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ÉTUDE DU RÔLE DE PML DANS LA RESTRICTION DES RÉTROVIRUS

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To my parents, family and all my friends, without whom none of my success would be possible

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RÉSUMÉ EN FRANCAIS

La pandémie de virus de l'immunodéficience humaine (VIH-1) continue d'être un problème global majeur en santé publique. Les thérapies anti-rétrovirales ont grandement amélioré le statut clinique de nombreux patients; cependant, elles ont des faiblesses importantes, notamment au niveau des effets secondaires, du coût ainsi que l'accumulation de mutations de résistance. De plus, ces thérapies ne peuvent cibler l'ADN du VIH-1 sous sa forme intégrée latente. Une meilleure connaissance des facteurs de l'hôte modulant l'infection pourrait probablement accélérer le développement de nouvelles molécules contre le VIH-1, et fournir une nouvelle perspective pour l'éradication du VIH-1. Les facteurs de restriction sont des protéines cellulaires spécialisées induites par les interférons de type 1 (IFN-I) qui bloquent les rétrovirus à différentes étapes du cycle viral. Cette thèse se concentre sur TRIM19, mieux connue sous le nom de la protéine de leucémie promyélocytaire (PML), un membre de la famille des protéines à motif tripartite (TRIM). PML est la protéine organisatrice d'une structure nucléaire appelée corps nucléaire de PML et est impliquée dans plusieurs fonctions cellulaires telles que l'apoptose, les modifications post-traductionnelles et la suppression de tumeurs. Alors que le rôle de PML dans l'immunité innée est bien documenté pour plusieurs virus, ses effets sur le VIH-1 sont peu clairs. De façon intéressante, il a été récemment proposé que PML régule l'expression des gènes dépendant de l'IFN (ISGs). Les travaux décrits dans cette thèse portent sur le rôle de PML dans la capacité des lentivirus à infecter les cellules humaines et de souris. Pour le modèle murin, nous avons utilisé des fibroblastes embryonnaires de souris (MEFs) PML-knockout (PML-KO). Dans nos premières études, nous avons observé que les MEFs étaient plus permissives à l'infection par des lentivirus en l'absence de PML. La surexpression de mPML ou d'isoformes de hPML a conféré le phénotype de restriction aux cellules PML-KO. Nous avons montré que hPML et mPML inhibent les étapes précoces de la réplication du VIH-1 dans les MEFs et que la restriction est saturable même en présence d'un excès de PML, suggérant que PML n'est pas l'effecteur direct. Nous avons également observé que PML contribuait à l'inhibition du VIH-1 par les IFN-I dans les MEFs. De plus, nous avons montré que PML réprime l'expression génique du VIH-1 dans les MEFs, suggérant un rôle dans la latence virale. Dans une deuxième étude, nous avons investigué le rôle de PML dans la restriction des lentivirus dans plusieurs lignées cellulaires humaines, dont des lymphocytes T, par knockout de PML à l'aide du système CRISPR-Cas9. Nous avons trouvé que le knockout avait peu ou pas d'effets sur l'infectivité des lentivirus, et que PML n'était pas impliqué dans l'inhibition du VIH-1 par les IFN-I, démontrant que PML n'a pas de rôle dans la restriction des lentivirus dans les cellules humaines. Pris ensemble, nos résultats démontrent que l'implication de PML dans l'immunité innée contre le VIH-1 et d'autres lentivirus est spécifique du contexte cellulaire ou de l'espèce. Également, nos travaux apportent un éclairage nouveau sur le rôle possible de PML dans la latence du VIH-1, suggérant que PML pourrait constituer une cible moléculaire pour des agents pharmacologiques visant à supprimer les réservoirs de virus latent.

Mots-clés: VIH-1, lentivirus, PML, interféron, latence, immunité innée.

ABSTRACT

The type 1 human immunodeficiency virus (HIV-1) pandemic remains a major global public health problem. Anti-retroviral therapy (ART) has improved dramatically the clinical status of many HIV patients; however, it has major drawbacks, including side effects, cost and the accumulation of drug-resistant variants. Moreover, ART is not able to target HIV-1 in its latent, integrated form. A better understanding of host factors modulating infection is likely to accelerate the development of new drugs and to provide a new perspective for HIV-1 eradication. Restriction factors are type I interferon (IFN)-induced specialized cellular proteins that block retroviruses at different steps of their life cycle. This thesis focuses on TRIM19, better known as promyelocytic leukemia (PML) protein, a member of the tripartite motif (TRIM) protein family. PML is the constitutive organizer of a nuclear domain termed nuclear body (NB) and is involved in many cellular activities including apoptosis, post-translational modifications and tumor suppression. While the role of PML in the innate immunity against several viruses is well documented, its effects on HIV-1 remain unclear. Interestingly, PML was recently proposed to regulate the expression of IFN-stimulated genes (ISGs). The following studies investigate the role of PML in the permissiveness of murine and human cells to infection with lentiviruses. PML knockout (KO) mouse embryonic fibroblasts (MEFs) were used as a murine cellular model. In early studies, we found that MEFs were significantly more permissive to lentiviral infection in the absence of PML. Overexpression of mPML or some hPML isoforms conferred the restriction phenotype to PML-KO cells. We showed that both hPML and mPML inhibit the early post-entry stages of HIV-1 replication in MEFs and that the restriction is saturable even in the presence of over-expressed PML, suggesting that PML is not the direct effector. We also observed that PML contributed to the IFN-induced inhibition of HIV-1 in MEFs. Moreover, we observed that PML repressed HIV-1 gene expression in MEFs, suggesting a role in latency. In the second study, the role of hPML in the restriction of lentiviruses in several human cell lines including T cells was investigated by knocking out PML using the CRISPR-Cas9 system. PML knockout had no or little effect on the infectivity of lentiviruses, and it was not involved in the IFN-induced restriction of HIV-1, demonstrating that PML does not restrict the early stages of lentiviral infection of human cells. Taken together, our results show that PML is involved in both innate and intrinsic immunity against HIV-1 and other lentiviruses in an isoform-specific, cellular context or species-specific fashion. In addition, our work provides new insights into the role of PML in HIV-1 latency, suggesting that PML is a potential target for new antiviral drugs aiming at purging latent reservoirs.

Keywords: HIV-1, lentivirus, PML, interferon, latency, innate immunity.

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AIDS	Acquired immunodeficiency syndrome
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
B-MLV	B-tropic murine leukemia virus
CA	Capsid protein, p24
Cas9	CRISPR-associated protein 9
CB	Cytoplasmic body
CBP	CREB binding protein
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CD4+	Cell bearing the CD4 receptor
CDC	United States Centers for Disease Control and Prevention
cDNA	Complementary DNA
CRFs	Circulating recombinant forms
CRISPR	Clustered regularly interspaced short palindromic repeat
CTD	C-terminal domain
CTL	Cytotoxic T-lymphocytes
CXCR4	Chemokine receptor type 4
CypA	Cyclophilin A
DHC	Dynein heavy chain
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

dsRNA	Double-stranded RNA
DSB	Double-strand break
EIAV	Equine infectious anemia virus
Env	Envelope
ESCRT	Endosomal sorting complexes required for transport
FIV	Feline immunodeficiency virus
Gag	Group specific antigens
HAART	Highly active antiretroviral therapy
HAT	Histone acetyltransferases
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HDR	Homology-directed repair
HFV	Human foamy virus
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HSV-1	Herpes simplex virus type 1
IF	Immunofluorescence
IFN	Interferon
IN	Integrase
IRF-3	IFN-regulatory factor 3
ISG	IFN-stimulated genes
JAK	Janus kinase
KO	Knockout

LTR	Long terminal repeat
MA	Matrix protein
MEF	Mouse embryonic fibroblast
MFI	Mean fluorescent intensity
MLV	Murine leukemia virus
MTOC	Microtubule-organizing center
MTs	Microtubules
NC	Nucleocapsid protein
Nef	Negative factor
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
N-MLV	N-tropic murine leukemia virus
NPC	Nuclear pore complex
NTD	N-terminal domain
p.i.	Post infection
PEP	Post-exposure prophylaxis
PIC	Pre-initiation complex
PML	Promyelocytic leukemia
PR	Protease
PrEP	Pre-exposure prophylaxis
P-TEFb	Positive transcription elongation factor b
PV	Papillomavirus
RBCC	RING finger, B-box, and coiled-coil
Rev	Regulator of expression of virion
RING	Really interesting new gene

RNA	Ribonucleic acid
RT	Reverse transcriptase
SAHA	Suberoylanilide hydroxamic acid
SAMHD1	SAM domain and HD domain-containing protein 1
shRNA	Short hairpin RNA
SIM	SUMO-interacting motif
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
SUMO	Small ubiquitin modifier
TasP	Treatment as prevention
Tat	Trans-activator of transcription
TLR	Toll-like receptor
TM	Transmembrane protein, gp41
TRIM	Tripartite motif
TRIM5α	Tripartite motif-containing protein 5α
UNAIDS	United Nation program on HIV/AIDS
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein unique
Vpx	Viral protein X
VSV	Vesicular stomatitis virus
WT	Wild type

CHAPTER I

INTRODUCTION

1.1 An introduction to HIV/AIDS

The first cases of acquired immunodeficiency syndrome (AIDS) were reported in the United States in the spring of 1981 (Centers for Disease Control 1981b, a). The largest group of early AIDS cases comprised homosexual and bisexual men as well as injection drug users. Outside of scientific communities, AIDS was sometimes justified as God's punishment for homosexual men and drug users (Shilts 1987). Nevertheless, doubts about the viral cause remained until the causative virus, later termed human immunodeficiency virus (HIV), was isolated from patients in 1983-4 (Barre-Sinoussi *et al.* 1983; Gallo *et al.* 1984).

1.1.1 HIV classification

HIV is a member of the genus Lentivirus of the Retroviridae family. Two main types of HIV have been characterized: HIV-1 and HIV-2. They are two distinct viruses that can both lead to AIDS. HIV-1 was discovered first and it is the most widespread type. HIV-2 was isolated in 1986 and it is a morphologically similar but antigenically different virus (Clavel *et al.* 1986). Humans are not the natural hosts of either HIV-1 or HIV-2. Instead, these viruses have entered the human population as a result of zoonotic or cross-species transmission. It was found that HIV-2 is closely related to a simian virus causing immunodeficiency in sooty mangabeys termed SIVsmm (Hirsch *et al.* 1989). Meanwhile, HIV-1 is closely related to a lentivirus in chimpanzees (*Pan troglodytes*), SIVcpz (Peeters *et al.* 1989; Huet *et al.* 1990).

HIV-2 is associated with significantly lower viral loads in plasma, slower rates of CD4+ T cell destruction, and reduced rates of disease progression compared to HIV-1

(Marlink *et al.* 1994; Andersson *et al.* 2000). Therefore, the majority of individuals infected with HIV-2 are long-term non-progressors and undetectable plasma viral load predicts normal survival in infected people (van der Loeff *et al.* 2010).

Due to extensive and dynamic genetic diversity, HIV-1 can be also classified into a major group (M) and three more minor groups: N, O, and P (Fig. 1.1). Within group M, there are at least 9 distinct clades (subtypes) based on genetic sequence data as well as recombinant forms known as circulating recombinant forms, CRFs (Hemelaar *et al.* 2006; Hemelaar 2012).

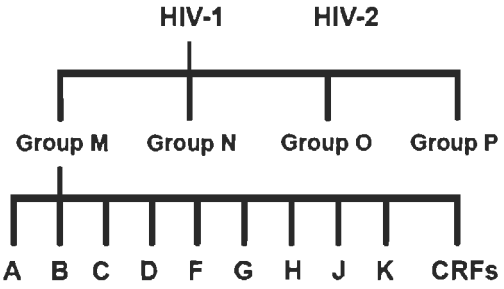

Figure 1.1 HIV classification.

Worldwide, HIV-1 group M is the predominant type and is the main causative agent of the AIDS pandemic, one of the most devastating infectious diseases in the last 35 years. In this study, we have focused on HIV-1 and mention of "HIV" refers to this type.

1.1.2 Infection

HIV gradually attacks the immune system, which is the human natural defense against infections and some types of cancer. The virus infects vital cells in the human immune system such as CD4+ helper T cells, macrophages, and dendritic cells (Klatzmann *et al.* 1984; Koenig *et al.* 1986; Steinman *et al.* 2003). HIV-1 causes the destruction of CD4+ lymphocytes with a half-life of less than two days (Ho *et al.* 1995; Wei *et al.* 1995; Perelson *et al.* 1996). This is the hallmark of HIV infection and predicts an individual's risk for infection with opportunistic pathogens as well as neurologic complications of HIV-1 infection such as HIV-1-associated dementia and related cognitive and motor disorders (Masur *et al.* 1989; Ho *et al.* 1995; Hellerstein *et al.* 1999; Spudich & Gonzalez-Scarano 2012). T cell depletion seen in AIDS is primarily a consequence of increased cell death, not decreased cellular production (Mohri *et al.* 2001), through three mechanisms: direct lysis of the infected cell, killing of the infected cell by

CD8 lymphocytes or promotion of apoptosis of uninfected bystander cells (Garg *et al.* 2012).

There are three clinical stages of HIV infection (Fig. 1.2): acute HIV infection, clinical latency, and AIDS. Generally within 1-4 weeks after infection, the patient may develop flu-like symptoms that can include headache, muscle aches, sore throat, low- or high-grade fever, swollen glands, rush, etc. (Cooper *et al.* 1985). During the acute stage of HIV infection, the levels of the virus in blood stream are very high, thus there is a high risk of transmission to others. At this stage, the immune system begins to respond to the virus by producing antibodies and cytotoxic lymphocytes.

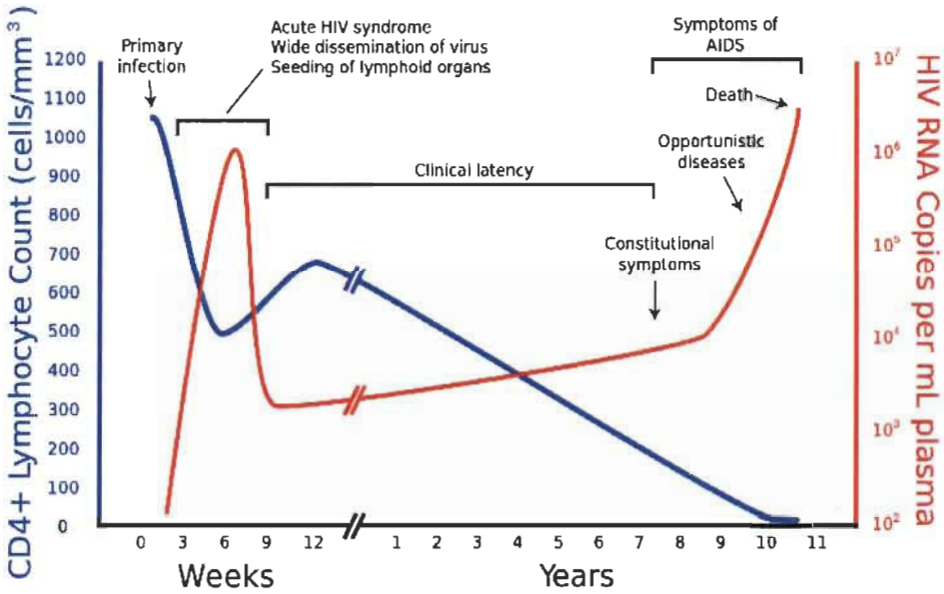

Figure 1.2 **Clinical stages of HIV infection.**
Blue line: CD4+ T cell count (cells/mm^3).
Red line: HIV RNA copies per ml of plasma.
(Pantaleo *et al.* 1993)

Following acute infection, the disease moves to the next stage termed clinical latency, which may be long (on average 10 years) even in the absence of treatment. During this period very low levels of infected cells and viremia in blood make detection of the virus expression extremely difficult, although the virus is still active and continues to reproduce at very low level (Fauci *et al.* 1991; Pantaleo *et al.* 1993). However a

progressive loss of CD4+ cells, on average 50-90 cells/mm^3 of blood per year, will be observed in most infected individuals (Bacchetti & Moss 1989; Phillips 1992).

AIDS is the final step of HIV infection if it is not treated. According to the Centers for Disease Control (CDC) criteria, AIDS is diagnosed when a person with HIV has a CD4+ count of < 200 cells/mm^3 of blood and/or one or more opportunistic infections by bacteria, fungi, viruses, etc., that are normally controlled by the immune system (Centers for Disease Control 1992).

1.1.3 Structure

HIV is spherical in shape (McGovern *et al.* 2002) and has a diameter of 110 to 128 and 132 to 146 nm for the mature and immature virus, respectively (Gentile *et al.* 1994) (Fig. 1.3). Two copies of the viral positive single-stranded RNA are incorporated into the envelope that is composed of two layers of phospholipids originating from the membrane of the producer host cell and a complex of surface projections containing a viral glycoprotein, gp120 trimers (Ozel *et al.* 1988; Chan *et al.* 1997; Lu *et al.* 2011). This structure in addition to three trans-membrane glycoproteins, gp41, forms the spikes that project from HIV particle and enable the virus to attach to the CD4 receptor and fuse with the host cell (Chan *et al.* 1997; Zhu *et al.* 2006a; Zhu *et al.* 2008). Inside the envelope, the matrix protein (MA, p17) lines the inner surface like a shell and the capsid protein (CA, p24) is located in the center forming a viral core and enclosing the viral genome which is tightly covered with nucleocapsid protein (NC, p7). Additionally, three essential virally encoded enzymes: protease (PR), reverse

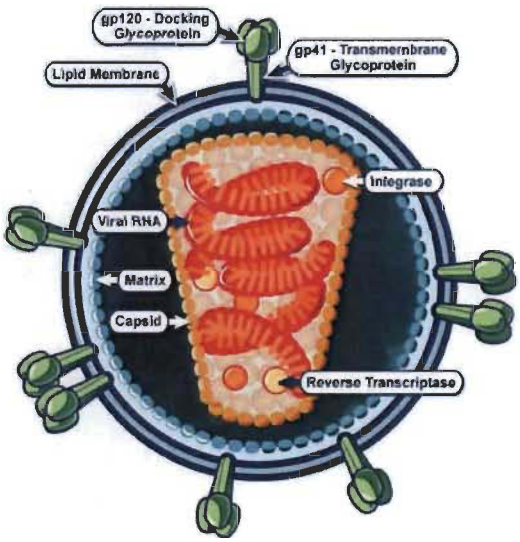

Figure 1.3 **HIV-1 virion.** This illustration of HIV structure shows the envelope, capsid, two RNA strands, and outer and inner proteins. (worldofviruses.unl.edu)

transcriptase (RT), and integrase (IN) along with the auxiliary proteins: Nef, Vif, and Vpr are packaged in the mature HIV virion (Turner & Summers 1999).

1.1.4 Genome

HIV is a complex retrovirus which is composed of two copies of unspliced RNA (Fig. 1.4). The genome of the integrated provirus is about 9.7 kb in length (Muesing *et al.* 1985) and both ends are flanked by a repeated sequence known as the long terminal repeats (LTRs) (Gallo *et al.* 1988). It contains 9 open reading frames and encodes 15 distinct proteins through multiple splicing (Frankel & Young 1998). These proteins are divided in three classes:

1- The core structural proteins Gag (group specific antigens), Pol, and Env

2- The regulatory proteins Tat (trans activator) and Rev

3- The auxiliary proteins Vpu, Vpr, Vif, and Nef

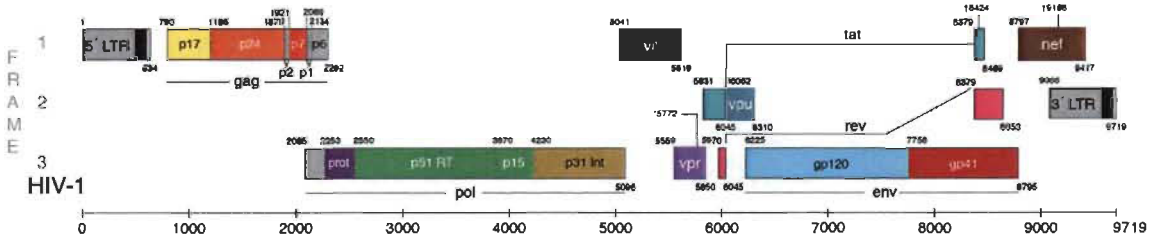

Figure 1.4 HIV-1 genome.
The three different reading frames are determined as numbered. (hivsystemsbiology.org)

To keep their genome as small as possible, viruses such as HIV often develop new strategies to synthesize multiple proteins from a single RNA. HIV utilizes a unique mode of gene regulation in which two genes, *gag* and *pol*, lie in different translational reading frames. The 3' end of *gag* overlaps the 5' end of *pol* resulting in two different products, Gag and Gag-Pol polyproteins (Jacks *et al.* 1988). The Gag polyprotein (also known as p55) is proteolytically processed to MA (p17), CA (p24), NC (p7), and p6 proteins by the

viral protease (PR), while the Gag-Pol polyprotein contains PR, RT, and IN but not p6. However, the Env glycoproteins gp120 and gp41 are expressed from a singly spliced mRNA. They are initially encoded as a precursor, gp160, which is then cleaved in the Golgi by the host cell protease furin into gp120 and gp41 (Hallenberger *et al.* 1992; Decroly *et al.* 1994). Other sequences encode for auxiliary proteins (Tat, Rev, Nef, Vif, Vpr and Vpu) that play important roles in the host-pathogen interaction and have significant impact on HIV infection (reviewed in (Li *et al.* 2005)). Briefly, Tat is essential for efficient transcription of viral genes and for viral replication. Rev is involved in the transportation of single-spliced and unspliced viral mRNAs to the cytoplasm, a step required for the expression of HIV structural proteins and the production of genomic RNA. Nef has multiple activities during virus replication and enhances virion infectivity and viral replication. Nef down-regulates cell surface expression of proteins including CD4 and CTLA-4 within the infected cell, helping HIV-1 to evade the host immune response (Garcia & Miller 1991; El-Far *et al.* 2013). Moreover, Nef was shown to promote HIV-1 infection by excluding SERINC5 (a host transmembrane protein) from virion incorporation (Rosa *et al.* 2015).Vif binds directly to APOBEC3G (an innate host defense factor against HIV infection (Mangeat *et al.* 2003)) and counteracts its anti-HIV activity by promoting its degradation. Vpr has been shown to have multiple activities during virus replication, including effects on the nuclear import of the proviral DNA, cell cycle G2/M progression, regulation of apoptosis, and transactivation of the HIV-1 LTR as well as host cell genes. Vpu is a membrane protein that enhances the release of progeny virions from infected cells and induces the degradation of the CD4 receptor. Vpu is known to down-modulate cell surface tetherin, a host restriction factor, thus removing tetherin molecules from the site of virus budding (Douglas *et al.* 2009; Guo & Liang 2012).

Infection by HIV-1 most often (in 60-80% of mucosal infections) results from the successful transmission and propagation of a single virus variant, termed the transmitted/founder virus (Keele *et al.* 2008). However, there are many different forms of the virus even in the body of a single infected person that are termed quasispecies. This is because of small mutations that may be introduced at each HIV replication cycle in the

host cell (Abram *et al.* 2010). This high genetic variability causes antigenic diversity and is one of the obstacles to eradicate HIV (Nowak *et al.* 1991; Robertson *et al.* 1995).

The existence of an antisense protein (ASP) expressed from an antisense polyadenylated transcript of *env* has been suggested. Although the ASP gene has still not been completely accepted by the HIV-1 research community, it was found to play a role in autophagy and HIV-1 replication (Torresilla *et al.* 2013).

1.1.5 Life cycle

The life cycle of HIV-1 can be divided into two phases: the early stage occurs between entry into the host cell and integration into its genome, and the late phase occurs from the state of integrated provirus to the release of infectious virion. Figure 1.5 shows a schematic representation of the HIV-1 life cycle that begins with the virus binding to the

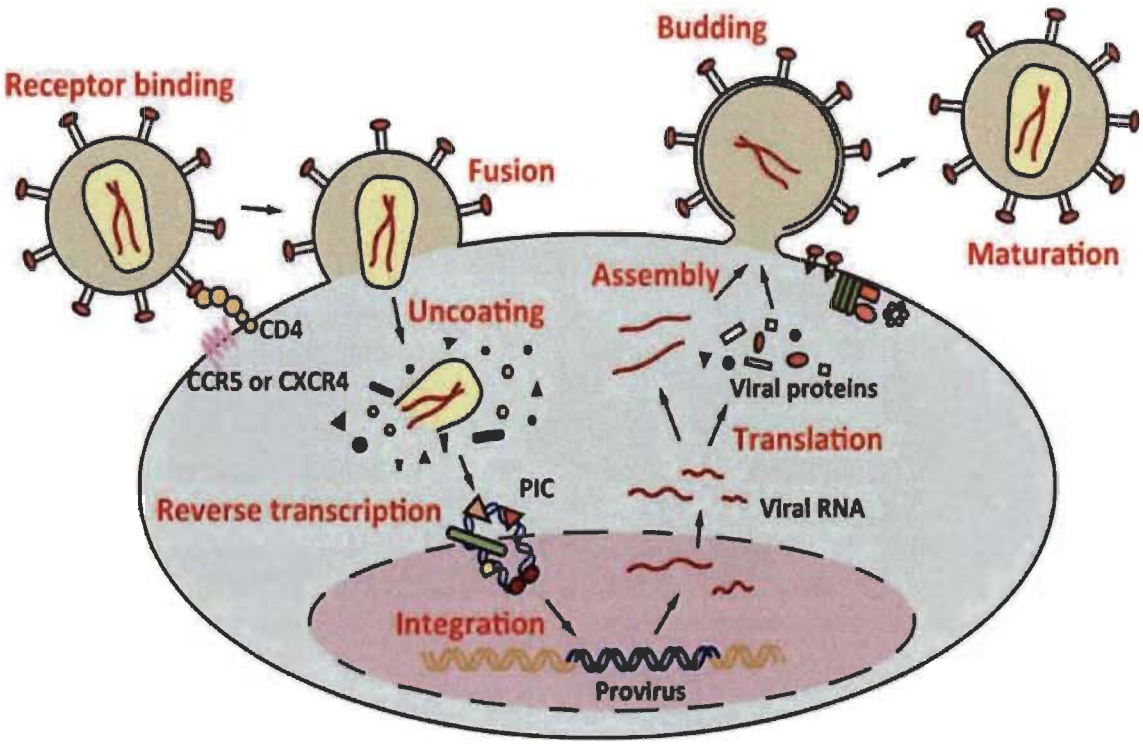

Figure 1.5 **Schematic drawing of the HIV-1 life cycle.**
HIV is able to dump its contents into the cell, reverse transcribe its RNA, integrate it into host DNA, and use the host to create new viral copies. The different steps of HIV life cycle are indicated in red (Suzuki & Suzuki 2011).

CD4 receptor and a co-receptor, CCR5 or CXCR4, on the surface of the host cell. The glycoproteins of the virus envelop spikes, gp120 and gp40, play the main role in this step. The binding step determines the ability of HIV to invade the cells of the human immune system, and blocking the receptors by inhibitors or inducing mutation in them confers protection against this virus (Mueller & Bogner 2007; Hutter *et al.* 2009). After binding, the spikes undergo a cascade of conformational changes that ultimately lead to the fusion between the host cell and the virus membranes, and releasing of the viral core in the cytoplasm (Gallo *et al.* 2003; Moore & Doms 2003).

Following entry, the viral core undergoes partial and progressive disassembly during a process known as capsid uncoating (Ambrose & Aiken 2014). Uncoating of the HIV-1 core is highly regulated and plays a critical role in early stages of infection; however, very little is known about the mechanism of this step. Nevertheless, it has been shown that optimal stability of the viral core regulates this step which is required for proper reverse transcription and nuclear import of viral genome, a hallmark of retroviral infection (Forshey *et al.* 2002). Uncoating is completed during reverse transcription or later in the virus life cycle and some host factors are involved in regulating this step (Friedrich *et al.* 2011; Ambrose & Aiken 2014). For example, HIV-1 uncoating was recently shown to be promoted by host microtubules (MTs) and cytoplasmic dynein, since disruption of MTs with Nocodazole or depletion of dynein heavy chain by RNAi substantially delay uncoating (Lukic *et al.* 2014; Pawlica & Berthoux 2014). These results demonstrate the importance of stable MTs in proper viral uncoating and subsequently infection. Accordingly, the virus enhances the early steps of its infection by inducing the formation of stable MTs (Sabo *et al.* 2013).

Concomitantly to uncoating, the viral RNA genome is subjected to the next step, reverse transcription into cDNA. This conversion is mediated by the viral enzyme reverse transcriptase and occurs in the cytoplasm of the infected cell. The host $tRNA^{(Lys3)}$ is used as a primer to initiate the proviral minus-strand cDNA synthesis (Brule *et al.* 2002) and the viral enzyme RNase H mediates the degradation of the viral RNA in the resulting RNA-cDNA hybrid (reviewed in Ref (Beilhartz & Gotte 2010)). Afterward, the second

strand of cDNA is synthesized by RT (Hu & Hughes 2012). The newly synthesized double-stranded cDNA remains associated with viral (PR, RT, IN, and Vpr) and cellular proteins (Farnet & Bushman 1997) as a high molecular weight complex termed pre-integration complex (PIC) that is responsible for the nuclear import of the genome (reviewed in (Jayappa *et al.* 2012)). This transport to the nucleus occurs through nuclear pore complexes and uses active transport mechanisms, implying that HIV can infect nondividing cells (Woodward & Chow 2010).

To achieve productive infection, the HIV cDNA is inserted into the host cell genome, and this integrated form is termed provirus. Integration is mediated by the virus-encoded protein IN as part of the PIC (Panganiban & Temin 1984) and involves the two long terminal repeats (LTR) flanking the viral cDNA, termed LTRs (Shine *et al.* 1977; Czernilofsky *et al.* 1980). In addition, several cellular co-factors are recruited by the virus in this step (Van Maele *et al.* 2006). The two LTRs in an unintegrated viral DNA can be ligated to each other to yield 2-LTR circles that are not competent for infection and thus represent dead-ends for the virus (Pauza *et al.* 1994). HIV integration is known to be favored in active transcription units, which promotes efficient transcription of the viral genes (Wang *et al.* 2007). A recent study has shown that HIV-1 integration occurs in transcriptionally active regions in the outer shell of the nucleus in close correspondence with the nuclear pores (Marini *et al.* 2015). Therefore, nuclear topography is a relevant factor in HIV-1 integration site selection.

Once integrated, the provirus serves as the template for transcription of viral RNAs by the host RNA polymerase II. The transcribed RNA may be translated into viral proteins or packaged as the genomic RNA in progeny virions. Low or absent viral gene transcription may lead to latency (see section 1.2). The 5' LTR functions as a promoter to promote proviral transcription, while the 3' LTR is required for efficient polyadenylation of the resulting transcripts (Brown *et al.* 1991; Klaver & Berkhout 1994). Although 3' LTR transcription can be triggered by inactivation of the 5' LTR promoter (Klaver & Berkhout 1994). HIV-1 recruits a complex of cellular transcription factors and the viral encoded trans-activator of transcription, Tat, to regulate its gene transcription (Cullen

1991). Although the cellular transcription factors are sufficient to induce a relatively low level of HIV-1 gene transcription, Tat is required for high levels of viral RNA and protein synthesis (Debaisieux *et al.* 2012). Tat interacts with several transcription factors and co-activators, including cyclin T1 and CDK9 and induces the phosphorylation of RNA polymerase II, releasing it from pausing and increasing transcription from HIV-1 LTR (Sobhian *et al.* 2010). Three types of viral RNA variants are transcribed: early fully spliced, late partially spliced and unspliced RNA (Felber *et al.* 1990). The fully spliced viral transcripts are exported from the nucleus by the same mechanism as cellular mRNAs, while the partially spliced transcripts export is dependent on the host Crm1 nuclear export factor that is recruited by the viral protein Rev (Zapp & Green 1989; Cullen 2000; Yi *et al.* 2002).

Successful transcription leads to the production of approximately 30 different HIV-1 RNAs that are all derived from a single full-length transcript by alternative splicing. The fully spliced and incompletely spliced mRNAs code for auxiliary proteins as well as Env. The unspliced RNA plays a dual role as a mRNA to code for the Gag and Gag/Pol polyproteins and as genomic RNA to assemble in viral progeny (Coffin *et al.* 1997). Like all other viruses, HIV-1 is dependent on the host translation machinery for its translation, and Tat is thought to act as a translational activator of HIV-1 mRNAs as well (SenGupta *et al.* 1990; Braddock *et al.* 1993; Charnay *et al.* 2009; Burugu *et al.* 2014).

In a productive infection, the provirus assembles all of the components required for infectivity into particles that can leave the infected cells and spread the infection to other host cells. These components include: two copies of the positive sense unspliced RNAs, the cellular $tRNA^{(Lys)}$, Vpr, the Gag polyprotein, the viral Env, the viral PR, IN, and RT (Müller *et al.* 2000; Kleiman *et al.* 2010; Sundquist & Krausslich 2012). Assembly occurs mainly at the plasma membrane; thus, all these components need to gather around the sites of assembly. The Gag protein of HIV-1 mediates assembly by binding other virion components through direct protein-protein and protein-RNA interactions (Freed 1998; Jouvenet *et al.* 2009) and on the other hand, specific association with cholesterol-enriched microdomains, termed rafts, at the plasma membrane (Ono & Freed 2001).

Assembly kinetics are rapid, as the assembly of a single virion is completed in about 10 min (Jouvenet *et al.* 2008; Ivanchenko *et al.* 2009).

To promote virus budding from the plasma membrane, the HIV-1 Gag binds directly to components of a cellular budding machinery, termed the ESCRT (endosomal sorting complex required for transport) pathway which is comprised of more than 30 different proteins (Demirov & Freed 2004; Bieniasz 2006). Moreover, the virus requires its lipid membrane as well as the Env glycoproteins, gp120 and gp41, to produce fully infectious particles. Since the virion buds at the plasma membrane, the viral membrane is therefore derived from the host cell. The mechanism by which the Env glycoproteins are incorporated into budded virions, however, remains poorly understood. Nevertheless, several models have been proposed: 1) random incorporation as a result of its expression on the plasma membrane, 2) a direct Gag-Env interaction recruiting the spikes into the virion, 3) the Gag-Env co-targeting model, a cellular structure recruits both Gag and Env in an assembly position resulting in incorporation of Env glycoproteins into the virion, and 4) the indirect Gag-Env interaction mediated by a host cell protein (reviewed in (Checkley *et al.* 2011)).

HIV-1 particles are initially released from the infected cells as immature virions that are not infectious yet and contain uncleaved precursors of Gag and Gag-Pol polyproteins. The proteolytic processing of the precursors, driven by the retroviral PR, occurs at ten different sites during or shortly after budding, yielding the fully processed MA, CA, NC, p6, RT, IN, and PR and leading to virus maturation (Kohl *et al.* 1988; Hill *et al.* 2005). MA remains associated with the viral membrane, while CA surrounds the NC that encloses the RNA genome in the center of the virion (Briggs *et al.* 2004; Briggs *et al.* 2006).

The life cycle of HIV-1 and its interaction with host restriction factors have been widely studied (Bushman *et al.* 2012). The virus completes its replication in approximately 24 hours (Mohammadi *et al.* 2013). Each step is targeted by the host inhibitors or antiviral drugs.

1.1.6 Transmission

A new host can be infected by the transfer of semen, breast milk, blood or blood clotting factors, vaginal fluid, or through the placenta. Unprotected sexual intercourse is the primary mode of HIV transmission (Rom & Markowitz 2007). HIV has also been found in saliva, tears, and sweat from some AIDS patients in very low quantities, but it is generally not transmitted by those body fluids. Moreover, oral transmission of HIV by the millions of HIV-infected individuals is a rare event and saliva of viremic individuals usually contains only non-infectious components of HIV. Due to its hypotonicity, saliva disrupts 90% or more of the cells including infected ones. This may be the major mechanism by which saliva kills infected blood mononuclear leukocytes and inhibits the multiplication of HIV, and also attachment of infected leukocytes to mucosal epithelial cells and thereby oral acquisition of the virus (Baron *et al.* 1999).

HIV is not transmitted by mosquitoes due to several reasons. The virus cannot replicate inside insects such as mosquitoes due to the lack of CD4 receptor on cell surface. Moreover, it is rapidly digested in the mosquito's gut and consequently has a short life-time. Considering low levels of the circulating virus in blood and insect's blood-sucking mechanism, the risk of HIV transmission by insects is practically zero (Iqbal 1999).

1.1.7 AIDS, a global epidemic

The most advanced stage of HIV infection is AIDS, which takes from 2 to 15 years to develop depending on the individual and has become a chronic disease in many areas of the world. To date, AIDS is the 6th leading cause of death among people among the 25-45 years old in the US. AIDS continues to be a major global public health issue, having claimed more than 34 million lives so far (Fig. 1.6). According to the most recent UNAIDS fact sheet (2016), 1.2 million people died from AIDS-related causes and about 37 million were living with HIV globally in 2015. Two million newly infected people have been recorded in 2015 which is a 35% reduction since 2000. Moreover, AIDS-related deaths have fallen by 42% since the peak in 2004. Sub-Saharan Africa is the most affected

region with 25.8 million people living with the virus in 2015, accounting to almost 70% of the total new infections worldwide.

