the control shRNA. Selected cell lines were then challenged with VSV-G pseudotyped HIV-1 BH10, and the production of infectious HIV-1 particles was assessed by infecting TZM-bl indicator cells (Figure 26). Since the shRNA library was generated without confirmation for most of the shRNA sequences, we considered a helicase as a candidate if at least one of its shRNA clones affected the production of infectious viral particles by more than 3.5-fold compare to the control shRNA, in duplicates.

This step allowed us to isolate a list of 35 genes with at least one shRNA displaying this effect, accounting for 27% of the initial tested targets (Figure 27).

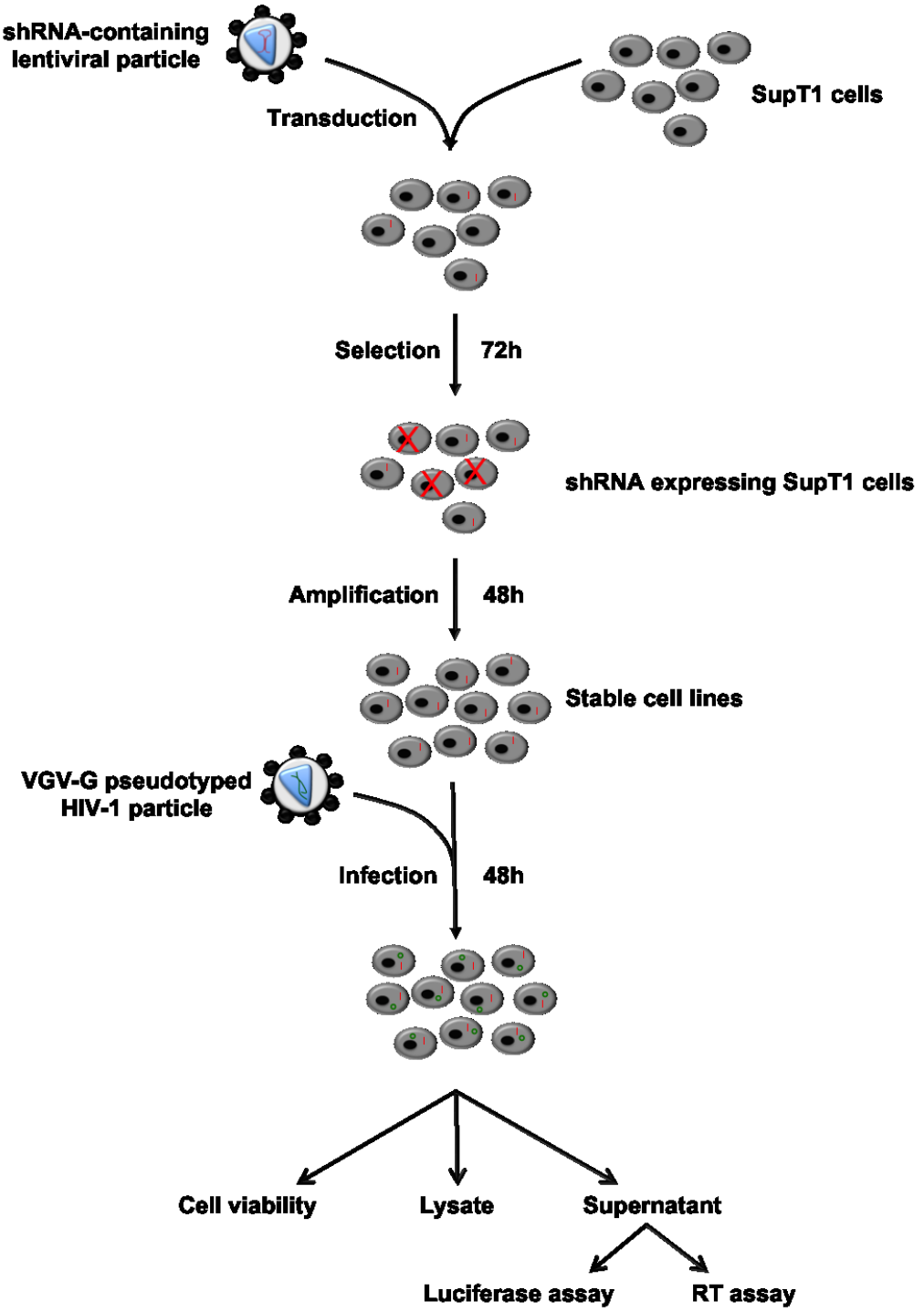

Figure 25 | Screening protocol
shRNA-containing virus particles were used to transduce SupT1 cells. Each lentiviral particle contained a specific shRNA sequence inserted into the pLK0.1 vector (Sigma) targeting the corresponding cellular helicase. Two days post-transduction, SupT1 cells were treated with 2µg/mL puromycin to allow for selection of stable cell lines. After three days selection, stable cell lines were grown for an additional two days. VSV-G pseudotyped HIV-1 particles were used for spin-infection of the stable cell lines. The cells were spun for 45 minutes at 1800 rpm with 5µg/mL polybrene. Forty-eight hours post infection, the supernatant was harvested to monitor virus production (reverse transcriptase assay) and infectivity (luciferase assay). Cell were lysed and the levels of viral protein expression and knockdown efficiency were monitored by Western-blot. Cell viability was also monitored using the CellTiter $^{96®}$ AQ_{eous} one solution cell proliferation assay kit (Promega).

Table 3 | List of helicases tested, from SIGMA shRNA library

Group 1	DDX3X	DHX15	DDX3Y	DHX8	RAD54L	HELLS	DHX16
	DDX5	DHX38	DHX9	INTS6	DDX41	DHX35	WRN
	SMARCA3	PIAS1					
Group 2	BLM	DDX47	DDX20	G3BP	SMARCA5	BTAF1	CHD1L
	ATRX	SMARCA4	SHPRH	FLJ20105	CHD7	CHD8	ERCC6
	LOC375748	RUVBL1					
Group 3	IGHMBP2	MCM6	MCM7	SMARCA2	GTF2F2	CHD1	CHD2
	SRCAP	CHD4	CHD5	ERCC2	ERCC3	TTF2	UPF1
	XRCC6	XRCC5					
Group 4	BRIP1	DDX54	MOV10	DDX51	DDX27	SMARCAD1	FLJ20035
	DDX19A	EP400	DDX24	DDX53	SUPV3L1	DDX31	DDX1
	DDX18	SMARCA1					
Group 5	DDX39	TDRD9	DHX36	IFIH1	DDX59	DDX37	DDX49
	DDX23	DDX50	DDX42	DDX19B	DDX4	RECQL4	DDX21
	DDX55	DXH29	DICER1	LGP2			
Group 6	LGP2	RAD54B	FANCM	DHX40	RECQL5	DHX33	SETX
	HEL308	RUVBL2	FBXO18	RTEL1	DDX56	DDX28	SKIV2L
	ASCC3L1	EIF4A2	SKIV2L2				
Group 7	RECQL	DHX30	MOV10L1	DDX52	ASCC3	EIF4A1	POLQ
	TNFRSF6B	PRIC285	DDX48	DHX34	DHX57	MCM4	BAT1
	DDX17	DDX6					
Group 8	DDX43	PEO1	SMARCAL1	CHD6	INOC1	CHD3	CHD9
	CHTF18	ZRANB3	NAV2	HELB	DDX25	YTHDC2	DDX11
	DDX58	HFM1					

SIGMA shRNA-containing lentiviral particles were provided in eight 96-well plates (Group 1-8), conserved at -80°C.

Relative virus production
100
10
1
0.1
0.01
DDX1
DDX3X
DDX4
DDX6
DHX8
DHX9
DDX11
DHX15
DHX16
DDX19A
DDX19B
DDX20
DDX21
DDX23
DDX25
INTS6
DDX27
DDX28
DHX29
DHX30
DDX31
DHX33
DHX34
DHX35
DDX36
DHX37
DDX38
DHX40
DDX41
DDX42
DDX43
DDX47
DDX49
DDX51
DDX52
DDX53
DDX54
DDX55
DDX56
DHX57
DDX58
DDX59
LGP2
FLJ20035
RECQL
BLM
WRN
RECQL4
RECQL5
EIF4A1
EIF4A2
DDX48
MOV10
MOV10L1
DDX5
DDX17
XRCC5
XRCC6
CHD1
CHD1L
CHD2
CHD3
CHD4
CHD5
CHD6
CHD7
CHD8
CHD9
ERCC2
ERCC3
ERCC6
FLJ20105
SMARCA1
SMARCAD1
SMARCAL1
SMARCA2
SMARCA3
SMARCA4
SMARCA5
HELLS
INOC1
MCM4
MCM6
MCM7
DDX18
DDX21
DDX24
DDX50
CHTF18
RUVBL1
RUVBL2
SKIV2L
SKIV2L2
RAD54L
RAD54B
ASCC3
ASCC3L1
BRIP1
FANCM
POLQ
HELS308
DDX39
BAT1
GTF2F2
SHPRH
G3BP
PIAS1
BTAF1
LOC37548
FBXO18
IGHMBP2
SRCAP
TTF2
UPF1
EP400
SUPV3L1
TDRD9
IFIH1
DICER1
SETX
RTEL1
TNFRSF6B
PRIC285
PEO1
ZRANB3
NAV2
HELB
YTHDC2
HFM1
ATRX

Figure 26 | Effect of helicases knockdown on HIV-1 production in SupT1 cells

Fold of change in the production of infectious HIV-1 particles. Relative viral production was measured by luciferase activity using the HeLa-TZM-bl reporter cells, as described in the material and methods.

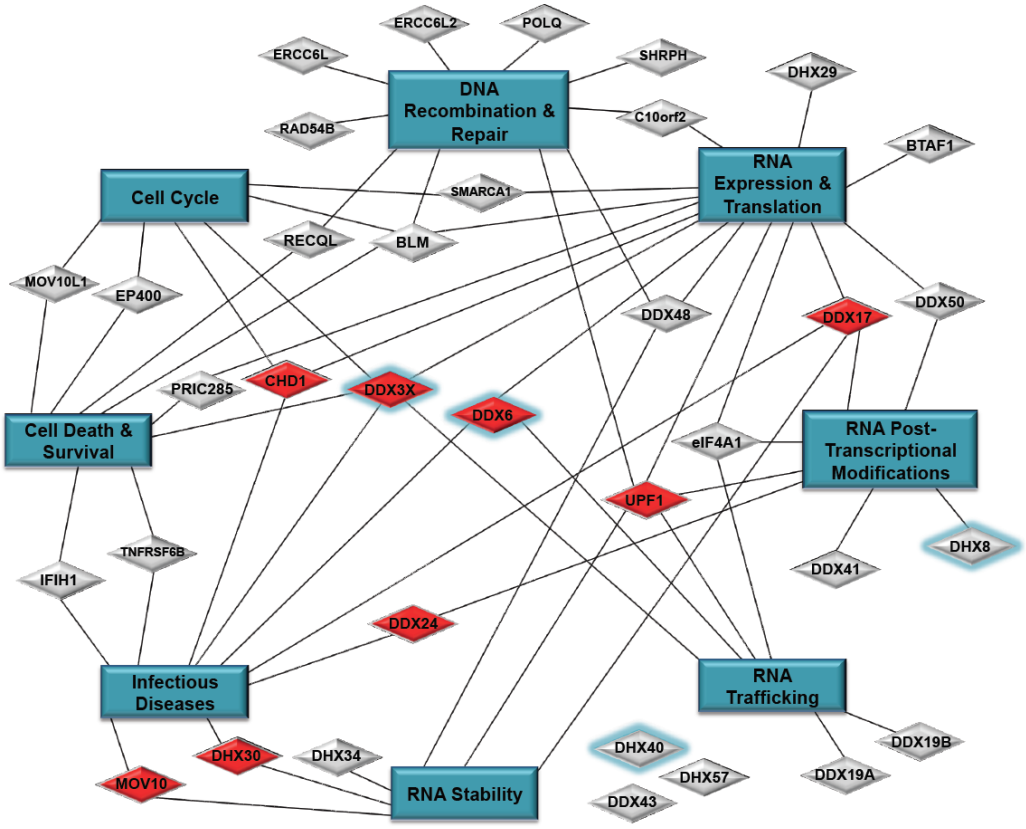

Figure 27 | Pathway analysis scheme of the 35 helicases candidates

Classification of the helicases in eight pathways, using the IPA (Ingenuity Pathway Analysis) software. Helicases in red have been individually studied for their effect on HIV-1 replication. A blue halo indicates a helicase that has been indentified in at least one genome-wide study.

3.3.2 Pathway Analysis of the Helicases Hits

To classify our 35 helicases candidates into cellular pathways that are involved in HIV replication, we performed a statistical analysis using the Ingenuity Pathways Analysis (IPA®) software. This revealed 8 putative pathways, including 1) Cell Cycle, 2) DNA Repair, 3) Cell Death and Survival, 4) RNA Expression and Translation, 5) RNA Post-Transcriptional Modifications, 6) RNA Trafficking, 7) RNA Stability and 8) Infectious Diseases (Figure 27). Multiple helicases belong to more than one pathway, which reflects the functional diversity of these protein family members (Table 4).

Table 4 | List of the 35 identified helicases candidates classified in pathways

Helicase	DNA Recombination & Repair	RNA Expression & Translation	RNA Post-Transcriptional Modifications	RNA Trafficking	RNA Stability	Infectious Diseases	Cell Death & Survival	Cell Cycle
BLM	DNA Repair	Transcription					Apoptosis - T Cell Proliferation	
BTAF1		Transcription						
C10orf2	Nuclear and Mitochondrial DNA Replication	Transcription						
CHD1		Transcription				HIV-1 Reactivation		Chromatin Remodeling
DDX17		Transcription	Splicing	Nuclear Export	HIV-1 RNA Exosomal Degradation	HIV-1 RNA Packaging		
DDX19A				Nuclear Export				
DDX19B				Nuclear Export				
DDX24			HIV-1 RNA Processing			HIV-1 RNA Packaging		
DDX3X		Transcription Translation		HIV-1 RNA Nuclear Export		Replication of RNA Viruses	Apoptosis	Interphase
DDX41			Splicing					
DDX43								
DDX48	DNA Repair	Translation			NMD			
DDX50			Unwinding					
DDX6		Translation		Accumulation of HIV-1 RNA		Production of Retroviridae		
DHX29		Translation						
DHX30						HIV-1 RNA Packaging		
DHX34					NMD			
DHX40								
DHX57								
DHX8			Splicing					
EIF4A1		Translation	Unwinding	Recruitment of RNA				
EP400	DNA Repair						Apoptosis	Chromatin Remodeling
ERCC6L	DNA Replication							
ERCC6L2	DNA Recombination DNA Repair							
IFIH1						Production of VSV - IFN Response	Cell Viability	
MOV10					mRNA Cleavage	Production of Retroviridae		
MOV10L1							Death of Spermatocytes	Arrest in Meiosis
POLQ	DNA Repair							
PRIC285		Transcription						
RAD54B	DNA Recombination DNA Repair							
RECQL	DNA Recombination						Apoptosis	
SHPRH	DNA Repair							
SMARCA1		Transcription						Chromatin Remodeling
TNFRSF6B						Maturation of DCs	Apoptosis	
UPF1	DNA Repair	Translation		Nuclear Export	NMD			

Green indicates a helicase that potentially promotes HIV-1 replication (inhibitory effect of the knockdown). Red indicates a helicase that potentially inhibits HIV-1 replication (stimulatory effect of the knockdown).

