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# Investigating the relationship between structural elements of the inner nuclear membrane and HIV infection

Anvita Bhargava

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**THÈSE DE DOCTORAT**  
**DE L'UNIVERSITÉ PSL**

Préparée à Institut Curie

**Investigating the relationship between structural elements of  
the inner nuclear membrane and HIV infection**

**Investigation de la relation entre les éléments structuraux de la  
membrane nucléaire interne et l'infection par le VIH**

Soutenue par

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**Immunologie**

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\*\*\*

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*"O frati," dissi, "che per cento milia  
perigli siete giunti a l'occidente,  
a questa tanto picciola vigilia*

*d'i nostri sensi ch'è del rimanente  
non vogliate negar l'esperienza,  
di retro al sol, del mondo senza gente.*

*Considerate la vostra semenza:  
fatti non foste a viver come bruti,  
ma per seguir virtute e canoscenza".*

***Inferno***, Canto XXVI, v. 112-120

Dante Alighieri

## **Abstract**

During infection by Human Immunodeficiency Virus (HIV), the virus crosses the nuclear envelope (NE) to invade the host genome. HIV gets access to the nucleus by passing through the nuclear pore complex (NPC). However, the underlying mechanism is not fully understood. It was recently described that SUN1 and SUN2, structural proteins of the inner nuclear membrane, play a role in the nuclear import of HIV. Modulating the levels of SUN1 or SUN2 inhibits HIV infection, revealing that the virus depends on a sweet-spot of SUN protein levels in cells. Intriguingly, increasing SUN protein levels in various cell types doesn't impact cell viability but causes deformation of the nucleus and ruffling of the NE.

We observed that overexpression of SUN1 and SUN2 led to reduced infection by both HIV-1 and HIV-2 in HeLa cells, primary monocyte-derived macrophages and CD4<sup>+</sup> T cells, with the last two being physiologically relevant HIV target cells. We further validated that SUN proteins and Cyclophilin A (CypA) functionally interact in HIV infection.

A strain-specific selectivity was observed in the fact that SUN1 shows stronger restriction of HIV-1 while SUN2 preferentially inhibits HIV-2. These preferential antiviral activities were mapped to the N-terminal, lamin-binding domains of SUN proteins. However, endogenous lamins are not required for SUN-mediated antiviral activity.

By using lamin A/C knock down cells as a positive control of nuclear deformation, no simplistic correlation between deformation and infection was found: The absence of lamin A/C, unlike SUN1/2 overexpression, showed no anti-viral activity. Instead, we identified properties that were unique to SUN1 overexpressing nuclei: reduced chromatin mobility and a reduced DNA damage signature. We find that induction of exogenous DNA damage is beneficial for HIV-1 infection (but not HIV-2) in cells. This is not the case for SUN1 overexpressing cells where additional damage does not lead to increased infection, suggesting that SUN1 modulates HIV infection downstream of the DNA damage events.

Overall our results suggest a role of SUN1 in modulation of nuclear dynamics, with subsequent interplay with the DNA damage pathway, that leads to control of productive HIV-1 infection.

Index

<b>1. LIST OF FREQUENTLY USED ABBREVIATIONS .....</b>	<b>9</b>
<b>2. INTRODUCTION.....</b>	<b>13</b>
2.1. HUMAN IMMUNODEFICIENCY VIRUS.....	13
2.1.1. <i>HIV and disease: AIDS.....</i>	13
2.1.2. <i>Virion structure and Genome organization.....</i>	17
2.1.3. <i>Viral Replication.....</i>	19
2.2. FOCUS ON HIV NUCLEAR ENTRY COFACTORS .....	21
2.2.1. <i>Viral determinants in nuclear import.....</i>	22
2.2.2. <i>HIV and NUPs.....</i>	23
2.2.3. <i>HIV, TNPO3 and CPSF6.....</i>	24
2.2.4. <i>HIV and CypA.....</i>	25
2.2.5. <i>HIV and SUN.....</i>	28
2.2.6. <i>HIV and MX2.....</i>	32
2.3. LINC COMPLEX.....	34
2.3.1. <i>INM proteins: SUN1 and SUN2.....</i>	34
2.3.2. <i>ONM proteins: Nesprins.....</i>	37
2.4. DNA DAMAGE RESPONSE .....	40
2.4.1. <i>Overview.....</i>	40
2.4.2. <i>DNA damage response signaling.....</i>	42
2.4.3. <i>DDR and LINC complex.....</i>	45
2.4.4. <i>HIV and DDR: Vpr as a key player.....</i>	46
2.5. AIM OF THE THESIS .....	49
<b>3. MATERIALS AND METHODS.....</b>	<b>51</b>
3.1. CONSTRUCTS .....	51
3.2. CELLS .....	52
3.3. VIRUS PRODUCTION .....	53
3.4. CELL TRANSDUCTION FOR PROTEIN OVEREXPRESSION OR KNOCKDOWN.....	54
3.5. CELL TRANSFECTION OF siRNA .....	55
3.6. CELL INFECTION.....	55
3.7. HIV REAL TIME qPCR.....	55
3.8. WESTERN BLOTTING .....	57
3.9. CONFOCAL IMAGING .....	58
3.10. FLUORESCENCE RECOVERY AFTER PHOTBLEACHING.....	59
3.11. INTRACELLULAR AND INTRANUCLEAR STAINING FOR FLOW CYTOMETRY .....	59
3.12. ELECTRON MICROSCOPY.....	60
3.13. MICROPIPETTE ASPIRATION MICROSCOPY .....	61
3.14. MICROARRAY GENE EXPRESSION (AFFYMETRIX®) .....	62
3.15. MOUSE DATA .....	62
3.16. STATISTICAL ANALYSIS .....	63
<b>4. RESULTS.....</b>	<b>65</b>
4.1. MAIN STORY .....	65
4.2. COMPLEMENTARY RESULTS.....	93
4.2.1. <i>Mouse data.....</i>	97
4.3. SIDE STORY: ATR.....	100



<b>5. DISCUSSION .....</b>	<b>106</b>
5.1. A MODEL IN HELA CELLS .....	112
5.2. VALIDATION IN PRIMARY CELLS AND LIMITATIONS.....	113
5.3. PERSPECTIVES AND IMPLICATIONS .....	114
<b>6. RÉSUMÉ DES CHAPITRES .....</b>	<b>117</b>
6.1. INTRODUCTION .....	117
6.1.1. <i>Objectif de la thèse</i> .....	120
6.2. RÉSULTATS ET DISCUSSION.....	121
<b>7. REFERENCES.....</b>	<b>127</b>
<b>8. APPENDIX .....</b>	<b>146</b>

## **1. List of frequently used abbreviations**

AIDS: Acquired Immune Deficiency Syndrome

ART: Anti-Retroviral Therapy

ATM: Ataxia Telangiectasia Mutated kinase

ATR: Ataxia Telangiectasia and Rad3 Related kinase

AZT: Azidothymidine or Zidovudine

BER: Base Excision Repair

BFP: Blue Fluorescent Protein

BSA: Bovine Serum Albumin

CA: Capsid

CCR5: C-C motif Chemokine Receptor type 5

CD4: Cluster of Differentiation 4, glycoprotein

Cdk1/2: Cyclin dependent kinase 1/2

CH: Calponin Homology domain

cPPT: Central Polypurine Tract

CsA: Cyclosporin A

CXCR4: C-X-C motif Chemokine Receptor type 4

CypA: Cyclophilin A

DC: Dendritic Cells

DDR: DNA Damage Response

DMSO: Dimethyl Sulfoxide

DSB: Double Strand Break

ER: Endoplasmic Reticulum

ESN: Exposed Seronegative Individuals

FACS: Fluorescence-Activate Cell Sorter scan

FRAP: Fluorescence Recovery After Photobleaching

GFP: Green Fluorescent Protein

HIV: Human Immunodeficiency Virus

HR: Homologous Recombination

IN: Integrase

INM: Inner Nuclear Membrane

KASH: Klarsicht-ANC1-Syne-homology domain

KD: knock-down

KO: knock-out  
LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry  
LINC: Linker of Nucleoskeleton and Cytoskeleton  
LTNP: Long Term Non-Progressors  
LTR: Long Terminal Repeats  
MA: Matrix protein  
MDDC: Monocyte-Derived Dendritic Cells  
MDM: Monocyte-Derived Macrophages  
MLV: Murine Leukemia Virus  
MX1/2: Myxovirus Resistance 1/2  
NC: Nucleocapsid protein  
NE: Nuclear Envelope  
NER: Nucleotide Excision Repair  
NHEJ: Non-Homologous End Joining  
NLS: Nuclear Localization Signal  
NPC: Nuclear Pore Complex  
NUP: Nucleoporin  
NVP: Nevirapine  
ONM: Outer Nuclear Membrane  
PBMC: Peripheral Blood Mononuclear Cell  
PBL: Peripheral Blood Leucocytes  
PSB: Phosphate Buffered Saline  
PCR: Polymerase Chain Reaction  
PFA: Paraformaldehyde  
PHI: Primary HIV Infection  
PIC: Pre-Integration Complex  
PNS: Perinuclear Space  
PrEP: Pre-Exposure Prophylaxis  
RFP657: Red Fluorescent Protein in far red  
RPA: Replication Protein A  
RT: Reverse Transcriptase and/or Reverse Transcription  
RT-qPCR: Real Time quantitative Polymerase Chain Reaction  
shRNA: short-hairpin RNA  
siRNA: small-interfering RNA

SIV: Simian Immunodeficiency Virus

SR: Spectrin-Repeat

SSB: Single Strand Break

ssRNA: single strand RNA

SUN: Sad1p and UNC-84 domain containing

TAR: Trans-Activation Response Element

Tat: Trans-activating regulatory protein

TRIM5 $\alpha$ : Tripartite motif-containing protein 5

VLP: Viral-Like Particle

VSV-G: Vesicular Stomatitis Virus Glycoprotein

# **INTRODUCTION**

## 2. Introduction

### 2.1. Human Immunodeficiency Virus

The Human Immunodeficiency Virus (HIV) comprises two distinct diploid ssRNA viral species, HIV-1 and HIV-2, that are classified as human lentiviruses, belonging to the family *Retroviridae*, subfamily *Orthoretrovirinae*. As such, upon entry into the host cell, its genome is rapidly reverse-transcribed into DNA by the viral reverse transcriptase [1]. A nucleoprotein complex containing the newly synthesized viral DNA, known as the pre-integration complex (PIC), is then translocated into the cell nucleus, where the viral integrase mediates successful integration of the viral DNA into the host genome [2].

Once integrated into the host, it can exist indefinitely as a provirus, thus establishing a latent viral reservoir comprising of a small percentage of infected cells [3]. The existence of such a reservoir is one of the main reasons for which it has been impossible, after almost 40 years of scientific research, to develop a definitive cure for HIV infection.

#### 2.1.1. HIV and disease: AIDS

HIV is a pathological agent transmitted via bodily fluids, more specifically through sexual, percutaneous and perinatal routes: it attacks the cells of the immune system, in particular CD4<sup>+</sup> T lymphocytes, leading progressively to their depletion and to the failure of the immune system itself. If an infected individual is left untreated, his or her condition can progress to Acquired Immune Deficiency Syndrome (AIDS), a disease in which opportunistic infections are left to thrive and take over, in the absence of a functional immune response from the host. No definitive cure from HIV infection has been developed to date: the virus can be kept under control with antiretroviral therapy (ART) but cannot be entirely eradicated from the host.

HIV is the causative agent of the global pandemic that was observed for the first time at the beginning of the 1980s [4]. Two different viruses causing a similar disease were identified: HIV-1 and HIV-2. HIV-1 was identified first, in 1983 [5] and it was found to be closely related to the simian immunodeficiency virus (SIV) from chimpanzees [6]. It actually comprises of four individual lineages: groups M, N, O and P, each of which is the result of a distinct cross-species transmission event [7]. Group M is the virus largely responsible for the widespread pandemic. HIV-2 was identified in 1986, as a morphologically similar yet genetically distinct etiological agent behind a disease that highly resembled AIDS, restricted mostly to West Africa [8]. HIV-2 was found to be closely related to the SIV from sooty mangabeys [9]. Interestingly,

though the two viruses share structural and functional similarities, HIV-2 is significantly less pathogenic than HIV-1 [10].

HIV shows tropism for the transmembrane surface glycoprotein CD4 [11, 12] that is present on the surface of many immune cells such as T helper cells, monocytes, macrophages and dendritic cells. CD4 in fact, is the main receptor that allows binding of both HIV-1 and HIV-2 and their subsequent entry into the cell, explaining why, HIV-infected patients face a specific, progressive loss of CD4<sup>+</sup> T lymphocytes, if the infection is left untreated. HIV also requires distinct co-receptors to be present on the surface of target cells: CXCR4 and CCR5 [13-15], both transmembrane chemokine receptors that are expressed on the cell surface of a broad range of leucocytes. While co-receptor usage is more strictly limited to these two proteins by HIV-1, evidence suggests that HIV-2, on the other hand, may actually be able to use a wider range of surface co-receptors [16].

Intriguingly, approximately 1% of the human Caucasian population is homozygous for a mutation in the CCR5 gene, consisting of a 32-base pair deletion (CCR5-Δ32) that in turn leads to mis-folding of the protein and its subsequent absence from the cell surface. People carrying this mutation are highly resistant to HIV infection [17, 18] and fall in the category of Exposed Seronegative Individuals (ESN).

In an HIV infected individual, three distinct phases of disease may occur (*Figure 1*, also reviewed in [19]). These phases (described in further detail below) are defined by total viremia and CD4<sup>+</sup> T lymphocyte count in the blood and are classified as

- 1) Primary infection (PHI) or acute phase (lasting the first few weeks after initial infection)
- 2) Asymptomatic or chronic phase of clinical latency (that can last up to many years)
- 3) Symptomatic phase corresponding to actual AIDS (leading to death)

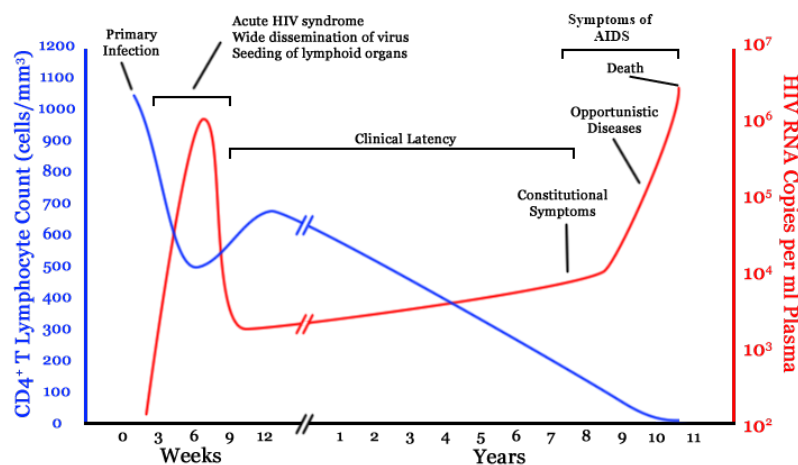


Figure 1: The three stages of disease of untreated HIV infection. The stages are identified and defined by viral load measured as HIV RNA copies/mL of plasma and by total blood CD4<sup>+</sup> T cell count (adopted from [20])

During PHI, an initial burst of plasma viremia occurs and viral load can go well above  $10^6$  copies of viral RNA/mL of plasma, after 2-4 weeks of infection [21]. During this stage, the number of circulating and resident  $CD4^+$  T cells can decrease temporarily [22, 23], sometimes allowing other opportunistic infections to occur at the same time. Indeed, patients may or may not develop a variety of flu-like or dermatological symptoms that are often therefore misdiagnosed. The virus spreads through the organism and establishes viral reservoirs within multiple lymphoid organs such as thymus, spleen, lymph nodes and gut. Eventually, the step of seroconversion is attained in which a virus-specific immune response is mounted and specific T cells proliferate, partially restoring depleted cells and reducing viral replication [24, 25].

The asymptomatic or chronic phase is characterized by a stable viral load called viral set point, (which varies greatly between patients and can predict potential disease progression), by a slow yet steady decline of  $CD4^+$  T cells and by the fundamental absence of major symptoms. The duration of this phase can last up to many years, in a patient-dependent manner. It is worth pointing out that up to 2% of untreated HIV-1 infected patients never progress to AIDS because they spontaneously control their viremia and  $CD4^+$  T cell counts. These patients are called HIV controllers or Long-Term Non Progressors (LTNP) [26]. The favored hypothesis for the gradual loss of  $CD4^+$  T cells is that new  $CD4^+$  T cell production fails to match HIV-driven  $CD4^+$  T cell death, a phenomenon dubbed the “bath tub effect” (reviewed in [19]). The reasons however are probably more complex than such a simple equation and may actually be multifactorial.

The last and final phase is the symptomatic or AIDS phase of the disease which is characterized by a peripheral blood  $CD4^+$  T cell count of  $<200$  cells/ $\mu$ L and/or the presence of secondary opportunistic infections or tumors. During this stage, patients undergo strong immune suppression and loss of anti-HIV specific cytotoxic T lymphocytes and neutralizing antibodies, with subsequent increase in viremia, eventually leading to death.

Both the “ESN” and “LTNP” mentioned previously are exceptions and do not follow through the described three-stage path of AIDS disease progression, even in the absence of treatment. Their exposure to the virus results either in resistance to infection or a spontaneous control of viral replication.

It is important to highlight that though the three-stage disease progression scheme holds true for HIV-1, patients infected with HIV-2 don't necessarily follow through the above order of events. As a matter of fact, the two viruses differ greatly in their pathogenesis because the majority of HIV-2 infected individuals are LTNP. Less than 25% of untreated, HIV-2 infected



patients develop AIDS. HIV-2 infected patients generally exhibit more favorable immune response characteristics. The viremia of HIV-2 patients is lower while blood CD4<sup>+</sup> T cell counts are generally higher compared to HIV-1 [10, 27, 28] and this has been correlated to lower rates of transmission [29]. Furthermore, infection with HIV-2 prior to HIV-1 in dual-infected patients leads to a much slower progression to AIDS, suggesting that HIV-2 provides partial cross-protection from HIV-1 [30].

After decades of fundamental research aimed at understanding the virus' complex biology and the parallel advances of translational science, the tools we have today for HIV prophylaxis and treatment are highly sophisticated and efficient [31]. HIV anti-retroviral therapy (ART) in fact targets multiple “vulnerable” yet essential steps within the viral replicative cycle such as reverse transcription (e.g. Zidovudine or AZT, Nevirapine), vDNA integration (e.g. Raltegravir), viral maturation (e.g. protease inhibitors like Saquinavir, Lopinavir) and more recently viral fusion (e.g. Enfuvirtide) (see *Figure 2*). Note that a more comprehensive description of the viral replication cycle shall be provided further on in subsection 2.1.3. These different classes of drugs, used consistently and continuatively in patient-tailored combinations, lead to suppression of viral replication, allowing patients to live a close-to normal life with no detectable viral load (<50 copies/mL of plasma), no symptoms and with a next to zero chance of transmission among serodiscordant persons.

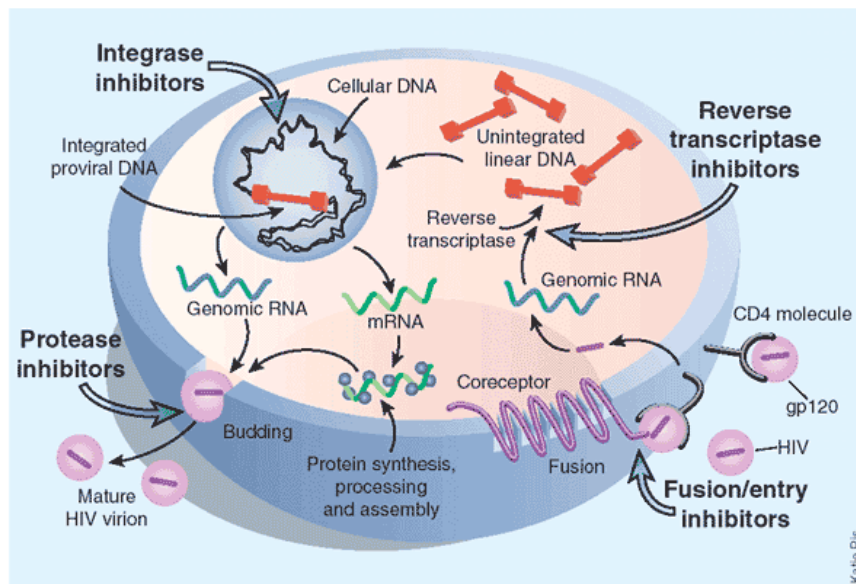


Figure 2: Different steps of the HIV replication cycle are used as targets for anti-retroviral therapy (adopted from [32])

Anti-viral drugs are also used in some cases and some countries as strategies for prevention. People who are seronegative but have a higher risk of contracting the disease such as sexually

active adults with high risk of HIV exposure, sex workers, injectable drug users and partners belonging to serodiscordant couples are encouraged to resort to this route of prophylaxis, generally known as Pre-Exposure Prophylaxis (PrEP), either daily or before/after exposure in what is known as an “event-driven” use. The use of PrEP with an optimal adherence regime was shown to significantly decrease the risk of acquiring HIV infection compared to placebo or no-PrEP treated individuals [33].

Though the advancement of technologies regarding prevention and treatment are quite encouraging, the real challenge has been ensuring that all patients currently infected with HIV actually have access to them. This has proven to be particularly hard for poor rural areas of countries with limited resources and high rates of infection such as those in Sub-Saharan Africa. Though gross efforts have been made in the fields of education, communication, medicine and infrastructure, the road doesn’t end here. As of 2019, according to the latest statistics reported by the World Health Organization, 38 million people were living with HIV around the world and of these, only 67% had access to ART ([www.who.int/health-topics/hiv-aids](http://www.who.int/health-topics/hiv-aids)).

### **2.1.2. Virion structure and Genome organization**

HIV is an obligate parasite that can exist in two forms: either as a viral particle or virion, budding out from infected cells and circulating in search of new target cells to infect, or as a provirus, in which case the viral DNA has been integrated in the host genome, awaiting transcription to produce new virions at any time.

Virions have a spherical morphology with a diameter of about 120 nm. The particle cores are surrounded by a lipid bilayer envelope that derives from the host cell and is enriched at the surface with viral glycoproteins gp120 and gp41 Env for HIV-1 [34] and gp125 and gp36 Env for HIV-2. Underneath the envelope, a mesh of matrix protein (MA) can be found [35]. The nucleoprotein cores themselves consist of a conical shaped capsid (CA) made up of p24 monomers (p26 for HIV-2), assembled as pentamers and hexamers to create the electrodense cone [36]. The core contains the viral genome: two copies of single-strand RNA that are protected and stabilized by the nucleocapsid (NC) protein. Other viral proteins such as the viral enzymes Reverse Transcriptase (RT), RNase H, Integrase (IN), tRNA and accessory proteins are also harbored within the capsid core.

The genomes of HIV-1 and HIV-2 share overall organization and have multiple similarities (*Figure 3*). The former is 9.2 kb in length while the latter is 9.67 kb. Both genomes encode for

three main elements: *gag*, *pol* and *env*. The *gag* gene is responsible for encoding the Pr55gag precursor polyprotein that is cleaved by the protease encoded by *pol* into the mature proteins of the matrix (MA or p17/p15), capsid (CA or p24/p26), nucleocapsid (NC), p6 and two spacer proteins (p1 and p2) [37]. A ribosomal frameshift leads to expression of viral enzymes (RT, IN and the protease) from a *gag-pol* precursor [38] while the envelope glycoproteins derive from the precursor polyprotein gp160 (gp140 for HIV-2), encoded by *env*, that is then processed by the cellular protease furin [39]. HIV genomes are flanked on both sides by Long Terminal Repeat (LTR) sequences, cis-acting elements that are required for HIV replication.