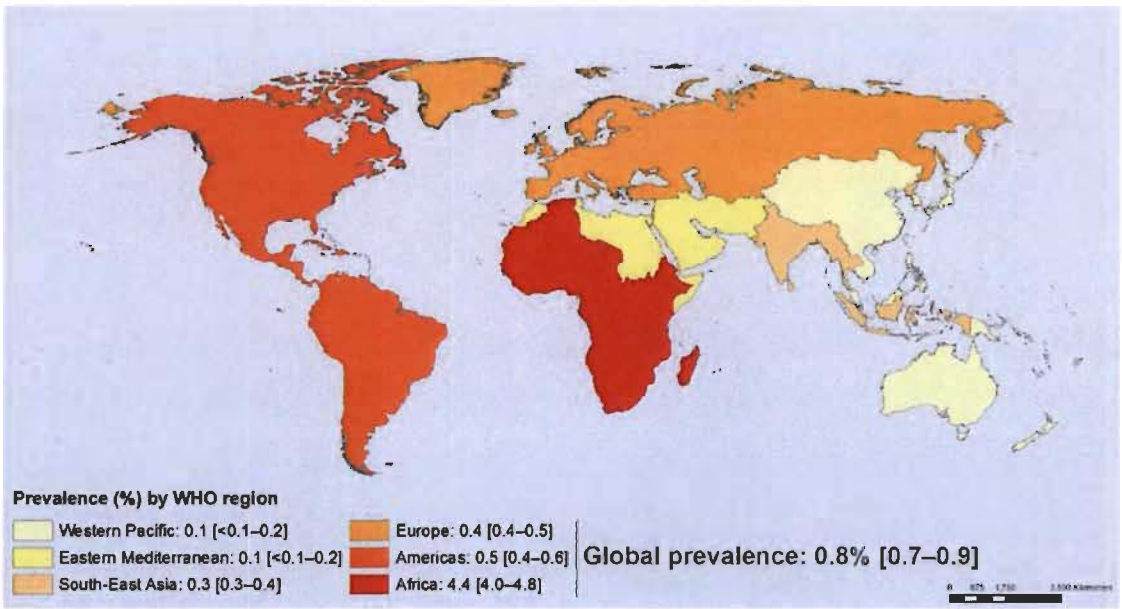

Figure 1.6 HIV prevalence rate.
Globally, 36.7 million [34.0–39.8 million] people were living with HIV at the end of 2015. An estimated 0.8% [0.7-0.9%] of adults aged 15–49 years worldwide are living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions. Sub Saharan Africa remains most severely affected, with nearly 1 in every 25 adults (4.4%) living with HIV and accounting for nearly 70% of the people living with HIV worldwide (WHO 2015).

According to 2015 national estimates, the number of people living with HIV is increasing in Canada. It is estimated that 75,500 Canadians are living with the virus (a prevalence rate of 212 per 100,000), representing a 9.7% increase since 2011. In addition, about 20% of HIV-positive people remained undiagnosed in 2015. The increase in prevalence is a result of both new infections and fewer deaths due to effective treatment. The prevalence is concentrated in specific populations; 53% of all people living with HIV in Canada are homosexual men, 19% are people who use injection drugs, 31% of people were infected due to heterosexual sex, and less than 1% of people were likely infected by blood transfusion or clotting factors, mother to child transmission, or needle-stick injuries in the workplace. Almost 25% of the infected people are women.

Over 26,000 people living with HIV have died due to AIDS-related illnesses since the beginning of the epidemic in Canada. In 2014, 2,570 people became infected with HIV, meaning that a new infection occurred every 3 hours. Thus, the HIV/AIDS pandemic still represents a major public health challenge in Canada.

1.1.8 Treatments and Prevention

Since HIV was characterized in 1983 and in spite of numerous trials by pharmaceutical companies and academic institutions around the world to develop drugs or vaccines, there is neither an effective HIV vaccine nor a cure for AIDS. However, a variety of treatments are available that can delay progression of the symptoms and improve the quality and length of patients life. In the absence of treatment, almost all infected people develop AIDS. However, a small group of patients called long-term non-progressors, develop AIDS very slowly or never at all in the absence of antiretroviral therapy (ART) over many years of infection (Sheppard *et al.* 1993; Lisziewicz *et al.* 1999). It seems that these patients have genetic particularities that significantly prevent the virus from destroying their immune system (Cohen *et al.* 1998; Kindberg *et al.* 2006).

There are different approaches and strategies toward the treatment or prevention of HIV infection; drug treatments, prevention of mother-to-child transmission, pre-exposure prophylaxis (PrEP), post exposure prophylaxis (PEP), and vaccines.

Currently, however, there is no effective vaccine against HIV. HIV is unusual in having a low density of envelope protein spikes on its surface (Fig. 1.3) (Zhu *et al.* 2006b). Therefore, effective neutralizing antibodies can't be generated through self-reactive intermediates due to low-density epitope display (Schiller & Chackerian 2014). Moreover, the high error rate of HIV reverse transcriptase combined with the estimated in vivo HIV-1 replication rate of ten billion new virions each day leads to extraordinary genetic diversity of HIV (see section 1.1.4). This contributes to escape immune pressures and also to the difficulties in producing a vaccine.

The first approved anti-HIV drug, AZT, was presented on 1988 that targets RT (Young 1988). By using HIV medicines (ART) consistently, progression to AIDS is prevented in most cases. ART helps control the virus so that infected people live longer and healthier and reduce the risk of transmitting HIV to others. The standard treatment, called highly active antiretroviral therapy (HAART), consists of a combination of antiretroviral drugs against different viral targets. HAART prevents drug resistance and increases the chances of survival (Pomerantz & Horn 2003). After viral replication is suppressed by HAART for 12 weeks, the number of circulating CD4+ T cells are considerably elevated.

Although HAART improves chances of survival by decreasing the HIV-1 burden on the immune system and preventing opportunistic infection, it is expensive (Levy *et al.* 2006). The UNAIDS Fast-Track strategy (90-90-90), launched in 2014, aims to greatly step up the HIV response in low- and middle-income countries to end the epidemic by 2030. The strategy calls for 90% of HIV-infected individuals to be diagnosed by 2020, 90% of whom will be on ART and 90% of whom will achieve sustained virologic suppression. The strategy sets targets for prevention, treatment and human rights (Jamieson & Kellerman 2016). Another important concern is the appearance of drug-resistant strains of HIV due to the expansion of HAART (Celum 2011).

PrEP is a prevention approach for people who are considered at high risk of HIV infection. It consists in providing them with antiretroviral drugs in order to prevent infection by any possible exposure to the virus (WHO 2011). When taken consistently, PrEP has been shown to reduce the risk of HIV infection in people who are at high risk by up to 95% (Grant *et al.* 2010). However, PrEP is much less effective if it is not taken consistently. Health care workers are offered post exposure prophylaxis (PEP) if they have been exposed with HIV during their works. It may be effective to decrease the risk of infection (Cohen *et al.* 2005; Smith *et al.* 2005).

Three different studies were done in South Africa (Auvert *et al.* 2005), Uganda (Gray *et al.* 2007), and Kenya (Bailey *et al.* 2007) to investigate whether male

circumcision reduces the risk of HIV infection. All three trials showed that circumcision reduced vaginal-to-penile transmission of HIV by 60%. Accordingly, a voluntary medical male circumcision is in progress in 14 priority countries in east and southern Africa (WHO/HIV/2016.14).

Timothy Brown, also known as the Berlin patient, is thought to be the only individual cured of HIV. Having contracted HIV in 1995, he was also diagnosed with acute myeloid leukemia in 2006 and later in 2008 received a hematopoietic stem cell transplant from a donor with the delta 32 mutation in the CCR5 receptor (Hutter *et al.* 2009). The mutation introduces a premature stop codon into the CCR5 receptor locus, resulting in a nonfunctional receptor that is not able to locate on the cell surface (Samson *et al.* 1996). The mutation offers a natural resistance to HIV and he is still free of readily detectable HIV after discontinuation of antiretroviral therapy. This has renewed hopes for a cure, especially in the gene therapy field.

1.2 HIV-1 latency

A successful antiretroviral therapy reduces the HIV load in plasma to undetectable levels but it is not able to eradicate the virus. The mechanism by which HIV-1 persists in the presence of ART is not clear yet. The most widely accepted mechanism is that the virus can establish a state of latent infection in CD4+ T cells, in which the provirus is integrated into the host cell genome but is transcriptionally inactivated and thus cannot be targeted by antiretroviral drugs or the immune system (Van Lint *et al.* 2013). Thus, this type of cells carry a viral reservoir and are responsible for HIV infection being a chronic rather than acute disease. The latent reservoir is now recognized as the major barrier to HIV-1 eradication because it persists even in the presence of HAART and any treatment interruption results in rapid viral rebound following cellular activation (Jubault *et al.* 1998; Davey *et al.* 1999; Richman *et al.* 2009).

The latent form of HIV-1 infection was first demonstrated in vivo in a small fraction of resting memory CD4+ T cells which were found to harbor the HIV reservoir (Chun *et*

al. 1995). The frequency of these cells is extremely low, typically around 1 per 10^6 cells (Chun *et al.* 1997). It was later shown that latency occurs early during acute infection, likely within 10 days of initial infection (Chun *et al.* 1998). Although more studies revealed that there are several potential cellular and anatomical reservoirs for HIV-1 that may contribute to long-term persistence of the virus, the most prominent one is CD4+ T cell subsets, primarily resting central memory T cells (TCM) and translational memory T cells (TTM) (Chomont *et al.* 2009).

HIV-1 entry in resting T cells may result in the accumulation of non-integrating viral DNA in the nucleus due to a block at reverse transcription, demonstrating that cellular activation is required for efficient infection and subsequently latency (Stevenson *et al.* 1990; Zack *et al.* 1990; Descours *et al.* 2012). Therefore, latently infected resting memory T cells may be generated from antigen-stimulated active T cells infected by HIV and survive long enough to revert back to a resting memory state (half-life of about 44 months) which is then non-permissive for viral gene expression (Pierson *et al.* 2000; Siliciano *et al.* 2003; Siliciano & Greene 2011).

A dynamic steady state of residual viremia is maintained in the plasma of patients on HAART, typically at levels lower than the limit of detection by current clinical assays, 50 copies/ml (Dornadula *et al.* 1999; Ramratnam *et al.* 2000; Havlir *et al.* 2003; Palmer *et al.* 2003). Because of the short half-life of free virus in plasma (2 days) (Wei *et al.* 1995), the presence of viral RNA suggests that ongoing viral replication is occurring despite the complete blocking of HIV-1 replication by HAART. Analysis of population structure demonstrated the presence of a residual viremia which is genetically distinct from proviruses in resting memory CD4+ T cells (Brennan *et al.* 2009), revealing the presence of additional reservoirs other than resting memory T cells (Sahu *et al.* 2009). Follow-up research showed that naïve T cells could be also as HIV-1 reservoir in patients on ART at a frequency lower than resting memory T cells. However they are quiescent in nature (Chomont *et al.* 2009; Wightman *et al.* 2010). This may be explained by transient activation of these cells by cytokines or other stimulators. Moreover, tissue macrophages constitute another primary target of HIV-1 infection, and could be a source of residual

viremia in plasma. Due to their ability to proliferate after infection, the proviruses are generally not completely silent in these cells (Abbas *et al.* 2015). The activity of some antiretroviral drugs on HIV-1 infection in macrophages was found to be several folds lower than T cells (Perno *et al.* 1998; Jorajuria *et al.* 2004). This allows continued HIV-1 replication which may result in the formation of HIV-1 reservoirs and the emergence of resistant virus as well.

In some multipotent hematopoietic progenitor cells (HPCs), latent infection was also detected that stably persisted in cell culture until viral gene expression was activated by differentiation factors (Carter *et al.* 2010). The detection of HIV genomes in HPCs isolated from patients on HAART showed that although HIV targets these long-life stem cells, activation of the provirus due to induction of differentiation kills the host cells rapidly. However, more recent studies have not detected latent HIV-1 in HPCs isolated from patients on ART (Durand *et al.* 2012; Josefsson *et al.* 2012). Further studies are therefore needed to determine whether HPCs are able to serve as reservoir for HIV-1.

The central nervous system, which is invaded by HIV shortly after infection (Schnell *et al.* 2009; Holman *et al.* 2010; Schnell *et al.* 2011), has been also suggested as another cellular reservoir for HIV. Astrocytes are the most abundant cell type in the brain. These cells perform vital functions to maintain brain homeostasis. Recently it has been shown that astrocytes can be latently infected by HIV and reactivation of the provirus results in release of infectious virions from these cells (Narasipura *et al.* 2014).

1.2.1 Mechanisms of latency

There are various mechanisms that likely underlie HIV latency (reviewed in (Battistini & Sgarbanti 2014)). These mechanisms use both cellular and viral factors and mostly act by suppressing the transcription of the viral promoter LTR (Fig. 1.7). One pathway toward latency is through integration downstream of a host gene in the same transcription polarity resulting in viral promoter occlusion. In this pathway, RNA polymerase II reads through the viral promoter that leads to silencing of the viral

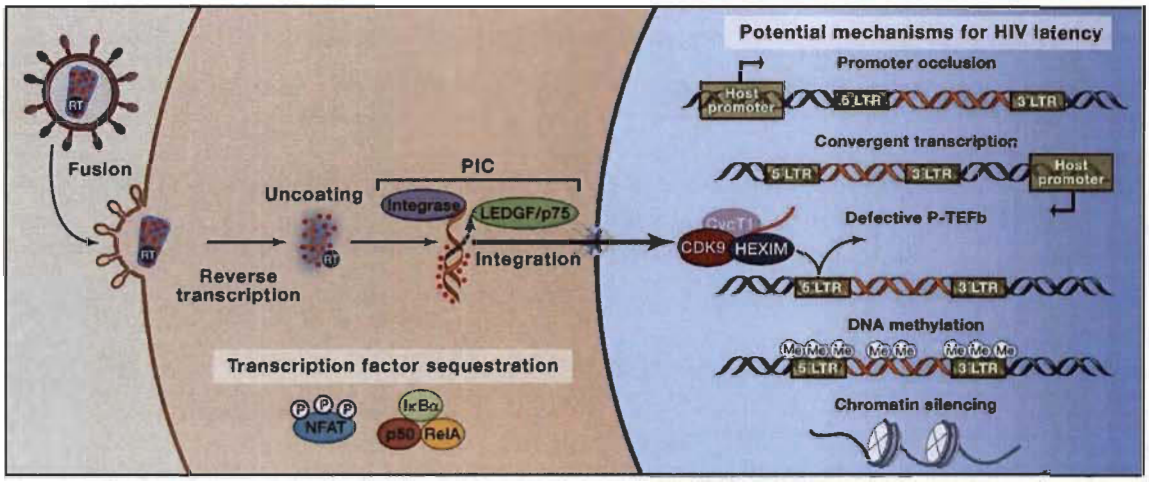

Figure 1.7 Major mechanisms of HIV-1 latency in T cells.
(Ruelas & Greene 2013)

transcription (Greger *et al.* 1998; Lenasi *et al.* 2008). On the other hand, viral transcription silencing may also occur due to convergent transcription, when the provirus integrates in the opposite direction of the host gene. In this context, a collision of the RNA pol II complexes from both the viral and host gene promoters can occur, leading to weaker or early arrest of transcription (Lewinski *et al.* 2006). Moreover, due to transcription from opposite directions, a complementary double-stranded RNA may form that leads to viral transcription silencing (Hu *et al.* 2004).

Since the 5' LTR acts as a promoter, it contains multiple sites for the binding of host transcription factors including NF-κB family members (Fig. 1.7) (Kinoshita *et al.* 1998; Kim *et al.* 2011). Therefore, sequestration of transcription factors in the cytoplasm can maintain latency in resting T cells. Moreover, in the nucleus, a transcriptionally repressive p50-p50 (a NF-κB member) homodimer binds the HIV 5' LTR constitutively and represses transcription upon recruitment of histone deacetylase 1 (HDAC1) (Williams *et al.* 2006).

Latency can also occur at the level of transcription elongation when the positive transcription elongation factor b (P-TEFb) complex (Fig. 1.7) that controls the elongation phase of transcription by RNA pol II (Peterlin & Price 2006) is inhibited by binding of

the 7SK snRNA to the complex (Michels *et al.* 2004). In addition, in resting, naïve, and memory CD4+ T cells and independent of their infection status, proteins of the complex are expressed at low levels and expression increases upon activation (Budhiraja *et al.* 2013); low expression of RNA pol II proteins helps maintaining HIV latency.

Histone posttranslational modifications in nucleosomes, such as acetylation and DNA methylation, regulate the level of gene expression in eukaryotic cells, including the integrated HIV provirus in T cells (Quivy & Lint 2002; Pearson *et al.* 2008). Acetylation of histones, catalyzed by histone acetyltransferases (HAT), results in reducing condensed chromatin into a more relaxed structure that is associated with greater levels of transcription (Eberharter & Becker 2002). This relaxation can be reversed following deacetylation of histones by HDACs, leading to chromatin silencing (Fig. 1.8) (Khochbin *et al.* 2001). Therefore, epigenetic silencing mediated by HDACs and HATs may also influence the establishment and maintenance of proviral quiescence by interfering with the expression of viral genes. However, this silencing can be reversed by the HIV-1 Tat protein that activates transcription independently of the chromatin environment (Jordan *et al.* 2001) or by pharmacological inhibitors (Lusic *et al.* 2003).

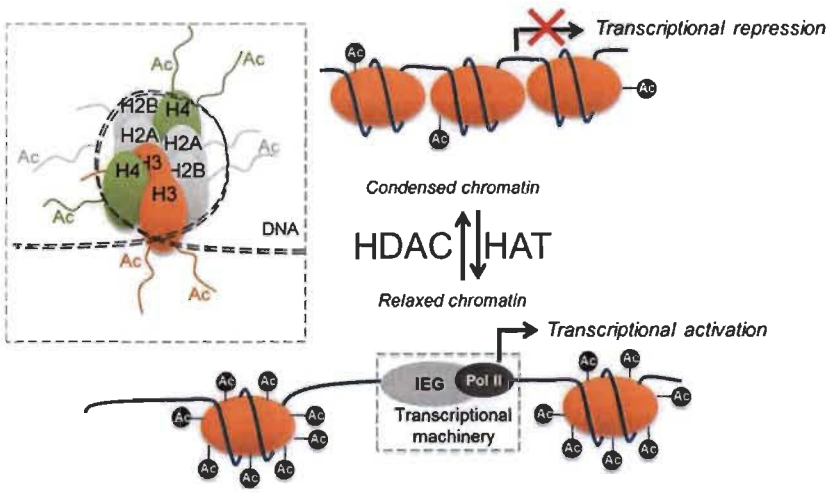

Figure 1.8 **Histone acetylation, a mechanism regulating gene transcription.** Acetylation of histone proteins is catalysed by the action of HATs and is reversed by the action of HDACs. Acetylation can promote gene transcription by causing direct structural changes to chromatin to result in a more relaxed state. Ac, acetylated lysine residues on histone tail proteins (Whittle & Singewald 2014).

Furthermore, CpG Methylation of the HIV-1 5′ LTR is an additional epigenetic restriction mechanism for the control of latent HIV-1 reactivation, and thus also constitutes a relevant factor in latency (Fig. 1.7) (Blazkova *et al.* 2009).

1.2.2 Therapeutic approaches to revert the latent state

Several therapeutic approaches, generally termed shock and kill strategy, aim at reactivating proviral expression to revert latency. Patients remain on standard anti-retroviral therapy (to block viral spread) while a second drug (shock agent; classes of drugs known as latency-reversing agents (LRAs)) is used to activate latent HIV, followed by the elimination of reactivated reservoirs through either natural means (e.g. immune response) or artificial means (kill agent; e.g. drugs, monoclonal antibodies, etc.) (Hamer 2004).

Of particular interest, some shock agents are HDAC inhibitors (HDACis) and are the most advanced drugs as HIV antilatency agents in clinical trials (Archin *et al.* 2012). Combination therapy with valproic, an HDACi, and intensified HAART safely accelerates clearance of HIV from resting CD4+ T cells in vivo (Lehrman *et al.* 2005). The suberoylanilide hydroxamic acid, SAHA (also known as vorinostat), another HDACi used in this study, has also shown potent activity in reactivating latent HIV (Archin *et al.* 2009; Contreras *et al.* 2009). However, a recent study revealed that SAHA increases the susceptibility of uninfected CD4+ T cells to HIV by increasing the kinetics and efficiency of postentry viral events (Lucera *et al.* 2014). Moreover, treatment with some HDACis including SAHA was found to suppress IFN-γ production at early time-points, whereby HDACis suppress the ability of cytotoxic T-lymphocytes (CTL) to kill HIV-infected cells (Jones *et al.* 2014). Also due to the mutagenic potential of SAHA in in vitro assays, its long-term administration in humans is not allowed (Margolis 2011). Novel HDACis are then needed to consider to reduce toxicity and increase the efficiency.

Histone methylation may also reinforce HIV-1 latency. Specific histone methyltransferase inhibitors such as BIX01294 have been reported to reactivate latent HIV-1 with minimal toxicity and causing T cells activation (Imai *et al.* 2010).

Although promoting viral transcription by shock agents results in the reactivation of latent virus, it is not sufficient to eliminate the HIV reservoir. Stimulation of CTLs with HIV-1 specific antigens such as Gag peptides and the cytokine IL-2 prior to virus reactivation in latent CD4+ T cells has led to rapid and effective killing of the infected cells. Therefore, immune-based therapy has been proposed in combination with other shock agents to enhance the clearance of the reactivated reservoir (Shan *et al.* 2012). Also, IL-15 has been introduced as shock agent that exposes HIV-Infected resting CD4+ T Cells to recognition by CTLs (Jones *et al.* 2016).

1.3 Innate immunity

Innate immunity refers to nonspecific defense mechanisms that come into play immediately or within hours of a pathogen invading the body. The innate immune responses are the first line of defense against invading pathogens and are also required to initiate antigen-specific adaptive immune responses mediated by lymphocytes and antibodies (Alberts *et al.* 2002). In mammals, these responses include a group of proteins and phagocytic cells that recognize conserved features of pathogens which are not present in the uninfected host cells (Fig. 1.9). Of particular interest are restriction factors that are members of the tripartite motif (TRIM) protein superfamily (Ozato *et al.* 2008).

Cytokines released from a wide variety of cells play key roles in the regulation of the immune responses against viral infections. Over 80 known cytokines are secreted by infected cells (reviewed in (Coondoo 2011)). Interferons, the first cytokines discovered, are a family of mediators critically involved in stimulating the cellular immune system in response to viral infections (reviewed in (Le Page *et al.* 2000)).

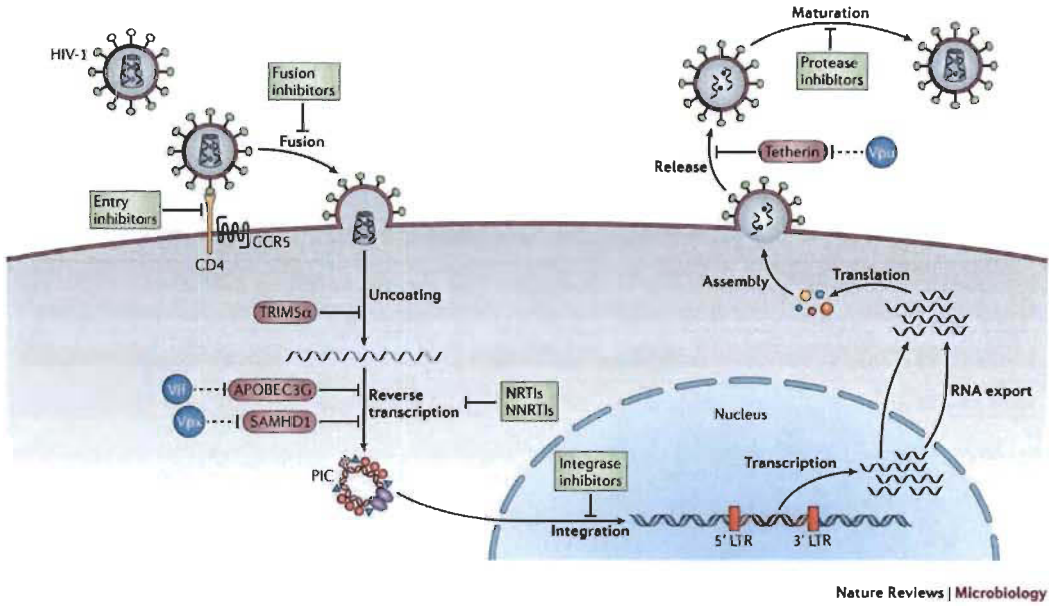

Figure 1.9 Stages of HIV-1 life cycles that are targeted by antiretroviral drugs or host restriction factors.
The figure illustrates the main steps in the HIV-1 replication cycle. The major families of antiretroviral drugs (green), and the step of the life cycle that they block, are indicated. Also shown are the key HIV restriction factors (TRIM5α, APOBEC3G, SAMHD1 and tetherin; red) and their corresponding viral antagonist (Vif, Vpx and Vpu; blue) (Barre-Sinoussi *et al.* 2013).

1.3.1 Interferons

Interferons (IFNs) were first described and named in 1957 by Issacs and Lindenmann (Isaacs & Lindenmann 1957). IFNs are a group of naturally occurring signal proteins that are made and released by host cells in response to pathogens (Abram *et al.* 2010) and activate multiple distinct signalling cascades inside the cells. IFNs are grouped in three major types (I, II, and III) according to the type of their cellular receptor. IFN-α, IFN-β, and IFN-ω are all belong to type I and bind to a specific cell surface receptor known as the IFN-α/β receptor (IFNAR) that contains two chains; IFNAR1 and IFNAR2 (de Weerd *et al.* 2007). IFN-I are produced by monocytes and fibroblasts generally in response to viral invasion (Parkin & Cohen 2001).

Upon binding to specific receptor, IFNs activate signal transducer and activator of transcription (STAT) complexes that regulate the expression of broad range of genes

named interferon stimulated genes (ISGs) which are involved not only in antiviral but also in immunomodulatory and antiproliferative activities (Platanias 2005). Many TRIM proteins are among ISGs and up-regulated by interferons, suggesting that they are involved in the innate immune response against viruses (Barr *et al.* 2008; Rajsbaum *et al.* 2008).

1.3.2 The TRIM protein family

Identified first in 1992 (Reddy *et al.* 1992), the conserved superfamily of TRIM proteins has more than 70 known members in humans, that share a highly conserved tripartite motif abbreviated as the RBCC, at the N-terminus: a RING domain, one or two B-box motifs, and a coiled-coil region (Fig. 1.10) (Reymond *et al.* 2001; Rajsbaum *et al.* 2014). This tripartite motif is associated with a variable C-terminal domain which often determines the specificity of interaction between TRIMs and associated proteins. TRIM proteins are involved in a wide array of intracellular functions such as apoptosis (Mandell *et al.* 2014), cell cycle regulation and antiviral responses (Meroni & Diez-Roux 2005; Short & Cox 2006). As a part of intrinsic immunity, TRIM proteins are the host restriction factors that provide the front line of defense against viral infection and their activity does not require any virus-triggered signaling or intercellular communication, although could, in principle, be upregulated in response to infection (Bieniasz 2004).

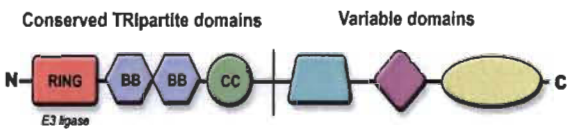

Figure 1.10 TRIM protein domains. TRIM proteins share a common organization: an N-terminal RING domain, followed by one or two B-boxes domain, a coiled-coil domain, and a variable C-terminal domain.

Ubiquitination is a post-translational modification used by eukaryotic cells to control the level of proteins through proteasome-mediated proteolysis. Three classes of enzymes are involved in this process; ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligase enzymes (E3) (Meroni & Diez-Roux 2005). The RING domain of many TRIM proteins is an E3 ligase, mediating the

conjugation of proteins with ubiquitin (Joazeiro & Weissman 2000) which is now known to be crucial for the anti-HIV functions of some TRIM proteins (Lienlaf *et al.* 2011). Ubiquitination of proteins can label them for degradation through a proteasome-dependent pathway (Lecker *et al.* 2006).

TRIM proteins can contain one or two consensus zinc-binding B-box domains which contribute to the restriction activity of TRIM proteins against HIV (Li *et al.* 2007). The B-box domains are almost always followed by a coiled-coil domain that mediates self-association of TRIM proteins collectively with B-box domain or heteromeric interactions with other proteins (Minucci *et al.* 2000; Reymond *et al.* 2001). Self-association of TRIM proteins through CC domains promotes the generation of higher order molecules that establish specific subcellular structures termed nuclear or cytoplasmic bodies. These high-molecular mass complexes are involved in many cellular activities including the restriction of viruses (Stremlau *et al.* 2004; Mische *et al.* 2005; Everett & Chelbi-Alix 2007).

Based on a consensus C-terminal domain organization, human TRIM proteins are classified into nine subsets, C-I to C-IX (Fig. 1.11) (Short & Cox 2006). However, the C-terminal sequences that are the most common in TRIM family members are the PRY (around 61 amino acids) and the SPRY (around 140 amino acids) domains (involved in 40 human TRIM proteins) (Grutter *et al.* 2006; Woo *et al.* 2006). In 39 human TRIMs, the PRY domain is fused to a SPRY to form the PRY/SPRY domain that is also termed B30.2 (Ozato *et al.* 2008) and is involved in protein-protein interactions between TRIM5 and retroviral capsids (Sebastian & Luban 2005; Perron *et al.* 2006).

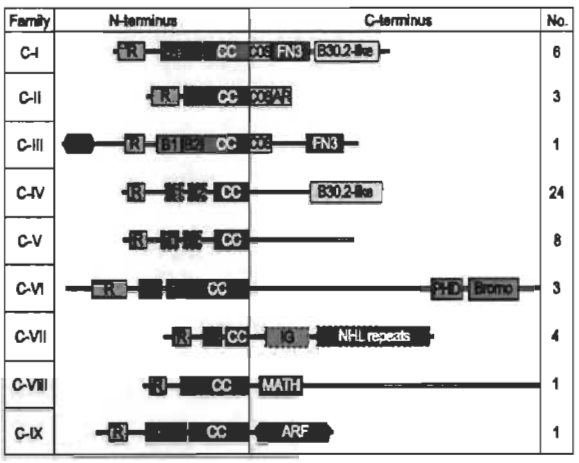

Figure 1.11 The domain based classification of human TRIM proteins.
(Short & Cox 2006)

Within the vast family of TRIM proteins, some have been studied and characterized as antiviral factors. TRIM32, for example, has been reported to bind HIV-1 Tat (Fridell *et al.* 1995). TRIM22 down-regulates transcription directed by the HIV-1 LTRs (Tissot & Mechti 1995) and TRIM1 isolated from humans restricts N-MLV in a CA-dependent way (Yap *et al.* 2004). Yet, the best studied members are TRIM5α and TRIM19 (PML) which are implicated in the restriction of many DNA and RNA viruses (Fig. 1.12).

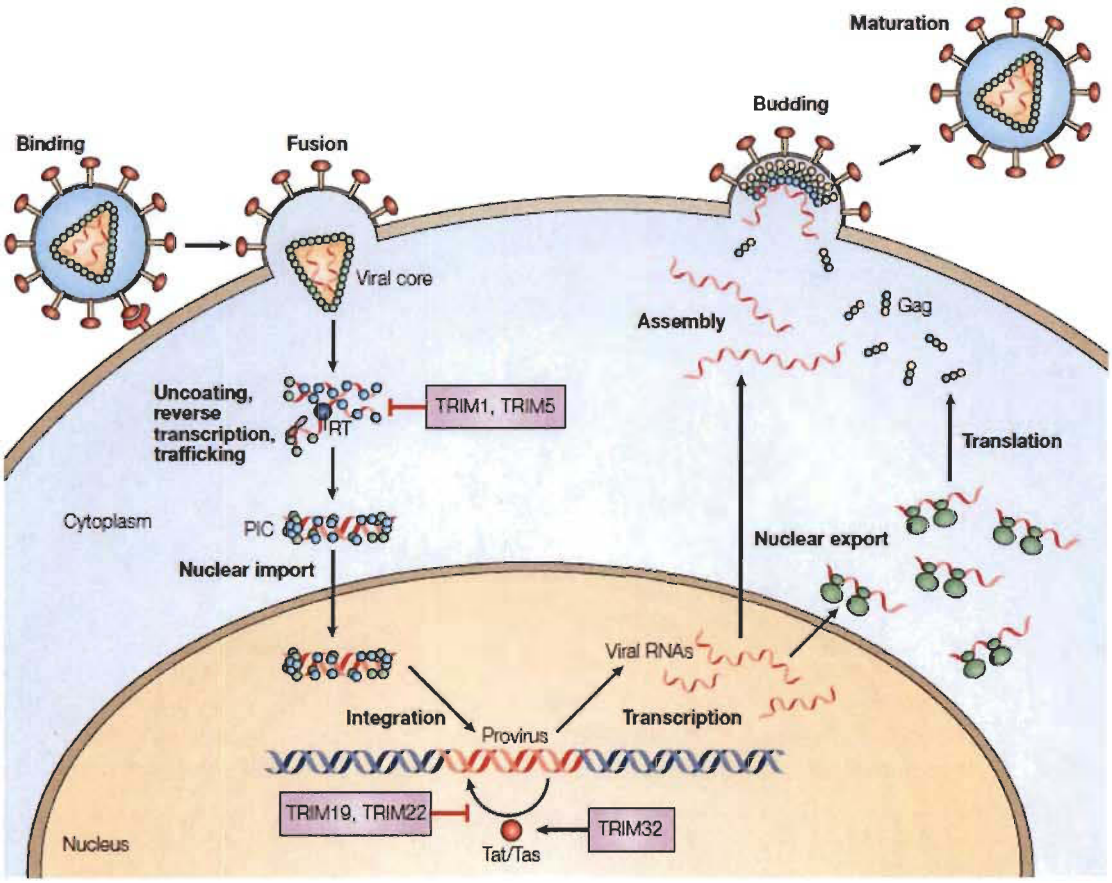

Figure 1.12 TRIM proteins interfering with the retroviral replicative cycle. Schematic representation of the replicative cycle of retroviruses. Arrows show at which stage of the cycle TRIM proteins are believed to interfere. PIC, pre-integration complex; RT, reverse transcriptase; Tat, transactivator (Nisole *et al.* 2005).

1.3.3 TRIM5α

TRIM5α is the largest isoform encoded by the *TRIM5* gene (Reymond *et al.* 2001) and belongs to subset C-IV that is characterized by the presence of a C-terminal B30.2 domain in addition to the RBCC motif. TRIM5α was found to restrict diverse retroviruses at early steps of the infection in a species-specific manner.

Whereas Human TRIM5α (huTRIM5α, formerly named Ref1 (Keckesova *et al.* 2004)) inhibits infection by the N-tropic murine leukemia virus (N-MLV) (Perron *et al.* 2004; Yap *et al.* 2004) and the equine infectious anemia virus (EIAV) (Hatziioannou *et al.* 2003), human cells are susceptible to infection by HIV, SIVmac (the simian immunodeficiency virus of macaques), and B-MLV (Besnier *et al.* 2003). On the other hand, TRIM5α protein from Old World monkeys such as rhesus macaque (rhTRIM5α) potently restricts HIV-1 infection (Stremlau *et al.* 2004; Sakuma *et al.* 2007; Black & Aiken 2010). SIVmac, HIV-1, and N-MLV also encounter a block in cells from many New World monkeys, caused by their respective TRIM5α orthologs (Hofmann *et al.* 1999; Yap *et al.* 2004). These species-specific restrictions against retroviruses are explained by different TRIM5α affinity to the viral capsid protein (Stremlau *et al.* 2004).

The viral target of TRIM5α is the N-terminal domain of capsid proteins, supporting the importance of capsid binding for restriction. For instance, huTRIM5α binds the retroviral capsid from the restricted N-MLV but not the non-restricted B-MLV (Sebastian & Luban 2005). Only TRIM5α, which is the longest isoform and possesses B30.2, exhibits antiretroviral activity. For example, rhTRIM5γ (another splice-variant of TRIM5) which lacks this domain, is inactive against HIV-1 (Stremlau *et al.* 2004). Replacement of a small segment in the B30.2 domain confers potent anti-HIV-1 activity to huTRIM5α (Perez-Caballero *et al.* 2005). Moreover, a single amino acid change, P332R, G330E or R335G, in the B30.2 domain of huTRIM5α is sufficient to restrict HIV-1 (Yap *et al.* 2005; Pham *et al.* 2010; Pham *et al.* 2013). Together, these observations demonstrate the importance of the B30.2 domain in targeting the specific retrovirus capsid. The contribution of the rhTRIM5α RING domain to the restriction of HIV is still under investigation; however,

mutations in this domain were shown to reduce the susceptibility of HIV-1 to rhTRIM5α (Stremlau *et al.* 2004). The TRIM5α CC domain is involved in the viral restriction activity as well. The CC domain also mediates the protein trimerization and changes that disrupt this structure proportionately affect the ability of TRIM5α to bind HIV-1 capsid complexes (Javanbakht *et al.* 2006). Mutating the B-box 2 domain also results in loss of retrovirus-restricting ability of rhTRIM5α (Diaz-Griffero *et al.* 2007).

One suggested model by which rhTRIM5α inhibits HIV-1 following capsid binding is by promoting its premature disassembly through the formation of a lattice or an array on top of the capsid, stemming from the ability of TRIM5α to undergo higher-order self-association (Fig. 1.13A) (Ganser-Pornillos *et al.* 2011). According to this model, TRIM5α assembly does not require the B30.2 domain, but both B-box 2 and CC domains are necessary to induce dimerization and higher-order assembly. The B30.2 domain is likely to make direct contact with capsid protein, providing specificity to TRIM5α by specific protein-protein interactions. The RING domain is localized at the surface of the lattice and mediates the lattice disassembly by ubiquitination of TRIM5α and subsequent degradation by the proteasome (Fig. 1.13B). This dissociation also correlates with an acceleration of HIV-1 capsid disassembly i.e. premature uncoating. Timely uncoating of HIV-1 core plays critical role in successful infection processes (Forshey *et al.* 2002).