3.3.2.1 Cell Cycle

The eukaryotic cell cycle is divided in four major steps, including mitosis (M) and interphase (G1, S and G2 phases). While the cells grow and display a high protein synthesis activity in G1, they replicate their DNA during the S phase

to eventually condense their chromatin in G2 and divide into two daughter cells in the M phase. During the interphase, the chromosomes exist as uncondensed chromatin, allowing RNA synthesis and DNA replication. CHD1, SMARCA1 (also known as SWI and SNF2L) and EP400 have been reported to be involved in chromatin remodeling [335-337]. In the context of HIV-1, CHD1 depletion increases chromatin accessibility at the HIV-1 promoter, resulting in the reactivation of the latent provirus integrated in intons [332]. The effect of SMARCA1 on HIV-1 replication remains undetermined.

Karyophilic properties of the uncoated PIC allow HIV-1 to successfully infect non-dividing cells [50]. In addition, HIV-1 Vpr induces a block in the G2 phase of the cell cycle, called the G2/M cell cycle arrest [338], likely through hyper-phosphorylating CDK1, resulting in the activation of the ATR (ataxia and telangiectasia mutated and Rad3 related) replication checkpoint [339]. Physiologically, this represents an advantage for the virus, since it inhibits the proliferation of T-cells, therefore suppressing part of the immune response [340]. Moreover, Vpr-induced G2 arrest provides the virus with a favorable environment for its replication, where the LTR is most active [341]. DDX3 was reported to interact with DDX5 during the G2/M phase, promoting RNP remodeling and splicing [342]. Considering that HIV-1 RNA undergoes extensive and highly regulated splicing events, it is not surprising that the virus gains to extend the G2/M phase. In addition to its multiple roles in HIV-1 replication, DDX3 involvement in viral RNA splicing would constitute a new function for this helicase in the virus lifecycle.

MOV10L1 has been reported to protect spermatocytes against retrotransposons through inhibiting LTR and LINE-1 retrotransposons [343]. The role of MOV10L1 in HIV-1 replication has not yet been investigated.

3.3.2.2 DNA Repair

The eukaryotic genome is constantly subject to modifications and repair. Cells possess complex mechanisms that allow them to overcome DNA damage imposed by mutagenic agents such as chemicals, UV or viruses. HIV-1 integration is triggered by the viral integrase that induces a double strand break to the cellular DNA, eventually leading to the insertion of the provirus into the host genome. One study investigated the effect of DNA repair proteins on HIV-1 integration [344]. In this study, the authors highlighted the importance of the base excision repair (BER) pathway, and two helicases were characterized as potential HIV-1 integration regulators: ERCC3 and RECQL4 [344]. Interestingly, although ERCC3 and RECQL4 were not identified as hits in our screening, we found four candidate helicases belonging to the same two families: ERCC6L, ERCC6L2, RECQL1 and BLM (also known as RECQL2), also suggesting the importance of the DNA repair mechanisms in HIV-1 replication, possibly at the integration step (Figures 26 and 27). In addition, we also found the helicases DDX48, RAD54B, POLQ, SHPRH, UPF1, EP400 and C10orf2, already described for their effect on cellular DNA repair [345-350] and mitochondrial DNA replication [351], as potential modulators for HIV-1 production.

3.3.2.3 Cell Death and Survival

Cells have diverse regulation mechanisms in response to stress stimuli. The ultimate mechanism is the use of programmed cell death, also called apoptosis. In human cells, a few DNA helicases were reported to cooperate with p53 to induce the p53-mediated apoptosis pathway, including WRN [352], BLM [353], ERCC2 and ERCC3 (also known as XPD and XPB, respectively) [354]. Interestingly, while ERCC2 and ERCC3 are involved in the degradation of the newly imported HIV-1 cDNA to the nucleus [355], WRN affects the virus at rather the transcriptional level than DNA degradation [356]. On the contrary, the RNA helicase DDX5 is required for the p53-dependent p21 expression and cell cycle arrest after DNA damage, rather than p53-mediated apoptosis, suggesting a role in cell survival [357]. Although two studies identified DDX5 as a host cell factor potentially modulating HIV-1 replication [319, 328], we did not find this helicase as a hit in our screening study, but its paralog DDX17 (Table 4), also involved in cell survival [358]. However, the role of DDX17 in HIV-1 replication is, to date, limited to act as a cofactor in the ZAP-mediated HIV-1 multiply spliced mRNA degradation [359]. In addition, we identified MOV10L1, EP400, IFIH1 and TNFRSFB6 as potential regulators of HIV-1 replication. Considering that apoptosis is one of the major cause of CD4+ T cells depletion [360], determining how these helicases affect virus replication could help unravel some mechanisms behind the HIV-1-induced cytotoxicity.

3.3.2.4 RNA Expression and Translation

Gene expression is a multistep process that includes mRNA production and protein synthesis. Considering that HIV-1 genome carries only nine genes to express fifteen proteins, the virus requires complex transcriptional and translational regulatory mechanisms to produce the proper ratios of viral proteins that are necessary for the synthesis of newly infectious particles. Indeed, RNA expression and translation is the most represented pathway in our study, including 13 helicases (Table 4). Eight of these helicases are known for their cellular function in DNA transcription, including DDX3X, DDX17, BLM, CHD1, SMARCA1, BTAF1, PRIC285 and C10orf2 [361-369]. Although only CHD1 has been characterized for its direct implication in HIV-1 transcription [332], the large number of helicases isolated in our study emphasizes the importance of the cellular transcriptional machinery in the regulation of HIV-1 mRNA synthesis. Moreover, even though RHA and WRN were not considered as hits in our screening study, other groups have confirmed their stimulatory role in HIV-1 DNA transcription [215, 370].

HIV-1 recruits the cellular translation machinery to produce viral proteins from its mRNA, in both cap-dependent and cap-independent (IRES-dependent) manners. Due to the complex spatial organization of HIV-1 5'UTR, helicases are potential key players in viral protein expression, by overcoming structural restrictions and

allowing ribosomal scanning. Indeed, the ribosomal complex contains various RNA helicases [371], including DDX3, DDX6, DHX9 (also known as RHA), DHX29, eIF4A1 (also known as DDX2) and eIF4A3 (also known as DDX48) (reviewed in [371]). In addition, UPF1 was shown to enhance translation termination of aberrant mRNAs [372], a function that causes HIV-1 mRNA degradation (reviewed in [196]). With the exception of DHX9, the other 6 helicases were identified in our screening study, confirming their essential role in the cellular translation process, and in HIV-1 in particular. Further investigation will be needed to determine the specific role of each helicase in HIV-1 translation.

3.3.2.5 RNA Post-Transcriptional Modifications

Post-transcriptional modifications allow the conversion of a newly expressed RNA into a mature RNA. These modifications comprise 5'-capping, 3'-polyadenylation and splicing. The implication of helicases in post-transcriptional modifications is poorly described in human. However, splicing has been extensively documented regarding the involvement of helicases in yeast, and 85% of the yeast spliceosome components have been found a human ortholog [373, 374]. Recently, Cordin and Beggs highlighted the importance of eight helicases in yeast splicing events: Prp5, Sub2, Prp28, Prp2, Prp16, Prp22, Prp43 and Brr2 [374]. To each of these helicases corresponds a human ortholog (Table 5). Interestingly, we found that DHX8 potentially promotes HIV-1 production (Table 4). In addition to DHX8, we also found DDX17 and DDX41 as cellular splicing

factors influencing HIV-1 replication (Table 4). Other splicing-independent helicases (DDX50, eIF4A1 and DDX24) were also found to modulate HIV-1 production, possibly through remodeling the viral RNP complex.

3.3.2.6 RNA Trafficking

Transcription, splicing, export and translation are closely related events, and modifications at one stage can change the fate of RNA for the subsequent steps. For example, several studies reported that splicing and export are two coupled mechanisms [375, 376]. In addition to the complex splicing processes that HIV-1 RNA must undergo, export to the cytoplasm also represents a highly regulated step. Not only the fully spliced viral mRNA but also intron-containing (full length and singly spliced) mRNAs must be exported to the cytoplasm, whereas intron-containing cellular mRNAs must complete the splicing process before they can leave the nucleus. Therefore HIV-1 evolved specific mechanisms in order to export its three RNA species. DDX1 and DDX3 have been reported to play active roles in HIV-1 RNA export (reviewed in [196]). Recently, DDX6 was reported to promote the accumulation of HIV-1 RNA at the assembly site [377]. Consistently, we identified DDX3X (often called DDX3) and DDX6 as helicases that can promote the production of HIV-1 particles (Table 4). However, neither our study nor other genome-wide screens could pick up DDX1 as a candidate. Nonetheless, other helicases displayed interesting effects on HIV-1 production, including DDX17, DDX19A, DDX19B, eIF4A1 and UPF1. Interestingly, in

yeast, Dbp5 (human homolog DDX19A) interacts with Nup159 to promote the export of polyadenylated RNA [378, 379]. Similarly, in human, DDX19B interacts with Nup214 (yeast homolog Nup156) [380, 381], suggesting its role in polyadenylated mRNA nuclear export. UPF1 and eIF4A1 are likely to be recruited to the viral RNP complex, targeting the viral RNA to degradation or translation, respectively.

3.3.2.7 RNA Stability

Our study revealed that six helicases, belonging to three RNA degradation pathways, are involved in HIV-1 production: exosome degradation, nonsense-mediated mRNA decay (NMD) and miRNA (Table 4). DHX30 and DDX17 are already known to form a complex with ZAP to trigger exosome-mediated RNA degradation [382, 383]. DDX48 and UPF1 are known to be part of the nonsense-mediated mRNA decay pathway, a cellular surveillance mechanism that leads to the degradation of aberrant mRNAs [384, 385]. Interestingly, in zebrafish, the helicase Dhx34 (human homolog DHX34) has also been shown to function in the NMD pathway [386]. Finally, MOV10 is a member of RISC (RNA-induced silencing complex), also involved in the degradation of mRNA as part of gene expression regulation [387]. However, in the context of HIV-1, MOV10 seems to inhibit reverse transcription rather than targeting newly synthesized viral mRNA to the RISC-mediated degradation [196].

3.3.2.8 Infectious Diseases

In the past decade, the direct role of helicases in the replication of viruses started to be investigated in details. In our screen, we identified nine helicases that have been previously shown to promote or inhibit viruses' replication (Table 4). DDX3X, DDX6, DDX17, DHX30, DDX24, MOV10 and CHD1 were previously described for their effect on HIV-1 replication. In addition, we found TNFRSF6B, which is implicated in the maturation of dendritic cells [388], and IFIH1 (also known as MDA5), an interferon inducible member of the RLR family [389], as potential helicases regulating HIV-1 production. As a player of the innate immune response, IFIH1 has been extensively described for its broad antiviral effect [390-398]. However, the roles of TNFRSF6B or IFIH1 in HIV-1 replication remain to be investigated.

Table 5 | Human (alias) and Yeast (*Saccharomyces cerevisiae* homologs) helicases

Human Name (alias name)	Yeast homolog
ASCC3L1 (SNRNP200, BRR2)	Brr2
BLM (RECQ2)	Rqh1
BTAF1 (MOT1, TAF(II)170)	Mot1
C10orf2 (PEO1, TWINKLE)	N/A
CHD1	chd-1
DDX17 (p72, RH70)	N/A
DDX19A (DDX19, DBP5)	Dbp5p
DDX19B (DDX19, DBP5)	N/A
DDX23 (PRFP28)	Prp28
DDX24	Mak5p
DDX3 (DDX3X)	Dbp1p
DDX39B (BAT1, UAP56)	Sub2
DDX41	sacy-1
DDX43 (HAGE, CT13)	N/A
DDX46	Prp5
DDX48 (eIF4A3)	Fal1p
DDX50 (GU2, RH-II/GuB)	N/A
DDX6 (HLR2, P54, RCK)	Dhh1p
DHX15 (DBP1, HRH2)	Prp43
DHX16 (DBP2)	Prp2
DHX29 (DDX29)	N/A
DHX30 (RETCOR)	N/A
DHX34 (HRH1)	N/A
DHX38	Prp16
DHX40 (ARG147, PAD)	N/A
DHX57	N/A
DHX8	Prp22
EIF4A1 (eIF4A, DDX2)	Tif1p, Tif2p
EP400 (TNRC12)	Noc4p
ERCC6L (RAD26L, PICH)	N/A
ERCC6L2	N/A
IFIH1 (MDA5, DHX58)	Mfh2
MOV10	N/A
MOV10L1 (CHAMP)	N/A
POLQ	N/A
PRIC285 (HELZ2)	N/A
RAD54B	Rdh54p
RECQL (RECQ1, RECQL1)	N/A
SHPRH	N/A
SMARCA1 (SNF2L, SWI)	Isw2p
TNFRSF6B (DCR3)	N/A
UPF1 (NORF1, RENT1)	Nam7p

3.3.3 Possible Roles for BLM, eIF4A1 and DDX17 in HIV-1 Replication

Among the 35 candidates that we identified, 9 helicases showed a particularly strong effect on HIV-1 replication, more than 5-fold increase or decrease in the production of HIV-1 particles for at least two shRNAs (Table 6). We chose to investigate BLM, eIF4A1 and DDX17 and propose research axis to further determine their role in HIV-1 replication. We generated new VSV-G packaged shRNA particles to perform a second round of HIV-1 infection (Figure 4). In this second step, we used these lentiviral particles to transiently knockdown the helicase candidate genes in Jurkat or SupT1 cells. The knockdown cell lines were then challenged with VSV-G pseudotyped HIV-1 BH10 and we analyzed the cell lysates for the levels of cellular and viral proteins by western blot, as well as the supernatants for the levels of infectious particles by infecting TZM-bl indicator cells.