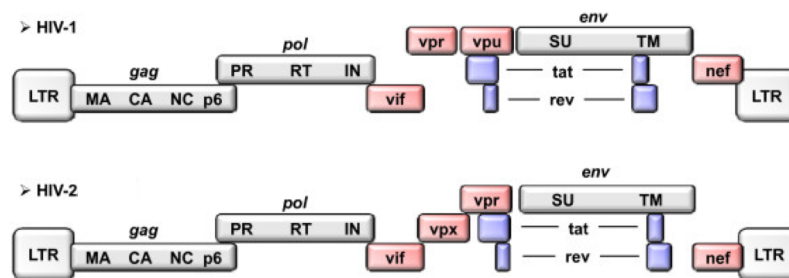


Figure 3: Genome organization of HIV-1 and HIV-2. Structural genes are shown in grey, regulatory proteins in blue and accessory proteins in red (adapted from [40]).

Other than structural elements and enzymes, the genome also encodes for multiple regulatory and accessory proteins. One essential regulatory protein is Tat, a transcriptional activator that binds to a specific sequence called Trans-Activation Regulatory element (TAR), that can be found at the 5' of viral transcripts. Tat binding is essential for transcriptional elongation [41]. Another regulatory protein is Rev, a RNA-binding protein required for the export of unspliced viral mRNA from the nucleus to the cytoplasm [42].

The HIV genomes also encode four accessory proteins, defined this way due to their non-essential nature for viral replication *in vitro*, despite being important virulence factors *in vivo*. These proteins are Nef, Vpr, Vif and Vpu for HIV-1, while HIV-2 encodes for the first three plus Vpx instead of Vpu. Nef's main role identified so far seems to be that of down-regulating CD4 expression from the host cells surface [43] and also reducing the surface expression level of both major histocompatibility complex classes I and II molecules (MHC-I, MHC-II) [44, 45] thus reducing immune detection of HIV-infected cells. Both Vif and Vpu have been shown to counteract host defense mechanisms against infection: Vif inhibits the antiviral activity of the host protein APOBEC3G [46] while Vpu has the dual function of inhibiting Tetherin (or BST-2) activity [47], a protein causing retention of budding virions to the cell membrane, and reducing cell surface expression of CD4 [48]. Vpr has been shown to be implicated in a vast and diverse plethora of viral and cellular processes and its role hasn't been fully understood

yet. A more detailed description of some of the main implications and functions of Vpr, characterized thus far, shall be provided further on in this manuscript. HIV-2 Vpx turned out to be an important factor for the infection of dendritic cells (DCs) which are usually refractory towards HIV infection due to the presence of SAMHD1, a deoxynucleoside triphosphate triphosphohydrolase (dNTPase) that depletes the cytosolic pool of available dNTPs and therefore interferes with the reverse transcription step of the viral life cycle [49]. Vpx is able to counteract the restriction mediated by SAMHD1 therefore leading to productive infections even in DCs [50, 51]. Additionally, Vpx is also able to enhance infection by HIV-1 in monocyte-derived dendritic cells (MDDCs), when added in trans [52].

### **2.1.3. Viral Replication**

The journey of HIV within the host cell (*Figure 4*) starts upon receptor recognition and binding by the viral glycoprotein surface subunit gp120. As mentioned above, the virus targets host CD4 and uses CXCR4 and CCR5 as coreceptors. Upon binding, gp120 protein undergoes a conformational change [53], thus prompting a subsequent conformational change in gp41, which then reveals its three peptide fusion domains. These then harpoon the host cell's membrane lipid bilayer, inducing fusion between the viral particle and the host cell itself, releasing the viral core within the cytoplasm [54].

Once within the cell, the step of reverse-transcription (RT) is initiated during which the viral genome is transcribed into double stranded cDNA using the host's available dNTP pool. As RT goes on, the viral capsid core is gradually destabilized leading to viral uncoating [55]. The viral cDNA in association with viral proteins CA, MA, RT, IN, NC and Vpr gives rise to the Pre-Integration Complex (PIC) [56-58], that uses cellular dynein and the microtubule network to translocate to the host cell nucleus [59]. The process, however, may not be so straightforward and the spatio-temporal distribution of the replication steps is probably less strictly compartmentalized. Indeed, recent emerging evidence shows that RT is actually still ongoing at nuclear import and is completed in the nucleus, along with uncoating [60, 61], challenging the previous dogma of RT and capsid shedding being a pre-nuclear entry process. Another study even goes as far as to say that intact cones are actually imported and uncoating only occurs in the nucleus, in the proximity of integration sites and just prior to integration [62].

The general and current view is that the PIC enters the nucleus through the Nuclear Pore Complex (NPC), although a detailed understanding of the mechanism is still unavailable. A more thorough description of this step and the viral and cellular elements involved shall be

provided in the next section. Once inside, the PIC makes its way towards host DNA and the viral integrase catalyzes proper integration of viral cDNA into the genome (reviewed in [63]). Host protein lens epithelium derived growth factor/p75 (LEDGF/p75) plays a key role in tethering the PIC to the host DNA and is indispensable for efficient integration [64]. Debates over the years have tried to shed light on whether HIV DNA integration demands some sort of integration site specificity: data from different viral strains on different target cell types have generated a complex information mosaic. It is, however, generally accepted that viral integration may favor the more open regions of chromatin underlying the NPCs, characterized by higher active transcription [65, 66]. Integration within host genome is not the only fate that viral cDNA can encounter: viral DNA can also undergo circularization events leading to the formation of 1 or 2- Long Terminal Repeat (1or 2-LTR) circles [67], that do not support subsequent replication even though they drive partial transcriptional activity. The above steps constitute the early phase of the HIV replication cycle.

At this point, the virus can either remain latent as an integrated provirus or it can enter a stage of active transcription driven by the 5'-LTR, thus initiating the late phase of the replication cycle. During the late phase, host RNA Polymerase II is exploited and short transcripts are produced. These are then spliced using the host cell machinery. Tat is synthesized during this step and is then recruited to ensure proper and stable elongation of both mRNA transcripts and replicated genome [68]. Unspliced viral mRNA is exported to the cytoplasm with the help of newly-synthesized Rev and is then translated into the structural proteins of HIV at the Endoplasmic Reticulum (ER). From here, they, along with two copies of viral RNA genome, are then shuttled for assembly at the plasma membrane to form the immature HIV viral particle. Assembly occurs preferentially in cholesterol and glycolipid-enriched microdomains of the plasma membrane known as lipid rafts [69] and budding through these domains confers HIV its cholesterol-rich bilayer membrane. The final step of the replication cycle occurs after release from the host cell and is called viral maturation: this involves cleavage of the immature Gag and Gag-Pol precursors by the viral protease and leads to generation of fully infectious, mature particles [70].

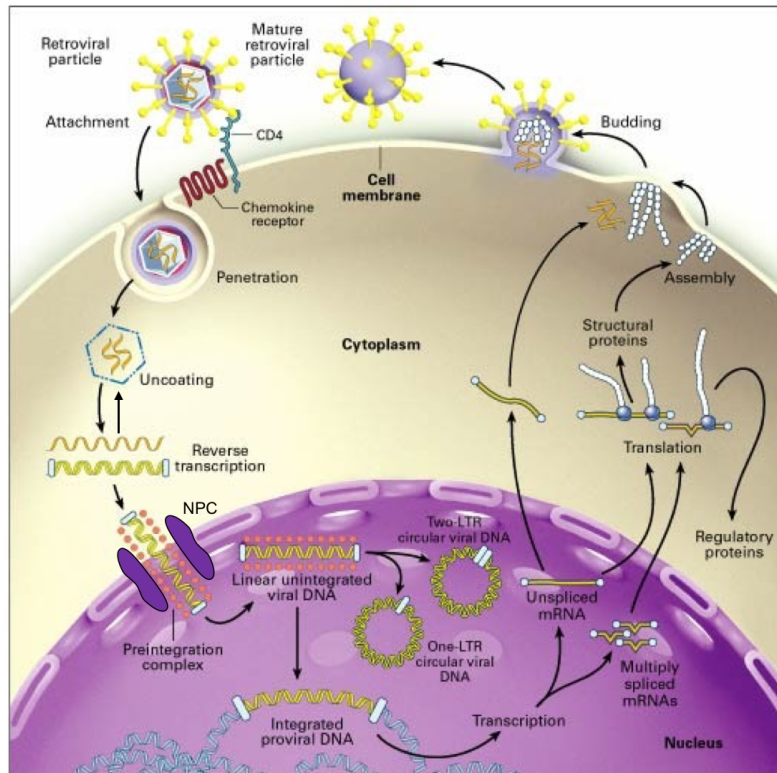


Figure 4: Schematic representation of the replication cycle of HIV (adapted from [71])

## 2.2. Focus on HIV nuclear entry cofactors

The section 2.2 and all of its subsections are an adaptation of the following review on HIV nuclear entry: “Let me in: control of HIV nuclear entry at the Nuclear Envelope”, authored by me and published in 2018 by Cytokine and Growth Factor Reviews [72].

One of the remarkable properties of HIV is its ability to infect non-dividing cells. In non-dividing cells, the nuclear envelope functions as a physical barrier to separate the nuclear content from the cytoplasm. The nuclear envelope is interspersed with NPCs, which are assembled at the end of mitosis and mediate both passive and highly regulated active transport through the nuclear envelope. The current view of HIV nuclear entry sees the virus passing through the NPC to reach the nuclear interior and to integrate into the host DNA, a view that is supported by a vast series of genetic, biochemical and imaging studies.

Despite the highly protective barrier that is the nuclear envelope, and the highly gated nature of NPCs, HIV is remarkable in its ability to thwart these natural defenses. Nonetheless, this ability comes at a cost: the virus has a narrow range of mutations that it can tolerate (in particular in the viral capsid) without losing this precious capability, and it depends in turn on multiple

cellular factors to successfully enter the nucleus, such as Cyclophilin A, NUP358, TNPO3, CPSF6 and SUN2. As part of their innate defenses, cells can also express type I interferon-inducible antiviral effectors that may limit HIV nuclear entry, such as MX2. Here, we focus with greater detail on known viral and host factors that modulate HIV entry into the nucleus.

### **2.2.1. Viral determinants in nuclear import**

The ability of HIV to enter the nucleus of non-dividing cells is not a characteristic shared by all retroviruses: Murine Leukemia Virus (MLV) requires mitosis to productively infect target cells [73]. To identify the elements specific to HIV that allow infection of non-dividing cells, chimeric viruses between HIV-1 and MLV were generated and it was observed that only viral particles containing HIV capsid (CA) protein retained the ability to infect aphidicolin-mediated cell cycle-arrested MAGI cells or terminally differentiated macrophages [74]. A later study also identified HIV-1 CA mutants that are defective in their ability to infect non-dividing cells; they also showed that sensitivities of these different mutants vary from one cell type to another [75]. Mutants T54A and N57A were consistent in their inability to infect non-dividing and arrested cells [75]. Overall, these reports suggest that CA plays a key role either during or after nuclear entry upon infection of non-dividing cells.

In addition to the capsid, the integrase (IN) also plays a role in nuclear import. A non-canonical Nuclear Localization Signal (NLS) located on the IN has been identified by protein fusion and translocation assays in HeLa cells and shown to be key in the nuclear import of Pre-Integration Complexes (PICs) [76]. Viruses harboring integrases that are mutated at the level of the NLS (IN mutants V165A and R166A) fail to infect both dividing and non-dividing cells. These IN NLS mutants are, however, not catalytically inactive as one may expect from this result because they were able to rescue integration of viruses harboring catalytically dead IN (with the D64A mutation) when present *in trans* as fusion proteins with Vpr [76]. Thus, the NLS of IN likely contributes to nuclear import of HIV, independently of integration.

Another determinant that plays a role at the level of nuclear import is the DNA Flap, a plus strand overlap of around 99 nucleotides produced by a strand displacement within the central poly-purine tract (cPPT) of unintegrated linear HIV DNA, during reverse transcription. While rates of viral DNA synthesis are not affected in cPPT mutants lacking this central DNA flap, 90% of the viral cDNA accumulates as unintegrated linear DNA, instead of progressing to viral integration [77]. Fluorescent In-Situ Hybridization of full length viral DNA is followed by confocal immunofluorescence imaging or Transmission Electron Microscopy in cell lines

showed that while signal from wild type virus at the nuclear envelope is lost between 12-24 hpi, concomitant with appearance of integrated viral DNA, signal from the Flap-defective mutant accumulates and persists at the cytosolic face of the nuclear envelope up to and beyond 72 hpi virus [77, 78]. The requirement for the DNA flap in infection of non-dividing cells was confirmed and appeared even more striking in primary cells including peripheral blood lymphocytes (PBLs), monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (MDDCs) [79, 80]. Interestingly, in viruses with combined cPPT and IN mutations, it was observed that re-introduction of the cPPT could partially rescue infectivity, suggesting it is sufficient for nuclear translocation [80].

### **2.2.2. HIV and NUPs**

Nuclear pore complexes are large multi-component protein structures that span the nuclear membrane and are responsible for active and passive nucleo-cytoplasmic trafficking [81]. Nucleoporins (NUPs) constitute a group of proteins that collectively compose the NPCs. Several NUPs contain phenylalanine-glycine (FG) repeats that branch out towards both the nucleoplasmic and cytoplasmic regions creating a gelatinous meshwork that serves as a docking platform for receptor-cargo complexes and confers dynamic selectivity to the pore [82]. NUP153, initially identified by three genome wide screens aiming to identify host factors implicated in optimal HIV-1 infection, is now well established to play an essential role in HIV-1 infection across cell types [83-85]. Other NPCs related proteins NUP358 (also known as RANBP2), NUP98 and NUP214 have also been proposed to participate to HIV nuclear entry, but their requirement is less firmly established.

NUP153 directly binds capsid through its FG repeats [86]. shRNA-mediated knockdown (KD) of NUP153 led to a reduction of HIV-1 infectivity in cell lines [87, 88]. While no change was observed in levels of viral Late RT products by RT-qPCR compared to control cells, levels of 2-LTR circles and integrated provirus were significantly lower upon NUP153 KD [87-89], suggesting that endogenous NUP153 plays a role in viral nuclear entry.

Depletion of NUP358 by siRNA or shRNA led to a reduction of HIV-1 infection in various cell lines [87, 90, 91] and RT-qPCR analysis on viral transcripts showed that the anti-viral effect is linked to nuclear entry considering that levels of 2-LTR circles were dramatically reduced upon knock-down of NUP358 compared to controls whereas levels of Late RT transcripts remained unaltered. NUP358 contains a Cyclophilin domain that can bind to the HIV-1 capsid, however, this domain is not required in NUP358 for infection [92].



Interestingly, the knockdown of NUPs 98 and 214 leads to decreased HIV-1 infectivity but only depletion of NUP358 and NUP153 affects nuclear entry, as assessed by measuring 2-LTR circles in infected cells [87]. Immunofluorescence analysis based on p24 staining at 6 hpi performed in HeLa cells showed that depletion of NUP358 also leads to loss of signal at the nuclear rim suggesting that this nucleoporin may be involved in the optimal docking of viral CA at the NE [87]. This hypothesis is consistent with the fact that NUP358 is the main cytoplasmically-oriented component of the NPC. Overall, it can be hypothesized that both NUP358 and NUP153 play distinct roles in HIV-1 nuclear entry. While NUP358 may be required for docking of the viral complex at the NE, NUP153, which is nucleoplasmically-oriented unlike NUP358, may play an active role in viral nuclear entry.

The NUPs required by HIV-2 for its nuclear entry are not yet extensively defined: loss of infectivity by a laboratory-adapted strain of HIV-2 was observed upon knock-down of NUP153 and NUP358 in cell lines [86, 93] while knock down of NUP153 and NUP358 led to reduced infectivity by HIV-2 primary isolates, with results showing patient to patient variability [94]. While the role of NUPs in HIV-1 infection has been extensively studied in cancer cell lines, data in relevant primary CD4<sup>+</sup> target cells is sparse. It is now well recognized that the NPCs are heavily remodeled in cancer [95]. Critical information regarding the role of NUPs in HIV-1 infection in relevant primary immune cells is still lacking.

### **2.2.3. HIV, TNPO3 and CPSF6**

Two other factors that have been shown to be required for HIV-1 infection and whose requirement has been mapped to the CA protein are the importin- $\beta$ -like karyopherin TNPO3, which promotes the nuclear import of serine/arginine-rich splicing factors (SR proteins) and the Cleavage and polyadenylation specific factor 6 (CPSF6) which is a component of the RNA maturation machinery [85, 96-98].

In the case of TNPO3 knock down, infection by wild type HIV-1 is compromised in cell lines and primary CD4<sup>+</sup> cells and the inhibitory effect has been correlated to the reduction of 2-LTR circle formation [99]. However, a decrease in integrated provirus that correlated to decreased infectivity was not consistently detected [99, 100]. These results suggest that TNPO3 may not directly regulate nuclear import of the virus.

CPSF6 was found to be involved in HIV-1 infection through a cDNA expression screen for host restriction factors [96]. The screen identified a splice variant of CPSF6 that was truncated at the C-terminus, mCPSF6-358, capable of restricting infection by HIV-1 when expressed

exogenously. Truncation of the protein at its C-terminus beyond residue 526 is enough for loss of nuclear localization and induction of its antiviral activity. Intriguingly, upon CPSF6 depletion, infection levels were either unaffected or marginally affected in some cell lines, suggesting that endogenous CPSF6 is not fully essential for HIV infection in the cell types tested [93, 96]. The effect of mCPSF6-358 was mapped to HIV-1 CA. The viral mutant N74D is, however, capable of replicating efficiently even in the presence of stable mCPSF6-358 expression.

Interestingly, it should be kept in mind that CA mutant N74D is also capable of escaping the antiviral activities of TNPO3 KD, NUP358 KD and NUP153 KD in HeLa cells [88, 96, 101], suggesting that this mutant is perhaps transported to the nucleus using a different pathway than wild-type HIV-1 and thus interacts with different host partners.

The roles of TNPO3 and CPSF6 are now considered linked: TNPO3 is a karyopherin that imports proteins with SR domains and the truncated mCPSF6-358 lacks precisely that. When TNPO3 is depleted, endogenous CPSF6 fails to localize at the NE and is mostly cytoplasmic, like its truncated mutant [99]. When ectopically-expressed CPSF6 is retargeted to the NE in TNPO3-depleted T2M-bl HeLa cells, infectivity of HIV-1 is rescued. This suggests that infectivity decrease in TNPO3-depleted cells might actually be a consequence of cytoplasmic re-localization of endogenous CPSF6.

#### **2.2.4. HIV and CypA**

Cyclophilin A (CypA) is a cytoplasmic peptidyl-prolyl isomerase known to interact with the CA protein of HIV-1 [102] and be incorporated into nascent viral particles during virion assembly [103, 104]. Interaction with CypA maps to HIV-1 CA residues Gly89 and Pro90 that are found within a proline-rich loop. Disruption of the CypA-CA interaction using multiple experimental techniques including treatment with the competitive inhibitor immunosuppressive drug cyclosporine A (CsA), knock-out or knock-down of endogenous CypA, use of CA mutants that are defective for CypA binding such as G89A and P90V, has consistently shown that CypA plays a key role in promoting HIV-1 infection in certain cell types but not others. CypA is required for optimal HIV-1 infection in Jurkat T cells, 293T, MT4, CEM, HOS, TE671 and human primary PBMCs, CD4<sup>+</sup> T cells and MDM [105-112]. However, infection is CypA independent in other target cells such as HeLa and H9 T cells, for treatment with CsA does not significantly impact infection levels of wild type HIV-1 compared to untreated cells [108, 113,

114]. Intriguingly, the positive modulation of HIV-1 infection is a consequence of the presence of CypA in the target cells rather than the CypA incorporated in the virion [107, 109].

Repeated passages of infection in CD4<sup>+</sup> HeLa cells treated with CsA selected viral mutants that are dependent on CsA for replication in this cell type [115]. Two such mutants are A92E and G94D, which are sensitive to CypA in HeLa and H9 cells and require CsA to replicate more efficiently. In other cell types however, they are CsA-resistant because they can replicate even in presence of the drug, unlike wild type HIV-1 [107, 113, 115]. Interestingly, CA mutants N74D and A105T are sensitive to CsA treatment also in HeLa and H9 cells, unlike wild type HIV-1. When encoded in cis, they are not only capable of rescuing A92E's infectivity in HeLa cells in absence of CsA, but they also render the virus sensitive towards CsA treatment, thus exercising a dominant effect [113, 116, 117].

Several attempts at understanding the mechanism behind CypA-mediated promotion of HIV-1 infection have been made but no unambiguous model has been identified, perhaps owing to the complexity of the cell type-specific phenotypes observed. Multiple studies have correlated the effect of CypA to an early phase of infection and to enhancement of reverse transcription, possibly through stabilization of HIV-1 CA cores [105, 118]. However, a later study that included a panel of 27 cell lines concluded that the effects of CypA on viral core stability correlate better with nuclear entry rather than with reverse transcription [113]. Two very recent studies have also finally demonstrated the long-suspected role of CypA-CA interaction to be necessary for the protection from human restriction factor tripartite motif 5a (TRIM5 $\alpha$ ) [119, 120]. This protection occurs prior to reverse transcription. CypA association to the core in the cytoplasm shields it from recognition and association with TRIM5 $\alpha$ . These results were observed in primary human CD4<sup>+</sup> T cells and macrophages.

Coming back to nuclear import, viral mutants that are defective in binding CypA (G89V and P90A) are also insensitive towards NUP358 [90]. This suggests that the CypA-binding mutants may perhaps use a NUP358-independent route of entry in the nucleus. Furthermore, pyrosequencing analysis on HIV-1 integration sites showed that wild-type virus treated with CsA, or G89V and P90A mutations, directed integration towards sites with increased density of transcription units, defined as regions enriched in genes and associated features such as CpG islands, DNAaseI hypersensitive sites and high GC content [90] compared to wild type HIV-1. It is thus inferred that disruption of CypA-CA interactions (and possibly NUP358-CA interactions) guides viral nuclear entry through a route that enhances integration in regions with high transcription unit density. However, it must be kept in mind that these mutants show reduced infectivity in most cell types, as described above: it could be speculated that since these

mutants show reduced infectivity, as measured by viral expression, in most cell types, inefficient viral gene expression from these altered sites of integration could also contribute to the reduction.

Importantly, addition of CsA on infected HeLa cells rescued the infectivity defect of wild-type HIV-1 induced by shRNA-mediated depletion of NUP358 [90]. This suggested that the activities of CypA on HIV-1 guide the virus to use NUP358 for faithful nuclear entry and integration. It must be pointed out that these results were obtained in HeLa cells, in which CsA treatment has no inhibitory effects on wild type viral replication. In primary cells such as MDM, CsA prevents replication of wild type HIV-1 [90, 111], suggesting that productive infection of a relevant target cell type relies on the presence of CypA. If the idea suggesting that CypA inhibition drives the virus to use alternative pathways of nuclear entry stands true, it must mean that these alternative pathways are more efficient in HeLa cells rather than primary cells in which HIV-1 has naturally evolved to use co-factors like NUP358/NUP153 and TNPO3 for productive infection.

This highlights how important it is in HIV biology to distinguish between cancer cell lines and primary HIV target cells: clearly the mechanisms involved and the pathways exploited can strictly differ and one conclusion, though true for a specific cell type, may not be physiologically relevant at all.

Capsid mutant viruses that showed a consistent CsA-dependency in all cell types tested, including primary CD4<sup>+</sup> target cells of human and macaques, were also identified: P86HA in HIV-2 (short HIVac-2; HIV CypA affinity-enhanced capsid) and V86I-IAP91LPA-M96L in HIV-1 (short HIVac-1) [93]. These mutated viruses, and most dramatically HIVac-2, show an enhanced affinity for CypA, enhanced viral incorporation of CypA, and exhibit a profound defect in infection that is consistently rescued by inhibiting or disrupting CypA across cell types and species. For HIV-2, the mutated HIVac-2 had no defect in reverse transcription, but showed reduced levels of integrated DNA and 2LTR circles, which is considered a marker of nuclear import, in non-cycling CD4<sup>+</sup> dendritic cells. Interestingly, when combined with second-site mutation N74D (N73D in HIVac-2), infectivity of HIVac-1 and -2 were restored and were rendered insensitive towards CsA in HeLa, GHOST and MDDCs. N74D and N73D are thus second-site mutations capable of escaping CypA's antiviral activity. Intriguingly, the rescue mutation did not reduce CypA incorporation in viral particles, suggesting that other host factors might be implicated in the process.