Arsenic trioxide (As_2O_3) stimulates retroviral infectivity when added to the target cells at the time of infection. This effect of As_2O_3 has been observed only in cells bearing TRIM5α-mediated restriction activity (Berthoux *et al.* 2003). Moreover, knockdown of human TRIM5α by RNA interference eliminated the As_2O_3 effect, demonstrating that the drug acts by modulating the activity of this retroviral restriction factor. Later it was shown that the stimulatory effect of As_2O_3 on HIV-1 infection involves the suppression of TRIM5-mediated restriction activity but is also dependent on other cell line-specific factors (Sebastian *et al.* 2006).

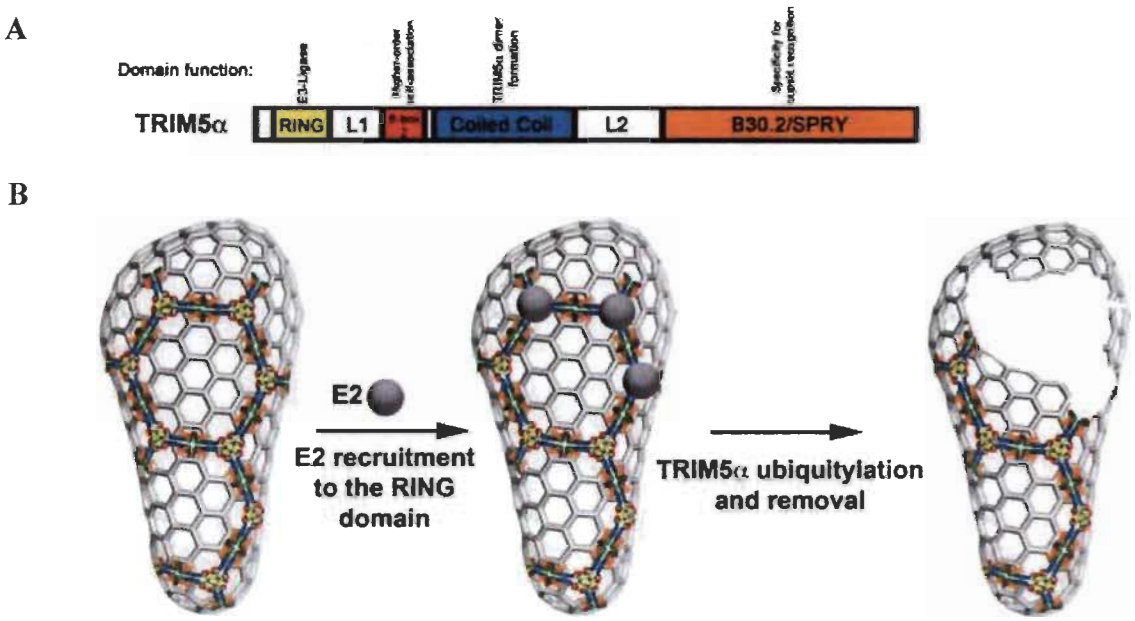

Figure 1.13 A model for acceleration of uncoating mediated by TRIM5α. (A) The different domains of the TRIM5α protein with their known functions. L1 and L2, linker. (B) The proposed model of TRIM5α-mediated uncoating of HIV-1 (Diaz-Griffero 2011).

1.3.4 TRIM19/PML

The human promyelocytic leukemia (PML, also known as TRIM19) gene was initially identified as the fusion partner of the retinoic acid receptor α (RARA) gene due to a chromosomal translocation found in patients with acute promyelocytic leukemia (APL) (de The *et al.* 1990; de The *et al.* 1991). *PML* is located on chromosome 15 and is approximately 35 kb in length, and composed of nine exons. The expressed proteins belong to subset C-V of TRIM proteins (Fig. 1.14A) (Fagioli *et al.* 1992). The gene is alternatively spliced in exon 6 to 9 leading to the expression of seven isoforms, I to VII (Fagioli *et al.* 1992; Jensen *et al.* 2001). All isoforms share the N-terminal TRIM domains, RBCC, encoded by exons 1 to 3 (Fig. 1.14B) (Reymond *et al.* 2001). The nuclear localization signal (NLS) is located in exon 6 which is skipped in isoform VII, yielding the cytoplasmic isoform (Lin *et al.* 2004). The other six isoforms localize mostly in the nucleus and are found either in the soluble fraction or within insoluble subnuclear structures known as nuclear bodies (NBs). The most abundant isoform is PML-I (Lallemand-Breitenbach & de The 2010).

In its NB-associated form, PML is involved in a wide range of biological processes, including transcription (Kentsis *et al.* 2001), apoptosis (Krieghoff-Henning & Hofmann 2008), cell cycle control (Gamell *et al.* 2014) and genome integrity (Seker *et al.* 2003; Xu

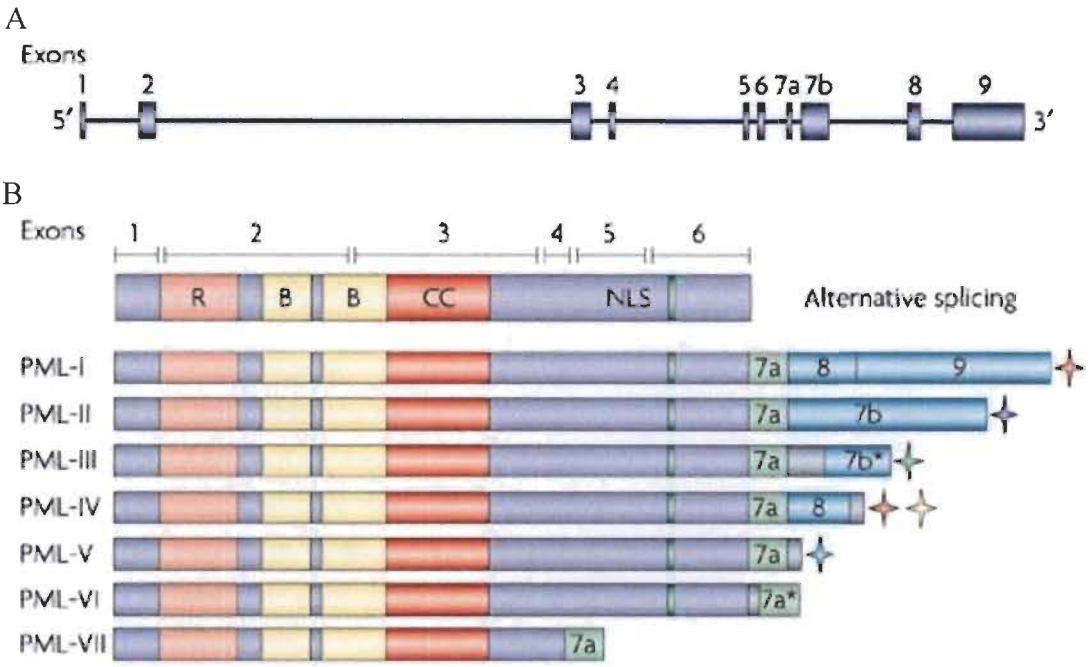

Figure 1.14 The PML gene and protein isoforms.
(A) The PML gene is located on chromosome 15q22 and contains 9 exons. (B) Alternative splicing at C-terminal leads to generation of 7 different isoforms. Six isoforms are predominantly nuclear due to presence of a NLS in exon 6. However, isoform VII is located in cytoplasm due to lack of exon 6. Asterisk represents frameshift (Bernardi & Pandolfi 2007).

et al. 2003; Everett 2006; Yeung *et al.* 2012). PML is associated with more than 150 proteins in NBs that are involved in a vast array of functions (Van Damme *et al.* 2010). Interestingly, TRIM5α proteins of two Old World primates, humans and rhesus monkeys, are transported into the nucleus and accumulated in nuclear bodies that contain PML as well (Diaz-Griffero *et al.* 2011). However, it is not clear yet why TRIM5α colocalizes with PML in NBs and what would be the impact of this colocalization on TRIM5α-mediated restriction of HIV-1.

Expression of PML is significantly up-regulated by type I and II IFNs (Chelbi-Alix *et al.* 1998; Regad & Chelbi-Alix 2001). PML is involved in IFN-induced antiviral responses (Kim & Ahn 2015) and its expression is essential for the ability of type I and II IFNs to induce programmed cell death (Wang *et al.* 1998b).

1.4 PML NBs

PML NBs, also known as nuclear domain 10 (ND10), are matrix-associated domains found typically as 10 to 30 small dots in the nucleus of most mammalian cell-lines and tissues and are spheres of 0.1-1.0 μm in diameter (Lallemand-Breitenbach & de The 2010). NBs recruit a variety of proteins other than PML such as Daxx, Sp100 and p53. However, PML is the main constitutive member and is required for the formation and integrity of NBs (Ishov *et al.* 1999). PML-KO cells do not contain PML NBs and other NB proteins fail to co-localize in NBs and instead exhibit more diffuse patterns (Ishov *et al.* 1999; Zhong *et al.* 2000). In a mature PML NB, PML forms the outer shell and creates a partition within the nucleoplasm, and other proteins are found within its clear core (Fig. 1.15) (LaMorte *et al.* 1998; de The *et al.* 2012). The number, size and composition of the PML NBs change during the cell cycle (Bernardi & Pandolfi 2007).

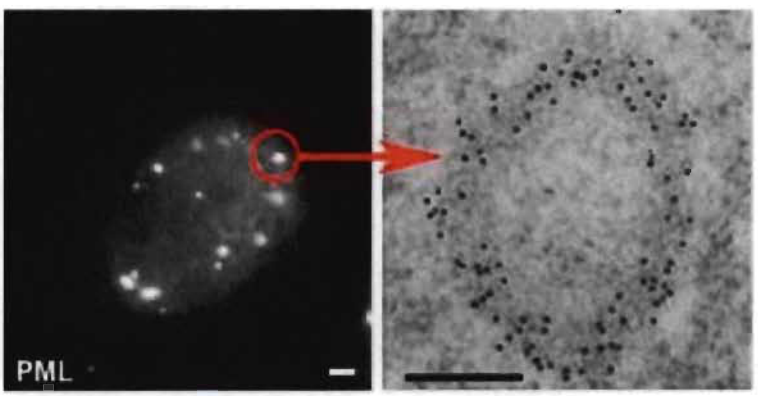

Figure 1.15 PML NB.
From left to right: immunofluorescence and electron microscopy views in CHO cells stably expressing PML. PML is both diffusely distributed in the nucleoplasm and aggregated in NBs. The red arrow points to an individual body, analyzed by electron microscopy. Bar, 1 μm (left). Electron microscopy show PML distribution in the electron-dense NB shell. Bar, 0.5 μm (right) (de The *et al.* 2012).

1.4.1 Formation and disruption

SUMOylation is a post-translational modification in which one of the four isoforms of SUMO (Small Ubiquitin MOdifier) protein is conjugated to a target protein as a single SUMO molecule (SUMO-1) or in polymeric chains (SUMO-2/3) (Vertegaal 2007). SUMOylation can affect a protein structure and subcellular localization. PML is conjugated with SUMO-1 on 3 Lysine residues; K65 in the RING, K160 in the B-box1, and K490 in the NLS (Kamitani *et al.* 1998). The RING domain was shown to be essential for PML self-SUMOylation (Quimby *et al.* 2006; Shen *et al.* 2006). PML also includes a SUMO-interacting motif (SIM) close to its C-terminus (Cho *et al.* 2009). SUMO modification regulates the incorporation of PML in NBs (Muller *et al.* 1998; Shen *et al.* 2006). Moreover, several protein partners are not recruited to NBs in the absence of SUMOylation (Muller *et al.* 1998; Fu *et al.* 2005; Lallemand-Breitenbach *et al.* 2008). However, a SUMOylation-deficient mutant of PML (PML 3K) which bears mutations in the three SUMOylation positions still shows distinct punctate localization in the nucleus. Subsequent analyses revealed that the SIM is also involved in the formation of NBs through its binding to the SUMOylated form of NB-associated partners (Fig. 1.16) (Shen *et al.* 2006). Regarding these observations, a proposed model for the formation of NBs is based on the SUMOylation of PML and non-covalent binding of the SUMOylated protein

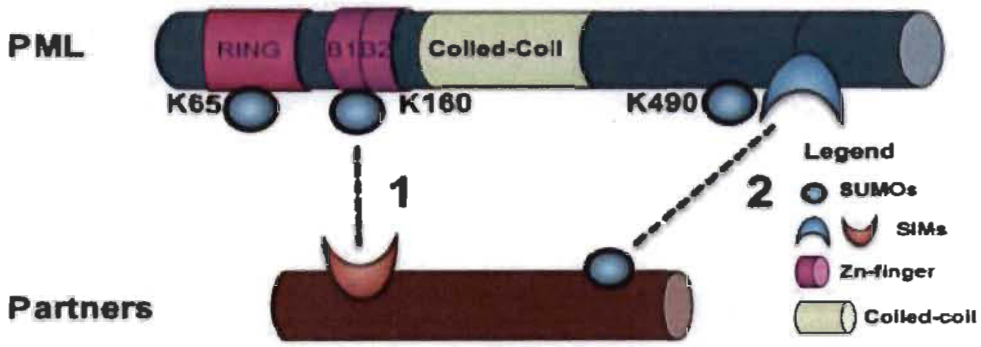

Figure 1.16 Interactions of partner proteins with PML through SUMO–SIM links.
The interactions are shown as dotted lines. PML primarily interacts with the partner protein through K160-SIM (1), however the SUMOylated partner protein interacts also with the PML SIM domain at C-terminal (2) (de The *et al.* 2012).

to another PML molecule through its SIM. This process constitutes the nucleation event of the PML NB formation, subsequently resulting in the recruitment of SUMOylated and/or SIM-containing NB partners to the PML NBs (Fig. 1.17) (Shen *et al.* 2006).

Similar to their formation, disruption of PML NBs also involves the SUMOylation pathway. Polysumoylated forms of PML are specifically recognized and ubiquitinated by ubiquitin ligases RNF4 and RNF111 in mammals (Tatham *et al.* 2001; Erker *et al.* 2013), leading to the ubiquitination-induced degradation of PML by the proteasome (Rock *et al.* 1994).

Disruption of PML NBs has also been observed in a variety of diseases such as neurodegenerative disorders, viral infections, or APL. In the latter, a t(15;17) chromosomal translocation frequently associated with this disease. The translocation breakpoint always lays between exons 3 and 6 of PML leading to loss of SIM (located in exon 7) in the oncogenic chimeric protein PML/RARA (Shen *et al.* 2006). The fusion protein is not SUMO-1 modified at its C-terminal K490 as well (Shen *et al.* 2006). The fusion protein PML/RARA disrupts NBs and exhibits altered transcription factor

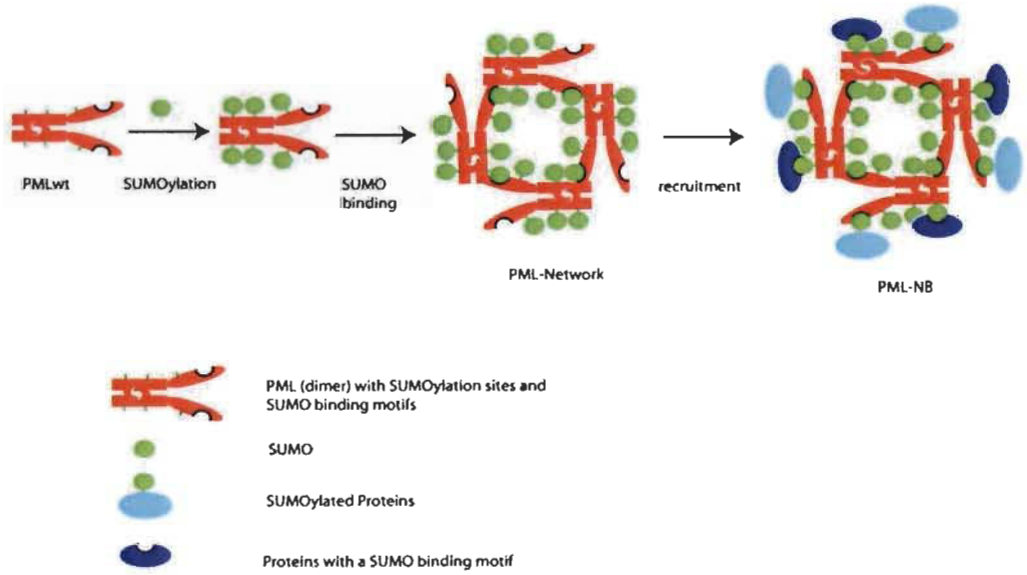

Figure 1.17 A proposed model of PML NB formation.
The model shows SUMOylation of PML and non-covalent binding of the SUMOylated protein to another PML molecule through its SIM, resulting formation of PML network and PML NB (Shen *et al.* 2006).

behavior compared to normal cells demonstrating the key role of PML in tumor suppression (Koken *et al.* 1994; Salomoni & Pandolfi 2002). Moreover, overexpression of PML/RARA disrupts normal PML NBs in human cells due to the negative dominant effect of the fusion protein (Yeung *et al.* 2012). Loss of SIM and of C-terminal SUMOylation may explain why the fusion protein fails to form the NBs.

The traditional Chinese medicine arsenic trioxide (As_2O_3) has been used as a therapeutic agent to cure APL (Fig. 1.18A). As_2O_3 was shown to trigger the proteasome-dependent degradation of PML and PML/RARA by promoting specific SUMOylation of PML at K160 (Lallemand-Breitenbach *et al.* 2001). More precisely, arsenic binds directly to cysteine residues in zinc fingers located within the RBCC domain of PML/RARA and PML, inducing PML oligomerization and increasing its interaction with the SUMO-conjugating enzyme UBC9, resulting in enhanced SUMOylation (Zhang *et al.* 2010). As described above, polysumoylated PML is ubiquitinated by RNF4 and consequently degraded by proteasome (Fig. 1.18B).

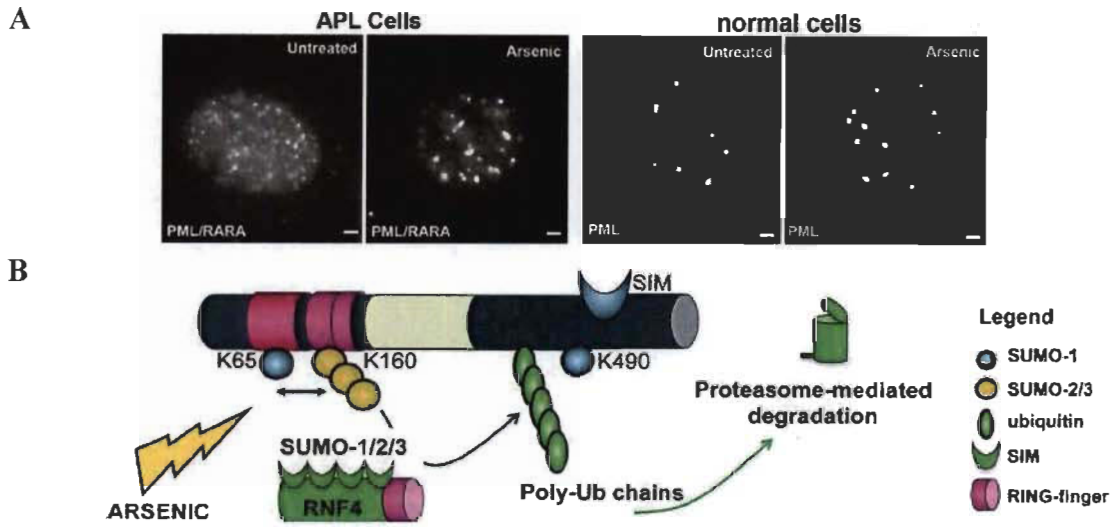

Figure 1.18 PML and PML/RARA degradation by As_2O_3.
(A) Immunofluorescence views of PML NBs in APL (left) and normal (right) cells before and after As2O3 treatment. As_2O_3 induces NBs aggregation in both normal and APL cell. Bar, 1 μm. (B) As_2O_3 enhances SUMOylation of PML on K65 and K160. The ubiquitin E3 ligase is then recruited onto hypersumoylated PML to induce its ubiquitination. PML is finally degraded by the proteasome machinery (de The *et al.* 2012).

1.4.2 Implications in regulation of chromatin structure and transcription

The observation that viruses transcribe their genomes adjacent to PML NBs suggests that PML NBs also function in regulating gene transcription (Ishov *et al.* 1997). Moreover, latent HSV-1 genome was found to associate selectively with PML NBs and the centromeres, supporting the idea that these nuclear domains may influence directly the behavior of latent viral genomes and their transcriptional activity (Catez *et al.* 2012). A recent study also revealed that in the nucleus of latent CD4+ T cells, the HIV-1 provirus is located in the vicinity of PML NBs, and this interaction inhibits HIV-1 gene expression (Lusic *et al.* 2013).

Highly acetylated chromatin can be found associated with PML NBs (Boisvert *et al.* 2000), which is consistent with the fact that PML NBs contain the histone acetyltransferase CBP (CREB binding protein) (LaMorte *et al.* 1998). Acetylation of

histones relaxes the facultative heterochromatin, resulting in gene transcription. On the other hand, another PML NBs component, Daxx, forms a chromatin remodeling complex with histone deacetylases, contributing to transcriptional silencing and gene suppression (Hollenbach *et al.* 2002). This regulation of acetylation may underlie the role of PML NBs in modulating virus latency (Lusic *et al.* 2013).

Several observations provide evidence supporting the involvement of PML NBs and their components in the regulation of transcription and chromatin architecture. For instance, single-stranded DNA (ssDNA) represents an intermediate in several DNA repair mechanisms and is thought to play a crucial role in checkpoint signaling. PML NBs recruit ssDNA molecules in response to UV-induced exogenous DNA damage (Boe *et al.* 2006). Moreover, several PML NB components have been shown to relocate to sites of DNA damage along with PML (Carbone *et al.* 2002; Barr *et al.* 2003; Davalos *et al.* 2004; Park *et al.* 2005).

1.4.3 Roles in antiviral defense

Viruses have evolved a variety of strategies that allow them to reach two goals: using the host machinery to replicate, and undermining the cellular defense mechanisms such as IFN-induced antiviral defenses. There are several indications that PML is linked to the IFN system and contributes to antiviral defenses. Both PML and Sp100, two constitutive components of NBs, are up-regulated by type I and II IFNs (Lavau *et al.* 1995; Stadler *et al.* 1995; Guldner *et al.* 1999). Moreover, the number, composition, and morphology of NBs is altered in response to IFNs and virus infection (Lavau *et al.* 1995; Everett & Chelbi-Alix 2007). PML has been shown to negatively affect the replication of many DNA and RNA viruses (reviewed in (Everett & Chelbi-Alix 2007)). Therefore, the disorganization of PML NBs induced by several viruses may represent a viral strategy to evade IFN-induced antiviral defenses. As detailed below, many studies revealed the potential activity of PML against several DNA and RNA viruses and the viral strategies to interfere with PML NBs.

Many viruses encode proteins that associate with PML NBs and cause the disruption or lysis of these nuclear domains, hence promoting viral gene expression and disabling the host antiviral defense. However, the genome of some nuclear-replicating DNA and RNA viruses associate preferentially with NBs with positive effects on viral gene expression and replication. The most widely studied example is herpes simplex virus (HSV)-1. Very soon after the discovery of PML, a pioneering study by Maul et al. showed that the outcome of HSV-1 infection was the disruption of PML NBs and that an immediate early gene 1 product of the virus, ICP0, was sufficient for this effect (Maul *et al.* 1993). Subsequent studies confirmed this observation and revealed that the ICP0 RING domain was necessary for the disruption of PML NBs (Everett & Maul 1994; O'Rourke *et al.* 1998). ICP0 acts as an ubiquitin E3 ligase and targets not only SUMO-modified PML, but also Sp100, resulting in the proteasome-dependent degradation of both (Chelbi-Alix & de The 1999; Boutell *et al.* 2011). ICP0-deficient viruses are not able to disrupt NBs and are particularly sensitive to IFN-induced inhibition (Mossman *et al.* 2000), suggesting that ICP0-mediated disruption of PML NBs constitutes a viral strategy to bypass the host defense systems mediated by IFNs.

In recent decades, more viral proteins causing the disruption of PML NBs have been identified. Of particular interest is the major immediate-early protein 1 (IE1) encoded by the human cytomegalovirus (HCMV) that abrogates the SUMOylation of PML and Sp100 and inhibits PML-mediated transcriptional repression (Muller & Dejean 1999). Interestingly, the IE1-mediated disruption of PML NBs does not lead to proteasome-dependent degradation (Xu *et al.* 2001). Another example is the Epstein-Barr virus (EBV) immediate-early protein BZLF1 that also reduces the amount of SUMO-modified PML. Disruption of PML NBs is important for efficient lytic replication of EBV (Adamson & Kenney 2001).

The high frequency of PML NB targeting by viral proteins suggests that depletion of these nuclear domains abolishes their antiviral activity. Evidence for such a function was obtained by using PML-/- primary mouse embryonic fibroblasts (MEFs). Compared to the wild-type control cells, the PML-knockout (KO) cells produced 20 times more

rabies virus and expressed viral proteins at higher levels (Blondel *et al.* 2002). PML-KO mice were also 10 times more susceptible to infection with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV) (Bonilla *et al.* 2002). Moreover, overexpression of PML was shown to interfere with the replication of VSV and influenza virus in the absence of IFN (Chelbi-Alix *et al.* 1998). Thus, PML is a contributor to innate immunity against some viruses and modulates host susceptibility to viral infections.

Although most DNA viruses have evolved mechanisms to evade repression from PML, infection by papillomaviruses (PVs) was shown to be enhanced by expressing PML in PML-KO cells (Day *et al.* 2004). The presence of PML NBs was associated with increased papillomavirus transcription.

The antiviral activity of PML has also been observed in the case of RNA viruses. Overexpression of PML leads to a drastic decrease in gene expression of a complex retrovirus, the human foamy virus (HFV). PML represses HFV transcription by complexing the HFV transactivator, Tas, preventing its direct binding to viral DNA. This repression depends on PML, since it is not observed in IFN-treated, PML-deficient MEFs, also demonstrating the important role of PML in IFN-induced antiviral states. Moreover, this inhibitory effect does not necessitate the localization of PML in NBs (Regad *et al.* 2001). Although these observations suggest a negative role for PML NBs in the viral life cycle, the propagation of the hepatitis C virus (HCV) was recently shown to be dependent upon the presence of PML (Kuroki *et al.* 2013). PML down-regulation did not affect HCV RNA levels in human cells, whereas the release of viral core proteins into the cell culture supernatant was significantly suppressed in PML knock-down cells. This suggested that PML affected a step other than the genome replication of HCV. Immunofluorescence and confocal microscopy analyses of the viral core protein localization determined that PML is required for the late steps of the HCV life cycle (Kuroki *et al.* 2013). Therefore, the function of PML in RNA virus infection is sometimes detrimental or beneficial.

1.5 Importance, hypothesis, and objectives

Despite significant advances in the control and prevention of the spread of the virus, the HIV pandemic still represents one of the greatest plagues in human history and a major challenge for medicine, public health and biological research. Although HAART suppresses the replication of the virus and improves the quality of the patient's life dramatically, a cure has not yet been achieved. Drug side effects, drug resistance, drug toxicity, and low levels of continued viral replication in the setting of HAART are some of the most important remaining challenges to overcome. HIV-1 persistence in reservoirs represents the major barrier for its eradication (Marcello 2006) as virus reactivation from latency occurs 5-8 days after ART interruption (Pinkevych *et al.* 2015) and leads to the viral load rebound. The molecular mechanisms that lead to HIV-1 reactivation are relatively well characterized, but the determinants of viral latency itself remain incompletely known. Importantly, continuing viral replication in reservoirs in the presence of current antiviral drugs leads to accumulation of drug resistant mutants. For that reason, developing new drugs that target different steps of the HIV life cycle is of high priority.

Because eradication of HIV-1 from an infected individual cannot be achieved by current drug regimens, identification of new host factors that prevent HIV-1 infection and/or regulate its gene expression can provide future targets for developing new drugs which either inhibit HIV-1 infection or target the latent provirus. Of particular interests are some TRIM proteins that are known to constitute a line of defense against viral infection in mammals (Nisole *et al.* 2005). TRIM5α and TRIM22, for instance, interfere with incoming HIV-1 at different stages of infection to inhibit its propagation (Tissot & Mechti 1995; Barr *et al.* 2008; Nakayama & Shioda 2010; Merindol & Berthoux 2015). TRIM19, better known as PML, is a TRIM member whose antiviral activity has not been well studied in lentivirus infections yet.

As most DNA viruses and retroviruses replicate in the nucleus, it is not unexpected that these viruses hijack nuclear structures such as NBs for their own replication (Moller

& Schmitz 2003). PML is the main constitutive protein of the NBs, thus it can be a potential target for HIV-1. However, very little is known about the possible role of PML in HIV-1 infection. A better understanding about this under-explored area might help the development of new drugs targeting different steps of the virus infection and might make important contributions to the global efforts to find a cure for HIV/AIDS.

1.5.1 Objective I: To study the role of PML (TRIM19) in lentiviral infections

PML was shown to interfere with the replication of HFV, a complex retrovirus like HIV, in human cells by repressing viral transcription, even in the presence of IFN (Regad *et al.* 2001). It was reported that HIV-1 infection induces a rapid and transient redistribution of PML into the cytoplasm, where PML may transiently colocalize with incoming HIV-1 PICs (Turelli *et al.* 2001; Dutrieux *et al.* 2015; Kahle *et al.* 2015). Also, a recent study revealed that in the nucleus of some constitutively HIV-latent CD4+ J-Lat T cells, the HIV-1 provirus associates with PML NBs and this proximity to PML NBs regulates latency (Lusic *et al.* 2013). Thus, we hypothesized that PML might be a part of innate immunity mechanisms against HIV that inhibits its infection. We also hypothesized that PML promotes HIV latency in nucleus. We first investigated the role of PML in lentivirus infectivity in Sup-T1, a human T cell line, and in primary murine cells, MEF (mouse embryonic fibroblast). Interventions that down-regulated PML in Sup-T1 moderately increased the lentiviral infection. Moreover, knocking out PML in MEFs led to a drastic increase in infection of lentiviruses. We showed that this PML-mediated inhibition was relevant to the IFN-induced restriction against lentiviruses. In addition, we demonstrated that both murine and human PML participated in HIV-1 transcriptional silencing. PML inhibited HIV-1 and other lentiviruses, as part of the IFN-induced pathways and at two distinct steps; reverse transcription and transcription.

Based on the results presented in chapter II, we hypothesized that the slight effect of PML knock down on HIV-1 infectivity in Sup-T1 might be due to incomplete depletion of the protein that was in adequate to restrict HIV-1. Accordingly, we were tempted to generate PML-KO cell lines that provided a model to study the effect of PML deletion on

retrovirus infection in human. The CRISPR-Cas9 system is an adaptive immune system that exists in a variety of microbes to degrade invading viral and plasmid DNA (Cong & Zhang 2015). Recently, a new version of this system has been created to use for genome-editing in mammalian cells (Mali *et al.* 2013) and it has been widely used to knock out targeted genes using specific guide RNA (gRNA) in variety of cell lines (Cho *et al.* 2013; Shin *et al.* 2016; Shinmyo *et al.* 2016). We benefited and optimized this system to make PML inoperative in several human cell lines, including T cells and myeloid cells. The infectivity of retroviruses was then assessed in WT and generated PML-KO cells. Our results revealed that PML had no role in intrinsic immunity against retroviruses in human cells. Also, we found that PML was not implicated in the IFN-induced innate immunity against retroviruses in human cells. Overall, we demonstrate that although hPML is involved in the restriction of lentiviruses in mouse cells, it does not generally act as an HIV-1 restriction factor in human cells, suggesting an isoform-specific and strongly cellular context-specific mechanism of restriction.

1.5.2 Objective II: To study the implication of PML (TRIM19) in TRIM5α-mediated restriction of HIV-1

As_2O_3 increases the SUMOylation of PML, promoting its degradation through proteasome-dependent pathway. Also, As_2O_3 has been shown to interact with TRIM5α-mediated restriction of retroviruses (Berthoux *et al.* 2003; Sebastian *et al.* 2006) in a TRIM5α-independent way (Pion *et al.* 2007). Yet, the mechanism by which arsenic counteracts with retroviral infections is not well understood. Recently, several TRIM5 proteins were found to shuttle into the nucleus of human cells and participate in NBs that include PML as well (Diaz-Griffero *et al.* 2011). This observation led us to hypothesize that PML may be involved in the TRIM5α-mediated restriction of retroviruses and that As_2O_3 may interfere with the restriction by triggering PML degradation. To address this, we overexpressed human and rhesus TRIM5α in WT and PML-KO MEFs followed by infection with lentiviruses. Our results demonstrate that rhTRIM5α restricted HIV-1 potently in the presence or absence of PML. We next investigated the effect of As_2O_3 treatment on lentivirus infectivity in human cell lines that were WT or knocked out for

PML. Our results show that As_2O_3 rescues retroviral infections to levels which were similar for both WT and PML-KO cells, suggesting that another cellular factor was targeted by As_2O_3. In conclusion, our findings reveal that PML inhibits lentiviruses depends on which cell context it is expressed. Moreover, PML is not involved in the rhTRIM5-mediated restriction of HIV-1.

Chapter II contains a study showing that PML (TRIM19) is involved in innate and intrinsic immunity against lentiviruses and promotes viral latency in mouse.

CHAPTER II

THE INTERFERON-INDUCED ANTIVIRAL PROTEIN PML (TRIM19) PROMOTES THE RESTRICTION AND TRANSCRIPTIONAL SILENCING OF LENTIVIRUSES IN A CONTEXT-SPECIFIC, ISOFORM-SPECIFIC FASHION

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2.1 Contributions

Masroori, Merindol and Berthoux designed the study. Masroori and Merindol performed the experiments. Masroori, Merindol and Berthoux analyzed the data and prepared the manuscript.

2.2 Abstract

The promyelocytic leukemia (PML) protein, a type I interferon (IFN-I)-induced gene product and a member of the tripartite motif (TRIM) family, modulates the transcriptional activity of viruses belonging to various families. Whether PML has an impact on the replication of HIV-1 has not been fully addressed, but recent studies point to its possible involvement in the restriction of HIV-1 in human cells and in the maintenance of transcriptional latency in human cell lines in which HIV-1 is constitutively repressed. We investigated further the restriction of HIV-1 and a related lentivirus, SIVmac, by PML in murine cells and in a lymphocytic human cell line. In particular,

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we studied the relevance of PML to IFN-I-mediated inhibition and the role of individual human isoforms.

We demonstrate that both human PML (hPML) and murine PML (mPML) inhibit the early post-entry stages of the replication of HIV-1 and a related lentivirus, SIVmac. In addition, HIV-1 was transcriptionally silenced by mPML and by hPML isoforms I, II, IV and VI in MEFs. This PML-mediated transcriptional repression was attenuated in presence of the histone deacetylase inhibitor SAHA. In contrast, depletion of PML had no effect on HIV-1 gene expression in a human T cell line. PML was found to contribute to the inhibition of HIV-1 by IFN-I. Specifically, IFN-α and IFN-β treatments of MEFs enhanced the PML-dependent inhibition of HIV-1 early replication stages.

We show that PML can inhibit HIV-1 and other lentiviruses as part of the IFN-I-mediated response. The restriction takes place at two distinct steps, i.e. reverse transcription and transcription, and in an isoform-specific, cellular context-specific fashion. Our results support a model in which PML activates innate immune antilentiviral effectors. These data are relevant to the development of latency reversal-inducing pharmacological agents, since PML was previously proposed as a pharmacological target for such inhibitors. This study also has implications for the development of murine models of HIV-1.

2.3 Introduction

In mammals, many effectors are involved in the innate immune response to pathogens, including viruses. Of particular interest are restriction factors that are members of the tripartite motif (TRIM) protein superfamily. Several of the TRIM superfamily members are upregulated by IFN-I, suggesting that they might be involved in antiviral innate immunity (reviewed in (Ozato et al., 2008)). PML, also known as TRIM19, is a member of this family of proteins. PML was initially identified as part of a hybrid protein that also contains retinoic acid receptor α (RARα) and that causes acute promyelocytic leukemia (de The et al., 1991; Goddard et al., 1991; Pandolfi et al., 1991). PML is

expressed in all cell lines tested (Stadler et al., 1995) and localizes to the nucleus; it is found both in the nucleoplasm and in association with a nuclear multiprotein structure called the nuclear body (NB) (Borden, 2002; Lallemand-Breitenbach & de Thé, 2010). In addition to PML, NBs include several other proteins, but the integrity of this structure depends on the presence of PML (Salomoni & Pandolfi, 2002). The transcription of some PML NB proteins, including PML and Sp100, is upregulated by interferons (Chelbi-Alix et al., 1995; Guldner et al., 1992; Stadler et al., 1995) and contributes to cellular defense mechanisms (Everett, 2006).