Table 6 | Top 9 candidates from stable knockdown cell lines

Helicase	Fold Change	Effect of Knockdown	Number of shRNAs
BTAF1	5.37	Decrease	2
ERCC6L2	6.51	Decrease	2
BLM	10.88	Decrease	3
DDX48	5.35	Increase	3
TNFRSF6B	6.43	Increase	3
POLQ	6.57	Increase	2
RECQL	7.86	Increase	3
DDX17	9.1	Increase	2
EIF4A1	10.3	Increase	3

Green indicates a potential inhibitory effect of the helicase on HIV-1 replication. Red indicates a potential stimulatory effect of the helicase on HIV-1 replication. The indicated fold-change represents the average of the shRNAs that have been selected (above the threshold with no cytotoxic effect) – right column.

3.3.3.1 Possible Role of BLM in Promoting HIV-1 Integration

Our screening study revealed that BLM knockdown cell lines led to a 10-fold decrease in HIV-1 production, suggesting an important role for BLM in promoting viral replication (Table 4). Consistently, transient knockdown of BLM in SupT1 cells leads to up to 10-fold decrease in the production of infectious particles (Figure 28A), which correlates with a decrease in the p24 levels in the cell lysate (Figure 28B), suggesting that BLM restricts HIV-1 after entry and prior to translation. BLM is a member of the RECQ family that comprises four other helicases, including RECQL1, WRN, RECQL4 and RECQL5. RECQ helicases are key players in double strand break DNA repair and telomere maintenance, therefore being involved in genome stability [399]. Although WRN has

previously been reported to facilitate HIV-1 transcription through stimulating the DNA-unwinding activity of DNA topoisomerase I (reviewed in [196]), only very little is known about the potential role of BLM during HIV-1 infection. One study from Bordi and colleagues reported that BLM and WRN are differentially regulated in HIV-1 infected PBMCs, where BLM mRNA levels are up-regulated at 24h post infection [370], suggesting a possible role for BLM in HIV-1 replication. Interestingly, WRN interacts with the heterodimer formed by the two cellular helicases Ku70 and Ku80, that are involved in double strand break DNA repair [400]. Furthermore, in *Drosophila*, *DmKu70* (homolog of human Ku70) was shown to interact with *Dmblm* (homolog of human BLM) and rescue the sterility phenotype imposed by mutated *Dmblm*, suggesting functional redundancy between *Dmblm* and *DmKu70* [401]. Moreover, in the context of HIV-1, Ku70 protects HIV-1 IN from proteasomal degradation [402] and Ku70/Ku80 heterodimer facilitates the integration of HIV-1 proviral DNA [297]. Altogether, we hypothesize that BLM might act alone or as a member of the DNA repair machinery to facilitate late HIV-1 DNA integration, potentially through repairing the double strand break induced by HIV-1 IN.

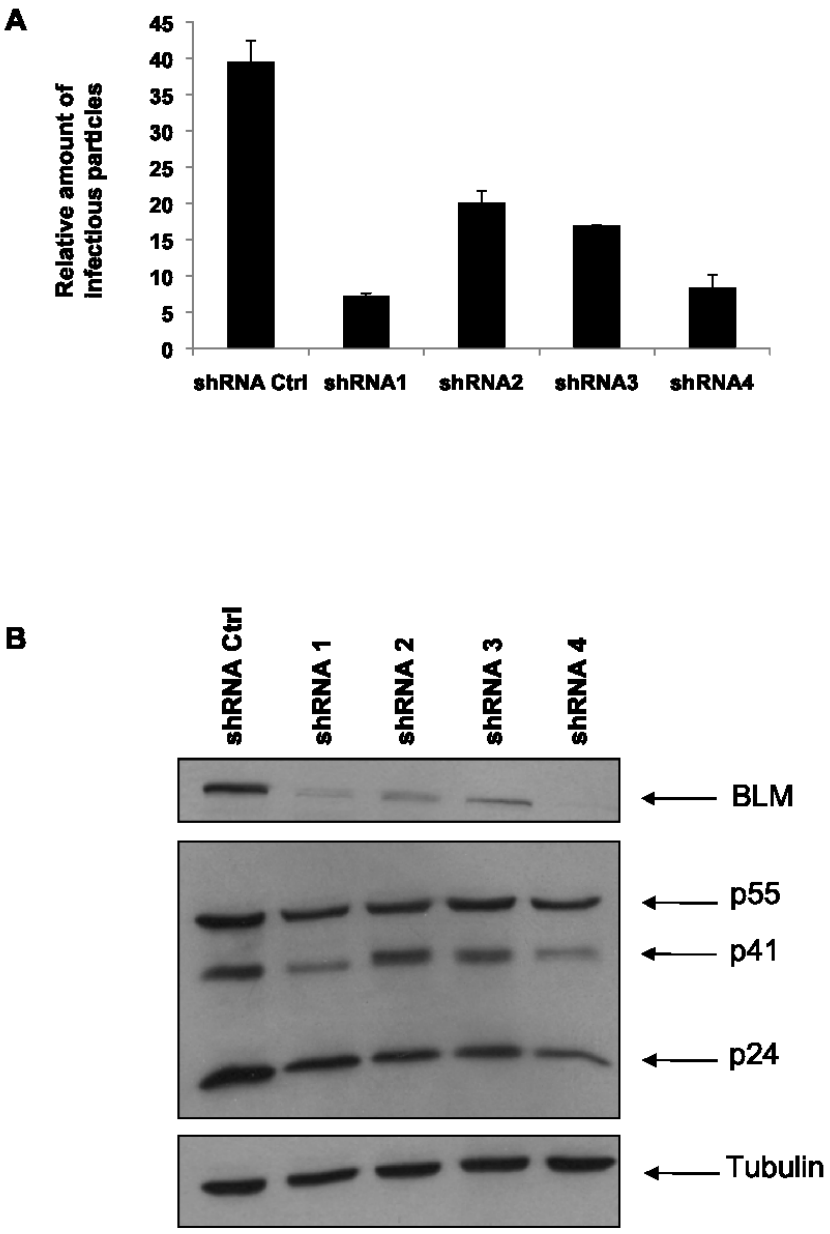

Figure 28 | Effect of BLM knockdown on HIV-1 production
SupT1 cells (1.5 x 10^6) were infected with 0.5mL BLM-shRNA-containing lentiviral particles. 48h post shRNA transduction, SupT1 cells were challenged with VSV-G pseudotyped BH10. Supernatant was harvested 48h post BH10 infection. The production of infectious viruses was assessed by measuring luciferase activity using 50μL supernatant to infect HeLa TZM-bl reporter cells (**A**). Protein expression levels in cell lysate were analyzed by Western blot (**B**).

3.3.3.2 Possible Role of eIF4A1 in Inhibiting HIV-1 Translation

In eukaryotes, eIF4A1 is a well-established translation factor known to initiate the cap-dependent translation of mRNAs, likely through unwinding the secondary structure of mRNA 5'UTR [403, 404]. Similarly to cellular mRNAs, full length HIV-1 mRNA recruits the eIF4 machinery to allow protein synthesis [252]. However, there is no evidence for a specific role of eIF4A1 in HIV-1 RNA translation. Unexpectedly, our screen identified eIF4A1 as a potential repressor of HIV-1 replication, with about 10-fold increase in virus production in stable knockdown cell lines (Table 6). Consistently, transient knockdown of eIF4A1 in Jurkat cells led to a 7-fold increase in the production of infectious HIV-1 particles (Figure 29A). Western-blot analysis of the cell lysate revealed a slight increase in the level of p24 Gag (Figure 29B), suggesting that eIF4A1 knockdown might have a stimulatory effect on HIV-1 translation or at a step prior to translation. HIV-1 has evolved several specific mechanisms to promote the translation of its mRNA, including the use of an IRES motif and the ribosomal frameshift [81, 252]. One hypothesis could be that eIF4A1 knockdown favors the cap-independent translation of HIV-1 mRNA. Using a dual luciferase reporter construct, we investigated the effect of eIF4A1 knockdown on cap-dependent and IRES-dependent translation (Figure 29C). Surprisingly, we observed that eIF4A1 depletion induced a slight increase in cap-dependent translation, but had no effect on IRES-dependent translation. Another hypothesis would be an indirect effect where eIF4A1 knockdown would prevent the expression of cellular genes

involved in HIV-1 restriction, therefore resulting in an increase of HIV-1 production. Furthermore, the dramatic increase in the amount of infectious particles compare to the moderate effect on translation could suggest a role for eIF4A1 at the late stage of the virus lifecycle. However, such an effect remains unlikely due to the cellular function of this helicase.

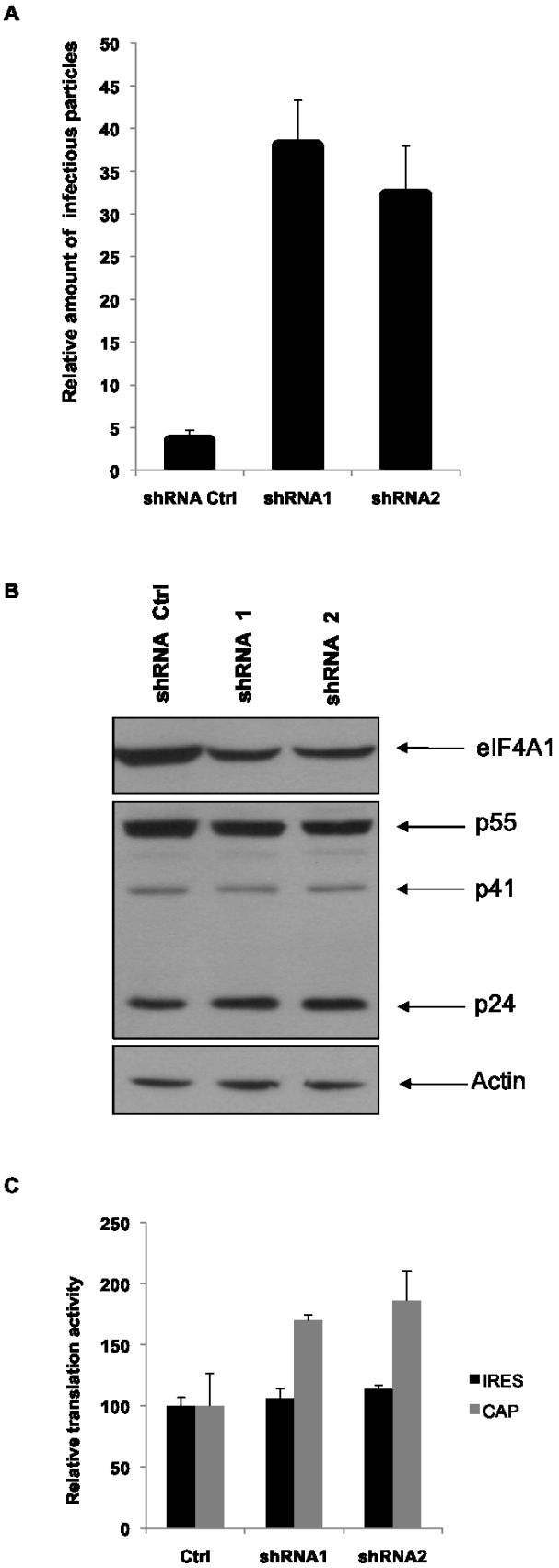
A
Relative amount of infectious particles
50
45
40
35
30
25
20
15
10
5
0
shRNA Ctrl
shRNA1
shRNA2
B
shRNA Ctrl
shRNA 1
shRNA 2
eIF4A1
p55
p41
p24
Actin
C
Relative translation activity
250
200
150
100
50
0
Ctrl
shRNA1
shRNA2
IRES
CAP

Figure 29 | Effect of eIF4A1 knockdown in HIV-1 production
SupT1 cells (1.5 x 10^6) were infected with 0.5mL eIF4A1-shRNA-containing lentiviral particles. 48h post shRNA transduction, SupT1 cells were challenged with VSV-G pseudotyped BH10. Supernatant was harvested 48h post BH10 infection. The production of infectious viruses was assessed by measuring luciferase activity using 50μL supernatant to infect HeLa TZM-bl reporter cells (**A**). Protein expression levels in cell lysate were analyzed by Western blot (**B**). HEK293 cells (5 x 10^5) were infected with 0.5mL eIF4A1-shRNA-containing lentiviral particles. 48h post shRNA transduction, HEK293 cells were transfected with 0.1**μ**g dual luciferase reporter plasmid (obtained from Dr N. Sonenberg). Luciferase activity was measured 48h post-transfection using the dual luciferase kit (Promega) (**C**).

3.3.3.3 Possible Role of DDX17 in Preventing HIV-1 Packaging

We first identified DDX17 in our study as a helicase that can inhibit HIV-1 replication, with a 9-fold increase in the production of viral particles in stable knockdown cell lines. DDX17 was previously reported to act as a cofactor of ZAP, involved in the exosome-mediated degradation of multiply spliced HIV-1 mRNA [359]. Unexpectedly, transient knockdown of DDX17 in Jurkat cells induced an opposite phenotype, where the viral production was reproducibly diminished by ~2-fold (Figure 30A). Analysis of viral protein expression revealed no effect on the production of HIV-1 Gag in Jurkat cells (Figure 30B), suggesting a role for DDX17 at the late stage of HIV-1 lifecycle. One explanation for the discrepancy that we observed regarding HIV-1 production in the context of stable *versus* transient knockdown could result in the ability of the cells to adapt and compensate for the loss of DDX17. For example, it is known that DDX17 and its paralog DDX5 play redundant roles in cellular metabolism [405]. Interestingly, DDX5 was identified as a potential modulator of HIV-1 integration [319] and

described to promote the nuclear export of HIV-1 mRNA [328]. The role of DDX17 in HIV-1 replication is further investigated in Chapter 4.