Altogether, there are two main possibilities to explain the diverse activities of CypA on HIV infection: either CypA has multiple and truly independent effects on HIV infection at various

steps of the replication cycle (uncoating, reverse-transcription, nuclear import, integration, etc), or CypA regulates a singular molecular mechanism of HIV, that manifests itself variably according to context. Considering this latter possibility, it was proposed that reverse transcription and uncoating of HIV-1 is likely delayed until docking to the nuclear envelope in true primary cells [121].

We acknowledge, yet again, that in cell lines, considering that these are cycling cancer cells, the kinetics and modalities of reverse transcription and nuclear import could be imperfect and misleading representations of the behavior of the virus in primary cells, thus resulting in inconsistent manifestations of contributions by CypA to HIV-1 infection. This strongly suggests that grasping the true nature of CypA activities on HIV infection will eventually demand a rationalized model that is relevant for primary target cells, instead of cycling cancer cells.

### **2.2.5. HIV and SUN**

SUN1 and SUN2 (Sad1p and UNC-84 domain containing 1 and 2), initially identified as UNC84A and UNC84B [122], are type II integral transmembrane proteins present in the Inner Nuclear Membrane (INM) of the NE. They possess a conserved carboxy-terminal SUN domain that stretches out in the perinuclear space in which it binds the Klarsicht-ANC1-Syne-homology (KASH) domain of proteins known as Nesprins, trans-membrane proteins of the Outer Nuclear Membrane that branch out of the nucleus into the cytoplasm to bind elements of the cytoskeleton such as actin, microtubules and intermediate filaments [123]. This protein complex is known as the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. In the nucleoplasm, SUN proteins interact with the Lamin meshwork underlying the NE through its N-terminal domain [124, 125]. A more detailed description of the LINC complex structure and function shall be provided further on in this manuscript.

SUN2 was initially included in a screen for Interferon Stimulated Genes (ISG) as a result of its expression in a gene-expression study [126]. SUN2 remarkably turned up as a positive hit exhibiting selective antiviral activity against HIV-1 infection, without any significant effect on infection by other viruses tested (HCV, Yellow Fever Virus, West Nile Virus, Venezuelan Equine Encephalitis Virus and Chikungunya Virus).

However, further study showed that SUN2 expression is minimally or not at all induced by IFN- $\alpha$  in various cell types or by IFN- $\alpha$ , - $\beta$ , or - $\gamma$  in primary CD4<sup>+</sup> T cells [93, 127]. Thus, SUN2 should not be considered an ISG.

On the other hand, the antiviral activity of ectopically expressed SUN2 was confirmed [93, 127], considering that overexpression of SUN2 indeed led to reduced infection by HIV-1 and HIV-2 in HeLa cells, by HIV-1 in CHME cells and in primary MDDCs. Overexpression of the homolog SUN1 also led to a decrease in HIV-1 infection in U87 MG CD4/CXCR4 and by HIV-1 and HIV-2 in HEK293A cells [128, 129].

The infection block is independent of the route of virus entry considering that infection by both Env and vesicular stomatitis virus envelope glycoprotein (VSV-G)-pseudotyped viruses was blocked by SUN1 and SUN2. The antiviral effect of SUN2 on HIV-1 did not require viral integration [127]. RT-qPCR performed at 28 hpi on the various viral DNA species showed that SUN2 blocks the virus before or at the level of nuclear entry [127]. Similarly, qPCR performed on control and SUN1 overexpressing cells infected with HIV-1 suggested that even the SUN1-mediated block of viral infection in this cell type occurs at or just prior to nuclear entry [128, 129]. However, original data presented in this manuscript, obtained in HeLa cells and primary macrophages, suggests differently (see Results section).

Donahue *et al.* identified a HIV-1 CA mutant in position 207 that is resistant to the antiviral activity of SUN2 towards NL4-3: when infected with this P207S mutant, SUN2 overexpressing CHME cells show <2-fold reduction of infection as opposed to the 8-fold reduction observed with NL4-3 wt [127]. This particular mutant had already been identified previously to be an escape-mutant towards MX2-mediated restriction [130], suggesting that the two antiviral activities may be partially overlapping. Intriguingly, the BRU strain of HIV-1 and several transmitter-founder viruses were also naturally insensitive to SUN2 antiviral activity in the study by Donahue *et al.* HIV-1 CA was also showed to play a determining role in conferring sensitivity towards overexpression by SUN1 because experiments based on chimeric vectors between HIV, SIV and MLV highlighted the importance of HIV CA for susceptibility towards SUN1 overexpression [129].

Strikingly, SUN2 over-expression, though not impacting cell viability, led to a less circular, ruffled nuclei phenotype in CHME and HeLa cells, up to the point of reaching a lobulated, flower-like nuclear shape in CHME cells [93, 127]. No changes in nuclear morphology upon SUN1 overexpression have been reported so far.

Both changes in nuclear morphology and antiviral activity mapped to the N-terminal domain of the NE protein: SUN2 deletion mutants lacking the entire SUN domain retained their antiviral activity and the capacity to induce flower-like nuclei when over-expressed. On the contrary, the lamin-binding N-terminus of SUN2 is required both for the changes in nuclear shape and for the antiviral activity upon overexpression: indeed, overexpression of deletion

mutants lacking the N-terminus but retaining the trans-membrane domain (SUN2  $\Delta$ 1-158) led to cells with round nuclei and no observed antiviral activity [127]. Also in the case of SUN1, deletion of the first 90 amino acids in the N-terminal domain abrogated all antiviral activity, while deletion of the carboxy-terminal SUN domain did not have an impact on reduction of infectivity [128, 129]. This kind of experiment suggested that the anti-viral activity of the over-expressed protein is independent of its interaction with KASH-proteins and thus with the cytoskeleton.

On the other hand, manipulation of endogenous expression levels of SUN2 also led to an antiviral activity towards HIV in a cell-type dependent manner [93, 127, 128]. Importantly, when SUN2 was depleted in primary cells such as MDDCs and CD4<sup>+</sup> T cells, infection levels were reduced for both single-round and replication-competent HIV-1 and HIV-2 viruses [93]. Furthermore, disruption of SUN2 in CD4<sup>+</sup> T cells led to reduced viability, cell proliferation and activation compared to control cells [131]. This was striking because SUN2 KO mice are viable, and this could suggest that the requirement for SUN2 in HIV infection may be intimately linked with an essential process of lymphocyte physiology. Contrasting with these findings, a recent study however reports a baseline antiviral role for endogenous SUN2, suggesting that the interaction between SUN2 and the underlying Lamin A/C helps maintain chromatin in a repressed state thus inhibiting HIV-1 LTR driven gene expression [132]. Indeed, shRNA-mediated knockdown of SUN2 in primary CD4<sup>+</sup> T cells actually led to increased HIV-1 infection in their hands and RT-qPCR mapped this effect to occur at the level of viral gene expression. Furthermore, SUN2 knockdown increased levels of proviral reactivation, as measured in the HIV-1 latently infected Jurkat T-cell clone C11, after treatment with latency reversal agents, suggesting endogenous SUN2 may play a role in repression of gene expression from proviral DNA.

In contrast to SUN2, CRISPR/Cas9 mediated SUN1 gene disruption in THP-1 cells or siRNA mediated downregulation in HEK293A cells didn't show any changes in infectivity, pointing to a SUN2-specific role on HIV infection when it comes to the endogenous protein [128, 129]. SUN2 also plays a role in the CypA-mediated regulation of HIV-1. In the context of SUN2 overexpression in CHME cells, the antiviral effects of SUN2 overexpression and CypA inhibition (either by CsA treatment or by protein knockdown) on HIV-1 infection weren't additive considering infectivity didn't decrease further (this held true also for the N74D CA mutant in Hela cells) [93, 127]. The endogenous levels of SUN2 also contributed to CypA's activities. In primary CD4<sup>+</sup> T cells, to address this question, CBS1, a non-immunosuppressive analog of CsA, was used [133]. Indeed, CsA also targets the Calcineurin pathway, and in

primary CD4<sup>+</sup> T cells this results in immunosuppression and a strong inhibition of proliferation following T cell receptor activation [134]. Consistent with what observed upon inhibition of CypA in primary CD4<sup>+</sup> T cells, it was found that CBS1 decreased infectivity by HIV-1. Even in this case, the combination of SUN2 depletion with CypA inhibition did not decrease HIV-1 infectivity further, confirming that SUN2 mediates the positive effects of CypA on HIV-1 replication in CD4<sup>+</sup> T cells.

Importantly, the role of endogenous SUN2 in mediating CypA activities was further confirmed in mouse cells by infection by HIVac-1 in BMDCs derived from *Sun2*<sup>-/-</sup> mice [93]. In wild-type control cells, infectivity of HIVac-1 was impaired compared to wild-type HIV-1 and could be rescued by CsA treatment. In *Sun2*<sup>-/-</sup> BMDCs, infection by HIVac-1 was largely rescued even without CsA treatment, proving that SUN2 is a cofactor for CypA mediated restriction of the HIVac capsid in mouse primary cells. Intriguingly, transient depletion of SUN2 in human MDDCs, CD4<sup>+</sup> T cells and THP-1 cells was not sufficient to rescue HIVac-1 infectivity [93, 128] suggesting that a different regulation may be at play in these cells compared to mouse primary cells.

SUN1's antiviral activity was also shown to be dependent on the interaction between HIV-1 CA and CypA because the fold-inhibition of HIV-1 NL4-3 by SUN1 overexpression in HEK293A cells decreased with increasing doses of CsA, an inhibitor of CypA, suggesting the effects were not additive [129].

In summary, SUN1 and SUN2 emerge as critical regulators of HIV infection, both upon over-expression and at the endogenous level. This may be due to their strategic position and function at the NE. Whether or not subsequent morphological changes of the nuclear envelope, caused by SUN level modulation, are directly or indirectly implicated in the antiviral effect needs further elucidation and is, in part, the object of this study.

It is also worth mentioning that a study from 2006 [135] identified another NE associated structural component as necessary for optimal HIV-1 infection in HeLa cells and primary macrophages: the INM protein emerin. It was shown that HIV-1 failed to optimally integrate in cells that had been depleted of emerin and this was correlated to inefficient association of the viral cDNA with host chromatin in these cells. This study, however relied strongly on in vitro manipulation and two further studies disproved HIV-1's stringent requirement for emerin *in vivo* [136, 137], suggesting it may be cell-type and strain specific.

Overall, it cannot be denied that the NE and its components, at the endogenous level, create a delicate equilibrium that, if perturbed, can easily compromise HIV infectivity.



### 2.2.6. HIV and MX2

The *Myxovirus resistance (Mx)* genes, initially identified in mice as type I IFN $\alpha$  induced influenza virus resistance genes (reviewed in [138]), encode conserved high-molecular-weight guanosine triphosphatases (GTPases) belonging to the dynamin superfamily. Humans encode two different Mx proteins, MX1 and MX2, that differ in localization and activity. While MX1 has been shown to have broad antiviral activity against RNA and DNA viruses [138, 139], screens have identified MX2 to be restrictive only towards certain viruses such as VSV, mouse herpes virus type 68 (MHV-68), and HIV-1 [126, 140].

Similar to SUN2, MX2 was first identified as an antiviral gene in a ISG screen [126]. Later studies showed that MX2 plays an important role in the IFN $\alpha$  induced antiviral activity towards HIV-1 infection and is an HIV-1 inhibitor per se when over-expressed in cancer cell lines [141-143]. As was the case for SUN2, the effect of MX2 was independent of the route of viral entry. Most studies reported that the inhibitory action of MX2 occurs prior to or at the level of nuclear import [141, 142]. However, defects in nuclear import do not fully explain the MX2-induced reduction in HIV-1 infectivity, suggesting that the block could be occurring at multiple steps in a cell-type specific manner [130].

Overexpression experiments with various MX2 mutants that were deficient for GTP binding and hydrolysis showed that MX2's antiviral activity against HIV-1 was retained and was independent of its GTPase domain [141, 142]. This is in contrast to what was observed for MX1, which requires GTPase activity for an efficient antiviral response against Influenza A virus [144].

The antiviral effect maps to the first 29 amino acids in the N-terminal region of MX2 [130]. This region is required as a Capsid binding motif [145, 146]; as a matter of fact, a triple-arginine motif in the first 25 residues of the N-terminus of MX2 is required for interaction with the capsid and subsequent restriction [146, 147]. Furthermore, results point to a clear requirement for the oligomerization of MX2 for antiviral activity against HIV-1 [148].

Liu et al. attempted to identify HIV-1 mutants that were resistant to MX2 restriction using repeat viral passaging in the presence of MX2. They discovered that the CA mutation in position A88 allowed the virus to completely resist the antiviral activity of overexpressed MX2. The fact that this mutation occurs within the CypA binding loop suggested that CypA may play a role in viral sensitivity towards MX2. In fact, disrupting HIV-1-CypA interaction using CsA or by targeting CypA using shRNA abrogated the antiviral activity of MX2 in SupT1 cells,

implying that MX2 depends on CypA for restriction. In support of this notion, other CypA binding deficient mutants, G89V and P90A were also found to be insensitive towards MX2 activity [141, 142]. Interestingly, co-immunoprecipitation experiments showed that CypA interacts with MX2 but not with MX1 [143]. However, a later study showed that MX2 does not require CypA for antiviral activity in all cell types as the same set of experiments performed in a different CD4<sup>+</sup> cell line produced entirely different results [130].

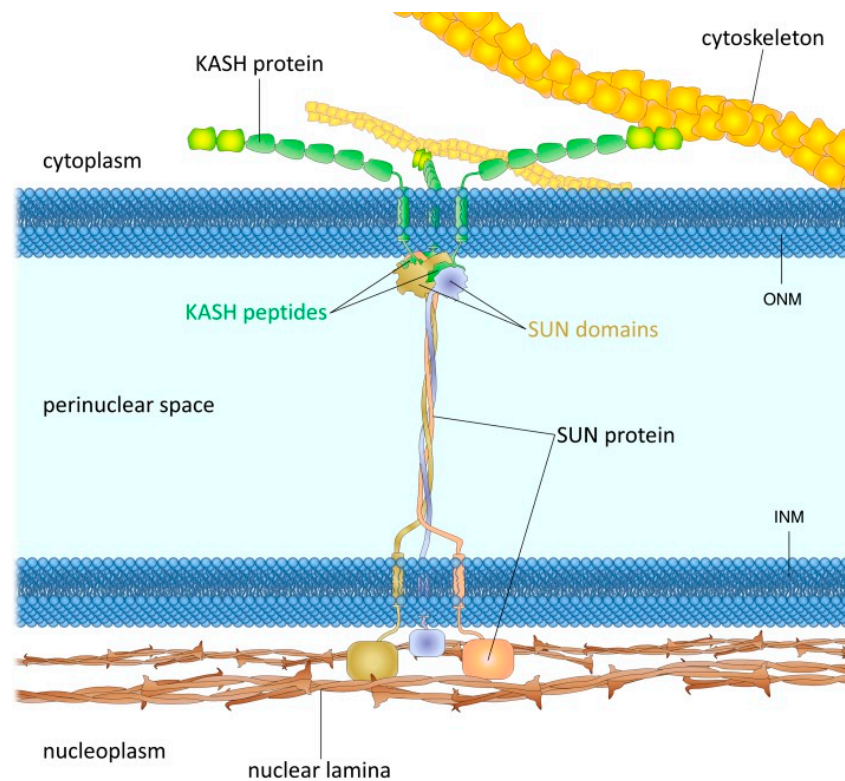
Further studies by Busnadiego et al. led to the identification of many other MX2 resistant CA mutations lying both within and outside the CypA binding loop; amongst the latter the most notable are the previously mentioned P207S, G208R and T210K. P207S is particularly interesting due to a species-specific MX2 resistance/sensitivity profile.

Moreover, the N74D capsid mutant, which is incapable of binding CPSF6 and NUP153 [96], was also reported to have reduced sensitivity towards MX2 in cell lines [141, 142].

It can be concluded that HIV-1 CA governs the sensitivity towards MX2 and co-factors that interact with CA at the level of nuclear import may impact, though not exclusively control, the MX2-mediated restriction of HIV-1. However, whether the antiviral effect of MX2 topologically occurs at the nuclear envelope remains unresolved. Furthermore, to what extent MX2 is relevant for IFN-induced antiviral activities in primary CD4<sup>+</sup> target cells has not been reported.

### 2.3. LINC Complex

In eukaryotic cells, the Nuclear Envelope is a physical barrier that separates the contents of the nucleus from the cytoplasm of a cell. It is composed of the Inner and Outer Nuclear Membranes (INM and ONM) that are separated by a thin lumen called Perinuclear Space (PNS), with the ONM being contiguous with the ER. Linker of Nucleoskeleton and Cytoskeleton (LINC) complexes are structures that physically connect the nuclear interior of a cell to the cytoskeleton. They are constituted by multiple proteins that bridge across the INM and ONM of the Nuclear Envelope (*Figure 5*). Defects in organization and function of any of the components of the LINC complex have been associated to multiple diseases of varying severity [149].



*Figure 5: The LINC complex is a protein complex that bridges across the NE. It is constituted by INM transmembrane SUN proteins that interact with the nuclear lamina at their N-terminus and carry a SUN domain at their C-terminus. In the PNS, the SUN domain binds the KASH peptide of ONM transmembrane proteins called nesprins. These in turn branch out into the cytoplasm where they bind cytoskeletal elements (adopted from [150]).*

#### 2.3.1. INM proteins: SUN1 and SUN2

The prototypical LINC components of the INM are the SUN (Sad1p and UNC-84 domain containing) proteins, type II integral transmembrane proteins first identified in *C. Elegans*

[122], that are evolutionarily highly conserved across species, especially in their C-terminus SUN domain that lies in the perinuclear space. In mammals, SUN1 and SUN2 are the most common isoforms expressed in the NE while SUN3 is mostly localized in the ER of the testes [151]. SUN proteins have an N-terminal domain that lies within the nucleoplasm, a transmembrane region that allows spanning across the INM [151, 152], a coiled coil region that allows formation of SUN1/SUN2 homo and heterotrimers [153] and the conserved SUN domain at the C-terminus that interacts with and binds proteins of the ONM [151, 152]. The overall morphology of SUN proteins resembles a flower on a stalk within the PNS.

Underlying the NE, on the nucleoplasmic face, lies a meshwork of structural elements called lamins and lamin-associated proteins, whose main role is that of providing anchoring support to chromatin and structure to the NE itself (reviewed in [154, 155]). In mammals, three different lamins are encoded: lamin B1, lamin B2 and A-type lamins that produce the alternatively spliced lamin A, lamin C and lamin C2. It was shown through yeast two-hybrid screens and in vitro pull-down assays that SUN proteins interact with lamins, in particular A-type lamins, through their nucleoplasmic N-terminal domain [124, 151]. Interaction was mapped to residues 1-138 of SUN1 and residues 1-129 of SUN2 [125]. Consistently, both SUN1 and SUN2 showed increased mobility and diffusion in mouse embryonic fibroblasts lacking functional lamin A/C, compared to wild type [156] even though data in cell lines showed that lamins are not strictly required for the NE retention of SUN [124]. This suggests that effects of A-type lamins on SUN protein anchoring may be indirect or incomplete because other chromatin-associated proteins may be involved. As a matter of fact, there is evidence of direct interaction between SUN proteins and chromatin, especially with the telomeric regions, a fact initially observed in yeast and then confirmed in *C. Elegans* and mammalian cells (reviewed in [157]).

The N-termini of SUN1 and SUN2 (through a site that is distinct from the one required for Lamin A/C interaction) also interact with emerin, a transmembrane NE protein, belonging to the lamin-associated protein family [125].

The C-terminus of SUN proteins, on the other hand contains the ~175 amino acid SUN domain, that was shown on multiple occasions to interact with the Klarsicht-ANC1-Syne-homology (KASH) domain of ONM proteins such as nesprins [124, 151, 158]. These are large proteins that present multiple isoforms and have been shown to localize mainly at the cytoplasmic face of the NE. Co-depletion of SUN1 and SUN2 in HeLa cells led to loss of NE localization for the nesprin giant isoform 2G. Furthermore, in these cells, an increase in the spacing between the INM and the ONM with subsequent enlargement of the PNS was also observed (>100nm as opposed to 50nm in control cells), implying a “molecular ruler” function of the SUN-nesprin

association [151]. Co-immunoprecipitation results further confirmed the interaction between SUN1/2 and the KASH domain of nesprin 2A, 2B and 2G [124, 151]. Further structural and biochemical analyses revealed the formation of a hexameric complex between SUN domain trimers and three independent KASH peptides [159]. This interaction represents the actual physical bridge between the nucleus and the cytoplasm, considering the ONM proteins then span out into the latter, establishing a connection with cytoskeletal elements (see next section).

Overall, the function of SUN proteins and their strategic positioning at the NE are cause for their implication in multiple nuclear processes. Many studies over time have also highlighted differential roles for SUN1 compared to SUN2, thus challenging the idea of the two being redundant homologs. As mentioned above, SUN has been found to interact with telomeric regions of chromatin and this interaction has been best described in yeast: in *S. Cerevisiae*, SUN domain containing Mps3 tethers the telomeres at the NE during S phase [160]. Telomere tethering is an important aspect of meiosis across species. Indeed, SUN proteins have been shown to play a role during this process in *S. Cerevisiae*, *S. Pombe* (reviewed in [157]) and SUN1 has been implicated in chromosome tethering and homolog pairing in *C. Elegans* [161] and in telomere attachment and homologous recombination in mice [162].

SUN1 is also important in mitosis: in *S. Pombe*, Sad-1 mediates centromere clustering at the NE and ensures proper chromosome segregation and mitotic progression [163]. Evidence for a role of SUN1 in mitosis of mammalian cells came from a study showing that human SUN1 and a membrane associated histone acetyl transferase called hALP cooperate in the decompaction of DNA at the end of mitosis, as demonstrated in HeLa cells [164]. Furthermore, results in synchronized HeLa cells showed that the SUN1-Lamin A/C and SUN1-Emerin interactions were disrupted in mitotic cells [165]. This disruption was correlated to a concomitant phosphorylation of three phosphorylation sites within the N-terminus of SUN1, identified by liquid chromatography tandem mass spectrometry (LC-MS/MS), specifically in mitotic and not in asynchronous HeLa. The mutated residues identified were S48 and S333, revealed to be targets of Cyclin-dependent kinase 1 (Cdk1) and S138, putative target of polo-like kinase 1 (Plk1). Note that both these kinases are implicated in cell cycle regulation and mitosis. The study concluded by hypothesizing a model in which SUN1 N-terminus phosphorylation upon onset of mitosis loosens its connection with the nuclear lamina, favoring disassembly of lamins and their interaction with chromatin. On the other hand, SUN1-Nesprin2A interaction is maintained suggesting that the LINC complex is not disrupted at this stage of mitosis.

An active role of SUN proteins has been identified also in DNA damage repair (DDR): indeed, both SUN1 and SUN2 have been shown to play a role in maintenance of genome integrity, mobility of damaged DNA sites and optimal DDR. A more detailed description of these mechanisms, along with bibliographical references, shall be provided in section 2.4.3.

SUN1 (and not SUN2) has been shown to functionally associate with Nuclear Pore complexes, via both its nucleoplasmic and luminal domains [166]. SUN1 depletion leads to NPC clustering, suggesting this interaction may be necessary for optimal distribution of NPCs across the surface of the NE.

SUN1, perhaps through this association with NPCs, is also implicated in the export of mRNA from the nucleus to the cytoplasm as it was shown that depletion of SUN1 led to an accumulation and impaired export of both heterogeneous nuclear ribonucleoproteins (hnRNPs) and poly(A)+RNA in the nucleus of HeLa cells [167]. This function was shown to be partially regulated by phosphorylation of SUN1 at its S113 by Protein Kinase C (PKC) [168].