The interactions between PML and/or PML NBs and viruses have been well documented. Soon after the discovery of PML NBs, Maul and colleagues showed that herpes simplex virus type 1 (HSV-1) causes the cellular redistribution of PML from PML NBs (Maul et al., 1993). Further investigations demonstrated that the HSV-1 immediate-early (IE) gene product ICP0 localizes to and disrupts PML NBs, resulting in an increase in viral gene expression (Maul & Everett, 1994). In human cytomegalovirus (HCMV)-infected cells, the PML NB-associated protein Daxx (Death domain-associated protein) silences viral immediate-early gene expression, but this antiviral mechanism is counteracted by the HCMV protein pp71 (Saffert & Kalejta, 2006; Woodhall et al., 2006). It has also been reported that constitutive overexpression of PML in mouse cells induces resistance to infection by RNA viruses, such as vesicular stomatitis virus (VSV) and influenza A (Chelbi-Alix et al., 1998). Furthermore, IFN-induced overexpression of PML in wild-type (WT) mouse embryonic fibroblasts (MEFs) represses the transcription of human foamy virus (HFV), a retrovirus, by forming a complex with the HFV transactivator, Tas, thereby preventing the direct binding of Tas to viral DNA (Regad et al., 2001). Accordingly, this inhibitory mechanism is not observed in PML knockout (KO) cells. In contrast to the antiviral activities often associated with PML, it was recently shown that depleting PML reduces the production of infectious hepatitis C virus particles, indicating that PML may enhance virus particle production (Kuroki et al., 2013). Likewise, establishment of human papillomavirus (HPV) is enhanced by PML expression in the early part of the life cycle (Day et al., 2004). Whether PML modulates the permissiveness to HIV-1 and other lentiviruses has been controversial (Berthoux et al.,

2003; Turelli et al., 2001), but recent reports have converged toward an inhibitory role for PML (Dutrieux et al., 2015; Kahle et al., 2015).

Although antiretroviral therapy (ART) is capable of decreasing HIV-1 viral load to levels below the limit of detection in many patients, the virus is not eliminated and interruption of ART almost always leads to a rapid viral rebound and progression to AIDS (Holkmann Olsen et al, 2007). HIV-1 is capable of establishing a state of latent infection when activated CD4+ T-cells (the major target of HIV-1) become infected and then revert back to a resting memory state (Chomont et al., 2009; Han et al., 2004; Soriano-Sarabia et al., 2014). These infected resting T-cells show low or absent viral gene expression and provide a viral reservoir that is protected from immune clearance and ART (reviewed in (Ruelas & Greene, 2013)). Current strategies to eradicate this reservoir aim at reactivating the latent proviruses by using various agents such as the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA; Vorinostat) (Archin et al., 2009) and the acetaldehyde dehydrogenase inhibitor disulfiram (Xing et al., 2011), often combined with protein kinase C agonists (Laird et al., 2015). Despite the current interest in pharmacological strategies to disrupt the quiescence of latent proviruses, the mechanism by which HIV-1 persists in the presence of ART is not well understood. In a recent study, the proximity of HIV-1 proviruses to PML NBs was found to correlate with the extent of HIV-1 gene expression silencing in a T cell-based HIV-1 latency model. Accordingly, PML degradation resulted in the activation of viral transcription following proviral displacement from PML NBs (Lusic et al., 2013). Here, we examined the involvement of PML in the restriction of HIV-1 in human and murine cells. Our results provide evidence that PML is a component of the innate immune response to lentiviruses and may participate in HIV-1 gene silencing and latency.

2.4 Materials and methods

Cell culture. Immortalized PML-KO and WT MEFs were a generous gift from Pier P. Pandolfi (Wang et al., 1998). Crandell-Rees feline kidney (CRFK), human embryonic kidney (HEK) 293T and MEF cells were maintained in Dulbecco's modified

Eagle's medium (DMEM; HyClone, Thermo Scientific, USA). SupT1 cells were maintained in RPMI 1640 (HyClone). All culture media were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (HyClone).

Plasmids, transfections and transductions. To transduce mPML using a retroviral vector, total RNA was extracted from WT MEF cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA synthesis was conducted using 2 μg of total RNA, random hexamers and the SuperScript III first-strand synthesis kit (Invitrogen) following treatment with DNase I (NEB), as described in the manufacturer's protocol. Mouse PML (mPML) cDNA was then amplified by PCR using the oligodeoxynucleotide (ODN) primers whose sequences are provided in the Additional file 3. The resulting 2.65-kb cDNA fragment was cut with BamHI-MfeI and then inserted into the MLV-based retroviral vector pMIP (Berthoux et al., 2005), and cut with BglII and EcoRI, yielding pMIP-mPML. The cloned PML cDNA was sequenced and determined to be a variant of isoform 2 (GenBank accession No. KJ650238). To transduce N-terminally FLAG-tagged versions of hPML isoforms I to VI using a retroviral vector, individual isoforms were PCR amplified from the corresponding pLNGY-hPML constructs generously provided by Roger D. Everett (Cuchet et al., 2011), using the ODNs shown in Table A.1, and cloned into pMIP, which had been cut with BglII-EcoRI, yielding pMIP-hPML-I to -VI.

Retroviral vectors expressing mPML or hPML were prepared by cotransfection of 293T cells plated at 70% confluency in 10 cm dishes with 10 μg of pMIP-m(h)PMLs together with 5 μg of pMD-G (Zufferey et al., 1997) and 10 μg of pCl-Eco (Naviaux et al., 1996) using polyethylenimine (PEI; Polyscience, Niles, IL). Virus-containing supernatants were collected 2 days later and clarified by low-speed centrifugation, as described previously (Berthoux et al., 2003; Bérubé et al., 2007). Stable mouse or human PML-expressing MEFs were obtained by spinfection of 2×10^5 cells with 2 ml of retroviral vector-containing supernatants for 50 min at 400 x g in the presence of 8 μg/ml polybrene (Sigma-Aldrich, MO, USA) (Berggren, 2012) and followed by a 24 h incubation at 37 °C. In order to eliminate the non-transduced cells, puromycin

(Calbiochem, CA, USA) was then added to the cultures at a final concentration of 2 µg/ml for 5 days. The relevant "empty" (non-PML-expressing) vector was transduced as a control in all experiments.

To produce GFP-expressing retroviral vectors, 293T cells were seeded in 10 cm culture dishes and transiently cotransfected as described above. The plasmids used were as follows: pMD-G, pCNCG and pCIG3-B to produce B-MLVGFP (Bock et al., 2000; Neil et al., 2001); pMD-G and pNL-GFP to produce HIV-$1_{NL\text{-}GFP}$ (Berthoux et al., 2003; He et al., 1997); pMD-G and pSIVmac239GFP to produce SIVmac-GFP (Cowan et al., 2002); or pONY3.1, pONY8.0 and pMD-G to produce EIAVGFP (Mitrophanous et al., 1999). The supernatants were replaced with fresh medium after 6 h and the retroviruses were harvested 24 h later. The retroviruses were clarified by centrifugation at 3,000 rpm and stored in aliquots at -80 °C. The viral stocks were titered by serial dilution on CRFK cells.

RNA interference. ODNs were designed to create pAPM-based, shRNA-expressing constructs targeting hPML, as described previously (Malbec et al., 2010; Pertel et al., 2011). The shRNAs expressed targeted the following sequences, present in all hPML isoforms: shPML1, AAGATGCAGCTGTATCCAAGA; shPML2, GCAAGACCAACAACATCTTCT; shPML3, GCACACGCTGTGCTCAGGATG. The full sequences of the ODNs used to generate these constructs are provided in table A.1. SupT1 cells were stably transduced with shRNAs targeting hPML or Luciferase as a control via lentiviral gene transfer. Briefly, lentiviral vectors were prepared by cotransfection of HEK293T cells with 10 µg of either pAPM-shLuc (Pertel et al., 2011) or pAPM-shPML1-3, together with 5 µg of pMDG and 10 µg of pΔR8.9 (Zufferey et al., 1997), as described above. The viral supernatants were used for transduction of shPMLs into SupT1 cells, as detailed above. Stably transduced cells were selected by addition of 5 µg/ml puromycin to the medium at 2 dpi and for 5 d.

Antibodies and WB analyses. The cells were lysed at 4 °C in RIPA lysis buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 8.0).

The lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by WB analysis using mouse anti-mPML mAb (36-1-104, Enzo life sciences, NY, USA), rabbit polyclonal anti-hPML (H-238, Santa Cruz, TX, USA), anti-FLAG (Cell Signaling, MA, USA), or anti-β-actin antibody (Sigma, MI, USA). The p24 capsid protein of HIV-1 (CA, p24) was detected using a mouse monoclonal antibody (clone 183, AIDS Research and Reference Reagent Program Cat. No. 3537).

Immunofluorescence microscopy. PML-KO cells stably transduced with FLAG-tagged hPML-I to VI isoforms or WT MEFs were seeded on glass coverslips placed in 3.5-cm wells. After 24 h, the cells were permeabilized and fixed for 10 min in Triton X-100/4% formaldehyde at room temperature (RT), followed by 4 washes with PBS. The cells were then treated with 10% goat serum (Sigma) for 30 min at RT followed by 4 h of incubation with antibodies against FLAG (Sigma, 1:150) or hPML (Santa Cruz, 1:150) or mPML (Enzo Life Sciences, 1:150) in 10% goat serum at RT. They were then washed 4 times with PBS and fluorescently stained with Alexa Fluor 488-conjugated goat anti-mouse (Molecular Probes, Eugene, OR) diluted 1:100 in 10% goat serum for 1 h at RT. The cells were then washed 4 times with PBS before mounting in Vectashield (Vector Laboratories, Peterborough, UK). Hoechst 33342 (0.8 μg/ml; Molecular Probes) was added along with the penultimate PBS wash to reveal DNA. Z-stacks were acquired on an AxioObserver Microscope (Carl Zeiss Canada, Toronto, ON) equipped with the Apotome module, and the median optical slice of each Z-stack was analyzed.

Pharmacological treatments. SAHA (Sigma-Aldrich) was resuspended in DMSO and used at a final concentration of 5 μM for 48 h prior to flow cytometric analysis. Recombinant murine IFN-α (11200-2) and IFN-β (12405-1) were obtained from PBL Interferon Source (NJ, USA) and added to the cells 16 h prior to infection with retroviruses. 24 h after infection, the supernatants were replaced with fresh IFN-containing medium. To block the extracellular domain of the IFN-I receptor in MEFs, the cells were treated with purified anti-mouse IFNAR-1 antibody (MAR1-5A3, BioLegend, UK), at a concentration of 650 ng per 20,000 cells, 1 h prior to infection with HIV-$1_{NL\text{-}GFP}$. Where applicable, the supernatants were replaced with fresh drug-containing medium 24 h after infection.

Viral challenges and flow cytometric analysis. The cells were seeded into 24-well plates at 2×10^4 cells/well (MEF) or 1×10^5 cells/well (SupT1) and infected the following day with GFP-expressing retroviral vectors. MEF cells were trypsinized at 2 dpi and fixed in 3% formaldehyde (Fisher Scientific, MA, USA). The percentage of GFP-positive cells and MFI were then determined by analyzing 1×10^4 cells on a FC500 MPL cytometer (Beckman Coulter, CA, USA) using the CXP Software (Beckman Coulter). MFI analysis was restricted to the GFP-positive cells.

Quantitative real-time PCR. The late RT products, 2-LTR circles, and HIV-$1_{NL\text{-}GFP}$ mRNA expression levels in infected cells were measured by either qPCR or qRT-PCR using the Stratagene Mx3000P system (Agilent, CA, USA). The cells were plated in 12-well plates at 3×10^5 cells/well and infected with HIV-$1_{NL\text{-}GFP}$. The retrovirus was pretreated with 20 U/ml DNase I (NEB) for 1 h at 37 °C and control infections were performed in the presence of 80 μM nevirapine (Sigma), as described previously, to demonstrate the absence of carry-over contaminating plasmid DNA (Veillette et al., 2013). Total cellular DNA was collected after 6 h of infection (late RT products) or 6 h of infection followed by 18 h incubation in virus-free medium (2-LTR-circles) using the QIAamp DNA mini kit (Qiagen, CA, USA). Sequence data for the ODNs used in the PCR reactions (GFP forward and reverse, 2-LTR circles forward and reverse, actin forward and reverse) is provided in Additional file 3. The reactions contained 1x SensiFast SYBR Lo-ROX mix (Bioline, UK), 400 nM forward and reverse primers, and 5 μl template (150-400 ng) in 20 μl final volume. After 3 min incubation at 95 °C, 40 cycles of amplification were performed as follows: 5 sec at 95 °C, 10 sec at 62 °C (GFP) or 65 °C (2-LTR), 15 sec at 72 °C.

For qRT-PCR, total RNAs were purified from infected or uninfected MEFs using the AllPrep RNA/Protein kit (Qiagen). Reverse transcription of 200 ng of each RNA sample followed by real time PCR were performed in a final volume of 20 μl using the SensiFAST SYBR Lo-ROX One-Step kit (Bioline) according to the manufacturer's instructions. The primer sets to detect GFP and actin in the PCR reactions were as mentioned above. The reaction conditions were: 48 °C for 30 min, 95 °C for 10 min,

40 cycles of amplification: 95 °C for 15 sec, 60 °C for 30 sec. Primers were validated by performing a standard curve and through dissociation curves analysis. Plasmid copy numbers dilutions ranging from 5.5×10^5 down to 14 were used for the GFP standard curve. Results were then analyzed with the MxPro software (Agilent). Absolute counts were determined using the equation of the standard curve $\log(y) = ax + b$ where copy number was 10((Ctsample-b)/-a).

Each PCR was performed in duplicate and the threshold cycle (Ct) was determined using the MxPro software (Agilent). In each experiment, a standard curve was run in duplicate, ranging from 300 to 3×10^5 copies plus a no-template control. The levels of HIV-1 transcript were normalized to those of GAPDH, which was quantified in parallel as an endogenous control.

2.5 Results

PML depletion increases the susceptibility of human T cells to lentivirus infection. We first investigated the effect of endogenous hPML depletion on HIV-1 and SIVmac infectivity in SupT1 cells, a human T lymphoblastoid cell line. The cells were stably transduced with lentiviral vectors expressing shRNAs targeting all hPML isoforms or expressing an shRNA against luciferase as a control, and conferring puromycin resistance. The untransduced cells were eliminated by puromycin treatment and PML knockdown was analyzed by WB (Fig. 2.1A). The results showed that both shPML2 and shPML3 efficiently decreased PML expression in SupT1 cells, whereas shPML1 had no significant effect. We next infected the 4 SupT1 pools with low viral doses of VSV protein G (VSV-G)-pseudotyped, green fluorescent protein (GFP)-expressing lentiviral vectors based on HIV-1 strain NL43 (HIV-$1_{NL\text{-}GFP}$) and simian immunodeficiency virus strain mac239 (SIVmac-GFP) for 2 days, followed by fluorescence-activated cell sorting (FACS) analysis. In these vectors, GFP is inserted in the Nef ORF and HIV-1 Env expression is disrupted (Cowan et al., 2002; He et al., 1997). The results showed that PML depletion, mediated by shPML2 and shPML3, increased the percentage of cells infected with HIV-$1_{NL\text{-}GFP}$ (2.4-fold and 3.7-fold, respectively), whereas shPML1 had no

significant effect (Fig. 2.1B, left panel). Similarly, the permissiveness of SupT1 cells to SIVmac-GFP was increased 4.3-fold and 3.9-fold by expression of PML shRNA2 and shRNA3, respectively, whereas shRNA1 had no effect (Fig. 2.1B, right panel).

GFP is routinely used as a reporter protein to study the activity of promoters; in particular, quantitation of GFP fluorescence intensity is a robust marker for expression levels, as it has been shown to directly correlate with mRNA levels in individual cells (Soboleski et al., 2005). Recently, GFP fluorescence intensity was used to analyze the Cas9 nuclease-mediated knockout of latently integrated HIV-1 genomes in human cells (Liao et al., 2015). GFP expression by the HIV-1-based vector HIV-$1_{NL\text{-}GFP}$, which is used in our study, is under the control of a natural 5'-LTR that acts as an enhancer and a promoter. This allowed us to investigate whether hPML interferes with HIV-1 gene expression by quantifying GFP mean fluorescence intensity (MFI) in infected cells using FACS. None of the shRNAs used had any significant effect on the GFP MFI following infection with HIV-$1_{NL\text{-}GFP}$ or SIVmac-GFP (Fig. 2.1C). Thus, PML restricts the early stages of HIV-1 and SIVmac infection but does not affect viral gene expression in SupT1 cells.

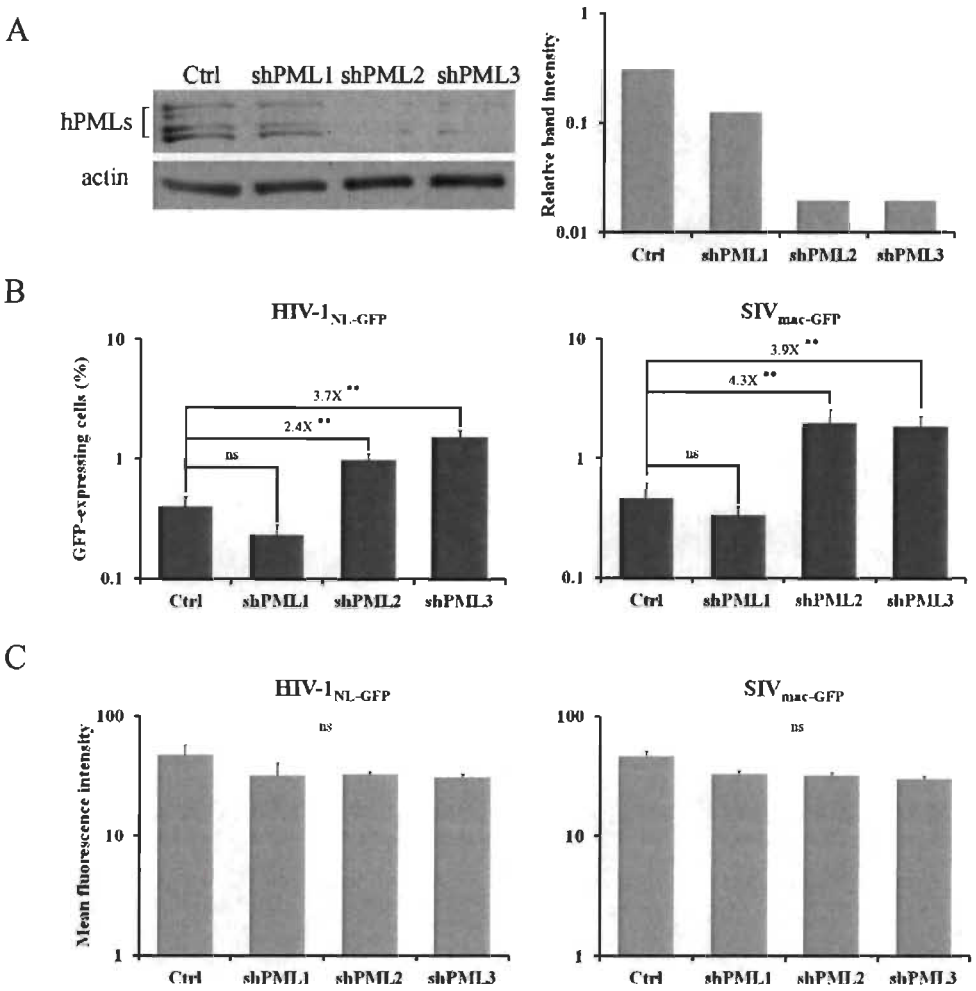


Figure 2.1 **PML-mediated restriction of HIV-1 and SIVmac infection in SupT1 cells.**

(A) WB analysis of human SupT1 cells stably transduced with shPMLs. Cells were stably transduced with either the control shRNA targeting luciferase or with shRNAs targeting all hPML isoforms. PML expression levels were analyzed by WB using a polyclonal antibody (upper panel). The same blot was reprobed with an anti-actin antibody as a loading control. The graph on the right shows the ratios of PML compared to actin following densitometry analysis. (B) Effects of shRNA-mediated depletion of hPML on HIV-1 and SIVmac infectivity. The cells stably expressing shPMLs or control shRNAs were infected with HIV-$1_{NL\text{-}GFP}$ (left) or $SIV_{mac\text{-}GFP}$ (right) (MOI of 0.1). Two days later, the percentages of infected cells were measured by FACS. The values represent the means of three independent experiments with standard deviations (**$P < 0.01$, two-tailed Student's t-test). (C) Effects of shRNA-mediated depletion of hPML on HIV-1 and SIVmac LTR-driven GFP expression. GFP MFI values are shown for the experiments in panel B (ns, non-significant in the two-tailed Student's t-test).

PML confers resistance to infection of murine cells by lentiviruses. Restriction factors such as TRIM5α, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) and Tetherin often function in a species-specific, virus-specific fashion (Browne & Littman, 2008; Jia et al., 2009; Stremlau et al., 2005). In order to analyze the antiretroviral potential of PML in a non-human context, PML-KO MEFs (Wang et al., 1998) and corresponding WT cells were challenged with increasing doses of HIV-$1_{NL\text{-}GFP}$, $SIV_{mac\text{-}GFP}$ and a GFP-expressing vector based on equine infectious anemia virus ($EIAV_{GFP}$). The percentage of infected (GFP-positive) cells was then measured by FACS. We found that MEF cells were up to 30 times more permissive to infection by the HIV-1 vector in the absence of PML (Fig. 2.2A). Similarly, the infectivity of the SIVmac and EIAV vectors was increased in PML-KO cells by up to 8-fold and 12-fold, respectively. This PML-dependent restriction phenotype decreased at higher virus doses (Fig. 2.2A), suggesting the presence of a saturation effect previously seen with TRIM5α (Hatziioannou et al., 2003), whereby large amounts of incoming retroviral cores "soak up" the restriction factor, resulting in attenuated or abrogated restriction. These data suggest that mPML is involved in a restriction mechanism targeting the early stages of infection by non-cognate lentiviruses. We used quantitative PCR (qPCR) to investigate the effects of PML on HIV-1 DNA synthesis and nuclear import, two early infection steps frequently affected by previously discovered restriction factors. When WT and PML-KO MEFs were infected with identical amounts of HIV-$1_{NL\text{-}GFP}$, we observed ~5-fold more reverse transcribed DNA in the PML-KO cells (Fig. 2.2B). We also observed significantly more 2-LTR circles (a marker of nuclear import) in PML-KO cells (Fig. 2.2B). However, the effect of PML on 2-LTR circle levels was not greater than its effect on total reverse transcribed DNA, suggesting that the PML-dependent restriction of HIV-1 in MEFs affects mainly the reverse transcription step, consistent with recent findings from other groups (Dutrieux et al., 2015; Kahle et al., 2015).

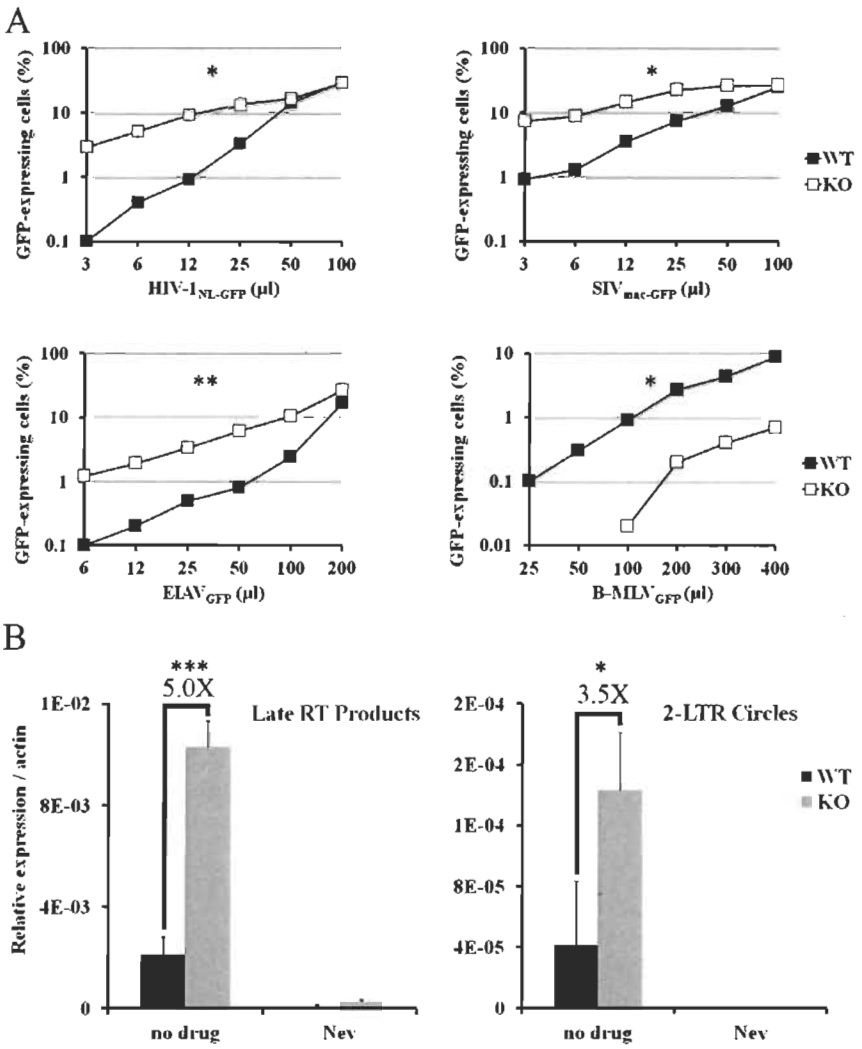


Figure 2.2 **Murine PML confers resistance to infection with lentiviruses.**
(A) Dose-dependent analysis of retrovirus infectivity. WT and PML-KO MEF cells were infected with increasing doses of HIV-$1_{NL\text{-}GFP}$, $SIV_{mac\text{-}GFP}$ and $EIAV_{GFP}$. The percentage of infected (GFP-expressing) cells was measured 2 d later by FACS. Shown is one experiment representative of ≥3 independent experiments that yielded comparable results (*$P < 0.05$, **$P < 0.01$, one-tailed paired Student's t-test). (B) Effects of PML on the early stages of HIV-1 replication. WT and PML-KO MEF cells were infected with HIV-$1_{NL\text{-}GFP}$ at a low MOI (0.01 as measured on CRFK cells, see Methods). Total cellular DNA was extracted 6 h and 24 h post-infection and subjected to qPCR of HIV-1 late reverse transcription products and 2-LTR circles. Data are shown as relative viral products levels compared to actin. An RT inhibitor (nevirapine, Nev) was included as a control to show the absence of contaminating DNA. The values represent the means of three independent experiments with standard deviations (*$P < 0.05$, ***$P < 0.001$, two-tailed Student's t-test).

PML promotes the down-regulation of HIV-1 LTR-driven GFP expression in MEFs. MEF WT and PML-KO cells were infected with increasing doses of HIV-$1_{NL\text{-}GFP}$, as described above, followed by FACS. The percentage of infected cells and MFI (within the GFP+ population) were measured at 2 and 10 days post infection (dpi) (Fig. 2.3A and 2.3B, respectively). Performing the analyses at 10 dpi ensured that any GFP detected would have been expressed from integrated proviral DNA (Butler et al., 2001). We found that PML knockout resulted in not only an increase in the percentage of GFP-expressing cells, but also an increase in the GFP MFI in these infected cells. Similar to what we observed in figure 2.2A, the effects of mPML knockout on viral infectivity were greatest when a low dose of virus was used and were abrogated at high virus doses (>25 µl of HIV-$1_{NL\text{-}GFP}$ in this experiment). In contrast, the effects of mPML knockout on the GFP MFI were relatively more constant across multiple doses of virus (~4-fold increase at 2 dpi, ~10-fold at 10 dpi). At 10 dpi, however, we observed a decrease in GFP MFI at the two highest virus doses used (50 and 100 µl) in PML-KO cells, perhaps reflecting the existence of an additional PML-independent mechanism of inhibition. Altogether, results from the experiments shown in figure 2.2 and figure 2.3 suggest that PML expression in MEFs is associated with at least two distinct HIV-1 restriction mechanisms; one takes place at early post-entry stages, whereas the second results in a decrease in LTR-driven gene expression. The first inhibitory mechanism can be abrogated at high virus doses, whereas the second is not inhibited at these high doses, supporting a model where these activities are independent from each other.

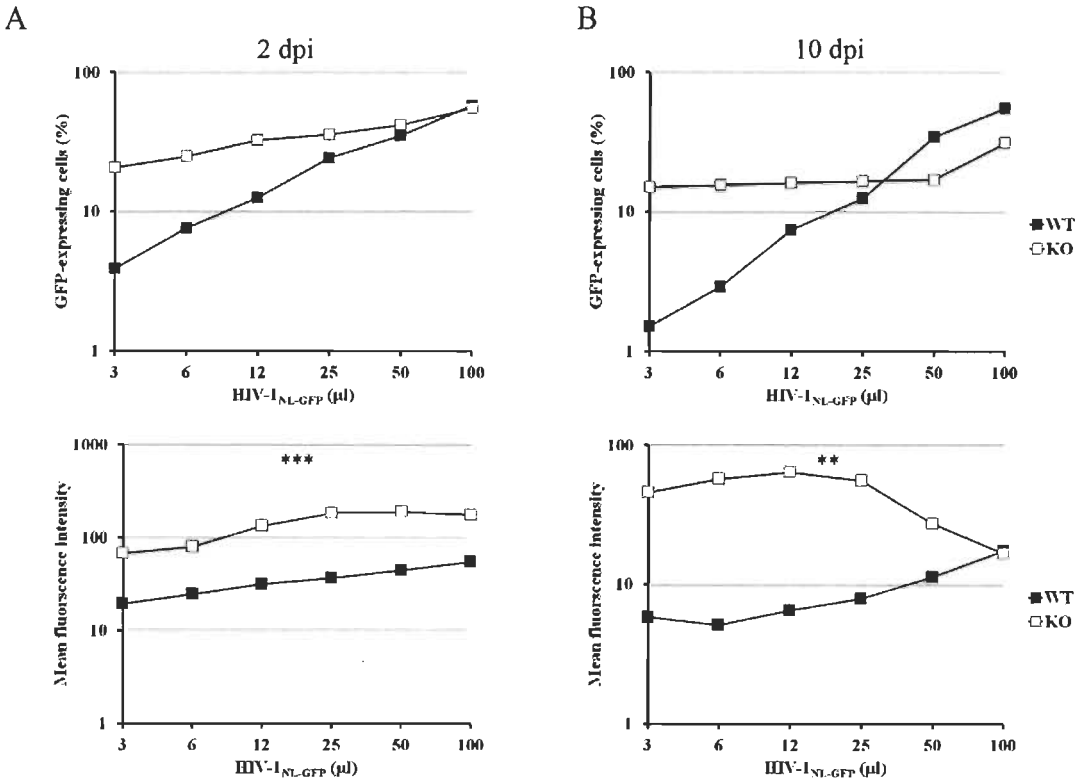


Figure 2.3 **mPML knockout increases HIV-1 LTR-driven GFP expression.** PML-KO and WT MEFs were infected with increasing doses of HIV-$1_{NL\text{-}GFP}$ and cells were maintained in culture for 2 days (A) or 10 days (B), followed by FACS analysis. The percentage of infected (GFP-expressing) cells and the mean fluorescence intensity (MFI) were measured at each time point (top and bottom panels, respectively) (**P < 0.01, ***P < 0.001; one-tailed paired Student's t-test).

Overexpression of mPML restores restriction of HIV-1 and SIVmac in PML-KO MEFs. Because the data reported above implicate mPML as a possible intrinsic defense factor against lentiviruses, we next examined whether its overexpression would restore the restriction of HIV-1 and SIVmac in PML-KO MEFs. For this, we cloned the mPML cDNA from MEFs into the murine leukemia virus (MLV)-based vector pMIP (Berthoux et al., 2005) and transduced it in both WT and PML-KO MEFs, together with the empty vector (EV) as control. Our cloning strategy allowed for the isolation of both main isoforms (1 and 2) of mPML, but 3/3 sequenced clones corresponded to mPML isoform 2, which is the longest of the two (GenBank accession No. KJ650238).

After puromycin selection of transduced cell populations, mPML expression was analyzed by Western blotting (WB). As shown in figure 2.4A, a band consistent with the expected size for mPML (110-120 kDa) was detected in the mPML-transduced cells, and a weaker band of the same size was seen in WT but not PML-KO MEFs. Additional bands corresponding to heavier proteins were also detected and could be SUMOylated forms. We then challenged the transduced cells with multiple doses of HIV-$1_{NL\text{-}GFP}$ (Fig. 2.4B, left panel) and SIVmac-GFP (Fig. 2.4B, right panel). Transduction of mPML into PML-KO MEFs decreased the infectivity of HIV-$1_{NL\text{-}GFP}$ and SIVmac-GFP by up to ~9-fold (Fig. 2.4B left panel) and ~14-fold (Fig. 2.4B right panel), respectively, similar to the levels seen in WT cells. In contrast to the PML-KO cells, overexpression of mPML in WT MEFs had no effect on infection with the HIV-1 and SIVmac vectors. Like before, the magnitude of change in infectivity by PML knockout was greatest at the lowest viral doses. These results demonstrate that PML can inhibit the early stages of lentivirus infection in MEFs and suggest that endogenous mPML levels are sufficient to accomplish this function.

To provide further insights into the possible role of mPML in inhibiting lentiviral gene expression, we also measured the GFP MFI (Fig. 2.4C, D). As shown in representative FACS dot plots in figure 2.4C, overexpression of mPML in PML-KO MEF cells not only decreased HIV-$1_{NL\text{-}GFP}$ infectivity from 76% to 14.6% but also reduced the GFP MFI by 13.3-fold. In contrast, overexpression of mPML in WT MEF cells had only a small effect on the GFP MFI (less than 2-fold). Figure 2.4D summarizes the GFP MFI results obtained upon infection of mPML- or empty vector-transduced WT and PML-KO MEFs with HIV-$1_{NL\text{-}GFP}$ and SIVmac-GFP. We found that overexpression of mPML in PML-KO MEFs strongly decreased the GFP MFI following infection by HIV-$1_{NL\text{-}GFP}$ and SIVmac-GFP (up to 13.3-fold and 22.3-fold, respectively). In contrast, overexpression of mPML in WT MEFs decreased GFP MFI by a much smaller magnitude following infection by HIV-$1_{NL\text{-}GFP}$ and SIVmac-GFP (up to 1.7-fold and 3.1-fold, respectively). As expected, the PML-induced reduction in GFP MFI was not dose dependent, thereby distinguishing the effects of mPML on infectivity and GFP MFI. As an additional control, we also infected the 4 cell pools with a GFP-expressing, "B-tropic" MLV-based vector

(Besnier et al., 2003), MLVGFP (Fig. 2.4D, right panel) and similarly measured GFP expression levels. As anticipated, we found that the GFP MFI did not significantly vary in response to expression of mPML (either endogenous or exogenous). Collectively, the data shown in figures 2.2-2.4 demonstrate that expression of mPML in murine cells inhibits both the infectivity and LTR-driven viral gene expression of non-murine lentiviruses.