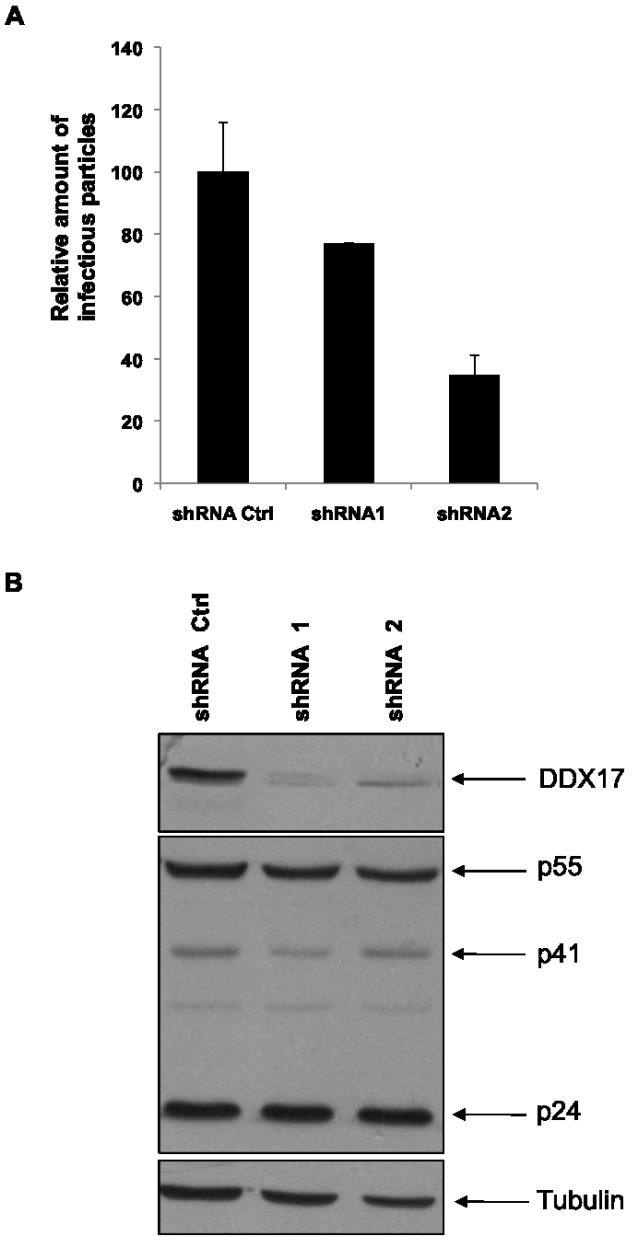

Figure 30 | Effect of DDX17 knockdown on HIV-1 production
SupT1 cells (1.5 x 10^6) were infected with 0.5mL DDX17-shRNA-containing lentiviral particles. 48h post shRNA transduction, SupT1 cells were challenged with VSV-G pseudotyped BH10. Supernatant was harvested 48h post BH10 infection. The production of infectious viruses was assessed by measuring luciferase activity using 50μL supernatant to infect HeLa TZM-bl reporter cells (**A**). Protein expression levels in cell lysate were analyzed by Western blot (**B**).

3.4 Conclusion

In this study, we present the effect of the stable shRNA-mediated knockdown of 130 helicases in SupT1 cells on HIV-1 production (Figure 26). Our study design allowed for the selection of candidates based on their effect on HIV-1 production in stable cell lines. Interestingly, DDX17 is a particular helicase which knockdown did not trigger the same outcome whether it was studied in the context of stable or transient knockdown. Not only this raises the importance of the capacity for the cells to adapt in the context of a stress induced by the loss of a protein, but also the possibility to obtain false negatives. Indeed, the loss of a helicase which function is compensated by the overexpression of another cellular protein would not appear as a candidate in our study. Beside this limitation, we identified 35 candidates that potentially modulate HIV-1 lifecycle, grouped into eight main cellular pathways (Figure 27). We observed that two pathways are more highly represented, with 9 helicases belonging to the DNA repair machinery and 12 involved in the regulation of gene expression. Interestingly, this correlates with the importance and the complexity of the mechanisms that are involved in HIV-1 integration and gene expression, highlighting the crucial role of helicases of these RNA processing pathways in the context of HIV-1 infection. Moreover, the multifunctional properties of helicases allow some members to be part of distinct RNA processing machineries (Table 4), therefore offering the possibility for one helicase to modulate more than one step of HIV-1 replication, making the identification of the viral step affected by the helicase more difficult. The role of

RHA in HIV-1 reverse transcription, transcription and translation is such an example of the polyvalent aspect of the helicase family proteins [196]. An increasing number of helicases has been reported to affect HIV-1 replication. Surprisingly, in the context of HIV-1 infection, the role of many of them differs from the function they normally have in the cell. For example, the functions of DDX17 in RNA degradation and WRN in transcription have been described in the context of HIV-1 but are not shared by the cell. Furthermore, specific viral requirements involve helicases, such as DDX24 and DHX30 in HIV-1 RNA packaging [291, 292]. Altogether, this emphasizes the fact that the virus is both able to recruit cellular proteins to support viral function and subvert cellular defense mechanisms. This study confirms the key roles of helicases and provides a list of potential key players in HIV-1 replication.

Authors' contribution

RL wrote the manuscript and made the figures. QP made the first round of stable cell lines for 30 helicases. RL made the stable cell lines for 100 helicases and performed the confirmation (second round of knockdown), transient knockdown, statistical analysis and individual candidate investigation.

Chapter 4 –DDX17 promotes the production of infectious HIV-1 particles by modulating viral RNA packaging and frameshift (Research Article)

RNA helicases are a large family of proteins that rearrange RNA structures and remodel ribonucleic protein complexes using energy derived from hydrolysis of nucleotide triphosphates. They have been shown to participate in every step of RNA metabolism. In the past decade, an increasing number of helicases were shown to promote or inhibit the replication of different viruses, including human immunodeficiency virus type 1. Among these helicases, the DEAD-box RNA helicase DDX17 was recently reported to modulate HIV-1 RNA stability and export. In this study, we further show that the helicase activity of DDX17 is required for the production of infectious HIV-1 particles. Overexpression of the DDX17 mutant DQAD in HEK293 cells reduces the amount of packaged viral genomic RNA and diminishes HIV-1 Gag-Pol frameshift. Altogether, these data demonstrate that DDX17 promotes the production of HIV-1 infectious particles by modulating HIV-1 RNA metabolism.

4.1 Introduction

RNA helicases are a large family of proteins that are involved in virtually all the steps of RNA metabolism [329, 330]. They utilize the energy derived from nucleotide triphosphates (NTPs) hydrolysis to unwind nucleic acids and remodel ribonucleic protein (RNP) complexes [331]. RNA helicases are classified into five superfamilies (SF1 to SF5) on the basis of their conserved motifs. They are prevalent in eukaryotes, prokaryotes and viruses [309, 406]. The SF2 has the most members, and includes the DEAD-box helicases that harbor the Asp-Glu-Ala-Asp signature motif [407]. In humans, malfunction of helicases causes various types of cancers [408-410].

Viruses need helicases to successfully replicate their nucleic acid genome. Some viruses carry their own helicase, such as Hepatitis C virus that encodes the NS3 protein [411, 412], while others need to hijack cellular ones to assist viral replication. Human immunodeficiency virus type 1 (HIV-1), like all retroviruses, does not encode a viral helicase. Over the past decade, many cellular RNA helicases have been reported to promote or inhibit HIV-1 replication at distinct steps. These include RNA helicase A (RHA), Moloney leukemia virus 10 (MOV10), XPB/XPD, Ku70/Ku80, Werner syndrome (WRN) helicase, DDX1, DDX3, DDX17, DDX24, Upf1 and DHX30 (reviewed in [196, 200, 413, 414]). RHA promotes HIV-1 reverse transcription, transcription and translation [39, 215, 415, 416]. MOV10 inhibits viral reverse transcription [417, 418]. DDX1, DDX3

and DDX17 promote Rev-dependent HIV-1 RNA export [199, 328, 419]. In addition to its role in HIV-1 RNA export, DDX3 also enhances HIV-1 translation [226, 420-423]. Upf1 and DDX17 are involved in HIV-1 RNA degradation [273, 359]. DDX24 promotes HIV-1 RNA packaging [291], while DHX30 was reported to inhibit this step [292]. Recently, SLFN11 was shown to inhibit HIV-1 translation in a codon-usage-dependent manner [268], adding a new layer of viral RNA regulation by cellular helicases.

DDX17, also known as RH70, has two isoforms, p72 and p82, as a result of alternative translation of its messenger RNA (mRNA) [424]. DDX17 can form heterodimers with its paralog DDX5 [425] and is involved in multiple aspects of RNA metabolism. Through interaction with other cellular factors, including HDAC1 [362], estrogen receptor alpha [426] and U1snRNP [427], DDX17 regulates gene transcription and alternative splicing. Its dysfunction can lead to cancer development [428-430]. Recently, DDX17 was reported to act as a co-factor of the zinc finger antiviral protein ZAP [383], which is involved in the degradation of the multiply spliced HIV-1 mRNA [359]. Another study by Naji *et al.* showed that DDX17 associates with HIV-1 Rev protein, and promotes the nuclear export of HIV-1 transcripts [328]. We now further show that DDX17 affects the production of infectious HIV-1 particles. Knockdown and overexpression of DDX17 change the ratios of unspliced *vs* spliced HIV-1 RNA. Furthermore, overexpression of the DDX17 DQAD mutant leads to a dramatic decrease in the amount of packaged viral RNA. Moreover, DDX17 is required for efficient Gag processing through its effect on HIV-1 Gag-Pol frameshift.

Altogether, our results demonstrate new roles for DDX17 in promoting the production of infectious HIV-1 particles through regulating HIV-1 Gag-Pol frameshift and genomic RNA packaging.

4.2 Materials and Methods

4.2.1 Plasmid DNA, Viruses and Antibodies

The cDNA clone of DDX17 gene was purchased from ATCC (MGC-2030). Short and long isoforms of DDX17 cDNA were amplified by PCR and a Flag tag was added at the N terminus using primer pairs DDX17-SS/DDX17-A, DDX17-SL/DDX17-A, respectively (Table 7). The PCR products were digested with BamHI and MulI and inserted into the pRetroX-Tight-Pur retroviral vector (Clontech) to create DNA plasmids Tet-DDX17S and Tet-DDX17L that are expressed in response to doxycyclin. DDX17 mutants were generated by site-directed mutagenesis using primers DQAD-S and DQAD-A (Table 7). The infectious HIV-1 proviral DNA clone NL4-3 was obtained from the NIH AIDS Research and Reference Reagent Program. Tat plasmid was kindly provided by Dr. Gatignol [431]. The frameshift luciferase reporter constructs FS (0) and FS (-1) were previously described [432] and provided by Dr. Brakier-Gingras. HIV-1 viruses were generated by transfecting HEK293T cells with the proviral DNA

clone NL4-3, using lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. When indicated, the vesicular stomatitis virus (VSV) glycoprotein (G) was used to pseudotype HIV-1 particles.

Anti-Flag and anti-tubulin antibodies were purchased from Sigma; anti-HIV-1 p24 antibody from ID Lab Inc.; anti-DDX5 and anti-DDX17 antibodies were purchased from Abnova (H00001655-B01) and Novus Biological (NB200-352), respectively.

4.2.2 Cell Culture and Transfections

TZM-bl, HeLa and HEK293 cells were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Plasmids and short interfering RNA (siRNA) were transfected into cells using lipofectamine2000 according to the manufacturer's protocol.

4.2.3 siRNA Knockdown of DDX5 and DDX17 in HeLa Cells

siRNA oligonucleotides targeting DDX17 (nt 1694-1713) were purchased from Qiagen (5'-AAT ACA CCT ATG GTC AAG GCA-3') (Cat. no. 1027423). siRNAs oligonucleotides targeting DDX5 and the DDX5-DDX17 subfamily were previously described [433]. Control siRNA was purchased from Qiagen (Cat. No. 1027281). shRNA targeting DDX17 (nt 1525-1545) was purchased from SIGMA (5'-CCC AAT CTG ATG TAT CAG GAT-3'). Control shRNA was purchased form SIGMA (Cat. No. SHC016). HeLa cells ($2x10^5$ per well) were seeded in 6-well plates 1 day before siRNA transfection. DDX5 and DDX17 were either knocked down individually, or simultaneously. 10nM of each siRNA was used in transfection. After two sequential siRNA transfections, the cells were transfected with HIV-1 NL4-3 DNA. Cells and supernatants were harvested 40h post HIV-1 NL4-3 transfection. Protein levels were analyzed by Western blotting, virus production was assessed by infecting the TZM-bl indicator cells.

4.2.4 DDX17 Overexpression in HEK293 Cells

HEK293 cells ($5x10^5$ per well) were seeded in 6-well plates 1 day before transfection. 500ng of DDX17 DNA constructs were co-transfected with 100ng Tet-ON plasmid and 100ng HIV-1 NL4-3. Cells were washed in 1x phosphate

buffered saline the next day and fresh DMEM containing 100ng/mL doxycyclin was added to induce DDX17 expression. Cells and supernatants were harvested 40h post HIV-1 NL4-3 transfection. Protein levels were analyzed by Western blotting and virus production was measured by infecting TZM-bl indicator cells.

4.2.5 Viral RNA analysis

Levels of viral RNA in cells were assessed by Northern blots as previously described [39, 292] (and RT-PCR followed by Southern blot, described below). Briefly, transfected cells were washed with 1x phosphate buffered saline and lysed in Trizol (Invitrogen). RNA was extracted according to the manufacturer's instructions. Northern blot analysis was performed as previously described by Zhou *et al.* [292]. Briefly, RNA was subjected to electrophoresis on a 1% denaturing agarose gel. RNA was transferred to nylon membranes (GE Healthcare UK Limited) and incubated with labeled HIV-1 DNA. Membranes were washed with 1X saline-sodium citrate (SSC) buffer containing 0.1% SDS and viral RNA signals were visualized by exposure to X-ray films.

Levels of virion-associated viral RNA and total viral RNA were determined by RT-PCR followed by Southern blots. RNA was first extracted from viruses using Trizol-LS (Invitrogen), followed by DNase I (Amp Grade) digestion at room temperature for 15 min to remove any contaminating plasmid DNA. DNase I (Amp Grade) was inactivated at 65 °C for 10 min. The effectiveness of DNase I

(Amp Grade) treatment was tested by amplifying the treated RNA samples by PCR with the primers FP and FL (Table 7). This primer pair amplifies HIV-1 cDNA sequence spanning nucleotides 665 to 1729. Reverse primers RP1 and RP2 were used for the amplification of singly spliced and multiply spliced HIV-1 transcripts, respectively, and were previously described [359]. RNA equivalent to 25ng of p24 was reverse transcribed and amplified with the primers FP and FL or RP1 or RP2, using the Titan One Tube RT-PCR System (Roche) according to the manufacturer's instruction and 20 PCR cycles (Reverse transcription at 50°C for 1h, followed by reverse transcriptase inactivation at 94°C for 5min; PCR: denaturation at 95°C for 30s, annealing at 45°C for 30s and elongation at 68°C for 2min). RT-PCR products were subjected to electrophoresis on a 1% agarose gel (0.5X Tris Borate EDTA) for 2h at 60V. The gel was treated in 0.2M HCl for 13 minutes, NaOH 0.5M for 30 minutes, NaCl 0.5M (pH 7.5) for 30 minutes and double-distilled H_2O for 10 minutes. The DNA was transferred to a nylon Hybond-N membrane (GE Healthcare UK Limited) with 10X SSC overnight. The next day, the membrane was washed in 6X SSC for 5 minutes at room temperature and RNA was fixed by UV cross-linking. Next, the membrane was incubated with 10mL pre-hybridization buffer (Ambion) at 42°C for 2h followed by overnight hybridization at 42°C with a [α-^{32}P] labeled HIV-1 probe. The next day, the membrane was washed with 1X SSC, 0.1% SDS at room temperature for 15 minutes. Signals were quantified using a phosphoscreen and the Phosphoimager (Amersham).