### **2.3.2. ONM proteins: Nesprins**

Typical ONM proteins, as mentioned above, are nesprins (Nuclear Envelope SPectrin Repeat, also called Syne-1/2, enaptin, Myne-1 or NUANCE). In mammals, these constitute a family of spectrin-repeat, transmembrane proteins that exist as various isoforms obtained by alternative splicing of four genes [169] and, like SUN proteins, they are evolutionarily conserved. These proteins contain a C-terminal Klarsicht-ANC1-Syne-homology (KASH) domain that lies in the lumen of the PNS and is sufficient for protein localization to the ONM, a trans-membrane region, a cytoplasmic rod-like extension containing variably-sized stretches of long spectrin-repeats (SR) followed, in most cases, by an N-terminal  $\alpha$ -actinin-type calponin homology (CH) actin binding domain [170, 171]. In fact, nesprins vary in size depending on the lengths of their SR, ranging from <30 kDa to >1MDa and may also differ in domain composition, expression pattern and potentially, in their functional properties.

The two main mammalian isoforms of nesprins are the giant nesprin-1G and nesprin-2G, respectively weighing 1.01 MDa and 796 kDa, first cloned using mRNA from mouse brain [172] and from human Burkitt's Lymphoma cells BL-60 [171]. Multiple shorter isoforms of both nesprins 1 and 2, obtained either by alternative splicing or by alternative transcriptional initiation, also exist, with some entirely lacking either their N-terminal domain or their C-terminal KASH domain. For example, nesprins-1 $\alpha$  or 2 $\alpha$  fall into the former category and have

been shown to localize deeper into the NE and interact directly with lamin A/C and emerin [173, 174].

Additional members of the nesprin family are nesprin-3, nesprin-4 and KASH5 that have a KASH domain, variable number of SR but lack the CH actin-binding domain. Nesprin-3 interacts with the actin-binding domain of plectin that in turn binds to intermediate filaments [175], nesprin-4 was shown to bind kinesin-1 and it was speculated to play a role in microtubule-dependent nuclear positioning [176] while KASH5 turned out to be germ-cell specific and to interact with SUN1, telomeres and with the microtubule associated dynein-dynactin complex to ensure proper chromosome anchoring during meiosis [177].

As expected, nesprins play an extremely essential role in nuclear scaffolding and positioning within the cell and evidence for this function came initially from a study in *C. Elegans* and *D. Melanogaster*: loss of nesprin homologues ANC-1 and MSP-300 in these organisms led to incorrect syncytia formation and improper nuclei distribution in hypodermal and nurse cells [170, 178]. This was confirmed *in vivo* in transgenic mouse models where the dominant negative KASH domain from nesprin-1 was specifically expressed in skeletal muscle cells, leading to a loss of synaptic nuclear distribution beneath the neuromuscular junction [179]. Furthermore, interaction of KASH proteins with molecular motors (as is the case for nesprin-4 for example) pinpoints towards an active role in nuclear kinetics. Indeed, it has been observed that the KASH protein UNC-83 of *C. Elegans* binds to the kinesin-1 light chain KLC-2 for optimal nuclear migration [180, 181]. Later studies also showed that nesprin-2, along with SUN1 and SUN2, plays a key role in nuclear migration during mouse retinal development [182] as well as in nuclear migration on retrograde flowing actin cables during fibroblast polarization (nesprin-2G and SUN2) [183].

There is evidence suggesting that the long rod-like SR domains of nesprins also serve as a docking platform for multiple cytosolic proteins and complexes including those of essential signaling pathways: for example, a KASH-less nesprin-2 colocalizes with mitogen-activated protein kinases (MAPK1/2 or ERK1/2) and promyelocytic leukemia bodies (PML) to regulate signaling [184] and loss of nesprin-2 in human cells in this study led to sustained activation of the pathway and increased proliferation.

In addition to these studies, there is plenty of evidence pointing to nesprin alteration as cause for insurgence of pathologies: in fact, missense mutations in KASH protein genes leading to protein truncation and/or loss of function have been reported in several diseases, in particular

in cardiomyopathies (these and other LINC-complex associated diseases are reviewed in [149, 185, 186]).

On top of the roles described thus far of LINC complex components in nuclear anchorage/positioning, nuclear migration, signal transduction, DDR and chromosome tethering especially during meiosis and mitosis, another crucial pathway needs to be mentioned: signal mechanotransduction and force transmission from the cytoplasm to the nucleus. In fact, disruption of the LINC complex in MEF cells leads to impaired propagation of intracellular forces and disorganization of perinuclear actin, as measured by biophysical assays [187]. Another study on isolated nuclei also showed that directly applying force on nesprin-1 leads to modulation of nuclear stiffness as a response [188], suggesting yet again that the LINC complex plays a role in biochemical and biophysical adaptation of the cell to mechanical signals.



## **2.4. DNA damage response**

### **2.4.1. Overview**

As all biological molecules, even DNA is subject to time-dependent deterioration. The genomic material within each cell supposedly receives tens of thousands of lesions per day [189]. Some of these aberrations arise via physiological processes such as mismatches during DNA replication or strand breaks caused by improper topoisomerase I and II activity. Others can be the fruit of spontaneous chemical reactions such as base hydrolysis or non-enzymatic methylations. Furthermore, replication enzymes themselves are error-prone and can introduce mutations during their reactions while other enzymatic processes such as oxidative respiration or inflammation can generate toxic byproducts, like reactive oxygen species, that can induce severe lesions to DNA. DNA damage can arise also due to exogenous causes of environmental origin: the most common causative agents that fall in this category are ultra-violet light (UV), that can cause formation of pyrimidine dimers, and ionizing radiation that can result both from decay of naturally occurring radioactive compounds and from exposure to man-made radioisotopes [189].

Common types of lesions are chemical modifications of bases, formation of adducts that block transcription and/or replication progression, base loss, single-strand breaks (SSBs) and, the most severe lesion of all, double-strand breaks (DSBs). Considering the importance of DNA as a biological molecule and the fact that it is represented as a unique copy per cell, it is of fundamental importance that any lesion be detected and repaired straight away, to avoid its propagation to daughter cells, thus triggering the onset of diseases such as cancer. In order to preserve genomic integrity, cells are equipped with a sophisticated network known as DNA damage response (DDR). This consists of multiple repair pathways, each depending on the category of the lesion, and several checkpoints and signal transduction mechanisms that all together make up a maintenance system that decides the cell's fate: survival, replicative senescence or death [190].

Chromatin remodeling is the first and foremost step in DDR because when DNA is tightly packed as chromatin, access to DNA by the repair machinery is heavily blocked. Evidence shows that chromatin relaxation occurs within seconds after appearance of a DNA lesion [191, 192] and histone modifications follow shortly after.

One of the most classical histone modifications (that is in fact used as an experimental readout for DNA damage and DDR initiation), is the phosphorylation of histone H2AX at its serine in position 139 [193] (referred to as  $\gamma$ -H2AX). This phosphorylation can be carried out by any one

of the following kinases: ATM, ATR or DNA-PK (described later in further detail). The primary function of this histone modification is that of acting as a platform for the recruitment and concentration of DDR effector proteins [194, 195] and loss of H2AX in mammalian cells has been linked with multiple genomic integrity defects [196].

When only one of the two strands of the double helix encounters a lesion (single-strand damage), such as a mismatch or a chemical modification for example, the affected nucleotide can be removed in a process called excision repair and the second strand can be used as complementary template for correction in a “cut and patch” mechanism.

If bases undergo small modifications that do not impact the overall structure of the double helix, the excision process used is called Base Excision Repair (BER). BER involves removal of the single damaged base by a glycosylase enzyme, cleavage of the DNA backbone by an AP endonuclease, correction of the region by a 5' to 3' exonuclease/polymerase activity and sealing of the gap by a DNA ligase [197, 198]. If the damage is more complex and involves extensive distortion of the double helix structure (as could be the case during formation of adducts such as UV-induced pyrimidine dimers), the repair pathway used is the Nucleotide Excision Repair (NER), a multi-step process involving a large family of proteins [199]. During NER, after recognition of the damage by various factors depending on the subcategory of lesion type, Transcription Factor II (TFH-II) is recruited to the site. Here, it acts as a helicase that unwinds the DNA strands [200]. The exposed single-strand recruits Replication Protein A (RPA) that is essential for correct positioning of endonucleases [201] such as XPG (belonging to the Xeroderma Pigmentosum group), that remove 12-24nt stretches of nucleotides both upstream and downstream of the lesion [202]. Finally, DNA polymerases are recruited to the strand and the gap is filled by synthesis of a new fragment [203], which is then ligated by DNA ligases I and III [204].

A much more severe type of damage occurs when the lesion causes both the strands of DNA to break: this is defined as a Double-Strand Break (DSB) and repair in this case needs to occur without any complementary template strand available. These are particularly dangerous because a severed double strand can lead to rearrangements in the genome. The two main pathways exploited by eukaryotic cells to repair DSBs are Non-Homologous End Joining (NHEJ) [205] and Homologous Recombination (HR) [206] and the choice between one or the other depends on the phase of the cell cycle the damaged cell is currently in. Indeed, HR takes advantage of the sister chromatid of the damaged chromosome and uses it as a template for repair. Therefore, HR can occur only after DNA replication, during the S or the G2 phase.

NHEJ can function throughout the cell cycle. It is known to be an imprecise process because joining of resected ends is always coupled to loss of nucleotides surrounding the site and addition of random bases during the process. Upon onset of a DSB during G<sub>0</sub>, G<sub>1</sub> or early S phase, the Ku heterodimer (Ku70/Ku80) is recruited to the site and binds to DNA ends [205]. Ku then recruits the protein kinase DNA-PK and the Artemis nuclease: these two proteins exist as a complex and upon binding to DNA, the complex gets phosphorylated by DNA-PK and the endonuclease activity of Artemis is activated, creating overhangs [207]. Gaps are filled by polymerases  $\mu$  or  $\lambda$  and the ends eventually re-ligated by the XRCC4/Ligase IV complex [208, 209].

HR is initiated when components of the MRN complex (consisting of Mre11, Rad50 and Nbs1) bind to DSBs to tether them together [210]. Subsequently, DNA end resection is promoted by the MRN/CtIP complex and this leads to exposure of ssDNA that is then able to recruit RPA [211, 212]. After an intricate process mediated by multiple proteins, a nucleoprotein filament between Rad51 and the single strand is formed: this presynaptic filament then scans DNA for sequences homologous to the 3' overhang (for example a sister chromatid during mitosis or the homologous chromosome during meiosis) and subsequently coordinates strand invasion into the second, identified duplex [213, 214]. A structure where four strands of two duplex DNA are crossed, known as the Holliday Junction, is thus obtained, with the invading strand 3' being extended along the recipient duplex by DNA polymerases. Multiple different pathways of recombination and junction resolution have been described with a vast plethora of mediators shown to be involved [213]. The end result is a sealed double-strand that has been repaired with moderate to high fidelity.

#### **2.4.2. DNA damage response signaling**

DDR is not an isolated process. It triggers a concerted response of multiple signaling pathways and checkpoints, that lead to cell cycle arrest. The blockade is ideally not released until the damage has been resolved. A functional signaling and checkpoint machinery is critical for avoiding aberrant proliferation of cells with unrepairable mutations, a fact that can easily lead to tumorigenesis [215]. Cells can be arrested at the G<sub>1</sub>/S or G<sub>2</sub>/M transition or within the S phase itself. In order for the arrest to occur, coordinated, hierarchical action ranging from sensors of damage, to mediators/transducers and finally down to effectors, is required [216] (*Figure 6*). In this section, I shall focus a bit more on the damage sensors rather than the latter.

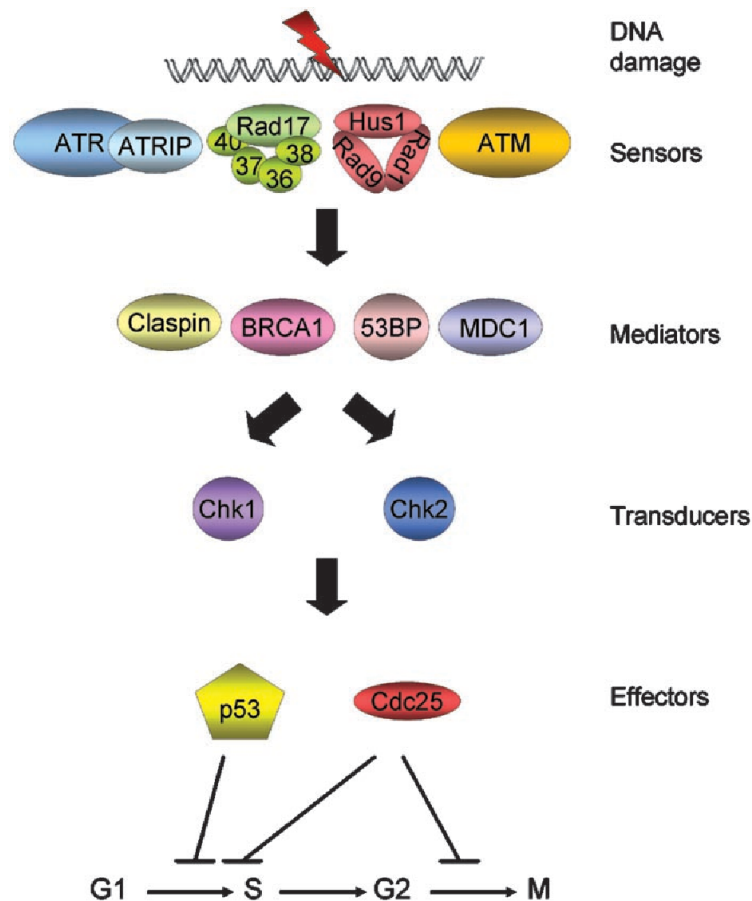


Figure 6: Components of the DNA damage checkpoint signaling pathways in human cells. The damage is sensed at various stages of the cell cycle by Sensors that in turn activate downstream Mediators and Transducers, eventually leading to regulation of Effectors that stall the cell cycle until the damage, if possible, is resolved (adopted from [216]).

In the case of DNA damage, checkpoints are activated by the two master phosphatidylinositol 3-kinases (PI3): Ataxia Telangiectasia mutated (ATM), that is directly recruited and activated by the DSB-recognizing protein complex MRN and ataxia telangiectasia and Rad3-related protein (ATR), that is recruited by RPA bound to ssDNA [217].

ATM is a 350 kDa oligomeric protein. It is a serine-threonine kinase whose activity is triggered by its recruitment to sites of DSB (or to recruitment to linear dsDNA in vitro) [218]. ATM recruitment to damaged chromatin leads to autophosphorylation and to downstream phosphorylation of effectors such as Chk2 and p53 [218-220].

ATR on the other hand is a 303 kDa protein with a C-terminal kinase, the loss of which confers embryonic lethality in mice [221]. It is recruited to DNA by the ssDNA coating protein RPA in complex with a binding partner called ATRIP [222] and was first identified in the context of DDR due to its enhanced activity observed in the presence of UV-induced DNA damage [223]. Absence of reaction with linear dsDNA in vitro suggested ATR doesn't really sense DSBs.

ATR activates a similar spectrum of effectors as ATM, plus the ATR-specific downstream kinase Chk1 [224].

Both signal transducers Chk1 and Chk2 are capable of phosphorylating downstream effectors, the most important being the phosphotyrosine phosphatases Cdc25 A, B and C, that dephosphorylate the different cyclin-dependent kinases involved in cell-cycle transitions.

When a G1/S arrest is required, Cdc25A is phosphorylated and inactivated either by the DSB induced ATM-Chk2 axis or by the UV induced ATR-Chk1 axis, leading to accumulation of inactive phosphorylated Cdk2, causing G1 arrest [225]. The arrest is then stabilized and maintained via phosphorylation of p53 [225].

Multiple pathways of intra-S phase arrest have also been described [216]. The S phase is also interesting for another reason: DNA replication, that characterizes this phase, is a source for potential DNA damage *per se* and this phenomenon is called replicative stress. It was observed that during DNA replication, ssDNA forms at the replication fork, subsequently recruiting RPA. In case of errors or aberrant replication, ATR is activated and acts as a checkpoint, inhibiting progression of the S phase [226]. The study used etoposide, a drug inhibiting the unwinding action of topoisomerase II on supercoiled replicating DNA, to cause tension-induced damage to replicative genomic regions (initially described in [227]) and block S phase progression in cell free *Xenopus laevis* egg extracts. The damage was shown to generate short ssDNA regions that recruited RPA. Furthermore, replication was rescued by inhibition of ATR [226]. Overall, results suggest that an ATR-dependent checkpoint, scanning for ssDNA lesions, is at play during DNA replication. This checkpoint activity also goes down the ATR-Chk1-Cdc25A axis [228] and its regulatory function in replication origin firing was also confirmed in mammalian cells [229]. Further evidence gathered over the years pointed out an overall essential role of ATR in guaranteeing replication fork stability [228].

The signaling for G2/M arrest is also carried out by the ATM-Chk2 or ATR-Chk1 axis (via phosphorylation of either Cdc25A or Wee1) but leads in this case to control of Cdc2/CyclinB activities, causing inhibition of cycle progression [230, 231].

Another mitosis-specific role, distinct from DNA damage and replication stress sensing, has recently been identified for ATR [232]. The study shows that ATR is recruited by mitotic regulator Aurora A at centromeres of mitotic chromosomes, where RPA coated R-loop structures lead to its activation. ATR then activates the mitotic spindle kinase Aurora B, which is essential for proper chromosome segregation, thus ensuring genomic stability during the vulnerable process that is mitosis.

### 2.4.3. DDR and LINC complex

The LINC complex and its components have been found to be implicated in the regulation of DDR in multiple ways. This is not at all surprising considering the role that NE proteins play in chromosome tethering and nuclear architecture in general. Evidence for this came initially from a study in *S. Cerevisiae* that focused on chromatin dynamics upon DSB induction [233]. This group used an imaging technique called Chromosome Conformation Capture (3C) to study the interaction of a chromosome harboring an inducible endonuclease induced DSB with neighboring intact chromosomes in asynchronous yeast cells. They discovered that sites with DSBs are rapidly translocated to the nuclear periphery and that this peripheral localization requires the SUN-domain containing NE protein Mps3. Furthermore, the effect mapped to the N-terminus of the protein. It is speculated that this translocation of DSBs to the nuclear periphery is required for optimal access by the repair machinery and has been observed on multiple occasions [234-236].

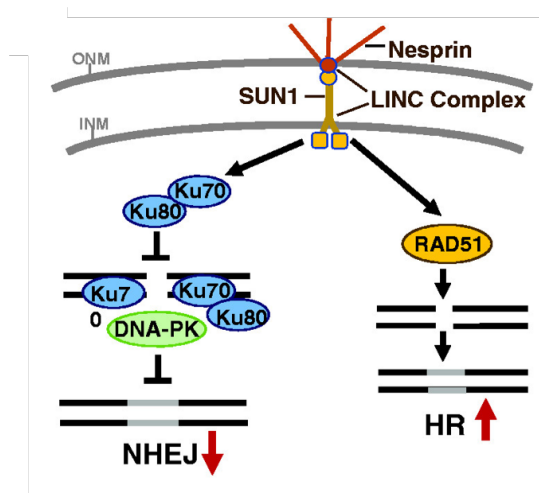
Another study in *S. Pombe* revealed the direct association of SUN domain protein Sad1 and KASH domain protein Kms1 with sites of induced DSBs [237]. This association is triggered by the persistence of the DSB and requires sensing by Rad3, an ATR homolog. Formation of Sad1-Kms1-DSB foci occurs at the proximity of the NE and is possibly required for the orchestration of optimal HR.

The importance of SUN proteins in HR was also highlighted in *C. Elegans* [238] in which loss of UNC-84 leads to defects in RAD51 loading upon DNA damage. As a consequence, stalled replication forks resort to NHEJ for repair. The defects are suppressed when the NHEJ pathway is inhibited, for example through codepletion of Ku70. The group also speculated that a similar mechanism could be at play in human cells. Indeed, siRNA mediated depletion of SUN1 in HeLa cells leads to increased sensitivity towards a DNA cross-linking agent. The sensitivity is suppressed and restored to control levels upon subsequent inhibition of DNA-PK. Further analysis in the worm also shed light on the importance of KASH proteins and microtubules in the process. The study concludes by speculating that the LINC complex may play a role in favoring HR over NHEJ, which it might be indirectly inhibiting.

SUN proteins also play a direct role in genome stability in the mouse [239]. MEF cells from *Sun1<sup>-/-</sup>Sun2<sup>-/-</sup>* mice display premature proliferative arrest in the S phase, increased DNA damage upon treatment with genotoxic agents and reduced DDR as measured by levels of phosphorylated ATM and  $\gamma$ -H2AX, considered to be hallmarks of an initiated repair process. Furthermore, co-immunoprecipitation revealed that SUN1 and SUN2 both associate to DNA-

PK, Ku70 and Ku80. This data can therefore strengthen the hypothesis that SUN proteins may be involved in the switch from NHEJ to HR (*Figure 7*).

Coming back to DNA mobility, there is constantly growing emerging evidence of the LINC complex being directly involved in the optimal re-localization of damaged sites [240, 241] in order to allow efficient downstream repair. The microtubule network also plays a role in the execution of the mobility [238, 240] and, more recently, it has emerged that nuclear actin polymerization is also essential for the mobility of DNA damage sites and for DSB clearance, also preferentially via HR [234, 241-243].



*Figure 7: SUN1 and the LINC complex modulate the DNA damage Response (DDR) by inhibiting Non-Homologous End Joining (NHEJ), perhaps via direct interaction with repair factors Ku70/Ku80 and promoting damage repair via Homologous Recombination (HR) (adapted from [244])*

Overall, it can be speculated that the LINC complex, in particular the INM SUN proteins and their homologs across species, mechanically modulates translocation of damaged DNA foci to the nuclear periphery in dividing cells, where it inhibits NHEJ, perhaps via direct interaction with the pathway components and favors the more high-fidelity repair via HR.

#### **2.4.4. HIV and DDR: Vpr as a key player**

One of the key features of DDR is cell cycle regulation. Pausing a cell's cycle, at a specific stage, gives the cell's repair machineries time to spring into action and repair any damage the genome may have suffered. One of the steps at which the cycle can be arrested, post damage sensing, is the G2/M transition. It has long been observed that multiple viruses can hijack a cell's checkpoint regulation mechanism to arrest proliferation [245]. The same holds true for HIV, known to arrest the cells in late G2 or at the beginning of the M phase [246, 247]. At the same time, it became clear that HIV accessory protein Vpr is necessary and sufficient to induce

this G2 block [246]. The G2 to M transition is governed by the action of the Cdc2/Cyclin B kinase complex and it became evident that the Vpr-induced block goes through the accumulation of the hyperphosphorylated form of the kinase Cdc2 [246, 248]. It was indeed shown that p34cdc2/Cyclin B complexes immunoprecipitated from Vpr-expressing cells were inactive as shown by a kinase assay. Furthermore, addition of a constitutively active kinase complex relieved the G2 block in these cells confirming that Vpr indeed exploits the checkpoint pathway [248]. In vitro binding of Vpr to Cdc25C, an activator of the Cdc2/Cyclin B complex, was speculated to play a direct role in this regulation [249]. It appears that Cdc25C and Cyclin B1 are mislocalized in cells expressing Vpr and this effect has been correlated to Vpr accumulating at the NE, where it induces herniations in the lipid bilayer and alterations in the underlying lamina, a phenomenon that could be contributing to cell cycle arrest signaling [250]. Multiple hypotheses have been put forth in an attempt at explaining why a G2 arrest would be beneficial for the virus. One such hypothesis is that Vpr arrests the cells at the stage where viral expression is the strongest [251]; indeed, the study shows that G2 is the stage where transcription from the LTR is most active, therefore Vpr is simply boosting viral production. Later on, another in vitro study exploiting biochemical techniques to arrest cells in G2, also confirmed that a G2 arrest promotes early steps of HIV infection in vitro and can be used to enhance cell transduction with HIV-based vectors [252]. Indeed, treatment with drugs arresting cell lines in the G2 phase leads to quite a significant increase in HIV DNA products compared to untreated controls.