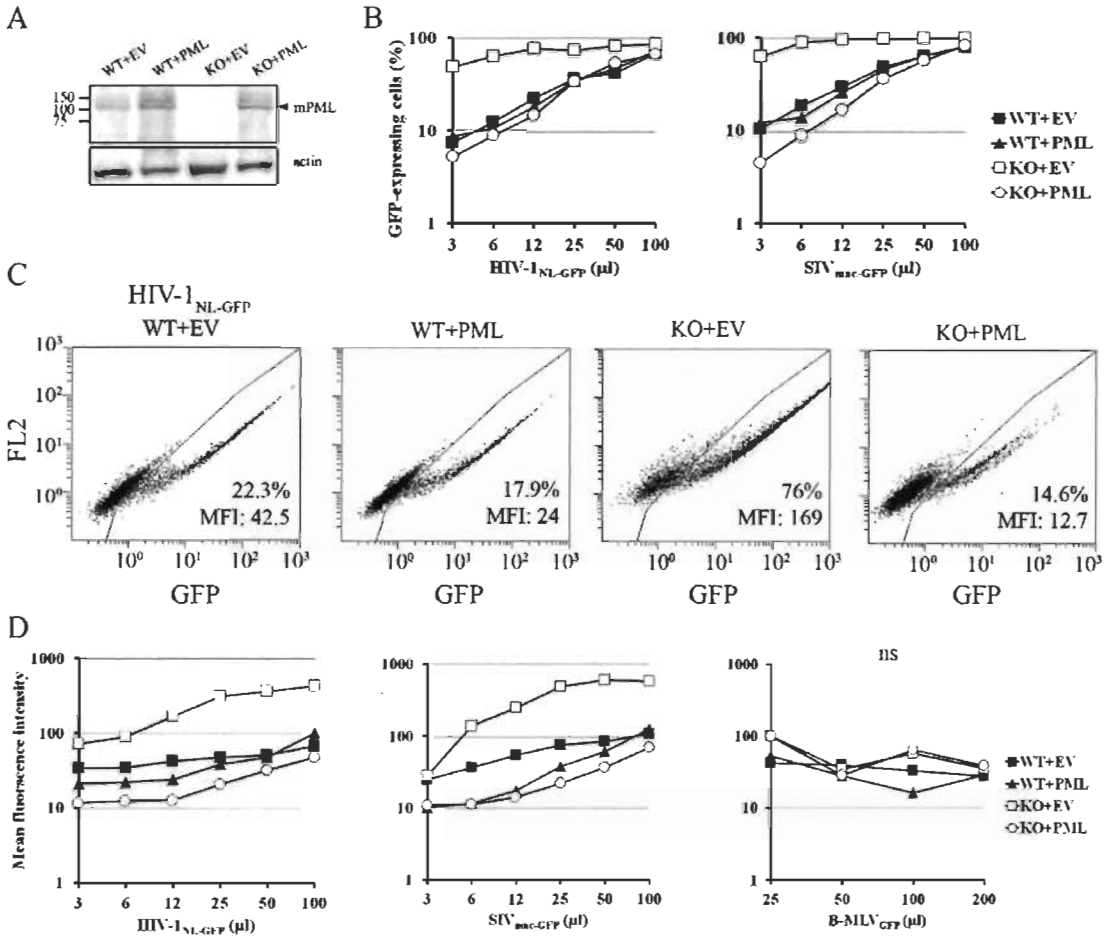


Figure 2.4 **Expression of mPML in PML-KO MEFs restores restriction of HIV-1 and SIV_{mac}.**

(A) Western blotting (WB) analysis of mPML overexpression in MEFs. PML cDNA PML cDNA was isolated from WT cells and transduced into both WT and PML-KO MEFs. The empty vector (EV) was transduced as a control. mPML expression was analyzed by WB of extracts from EV-transduced MEF-WT cells (WT+EV), mPML-transduced MEF-WT cells (WT+PML), EV-transduced PML-KO cells (KO+EV), and mPML-transduced PML-KO MEF cells (KO+PML). The WB was performed using an anti-mPML monoclonal antibody (upper panel) followed by an anti-actin antibody (lower panel) as a loading control. The arrow points to mPML, as judged from its expected size, whereas the heavier bands are presumably SUMOylated forms. The positions of the molecular size markers are indicated on the left. (B) Analysis of retrovirus infectivity in the transduced MEFs. The cells were infected with multiple doses of either $HIV\text{-}1_{NL\text{-}GFP}$ or $SIV_{mac\text{-}GFP}$, and the percentage of GFP-positive cells was measured at **2** dpi by FACS ($P \leq 0.001$, one-tailed paired Student's *t*-test for KO + PML vs. KO). (C) FACS plots from transduced MEFs infected

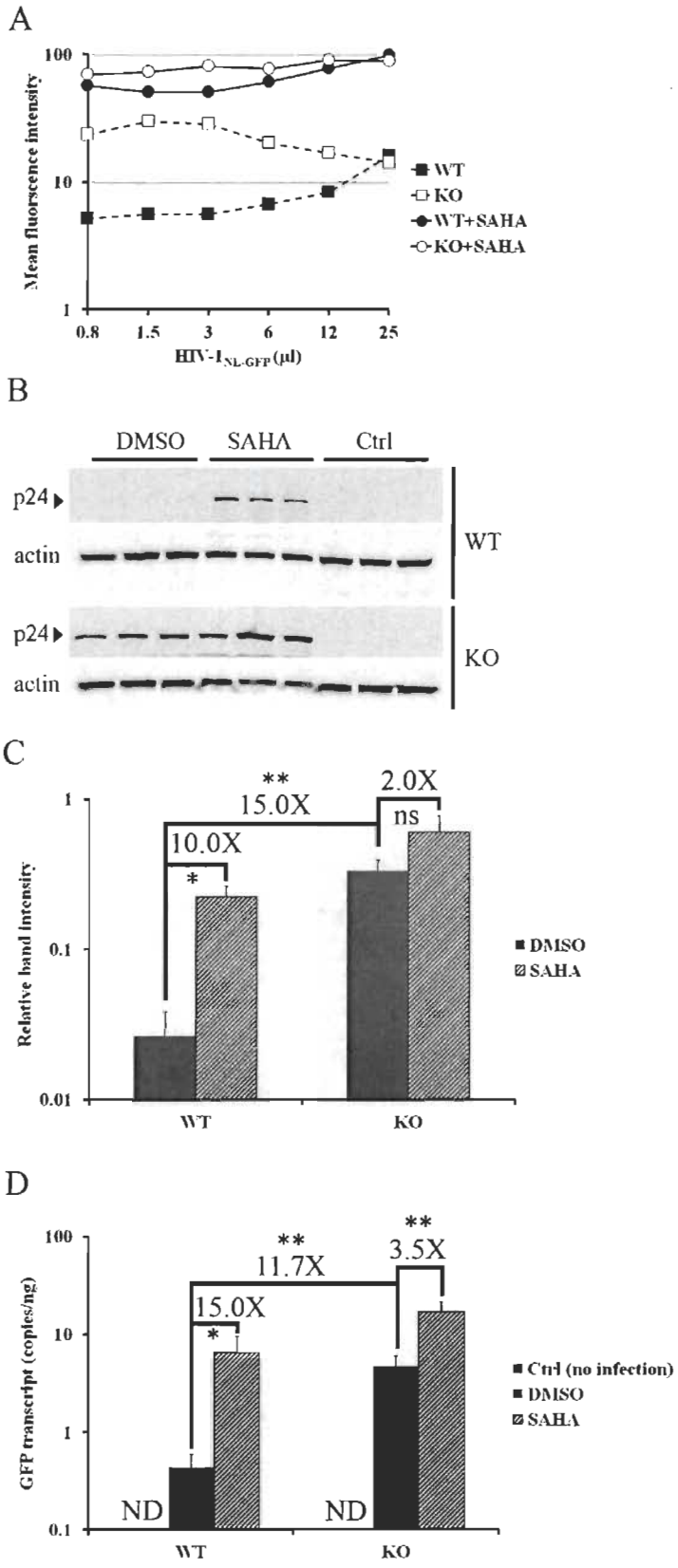
with HIV-$1_{NL\text{-}GFP}$. WT and PML-KO MEF cells transduced with either EV or mPML were infected with HIV-$1_{NL\text{-}GFP}$. The percentage of infected cells and mean fluorescence intensities determined at 2 dpi are indicated for each plot. (D) Down-regulation of LTR-driven GFP expression following overexpression of mPML in PML-KO MEFs. WT and PML-KO MEFs were stably transduced with either mPML or EV, as a control, then infected with multiple doses of HIV-$1_{NL\text{-}GFP}$ (left panel), $SIV_{mac\text{-}GFP}$ (middle panel) or B-MLV_{GFP} (right panel). The MFI was measured by FACS at 2 dpi ($P < 0.01$, one-tailed paired Student's *t*-test for KO + PML vs. KO after HIV-$1_{NL\text{-}GFP}$ or $SIV_{mac\text{-}GFP}$ infection).

PML-dependent transcriptional silencing of HIV-1 in MEFs. To further study the role of mPML in the regulation of the HIV-1 LTR-driven gene expression, we used SAHA, a HDAC inhibitor. HDACs act on histones within the nucleosome-bound promoter of HIV-1 to maintain proviral latency (He & Margolis, 2002). HDAC inhibition by SAHA leads to promoter expression and the escape of HIV-1 from transcriptional repression (Archin et al., 2009; Archin et al., 2012). Recently, SAHA was shown to affect the spatial distribution of hPML NBs (Lusic et al., 2013). We reasoned that if the HIV-1 LTR was repressed by mPML in MEFs in a fashion similar to its transcriptional repression in some human lymphocyte subpopulations (Chomont et al., 2009), then SAHA would counteract the effects of mPML in MEFs. We infected WT and PML-KO MEFs with multiple doses of HIV-$1_{NL\text{-}GFP}$ and maintained the cultures for 10 days. The cells were then treated with either 5 μM of SAHA or with DMSO as control for 48 h followed by FACS analysis of the GFP MFI, performed like before. As shown in figure 2.5A, the levels of GFP expression increased by up to ~11-fold in WT cells following treatment with SAHA, while a smaller increase (up to 3-fold) was observed in PML-KO cells. Therefore, SAHA counteracts the PML-mediated reduction in LTR-driven GFP expression in MEF cells, consistent with transcriptional repression of the LTRs as the underlying mechanism. Interestingly, at the highest dose of virus used, SAHA had the same effect on the GFP MFI in WT and PML-KO cells (Fig. 2.5A). This observation suggests that at high virus doses, HIV-1 LTR-driven gene expression may additionally become inhibited by a distinct mechanism independent of PML.

To insure that the inhibitory effect of PML on HIV-1 gene expression was not specific to the GFP reporter gene used in previous experiments, we also analyzed the

expression of the HIV-1 p24 capsid protein in similar settings. WT and PML-KO MEFs were infected with HIV-$1_{NL\text{-}GFP}$ in triplicate. 10 d later, the cells were treated or not with SAHA for 2 d and protein extracts were then analyzed by WB. As shown in figure 2.5B and figure 2.5C, p24 was barely detectable in DMSO-treated WT cells, whereas treatment with SAHA resulted in a 10-fold increase in p24 expression levels in these cells. In contrast, SAHA treatment caused only a 2-fold increase in p24 expression in PML-KO cells (Fig. 2.5C). Therefore, the results obtained in this experiment were consistent with those obtained for GFP.

To directly address whether the PML-dependent decrease in LTR-driven expression resulted from transcriptional repression, we used quantitative reverse transcription-PCR (qRT-PCR) to analyze the abundance of HIV-1 mRNA in MEF cells infected with HIV-$1_{NL\text{-}GFP}$ exactly as in figure 2.5B. Levels of HIV-1 mRNA (analyzed using primers specific to the GFP coding sequence) were less than 1 copy per ng of total RNA in WT MEFs, but were 11.7 times higher in PML-KO cells (Fig. 2.5D). In response to the treatment of WT cells with SAHA, we observed a 15-fold increase in the levels of viral mRNA, compared to an increase of only 3.5-fold in the PML-KO cells. A second qPCR analysis was performed, this time normalized to actin transcription level (Fig. A.1). The results were consistent with those shown in figure 2.5D, as HIV-1 transcription was found to be 13.3-fold higher in the absence of mPML and SAHA specifically rescued HIV-1 transcription in WT MEFs. Taken together, these results provide strong evidence that mPML interferes with the HIV-1 transcription in MEFs.


A
100
10
1
Mean fluorscence intensity
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1.5
3
6
12
25
HIV-1NL-GFP (μl)
WT
KO
WT+SAHA
KO+SAHA
B
DMSO
SAHA
Ctrl
p24
actin
WT
p24
actin
KO
C
**
15.0X
2.0X
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10.0X
*
1
0.1
0.01
Relative band intensity
DMSO
SAHA
WT
KO
D
**
11.7X
**
3.5X
15.0X
*
100
10
1
0.1
GFP transcript (copies/ng)
ND
ND
Ctrl (no infection)
DMSO
SAHA
WT
KO

Figure 2.5 SAHA counteracts the PML-dependent inhibition of HIV-1 gene expression.

(A) Effects of SAHA on HIV-1 LTR-driven GFP expression. PML-KO and WT MEFs were infected with increasing doses of HIV-$1_{NL\text{-}GFP}$. Ten days later, the cells were treated with 5 μM SAHA or with DMSO for 48 h and the MFI was then measured by FACS ($P = 0.0001$, one-tailed paired Student's *t*-test for SAHA vs. DMSO treatment in WT cells). (B) Analysis of HIV-1 p24 expression levels. PML-KO and WT MEFs were infected in triplicate with HIV-$1_{NL\text{-}GFP}$ at a CRFK MOI of 0.1 and then treated with either SAHA or DMSO at 10 dpi. Cellular lysates were prepared 48 h later and analyzed by WB using an anti-p24 antibody. Uninfected extracts were used as a negative control and actin was analyzed as a loading control. (C) The p24 and actin bands in the WB analysis shown in (B) were quantified by densitometry. The values represent the means of p24/actin ratios from the 3 data points for each condition with standard deviations (*$P < 0.05$, **$P < 0.01$, two-tailed Student's *t*-test). (D) qRT-PCR analysis of HIV-1 transcription. WT or PML-KO MEFs were infected with HIV-$1_{NL\text{-}GFP}$ in triplicate. Ten days after infection, the cells were treated with either DMSO or SAHA for 48 h. Total RNA was purified from the cells and the level of GFP transcript was quantified by qRT-PCR. Total RNA from uninfected cells was used as a negative control. The values represent the means of three independent experiments with standard deviations (*$P < 0.05$, **$P < 0.01$, two-tailed Student's *t*-test). ND, not detected.

mPML-mediated restriction of lentiviruses does not require IFN-I, but mPML contributes to IFN-I-induced antiviral responses. The hPML expression levels can be altered during infection with some viruses, such as HSV-1, HCMV and Epstein–Barr virus (EBV) (Salsman et al., 2008). Interestingly, we observed a significant increase in the levels of mPML expression in response to infection of MEFs with the HIV-$1_{NL\text{-}GFP}$, $SIV_{mac\text{-}GFP}$, and B-MLV_{GFP} vectors (Fig. 2.6A), suggesting an interferon-dependent mechanism. Accordingly, the expression of PML is known to be increased in response to both type I and II IFNs (Chelbi-Alix et al., 1995; Lavau et al., 1995). IFNs might be relevant to the observed HIV-1 restriction phenotype in MEFs in two ways. First, IFN treatment-mediated antiviral activities might be dependent on PML. Second, PML could indirectly interfere with HIV-1 infection and/or transcription by upregulating the production of type I IFN. To test the latter hypothesis, IFN-induced signaling in MEFs was prevented by using a blocking antibody against the mouse IFN-alpha/beta receptor subunit 1 (IFNAR-1) (Mogensen et al., 1999). The efficacy of this antibody was

determined by treating WT MEFs with murine IFN-β in the presence or absence of the blocking antibody and then measuring the levels of mPML expression by WB (Fig. 2.6B). The results show that, as expected, PML levels were greatly increased by IFN-β treatment, whereas treatment with the anti-IFNAR-1 antibody abrogated this effect in a dose-dependent fashion. Next, WT and PML-KO MEFs were treated with the blocking antibody (650 ng per 20000 cells) prior to infection with increasing doses of HIV-1 (Fig. 2.6C). Compared with control untreated cells, inhibition of IFN-induced signal transduction did not modulate HIV-1 infectivity (Fig. 2.6C, left panel) nor HIV-1 LTR-driven GFP expression levels (Fig. 2.6C, right panel) in either WT or PML-KO MEFs. These results indicate that PML-mediated restriction of HIV-1 in MEFs does not require activation of IFN-mediated pathways, even though PML itself is upregulated by IFNs.

To test whether PML is important for IFN-mediated antiviral activity, we treated both WT and PML-KO MEFs with IFN-β for 16 h, then challenged them with HIV-$1_{NL\text{-}GFP}$. As shown in figure 2.6D (left panel), treatment with IFN-β led to a 20-fold reduction in the percentage of infected cells in the presence of PML compared to only a 5-fold decrease in infectivity in PML-KO cells. However, IFN-β treatment did not modify the GFP MFI in either WT or PML-KO cells (Fig. 2.6D, right panel). To analyze further the importance of PML in IFN-β-mediated inhibition of HIV-1, we treated WT and PML-KO cells with murine IFN-α or IFN-β and then infected them with increasing doses of HIV-$1_{NL\text{-}GFP}$. We found that IFN treatment reduced the infectivity of HIV-1 by up to ~100-fold at low virus doses in WT cells. However, the inhibitory effect of IFNs was significantly more modest (up to ~10-fold) in PML-KO cells (Fig. 2.6E). Therefore, our data support a model where type I IFNs inhibit HIV-1 through mechanisms that partially involve the PML-mediated inhibition of early replication stages but are not relevant to the inhibition of LTR-driven gene expression. Thus, PML-mediated antilentiviral functions can be both induced and constitutive.

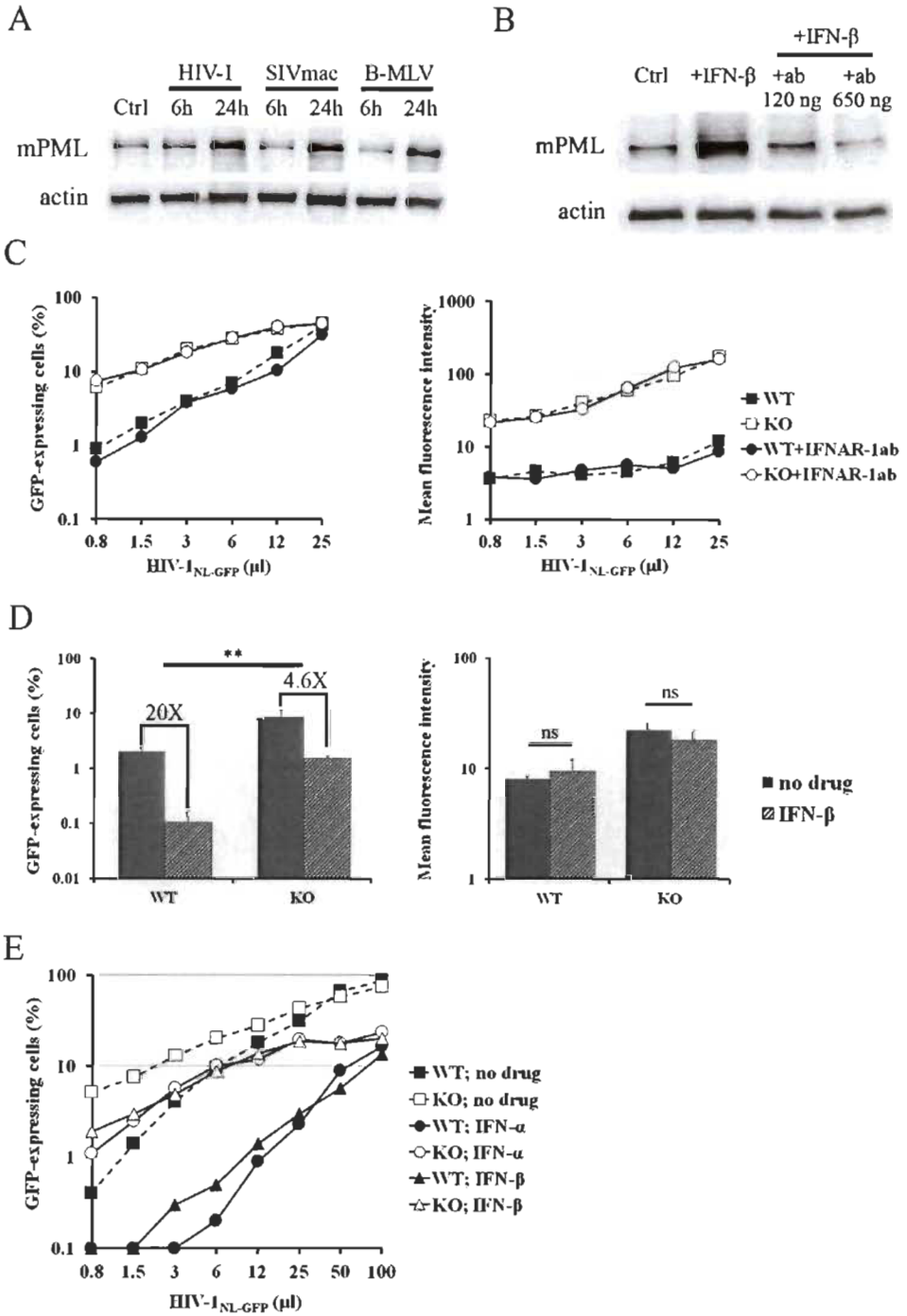

Figure 2.6 **HIV-1 restriction by mPML does not require a type I IFN-induced antiviral state, but efficient IFN-induced inhibition of HIV-1 in MEFs requires PML.**

(A) WB analysis of infection-induced up-regulation of PML in MEFs. WT MEF cells were infected with HIV-$1_{NL\text{-}GFP}$, $SIV_{mac\text{-}GFP}$, or B-MLV_{GFP} at an MOI of 1. Protein extracts were analyzed by WB at 6 h MOI of 1. Protein extracts were analyzed by WB at 6 h or 24 h post infection, along with a

no-infection control, using an anti-mPML monoclonal antibody (upper panel). Actin was analyzed as a loading control. (B) Expression of mPML was analyzed in WT MEFs left untreated (Ctrl), treated with IFN-β alone, or treated with a blocking antibody against IFNAR-1 at two different doses to block IFN-β-induced signal transduction prior to IFN-β treatment. mPML was detected using a monoclonal antibody (upper panel). Actin was analyzed as a loading control. (C) Blocking the IFN-I receptor does not alter HIV-$1_{NL\text{-}GFP}$ restriction by PML. PML-KO and WT MEFs were treated with the anti-IFNAR-1 antibody, or with PBS as a control, and were then infected with increasing doses of HIV-$1_{NL\text{-}GFP}$. The percentage of infected cells (left panel) and GFP MFI (right panel) were assessed 2 days later by FACS. (D) The effects of IFN-I and PML on the antiviral state. PML-KO and WT MEFs were treated with IFN-β for 16 h prior to infection with HIV-$1_{NL\text{-}GFP}$. The percentage of infected cells (left panel) and GFP MFI (right panel) were assessed 2 days later by FACS. The values represent the means of three independent infections with standard deviations (**$P < 0.01$, two-tailed Student's *t*-test; ns, non-significant). (E) Virus dose-dependent analysis of the role of PML in IFN-induced HIV-1 restriction. WT and PML-KO MEFs were treated with either IFN-α (500 U/ml) or IFN-β (100 U/ml) for 16 h, followed by infection with increasing doses of HIV-$1_{NL\text{-}GFP}$. The percentage of infected cells was assessed 2 days later by FACS.

Human PML expression induces restriction of HIV-1 and SIVmac in MEFs. To investigate the isoform specificity and the cellular context specificity of the restriction of HIV-1 by hPML, we expressed several hPML isoforms in PML-KO MEFs. We stably transduced FLAG-tagged versions of all six nuclear hPML isoforms (isoforms I to VI) (Cuchet et al., 2011) individually into these cells. The cells were selected in puromycin to eliminate untransduced cells. Immunofluorescence staining of the transduced MEF cells using an anti-FLAG antibody indicated that the different hPML isoforms were expressed in nuclei (Fig. 2.7A), though some cytoplasmic staining was detected for PML-V. WB analyses confirmed that all isoforms were expressed, albeit at various levels, with isoforms III, IV and V being expressed at apparently lower levels (Fig. A.2). The cells were then infected with increasing doses of HIV-$1_{NL\text{-}GFP}$ or $SIV_{mac\text{-}GFP}$ (Fig. 2.7B). HIV-1 was ~5- to 10-fold less infectious in PML-KO MEFs expressing hPML-I, II, IV and VI, compared with the empty vector-transduced control cells. PML-V had a more modest effect and PML-III did not impede HIV-1 infection (Fig. 2.7B, left panel). Transduction of hPML-I, II, IV, and VI in PML-KO MEFs also reduced the infectivity of $SIV_{mac\text{-}GFP}$, by up to 44-fold (Fig. 2.7B, right panel). Similar to HIV-$1_{NL\text{-}GFP}$, SIVmac-GFP infectivity was

modestly inhibited by hPML-V and was not affected by hPML-III. HIV-1 LTR-driven gene expression was assessed by measuring the GFP MFI in the infected cells. We observed that hPML-I, II, IV, and VI caused a reduction in GFP levels in MEFs infected with HIV-$1_{NL\text{-}GFP}$ or $SIV_{mac\text{-}GFP}$, while hPML-III and V had no effect (Fig. 2.7C). These data show that hPML can restrict HIV-1 and SIVmac in MEFs but in an isoform-specific fashion. In addition, hPML-V decreased HIV-1 infectivity but had no effect on HIV-1 LTR-driven GFP expression levels, implying that these two restriction mechanisms are genetically separable.

We next tested whether SAHA treatment would specifically rescue HIV-1 LTR-driven GFP expression in MEF cells expressing human PML isoforms. PML-KO MEFs stably expressing hPML isoforms I to VI were infected with low doses of HIV-$1_{NL\text{-}GFP}$ and kept in culture for 10 d. The cells were then treated with either 5 μM of SAHA or with DMSO as a control for 48 h, followed by FACS analysis. We observed that expression of hPML isoforms I, II and VI led to a decrease in HIV-$1_{NL\text{-}GFP}$ infectivity, although the magnitude of this inhibition was slightly smaller than what was observed 2 d post-infection. hPML-IV and V did not significantly decrease HIV-$1_{NL\text{-}GFP}$ infectivity, as seen at this time-point (Fig. 2.7D, left panel), perhaps due to the fact that these human isoforms delayed infection with HIV-1 rather than disrupting it entirely. hPML-I, II, III and VI caused a reduction in GFP MFI in these conditions (Fig. 2.7D, right panel), but this effect was also smaller than we had observed at 2 d post-infection (Fig. 2.7C). As expected, SAHA treatment had no significant effect on the infectivity of HIV-$1_{NL\text{-}GFP}$ in PML-KO MEFs expressing the various hPML isoforms (Fig. 2.7D, left panel). SAHA slightly increased (1.9-fold) the GFP MFI in the control cells (Fig. 2.7D, right panel), a result similar to what we had observed before (Fig. 2.5). The effect of SAHA was significantly greater in cells stably expressing hPML-I, III and VI (Fig. 2.7D, right panel), suggesting that the mechanism of inhibition of GFP expression by these isoforms was transcriptional silencing, similar to what we had demonstrated with mPML. Taken together, these data suggest that hPML can mediate the two inhibitory phenotypes also observed with mPML, although in an isoform-specific, cellular context-specific manner.

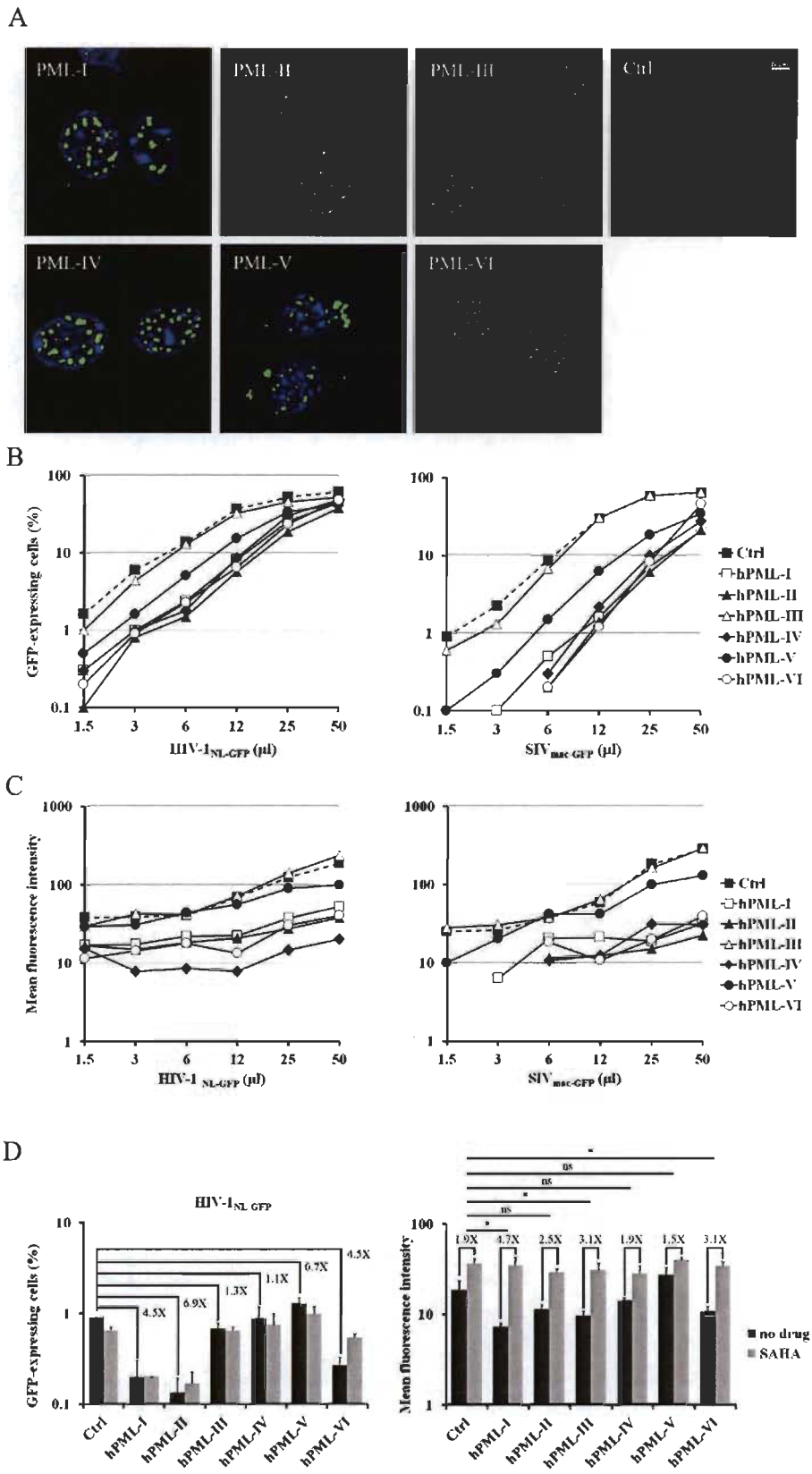
A
PML-I
PML-II
PML-III
Ctrl
PML-IV
PML-V
PML-VI
B
GFP-expressing cells (%)
HIV-1NL-GFP (µl)
SIVmac GFP (µl)
Ctrl
hPML-I
hPML-II
hPML-III
hPML-IV
hPML-V
hPML-VI
C
Mean fluorescence intensity
HIV-1 NL-GFP (µl)
SIVmac-GFP (µl)
D
HIV-1NL GFP
GFP-expressing cells (%)
Mean fluorescence intensity
no drug
SAHA

Figure 2.7 Expression of hPML isoforms in MEFs restricts HIV-1 and SIVmac. (A) Immunofluorescence staining of hPML in PML-KO MEFs stably transduced with FLAG-tagged hPML isoforms I to VI. Images are representative of multiple observations. hPMLs were stained with an anti-FLAG antibody (green) and nuclear DNA was stained using Hoechst 33342 (blue). Scale bar: 5 μm. (B) Effects of hPML isoforms on HIV-1 and SIVmac infectivity. PML-KO cells transduced with individual hPML isoforms were infected with increasing doses of HIV-$1_{NL\text{-}GFP}$ (left) or $SIV_{mac\text{-}GFP}$ (right). The percentage of infected cells was measured 2 days later by FACS. (C) Effects of hPML isoforms on HIV-1 and SIVmac LTR-driven GFP expression. GFP MFI values are shown for the experiments in panel B. (D) Effects of SAHA on hPML-dependent restriction of HIV-1 infectivity and LTR-driven GFP expression. MEF cells transduced with the individual hPML isoforms were infected with HIV-$1_{NL\text{-}GFP}$ at a CRFK MOI of 0.1. Ten days later, the cells were treated with either DMSO (no drug control) or SAHA for 48 h, followed by FACS. The percentage of infected cells (left panel) and GFP MFI (right panel) were assessed. The values represent the means of three independent experiments with standard deviations. The SAHA-dependent fold-increase in GFP MFI was compared between cells transduced with individual hPML isoforms and those transduced with the empty vector (Ctrl) using the two-tailed Student's t-test. The calculated p-values are indicated on the graph. ns, non-significant.

2.6 Discussion

The results from this study show that PML can interfere with at least two distinct steps in the replication of HIV-1 and other lentiviruses. The first block to replication occurs at early post-entry stages and was seen in both MEF and SupT1 cells, although the magnitude of the restriction was significantly higher in the murine cells. The existence of an early post-entry block to HIV-1 replication in murine cells has long been known (Bieniasz & Cullen, 2000; Hofmann et al., 1999). This restriction of HIV-1 infection was seen in all murine cell types analyzed by these investigators, although it was stronger in lymphocytes compared to fibroblasts (Baumann et al., 2004). On the basis of viral DNA analyses, the block was found to occur prior to integration (Baumann et al., 2004; Noser et al., 2006; Tsurutani et al., 2007), consistent with recent results from other groups (Dutrieux et al., 2015; Kahle et al., 2015). Here we show that in addition to HIV-1, the early post-entry replication of two other lentiviruses, SIVmac and EIAV, is restricted in murine MEFs. Our results indicate that PML is required for this early phase of restriction

to occur. Another team recently reported that the PML body component Daxx was involved in the PML-mediated inhibition of HIV-1 (Dutrieux et al., 2015), though this finding was contradicted in a report from another group (Kahle et al., 2015). We find that the PML-dependent restriction of early-stage HIV-1 infection was increased by treatment with IFN-α or IFN-β, which suggests that PML is relevant to the intrinsic cellular defenses against retroviral infections. Type I IFN treatment increased expression of mPML itself (Fig. 6), yet mPML overexpression was not sufficient to increase the restriction of incoming HIV-1 or SIVmac in MEFs (Fig. 4). These various observations are consistent with a model thereby IFN-I inhibits HIV-1 in mouse cells by increasing the expression of a restriction factor that acts downstream of PML and directly targets incoming HIV-1. Along these lines, restriction of HIV-1 appeared to be saturable in several of our experiments, supporting a model where an antiviral effector is present in limiting concentrations, which is not consistent with PML being this effector. Also in support of an indirect effect of PML is the fact that HIV-1 infection of MEFs was inhibited at reverse transcription, a step that takes place in the cytoplasm while PML is predominantly nuclear. Taken together, these observations suggest that PML promotes the restriction of multiple lentiviruses by activating a downstream effector whose identity and viral target(s) remain to be determined. Interestingly, PML was recently found to be involved in the transcriptional activation of interferon-stimulated genes following treatment with IFN-I (Kim & Ahn, 2015), supporting the idea that PML plays an activating role upstream of innate immune effectors.

In addition to its effects on early stage viral replication, PML also caused transcriptional silencing of HIV-1 in MEFs, a result consistent with previous observations that HIV-1 transcription was low in murine cells, even in the presence of human cyclin T1 (hCycT1) (Zhang et al., 2008). Unlike the restriction of early stages of replication, the repression of HIV-1 gene expression was not enhanced by IFN-I treatment. Therefore, although both inhibitory mechanisms are dependent upon the presence of PML, they are differentially regulated. We found no evidence that hPML repressed HIV-1 LTR-driven gene expression in SupT1 cells. However, transfer of some hPML isoforms (hPML-I, II, IV and VI and to a lesser extent hPML-V) in PML-KO MEFs fully reconstituted the

restriction activities, supporting a conserved role for mPML and hPML. This discrepancy may result from the fact that the establishment of latency in human cells may be rare and may occur only in specific conditions, whereas the HIV-1 promoter is constitutively repressed in murine cells. Recently, two different teams used cell lines belonging to the "J-Lat" series, which are human T cell lines containing integrated but transcriptionally silent copies of an HIV-1-derived vector, to investigate whether PML and PML bodies have a role in latency. Both teams found that latent HIV-1 could be reactivated by treatment with the PML inhibitor arsenic trioxide (As_2O_3) (Lusic et al., 2013; Wang et al., 2013). Lusic and collaborators also observed that PML depletion similarly reactivated HIV-1 in the J-Lat 9.2 clone (Lusic et al., 2013). These results show that PML is required for the maintenance of transcriptional latency in these models, but they do not address the question of whether it is involved in the establishment of latency. MEFs and possibly other murine cell types in which HIV-1 transcription is constitutively repressed may provide valuable investigatory tools to identify the factors controlling the establishment and maintenance of viral latency and persistence.

As_2O_3 has been shown to interfere with several retroviral restriction pathways over recent years, including TRIM5α (Berthoux et al., 2004; Berthoux et al., 2003), TRIMCyp (Sebastian et al., 2006), APOBEC3G (Stalder et al., 2010), Lv4 (Pizzato et al., 2015) and possibly SAMHD1 (Pion et al., 2007). As_2O_3 is unlikely to directly inhibit those various restriction effectors. Therefore, the most straightforward explanation is that it acts upstream, by interacting with a factor that controls the global antiviral state of the cell. Clues that PML might be this factor come from imaging and biochemical studies that used fluorescent and biotin-labeled analogs of As_2O_3. These studies strongly suggested that PML was the major and perhaps the sole cellular target for this drug (Jeanne et al., 2010; Zhang et al., 2010). As_2O_3 promotes PML oligomerization, resulting in increased SUMOylation and ubiquitination, followed by proteasome-dependent degradation (Zhang et al., 2010). The picture emerging from these and other studies (Kim & Ahn, 2015) is that PML upregulates antiretroviral effectors that target viral replication at several steps. Changes in the expression patterns of these downstream effectors might explain the cellular context specificity observed for the effects of PML expression on HIV-1.

2.7 Conclusions

Taken together, our observations suggest that PML broadly upregulates the activity of innate antiviral effectors, through mechanisms that are yet to be dissected. It has been suggested that the PML inhibitor As_2O_3 could be tested as a pharmacological agent to counter HIV-1 latency in humans (Lusic et al., 2013; Wang et al., 2013). However, this study and previous ones (Berthoux et al., 2003; Dutrieux et al., 2015) show that targeting PML might enhance the early stages of HIV-1 replication by removing PML-controlled antiviral activities. Thus, As_2O_3 and other compounds targeting PML likely involve a trade-off between inhibition of latency and inhibition of innate immune mechanisms. Our results are also relevant to the development of murine models for HIV-1. Despite multiple attempts at introducing key human positive factors in murine cells, such as hCD4, hCCR5, hCycT1 or hCRM1 (Bieniasz & Cullen, 2000; Elinav et al., 2012; Zhang et al., 2010), murine cells remain non-permissive to HIV-1. Removing the endogenous mPML in the context of murine cells expressing key human factors might support HIV-1 propagation. The availability of PML knockout mice for crossing experiments (Wang et al., 1998) might finally open the door to the long sought-after human tissue-free murine model for AIDS.

2.8 Acknowledgements

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Chapter III contains a study showing that PML is dispensable for the restriction of lentiviruses in human cells and moreover, PML is not involved in IFN-induced restriction of lentiviruses. Additionally, we demonstrate that PML is not involved in the TRIM5α-mediated restriction of HIV-1.

CHAPTER III

GENE KNOCKOUT SHOWS THAT PML (TRIM19) DOES NOT RESTRICT THE EARLY STAGES OF HIV-1 INFECTION IN HUMAN CELL LINES

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3.1 Contributions

Masroori and Berthoux designed the study. Masroori, Cherry, Merindol, Li, Dufour, Poulain and B. Plourde performed the experiments. Masroori and Berthoux analyzed the data and prepared the manuscript.