4.2.6 Virus production assays

The amount of infectious viruses in culture supernatants was determined by infecting the TZM-bl cells. Cells were seeded in 24-well plate at a density of $4x10^4$ cells per well 1 day before infection. 50μL of supernatant from transfected cells were used to infect TZM-bl cells. Forty-eight hours after infection, cells were lysed in 1X passive lysis buffer (Promega), and levels of firefly luciferase activity in the cell lysates were measured using the Luciferase Assay kit and the Glomax luminometer (Promega). Virus production was determined by measuring viral reverse transcriptase (RT) activity in the supernatants [434].

Table 7 | List of primers

Primer	Sequence
DDX17-SS	5'-GCACGGATCCATGGATTACAAGGATGACGACGATAAGCGCGGAGGAGGCTTTGGGGAC-3'
DDX17-SL	5'-GCACGGATCCATGGATTACAAGGATGACGACGATAAGCCCACCGGCTTTGTAGCCCCG-3'
DDX17-A	5'-GCAGACGCGTCATTTACGTGAAGGAGGAGG-3'
DQAD-S	5'-CTTGTATTGGACCAAGCTGACAGAATGC-3'
DQAD-L	5'-GCATTCTGTCAGCTTGGTCCAATACAAG-3'
FP	5′-AGTAAAGCCAGAGGAGATCTCTCG-3′
FL	5′-TACCTCTTGTGAAGCTTGCTCGGCTCT-3′
RP1	5'-ATTGGTATTAGTATCAATCTTCAAATC-3'
RP2	5'-GCCACCCATCTTATAGCAAAATCC-3'

4.3 Results

4.3.1 Knockdown of DDX17 but not DDX5 Reduces HIV-1 Production and Infectivity

DDX17 was recently identified as a co-factor of ZAP that causes the degradation of HIV-1 multiple spliced mRNAs [383]. DDX17 was also reported to associate with Rev, and promote the export of incompletely spliced HIV-1 RNAs from the nucleus to the cytoplasm [328]. To determine the effect of DDX17 on HIV-1 production, we silenced DDX17 in HeLa cells using siRNAs, followed by transfection of HIV-1 NL4-3 DNA (Figure 31A). We observed a 2-fold decrease in the amount of viral particles upon DDX17 knockdown (Figure 31B), which correlates with a diminution of the viral p24 levels in the supernatant (Figure 31C). Furthermore, quantification of infectious viruses by infecting TZM-bl indicator cells revealed a 4-fold decrease (Figure 31D). Therefore, the infectivity of viral particles is decreased by 2-fold. Together with the work from other studies, our data suggest that DDX17 affects the amount and the quality of HIV-1 particles made. When DDX5, a paralog of DDX17, was silenced, we observed a slight increase in the amount of HIV-1 virions and no change in the infectivity of HIV-1 particles (Figure 31D). Interestingly, silencing DDX5 moderately increased DDX17 expression (including both the p72 and p82 isoforms) (Figure 31A). This suggests that cells compensate for the loss of DDX5 by increasing DDX17 expression, as reported by others [428]. Along this line,

overexpression of DDX5 or DDX17 also moderately diminished the level of DDX17 or DDX5 (Figure S1), which further indicates the mutual effects on their expressions. Knockdown of both DDX5 and DDX17 severely diminished cell viability, thus was not tested on HIV-1 production (data not shown). In order to confirm the observed inhibitory effect of DDX17 knockdown on HIV-1 production, we utilized an shRNA to deplete endogenous DDX17 in HeLa cells. A profound decrease in the generation of infectious HIV-1 particles was again observed (Figure 31E). Collectively, we conclude that DDX17 is required for HIV-1 production.

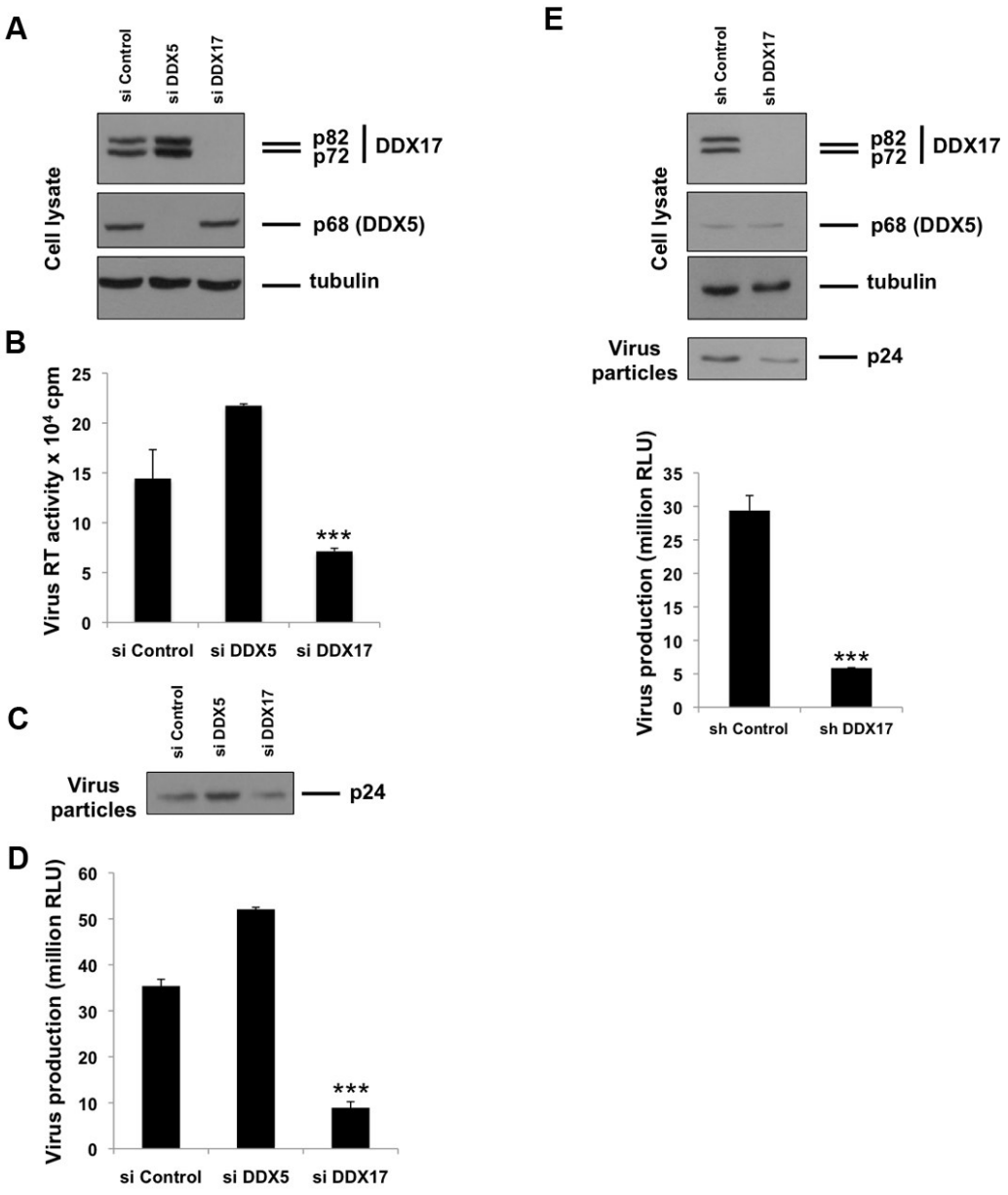

Figure 31 | Effect of DDX5 and DDX17 knockdown on HIV-1 production
(**A**) HeLa cells ($1.5x10^5$) were transfected with 0.1μg HIV-1 NL4-3 DNA and 20pmol/ml siRNAs directed against DDX17 or DDX5. Levels of endogenous DDX5 and DDX17 were determined by Western blotting. (**B**) Culture supernatants were harvested 48h post transfection and the amounts of virus particles were determined by measuring viral reverse transcriptase activity. (**C**) Levels of viral particles in the culture supernatants were analyzed by Western blotting with anti-HIV-1 p24 antibody. (**D**) Levels of infectious HIV-1 particles in the supernatants were determined by infecting the TZM-bl indicator cells. (**E**) HeLa cells ($1.5x10^5$) were transfected with 0.1μg HIV-1 NL4-3 DNA and 1.0μg shRNA directed against DDX17. Protein expression levels in cell lysate were analyzed by Western blot; culture supernatants were harvested 48h post

transfection and the levels of released viral particles were analyzed by the Western blotting 50μL supernatant with anti-p24 antibody the amounts of virus particles were determined by measuring viral reverse transcriptase activity; levels of infectious particles were determined by measuring luciferase activity *** represents a P-value < 0.001, n = 6. The P value was calculated with reference to the control siRNA data. p82, DDX17 L (long) isoform; p72, DDX17 S (short) isoform; p68, DDX5.

4.3.2 HIV-1 Production is Inhibited by DDX17 Mutant Carrying the Mutated DEAD box Motif

As a typical RNA helicase, DDX17 carries seven conserved motifs including the DEAD box that is critical for helicase activity (Figure 32A). To determine whether the helicase activity of DDX17 is required for its effect on HIV-1 production, we mutated the DEAD motif to DQAD in the context of both the long (p82) and short (p72) forms of DDX17 (Figure 32A). We then transfected HEK293 cells with various DDX17 constructs and the HIV-1 proviral DNA NL4-3 (Figure 32B), and analyzed viral particles released in the supernatant. DDX17 DQAD (L) mutant expression led to a 3.5-fold decrease in the amount of viral particles as being determined by measuring viral reverse transcriptase activity in the supernatants (Figure 32C). We further determined the levels of infectious HIV-1 particles by infecting the TZM-bl indicator cells, and observed that the DQAD (L) mutant caused a 10-fold reduction (Figure 32E). Considering the 3.5-fold decrease in the viral RT level (Figure 32C) and the diminution in p24 found in the supernatant (Figure 32D), we conclude that DQAD

(L) decreases the infectivity of HIV-1 particles by 3-fold. For the DQAD (S) mutant, we observed a 50% decrease in the viral RT (Figure 32C) and a 70% decrease in virus production (Figure 32E). We also ectopically expressed DDX17 and its DQAD mutants in HeLa cells and observed a more than 5-fold reduction in the production of infectious HIV-1 particles as a result of DQAD expression (Figure S2). Taken together, these results suggest that the DQAD mutant of DDX17 impairs the production of infectious HIV-1 particles. Considering the existence of endogenous DDX17 when DQAD mutant is ectopically expressed, we conclude that this mutant acts in a dominant negative fashion to prevent the endogenous DDX17 from promoting HIV-1 production.

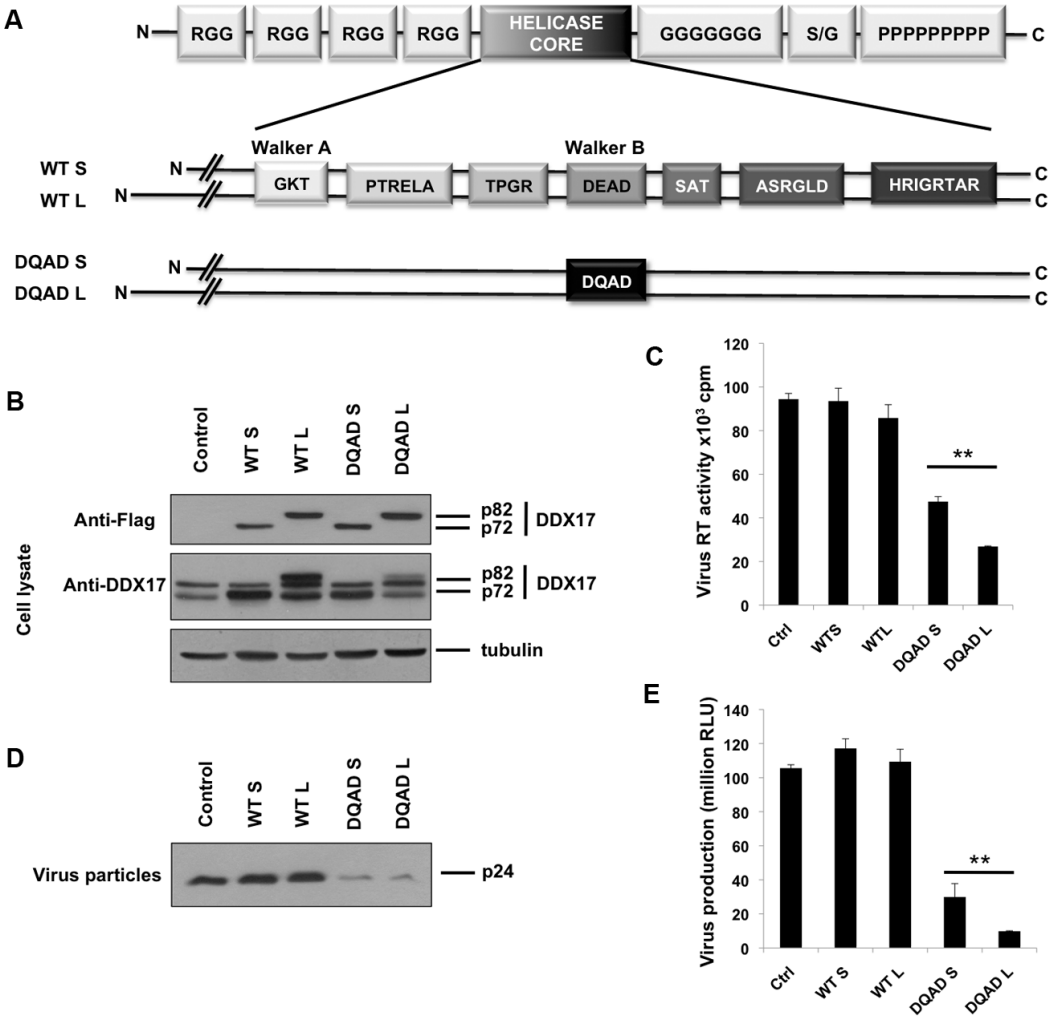

Figure 32 | Effect of DDX17 overexpression on HIV-1 production

(**A**) Illustration of DDX17 DNA constructs. Conserved helicase Walker B motif, or DEAD-box, was mutated into DQAD and inserted into both short and long isoforms of DDX17, DQAD (S) and DQAD (L). HEK293 cells ($5x10^5$) were transfected with 0.1μg HIV-1 NL4-3 DNA and 0.5μg DDX17. (**B**) Levels of DDX17 and its mutants in cell lysate were analyzed by Western blot using either anti-Flag or anti-DDX17 antibodies. (**C**) Levels of viral reverse transcriptase activity in the culture supernatants. (**D**) The amounts of HIV-1 particles in the supernatants were determined by Western blotting using anti-HIV-1 p24 antibody. (**E**) Levels of infectious HIV-1 particles were determined by infecting the TZM-bl indicator cells. Levels of luciferase activity reflect the relative infectiousness of the virus samples tested. ** represents a P-value < 0.01, n = 3. p82, DDX17 L isoform; p72, DDX17 S isoform; Ctrl, control.