The solid and direct link between Vpr and DDR however lies in the former's direct implication of the DNA damage sensor ATR. Depletion or pharmacological inhibition of ATR abrogates the Vpr induced block in G2 in HeLa cells. The same result is obtained in U2OS cells expressing a dominant negative construct of ATR [253, 254]. Vpr-induced increase of LTR transactivation is also reduced in these cells, suggesting that ATR activation leads to increased viral expression through Vpr. At the same time, an increase in phosphorylation of ATR's target mediator Chk1 is also observed upon Vpr transduction in HeLa cells. Depletion of Chk1 relieves the Vpr-induced G2 block, further confirming the exploitation of the ATR-Chk1 signaling axis by Vpr [253]. ATR knockdown relieves the G2 block also in primary CD4<sup>+</sup> lymphocytes infected with full length HIV-1<sub>NL4-3</sub>, while Vpr fails to activate the ATR/Chk1 axis in monocyte-derived macrophages, considering their post-mitotic, non-proliferative status [255].

$\gamma$ -H2AX, another downstream target of ATR and a hallmark of DNA damage signaling, is upregulated in the presence of Vpr in cell lines [254, 256] and in primary CD4<sup>+</sup> thymocytes upon infection by full length HIV-1<sub>NL4-3</sub> [256]. The link between Vpr and ATR is further



reinforced by looking at upstream events: indeed, Vpr expression leads to increased RPA loading on chromatin, which can explain ATR recruitment and activation, and this occurs probably via direct interaction between Vpr and chromatin [254, 257]. A more recent study even revealed, using cell-free assays and cell lines, that Vpr directly causes unwinding of dsDNA, thus altering the chromatin structure, leading to recruitment of RPA and DDR as a consequence [258].

Overall, these results create an obvious parallelism between Vpr activity and the DNA damage-induced regulation of the G2/M transition. In any case, whether Vpr itself induces DNA damage or simply mimics it to trigger signaling has been a well debated question. The study by Lai *et al.* shows that Vpr expression does not directly induce DSBs in HeLa cells, consistent with an absence of activation of DSB sensor ATM [254]. On the other hand, there have been other studies indicating that indirect damage induction by Vpr is indeed occurring. Tachiwana *et al.* observed that purified Vpr protein promotes DSBs when incubated with isolated nuclei, in a manner dependent on its ability to bind DNA [259]. Meanwhile, Iijima *et al.* speculate that DSBs arise as a result of the supercoiling caused by the local DNA unwinding induced by Vpr [258]. A more recent study reveals that Vpr may actually be acting at two independent steps: on one hand, it induces DNA damage and activates DDR, while on the other, it represses repair by inhibiting both NHEJ and HR [260]. Intriguingly, mutational analyses of Vpr shows that the DDR inducing activity is uncoupled from G2 arrest and repair inhibition. Note that this study is solely based on overexpression of exogenous Vpr in U2OS cells and is not confirmed by infection using full length HIV.

Another interesting fact regarding Vpr's cytostatic ability is the proven notion that this effect is mediated by the interaction and activation of the DDB1-CUL4A<sup>VPRBP</sup> E3 ubiquitin ligase complex, through which Vpr may be targeting key cellular factors for proteolysis, therefore stalling the cell cycle [261-263]. As a matter of fact, this complex has been shown to target multiple proteins involved in the regulation of DDR and maintenance of genomic stability [264-266]. Indeed, Vpr exploits this complex to target many DDR proteins for degradation, as has been demonstrated in numerous studies over the years.

## **2.5. Aim of the thesis**

Previous work from the lab had already highlighted the involvement of NE protein SUN2 in the context of infection by HIV-1 and HIV-2 [93]. Considering that it is a structural protein of the nuclear envelope and that the antiviral block occurs at or prior to viral nuclear entry, attention was focused on what could be the possible role of the NE itself, its constitutive elements and nuclear architecture in general in the regulation of HIV infection.

My thesis focused initially on understanding how SUN2, its homolog SUN1 and other related NE proteins regulate HIV infection. The project aimed to compare the SUN-mediated regulation of both HIV-1 and HIV-2 and dissect the requirement of CypA in this process in cell lines and in primary HIV target cells.

Furthermore, it aimed to establish whether there is a link between NE morphology/architecture and HIV infection, by studying the effects of SUN overexpression on the biophysical properties of nuclei and understanding their impact on HIV infection modulation. Indeed, SUN protein upregulation has a strong impact on nuclear shape. We therefore set out to explore whether this morphological phenotype has a mechanical consequence on the nucleus that disrupts the conditions for optimal productive infection.

Over the course of the PhD, results pointed out that the SUN-mediated modulation of infection is mediated by the nucleoplasmic N-termini of the proteins. This brought the focus of the project to the impact SUN proteins may be having on nuclear organization itself, in particular on chromatin mobility and DNA damage.

Considering the role LINC complex plays in maintenance of genome integrity, we set out to examine whether there is a SUN-mediated regulation of chromatin dynamics, that could impact HIV infectivity. Indeed, this turning out to be the case, we further set out to explore which pathways were altered, which steps of HIV infection were impacted and lastly, whether the same results could be recapitulated in primary cells that are natural targets of HIV.

## **MATERIALS AND METHODS**

### 3. Materials and methods

#### 3.1. Constructs

The plasmid constructs for lentiviral expression and HIV infection used in this study are listed in the table below.

*Table 1: Plasmids used in this study*

<b>Plasmid Name</b>	<b>Purpose</b>	<b>Insert/Target/ Components</b>	<b>Reporter/Selection</b>
pTRIP-SFFV-tagBFP-2A	Overexpression	/	BFP
pTRIP-SFFV-TagRFP657-2A	Overexpression	/	RFP-657
pTRIP-SFFV-EGFP	Overexpression	/	GFP
pTRIP-CMV-EGFP-2A	Overexpression	/	GFP
pTRIP-SFFV-tagBFP-2A-SUN1 (Dharmacon)	Overexpression	SUN1	BFP
pTRIP-SFFV-tagBFP-2A-NtSUN2	Overexpression	SUN2	BFP
pTRIP-SFFV-tagBFP-2A-SUN1 Dharmacon (1-298)-ntSUN2 (220-717)	Overexpression	SUN1-SUN2	BFP
pTRIP-SFFV-tagBFP-2A-NtSUN2 (1-219)-SUN1 Dharmacon (299-785)	Overexpression	SUN2-SUN1	BFP
pTRIP-SFFV-TagRFP657-2A-SUN1 Dharmacon	Overexpression	SUN1	RFP-657
pTRIP-SFFV-TagRFP657-2A-ntSUN2	Overexpression	SUN2	RFP-657
pTRIP-SFFV-EGFP-SR-KASH	Overexpression	KASH DN	GFP
pLKO1puro-shLACZ	Knock-down	LacZ	Puromycin
pLKO.1-Puro-LMNA sh2 TRCN0000061835	Knock-down	Lamin A/C	Puromycin
pLKO.1-Puro-LMNB2 sh5 TRCN0000072422	Knock-down	Lamin B2	Puromycin

pCMV-VSVG	Expression	VSV-G	/
psPAX2	Packaging vector	/	/
psPAX2 P86HA	HIVac-1 Packaging vector	/	/
HIVGFP (NL4-3 strain)	Infection	No accessory protein	GFP in Nef
HIVGFP N74D	Infection	No accessory protein	GFP in Nef
HIVGFP env-nef-	Infection	Vif, Vpu, Vpr	GFP in Nef
HIVGFP env- nef- vpr-	Infection	Vif, Vpu	GFP in Nef
HIV-mTagBFP2 (NL4-3 strain)	Infection	No accessory protein	BFP in Nef
pNL4-3 wt	Infection	Full length HIV-1	/
pNL4-3 P207S	Infection	Full length HIV-1	/
HIV-2 ROD9 $\Delta$ env $\Delta$ nef mTagBFP2+ sequenced (MASA)	Infection	Vif, Vpr, Vpx	BFP in Nef
HIV-2 ROD9 $\Delta$ env $\Delta$ nef GFP	Infection	Vif, Vpr, Vpx	GFP in Nef
pSIV3+	Helper plasmid	Vpx	/

### 3.2. Cells

GHOST (GHOST X4R5), 293FT and HeLa cells were cultured in DMEM with Glutamax, 10% fetal bovine serum (FBS) (Corning), and penicillin-streptomycin (Gibco). Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from normal human donors (approved by the Institut National de la Santé et de la Recherche Médicale ethics committee) using Ficoll-Paque PLUS (GE). CD14<sup>+</sup> cells were isolated by a positive selection with anti-human CD14 magnetic beads (Miltenyi) from PBMCs. To obtain macrophages (MDMs), CD14<sup>+</sup> cells were cultured in RPMI with Glutamax, 5% FBS (Eurobio), 5% human serum (Sigma), Penicillin-Streptomycin, Gentamicin (50  $\mu$ g/ml, GIBCO) and HEPES (GIBCO) in the

presence of recombinant human M-CSF (Miltenyi) at 50 ng/ml. Fresh media was added at day 5 or 6, and cells were treated/infected at day 9. CD4<sup>+</sup> T lymphocytes were isolated from CD14<sup>-</sup> fractions or total PBMCs using EasySep™ Human CD4<sup>+</sup> T cell Isolation Kit (Stem Cell) based on negative selection and cultured in X-Vivo medium (Lonza) supplemented with penicillin-streptomycin (Gibco) and 100 U/mL of recombinant human IL-2 (Immunotools). CD4<sup>+</sup> T lymphocytes were activated post isolation using Dynabeads® Human T-Activator CD3/CD28 (Fischer Scientific) added at 1:3 beads to cell ratio. Media was replaced at day 3 and cells were treated/infected at day 5. Drug treatments performed on cultured cells are listed in Table 2.

*Table 2: Drugs used in cell culture in this study*

<b>Name</b>	<b>Description</b>	<b>Cat. Reference</b>	<b>Company</b>	<b>Final Concentration</b>
Cyclosporin A	Inhibits CypA-CA interaction	S2286	Selleckchem	2 $\mu$ M
AZT	RT inhibitor	A2169	Sigma	24 $\mu$ M
NVP	RT inhibitor	SML0097	Sigma	10 $\mu$ M
Etoposide	Topoisomerase II inhibitor	E1383	Sigma	5, 50 or 500 $\mu$ M
Q-VD-Oph	Pan-caspase inhibitor	S7311	Selleckchem	50 $\mu$ M
AZD6738	ATR inhibitor	S7693	Selleckchem	1 $\mu$ M
DMSO	Diluent/Neg Ctrl	BDH1115	VWR Chemicals	adjusted

### **3.3. Virus production**

Viral particles were produced by transfection of 293FT cells in 6-well plates with 3  $\mu$ g DNA and 8  $\mu$ l TransIT®-293 Transfection Reagent (Mirus Bio) per well. For VSV-G pseudotyped SIVmac VLPs, 0.4  $\mu$ g CMV-VSVG and 2.6  $\mu$ g pSIV3<sup>+</sup> was used. For VSV-G pseudotyped HIV-1 and HIV-2 GFP or BFP-reporter viruses, 0.4  $\mu$ g CMV-VSVG and 2.6  $\mu$ g HIV DNA was used. For over-expression or sh-RNA mediated knock-down, 0.4  $\mu$ g CMV-VSVG, 1  $\mu$ g psPAX2 and 1.6  $\mu$ g of lentivector of interest were combined.

One day after transfection, media was removed, cells were washed once, and 3ml per well of RPMI medium with Glutamax, 10% FBS (Gibco), PenStrep (Gibco), 50µg/ml Gentamicin (Gibco) and 0.01M HEPES (Gibco) were added. Viral supernatants were harvested 1 day later, filtered using 0.45 µm pore filters, used fresh or aliquoted and frozen at -80°C. When required, the virus was purified and concentrated on a 20% sucrose cushion in PBS in Ultra Clear Centrifuge tubes (Beckman Coulter), via ultracentrifugation at 4°C at 31,000  $\times$  g in a SW32Ti swinging bucket rotor (Beckman Coulter). Viral pellets were then resuspended in complete medium at a 100-fold concentration compared to crude.

Viral titers were measured on GHOST cells (titration as previously described [267]) or by HIV-1 p24 ELISA (XpressBio). ELISA absorbance acquisitions were acquired on a FLUOstar OPTIMA (BMG Labtech) and data were analyzed and exported to Excel with MARS Data Analysis Software (BMG Labtech).

#### **3.4. Cell Transduction for protein overexpression or knockdown**

HeLa cells were counted and seeded in 6-well plates on the day prior to transduction. Purified virus was added at a 2:1 volume ratio on medium containing protamine at a final concentration of 1 µg/mL. CD14<sup>+</sup> monocytes were seeded in 10-cm dishes and transduced with purified SIVmac VLPs and lentiviruses carrying construct of interest, mixed at a 1:1 ratio. Human serum was added at day 1 post transduction and M-CSF was supplemented to induce differentiation into macrophages. CD4<sup>+</sup> T cells were seeded at 10<sup>5</sup> cells/well in a U-bottom 96-well plate. Purified virus was applied to cells at a 1:1 volume ratio and cells were spinoculated at 25 °C at 1200  $\times$  g for 2hrs.

Transductions of both monocytes and CD4<sup>+</sup> T cells were performed in the presence of protamine at a final concentration of 1 µg/mL.

HeLa and CD4<sup>+</sup> T cells were washed once in PBS and passaged at 48 hours post transduction with or without 2 µg/mL of puromycin. For MDMs, medium was replaced at day 5-6 post transduction. Over-expression was assessed by quantification of fluorescent reporter signal via flow cytometry on a BD FACS Verse Flow Cytometer. Both over-expression and protein knock-down were confirmed by Western Blotting at day of experiment.

### **3.5. Cell transfection of siRNA**

Hela cells were seeded at  $1.5 \times 10^5$  cells/well in a 6 well plate the day prior to the first round of transfection.

For each well, 240 pmol (12  $\mu$ L of 20  $\mu$ M) siRNA were premixed with 200  $\mu$ L Opti-MEM™ Reduced Serum medium (Gibco) and 4  $\mu$ L of Oligofectamine™ (Invitrogen) were premixed with 15  $\mu$ L of  $\mu$ L Opti-MEM™ (Gibco). Both mixes were incubated at room temperature for 5'. The mixes were combined, vortexed and incubated at room temperature for 20'. Finally, 200 $\mu$ L of complexes were added to the well, directly onto complete medium. The same procedure was repeated again, 24 hours later. 48 hours post-transfection, cell pellets were harvested for Western Blot and cells were infected as described above.

siRNA duplex against ATR was ordered from Eurogentec (NM\_hATR\_siRNA: CCU-CCG-UGA-UGU-UGC-UUG-A55; NM\_hATR\_siRNA\_antisense: UCA-AGC-AAC-AUC-ACG-GAG-G55). A negative control siRNA duplex was also provided by Eurogentec.

### **3.6. Cell infection**

HeLa, GHOST, MDMs (day 8-9 post transduction) and CD4<sup>+</sup> T cells (day 4 post-transduction) were seeded and infected in the presence of 1  $\mu$ g/mL of protamine with different dilutions of frozen viral stocks, produced as described above, in a BSL-3 laboratory. Mouse total T cells were infected with pTRIP-backbone based lentivectors, in a BSL-2 facility. Virus was removed at 48 hours post-infection (hpi), cells were washed, harvested, stained for viability or p24 where required, fixed in 1% paraformaldehyde (PFA; Electron Microscopy Sciences) and analyzed for GFP or p24 positivity via flow cytometry on a BD FACS Verse Flow Cytometer. Viral titers (measured as infectious units, i.u./mL) were calculated based on seeded cell number and the percentages of infected cells, within the exponential range of infection.

### **3.7. HIV Real Time qPCR**

HeLa cells and MDMs were infected as described, with the addition of infected wells treated with RT inhibitors as negative control. For this purpose, either 24  $\mu$ M of AZT (Sigma) or 10  $\mu$ M of NVP (Sigma) were used. After 24 hours, cells were washed in PBS and harvested. Total DNA was extracted from cell pellets using NucleoSpin® Tissue (Macherey-Nagel) kit, as per manufacturer's protocol.



Real-time PCR analysis was performed as previously described [79]. Each sample was measured in triplicate for all primers. For beta-globin, primers were bglobin-f and bglobin-r. For HIV-2 Late RT, primers were hiv2-3'U3-fwd and hiv2-psi-rev. For HIV-2 2LTR, primers were hiv2-3'U3-rev and hiv2-R-fwd. For HIV-2 integrated DNA two rounds of amplification were performed. For the first round, primers were alu1 and hiv2-r. For the second round, 1  $\mu$ l of first-round reaction was used as template, and primers were hiv2-f2 and hiv2-r2. For HIV-1 Late RT, primers were hiv1-3'U3-fwd and hiv1-psi-rev2. For HIV-1 2LTR JNCT, primers were Junct4-fwd and Junct2-rev. For HIV-1 integrated DNA two rounds of amplification were performed. For the first round, primers were alu1 and hiv1-psi-rev2. For the second round, 1  $\mu$ l of first-round reaction was used as template, and primers were hiv1-f2 and hiv1-r2. Cycling conditions were 1x 95°C for 5'; 35x 95°C for 10'', 65°C for 20'' (50°C for beta-globin) and 72°C for 30''. Relative concentrations of Late RT, 2LTR and integrated viral DNA were calculated relative to beta-globin using the  $\Delta$ Ct method. The primers used are listed in Table 3.

*Table 3: Primers used for HIV DNA species Real Time Quantitative PCR*

Strain	Amplification	Primer name	Sequence	Annealing
Human	Beta-globin	bglobin-f	CCCTTGGACCCAGAGGTTCT	50°C
Human	Beta-globin	bglobin-r	CGAGCACTTTCTTGCCATGA	50°C
HIV-1	Late RT	hiv1-3'U3-fwd	GCATGGAATGGATGACCCTGAGA	65°C
HIV-1	Late RT	hiv1-psi-rev2	CGTCGAGAGATCTCCTCT GGCTTTA	65°C
HIV-1	2-LTR	Junct4 -fwd	CAGTGTGGAAAATCTCTA GCAGTACTG	65°C
HIV-1	2-LTR	hiv1-psi-rev2	CGTCGAGAGATCTCCTCT GGCTTTA	65°C
HIV-1	Integrated DNA round 1	alu1	GCCTCCCAAAGTGCT GGGATTACAG	65°C

HIV-1	Integrated DNA round 1	hiv1- psi-rev2	CGTCGAGAGATCTCCTCT GGCTTTA	65°C
HIV-1	Integrated DNA round 2	hiv1-f2	CTGGGAGCTCTCTGGCTAACTA	65°C
HIV-1	Integrated DNA round 2	hiv1-r2	AACAGACGGGCACACACTACTT	65°C
HIV-2	Late RT	hiv2- 3'U3- fwd	GAAGGGATGTTTT ACCATTTAGTTA	65°C
HIV-2	Late RT	hiv2- psi-rev	GTTCCAAGACTTCTCAGTCTTCTTC	65°C
HIV-2	2-LTR	hiv2-R- fwd	GTTCTCTCCAGCACTAGCAGGTA	65°C
HIV-2	2-LTR	hiv2- 3'U3- rev	TAATAAATGGTA AAACATCCCTTC	65°C
HIV-2	Integrated DNA round 1	alu1	GCCTCCCAAAGTGCT GGGATTACAG	65°C
HIV-2	Integrated DNA round 1	hiv2-r1	AAGGGTCCTAACAGACCAGGGTCT	65°C
HIV-2	Integrated DNA round 2	hiv2-f2	GCAGGTAGAGCCTGGGTGTTC	65°C
HIV-2	Integrated DNA round 2	hiv2-r2	CAGGCGGCGACTAGGAGAGAT	65°C

### 3.8. Western Blotting

0.5 to 1 million cells were lysed in 100 µL of RIPA buffer (50mM Tris HCl, 150mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP-40, Protease inhibitor (Roche; 1187358001)). Lysis was performed on ice for 30'. Lysates were cleared by centrifugation at 8000 g for 8 minutes at 4°C, 20 µl of Laemmli 6x (12% SDS, 30% Glycerol, 0.375M Tris-HCl pH6.8, 30% 2-mercaptoethanol, 1% bromophenol blue) was added and samples were boiled at 95°C for 15'. Cellular protein lysates were resolved on Criterion or 4%–20% Biorad precast SDS-PAGE gels and transferred to PVDF membranes (BioRad). Membranes were saturated and proteins were

blotted with antibodies in 5% non-fat dry milk, PBS 0.1% Tween buffer. ECL signal was recorded on the ChemiDoc-XRS or ChemiDoc Touch Biorad Imager. Data was analyzed and quantified with the Image Lab software (Biorad). The antibodies used in this study are listed in Table 4.

### **3.9. Confocal Imaging**

For live imaging, HeLa cell lines were plated either in a glass bottom FuoroDish (World Precision Instruments) or in a glass-bottom Cellview™ Cell Culture Dish with 4 compartments (Greiner Bio-One), on the day prior to experiments. One hour prior to imaging, cells were incubated with 1 μM of SiR-DNA (Tebu Bio), directly in the culture medium, at 37°C. Cells were imaged with a Leica Dmi8 inverted microscope equipped with an SP8 confocal unit using a 20x objective and an additional zoom of 2x. Imaging was performed in an on-stage incubator chamber at 37°C, with 5% CO<sub>2</sub>. An image per field, per condition, was taken every 2 minutes. Particle Image Velocimetry analysis was performed on SiR-DNA staining over the first ten time-frames, using the appropriate plug-in in Image J, with the help of the unit's image analysis expert Mathieu Maurin. PIV is a basic optic flow analysis [268], that divides each image of a stack in small clusters of pixels (interrogation windows) and measures the displacement of each cluster between pairs of consecutive frames. The cross-correlation then generates a pattern of “movements” within the nucleus that are color-coded based on the amplitude of the vector corresponding to the displacement of each cluster. Red shades indicated higher amplitudes of displacement while violets correspond to quasi-immobile clusters. The plug-in generates a matrix for each nucleus for every time frame and gives an average value per nucleus. This value is used as a readout for chromatin mobility within each nucleus.

For immunofluorescence, HeLa cells were grown overnight onto 12 mm glass coverslips (Thermo Scientific) placed in 6-well plates. Cells were fixed with 4% PFA for 20 minutes at room temperature. Coverslips were washed multiple times with PBS and quenched with 0.1M Glycine (Life Technologies) for 10 minutes at room temperature. Coverslips were then blocked with PBS, 0.2% (w/v) BSA (Euromedex), 0.05% (w/v) Saponin from quillaja bark (SIGMA) for 30 minutes at RT. Cells were stained overnight with anti-NUP153 antibody (Sigma) at 2 μg/mL (1:50 dilution) or with Normal Rabbit IgG Isotype Control (Thermo Scientific) at corresponding concentration of the primary antibody, in PBS, 0.2% (w/v) BSA, 0.05% (w/v) Saponin + 10% goat serum (Sigma), at 4°C in a humidified chamber. The following day, cells were washed multiple times and incubated with the secondary antibody Alexa Fluor® 546 goat

anti-rabbit IgG (H+L) (Invitrogen; 1:200 dilution in PBS-BSA-Saponin) in the presence of 1  $\mu$ M of SiR-DNA for 2 hours in the dark, at room temperature. Coverslips were washed multiple times in PBS-BSA-Saponin and finally rinsed once in distilled water to remove any trace of salt. Coverslips were mounted onto glass slides using Fluoromount G (eBioscience) mounting medium. The slides were finally dried at 37°C for 1h and stored at 4°C. Cells were imaged with a Leica DmI8 inverted microscope equipped with an SP8 confocal unit using a 63x objective with applied Type F Immersion Liquid (Leica).

### **3.10. Fluorescence Recovery After Photobleaching**

HeLa cell lines were first transduced to express ubiquitous and constitutive GFP. They were then transduced to overexpress either the control vector, SUN1 or SUN2. Cells were seeded at  $2.5 \times 10^5$  cells/dish in a glass bottom FuoroDish (World Precision Instruments) on the day prior to the experiment. Cells were imaged with a Leica DmI8 inverted microscope equipped with an SP8 confocal unit using a 20x objective. Imaging was performed in an on-stage incubator chamber at 37°C, with 5% CO<sub>2</sub>. Two independent modules were used in a sequential manner: one for bleaching, one for imaging the signal recovery. During the application of the bleaching module, the 488 laser was focused at an intensity of 5% and with a gain of 0.1% on to an area within the nucleus of each cell at maximum zoom for 20 seconds. Immediately afterwards, the first sequence was manually cancelled, the resolution was optimized, zoom was restored to 5x for the second sequence. The laser power was thus set for optimal imaging level and images of the whole cell were acquired for 3 min ca at the rate of one image every 2.2 seconds.