3.2 Abstract

The PML (promyelocytic leukemia) protein is a member of the TRIM family, a large group of proteins that show high diversity in functions but possess a common tripartite motif giving the family its name. We and others recently reported that both murine PML (mPML) and human PML (hPML) strongly restrict the early stages of infection by HIV-1 and other lentiviruses when expressed in mouse embryonic fibroblasts (MEFs). This restriction activity was found to contribute to the type I interferon (IFN-I)-mediated inhibition of HIV-1 in MEFs. Additionally, PML caused transcriptional repression of the HIV-1 promoter in MEFs. By contrast, the modulation of the early stages of HIV-1

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infection of human cells by PML has been investigated by RNAi with unclear results. In order to conclusively determine whether PML restricts HIV-1 or not in human cells, we used CRISPR-Cas9 to knock out its gene in epithelial, lymphoid and monocytic human cell lines. Infection challenges showed that PML knockout had no effect on the permissiveness of these cells to HIV-1 infection. IFN-I treatments inhibited HIV-1 equally whether PML was expressed or not. Over-expression of individual hPML isoforms, or of mPML, in a human T cell line did not restrict HIV-1. The presence of PML was not required for the restriction of nonhuman retroviruses by TRIM5α, another human TRIM protein, and TRIM5α was inhibited by arsenic trioxide through a PML-independent mechanism. We conclude that PML is not a restriction factor for HIV-1 in human cell lines representing diverse lineages.

3.3 Importance

PML is involved in innate immune mechanisms against both DNA and RNA viruses. Although the mechanism by which PML inhibits highly divergent viruses is unclear, it was recently found that it can increase the transcription of interferon-stimulated genes (ISGs). However, whether human PML inhibits HIV-1 has been debated. Here we provide unambiguous, knockout-based evidence that PML does not restrict the early post-entry stages of HIV-1 infection in a variety of human cell types and does not participate in the inhibition of HIV-1 by IFN-I. Although this study does not exclude the possibility of other mechanisms by which PML may interfere with HIV-1, we nonetheless demonstrate that PML does not generally act as an HIV-1 restriction factor in human cells and that its presence is not required for IFN-I to stimulate the expression of anti-HIV-1 genes. These results contribute to uncovering the landscape of HIV-1 inhibition by ISGs in human cells.

3.4 Introduction

PML/TRIM19 belongs to the tripartite motif (TRIM) protein superfamily that shares a conserved tripartite architecture: a RING domain, one or two B-boxes, and a coiled-coil

domain (1). Due to the alternative splicing of the C-terminal domain, seven PML isoforms are present in human cells. Isoforms I to VI are primarily located in the nucleus, while PML VII is mostly cytoplasmic (2). PML is the major component of a nuclear substructure named PML nuclear body (PML NB). PML NBs are dynamic and their size, number, and composition change in response to cellular stresses or during the cell cycle. In addition to PML, these NBs recruit many other proteins in a transient fashion (3-6). TRIM5α, a cytoplasmic factor that restricts retroviruses in a species-specific, virus-specific manner (7), is actively shuttling between the cytoplasm and the nucleus and localizes to the PML NBs when present in the nucleus (8). PML is involved in many cellular activities including transcriptional regulation and tumor suppression (5, 9, 10).

IFNs are a multigene family of inducible cytokines released by host cells in response to pathogens, including viruses (11-13). IFN-I binding to its receptor leads to the transcriptional stimulation of a set of genes encoding antiviral proteins which inhibit the replication of a wide range of viruses (12, 14). The transcription of PML and of many NB-associated proteins (e.g. Daxx and Sp100) is up-regulated by IFN-I (15, 16). Conversely, it was recently proposed that PML is involved in the IFN-I-induced expression of ISGs by directly binding their promoter (17).

The involvement of PML in antiviral defense mechanisms against several DNA and RNA viruses has been extensively studied. PML was shown to restrict a complex retrovirus, the human foamy virus, by inhibiting viral gene expression (18). PML deficient cells are also more prone to infection with rabies virus (19). Moreover, PML was shown to interfere with the replication of poliovirus (20), encephalomyocarditis virus (EMCV) (21), herpes simplex virus type-1 (HSV-1), adeno-associated virus (AAV) (22), influenza virus, and vesicular stomatitis virus (VSV) (23). As a direct consequence, some viruses such as HSV-1 and the human cytomegalovirus have evolved mechanisms to counteract PML, either by disrupting PML NBs and/or by inducing PML degradation (24-26).

The role of PML in HIV-1 infection of human cells is controversial. As_2O_3, a drug that induces PML oligomerization and degradation (27), was shown to increase the

susceptibility of human cells to N-tropic murine leukemia virus (N-MLV) and HIV-1 (28). A recent study proposed that PML was an indirect inhibitor of HIV-1 early post-entry infection stages through its association with Daxx, a constitutive partner protein in PML NBs (29). However, another group found that the depletion of PML (but not that of Daxx) enhanced HIV-1 infection in human primary fibroblasts, while having no effect in T cell lines such as Jurkat (30). PML was also found to regulate HIV-1 latency. Specifically, PML degradation or NBs disruption resulted in the activation of HIV-1 provirus transcription in a lymphoid model of HIV-1 latency (31), although these results have not been independently confirmed. There is consensus, however, on the existence of a PML-dependent restriction of HIV-1 in MEFs. In these cells, PML inhibits the early post-entry stages of infection (32-34) and also promotes the transcriptional silencing of the integrated provirus (34). Human PML (hPML) was able to reconstitute both restriction activities in MEFs knocked out for the endogenous murine PML (mPML), in an isoform-specific fashion (34). In addition, the inhibition of lentiviruses by IFN-I in MEFs involves PML (34). In this study, we investigate the role of PML in the restriction of HIV-1 and other retroviruses in several human cell lines, including T cells and myeloid cells, by gene knockout. We also examine the role of PML in the IFN-induced restriction of lentiviruses in human cells. We show that PML is dispensable for the restriction of lentiviruses in human cells, is not involved in the IFN-I-mediated inhibition of infection, and is not relevant to the inhibition of TRIM5α by As_2O_3.

3.5 Results

CRISPR-Cas9-mediated knockout of PML in human cells. In order to stably and irreversibly deplete PML in human cells, we designed two guide RNAs (gRNAs), hPML1 and hPML2, to target the Cas9 nuclease towards exon 2 of PML (Fig. 1). Exon 2 is present in all hPML isoforms, and the algorithm used to design the gRNAs minimizes the risk of nonspecific targeting. The plasmid used in this study, pLentiCRISPRv2 (pLCv2), can mediate knockouts through transfection and also through lentiviral transduction. The control plasmid, pLCv2-CAG, targets the CMV-IE/chicken actin/rabbit beta globin hybrid promoter, a nonhuman sequence (35). We used the Surveyor assay (36) to reveal

the presence of insertions/deletions (indels) in the PML gene of HEK293T cells transiently transfected with pLCv2-hPML1 or pLCv2-hPML2. We could observe the presence of PML DNA digestion products of the expected size in cells transfected with each of the PML gRNAs but not in cells transfected with the control gRNA (Fig. 3.1A), indicating that both PML gRNAs generated double-strand breaks that were repaired by non-homologous end joining (NHEJ). To quantify the extent of DNA damage following stable lentiviral transduction of the CRISPR components, we transduced human monocytic THP-1 cells with the LCv2-hPML1 vector and, as a control, the irrelevant LCv2-CAG vector. Cells were treated with puromycin to eliminate non-transduced cells, and amplicons of the targeted PML region were then obtained and Sanger sequenced. A reference contig alignment of the sequencing plots revealed that a -1 deletion was the most prevalent mutation found in LCv2-hPML1-transduced cells, but other types of indels were present, as evidenced by the presence of additional peaks at each position (Fig. 3.1B). We further analyzed the sequencing data using the Tracking of Indels by Decomposition (TIDE) method available online (see Methods) (Fig. 3.1C). Computations using this assay showed that at least 96.3% of PML alleles contained an indel at the expected position in cells transduced with the hPML1 gRNA.

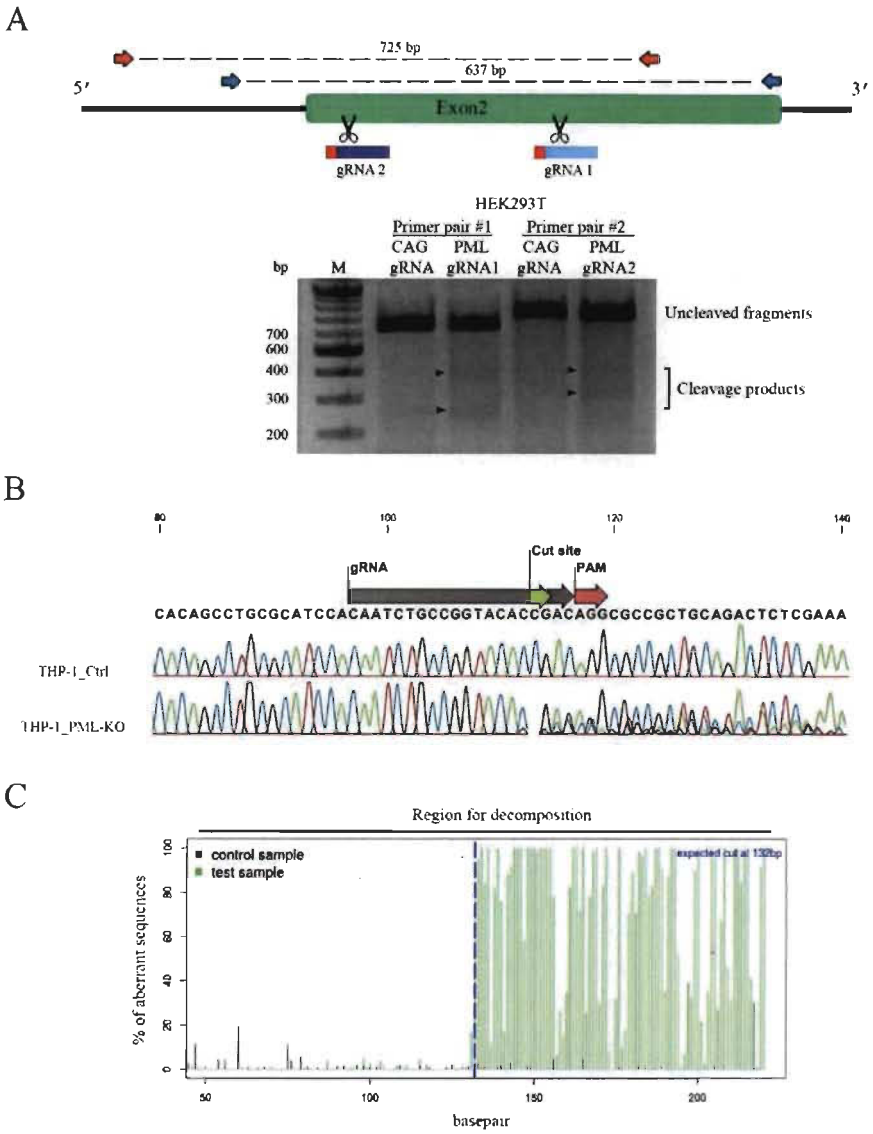


Figure 3.1 **CRISPR-Cas9-mediated genome editing of PML in human cell lines.** (A) The Cas9 nuclease was targeted to exon 2 of the PML gene (green) by two selected gRNAs whose binding sites are shown in blue (PAM motifs are in red). Arrows indicate the positions of the binding sites for the ODNs used in the PCR-Surveyor assay (blue arrows for gRNA1-, red arrows for gRNA2-guided cut sites). The Surveyor assay is shown in the lower panel. Briefly, PCR products amplified from cells transfected with pLCv2-hPML1, -hPML2, or pLCv2-CAG (Ctrl), were subjected to denaturation, re-annealing and digestion with the Surveyor enzyme. Arrowheads indicate cleavage products of the expected size. (B) Sanger sequencing analysis of PML in cells transduced with LCv2-hPML1. THP-1 cells were transduced with lentiviral vectors produced using pLCv2-hPML1 or the control pLCv2-CAG. Following puromycin selection, the targeted PML locus was PCR-amplified and the PCR product was Sanger sequenced. The figure

shows an alignment of the obtained sequence plots. (C) Decomposition of sequencing plots by TIDE assay. The graph shows the % of aberrant peaks upstream and downstream of the cut site in the sequencing reactions shown in panel B. The % of indel-containing alleles was computed by TIDE.

Knocking out PML in human monocytic cells has little to no effect on the permissiveness to HIV-1 in the presence or absence of IFN-I. THP-1 cells were stably transduced with lentiviral vectors produced using pLCv2-hPML1 and pLCv2-hPML2. Following puromycin selection, we performed a Western blotting (WB) analysis of PML levels in bulk populations (Fig. 3.2A). The levels of hPML were not sufficiently high to be detected in unstimulated cells (not shown), and therefore, the analysis was done using cells treated with IFN-β. In control cells we found several bands corresponding to hPML isoforms, as previously reported (2). In the cells transduced with the hPML gRNAs, PML was undetectable, showing that knockout was efficient with both gRNAs and affected all detectable isoforms. This result is consistent with the NHEJ-mediated mutagenesis observed in transfected HEK293T cells using both gRNAs shown in Fig. 3.1. As both gRNAs showed similar efficiency, all the subsequent experiments in this study were only performed with one gRNA, hPML1. We next infected PML knockout (hPML1 gRNA transduced) and control cells (CAG transduced) with a single dose of HIV-$1_{NL\text{-}GFP}$ (37), a VSV-G-pseudotyped, Δ-Envelope HIV-1 vector expressing GFP in place of Nef (Fig. 3.2B). The percentage of GFP-positive cells following HIV-$1_{NL\text{-}GFP}$ challenge is directly proportional to the cells' permissiveness toward infection by this virus. This system is thus well-suited to analyze restriction activities taking place during post-entry steps and until integration. These infections were performed in the presence or absence of IFN-β, owing to the reported role of PML in stimulating the transcription of ISGs (38). In the absence of IFN-β, we found that the PML-KO cells were slightly more permissive to infection by HIV-$1_{NL\text{-}GFP}$ compared with the control cells (less than 2-fold). The addition of IFN-β very strongly inhibited (>20-fold) the infection of THP-1 cells (Fig. 3.2B), and the low numbers of infected cells prevented a fine analysis of the role of PML in this inhibition. However, the absence of PML clearly did not prevent IFN-β from inhibiting HIV-$1_{NL\text{-}GFP}$, showing that PML was dispensable for this activity.

In order to obtain a more complete picture of the importance of PML in the permissiveness to retroviruses in this immune cell line, we performed additional infections with this HIV-1 vector as well as with GFP-expressing vectors derived from the macaque strain of the simian immunodeficiency virus (SIVmac-GFP), the equine infectious anemia virus ($EIAV_{GFP}$) and the B-tropic murine leukemia virus ($B\text{-}MLV_{GFP}$). EIAV is restricted by TRIM5α in human cells (39), making it possible to analyze whether PML modulates the restriction of retroviruses by this well-characterized restriction factor. Infectivity of the three lentiviral vectors (HIV-1, SIVmac, EIAV) was slightly higher in the absence of PML at most virus doses used, whereas infectivity of the B-MLV vector was unaffected by PML knockout (Fig. 3.2C). These results suggest that PML has a small, barely detectable inhibitory effect on the infection of THP-1 cells by lentiviruses and does not modulate TRIM5α activity. Treatment with IFN-β strongly decreased THP-1 permissiveness to all four vectors, preventing us from measuring the -fold decrease in infectivity with accuracy (Fig. 3.2C). However, it was clear that IFN-β efficiently inhibited infection in the presence or absence of PML, indicating that PML is not crucial for the IFN-I-mediated anti-retroviral response.

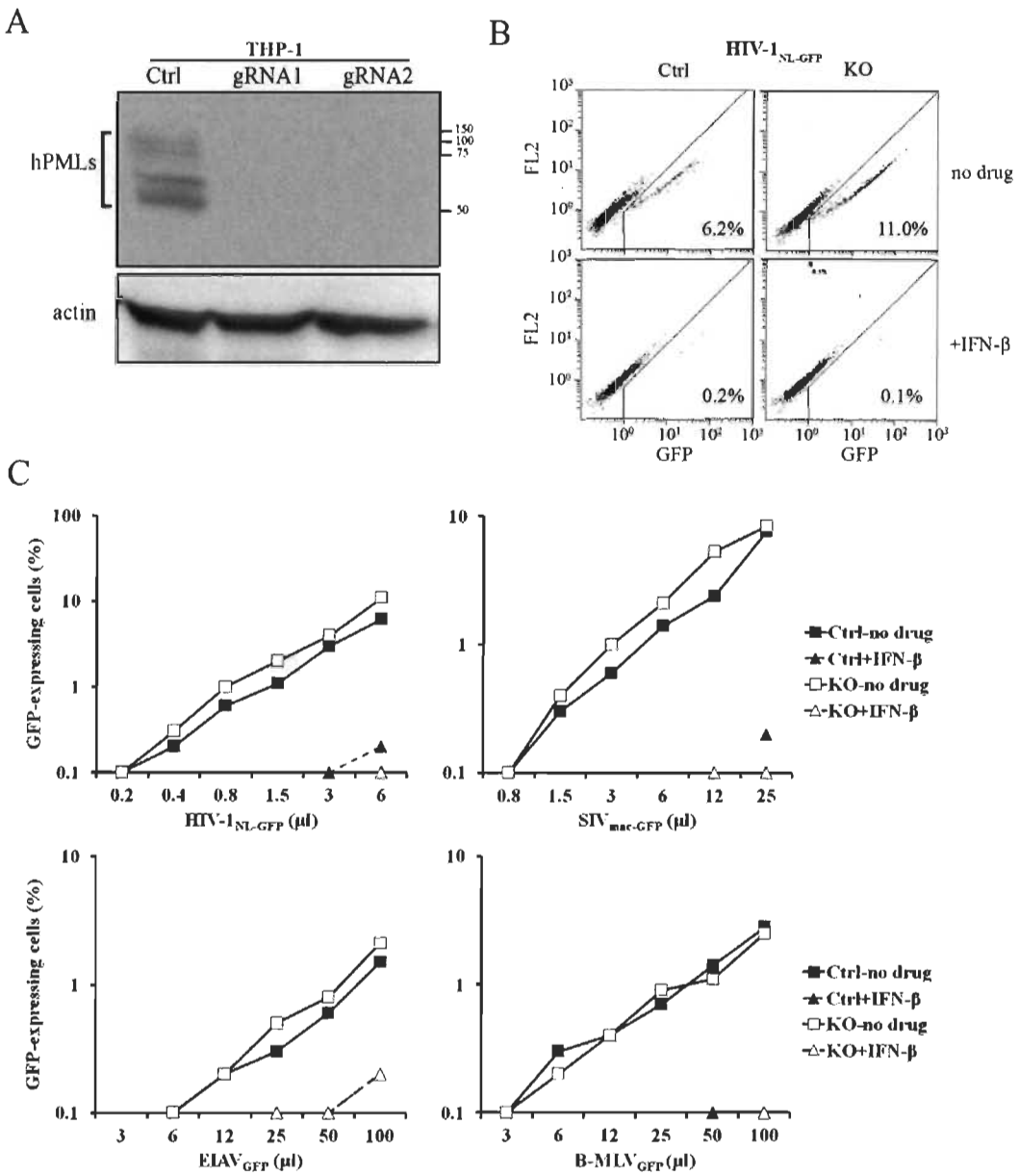


Figure 3.2 **PML knockout has negligible effects on intrinsic or IFN-I-induced restriction of retroviruses in THP-1 cells.**
(A) WB analysis of THP-1 cells transduced with pLCv2-based vectors expressing Cas9 and a gRNA targeting either hPML or CAG. Stably transduced, puromycin-resistant cells were treated with IFN-β (10 ng/ml). Cellular lysates were prepared 16 h later and analyzed by WB using antibodies against hPML and actin as a loading control. (B) FACS plots from PML-knockout (KO) and control (Ctrl, CAG gRNA-transduced) THP-1 cells infected with HIV-1NL-GFP. Control or PML-KO THP-1 cells were treated with IFN-β or left untreated and then exposed to HIV-1_{NL-GFP} (10 μl). 2 d later, cells were analyzed by FACS and the

percentage of infected (GFP-positive) cells observed is indicated on each plot. (C) Virus dose-dependent analysis of the role of hPML in the intrinsinc and IFN-I-induced restriction of retroviruses. Control and PML-KO THP-1 cells were treated with IFN-β (10 ng/ml) for 16 h, followed by infection with increasing doses of retroviral vectors. The percentage of infected cells was assessed 2 d later by FACS.

Knocking out PML in human epithelial cells has little to no effect on the permissiveness to retroviral infections in the presence or absence of IFN-I. We then transduced epithelial carcinoma HeLa cells with the CAG or PML gRNAs. PML was efficiently knocked out, as seen by WB (Fig. 3.3A). We also performed immunofluorescence microscopy to analyze the effect of PML knockout on PML and SUMO nuclear bodies. A large part (but not all) of SUMO-1 localizes to PML bodies in normal conditions (40). As expected, signal corresponding to PML nuclear bodies almost completely disappeared from the cells transduced with the PML gRNA (Fig. 3.3B). In addition, SUMO-1 punctate nuclear staining was strongly diminished but not abolished (Fig. 3.3B). We then challenged the stably transduced cells with GFP-expressing viral vectors like we had done in THP-1 cells. We found that susceptibility to HIV-1, SIVmac, EIAV and B-MLV vectors was identical whether PML was present or not (Fig. 3.3C-D). IFN-β inhibited all four viral vectors, although the magnitude of this effect (~2- to 3-fold) was much smaller than in THP-1 cells. IFN-β treatments had identical effects in PML-expressing and PML-knockout cells, again showing that PML does not modulate this inhibitory pathway in human cells.

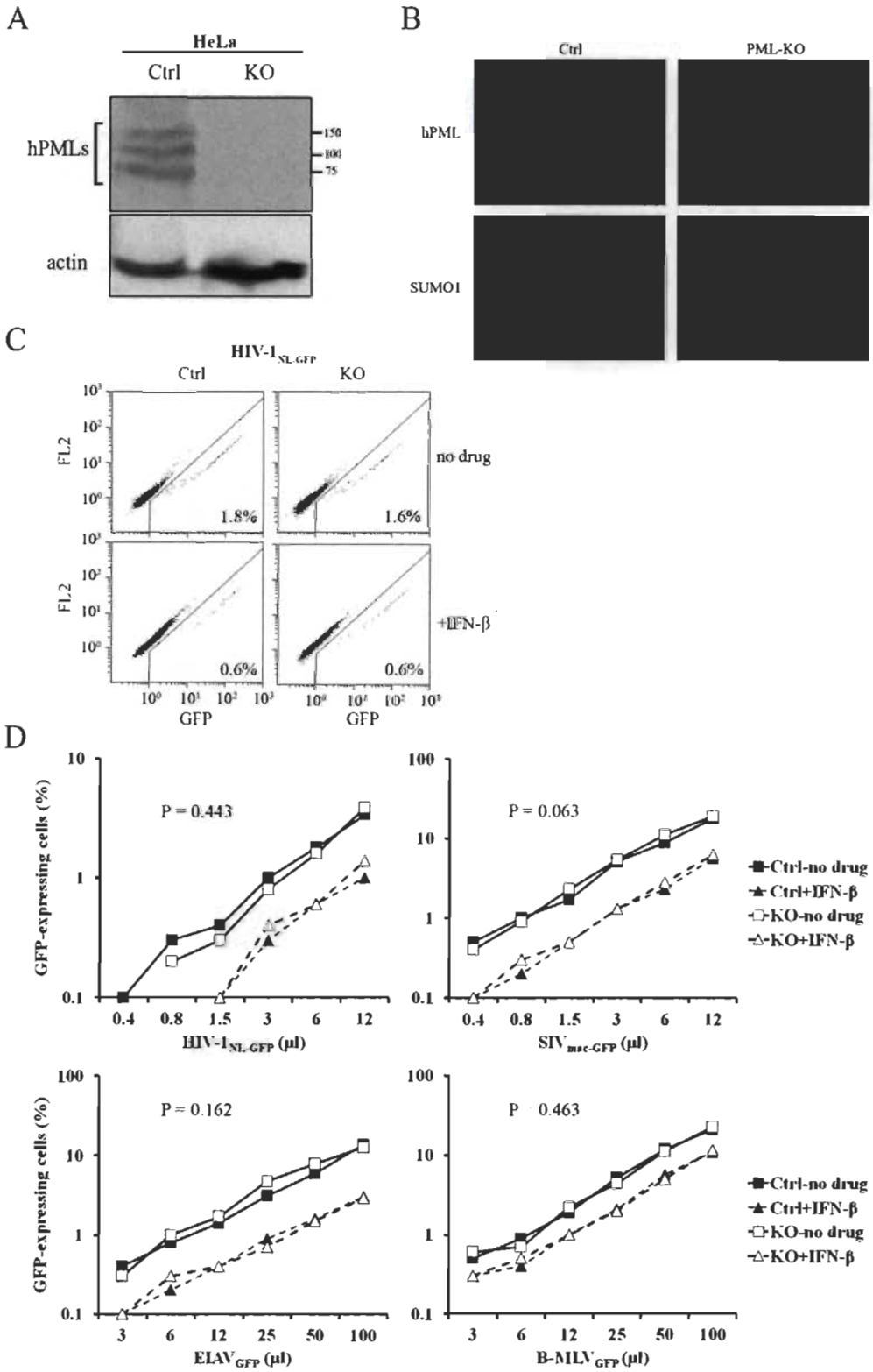

A
HeLa
Ctrl
KO
hPMLs
150
100
75
actin
B
Ctrl
PML-KO
hPML
SUMO1
C
HIV-1NL-GFP
Ctrl
KO
FL2
no drug
1.8%
1.6%
+IFN-β
0.6%
0.6%
GFP
GFP
D
GFP-expressing cells (%)
P = 0.443
HIV-1NL-GFP (μl)
P = 0.063
SIVmac-GFP (μl)
Ctrl-no drug
Ctrl+IFN-β
KO-no drug
KO+IFN-β
P = 0.162
EIAVGFP (μl)
P 0.463
B-MLVGFP (μl)

Figure 3.3 PML knockout has no effect on intrinsic or IFN-I-induced restriction of retroviruses in HeLa cells.

(A) HeLa cells lentivirally transduced with pLCv2 vectors expressing either the hPML gRNA1 or (as a control) the CAG-targeting gRNA were treated with IFN-β (10 ng/ml). Cellular lysates were prepared 16 h later and analyzed by WB using an anti-hPML antibody. Actin was analyzed as a loading control. (B) IF microscopy analysis of PML bodies in HeLa cells transduced with pLCv2-PML1 (PML-KO) or transduced with pLCv2-CAG as a control (Ctrl). Puromycin-selected cells were stained for PML (top) or SUMO-1 (bottom). Nuclei were stained with Hoechst33342. (C) FACS plots from transduced HeLa cells infected with HIV-$1_{NL\text{-}GFP}$. Control and PML-KO HeLa cells treated or not with IFN-β were infected with HIV-$1_{NL\text{-}GFP}$ (6 µl). The percentage of infected cells determined at 2 d post-infection is indicated for each plot. (D) Virus dose-dependent analysis of the role of hPML in IFN-I-induced restriction of retroviral infection. Control and PML-KO HeLa cells were treated with IFN-β, followed 16 h later by infection with increasing doses of the indicated retroviral vectors. The percentage of infected cells was assessed 2 d later by FACS.

Rhabdomyosarcoma-derived epithelial TE671 cells were similarly knocked out for PML by lentiviral transduction, and knockout was efficient (Fig. 3.4A). Similar to what we found in HeLa cells, infectivity of the four vectors tested was identical whether PML was present or not (Fig. 3.4B). IFN-β decreased the permissiveness of TE671 cells to all four vectors, although we noticed that IFN-β had a relatively smaller effect on HIV-$1_{NL\text{-}GFP}$ compared with the three other vectors in TE671 (Fig. 3.4B). The IFN-β-induced inhibition of the four retroviral vectors in TE671 cells was identical whether PML was present or not (Fig. 3.4B).

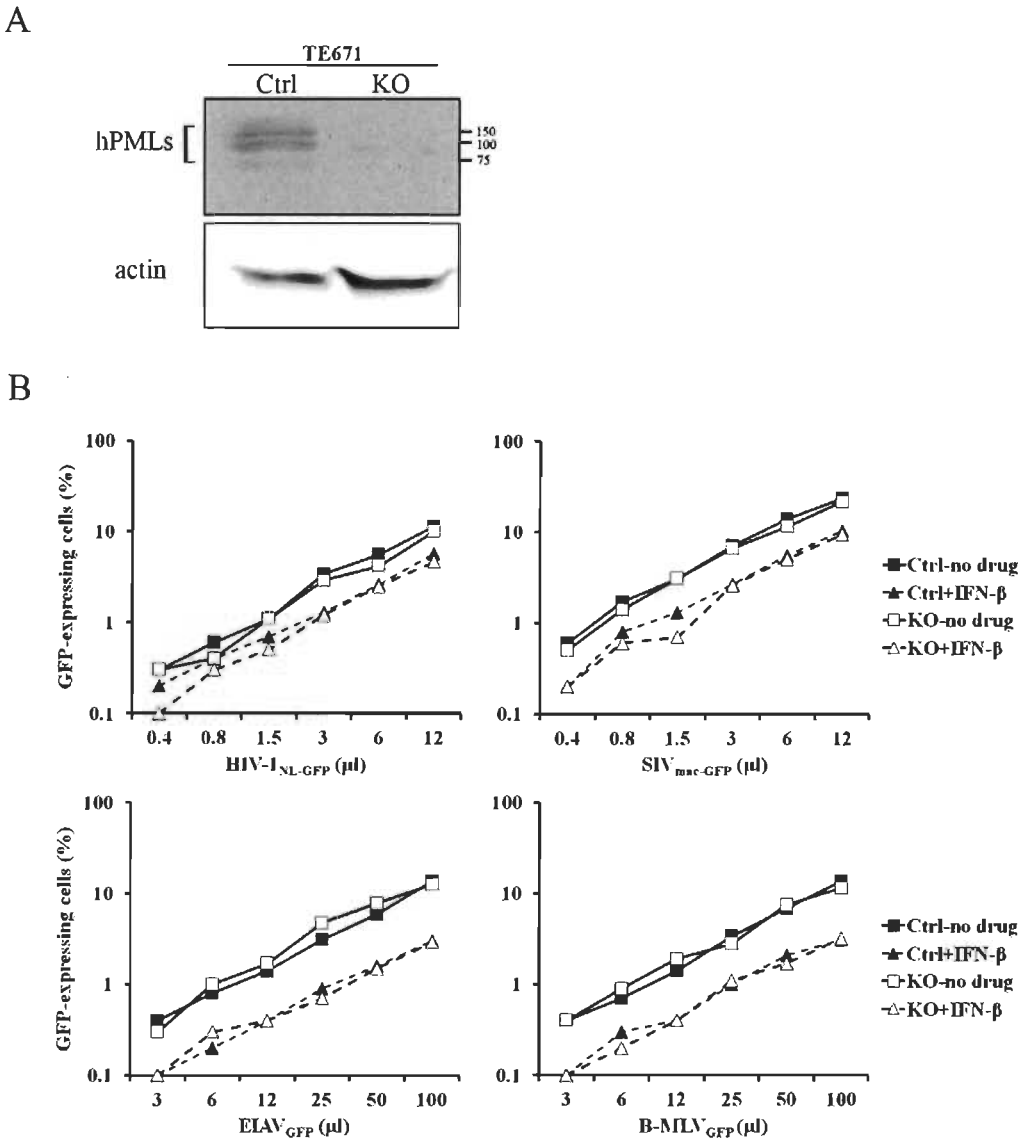


Figure 3.4 **PML knockout has no effect on intrinsic or IFN-I-induced restriction of HIV-1 in TE671 cells.**

(A) WB analysis. TE671 cells were stably transduced with pLCv2-based vectors expressing Cas9 and either the hPML-targeting gRNA1 or the CAG-targeting gRNA as a control. The cells were treated with IFN-β (10 ng/ml) or left untreated as a control. Cellular lysates were prepared 16 h later and analyzed by WB using an anti-hPML antibody along with actin as a loading control. (B) Infection assay. Control (CAG gRNA-transduced) and PML-KO TE671 cells were treated with IFN-β or left untreated. 16 h later, the cells were infected with increasing doses of the indicated retroviral vectors. The percentage of infected cells was assessed 2 d later by FACS.

A knock-in approach to suppress PML in human cells. In order to achieve efficient knockout by transient transfection without the need to isolate cellular clones by limiting dilution, we constructed a plasmid to serve as donor DNA in homology-directed repair (HDR). This plasmid contains two ~800bp-long PML homology arms surrounding a neomycin resistance cassette (Fig. 3.5A). It is expected that its co-transfection in cells along with Cas9 and the hPML gRNA1 would lead to the knock-in of NeoR in PML through HDR in a fraction of the cells. Selection in neomycin then eliminates cells in which the knock-in did not occur. Even if not all alleles of a given gene are successfully modified by knock-in, recent reports indicate that the remaining ones are usually knocked out through NHEJ-dependent mechanisms (41). We designed PCR primers for the specific amplification of the knock-in product and another pair to amplify the wild-type (WT) or the NHEJ-repair knockout alleles (Fig. 3.5A). To validate this system, we co-transfected TE671 cells with pLCv2.PML1 and the HDR donor plasmid, and randomly isolated a number of neomycin-resistant cell clones of which a representative analysis is shown in Fig. 3.5B. The knock-in product was detected as expected in all 7 clones while being absent in the parental cells. On the other hand, the band corresponding to WT or NHEJ-repaired alleles was less intense in these clones relative to the parental cells, but was always present, suggesting that HDR-mediated knockout did not affect all the *PML* alleles.

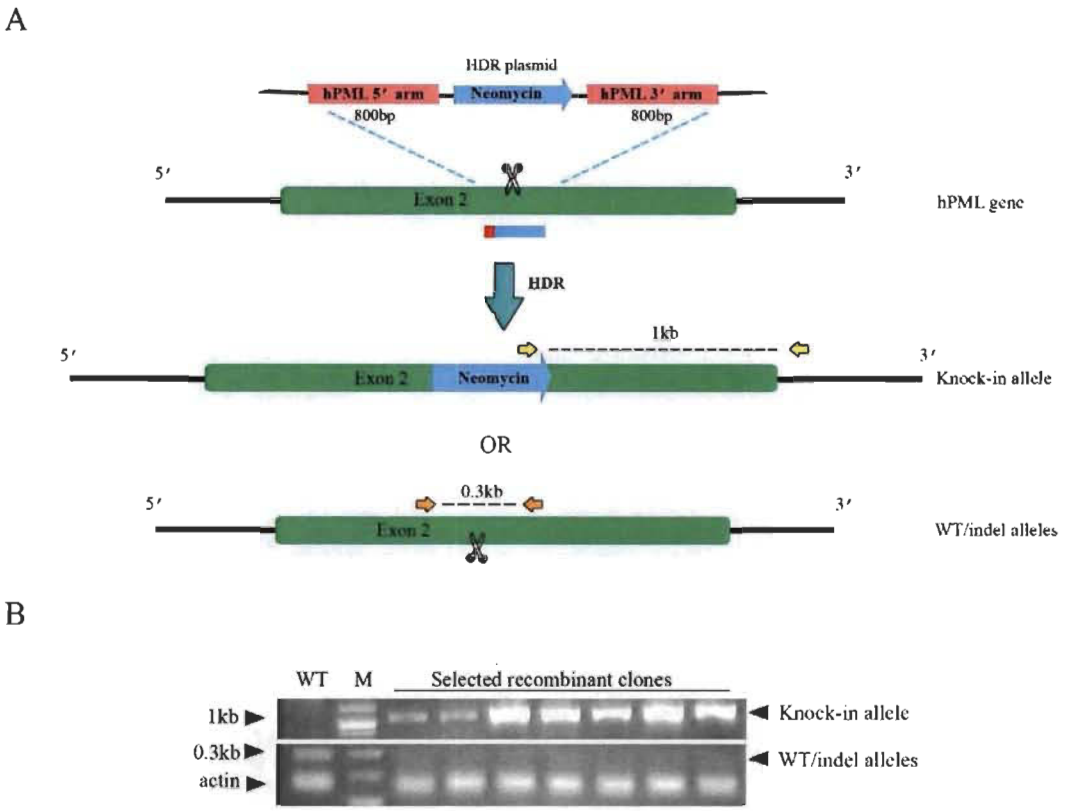


Figure 3.5 HDR-mediated knockout of PML.
(A) Schematic of the HDR plasmid and targeting strategy for the knock-in of the Neomycin resistance gene at the PML locus. Two 800 bp-long PML homology arms encompass the Neo^R expression cassette on plasmid pNMs-Neo.HDR-hPML. The arms are complementary to the PML regions on either side of the gRNA1-mediated Cas9 cleavage site. Co-transfection of pLCv2-hPML1 and pNMs-Neo.HDR-hPML may yield a knock-in allele as indicated if DNA is repaired by HDR. If DNA is repaired by NHEJ, WT or indel-containing alleles may be generated. Yellow and orange arrows indicate the binding sites for the primers used to detect knock-in and WT/indel alleles by PCR (1 Kbp and 0.3 Kbp products, respectively). (B) PCR analysis of Neomycin-resistant TE671 clones. TE671 cells were co-transfected with pLCv2-hPML1 and pNMs-Neo.HDR-hPML, then grown in presence of neomycin. Individual Neo^R clones were analyzed using the two primer pairs described in A.