4.3.3 DDX17 Expression increases HIV-1 genomic RNA packaging

To understand how the DDX17 DQAD mutant reduces the production of infectious HIV-1 particles, we first measured the levels of viral RNA associated with HIV-1 particles. We thus transfected HEK293 cells with the DDX17 and HIV-1 NL4-3 DNA clones. Viral particles from culture supernatants were harvested 2 days post transfection and amounts of particles were determined by ELISA against HIV-1 p24. Viral RNA was amplified by RT-PCR and further quantified in Southern blotting (Figure 33). We observed that the short isoform of wild-type DDX17 overexpression led to a 40% increase in the amount of RNA that is packaged into HIV-1 particles. In contrast, the DDX17 DQAD mutants resulted in a dramatic 5- to 10-fold decrease in the amount of packaged HIV-1 genomic RNA (gRNA). The stronger inhibition of HIV-1 RNA packaging by the longer DQAD mutant may result from its higher expression level than the shorter DQAD mutant. Alternatively, the extra 79 N-terminal amino acids in the DDX17 long isoform (p82), which are absent in DDX17 short forms (p72) may be involved in preventing HIV-1 RNA packaging. These results suggest that the DQAD mutant impedes HIV-1 RNA packaging and thus reduces virus infectivity.

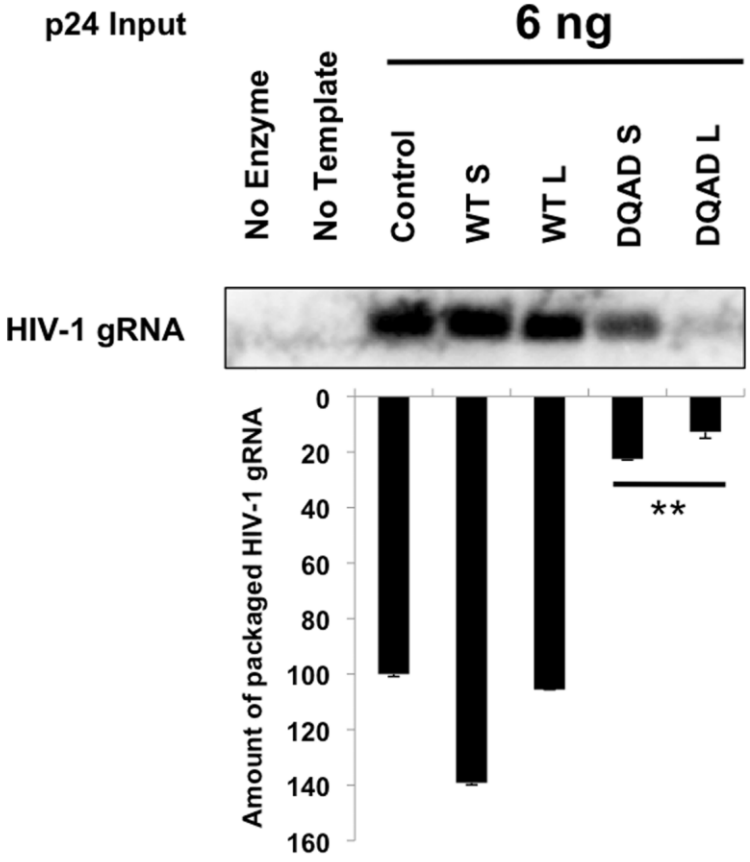

Figure 33 | Effect of DDX17 on HIV-1 gRNA packaging
HEK293 cells ($5x10^5$) were transfected in 6-well plates with 0.5μg DDX17 DNA and 0.1μg HIV-1 NL4-3. Culture supernatants were harvested 48h post transfection and the amounts of particles were quantified by ELISA against HIV-1 p24. Virus particles corresponding to 55ng of p24 were spun at 35,000 rpm for 1h and RNA equivalent to 10ng of p24 were used for RT-PCR assay. RT-PCR products corresponding to 6ng of p24 were analyzed by Southern blot, and radioactive signal intensity was quantified by Phosphoimager (Amersham). Results of one of the three independent transfection experiments are shown. ** represents a P-value < 0.01.

4.3.4 The DDX17 DQAD Mutant Disturbs the Balance of the Unspliced *vs* Spliced HIV-1 RNA Pools

A decrease in the amount of packaged HIV-1 RNA can be the result of multiple causes, including defects in viral RNA expression in the cell. We first tested whether DDX17 affects HIV-1 Long Terminal Repeat (LTR) promoter activity by transfecting DDX17 DNA and Tat into the TZM-bl indicator cells that contain the HIV-1 LTR-luciferase reporter (Figure 34A). We observed no detectable effect of DDX17 on Tat-dependent HIV-1 transcription. Transfection of 40ng HIV-1 Tat showed a further 10-fold increase in luciferase activity, attesting that the assay was not saturated (data not shown).

Recently, DDX17 was reported to act as a cofactor of ZAP and contribute to the degradation of multiply spliced HIV-1 RNA [359]. In addition, no noticeable effect was seen on the levels of full length viral RNA upon silencing of ZAP [359]. In another recent study, Naji *et al.* reported that DDX17 silencing led to a decrease in the levels of both multiply spliced and unspliced HIV-1 RNAs [328]. Naji *et al.* also reported that DDX17 associates with the viral protein Rev to promote the export of RRE-containing viral RNAs [328]. To further determine the effect of DDX17 on HIV-1 RNA expression, we transfected HEK293 cells with DDX17 plasmids and HIV-1 NL4-3, and examined HIV-1 RNA levels by Northern blot (Figure 34C). We found that overexpression of wild-type DDX17

led to a slight increase in HIV-1 full length RNA while overexpression of DDX17 DQAD mutants induced an increase in HIV-1 multiply spliced mRNA species and a decrease in HIV-1 full length RNA (Figure 34C). To confirm this observation, we silenced DDX17 and DDX5 in HeLa cells and analyzed HIV-1 RNA by Northern blot (Figure 34B). We observed that silencing DDX17 led to an increase in HIV-1 spliced transcripts and a decrease in the viral full length RNA (Figure 34B). Interestingly, DDX5 knockdown displayed an opposite effect, as we observed an increase in HIV-1 full length RNA and a decrease in HIV-1 RNA spliced forms (Figure 34B). Consistent with the effect of DDX17 knockdown on viral particles shown in Figure 32C, this pattern can be explained by the fact that the cells compensate for the loss of DDX5 by upregulating the expression of DDX17.

To distinguish the different spliced mRNA species and to quantify their relative amounts, we analyzed viral transcripts by RT-PCR followed by Southern blotting as described in [359] (Figure 34D-F). We observed that overexpression of DDX17 DQAD (L) led to a 3.5-fold increase in the amount of multiply spliced RNAs, and up to a 50% decrease in the full length RNA (Figure 34D and 34E), which results in 6.5-fold increase for the multiply spliced/full length HIV-1 RNA ratio (Figure 34F). Overexpression of wild-type DDX17 had no significant effect on the relative amounts of different HIV-1 RNA species. Supporting the work from other groups [328, 359, 383], our results suggest that DDX17 affects the balance of spliced and unspliced HIV-1 RNA populations.

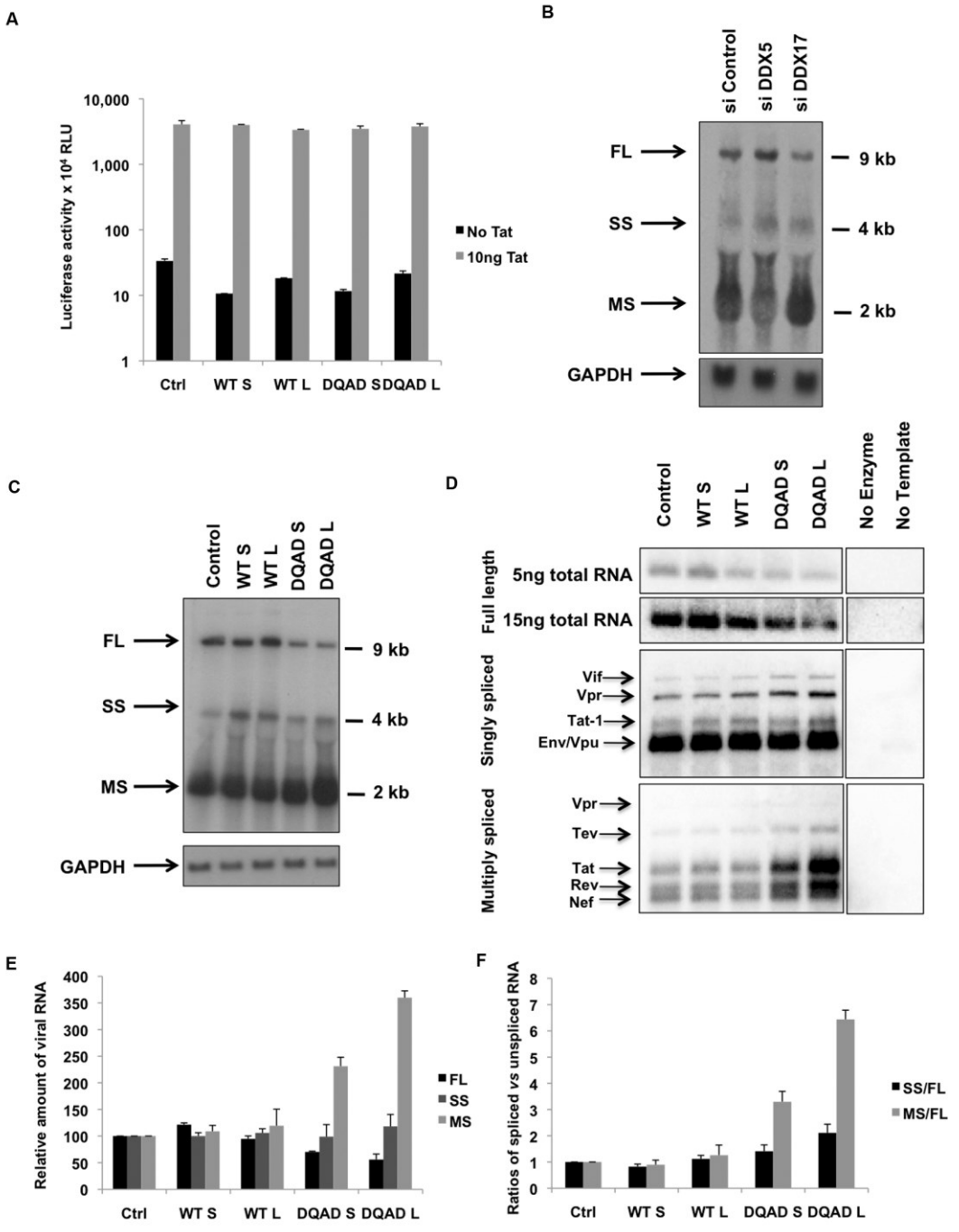

Figure 34 | Effect of DDX17 on HIV-1 RNA expression

(**A**) HeLa TZM-bl cells ($4x10^4$) were transfected in 24-well plates with 0.1µg DDX17 DNA and either 10ng of HIV-1 Tat or 10ng cDNA3.1. Luciferase activity was measured 24h post transfection (n = 4). (**B**) HeLa cells ($1.5x10^5$) were transfected with 0.1µg HIV-1 NL4-3 DNA and 20pmol/ml siRNAs against DDX17 or DDX5. Cells were lysed in Trizol (Invitrogen) 48h post transfection

and HIV-1 RNA profile was analyzed by Northern blot. (**C**) HEK293 cells ($5x10^5$) were transfected with 0.1μg HIV-1 NL4-3 DNA and 0.5μg DDX17. Cells were lysed in Trizol (Invitrogen) 48h post transfection and HIV-1 RNA profile was analyzed by Northern blot. (**D**) HIV-1 RNA samples from the co-transfected HEK293 cells were first amplified by RT-PCR using different primer pairs, then subject to Southern blot analysis. (**E**, **F**) Radioactive signals from the distinct viral RNA species were quantified from the Southern blot using phosphorescreen and Phosphoimager (Amersham) and splicing ratios were calculated. FL, full length; SS, singly spliced; MS, multiply spliced.

4.3.5 DDX17 Modulates HIV-1 Gag Processing

Expression of DDX17 DQAD mutant in HEK293 cells decreases the amount of viral p24 in the supernatant (Figure 31D). This defect may result from diminished HIV-1 protein expression in cells. To test this, we transfected HEK293 cells with DDX17 constructs and HIV-1 NL4-3 DNA and analyzed viral protein expression by Western blot (Figure 35A). We observed that expression of DDX17 DQAD decreased the amount of viral p24. However, the level of $Pr55^{Gag}$ was not affected, suggesting that the DDX17 mutant likely inhibits Gag processing rather than impeding Gag production (Figure 35A). Similarly, DDX17 knockdown in HeLa cells reduced the amount of viral p24 but not $Pr55^{Gag}$, suggesting the same defect in Gag cleavage (Figure 35B).

HIV-1 genome encodes two polyproteins $Pr160^{Gag\text{-}Pol}$ and $gp160^{Env}$. While $gp160^{Env}$ precursor is cleaved in gp120 and gp41 by the cellular protease Furin [23], the Gag-Pol polyprotein is self-cleaved by the viral protease (PR) [435]. HIV-1 *Pol* gene encodes the three viral enzymes, including protease (PR), reverse

transcriptase (RT) and integrase (IN) among which PR is responsible for the processing of $Pr160^{Gag\text{-}Pol}$ and $Pr55^{Gag}$ precursors. In order to assess the effect of DDX17 on total Gag expression, we transfected HEK293 cells with DDX17 DNA and treated the cells with 100nM duranavir, an HIV-1 PR inhibitor, and assessed the levels of $Pr160^{Gag\text{-}Pol}$ and $Pr55^{Gag}$ in the cell lysate (Figure 35C). For wild-type DDX17, DRV treatment led to the accumulation of $Pr160^{Gag\text{-}Pol}$, as a result of HIV-1 PR-dependent cleavage inhibition. No overall changes were observed for total Gag levels upon wild-type DDX17 expression (Figure 35C). Interestingly, overexpression of DDX17 mutants led to a slight decrease in the amount of $Pr160^{Gag\text{-}Pol}$ but no significant effect on $Pr55^{Gag}$ expression (Figure 35C). In addition, as seen in Figure 35A, p24 levels were dramatically diminished upon the expression of both DQAD (S) and DQAD (L) mutants. Our results suggest that the DDX17 DQAD mutant compromises Gag processing, and the decrease in the overall amount of Gag-Pol products suggests a possible defect in HIV-1 frameshift activity.