### **3.11. Intracellular and intranuclear Staining for Flow Cytometry**

Cell surface staining was performed in PBS, 1% BSA (Euromedex), 1mM EDTA (GIBCO), 0.01% NaN<sub>3</sub> (AMRESCO) (FACS Buffer) at 4°C. Viability staining (Live-Dead) with Fixable Viability Dye eFluor® 780 was performed in PBS at 4°C. Intracellular p24 staining was carried out as follows: 48 hours after infection, cells were extensively washed with PBS, harvested and fixed/stained using the BD Cytoperm/Cytofix™ kit, as per manufacturer's protocol, using the anti-HIV-1 core antibody clone KC57 coupled to Rhodamine. Cells were resuspended in FACS Buffer prior to final acquisition. Intranuclear staining of  $\gamma$ H2AX was performed using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience™) as per manufacturer's protocol. Cells were resuspended in FACS Buffer prior to final acquisition.

All flow cytometry acquisitions were performed on the FACS Verse (BD) using the FACS Suite software (BD) and analyzed on FlowJo v10. The antibodies used are listed in Table 4.

*Table 4: Antibodies used in this study for Western Blot, Confocal Imaging and Flow Cytometry in cell lines and primary human cells*

<b>Antibody target</b>	<b>Cat. Reference</b>	<b>Company</b>	<b>Application</b>
Actin	MAB1501 Clone C4	Sigma	WB (1:5000)
Vinculin	V9264	Sigma	WB (1:5000)
SUN1	ab124770	Abcam	WB (1:1000)
SUN2	HPA001209	Atlas antibodies	WB (1:1000)
SUN2	ABT272	Millipore	WB (1:1000)
Lamin A/C	SAB4200236	Sigma	WB (1:1000)
Lamin B2	ab8983 clone LN43	Abcam	WB (1:1000)
NUP153	HPA027896	Sigma	WB (1:1000); IF (1:50)
SiR-DNA staining	SC007	Tebu Bio	Live imaging (1 $\mu$ M)
HIV-1 p24	6604667 Clone KC57	Beckman Coulter	Coupled to RD1; FACS (1:50)
H2AX p-S139	562377 Clone N1-431	BD	Coupled to PE; FACS (1:100)
Rabbit-IgG (H+L)	A-11010	Invitrogen	Alexa Fluor® 546 for IF (1:200)
Rabbit-IgG	7074S	Ozyme	Conjugated to HRP for WB (1:10000)
Mouse IgG	7076S	Ozyme	Conjugated to HRP for WB (1:10000)

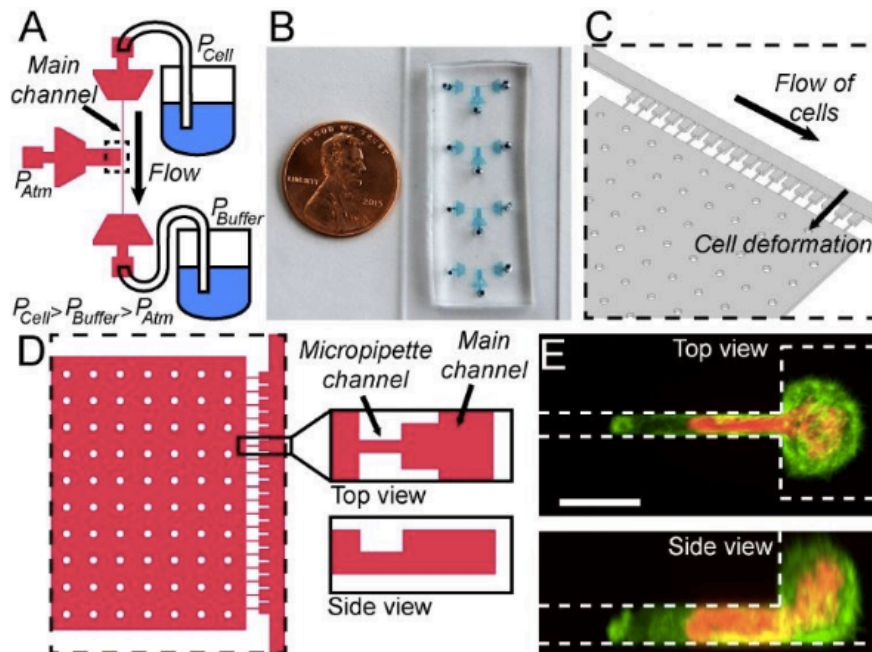
### **3.12. Electron Microscopy**

HeLa cells overexpressing either Ctrl, SUN1 or SUN2 were seeded at 5 x 10<sup>4</sup> cells/well in a 24w plate onto sterile 12 mm glass coverslips (Thermo Scientific) and left to adhere overnight.

The following morning, cells were washed in PBS and were fixed using 2 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1h, post fixed for 1h with 2% buffered osmium tetroxide, dehydrated in a graded series of ethanol solution, and then embedded in epoxy resin. Images were acquired with a digital camera Quemesa (SIS) mounted on a Tecnai Spirit transmission electron microscope (FEI Company) operated at 80kV. The experiment was performed by the Unit EM expert Mabel San Roman.

### 3.13. Micropipette aspiration microscopy

Prior to harvest, HeLa cell lines were incubated with  $1\mu\text{M}$  SiR-DNA dye from Tebu Bio for 1h30' at  $37^\circ\text{C}$  in cell culture medium. Cells were then washed, harvested and resuspended at a concentration of  $5 \times 10^6$  cells/mL in sterile 3% BSA in PBS-0.2% FBS. Cells were subjected to the experimental conditions described in [269] and shown in *Figure 8* and imaged in parallel at the Nikon Imaging Center at Institut Curie, with the assistance of Patricia Davidson.



*Figure 8: Overview of the micropipette devices. (A) Different pressures applied to the three ports. The dashed rectangle indicates the region shown as close-up in panels (C) and (D). (B) Photograph of the actual device. (C) and (D) Schematics showing direction of cell flow in main channel and micropipette constrictions that lead to cell deformation. (E) Representative image of a cell with histones labeled in red and cytoplasmic actin in green, going through the microchannel. Adopted from [269].*

### **3.14. Microarray Gene Expression (Affymetrix®)**

Total RNA was extracted from  $10^6$  HeLa cells using NucleoSpin® RNA and adjusted to 50ng/ $\mu$ L. A WT Plus amplification and labeling protocol was conducted with 100ng of total RNA. Samples passed the quality control with a high score.

The Affymetrix analysis was performed by the NGS platform at Institut Curie using the Human Gene 2.0 ST chip. Human Gene 2.0ST array were scanned using a Genechip 7G scanner, according to the supplier's protocol.

Micro-array analyses were processed with R using packages from Bioconductor. The quality control was performed using ArrayQualityMetrics package without detecting any outlier among the experiment. Data was normalized using the Robust Multi-Array Average algorithm from the Oligo package. Annotation of the probes was done using the hugene20 annotation data (chip hugene20sttranscriptcluster) from Bioconductor. Differential gene-expression analysis was performed with Limma. The analysis was performed by Nicolas Manel.

### **3.15. Mouse data**

Genotypes of the mice used in this study are listed in Table 5. The experiments were performed in collaboration with Nilushi De Silva.

Spleens and lymph nodes (inguinal and brachial) were dissected and mashed on a 40  $\mu$ m cell strainer with FACS buffer under sterile conditions. Red blood cell (RBC) lysis was performed using the RBC Lysis Buffer (Ozyme). T cell enrichment was performed using the Pan T Cell Isolation Kit II, mouse (Miltenyi) as per manufacturer's protocol.

T cell stimulation and infection strategy was adapted from [270]. Briefly, cells were stimulated post-enrichment, by plating on anti-CD3 (BD) coated plates with addition of anti-CD28 (BD) for 24 hours. Cells were then re-plated in the presence of anti-CD3, anti-CD28, 100U/mL of recombinant human Interleukin-2 (Immunotools) and 1  $\mu$ g/mL of protamine. Cells were infected as described above. 48 hours post-infection, cells were washed, stained for viability using Fixable Viability Dye eFluor® 780 and for surface markers as per the following panel: CD25-PE, CD69-PerCP-Cy5.5, CD4-PE-Cy7, CD8-APC, TCR- $\beta$ -APC-Cy7, all by BD (1:200). Cells were resuspended in FACS Buffer and fixed in 1% PFA prior to acquisition.

*Table 5: Description of mice used for generation of mouse T-cell data in this study*

<b>Mouse</b>	<b>Genotype</b>	<b>Number of mice used</b>	<b>Comments</b>
Wild-type control	SUN1 <sup>WT/WT</sup>	3	/
Wild-type control	SUN2 <sup>WT/WT</sup>	3	/
Wild-type control	Different line	2	Non-littermate control
SUN1 KO	SUN1 <sup>TM1a/TM1a</sup>	4	Full knock-out
SUN2 KO	SUN2 <sup>KO/KO</sup>	3	Full knock-out
SUN1/SUN2 double KO	SUN1 <sup>TM1c/TM1c</sup> SUN2 <sup>KO/KO</sup>	4	SUN1 conditional knock-out (CD4-Cre <sup>+/-</sup> ) SUN2 full knock-out

### 3.16. Statistical Analysis

Statistical analyses were performed in Prism 7 or 8 (GraphPad Software) as indicated in the figure legends.



## **RESULTS**

## 4. Results

### 4.1. Main Story

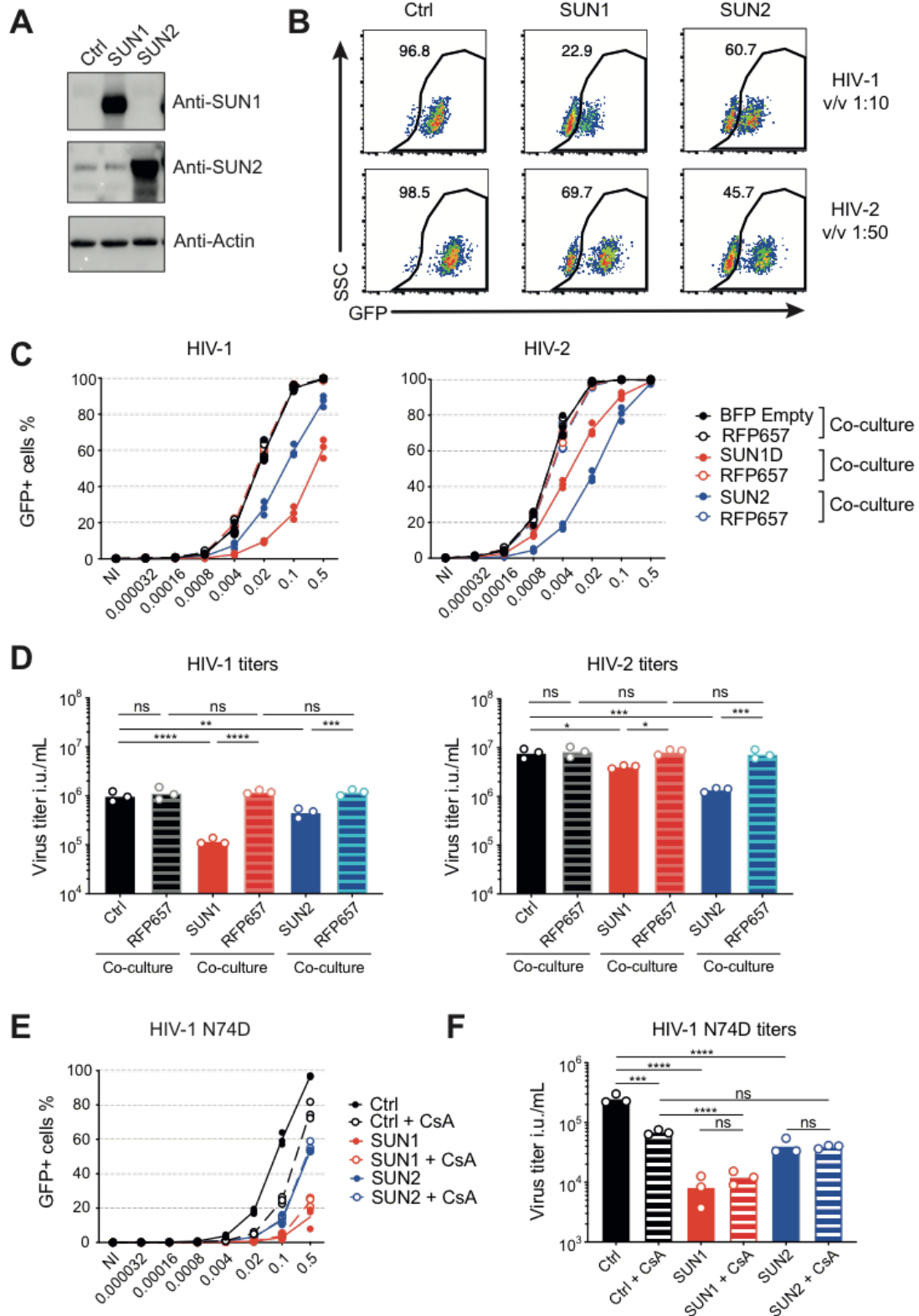
The lab had previously published a study reporting the anti-viral activity of SUN2 in HIV infection [93]. We therefore asked ourselves whether SUN2's homolog SUN1 plays a similar role. We generated HeLa cell lines overexpressing full length human SUN1 and SUN2 coupled to a BFP reporter that is expressed in a stoichiometric 1:1 ratio with the protein of interest. Transduction efficiency (based on BFP signal observed by flow-cytometry, not shown) was 100% and protein overexpression was strong, as assessed by Western Blot (Figure 1A). The cell lines were infected in single round with multiple dose-dilutions of VSV-G pseudotyped HIV-1 (NL4-3  $\Delta$ vif $\Delta$ vpr $\Delta$ vpu $\Delta$ env $\Delta$ nef) and HIV-2 (Rod9  $\Delta$ env $\Delta$ nef), both expressing a GFP reporter in the place of nef. Productive infection was assessed 48 hours post infection (hpi) by flow cytometry using GFP<sup>+</sup> cells as a readout. Results showed that overexpression of both SUN1 and SUN2 in HeLa cells leads to reduced infectivity by HIV-1 and HIV-2 compared to control cells at any given dose, as can be observed in the representative FACS plot showing GFP signal within BFP<sup>+</sup> cells (Figure 1B).

The above-mentioned cell lines were then co-cultured with control HeLa cells, expressing the fluorescent reporter RFP657, at a 50:50 ratio. Upon infection by HIV-1 and HIV-2 it was observed that the antiviral activity of SUN1 and SUN2 was restricted to the BFP<sup>+</sup> cell subpopulation and did not apply to the co-cultured RFP657<sup>+</sup> cells (Figure 1C shows the infection curves at multiple dilutions of viral input and figure 1D shows the computed titers based on percentage of GFP<sup>+</sup> cells), proving that the antiviral effect is intrinsic to cells actually overexpressing SUN1 or SUN2 and that these cells do not have a restrictive activity *in trans*.

One interesting observation that caught our attention since the beginning was the fact that the SUN protein-mediated antiviral activities showed a strain preference: SUN1 overexpression was more antiviral towards HIV-1 while SUN2 overexpression was more efficient at restricting HIV-2.

It had previously been shown that the effects of SUN2 overexpression are not additive to the antiviral effect of inhibiting the interaction between the CA of HIV-1 and Cyclophilin A [93]. We therefore asked whether SUN1 overexpression led to a similar phenotype. We infected our SUN-overexpressing HeLa cell lines with HIV-1 strains harboring either a WT CA or the CA mutant N74D. This mutant was chosen due to the fact that in HeLa cells, WT HIV-1 is resistant to inhibition of CypA-CA interaction, while the mutant N74D is susceptible to it [113]. Indeed, our results confirmed that inhibiting the interaction between CypA and HIV-1 using

Cyclosporine A leads to reduced infection by HIV-1 N74D compared to untreated. However, there is no further reduction of infection levels in cells overexpressing either SUN1 or SUN2 (Figures 1E and 1F). This lack of an additive effect suggests a functional overlap between SUN proteins and the role of CypA in HIV-1 infection.

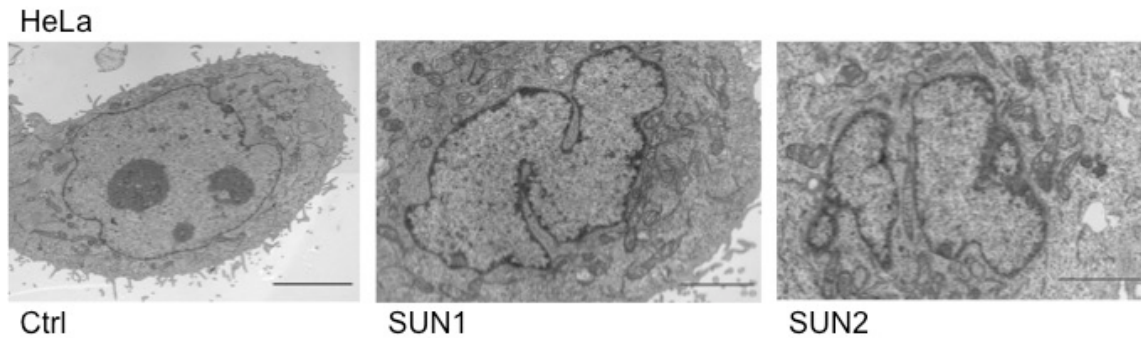


**Figure 1**

**Figure 1: Overexpression of SUN1 and SUN2 inhibits HIV infection in HeLa**

- (A) Western blot against SUN1 and SUN2 confirming overexpression in HeLa cells, transduced with lentivectors encoding expression of indicated proteins. Actin was used as housekeeping control.
- (B) GFP expression within the BFP-expressing subpopulation of transduced HeLa cells, 48 hours after infection with indicated v:v dilution of HIV-1 and HIV-2, encoding GFP in the place of Nef and pseudotyped with VSV-G (representative FACS data from one experiment at a given dose of virus).
- (C) BFP-2A Ctrl, BFP-2A-SUN1 and BFP-2A-SUN2 expressing HeLa cells were co-cultured at a 50:50 ratio with HeLa cells expressing RFP657-2A and infected with multiple dilutions of HIV-1 and HIV-2 (v:v ratios are indicated on the X axis). Percentage of GFP<sup>+</sup> cells 48hrs post infection at different viral dilutions within the BFP<sup>+</sup> or co-cultured RFP657<sup>+</sup> subpopulations are shown as infection curves (n=3 independent experiments).
- (D) Viral titers based on percentages of GFP<sup>+</sup> cells in given subpopulations (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean).
- (E) Percentage of GFP<sup>+</sup> HeLa cells expressing Ctrl, SUN1 or SUN2, 48 hrs after infection with different dilutions of HIV-1 or HIV-1 N74D capsid mutant, encoding GFP in the place of Nef, pseudotyped with VSV-G, with or without treatment with 2μM of CsA (n=3 independent experiments).
- (F) Viral titers in given cell lines with or without CsA treatment (n=3, paired RM ANOVA one-way test on LOG-transformed titers, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean)

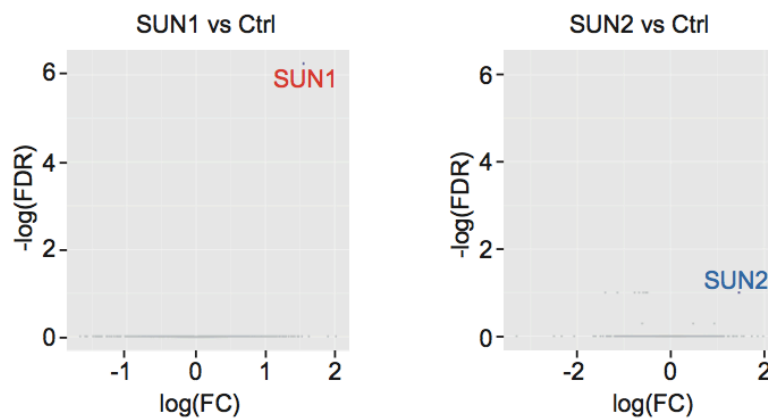
Though overexpression of either SUN1 and SUN2 in HeLa cells did not impact viability and/or proliferation rate, it did bring along profound morphological changes at the level of the NE. The nuclei of these cells appeared to be more deformed by confocal imaging (data shown in Figure 3) and the nuclear envelope appeared a lot more lobulated and full of invaginations, as can be seen in the representative images of electron microscopy shown in Figure S1.1.



**Figure S1.1: SUN1 and SUN2 overexpression deforms nuclei in HeLa**

Representative Electron Microscopy (EM) images showing largely deformed nuclei in control, SUN1 and SUN2 overexpressing HeLa cells (scale: 10, 2 and 5  $\mu\text{m}$  respectively).

A transcriptomic profiling analysis (Affymetrix®) was also performed on HeLa cells expressing either SUN1 or SUN2. One would expect that the overexpression of a structural protein and the profound morphological changes it leads to, would correlate to changes in the gene expression profile of these cells. Surprisingly enough, no genes were found to be either up or down-regulated upon the overexpression of SUN proteins (Figure S1.2). Only SUN1 and SUN2 themselves came up as significantly upregulated hits, serving as positive controls. This strongly suggested that the nature of the effects caused by SUN overexpression in HeLa are either mechanical, epigenetic or both.

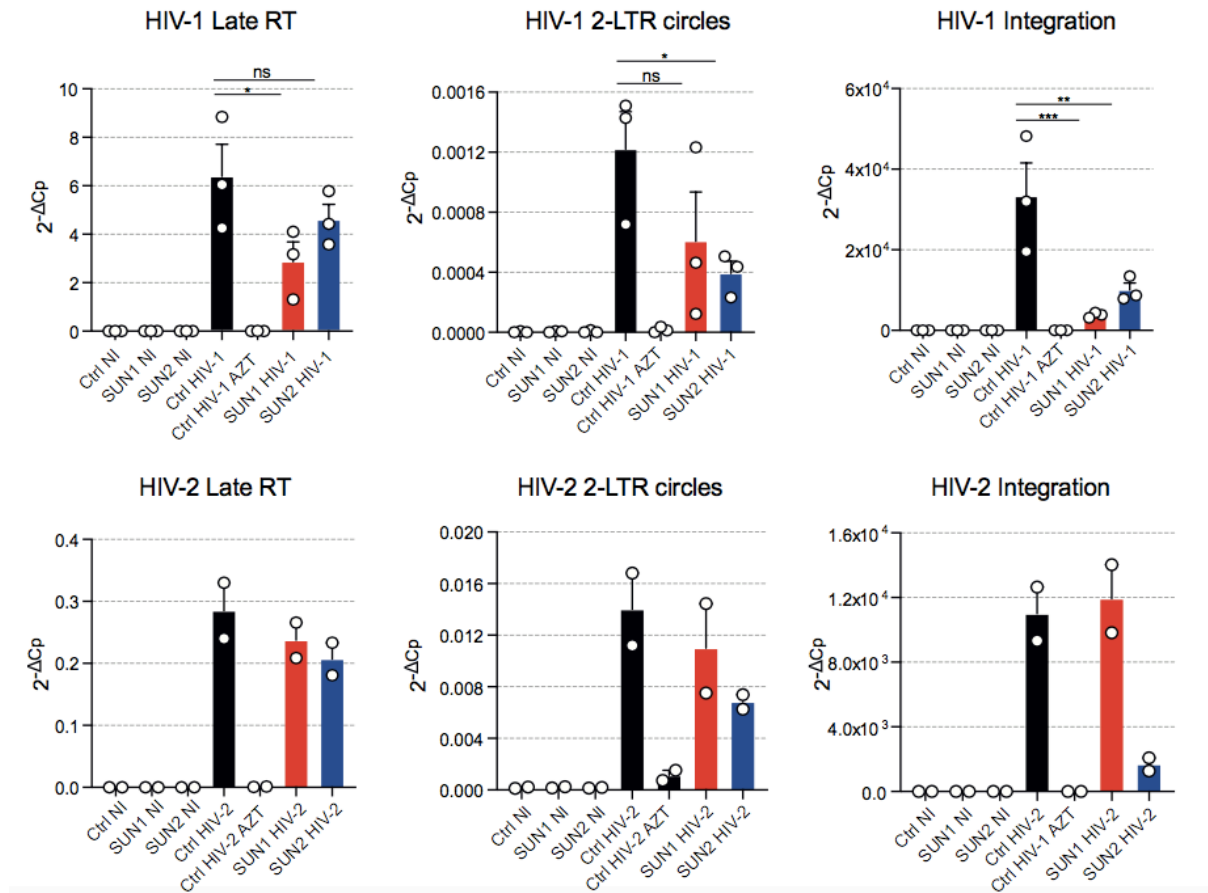


**Figure S1.2**

**Figure S1.2: Gene expression analysis reveals no changes upon SUN overexpression**

Gene expression analysis (Affymetrix® using Human Gene 2.0 ST chip) was performed on Ctrl, SUN1 or SUN2 overexpressing HeLa cells in triplicate. Left panel shows expression changes in SUN1 overexpressing cells vs Ctrl cells while right panel compares SUN2 overexpression to Ctrl (FC stands for fold change; FDR is False Discovery Rate).