PML is important for the efficient inhibition of SIVmac but not HIV-1 by IFN-I in lymphoid cells. We knocked out PML in Jurkat cells using the transfection approach that results in the insertion of *Neo*^{R} in *PML*, as described above. We performed WB analyses to assess knockout efficiency. (Fig. 3.6A). Treatment with the IFN-I species

IFN-α, IFN-β and IFN-ω stimulated PML expression in Jurkat cells. PML was efficiently knocked out (Fig. 6A), validating the HDR-based approach. In the absence of IFN-β, PML had little effect on the infectivity of all four vectors (<2-fold) (Fig. 3.6B). The effect of IFN-β treatment differed according to the retroviral vector used (Fig. 3.6B). IFN-β treatment decreased HIV-$1_{NL\text{-}GFP}$ infectivity by ~3.5-fold in both control and PML-KO cells. IFN-β similarly decreased the infectivity of $SIV_{mac\text{-}GFP}$ by about 4-fold, but only in the control cells. In the PML-KO cells, the inhibitory effect of IFN-β on $SIV_{mac\text{-}GFP}$ infectivity was smaller (<2-fold). Interestingly, we found the opposite situation upon challenge with the $EIAV_{GFP}$ vector: IFN-β treatment had no effect on $EIAV_{GFP}$ infectivity in the WT Jurkat cells, whereas it significantly inhibited this vector in PML-KO cells, especially at low vector doses. Finally, IFN-β decreased the infectivity of B-MLV_{GFP} in both WT cells and PML-KO cells, with no apparent specificity. Thus, Jurkat cells provided a more complex situation with respect to the importance of PML in the antiviral effects of IFN-β. In order to further study the contrasting phenotypes of the HIV-1 and SIVmac vectors in these cells, we also analyzed the effects of IFN-α and IFN-ω. (Fig. 3.6C). We found that in control cells, all three IFN-I species decreased infectivity of both the HIV-1 and the SIVmac vectors, by 2- to 4-fold; IFN-β appearing to be the most consistently inhibitory IFN-I in these cells, similar to what we had observed in other cell lines (not shown) and to what was reported in the literature (42). In PML-KO cells, HIV-$1_{NL\text{-}GFP}$ was inhibited by all three IFN-I species, similar to the control cells. In contrast, IFN-I inhibition of $SIV_{mac\text{-}GFP}$ was much less efficient in PML-KO cells (Fig. 3.6C, bottom right panel). Thus, PML is important for IFN-I to inhibit the early infection stages of SIVmac, but not HIV-1, in Jurkat cells.

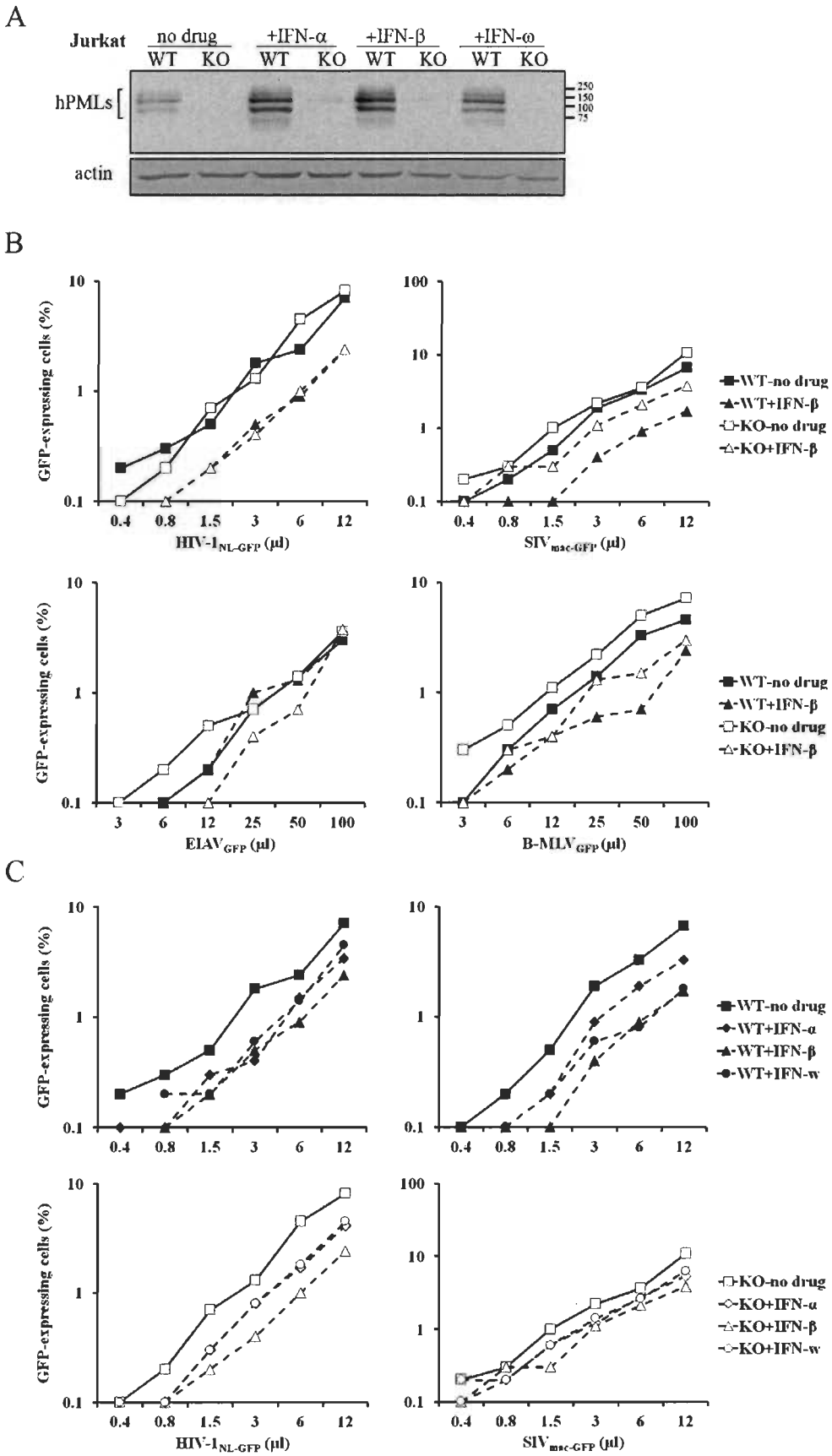

A
Jurkat
no drug
+IFN-α
+IFN-β
+IFN-ω
WT KO WT KO WT KO WT KO
hPMLs
actin
250
150
100
75
B
GFP-expressing cells (%)
HIV-1NL-GFP (μl)
SIVmac-GFP (μl)
EIAVGFP (μl)
B-MLVGFP (μl)
WT-no drug
WT+IFN-β
KO-no drug
KO+IFN-β
C
WT+IFN-α
WT+IFN-w
KO+IFN-α
KO+IFN-w

Figure 3.6 PML knockout has virus-specific effects on the restriction of retroviruses in Jurkat cells.
(A) Jurkat cells were co-transfected with pLCv2.hPML1 and pNMs-Neo.HDR-hPML. Neomycin-resistant cells (KO) and parental untransfected cells (WT) were treated with IFN-α, IFN-β or IFN-ω (10 ng/ml). Cellular lysates were prepared 16 h later and analyzed by WB using an anti-hPML antibody. Actin was analyzed as a loading control. (B) Virus dose-dependent analysis of the role of hPML in the intrinsic and IFN-I-induced restriction of retroviruses. PML-KO and control Jurkat cells were treated with IFN-β for 16 h, followed by infection with increasing doses of the indicated retroviral vectors. The percentage of infected cells was assessed 2 d later by FACS. (C) PML-KO and control cells were challenged with increasing doses of HIV-$1_{NL\text{-}GFP}$ following treatment with IFN-α, -β or -ω for 16 h. The percentage of infected cells was assessed 2 d later by FACS.

Over-expression of murine or human PML in Jurkat cells does not affect the infectivity of an HIV-1 vector. Unlike the PML-KO THP-1, HeLa and TE671 cells, the PML-KO Jurkat cells generated do not continuously express Cas9 or a PML-targeting gRNA. Thus, these cells provided an appropriate model to test whether the over-expression of specific hPML isoforms in a PML-KO background could inhibit HIV-1 or other retroviruses. In other words, this experiment was designed to reveal a possible cryptic restriction activity associated with specific PML isoforms that would normally not be apparent due to the presence of other isoforms. We retrovirally transduced the isoforms I to VI of hPML into PML-KO Jurkat cells, separately. Because HIV-1 is inhibited by mPML in MEFs (32-34), we also transduced mPML. A WB analysis showed that all six isoforms of hPML were expressed, as was mPML isoform 2 (Fig. 3.7A). We then challenged the various cell cultures with the HIV-1, SIVmac, EIAV and B-MLV vectors (Fig. 3.7B). We found that none of the PML isoforms had an effect on GFP transduction by HIV-$1_{NL\text{-}GFP}$. Interestingly, several hPML isoforms and mPML slightly increased permissiveness to SIVmac-GFP, by ~2-fold. Permissiveness to $EIAV_{GFP}$ was overall not modulated by over-expression of hPML or mPML, although a slight increase in infectivity was observed in presence of some hPML isoforms at the highest virus doses tested. Finally, the presence of hPML-VI slightly inhibited infection by B-MLV_{GFP} at least at some virus doses used (Fig. 3.7B). Thus, although individual PML isoforms modestly modulated the permissiveness to infection by the SIVmac, EIAV and B-MLV vectors in

a virus-specific fashion, none of them affected permissiveness to infection by the HIV-1 vector.

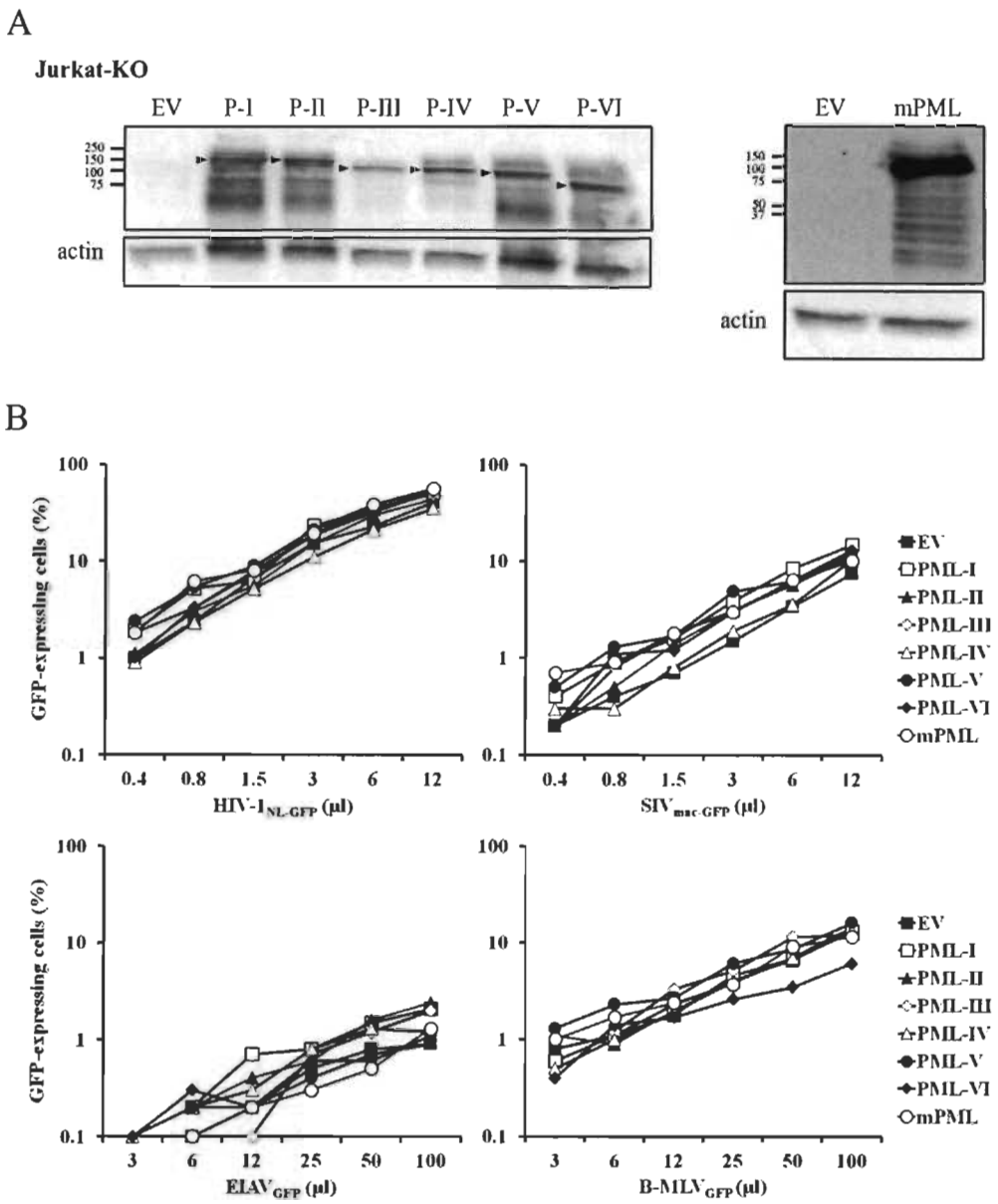


Figure 3.7 **Transduction of mPML or hPML isoforms in PML-KO Jurkat cells has virus-specific effects on the permissiveness to retroviral vectors.** (A) WB analysis of mPML and hPML expression. PML-KO Jurkat cells were stably transduced with mPML or with FLAG-tagged hPML-I to -VI separately. The empty vector (EV) was transduced as a control. Lysates prepared from the different cell populations were analyzed by WB with anti-FLAG (left panel) or anti-mPML (right panel) antibodies. Actin was probed as loading control. The arrowheads indicate the expected size

for each hPML isoform. (B) Susceptibility to transduction by retroviral vectors. The cells were infected with multiple doses of the indicated retroviral vectors and the percentage of GFP-expressing cells was determined 2 d later by FACS.

Restriction of N-MLV by TRIM5α and inhibition of TRIM5α by arsenic trioxide are independent of PML. Intriguingly, TRIM5α localizes at PML bodies when shuttling to the nucleus, as demonstrated by pharmacological treatment interfering with its nuclear export (43). The possibility of PML involvement in the inhibition of retroviruses by TRIM5α has been envisioned but not proven. The infectivity of the EIAV vector used here (which is restricted 5- to 10-fold by human TRIM5α (44)) was not significantly affected by knocking out PML (Fig. 3.2-3.4), suggesting that TRIM5α does not require PML. In order to increase sensitivity, we used an "N-tropic" strain of MLV, which is even more strongly restricted by human TRIM5α (39, 45) than EIAV, and of which restriction is counteracted by As_2O_3 in a cell context-specific fashion (46, 47). Thus, As_2O_3 greatly increases the infectivity of N-MLV but not B-MLV vectors in many human cell lines. The mechanism of action of As_2O_3 against TRIM5α has not been determined but it was thought to involve PML, since As_2O_3 is well-known as a specific inhibitor of PML (27, 48). Interestingly, As_2O_3 also enhances the infectivity of HIV-1 in human cells, although the magnitude of this effect is milder than what is found with N-MLV (46, 49). We infected HeLa, TE671 and Jurkat cells with HIV-$1_{NL\text{-}GFP}$, B-MLV_{GFP} and N-MLV_{GFP} in the presence of increasing As_2O_3 concentrations (Fig. 3.8A). In the absence of As_2O_3, N-MLV_{GFP} infectivity was barely detectable or undetectable in all three cell lines, reflecting the strong inhibition conferred by TRIM5α in human cells. At the same virus dose, B-MLV_{GFP} infected 3% to 5% of the cells. PML knockout had no effect on the infectivity of the two MLV vectors, implying that PML is not required for TRIM5α-mediated restriction of N-MLV. In presence of As_2O_3, N-MLV_{GFP} infectivity was greatly enhanced, although the stimulating effect was partly reversed at high As_2O_3 concentrations in HeLa and TE671 cells (Fig. 3.8A). As_2O_3 effectiveness at counteracting TRIM5α-mediated restrictions was found to decrease at high concentrations in previous studies as well (47, 49). In contrast to N-MLV_{GFP}, B-MLV_{GFP} was only slightly enhanced by As_2O_3. As reported before, As_2O_3 modestly increased HIV-$1_{NL\text{-}GFP}$ infection of HeLa and TE671 cells, although it had no effect on this vector in Jurkat cells (Fig. 3.8B).

Knocking out PML had no detectable effect on the As_2O_3-mediated stimulation of N-MLV_{GFP} and HIV-$1_{NL\text{-}GFP}$ in the three cell lines tested. We performed an additional infection of the HeLa cells with the N-MLV and B-MLV vectors, this time at a fixed As_2O_3 concentration and varying virus doses. Again, we observed that (i) PML had no effect on the infectivity of N-MLV_{GFP} and B-MLV_{GFP}, (ii) As_2O_3-mediated stimulation of N-MLV_{GFP} was significantly stronger than that of B-MLV_{GFP}, regardless of the virus dose, and (iii) knocking out PML had no impact on the effect of As_2O_3 on the MLV vectors. These data demonstrate that PML is not involved in the restriction of N-MLV by TRIM5α, nor is it involved in the mechanism by which As_2O_3 stimulates retroviral infections and counteracts TRIM5α.

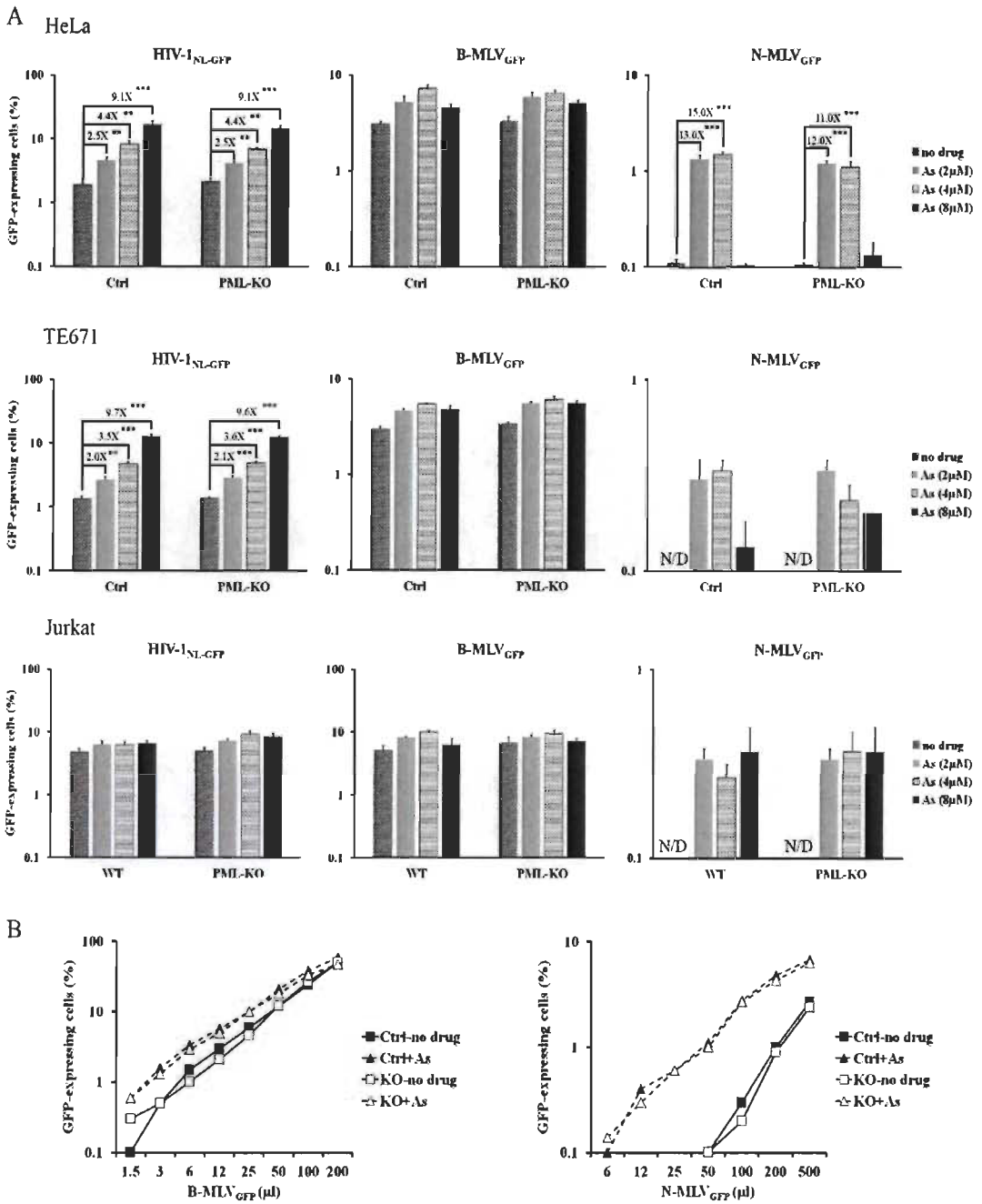


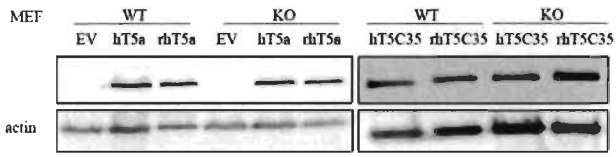
Figure 3.8 **PML is irrelevant for the As_2O_3-induced stimulation of retroviral infectivity in human cells.**

(A) Effect of As_2O_3 on the permissiveness to retroviral vectors in the presence or absence of PML. Control and PML-KO human cell lines were treated with the indicated amounts of As_2O_3 for 15 min prior to infection with HIV-1, B-MLV and N-MLV vectors expressing GFP (B-MLV_{GFP} and N-MLV_{GFP} have identical titers in non-restrictive CRFK cat cells). The percentage of infected cells was assessed 2 d later by FACS. The values represent the means of three independent infections with

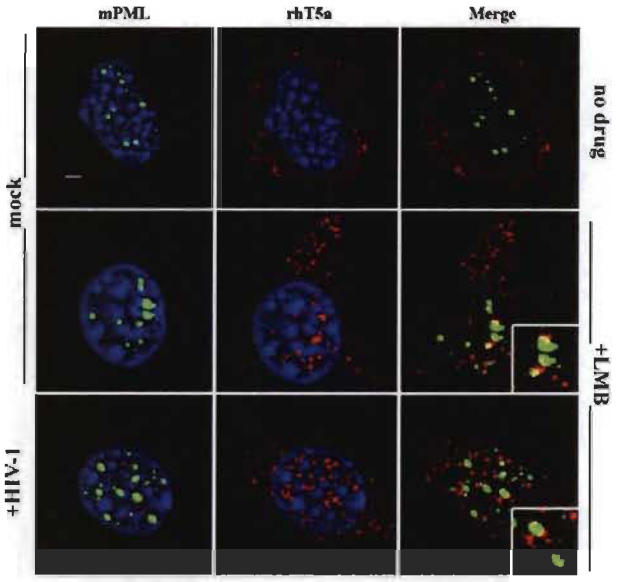
standard deviations (**P < 0.01, ***P < 0.001, N/D not detected). (B) Virus dose-dependent infections. Ctrl and PML-KO HeLa cells were infected with increasing doses of B-MLV$_{GFP}$ or N-MLV$_{GFP}$ vectors in the presence or not of 4 μM As_2O_3. 2 d later, the percentage of infected cells was determined with FACS.

PML is not required for TRIM5α-mediated restriction of HIV-1 in MEFs. MEFs provide a cellular environment in which PML restricts HIV-1, as seen by several laboratories (32-34). In addition, PML also inhibits HIV-1 transcription in MEFs, an effect that we did not observe in human cells (34). Thus, it would be conceivable for PML to have an impact on TRIM5α-mediated restriction of HIV-1 in this specific cellular environment. To test this hypothesis, we used PML-KO MEFs (34, 52). WT and PML-KO MEFs were stably transduced with the HIV-1-restrictive Rhesus macaque TRIM5α or the non-restrictive human TRIM5α as a control. The cells were also transduced with the C35A RING domain mutant of each TRIM5α ortholog, which abolishes the RING domain-associated ubiquitin ligase activity (53). WB analyses showed that the transduced TRIM5α variants were expressed at comparable levels (Fig. 3.9A). Colocalization of a fraction of TRIM5α with PML NBs was seen in the presence of the nuclear export inhibitor leptomycin B, consistent with published data obtained in human and canine cells (43), and exposure of the cells to HIV-1 did not modify this pattern (Fig. 3.9B). The cells were then challenged with HIV-1$_{NL-GFP}$ or with the relatively restriction insensitive SIVmac-GFP as a control (54), using virus doses at which PML has only mild effects on transduction by these lentiviral vectors in the absence of TRIM5α (34). HIV-1 was very strongly restricted by rhTRIM5α in both WT and PML-KO MEF cells (Fig. 3.9C). As expected, C35A rhTRIM5α and hTRIM5α (WT or C35A) had little to no effect on HIV-1$_{NL-GFP}$, although we observed slightly higher levels of HIV-1 restriction by C35A rhTRIM5 in the presence of PML, perhaps suggesting that the presence of PML could partially compensate for the loss of a functional TRIM5α RING domain. SIVmac-GFP was moderately restricted by rhTRIM5α, and PML knockout did not affect this inhibitory effect (in fact, restriction was slightly greater in the absence of PML) (Fig. 3.9C). In conclusion, PML is not required for rhTRIM5α to restrict HIV-1.

A


MEF
WT
KO
WT
KO
EV hT5a rhT5a EV hT5a rhT5a hT5C35 rhT5C35 hT5C35 rhT5C35
actin

B


mPML
rhT5a
Merge
mock
+HIV-1
no drug
+LMB

C

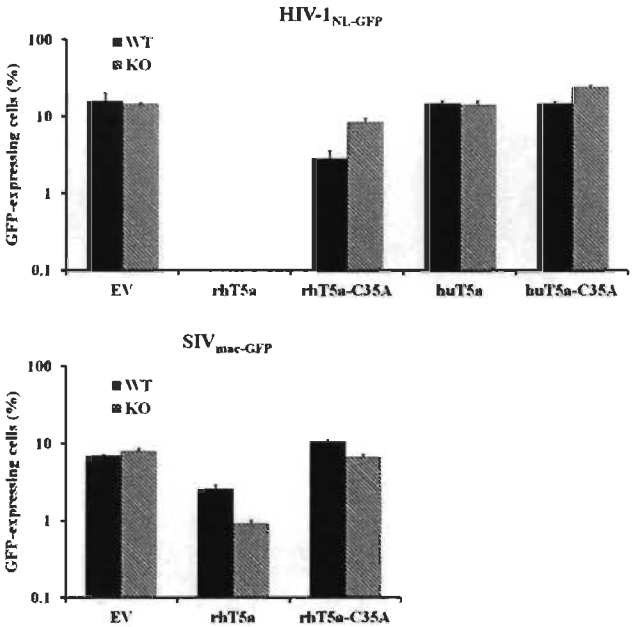

HIV-1NL-GFP
WT
KO
GFP-expressing cells (%)
100
10
1
0.1
EV
rhT5a
rhT5a-C35A
huT5a
huT5a-C35A
SIVmac-GFP
WT
KO
GFP-expressing cells (%)
100
10
1
0.1
EV
rhT5a
rhT5a-C35A

Figure 3.9 PML is not required for the TRIM5α-mediated restriction of HIV-1 in MEFs.

(A) WB analysis of WT and mutant TRIM5α. MEFs were transduced with retroviral vectors expressing WT and C35A variants of FLAG-tagged rhTRIM5α and hTRIM5α. Following puromycin selection, cell lysates were prepared from the various cell populations transduced with the indicated vectors or transduced with the empty vector (EV) as a control. TRIM5α was detected using an antibody against FLAG, with actin used as a loading control. (B) Immunofluorescence staining of mPML and rhTRIM5α in WT MEFs stably transduced with FLAG-tagged rhTRIM5α. The cells were treated with either LMB (20 ng/ml) or PBS as a control, 3 h prior to infection with HIV-$1_{NL\text{-}GFP}$ at a viral dose leading to approximately 10% infected cells. 6 h later, the cells were analyzed by immunofluorescence microscopy using anti-FLAG (red) and anti-mPML (green) antibodies. Nuclear DNA was stained using Hoechst 33342 (blue). Images are representative of multiple observations. Scale bar, 5 μm. (C) rhTRIM5α restricts HIV-1 in the presence or absence of PML. PML-KO or WT MEFs stably transduced with rhTRIM5α or huTRIM5α (WT or C35A mutant) were infected with HIV-$1_{NL\text{-}GFP}$ or SIVmac-GFP, using virus amounts leading to infection of about 10% of the parental cells. 2 d later, the percentage of infected cells was measured by FACS. The values represent the means of three independent infections with standard deviations.

3.6 Discussion

Whether PML has an impact or not on the infection of human cells by HIV-1 has been an open question for over 15 years. Trono and colleagues reported that PML is transiently exported in the cytoplasm following exposure to HIV-1 and co-localizes with the incoming virus in HeLa cells (55); however, this study did not include functional evidence for the involvement of PML in HIV-1 infection. Another team found no effect of HIV-1 infection on the distribution of PML bodies (56). As_2O_3, a known PML inhibitor, was found to enhance the infection of human cells with HIV-1 (46, 55) but it also stimulated the infection of MEFs with HIV-1 vectors whether PML was present or not (46). Interest for PML as a modulator of HIV-1 infection surfaced again in recent years, as it was proposed to act as an HIV-1 restriction factor in mouse and human cells (33). However, the data gathered so far by three different teams, including this study, suggest that the restriction activity in human cells, if it exists, is cell type-specific. Dutrieux and colleagues, using shRNAs, observed a modest inhibition of HIV-1 vector transduction

conferred by PML in HeLa cells (<2-fold). They also observed a small delay in HIV-1 propagation in peripheral blood mononuclear cells, but the decrease in infectivity was not quantified (33). We previously observed that knocking down PML in T-lymphoid Sup-T1 cells increases HIV-1 infectivity by 2- to 4-fold (34). On the other hand, Kahle and colleagues saw no effect of knocking down PML on the infectivity of an HIV-1 vector in T lymphoid cell lines including CEM, HuT78, Jurkat and Molt4 (32). They showed, however, that PML reduces HIV-1 infectivity in human foreskin fibroblasts by 2- to 3-fold (32). Taken together, those previous papers showed that knocking down PML has either no effect or modest effects on HIV-1 infectivity in human cells. We were not able to efficiently knock out PML in Sup-T1 cells, preventing us from drawing comparisons with our previous knockdown results. However, our knockout experiments in Jurkat, THP-1, HeLa and TE671 are not consistent with PML being an HIV-1 restriction factor in human cells.

A recent study by Kim and Ahn (38) uncovered an additional function for PML in human skin fibroblasts: the stimulation of ISG expression through a direct association with their promoter. Accordingly, we previously showed that PML was important for the efficient inhibition of HIV-1 by IFN-I in MEF cells (34). Although HIV-1 is also readily inhibited by IFN-I in a variety of human cell types, as illustrated in our study, we find that this effect is not affected by knocking out PML. However, we cannot exclude the possibility that PML is involved in regulating IFN-I-dependent transcription in specific cellular contexts such as skin fibroblasts (38). It is also possible that PML stimulates the transcription of some ISGs but not others. In support of this idea is our observation that SIVmac inhibition by IFN-I in Jurkat cells was significantly greater in the presence of PML. SIVmac, but not HIV-1, is inhibited by an unidentified restriction factor in Jurkat cells and other T cells, provisionally called Lv4 (51). It is conceivable that the gene encoding Lv4 is specifically stimulated by IFN-I in a PML-dependent fashion in Jurkat cells. This characteristic could be exploited to identify this gene, similar to the strategy that led to the identification of Tetherin as a retroviral restriction factor (57).

In our previous study (34), we showed that PML inhibited HIV-1 transcription in MEFs but not in Sup-T1 cells and in an IFN-I-independent fashion. We analyzed GFP mean fluorescence intensity in all our experiments for this study, as a surrogate for HIV-1 gene expression levels. Consistent with our previous findings, we observed no effect of PML on the GFP fluorescence intensity following infection of THP-1, Jurkat, HeLa or TE671 cells with our various vectors (not shown). We conclude that PML does not repress HIV-1 transcription in human cells. This apparently contradicts a report by Giacca and colleagues that PML inhibits HIV-1 transcription by directly binding the viral promoter (31). However, the latter study was based on the use of "J-Lat" clones, which are Jurkat cells in which the HIV-1 provirus has become constitutively repressed through unknown mechanisms (58). We propose that PML may be involved in the rare silencing events leading to HIV-1 latency in Jurkat cells, and that PML is important for the maintenance of silencing; however, PML is not a ubiquitous silencer of HIV-1 transcription.

Finally, our study shows that the As_2O_3-mediated stimulation of early retroviral infection stages is completely independent of PML, and so is the inhibition of TRIM5α by this drug. Our experimental system was tailored to study the effect of As_2O_3 on restriction by TRIM5α, and we cannot exclude that PML might be involved in other restriction activities known to be counteracted by As_2O_3 (50, 51). It is not entirely surprising that As_2O_3 inhibits TRIM5α in the absence of PML, considering that TRIM5α could target N-MLV in human cells, and HIV-1 in MEF cells, in the absence of PML. However, these results challenge conclusions from another paper that used radioactively or chemically labelled arsenate compounds to show that PML was the main target for this group of pharmacological agents (27). How, then, does As_2O_3 counteract TRIM5α and, to a lesser extent, stimulate HIV-1 and B-MLV vectors in human cells? Perhaps addressing this long-unanswered question will be helped by an observation that pre-dated the isolation of TRIM5α. Indeed, PK11195, a compound which, like As_2O_3, affects mitochondrial functions, also counteracts TRIM5α (49). Strikingly, these two drugs enhance autophagy (59, 60), an outcome possibly related to their effect on mitochondria. It is possible that As_2O_3-induced autophagy accelerates the lysosomal degradation of TRIM5α and other cytoplasmic restriction factors.

3.7 Materials and methods

Cell culture. Jurkat and THP-1 cells were maintained in RPMI 1640 medium (HyClone, Thermo Scientific, USA). Human embryonic kidney (HEK) 293T, HeLa, MEF and TE671 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone). All culture media were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (HyClone).

Plasmids and preparation of retroviral vectors. The pMIP retroviral vector plasmids containing individual isoforms of hPML (pMIP-hPML-I to VI) and the isoform 2 of mPML (pMIP-mPML) have been described in details in a recent publication (34) and make use of materials generously provided by Roger D. Everett (60). Retroviral vectors were prepared by co-transfection of 293T cells with pMIP-m(h)PMLs together with pMD-G and pCL-Eco using polyethylenimine (PEI; Polyscience, Niles, IL) as detailed previously (34). Virus-containing supernatants were collected 2 d later, clarified by low-speed centrifugation and kept at -80 °C.

To produce GFP-expressing retroviral vectors, 293T cells were seeded in 10 cm culture dishes and transiently co-transfected with the following plasmids: pMD-G, pCNCG and pCIG3-B or pCIG3-N to produce B-MLV$_{GFP}$ and N-MLV$_{GFP}$, respectively; pMD-G and pHIV-1$_{NL-GFP}$ to produce HIV-1$_{NL-GFP}$; pMD-G and pSIV$_{mac239-GFP}$ to produce SIV$_{mac-GFP}$; or pONY3.1, pONY8.0 and pMD-G to produce EIAV$_{GFP}$ (see (34, 61) and references therein).

Design of gRNAs and transduction of lentiviral CRISPR-Cas9 vectors. The lentiviral expression vector plentiCRISPRv2 (pLCv2) was a gift from Feng Zhang (Addgene plasmid # 52961) and can be used to simultaneously express a gRNA, Cas9 nuclease, and puromycin resistance, either by transfection or lentiviral transduction (62). Two gRNAs (hPML1 and hPML2) targeting *hPML* (NG_029036) were designed using the Zhang lab online software available at crispr.mit.edu. The sequences targeted are 5'CAATCTGCCGGTACACCGAC (hPML1) and 5'CACCGGGAACTCCTCCTCCGAAGCG (hPML2). A gRNA targeting the CAG

hybrid promoter (target: 5'GTTCCGCGTTACATAACTTA) was used as a negative control (35). The oligodeoxynucleotides (ODNs) needed for the generation of pLCv2-based constructs were designed according to the Zhang lab protocol (62, 63) and are shown in Table A.2.

The lentiviral vectors were prepared by co-transfection of 293T cells with 10 μg of the plentiCRISPRv2 construct together with 5 μg of pMD-G and 10 μg of pΔR8.9 (64). The viral supernatants were collected at 1.5 or 2 d post-transfection and used to transduce various cell lines. Stably transduced cells were selected by addition of 0.5 μg/ml puromycin (Thermo Fisher Scientific) to the medium at 2 d post-infection and for 5 d. Control untransduced cells were killed under these conditions.

Surveyor nuclease assay. To evaluate on-target modifications (indels) in *hPML*, a surveyor nuclease assay was performed. 293T cells were transfected with either plentiCRISPRv2-hPML1, -hPML2 or -CAG using PEI. 3 d later, the genomic DNA was extracted from the transfected cells using the QIAamp DNA mini kit (Qiagen, CA, USA). Two pairs of primers were designed to amplify 637 bp and 725 bp fragments on either side of Cas9 targets guided by gPML1 and gPML2 respectively (Fig. 1A). The sequences of these ODNs are included in Table A.2. PCR amplicons were heat-denatured at 95 °C, and re-annealed by slow cooling to promote formation of dsDNA heteroduplexes. The heteroduplexes were then cleaved by surveyor nuclease S (Integrated DNA Technologies, Coralville, IA), according to the manufacturer's instructions. Digestion products were visualized by agarose gel electrophoresis.