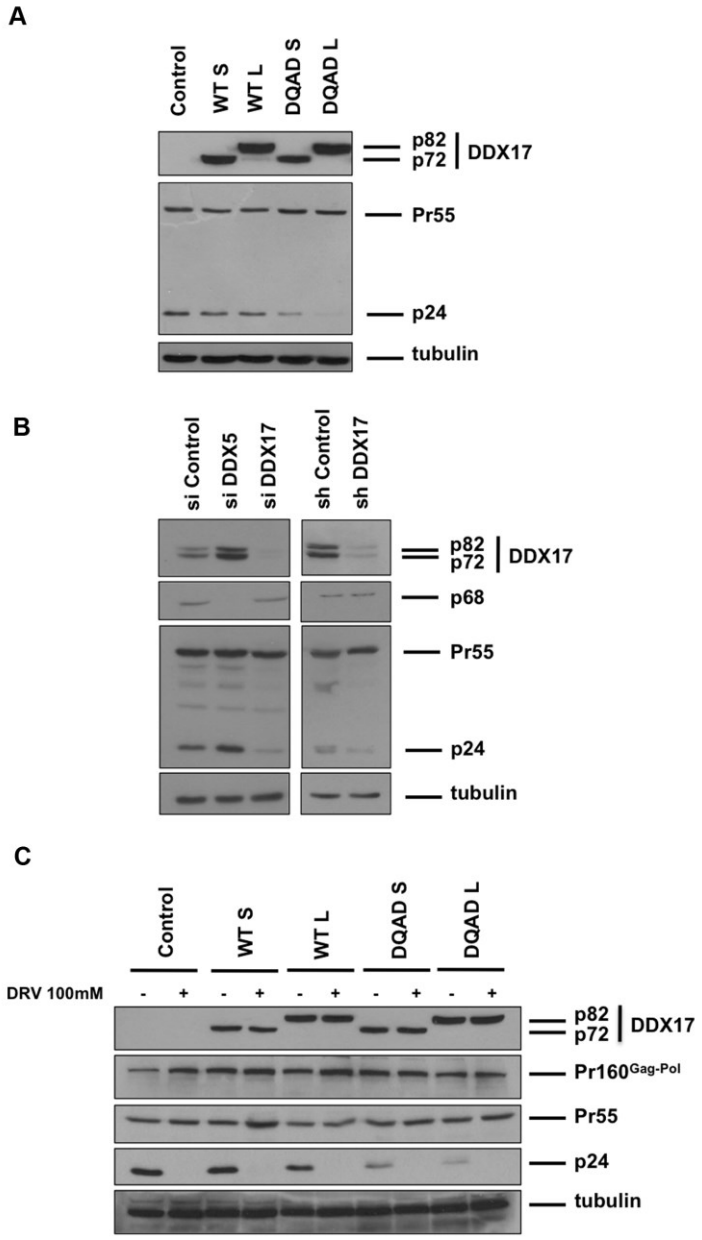

Figure 35 | DDX17 modulates HIV-1 Gag processing

(**A**) HEK293 cells ($5x10^5$) were transfected with 0.1µg HIV-1 NL4-3 DNA and 0.5µg DDX17. Protein expression levels in cell lysate were analyzed by Western blot using anti-p24, anti-FLAG and anti-tubulin antibodies. (**B**) HeLa cells ($1.5x10^5$) were transfected with 0.1µg HIV-1 NL4-3 DNA and 20pmol/ml siRNAs directed against DDX17 or DDX5. Protein expression levels in cell lysate were analyzed by Western blot using anti-p24, anti-DDX5, anti-DDX17 and anti-tubulin antibodies. **(C**) HEK293 cells ($5x10^5$) were transfected with 0.1µg HIV-1 NL4-3 clone and 0.5µg DDX17 DNA and treated with 100nM protease inhibitor duranavir (DRV). Cells were lysed 48h post transfection and protein levels were analyzed by Western blot using anti-p24 and anti-tubulin antibodies.

4.3.6 Effect of DDX17 on Gag-Pol Frameshift

To regulate the ratio of Gag and Gag-Pol expression, HIV-1 has evolved a slippery RNA sequence that causes -1 frameshift, which allows producing HIV-1 enzymes [81, 82]. Therefore, a decrease in HIV-1 frameshift efficiency implies a defect in the production of viral enzymes, including HIV-1 PR that is responsible for Gag cleavage, thus reduces virus particle infectivity. To test this hypothesis, we used two dual-luciferase reporter plasmids FS (0) and FS (-1) previously described by Grentzmann to measure HIV-1 Gag-Pol frameshift [432] (Figure 36A). We transfected HEK293 cells with DDX17 DNA with either FS (0) or FS (-1) reporter and quantified luciferase expression levels. We observed that overexpression of DDX17 DQAD mutant led to up to 40% decrease in frameshift efficiency (Figure 36B). No significant effect was observed for the wild type DDX17. These results suggest that the helicase activity of DDX17 is involved in maintaining the proper ratio of *Gag/Pol* gene expression required for optimal infectivity.

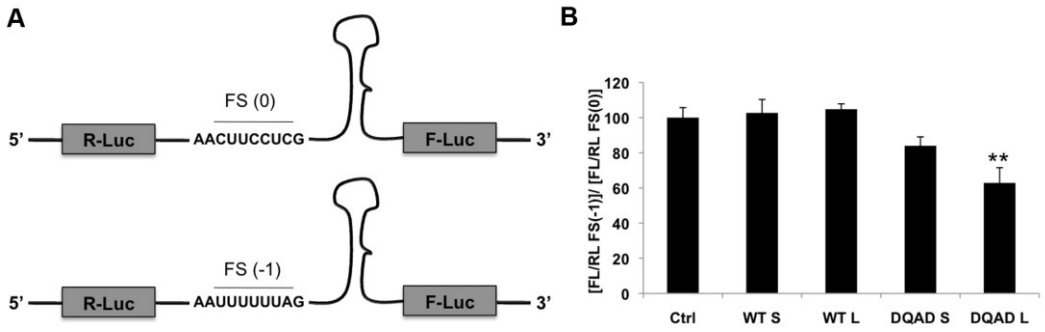

Figure 36 | Effect of DDX17 on Gag-Pol frameshift
(**A**) Depiction of the HIV-1 frameshift dual-luciferase reporter constructs FS (0) and FS (-1), described in [432]. (**B**) HEK293 cells ($5x10^5$) were transfected with 0.5μg DDX17 DNA and 75ng of either dual luciferase reporter FS(0) or FS (-1). FL/RL ratios were calculated and the FL/RL FS(-1) / FL/RL FS(0) was plotted to indicate frameshift efficiency. ** represents a P-value < 0.01, n = 3. (**C**) Levels of the ectopically expressed DDX17 and its DQAD mutants as determined by Western blotting.

4.4 Discussion

In this study, we found that DDX17 promotes HIV-1 particle infectivity by modulating HIV-1 RNA packaging and Gag-Pol frameshift. Viral RNA analysis also demonstrates that the overexpression of the helicase negative DDX17 DQAD mutant increases the multiply spliced/unspliced HIV-1 RNA ratio by up to 6-fold (Figure 34F). Recently, Chen *et al.* reported that the zinc finger antiviral protein ZAP interacts with DDX17 [383] that acts as a cofactor to potentiate the activity of ZAP in inhibiting the replication of a few viruses, including murine leukemia virus. The same group also showed that ZAP targets HIV-1 multiply spliced mRNA to exosomes for degradation [359]. The crystal structure of the N-terminal

domain of ZAP reveals the RNA binding region that is likely involved in HIV-1 RNA recognition [436]. In addition to its role in ZAP-mediated degradation of multiply spliced HIV-1 mRNA, DDX17 also has ZAP-independent functions in HIV-1 replication. Naji *et al.* found that DDX17 interacts with HIV-1 Rev and that DDX17 silencing reduced the amount of viral transcripts and inhibited the export of both unspliced and spliced HIV-1 mRNAs to the cytoplasm [328].

In our study, we also found that DDX17 silencing led to a decrease in full length HIV-1 RNA (Figure 34C). Interestingly, this decrease in full length RNA correlates with an increase in multiply spliced viral transcripts (Figure 34). We propose that several mechanisms may account for the accumulation of multiply spliced HIV-1 RNA upon DDX17 silencing or DDX17 DQAD overexpression. The first mechanism is simply the inefficient ZAP-mediated degradation of HIV-1 multiply spliced RNA [359]. A second mechanism involves excessive splicing due to sequestration of HIV-1 RNA in the nucleus, which leads to decreased amount of unspliced viral mRNA and increased amount of multiply spliced HIV-1 mRNAs. Lastly, DDX17 may directly modulate HIV-1 RNA splicing, as previously reported for DDX17 to affect alternative splicing of cellular genes [429]. HIV-1 splicing is a highly regulated process, which involves stem-loops regulatory elements called splicing silencers and splicing enhancers and generates more than 30 HIV-1 RNA species [72]. During splicing, RNA is remodeled in order to expose splicing silencers and splicing enhancer stem-loops that act as docking sites for distinct members of the spliceosome machinery, thereby influencing exon definition [72, 437]. One hypothesis would be the involvement

of DDX17 in remodeling HIV-1 RNA structure, leading to a change in exon definition, resulting in an increase of full length and a decrease in spliced HIV-1 RNAs, as seen in Figure 34. Generally, moderate effects were seen for the overexpression of wild type DDX17, which can be due to the fact that endogenous levels are sufficiently high to promote the production of infectious HIV-1 particles.

We found that expression of DDX17 mutants induce a dramatic decrease in the amount of packaged gRNA (Figure 33), which leads to the production of less infectious viral particles. Considering that the amount of full-length HIV-1 RNA decreased by only 2-fold in the cells for DQAD (L), we conclude that the packaging of gRNA was diminished by 5-fold. Similarly, we observed a 1.5 fold decrease in full length HIV-1 RNA for DQAD (S), but a 3.5-fold diminution in packaged gRNA (Figure 33). This data suggests that the helicase activity of DDX17 is necessary to promote HIV-1 gRNA packaging. HIV-1 RNA packaging is a complicated process that involves interactions of cellular and viral proteins with specific viral RNA motifs. HIV-1 5'UTR contains a number of stem-loop (SL) structures that act as regulatory sites for multiple processes, including splicing and packaging. Being a RNA helicase, it is possible that DDX17 remodels the viral RNP complex within the nucleus, therefore modifying its composition through the recruitment of various factors and changes the fate of the viral RNA. We do not lose sight of the possibility that perturbing the levels of DDX17 in cells may modulate cellular processes such as RNA transcription and splicing which in turn affects the process of HIV-1 packaging.

Interestingly, although no noticeable change in the level of $Pr55^{Gag}$ was observed, overexpression of DDX17 DQAD mutants in HEK293 cells and knockdown of DDX17 in HeLa cells lead to a dramatic decrease in viral p24 levels, which suggests that DDX17 DQAD mutants inhibit Gag processing. This effect on Gag processing correlated with a slight diminution of $Pr160^{Gag\text{-}Pol}$ (Figure 35C), suggesting that the helicase activity of DDX17 is required for frameshift to occur and for producing optimal ratios of Gag *vs* Gag-Pol. Indeed, results of experiments with the frameshift reporter constructs showed that DDX17 DQAD (L) led to a significant 40% decrease in frameshift efficiency (Figure 36B), which is expected to result in less production of $Pr160^{Gag\text{-}Pol}$. The decrease in $Pr160^{Gag\text{-}Pol}$ synthesis will lead to a reduction of viral protease concentration in the progeny virus particles and consequently, a defect in viral Gag processing.

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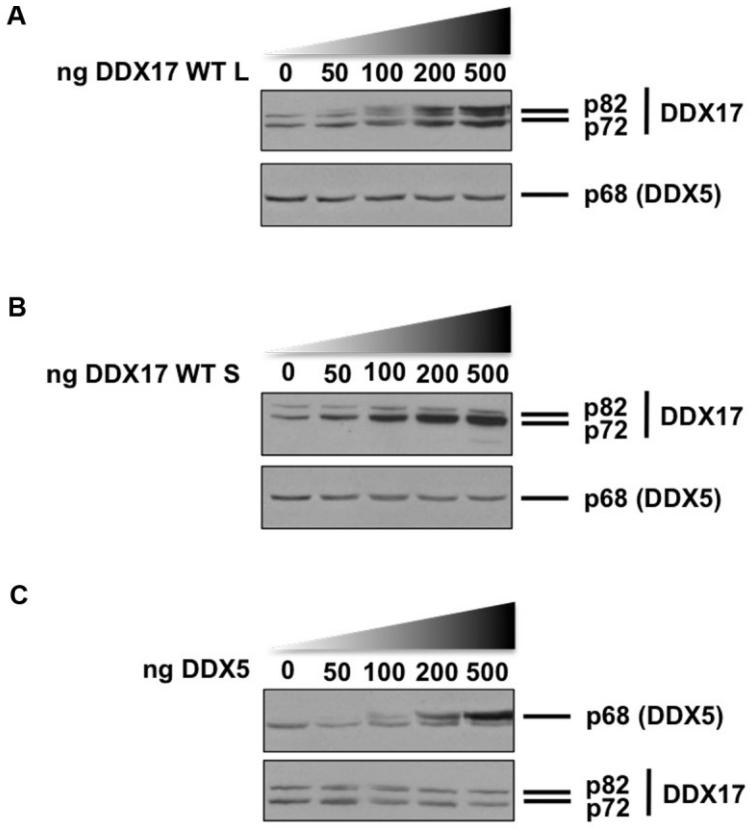
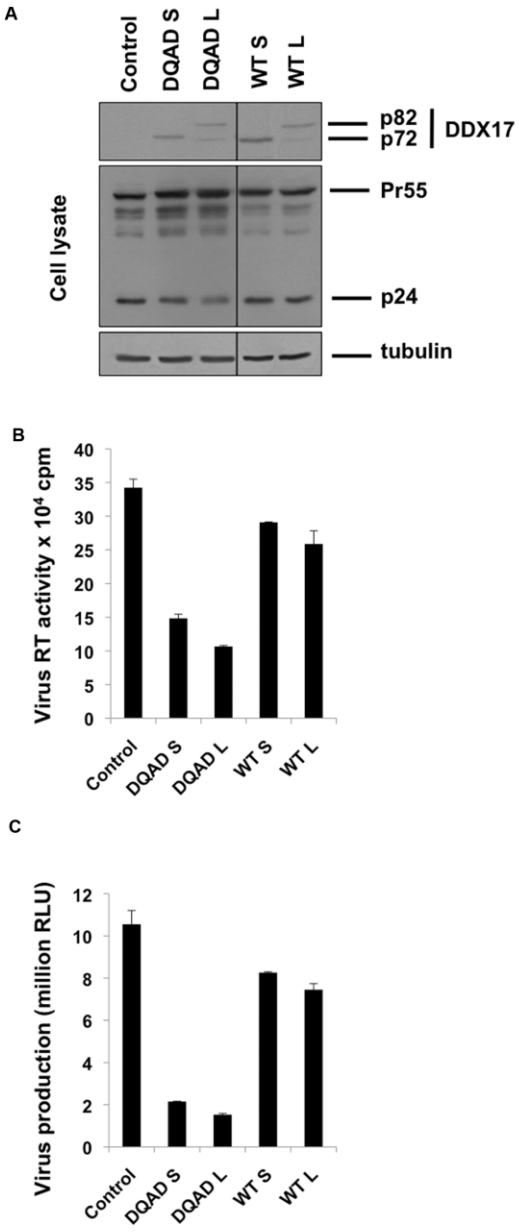
Authors' contribution

RL and CL conceived the project. RL wrote the manuscript and made the figures. QP performed the RT assay. CL and YL critically read the manuscript.