In order to understand which step of the viral replicative cycle is impacted by overexpression of SUN1 and SUN2 in HeLa cells, quantitative real time PCR of viral DNA species was performed on total DNA extracted from HeLa cells infected for 24 hours with either HIV-1 (at a v:v dilution of 0.1) or HIV-2 (0.02 viral dilution). Infection was assessed in parallel at 48hpi by flow cytometry within each individual experiment, confirming the antiviral activities of either SUN1 or SUN2 overexpression (data not shown). 24  $\mu$ M AZT treatment was included as a negative control. Results partially confirmed data from previous studies [127] showing that overexpression of SUN2 leads to a block of nuclear import of both HIV-1 and HIV-2, as shown by the reduced number of 2-LTR circles (Figure S1.3). As expected, this led to a subsequent reduction of levels of integrated viral DNA. The situation for SUN1 overexpression was however, much less clear. Though previous studies have reported a nuclear import block of HIV-1 infection caused by SUN1 overexpression [128, 129], our data doesn't necessarily recapitulate this result. Though a slight reduction of 2-LTR circle formation was observed for both HIV-1 and HIV-2, it was not enough to explain the antiviral activity of SUN1, especially against HIV-2. Furthermore, a significant reduction of Reverse Transcription was also observed for HIV-1 but not HIV-2. Finally, a striking decrease in SUN1 mediated viral DNA integration was observed, but only for HIV-1. Overall, results suggested a more complex role for SUN1 in regards to HIV-1 infection than a simple block of nuclear entry. As for HIV-2, the antiviral activity of SUN1 may be occurring downstream, at the level of viral expression.

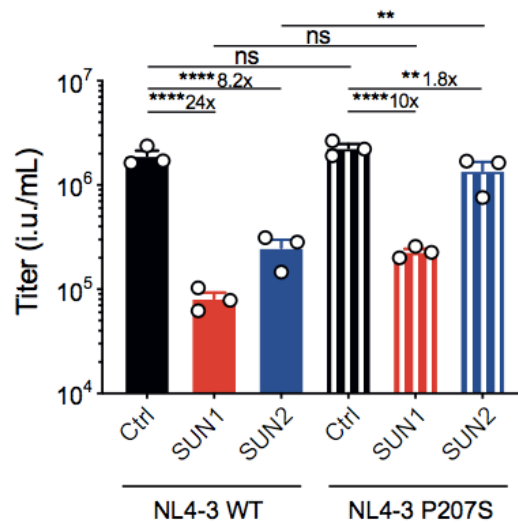


**Figure S1.3: SUN1 and SUN2 restrict HIV-1 and 2 at different steps of the viral life cycle**

Real Time qPCR performed on viral DNA species at 24hpi on total DNA extracted from HeLa cell lines, infected with a given dose of either HIV-1 (0.1 v:v) or HIV-2 (0.02 v:v). Treatment with 24  $\mu$ M of reverse transcriptase inhibitor AZT was used as negative control. n=3 for HIV-1, paired RM ANOVA one-way test, \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; ns, not statistically significant; line at mean  $\pm$  SEM. n=2 for HIV-2.

Donahue *et al.* showed that the CA mutant P207S, a previously characterized escape mutant of MX2 inhibition [130], was also resistant to the antiviral effect of SUN2 overexpression [127]. We asked whether the P207S mutation renders HIV-1 insensitive also towards SUN1 overexpression. SUN1 and SUN2 overexpressing HeLa cells were infected with either WT or P207S CA harboring full-length, VSV-G pseudotyped, NL4-3 viral strains. Analysis of p24<sup>+</sup> cells showed that infection by the P207S mutant was indeed rescued even in the presence of SUN2 overexpression. This was not the case for SUN1: even though there was a slight increase in P207S infection levels compared to WT, this was not statistically significant and the fold-reduction compared to control cells was still high (Figure S1.4). These results further

strengthened the notion that the antiviral activities of SUN1 vs SUN2 may be implemented via different mechanisms.



#### **Figure S1.4: Absence of rescue of SUN-1's antiviral activity by the CA mutant P207S**

HeLa cell lines were infected with different dilutions of full length HIV-1 NL4-3 WT or P207S, pseudotyped with VSV-G. The figure shows viral titers based on percentages of p24<sup>+</sup> cells obtained via KC57-FITC antibody staining at 48hpi (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean ± SEM).

We wanted to validate the antiviral activities of SUN1 and SUN2 overexpression in primary cells that are physiologically relevant for HIV infection. We decided to look at monocyte-derived macrophages, in which the nucleus and the LINC complex are possibly under the same kind of tension as HeLa cells, simplistically considering the two are both adherent cell types. We transduced monocytes isolated from peripheral blood with the lentivectors expressing SUN1 and SUN2. Transduction was performed in the presence of viral-like particles (VLPs), containing the Vpx protein from SIVmac, in order to target the restriction factor SAMHD1 and optimize transduction. After 9 days of differentiation into macrophages, overexpression of the proteins of interest was assessed by Western Blot (representative blot from one donor shown in Figure 2A) and cells were infected with VSV-G pseudotyped HIV-1 and HIV-2. Results showed that SUN1 and SUN2 overexpression led to reduction of HIV-1 and HIV-2 infection levels also in MDMs (Figure 2B, pooled data from 9 donors). Furthermore, the preferential antiviral activities towards HIV-1 or HIV-2 by the two homologs were maintained in this cell type. At the same time, we also confirmed the functional overlap between SUN proteins and

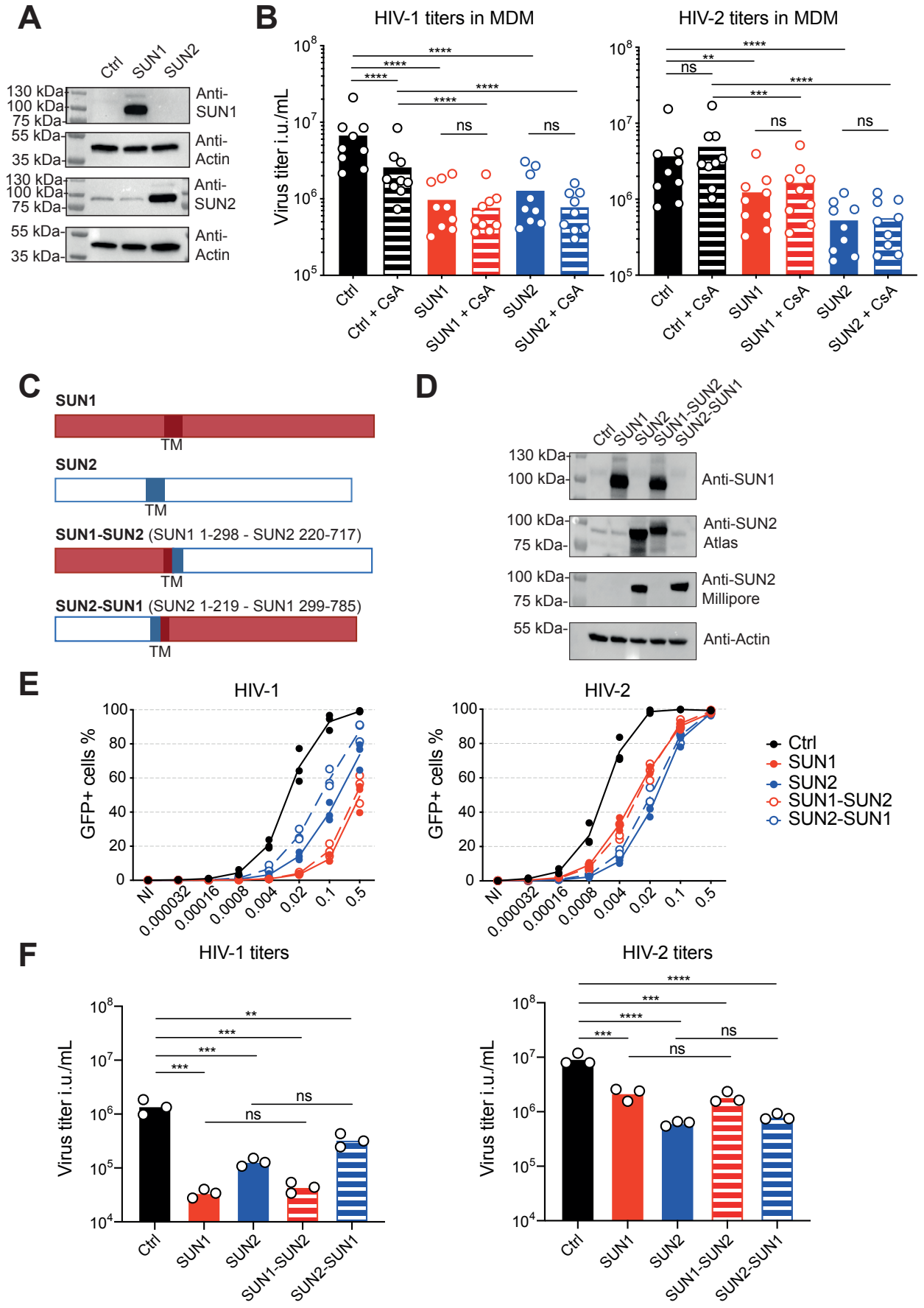


CypA requirement by adding CsA and observing no further reduction of HIV-1 infection levels in cells overexpressing SUN1 or SUN2, as opposed to controls. These results recapitulated what was observed in HeLa, this time with an HIV-1 strain harboring a WT capsid. As expected, titers of HIV-2 were not impacted by the drug.

Real Time qPCR on viral DNA species performed on total DNA extracted from infected macrophages confirmed what we observed in HeLa cells (Figure S2.1). SUN2 overexpression led to a block in infection by HIV-1 and HIV-2 at the level of nuclear import, while the overexpression of SUN1 showed a more diverse profile. While it blocked HIV-2 at the nuclear entry step, it restricted HIV-1 at the level of RT but mostly at the step of viral integration.

No significant differences were detected between untreated vs CsA treated conditions in SUN1 and SUN2 overexpressing macrophages.

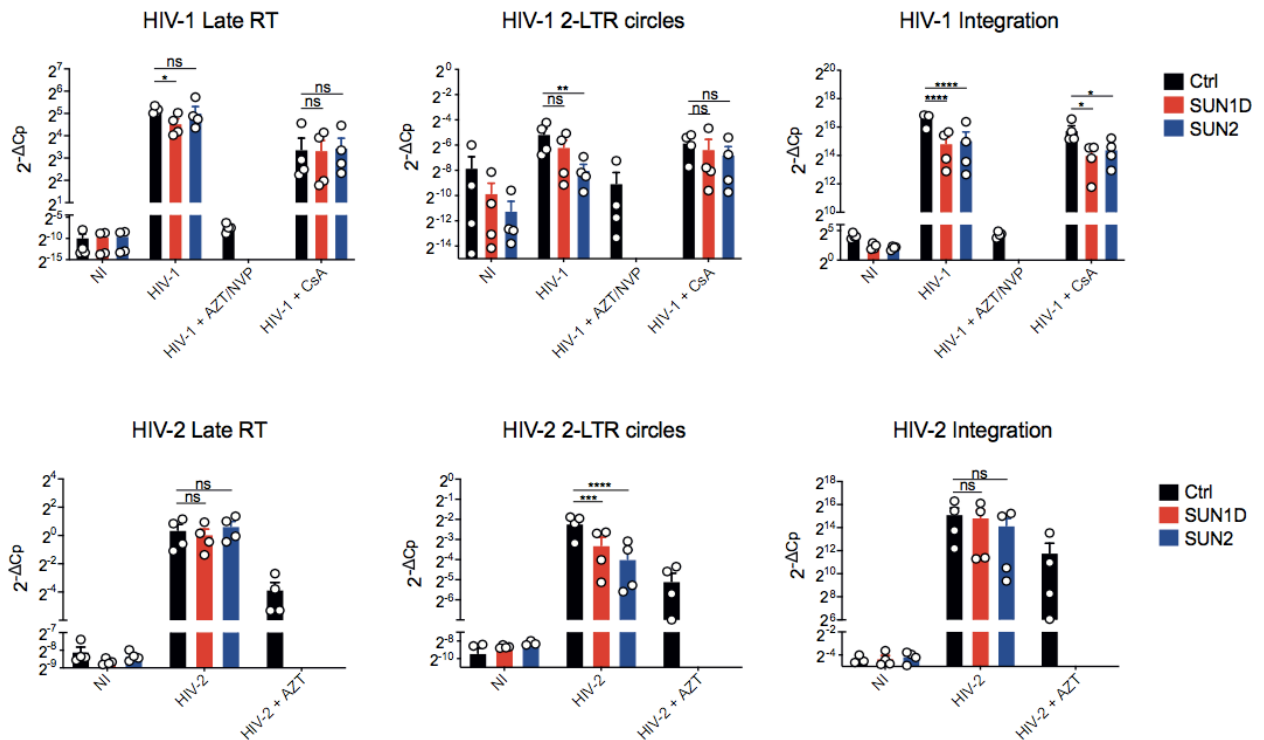
Profoundly intrigued by the preferential profiles shown by SUN1 and SUN2, we decided to generate chimeric proteins between SUN1 and SUN2. Using a PCR-based cloning strategy, we generated hybrid proteins harboring the N-terminus from one homolog and the C-terminus from the other, with the domain swap occurring at the level of the transmembrane region (see Figure 2C for schematics). We confirmed the overexpression of the chimeric proteins in HeLa, at expected size, by Western Blot (Figure 2D) and proceeded to infect with HIV-1 and HIV-2. Results showed us that the preferential antiviral activities of the proteins are driven by their nucleoplasmic N-terminal domains. Indeed, the chimera SUN1-SUN2 followed the trend of full-length SUN1 while the chimera SUN2-SUN1 showed a profile closer to that of full-length SUN2. This result confirmed previous studies mapping the antiviral activity of the two NE proteins to their N-terminus and further shed light on the potential differences there may be between how SUN1 restricts infection, compared to SUN2. These differences may indeed be linked to how SUN1 and SUN2 differentially interact with the underlying nucleus, via their N-termini.



**Figure 2**

**Figure 2: SUN overexpression is antiviral in monocyte-derived macrophages. The antiviral activity maps to the N-ter of SUN proteins in HeLa**

- (A) Western Blot against SUN1 and SUN2 confirming overexpression in transduced primary Monocyte-derived macrophages. Actin was used as housekeeping control (blot of one representative donor).
- (B) Viral titers based on percentages of GFP<sup>+</sup> macrophages 48 hours after infection with different dilutions of HIV-1 or HIV-2 encoding GFP in Nef and pseudotyped with VSV-G, with or without 2 $\mu$ M CsA (pooled data from 9 donors, paired RM ANOVA one-way test on LOG-transformed titers, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean)
- (C) Schematic representation of chimeric proteins obtained by PCR cloning of fragments from full-length SUN1 (red) and SUN2 (blue). Aminoacid residues retained in hybrid proteins are indicated within brackets.
- (D) Western Blot against SUN1 and SUN2 confirming proper size and overexpression of chimeric proteins in HeLa cells. Two different antibodies targeting SUN2 recognizing two different epitopes within distinct parts of the protein were used. Actin was used as housekeeping control.
- (E) Percentage of GFP<sup>+</sup> HeLa cells expressing Ctrl, SUN1, SUN2, SUN1-SUN2 or SUN2-SUN1, 48 hrs after infection with different dilutions of HIV-1 or HIV-2, encoding GFP in the place of Nef, pseudotyped with VSV-G (n=3 independent experiments, v:v ratios are indicated on the X axis).
- (F) Viral titers based on percentages of GFP<sup>+</sup> cells in given cell lines (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean).



**Figure S2.1: SUN1 and SUN2 restrict HIV-1 and HIV-2 at different steps of the viral life cycle in primary MDMs**

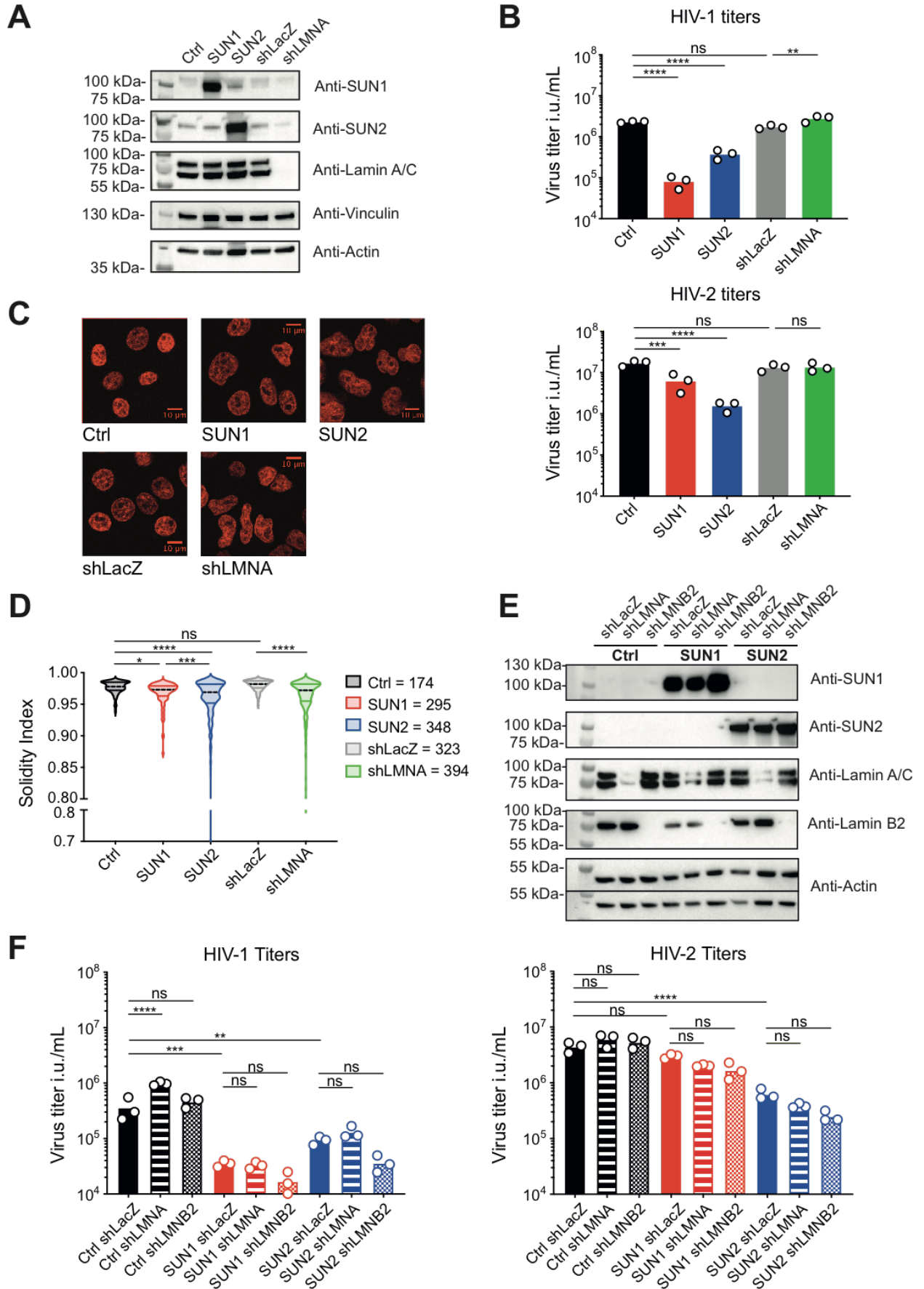
Real Time qPCR performed on viral DNA species at 24hpi on total DNA extracted from transduced monocyte-derived macrophages expressing either the Ctrl vector, SUN1 or SUN2, infected with a given dose (0.17 v:v dilution ratio) of either HIV-1 or HIV-2. Treatment with 24  $\mu$ M of AZT combined with 10  $\mu$ M of NVP was used as negative control in the case of HIV-1 infection. AZT alone was used for HIV-2. (n=4 donors, paired RM ANOVA one-way test, \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ; ns, not statistically significant; line at mean  $\pm$  SEM).

Underlying the Nuclear Envelope, lies a fibrous meshwork of structural proteins known as the nuclear lamina. As described in the first section of this manuscript, SUN proteins are known to physically interact with the lamins via their N-termini. Lamins confer rigidity and structure to the nucleus and guarantee its overall spherical architecture. Loss of nuclear lamina *in vitro* significantly weakens the nucleus and makes it more prone to damage and rupture. Loss of Lamin A/C in particular, alters the morphology of the nucleus, making it more plastic and deformable [271, 272].

Overexpression of SUN1 and SUN2 leads to nuclear deformation just as lamin A/C depletion does. Wondering whether the changes in nuclear shape are the reason behind the suboptimal

HIV infection induced by SUN overexpression, we included lamin A/C depletion in our panel of HeLa cell lines. Efficient depletion was obtained via shRNA mediated knock-down and was confirmed by Western Blot (Figure 3A). Upon infection with HIV-1 and HIV-2, it was observed that not only did lamin A/C knock-down not lead to decreased infection, on the contrary, it seemed to boost infection by HIV-1 (Figure 3B). This result seems to suggest that endogenous lamin A/C may actually be restrictive towards HIV-1 infection. We analyzed the nuclear shape across the five cell lines by performing confocal microscopy on the cells, with SiR-DNA stained chromatin (Figure 3C). The observations confirmed the presence of deformed nuclei in both SUN overexpressing cells and those with depleted lamin A/C (a quantification of deformation can be seen in Figure 3D). Based on these results, it can be concluded that nuclear deformation alone is not sufficient to inhibit infection by HIV-1 or HIV-2.

Considering the evidence for an antiviral role of endogenous lamin A/C, it was necessary to understand whether lamins are required for the antiviral activities of SUN1 and SUN2. We therefore co-transduced HeLa cells in order to have combined lamin-depleted and SUN-overexpressing cell lines. We knocked-down lamin A/C, lamin B2 but failed to obtain viable cells with depleted lamin B1. Efficient overexpression and protein knock-down were confirmed by Western Blot (Figure 3E). Upon infection by HIV-1 and HIV-2, it became clear, first of all, that infection by HIV-1 is enhanced by knock-down of lamin A/C and not lamin B2 (Figure 3F). Interestingly, it also became apparent that endogenous lamins A/C and B2 are not required for the antiviral activities of SUN overexpression, considering that loss of lamins, especially lamin A/C, was not able to rescue infection by HIV-1 and HIV-2 in SUN1 or SUN2 overexpressing HeLa cells.