Construction of the homology directed repair (HDR) plasmid and generation of PML-KO Jurkat cells. We used pcDNA3.1+ as the backbone plasmid to prepare a HDR "donor" plasmid containing a neomycin selection gene (Neo^R). First, the backbone plasmid was cut with BamHI and BglII, then self-ligated in order to remove the cytomegalovirus promoter from upstream of the multicloning site MCS1. Next, two ODNs were designed to introduce the second MCS (MCS2) (see Table A.2); these ODNs were annealed, and the resulting duplex ligated into the PciI cut site of the plasmid,

downstream of Neo^R, yielding pNMs-Neo.HDR. To construct the PML HDR plasmid, homology arms corresponding to 800 bp-long regions immediately upstream and downstream of the hPML gRNA1-mediated Cas9 cut site in *hPML* were designed. The arms were amplified by PCR from genomic DNA extracted from 293T cells using the QIAamp DNA mini kit (Qiagen). The sequences of ODNs used in the PCR reactions are provided in Table A.2. The 5' arm was cloned into MCS1 of pNMs-Neo.HDR which had been cut with NotI and XbaI. The plasmid was then cut with Mfe I and Sbf I in order to clone the 3' arm into MCS2, yielding pNMs-Neo.HDR-hPML.

Jurkat cells (300,000) were electroporated with 1.5 μg of pNMs-Neo.HDR-hPML together with 1.5 μg of pLCv2-hPML1 using an MP-100 microporator (Digital Bio Technology) according to the manufacturer's instructions. The parameters were 1300 V, 2 pulses, 20 ms. 48 h later, cells were placed in medium containing 1 mg/ml G418, and selection was carried out for 7 d.

Antibodies and WB analyses. Cells (1×10^6) were lysed at 4°C in RIPA lysis buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 8.0). The lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by WB analysis using mouse anti-mPML mAb (36-1-104, Enzo life sciences, NY), rabbit polyclonal anti-hPML (A301-167A, Bethyl Laboratories, TX), rabbit polyclonal anti-FLAG (Cell Signaling, MA, USA), or mouse anti-β-actin antibody (Sigma, MI).

Viral challenges and flow cytometric analysis. Cells were seeded into 24-well plates at 3×10^4 cells/well and infected the following day with GFP-expressing retroviral vectors. HeLa and TE671 cells were trypsinized at 2 d post-infection and fixed in 3% formaldehyde (Fisher Scientific, MA, USA). The percentage of GFP-positive cells was then determined by analyzing 1×10^4 to 5×10^4 cells on a FC500 MPL cytometer (Beckman Coulter, CA, USA) using the CXP Software (Beckman Coulter).

Pharmacological treatments. A 0.1 M stock solution of As_2O_3 (Sigma) was prepared in 1 N NaOH, as previously described (28), and diluted in the culture medium

immediately before use. Cells were treated for 15 min prior to infection. 16 h post-infection, the supernatants were replaced with fresh medium devoid of drug. Recombinant human IFN-α was obtained from Shenandoah biotechnology (Warwick, PA). Recombinant human IFN-β and IFN-ω were obtained from PeproTech (Rocky Hill, NJ). IFN-I was added to cell cultures 16 h prior to infection and at a final concentration of 10 ng/ml.

Immunofluorescence microscopy. HeLa or MEF cells were seeded on glass coverslips placed in 3.5-cm wells. MEFs were treated with LMB (20 ng/ml) 3 h prior to infection then infected for 6 h with HIV-$1_{NL\text{-}GFP}$. The cells were permeabilized and fixed for 10 min in Triton X-100/4% formaldehyde at room temperature (RT), followed by 4 washes with PBS. Cells were then treated with 10% goat serum (Sigma) for 30 min at RT followed by 4 h of incubation with antibodies against FLAG (Sigma, 1:150), hPML (Bethyl Laboratories, 1:150) or mPML (Enzo Life Sciences, 1:150) in 10% goat serum at RT. They were then washed 4 times with PBS and fluorescently stained with Alexa Fluor 488-conjugated goat anti-mouse or 594-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) diluted 1:100 in 10% goat serum for 1 h at RT. The cells were then washed 4 times with PBS before mounting in Vectashield (Vector Laboratories, Peterborough, UK). Hoechst 33342 (0.8 μg/ml; Molecular Probes) was added along with the penultimate PBS wash to reveal DNA. Images were acquired on an AxioObserver Microscope (Carl Zeiss Canada, Toronto, ON) equipped with the Apotome module.

3.8 Acknowledgements

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Chapter IV contains a discussion of the results achieved in Chapters II and III. In this chapter, new directions and perspectives of the studies are described as well.

CHAPTER IV

CONCLUSIONS

4.1 PML (TRIM19): involvement in the antiviral state against lentiviruses

To decipher the role of PML in the replication of HIV-1, we performed the study in two models, mouse and human cells. In the study presented in chapter II, we focused most of our attention on mouse cells and found that MEFs had a drastically reduced susceptibility to infection with lentiviruses in the absence of PML. The PML-mediated restriction occurred at two distinct phases; the first block was at early post-entry steps of infection, while the second block occurred at the level of post-integration transcription demonstrating that PML is involved in gene silencing of HIV-1 as well. We found that PML inhibits lentiviral infection independently of IFNs; however, PML is involved in IFN-induced restriction pathways in MEFs. In chapter III, we focused on the role of PML in human cells. Fully PML-deficient human cell lines were generated and the impact of PML on lentiviral infectivity was assessed. We found that PML had little or no effect on infectivity of lentiviruses including HIV-1 in human cells. Taken together, our results indicate a cell context-dependent isoform-specific anti-retroviral activity of PML that was not specific to HIV-1.

4.1.1 PML is involved in the pre-integration restriction of lentiviruses

Mouse cells have been used widely as a model to study infection by human viruses including HIV-1 (Chelbi-Alix *et al.* 1998; Regad *et al.* 2001; Baumann *et al.* 2004; Becker *et al.* 2004; El McHichi *et al.* 2010; El Asmi *et al.* 2014; Dutrieux *et al.* 2015; Kahle *et al.* 2015). Moreover, they are frequently used to study the physiological consequences of selective gene ablations. To study the potential role of PML in lentiviral infection, we first used PML-KO MEFs (see Annex A for more information about these cells). The cells were infected with pseudotyped lentiviruses encoding GFP as a marker of viral infection

and gene expression. We found that MEFs were more permissive to infection with lentiviruses in the absence of PML, providing evidence of the involvement of PML in mediating intrinsic immunity against lentiviruses. The block was at the reverse transcription step, before integration of the viral DNA into the host genome. The early post-entry block to HIV-1 was reported in mouse cells a long time ago (Hofmann *et al.* 1999; Bieniasz & Cullen 2000). Here, our findings indicate that PML is required for this block to HIV-1 and two other lentiviruses, SIVmac and EIAV.

There are only two isoforms of mPML and the one we isolated and overexpressed in MEFs was isoform 2. By contrast, seven isoforms have been characterized in human cells. Six isoforms (I-VI) localize in the nucleus and participate in NB, while hPML-VII is cytoplasmic. To determine whether hPMLs were also involved in restriction, we overexpressed hPML-I to VI in PML-KO cells individually. Our results suggest that PML-mediated restriction of HIV-1 is isoform-specific with hPML-I, II, IV, and VI being the most restrictive isoforms. PML-III had no restriction activity (Fig. 2.7B). Our immunofluorescence microscopy showed that the NBs formed by PML-III were smaller in size and number than the other isoforms (Fig. 2.7). That might influence the possible role of this isoform in restriction activity. One of the shared hallmarks of mouse and human PML-mediated resistance to lentiviruses in MEFs was that the block is most evident at low doses and it was saturable at high doses of the viruses, even in the presence of excessive PML. This abrogation of restriction is consistent with a model in which PML is not the direct antiviral mediator; however, the effector is reliant on PML and limited in concentration. Although, up-regulation of PML by exogenous expression or IFN treatment was shown to restrict replication of several RNA viruses including VSV, influenza virus, and HFV (Chelbi-Alix *et al.* 1998; Regad *et al.* 2001). Our hPML overexpression results in MEFs can also help to find the antiviral effector, since the NB formed by each isoform employs specific associated proteins. For example, the tumor protein p53 is activated when it co-localizes with NBs including PML-IV (Ivanschitz *et al.* 2015).

4.1.2 PML contributes to IFN-induced anti-HIV response, however the PML-mediated early block to HIV-1 is IFN-independent

PML and some other NB associated proteins such as Daxx and Sp100 are ISGs. We also found that infection with retroviruses caused a significant increase in the expression of PML (Fig. 2.6A). Regarding these observations, a suggested downstream effector would be an IFN-induced restriction factor so that its activity was dependent on the presence of PML. To address this suggestion, the IFN-induced signaling in PML-KO and WT MEFs was prevented prior to infection with HIV-1 using a blocking antibody against the IFN-α/β receptor. HIV-1 was still restricted in WT cells in the absence of IFN-induced signaling and it was saturable at high dose of the virus that reveals the IFN-induced pathway mediators are not incorporated in the PML-mediated early block to HIV-1 (Fig. 2.6C). This indicated that PML positively regulates type I interferon response by promoting transcription of ISGs through increasing the level of activated STAT1 and STAT2, two signal transducers and activators of transcription in IFN-induced pathway (Kim & Ahn 2015). We also showed that PML plays a role in innate immunity, so that the IFN-induced antiviral response against HIV-1 is more efficient in the presence of PML in MEFs (Fig. 2.6D).

4.1.3 Does restriction take place in the nucleus or the cytoplasm?

A rapid but transiently cytoplasmic relocalization of PML early after infection with HIV-1 (Turelli *et al.* 2001; Dutrieux *et al.* 2015; Kahle *et al.* 2015) suggested that PML likely intervenes with retroviral infection in the cytoplasm, while PML is mainly a nuclear protein. Additionally, our quantitative analysis of viral cDNA 6 h after infection of MEFs with HIV-1 revealed that PML inhibits the retroviral reverse transcription step (Fig. 2.2B) which occurs in the cytoplasm. Our hPML overexpression results provided further insights into the site of the restriction. PML-I is the most abundant and the longest isoform and harbors nuclear export signal (NES) at its C-terminal domain in addition to a NLS. That allows shuttling of all isoforms between the two cellular compartments, nucleus and cytoplasm, through heterodimer formation (Condemine *et al.* 2006). Since we overexpressed the hPML isoforms individually in PML-KO MEFs, formation of

heterodimers was not possible and thus the most restrictive isoforms (PML-II, IV, and VI) were all located in the nucleus (Fig. 2.7B). This observation indicates that the nuclear localization of PML is adequate for restricting HIV-1 and is consistent with a model whereby PML inhibits lentiviruses through a downstream effector which directly interacts with the incoming retrovirus in the cytoplasm. However, the reasons why PML localizes to the cytoplasm early after infection with HIV-1 and the identity and the viral target for the putative effector remain to be determined.

4.1.4 PML is involved in HIV-1 gene silencing

In the present study, we employed a pseudotyped GFP-expressing HIV-1 in which the reporter gene was under the control of WT LTR promoter. Thus, the intensity of GFP expression (MFI) in the infected cells was an indicator of viral gene expression levels. To measure only the level of expression of the integrated GFP reporter gene, the MFI was monitored on day 10 after infection. We found that PML induced a strong gene silencing of HIV-1 in WT MEFs that was not observed in PML-KO cells. In contrast to the pre-integration restriction, the PML-mediated repression of gene expression was not enhanced by IFN treatment in MEFs (Fig. 2.6B), demonstrating that the reverse transcription inhibition and the gene silencing were exerted by two different mechanisms which both employ PML.

HDACs regulate HIV latency directly by inducing histone deacetylation at HIV integrated sites. SAHA is a member of the hydroxamic acid class of HDACis, shown to induce spatial redistribution of PML NBs in J-Lat clones, a human T cell line latently infected with HIV-1 (Fig. 4.1), and resulted in strong activation of viral gene expression (Lusic *et al.* 2013). We used SAHA to further analyze the role of PML in HIV-1 gene silencing. We found a significant increase in the level of viral capsid protein upon treatment with SAHA in WT MEFs, which was comparable to PML-KO cells (Fig. 2.5D). Further quantitative analysis using RT-PCR revealed that PML suppresses the viral transcription step. It was already shown that the level of infectious HIV-1 is dramatically reduced due to low viral transcription in mouse cells, even in the presence of the

transcription enhancer hCyclin T1 (Zhang *et al.* 2008). Here, our findings shed light on this inhibition. We also observed a slight SAHA-induced up-regulation of GFP transcription in PML-KO cells that was probably related to global gene expression changes upon treatment with HDACis (Shirakawa *et al.* 2013). We also found that hPML has a similar inhibitory effect on HIV-1 transcription when individual hPML isoforms were overexpressed in PML-KO MEFs, suggesting that both mouse and human PML play a role in the establishment and/or maintenance of HIV-1 latency.

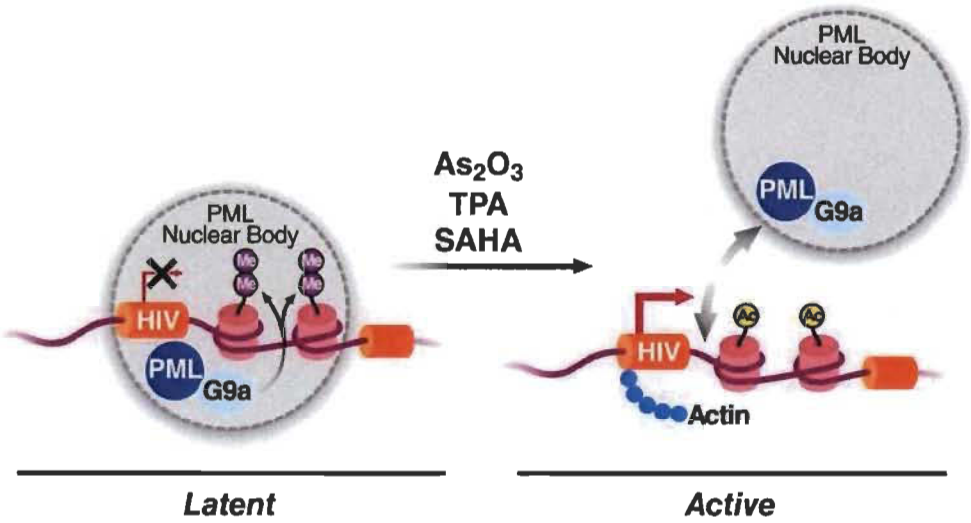

Figure 4.1 **Regulation of HIV latency by proximity to PML NBs.**
During latency, PML is located close to the HIV provirus and recruits the methyltransfrase G9a to the HIV promoter, leading to dimethylation of histone and transcriptional silencing. Upon inducing PML degradation with SAHA, As_2O_3, and TPA (a phorbol ester), the HIV provirus is relocated away from PML NB and transcription is reactivated (Ott & Verdin 2013).

PML inhibits HFV gene expression by complexing the viral transactivator, Tas, and prevents its direct binding to the proviral DNA (Regad *et al.* 2001). To determine if PML had a same function on the HIV-1 transactivator protein Tat, we infected WT and PML-KO MEFs with a different pseudotyped virus, HIV-$1_{CMV\text{-}GFP}$, that was bearing mutations in LTRs and the marker GFP was under the control of CMV promoter (Fig. A.3). PML was still able to suppress HIV-1 gene expression, demonstrating that the restriction mechanism was not through the viral Tat protein. This finding provides more evidence for

the suggested model by Lusic and colleagues in which spatial intervention between PML NB and the proviral chromatin results in viral gene silencing (Fig. 4.1). We found this intervention is isoform-specific and cell context-dependent.

4.1.5 Future experiments

The mechanism of the early PML-dependent block to lentiviruses in murine cells remains unclear. It is thought that PML indirectly, likely through a partner protein, inhibits viral reverse transcription. This may occur through interaction with a viral or cellular protein that modulates reverse transcription and/or PIC formation. Thus, this putative effector must be able to shuttle between nucleus and cytoplasm. Other major components of PML NBs, such as Daxx and Sp100, also exhibit antiviral activity through unknown mechanisms. Recently, cytoplasmic Daxx was reported to be involved in the inhibition of HIV-1 in both mouse and human cells (Dutrieux *et al.* 2015). Daxx locates in the vicinity of incoming HIV-1 capsid in the cytoplasm and inhibits reverse transcription. However, these findings were contradicted by another group shortly after publishing (Kahle *et al.* 2015). Yet, there is no study that investigates the role of PML NB associated proteins in details. Thus, murine Daxx or Sp100 are interesting candidates for the reverse-transcription block. Depletion of these proteins alone and/or in combination with mPML using shRNA, may give us more conclusive answers to how PML interferes with incoming lentiviruses early after infection with HIV-1. A knockout approach in MEFs would be also important to address more completely the role of associated proteins in the restriction activity against HIV-1.

PML is typically found concentrated in NBs. PML needs to be SUMOylated in order to localize in the NB. However, a fraction of unmodified PML is associated with the soluble nucleoplasmic phase (Muller *et al.* 1998). Interestingly, both forms of PML have been found to interfere with viral infections. For example, the PML-mediated restriction of HFV transcription does not necessitate PML localization in NBs (Regad *et al.* 2001). On the other hand, HCMV disrupts PML NBs at early stages of infection to promote its replication. The disruption is mediated by the viral protein IE1 (Ahn & Hayward 2000).

Whether localization in NB is necessary for PML to restrict lentiviruses is not clear yet. To answer this question, a PML mutant that can no longer be modified by SUMO-1 and forms aberrant aggregates will be helpful when transduced into PML-KO cells. The mutations should be introduced in SUMOylation sites as well as in the SIM. Alternatively, expression of HCMV protein IE1 in WT cells leads to disruption of NBs without degradation of PML. The latter method benefits from the presence of WT protein, which provides a situation with less modifications in the protein structure.

Due to lack of NLS, PML-VII is purely cytoplasmic and does not incorporate in NBs. The only unveiled function for PML-VII is as a critical regulator of transforming growth factor beta (TGF-beta), a cytokine which controls tumor suppressive functions (Lin *et al.* 2004). To date, no restriction activity or immune function has been reported for this isoform. Therefore, it would be of interest to overexpress this isoform of hPMLs in PML-KO cells as well and to investigate its role in intrinsic immunity against lentiviruses.

Finally, in order to further analyze whether the PML-mediated early block to lentivirus infection occurs in the cytoplasm or nucleus, it would be informative to express NLS deficient mutants of PML in PML-KO cells and assess the restriction activity.

4.2 The PML-mediated restriction of lentiviruses is cell context-dependent

In humans, the role of PML in the lentiviral replication has been controversially discussed for over 15 years. A possible connection between HIV-1 replication and PML stems from an early report proposing that incoming HIV-1 PICs trigger cytoplasmic export of PML in HeLa cells and suggesting that the efficiency of HIV-1 transduction increases in the presence of arsenic trioxide, a potent inhibitor of PML (Turelli *et al.* 2001). Recently, Dutrieux and colleagues found a modest increase in HIV-1 vector transduction upon depletion of PML in HeLa and peripheral blood mononuclear cells using shRNAs (Dutrieux *et al.* 2015). Shortly after, PML was shown to interfere with HIV-1 replication only in primary human fibroblasts but PML knockdown had no effect in myeloid and T cells (Kahle *et al.* 2015). Taken together, these studies showed that

knocking down PML has either no effect or a modest effect on HIV-1 replication in human cells. We investigated the effect of PML on lentiviral replication in several human cell lines including T cells using two approaches; shRNA-based knock down and for the first time, PML knockout. Consistently, our preliminary results showed that knockdown of PML in SupT1 cells resulted in a moderate increase in infectivity of HIV-1 and the related lentivirus, SIVmac (Fig. 2.1). The modest effect could be due to incomplete depletion of PML by shRNAs that was still adequate to prevent the infection. Thus, to generate a fully PML-deficient human cell lines, we aimed to knockout both alleles of hPML using a new tool based on a bacterial immune system termed CRISPR-Cas9. Following its initial demonstration in 2012 (Jinek *et al.* 2012), the CRISPR-Cas9 system has been widely adopted to target genes in many cell lines and organisms, including human (Mali *et al.* 2013). Interestingly, this genome editing tool has been also used to excise the HIV-1 provirus from the cellular genome by targeting integrated HIV-1 LTR in human cells (Ebina *et al.* 2013). Moreover, CRISPR-Cas9 complex has been used to deactivate HIV-1 proviral DNA by mutating *rev* DNA in latently infected Jurkat cells (Zhu *et al.* 2015). The CRISPR-Cas9 system is able to mediate site-specific DSBs, which can be repaired through either NHEJ or HDR. Editing by HDR is inefficient and can be corrupted by additional indels (Inui *et al.* 2014). Herein, we developed an HDR plasmid that provides a specific DNA repair template for a DSB. When co-transfected with the CRISPR-Cas9 plasmid, the HDR plasmid results in the incorporation of a Neo^R gene at Cas9-induced DNA cleavage sites. Using this technique, we successfully knocked out all PML alleles in HeLa, TE671, THP-1, and Jurkat. Unfortunately, our efforts to knockout PML in SupT1 were not successful, preventing us to generate a side by side comparison with our early knockdown data in this cell line. However, our data gained from knockout PML in other cell lines demonstrated that PML has no effect on the replication of HIV-1 in human cells. Yet, EIAV was slightly inhibited by PML in Jurkat cells. Additional experiments revealed that PML-IV and -VI were involved in the restriction activity. Interestingly, overexpression of mPML had no effect on HIV-1 in Jurkat. This observation is also consistent with a model in which PML is not the direct mediator and thus its activity is dependent on factors specific to the cell line in which PML is expressed.

4.2.1 Future experiments

As anti-HIV activity of PML was found to be dependent on the cell context in which the protein is expressed, manipulation of other proteins known to interact with PML, such as Daxx and Sp100, should be considered as well in human cells. One suggestion is knocking out Daxx and/or Sp100 in human cells using the same system which was used in this study to knockout PML followed by overexpression of murine Daxx or Sp100. It would be of interest to find how these murine PML NB associated proteins change the restriction activity of human cells against lentiviruses.

4.3 As_2O_3-mediated stimulation of retroviral infection is independent of PML

TRIM5α inhibits N-MLV in human cells (Hatziioannou *et al.* 2004; Yap *et al.* 2004) and As_2O_3 abrogates this inhibition by modulating TRIM5α (Berthoux *et al.* 2003). As_2O_3 was also found to directly bind PML and induce its degradation by promoting the protein SUMOylation (Zhang *et al.* 2010). The latter observation was consistent with our hypothesis that PML is likely involved in TRIM5α-mediated retroviral response and thus, As_2O_3 counteracts TRIM5α through its effect on PML. Contrary to our expectation, however, we found that PML is not relevant for this drug effect on retroviruses, since As_2O_3 blocks the restriction activity of hTRIM5α against N-MLV, either in the presence or absence of PML. Consistently, we later found that PML had no role in the TRIM5α-mediated restriction of retroviruses (described in the section 4.4).

4.4 PML is not involved in the rhTRIM5α-mediated restriction of HIV-1

Uncoating of the capsid is linked to reverse transcription, therefore modifications that delay or accelerate this process lead to a block in viral replication occurring prior to or during reverse transcription (Forshey *et al.* 2002; Leschonsky *et al.* 2007; Li *et al.* 2009). The host restriction factor TRIM5α provides early intrinsic defense against retroviral infections in mammalian cells in a species-specific manner. TRIM5α blocks HIV-1 infection by targeting the viral capsid after entry but prior to completion of reverse

transcription (Stremlau *et al.* 2006). The impact of TRIM5α on HIV-1 replication is similar to that we found in PML-KO cells, impairing reverse transcription. Interestingly, a recent study revealed that several TRIM5α proteins were transported into the nucleus and formed nuclear bodies that also contained PML (Diaz-Griffero *et al.* 2011). These data led us to hypothesize that TRIM5α-mediated restriction of retroviruses might be dependent on the presence of PML NBs. Thus, we overexpressed WT or mutant versions of rhesus and human TRIM5α in WT and PML-KO MEFs that were afterward infected with lentiviruses. rhTRIM5α showed the expected restriction activity against HIV-1 in the presence or absence of PML, demonstrating that rhTRIM5α-mediated restriction of HIV-1 is independent of the protein colocalization with PML NBs (Fig. 3.9). The trafficking ability of TRIM5α proteins and its colocalization with NB could be important for induction of IFN-I responses (Portilho *et al.* 2016) or an as-yet-unknown function of TRIM5α.

4.5 Perspectives: gene therapy targeting incoming or latent HIV-1

Latent HIV-1 reservoirs are established early during primary infection and constitute a major barrier to eradication, even in the presence of highly active antiretroviral therapy. The Berlin patient, the only individual that is considered to be cured of HIV, has reinforced the idea that genetic modifications could confer resistance to HIV infection after HAART interruption by blocking attachment of HIV to the cell (described in section 1.1.8). To date, several strategies, including gene therapy techniques, have been exploited to target the HIV latent reservoir. Recently, a population of pluripotent stem cells has been generated that bear a homozygous CCR5Δ32 mutation (Ye *et al.* 2014), just similar to the natural mutation in individuals who are resistant to HIV infections (e.g. the Berlin patient). Monocytes and macrophages differentiated from these mutated stem cells in vitro were resistant to HIV infection. In a separate study, a CRISPR-Cas9-based cure strategy has been exploited to mutate and inactivate HIV-1 proviral DNA successfully in latently infected Jurkat cells (Zhu *et al.* 2015). These findings suggest a promising avenue for developing gene therapy to treat HIV infection. PML and/or PML NB associated proteins are suggested to be a great target for gene therapy, since they are involved in restriction

of HIV-1 both at pre-integration and post-integration steps (Lusic *et al.* 2013). It could be accomplished by modifying the NB associated proteins in human cells or introducing of a mouse version of the proteins.

4.6 Perspectives: development of new antiretroviral drugs

Whereas the HIV-1 life cycle presents many potential opportunities for therapeutic intervention, only a few have been exploited. Among them is the reverse transcription step which still remains a potential target for developing new drugs. Of the 26 drugs currently approved to treat HIV-1 infections, 14 are RT inhibitors (Arts & Hazuda 2012; Hu & Hughes 2012). As with all antiretroviral therapies, treatment with any of these agents often results in the emergence of HIV-1 strains with reduced drug susceptibility. Furthermore, some cellular factors such as APOBEC3G (Mangeat *et al.* 2003), have been shown to interact with HIV-1 replication during the reverse transcription step. In this study, PML was shown as a double-edged sword. On one hand, PML also inhibits the HIV-1 reverse transcription, and on the other hand, PML helps the provirus to stay latent in the host cell. The increase in knowledge regarding the HIV life cycle, in particular the function of the HIV RT and its essential interactions with other host factors different from the ones of the already approved drugs, will reveal potential targets for drug development.

4.7 Final conclusions

Taken together, the present thesis introduces PML as a restriction factor participating in both innate and intrinsic immunity against lentiviruses. PML is shown to be involved in HIV latency as well, a new role that makes this nuclear protein a potential target for developing new antiviral drugs to purge latent reservoirs. Our results are also relevant to the development of a mouse model for studying HIV persistence and latency. We hope that sharing these observations will contribute to the future efforts to cure HIV/AIDS.

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ANNEX A

SUPPLEMENTARY DATA

Mouse embryonic fibroblast cells (MEFs)

By homologous recombination in murine embryonic stem (ES) cells obtained from a 129Sv mouse strain genomic library, part of exon 2 of the PML gene, which encodes the RING-finger domain, was substituted with a neomycin resistance gene cassette. Mice homozygous for the PML mutation ($PML^{-/-}$) were born with the expected Mendelian frequency, were indistinguishable at the gross phenotypic level from $PML^{+/+}$ and $PML^{+/-}$ littermates, and were fertile; however, the $PML^{-/-}$ mice were extremely susceptible to spontaneous Botryomycotic infections. Successful disruption of the PML gene was inferred from the lack of PML mRNA and PML NBs in mouse primary embryonic fibroblasts (MEFs) from $PML^{-/-}$ embryos (Gaboli *et al.* 1998; Wang *et al.* 1998a).

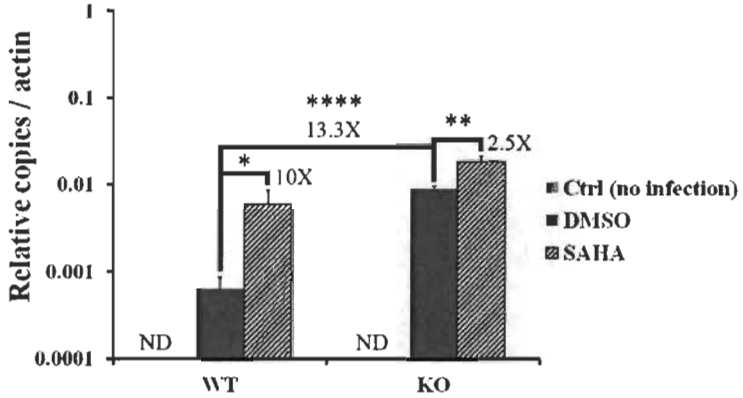

Figure A.1 Relative quantification of HIV-1 transcription.
WT or PML-KO MEFs were challenged with HIV-$1_{NL\text{-}GFP}$ in triplicate (CRFK MOI = 0.01). Ten days later, the cells were treated with either DMSO or SAHA for 48 h. Total RNAs were then purified from the cells and the levels of GFP and actin transcripts were determined by qRT-PCR. Data are presented as the ratios of GFP compared to actin mRNAs. Total RNAs from uninfected cells were used as negative control. The values represent the means of three independent experiments with standard deviations (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, two-tailed Student's t-test). ND, not detected.

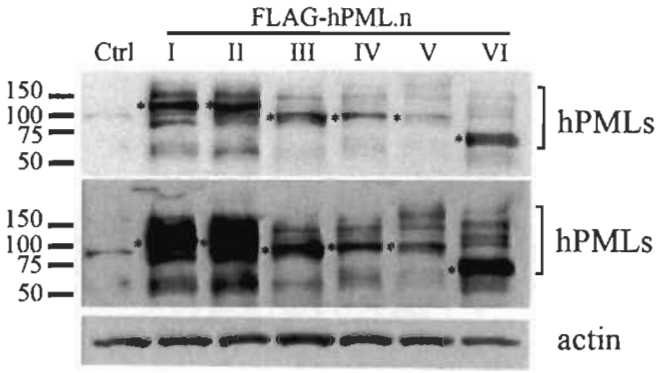

Figure A.2 Western blot analysis of hPML isoforms overexpressed in PML-KO MEFs.

Cells were stably transduced with hPML isoforms I to VI. The upper panel shows a WB analysis of hPML expression using a monoclonal antibody (H-238). The bands labeled with asterisks correspond to the expected isoforms according to their sizes. The heavier bands are likely to be SUMO-modified PML. The lower panel shows the same blot reprobed using an anti-FLAG antibody. Actin was used as a loading control.

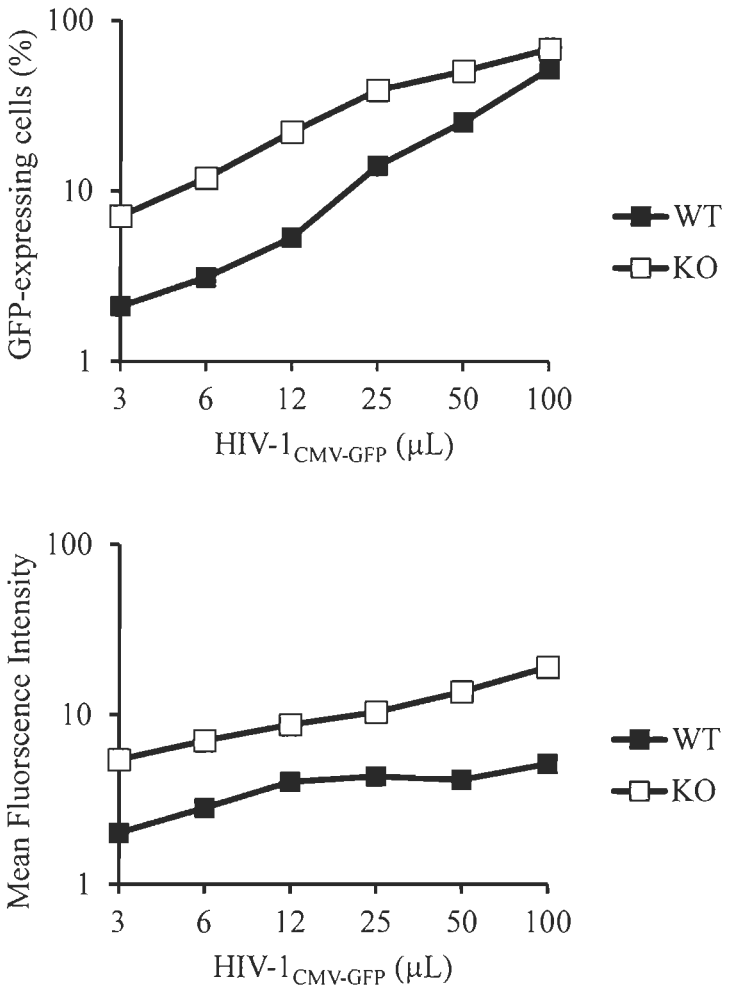

Figure A.3 **mPML knockout increases CMV promoter-driven GFP expression.** PML-KO and WT MEFs were infected with increasing doses of HIV-$1_{CMV\text{-}GFP}$ and cells were maintained in culture for 10 days, followed by FACS analysis. The percentage of infected (GFP-expressing) cells and the mean fluorescence intensity (MFI) were measured (top and bottom panels, respectively).

Table A.1 Sequences of ODNs used in chapter II.

Mouse PML forward	5'CACGGATCCACCATGGAAACTGAACCAGTTTCC 3'
Mouse PML reverse	5'ATCCAATTGCTAGGCCAGGCATCCCTTACTTTCAG C 3'
Human PML forward	5'AATTAGTCGACGGATCCACCATGGACTACAAAGA CGATGACGACAAGGAGCCTGCACCCGCCCGATC 3'
Human PML-I reverse	5'ATCCAATTGTCAGCTCTGCTGGGAGGCCCTCTCTG 3'
Human PML-II reverse	5'ATCGAATTCTCAGAGGCCTGCTTGACGGGCGCCTG 3'
Human PML-III reverse	5'ATCCAATTGTCAGCGGGCTGGTGGGGAGGCCAAG 3'
Human PML-IV reverse	5'AGCCAATTGCTAAATTAGAAAGGGGTGGGGGTAG C 3'
Human PML-V reverse	5'AGCCAATTGTCAATGCCTCACTGGAAAATTCCCCA G 3'
Human PML-VI reverse	5'ATCCAATTGTCACCACAACGCGTTCCTCTCCCTAC C 3'
shPML1	5'TGCTGTTGACAGTGAGCGCAAGATGCAGCTGTATC CAAGATAGTGAAGCCACAGATGTATCTTGGATACAG CTGCATCTTTTGCCTACTGCCTCGGA3'
shPML2	5'TGCTGTTGACAGTGAGCGAGCAAGACCAACAACA TCTTCTTAGTGAAGCCACAGATGTAAGAAGATGTTG TTGGTCTTGCGTGCCTACTGCCTCGGA3'
shPML3	5'TGCTGTTGACAGTGAGCGCGCACACGCTGTGCTCA GGATGTAGTGAAGCCACAGATGTACATCCTGAGCAC AGCGTGTGCATGCCTACTGCCTCGGA3'
GFP forward	5'GACGACGGCAACTACAAGAC3'
GFP reverse	5'CGGATCTTGAAGTTCACCTTG3'
2-LTR circles forward	5'AACTAGGGAACCCACTGCTTAAG3'
2-LTR circles reverse	5'TCCACAGATCAAGGATATCTTGTC3'
Actin forward	5'CCTCCCTGGAGAAGAGCTA3'
Actin reverse	5'ACGTCACACTTCATGGA3'

Table A.2 Sequences of ODNs used in chapter III.

Name	Sequence
PML gRNA1 top	5'CACCGCAATCTGCCGGTACACCGAC
PML gRNA1 bottom	5'AAACGTCGGTGTACCGGCAGATTGC
PML gRNA2 top	5'CACCGGGAACTCCTCCTCCGAAGCG
PML gRNA2 bottom	5'AAACCGCTTCGGAGGAGGAGTTCCC
CAG gRNA top	5'CACCGGTTCCGCGTTACATAACTTA
CAG gRNA bottom	5'AAACTAAGTTATGTAACGCGGAACC
Surveyor gRNA1 fwd	5'AATGGGGGTATTGGGGTGCTG
Surveyor gRNA1 rev	5'TGGTCAGCGTAGGGGTGC
Surveyor gRNA2 fwd	5'AAGAGTGGAATTTCTGGGTC
Surveyor gRNA2 rev	5'GAAGCACTTGGCGCAGAGG
PML 5'arm fwd	5'CTAGCGGCCGCATTTCATTTCTTTCTAAC
PML 5'arm rev	5'AATTCTAGAGCCGCTGCAGACTCTC
PML 3'arm fwd	5'TTACAATTGGGCTGTGTGCACCC
PML 3'arm rev	5'CGCCCTGCAGGCTGTACGAATGTATTAC
MCS2 top	5'CATGGCAATTGAAGCTTCCTGCAGGGGATCCA
MCS2 bottom	5'CATGTGGATCCCCTGCAGGAAGCTTCAATTGC
Knock-in fwd	5'TCTGGACGAAGAGCATCAGG
Knock-in rev	5'GATTGCACTCTCTCTCTCCTC
WT/indel fwd	5'ACACGCTGTGCTCAGGATGC
WT/indel rev	5'GTTGCGCAGCTCTGCTAGG