Supplementary Figure S1 | Mutual effect of DDX17 and DDX5 on their expression

HEK293 cells ($5x10^5$) were transfected with different amounts of DDX17 S (short form) (**A**), DDX17 L (long form) (**B**) or DDX5 (**C**). Levels of DDX5 and DDX17 were analyzed by Western blotting with anti-DDX17 and anti-DDX5 antibodies.



Supplementary Figure S2 | Effect of the overexpression of DDX17 and its DQAD mutants on HIV-1 production in HeLa cells

(**A**) Levels of DDX17 and HIV-1 Gag/p24 in the transfected HeLa cells as determined by Western blotting. Tubulin was probed as an internal control. The results shown are from one representative experiment. (**B**) Levels of viral reverse transcriptase activity in the supernatants of transfected HeLa cells. (**C**) Amounts of infectious HIV-1 particles in the culture supernatants were determined by infecting the TZM-bl indicator cells. The luciferase activity (RLU) represents the relative infectiousness of the virus samples tested.

Chapter 5 – Conclusion and Discussion

5.1 Helicases and HIV-1

5.1.1 Screening Studies: Advantages and Limitations

HIV-1 is a complex and highly regulated retrovirus. With its small 9kb genome coding for only 15 proteins, it must exploit the cellular machinery to successfully complete its lifecycle in the host. Moreover, HIV-1 has evolved to express accessory proteins that allow it to overcome the cellular restrictions imposed by the innate immune response, rendering the virus able to escape early immunity. In addition, the lack of proofreading activity of viral reverse transcriptase provides HIV-1 with a high mutation rate that confers it the ability to escape from the adaptive immune response. Overall, the difficulty to understand in detail the mechanisms underlying HIV-1 replication emphasizes the complexity of virus-host interactions. In the last decade, several groups have devoted their efforts to investigate the role of cellular factors that are involved in either promoting or inhibiting viral replication through the design of genome-wide studies. These studies aimed at identifying key cellular factors that are essential for HIV-1 replication. Surprisingly, the overlap between these large-scale screens appeared particularly weak and only very few human genes were found as potential HIFs in every study. Several factors could account for this poor overlap.

Firstly, not all the studies were performed using the same cell types. Although lymphocytes constitute the most relevant cell type to study HIV-1 replication, numbers of studies were conducted in HeLa [304, 321] or HEK293T [238, 318] cells, therefore contributing to some discrepancy due to initial distinct gene expression profiles. Secondly, the diversity of techniques that were used can lead to different outcomes. Indeed, some studies adopted a gene knockdown approach (siRNA [304, 318, 321] or shRNA [317, 320]), while others preferred mutagenesis [316] or immunoprecipitation [238, 328]. Thirdly, the duration of the protocol differs among the studies upon the techniques that are used. Some groups investigated the effect of a gene on HIV-1 replication 48h post knockdown [304, 317], while other experimental settings required up to three weeks selection to generate stable knockdown cell lines [320]. However, rather than identifying specific cellular targets, screening studies allowed for the characterization of cellular pathways that seem to be highly exploited by HIV-1, such as DNA repair, nuclear import/export, RNA expression and processing [149, 238, 304, 316-321, 328, 344]. Helicases isolated from these genome-wide screens have been recently reviewed [438].

Helicases are a family of cellular enzymes involved in the processing of nucleic acids. As a consequence, it is not surprising that they are also involved in the processing of HIV-1 RNA and DNA. Moreover, HIV-1 RNA requires specific mechanisms that are not shared by cellular RNA, such as reverse transcription, dimerization and packaging. The involvement of helicases in some of these steps suggests that HIV-1 can turn the cellular machinery away from its endogenous

function, with the outcome to sometimes promote and sometimes inhibit viral replication. The project of this thesis was to investigate the role of this family of proteins in the context of HIV-1 infection. Among 130 helicases that we tested in our study, 35 were found to positively or negatively modulate HIV-1 replication in SupT1 cells, grouped in eight different pathways (Table 4). Deeper analysis demonstrated that DNA repair and RNA expression are the two major pathways that seem to be recruited by HIV-1 during its lifecycle. Considering the function of each candidate in the cellular context, further investigation will allow to characterize the specific effect of these helicases in viral replication, such as BLM in viral integration or eIF4A1 in translation. In the end, we provide a list of 35 cellular genes coding for helicases that have the potential to considerably affect HIV-1 replication.

5.1.2 DDX17: a Multifunctional Helicase

In our screening study, we generated stable knockdown cell lines to monitor the effect of helicases on HIV-1 replication. We rapidly identified DDX17 as a promising candidate, since we observed a ~10-fold increase in the production of HIV-1 particles in SupT1 knockdown cell lines (Figure 26), suggesting an inhibitory effect of DDX17 on HIV-1 replication. Indeed, DDX17 was found to associate with ZAP to initiate the exosome-mediated degradation of multiply spliced HIV-1 mRNA [359, 383]. Unexpectedly, further investigation revealed that transient knockdown of DDX17 in both lymphocytes (SupT1 and

Jurkat cells) and adherent cells (HEK293 and HeLa) diminished the production of infectious HIV-1 particles, suggesting that DDX17 can promote HIV-1 replication, and that this effect was independent of ZAP (data not shown). Transient knockdown of DDX17 in Jurkat cells led to a diminution in the production of infectious HIV-1 particles, but no effect was observed on Gag expression, suggesting a late stage effect of DDX17 (Figure 30). Interestingly, siRNA knockdown of DDX17 in HeLa cells and overexpression of DDX17 DQAD mutants in HEK293 cells correlate with a change in Gag expression profile and a dramatic decrease in the infectivity of HIV-1 particles (Figures 31A and 32B). Altogether, our data suggest that DDX17 exerts multiple effects on HIV-1, including viral RNA frameshift and packaging.

It is the first time that a helicase is reported to affect HIV-1 frameshift, with all the possible downstream consequences that it entails. Indeed, since the three viral enzymes are synthesized from the *Pol* gene, which expression depends on the viral frameshift, it would be interesting to further test the effect of DDX17 on HIV-1 PR, RT and IN. We already tested the effect of DDX17 of viral reverse transcriptase activity and observed that DDX17 is required for optimal RT activity (Figure 31B and 32C). A decrease in HIV-1 frameshift could also result in lower epression levels of HIV-1 IN, leading to a diminution of the provirus integration in newly infected cells. Furthermore, we used the reverse transcriptase assay to quantify the amount of viral particles that were produced. Considering that DDX17 modulates the overall amount of HIV-1 RT, it would be interesting to quantify the amount of particles produced by ELISA against p24 in the supernatant and to further normalize this value to the intracellular HIV-1 p24

(also measurable by ELISA). Moreover, DDX17 and DDX5 are paralogs that share numbers of structural and functional features [433, 439]. Interestingly, DDX5 was identified as an HIV-1 IN partner [318], a particularity that may be shared with DDX17. Therefore, an effect of DDX17 in HIV-1 integration could be either direct (as an IN partner) or indirect through the regulation of HIV-1 IN synthesis. Investigation of the role of DDX17 in HIV-1 PR activity would be more difficult, since HIV-1 PR is a cleavage product of the polyprotein Gag-Pol, thus regulating its own synthesis.

HIV-1 gRNA packaging is facilitated by the Ψ signal, located in the 5'UTR of the viral RNA [440]. Recently, Chamanian *et al.*, identified the GRPE (gRNA packaging enhancer) element, a new 200-nucleotides region overlapping the frameshift sequence in HIV-1 RNA [441]. Interestingly, mutation of the GRPE led to a decrease in Gag-Pol expression, a decrease in viral packaging activity and a loss of up to 50-fold in HIV-1 infectivity [441]. Similarly, we observed that the expression of DDX17 DQAD dominant negative mutants was also leading to a decrease in Gag-Pol expression, HIV-1 gRNA packaging and viral infectivity (Chapter 4). It would be interesting to investigate the role of DDX17 in the context of the GRPE. Being a helicase, DDX17 could promote the proper folding of this specific region, therefore regulating HIV-1 frameshift, gRNA packaging and viral infectivity.

5.1.3 Helicases as Potential Drug Targets

Considering the high ability of HIV-1 to counteract cellular restriction factors and to resist to antiviral treatments through mutating its genome, finding new targets to inhibit viral replication is a constant challenge. Two approaches can be envisaged, as it is theoretically possible to target both viral and cellular components that are essential for the virus to replicate. While viral components - and especially viral enzymes - have been the main target for the development of anti-HIV-1 treatments, a new focus is to investigate a new category of compounds that would specifically target cellular proteins used by HIV-1 to complete its replication cycle. Since helicases are critical for HIV-1 replication, this family of proteins is of particular interest [309]. Among the many helicases that are involved in HIV-1 replication, DDX3, that is essential for the nuclear export of HIV-1 RNA [199], has been revealed to be a promising candidate for the development of this new generation of anti-HIV-1 drugs [442, 443].

Other helicases are considered as putative targets for the development of anti-HIV-1 and anti-cancer drugs, including BLM and WRN [444]. However, while compounds that are currently being tested against DDX3 confirm this helicase as a valid candidate [442], we should remain cautious when it comes to target cellular proteins regarding to toxicity issues. For example, although BLM and WRN play important roles in HIV-1 replication [356, 370, 445], their dysfunction is also associated with metabolic disorders in human, including all types of cancers predisposition [446].

5.2 More Challenges

5.2.1 Prevention of HIV-1 Infection

5.2.1.1 Behavioral Characteristics

One of the reasons, if not the major one, that causes the rapid and global spread of HIV-1, lies in the principal mode of transmission of the virus, sex, and therefore human behavior. Among the populations that are at risk, we often find men who have sex with men, sex workers, prisoners, as well as drug users. There are several ways of preventing HIV infection. If the best one is obviously the use of condoms, this practice remains less evident when it comes to different social and life contexts. Therefore, although not sufficient, counseling is today one of the main priority. Counseling is the approach that consists in educating a population about the HIV and the disease that it eventually causes, resulting in making the community members aware of their responsibilities. This raises the importance of social workers that are in the first line of action such as the streets and the testing and counseling centers. In addition, together with the promotion of safer sex behaviors, male circumcision has been shown to reduce HIV-1 infection by 60% in heterosexual men [447].

5.2.1.2 Mother-to-child Transmission

Pediatric infection during pregnancy, delivery or breastfeeding makes mother-to-child HIV transmission another challenge. In the absence of treatments, the newly born child has 15% to 45% chances to be infected with HIV. The use of adequate treatments lowers the risk of contamination as low as 5%. The actual goal is to eliminate new mother-to-child HIV transmission by 2015 [447]. Recently during CROI 2013 conference in Atlanta (USA), Persaud et al. reported the case of a child born with HIV that appears, 20 months later, to be "functionally cured" through antiretroviral treatment 30h after birth. Considering that more than 300 000 children are newly infected each year, this case represents a potential outbreak [447]. However, the scientific community remains cautious, as more studies and time will be needed to verify the HIV status of the child.

5.2.2 Treatments of HIV

5.2.2.1 Preventive treatments

In the last 30 years, we have found ~30 anti-HIV drugs that transformed HIV from a deadly infection to a chronic disease, when combined in a multi-drug regimen. Such advance is however limited since current anti-HIV therapies require daily medication uptakes for life-long treatments. Recently, a new approach has been envisaged with the use of antiretroviral drugs for both treatment and prevention, therefore reducing the amount of new infections.

Guidelines and recommendations for this new use of antiviral drugs should be announced in July 2013 by the WHO [447]. However, providing universal access to HIV/AIDS treatments remains a challenge.

5.2.2.2 Co-infections

In addition to opportunistic diseases that emerge during the AIDS phase, many patients face co-infections, mainly caused by HCV [448], tuberculosis (TB) [449] and HBV [450]. These co-infections both worsen the progression of the disease and account for increased number of complications leading to liver- and pulmonary-related deaths. Several complications emerge from the co-infection profile of a patient. For example, HCV is currently treated with interferon, and treatments for HIV/HCV co-infection only show 30-70% cure for HCV, depending on HCV genotypes [451]. Therefore new drugs are needed for the treatment of HCV in HCV/HIV co-infected patients. However, similar challenges to the ones observed in the context of HIV infection would arise for HCV. Due to HIV/HCV treatments contraindications, poor adherence caused by polymedication, low tolerance, toxicity and higher risk for selection of drug-resistant HCV strains, many patients would fail to follow anti-HCV therapy. This further highlights the issue of co-infections, the importance of promoting prevention as well as the challenge to development vaccines against HCV.

5.2.2.3 Latency

Lots of progresses have been made in the course of finding effective therapies, and the use of the combination of antiretroviral drugs managed to pause the viral infection and stop the evolution of the disease. However, the capacity for the virus to become dormant and invisible to the host by inducing latency adds a new obstacle to the route towards HIV-1 eradication. With only 1 CD4+ lymphocyte out of a million considered as a latent cell, the study of viral reservoirs becomes very challenging. In fact, both the nature of viral reservoirs and the mechanisms underlying latency remain poorly known [452, 453]. Studying latency in animal (and more specifically monkeys) models will allow unraveling the mechanisms of latency in humans. Combined with the use of antiretroviral therapy, new treatments targeting latent cells and reservoirs are expected be the last step towards a cure for HIV.

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