**Figure 3**

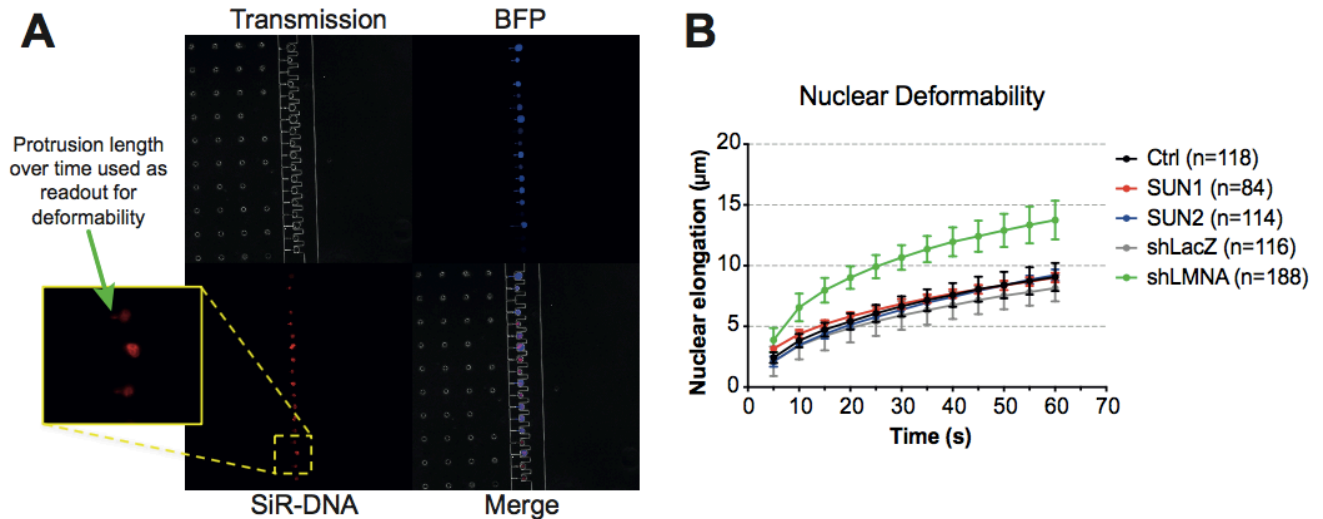
**Figure 3: SUN1 and SUN2 overexpression deforms nuclei in HeLa but shape doesn't correlate directly to reduced infection. The anti-viral activities of SUN1 and SUN2 are independent of endogenous lamins.**

- (A) Western Blot against SUN1 and SUN2 confirming overexpression and against Lamin A/C confirming specific protein knock-down, in HeLa cells. Actin and vinculin were used as housekeeping controls.
- (B) HIV-1 and HIV-2 viral titers based on percentages of GFP<sup>+</sup> cells in given cell lines (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean).
- (C) Representative images of nuclei of indicated HeLa cells lines obtained via live confocal imaging of DNA staining using SiR-DNA dye. Images show signal from an individual, central plane, scale bar is at 10  $\mu$ m.
- (D) Quantification of object solidity using shape descriptor analysis by Image J as a measurement of nuclear deformation (combined results from 2 independent experiments, legend indicates total number of nuclei analyzed per cell line, unpaired ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at median).
- (E) Western Blot against SUN1 and SUN2 confirming overexpression and against Lamin A/C and Lamin B2 confirming specific protein knock-down, in HeLa cells. Actin was used as housekeeping control.
- (F) HIV-1 and HIV-2 viral titers based on percentages of GFP<sup>+</sup> cells in given cell lines (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean).

Intrigued by the deformed nuclei, we wanted to better characterize the intrinsic nature of these morphological changes. Considering that both SUN overexpression and Lamin A/C knockdown lead to nuclear deformations yet have opposite activities on HIV-1 infection, we asked ourselves whether the deformations are linked to differential effects on nuclear envelope rigidity. Indeed, one could assume that a depleted lamina meshwork may render the nuclei softer, while overcrowding of the NE by SUN proteins may have the opposite effect, thus explaining the opposite effects on infection. In order to characterize nuclear rigidity or deformability, we resorted to a microfluidics device and protocol developed by Patricia Davidson and described in [269]. This protocol combines a multiplex micropipette aspiration approach with an automated image analysis platform, allowing robust and reproducible measurement of the viscoelastic properties of the nucleus in time (see section 3.13 for schematic overview). In a nutshell, SiR DNA-stained cells in suspension are forced to go through constricted channels at a given pressure and are imaged live using confocal microscopy. The nucleus is the limiting organelle during this migration and its progress through the channel can be tracked by measuring the length of the SiR-DNA “protrusion” or “tail” within the microchannel over time (Figure S3.1A shows an example field of Ctrl BFP<sup>+</sup> HeLa cells, with SiR-DNA stained chromatin, going through the microchannels). This protrusion length is then used as a readout for nuclear deformability.

By performing the experiment on our HeLa cell lines from figure 3A (with the help of Patricia Davidson herself), we showed that while Lamin A/C depletion indeed renders the nuclei more deformable, SUN1 or SUN2 overexpression doesn't alter nuclear rigidity within cells, compared to Ctrl (Figure S3.1B). Therefore, it can be concluded that the SUN overexpression-mediated block of HIV infection is not linked to an increased rigidity of the nuclear envelope.



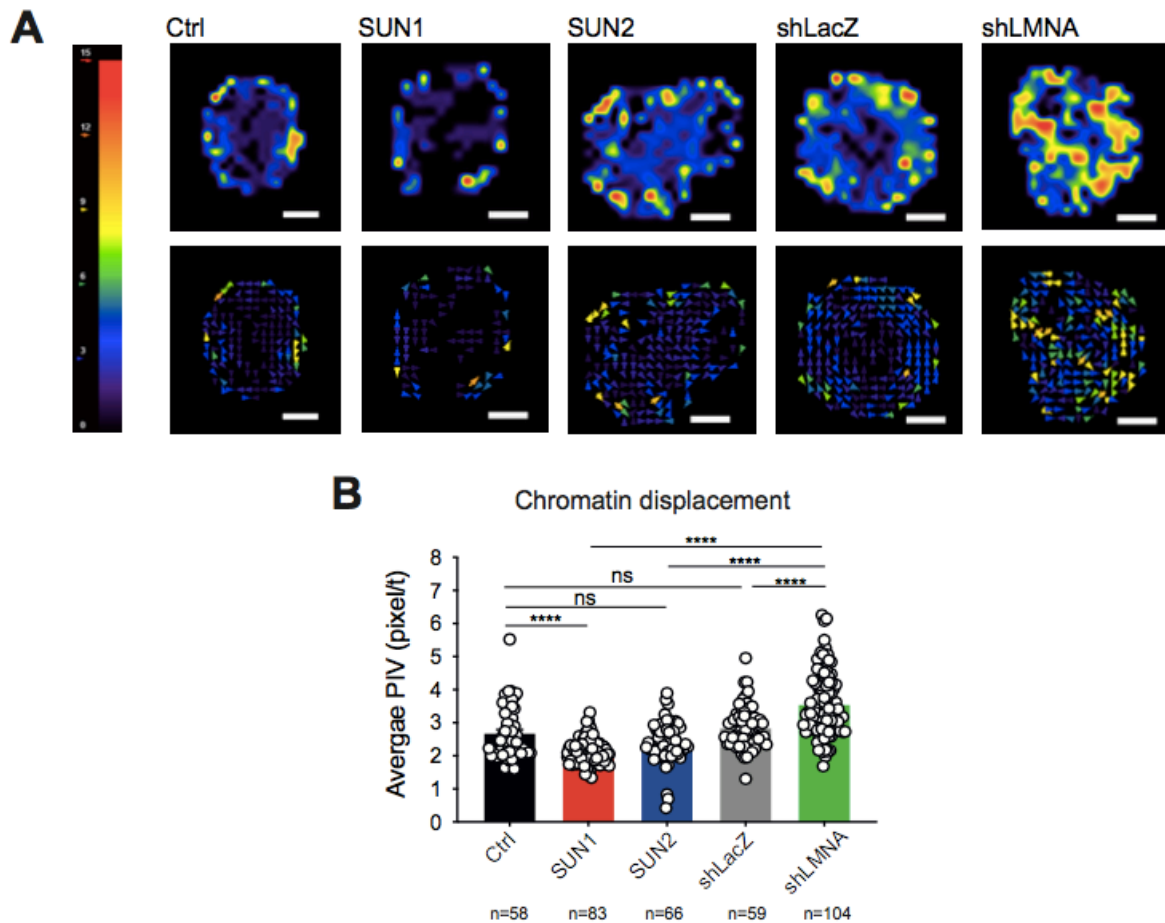


**Figure S3.2: Microchannel experiment reveals that SUN overexpression doesn't alter nuclear rigidity in HeLa**

- (A) Representative image of a field showing BFP+ Ctrl HeLa cells going through  $3 \times 3 \mu\text{m}$  microchannels under applied pressure. Nuclear deformation can be observed as the elongation of SiR-DNA staining (bottom left) within the channels itself. The green arrow indicates the protrusion that is measured over time as a readout for nuclear deformability.
- (B) Quantification of nuclear deformability across indicated HeLa cell lines over time (the number of nuclei measured per cell line is indicated within brackets, n=3 independent experiments).

We next took our analysis of nuclear morphology a little step further. Thus far, we have only made the observation that at a given point in time, nuclei within our cell lines appear deformed. We next asked ourselves how the nuclei behave dynamically over a given length of time. We resorted to live imaging of SiR-DNA stained cells in order to answer this question. DNA staining in live cells allowed us to monitor the overall shape of the nucleus and whether this changed in time. However, we were also able to characterize another intriguing property: chromatin mobility or DNA displacement within the nucleus.

It became apparent immediately that DNA in SUN overexpressing nuclei didn't behave the same way as in nuclei with reduced lamin A/C. Lamin A/C KD nuclei appeared “floppier” and their DNA content seemed to “flow” within the nucleus at a high rate. On the contrary, nuclei overexpressing SUN1 or SUN2 seemed stuck in their shapes and their chromatin appeared more immobile. In order to quantify these differences, we set up an automated analysis system on Image J based on the calculation of Particle Image Velocimetry (PIV) which allowed us to track the displacement of clusters of pixels based on their intensities between one time frame and another. Displacements can be visualized via a color code based on their amplitudes. Immobile patches of the nucleus thus appear in blues and violets while greater mobility is indicated by the orange-red end of the spectrum (Figure 4A, color scale on the left). As can be observed in the representative images in Figure 4A, a Lamin A/C depleted nucleus displayed higher intensities of chromatin displacement over time while SUN-overexpressing nuclei were more skewed towards the blues. Quantification results of PIV confirmed that chromatin within lamin A/C depleted nuclei was significantly more mobile than in the shLacZ control (Figure 4B). On the other hand, SUN1 (but not SUN2) overexpression seemed to “lock down” chromatin in its place, by reducing its overall mobility, compared to control nuclei. SUN2-overexpressing nuclei, though more deformed than both control and SUN1 nuclei, did not show this phenotype of reduced chromatin mobility.



**Figure 4: Particle Image Velocimetry reveals that SUN1 overexpression leads to reduced chromatin mobility**

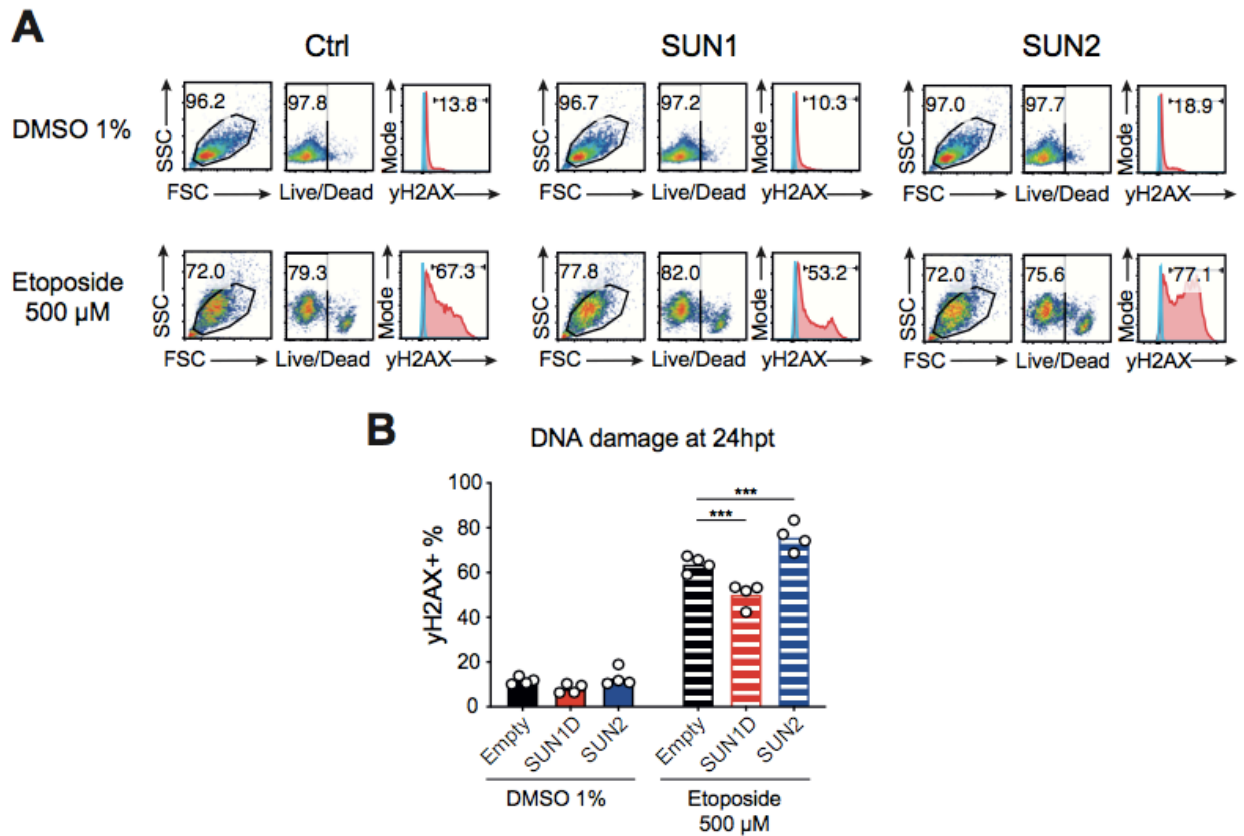
(A) Representative images for Particle Image Velocimetry (PIV) analysis (Image J) on individual nuclei from given HeLa cell lines. DNA was stained by SiR-DNA and imaged live by confocal microscopy; top images show overall flow per time frame per nucleus, bottom images show individual vectorial displacements per given time frame. Scale bar corresponds to 5  $\mu\text{m}$ . Reference color scale for pixel displacement per time frame is shown on left.

(B) Quantification of pixel displacement in individual nuclei by PIV measured over 20 minutes of live confocal imaging of SiR-DNA staining of given cell lines (combined results from 2 independent experiments, unpaired ANOVA one-way test, \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; ns, not statistically significant; line at mean).

We were stunned by the observation of such a direct impact of SUN1 overexpression on the chromatin itself. We therefore wondered whether SUN1 overexpression could be affecting DNA damage and altering patterns of DDR, especially considering the active role it plays in these pathways (see section 2.4.3 of this manuscript). In order to understand whether DDR was being impacted, we used staining of phosphorylated H2AX at position Ser139 ( $\gamma$ H2AX) as a readout for initiation of DDR. As for damage induction itself, we resorted to treating the cells overnight with a high dose of etoposide, an inhibitor of topoisomerase II known to cause DSBs due to tension induced by DNA supercoiling [227]. The experiment was performed on Ctrl, SUN1 and SUN2 overexpressing HeLa cells, that were treated for 24h with either 500  $\mu$ M etoposide or with corresponding DMSO control. Cells were harvested and stained at 24hpt with a viability marker and for intra-nuclear  $\gamma$ H2AX and analyzed by flow cytometry.

Results confirmed massive upregulation of  $\gamma$ H2AX signal in cells treated with etoposide (Figure 5A shows representative FACS plots for viability and  $\gamma$ H2AX staining from one experiment). Intriguingly, SUN1 vs SUN2 overexpression gave us differential trends once again: SUN1 overexpression led to an overall reduced percentage of  $\gamma$ H2AX<sup>+</sup> cells compared to Ctrl while SUN2 overexpression seemingly led to an increase in this population (representative results are shown in Figure 5A while quantification can be found in Figure 5B). Consistent shifts in fluorescence intensity were also observed (data not shown).

Overall, these results suggest that SUN1 and SUN2 overexpression may interfere with DDR pathways in manners that may be independent of each other. One can be tempted to conclude that SUN1 overexpression is somehow protecting the cells from DNA damage, perhaps via limitation of chromatin mobility, while SUN2 overexpression is enhancing it due to reasons unknown. It must be, however, kept in mind that  $\gamma$ H2AX is not a marker of DNA damage *per se* but rather of initiation of the repair response. We are therefore simplifying a bit by referring to it as a readout of “DNA damage”, the actual effect is probably more intricate.



**Figure 5: SUN1 and SUN2 overexpression show opposite profiles in H2AX phosphorylation upon exogenous DNA damage induction**

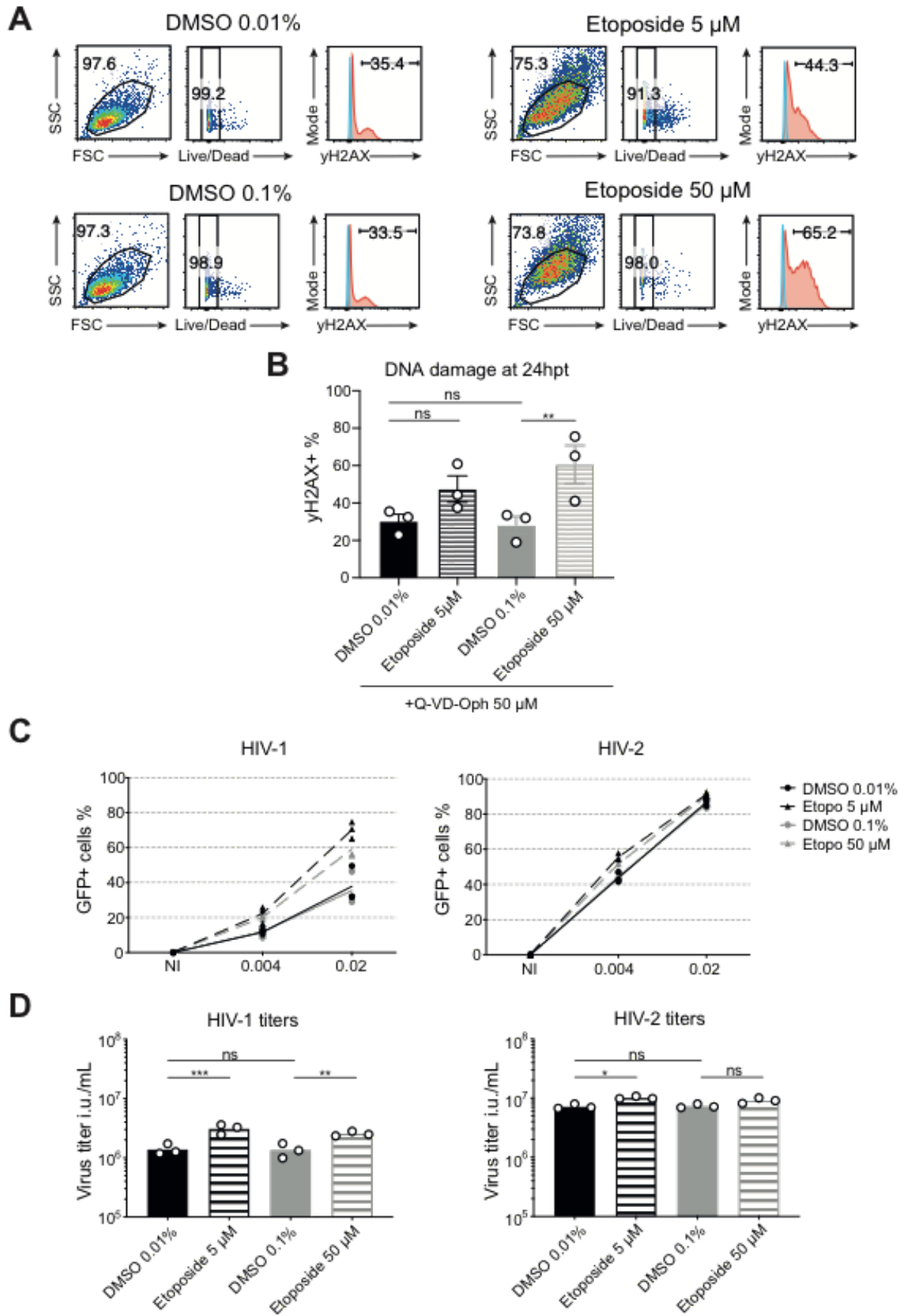
(A) Representative FACS plots from one experiment showing  $\gamma$ H2AX intra-nuclear staining in given HeLa cell lines, 24h post treatment with either 500  $\mu$ M of etoposide or 1% DMSO as control. Viability was assessed via incorporation of Fixable Viability Dye eFluor® 780 and gating on the negative population.

(B) Quantification of  $\gamma$ H2AX<sup>+</sup> cells across cell lines and conditions (n=4, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean).

These results immediately made us wonder whether the SUN-mediated effects on HIV are perhaps in part mediated by the interplay of these proteins with the DDR pathways. After all, the modulatory effects on HIV infection have indeed been mapped to the nucleoplasmic N-termini of SUN proteins, known to be interacting with DNA and chromatin across various physiological processes.

To explore this, we initially asked whether the exogenous induction of DNA damage has an impact on infection by HIV-1 or HIV-2 in our system. We continued the use of etoposide to induce DNA damage. However, etoposide is an irreversible inhibitor and the induced DNA damage leads to cell death by apoptosis. In order to treat the cells, infect them and keep them alive for 48 hours to be able to measure the infected GFP<sup>+</sup> cells, we opted for a shorter treatment with lower doses of etoposide and had to include the pan-caspase inhibitor Q-VD-Oph to inhibit apoptosis [273]. The experiment was initially performed in non-transduced HeLa cells. Cells were treated with either 5 or 50  $\mu$ M of etoposide and corresponding DMSO controls and concomitantly infected with two doses of HIV-1 or HIV-2. Both the drug and the virus were washed off at 4h post treatment/infection. Induction of  $\gamma$ H2AX was measured at 24hpt and occurred at both doses of etoposide treatment, in a dose-dependent manner (Figures 6A and 6B). Infection results showed that DNA damage induction via etoposide treatment led to significantly increased infection by HIV-1 in HeLa cells (Figures 6C and 6D). This was not the case for HIV-2 whose infectivity was only slightly impacted by etoposide-induced DNA damage.

This result provided us with yet another correlative element: DNA damage increases HIV-1 infection, SUN1 overexpression is strongly antiviral towards HIV-1 (much more than HIV-2) and SUN1 overexpression seems to be limiting the formation of  $\gamma$ H2AX foci, thus interfering with DDR. We therefore speculate that SUN1 overexpression could be interfering with the proviral role of DNA damage in HIV-1 infection. For this reason, we decide to focus only on HIV-1 from this point onwards.



**Figure 6**

**Figure 6: Exogenous DNA damage induction leads to increased HIV-1 infection**

- (A) Representative FACS plots from one experiment showing  $\gamma$ H2AX intra-nuclear staining in HeLa cells, 24h post treatment with 5/50  $\mu$ M of etoposide or 0.01/0.1% DMSO as control. Viability was assessed via incorporation of Fixable Viability Dye eFluor® 780 and gating on the negative population.
- (B) Quantification of  $\gamma$ H2AX<sup>+</sup> cells across conditions (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean  $\pm$  SEM).
- (C) Percentage of GFP<sup>+</sup> HeLa cells 48 hrs after infection with different dilutions of HIV-1 or HIV-2, encoding GFP in the place of Nef, pseudotyped with VSV-G (n=3 independent experiments, v:v ratios are indicated on the X axis).
- (D) Viral titers based on percentages of GFP<sup>+</sup> cells (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean).



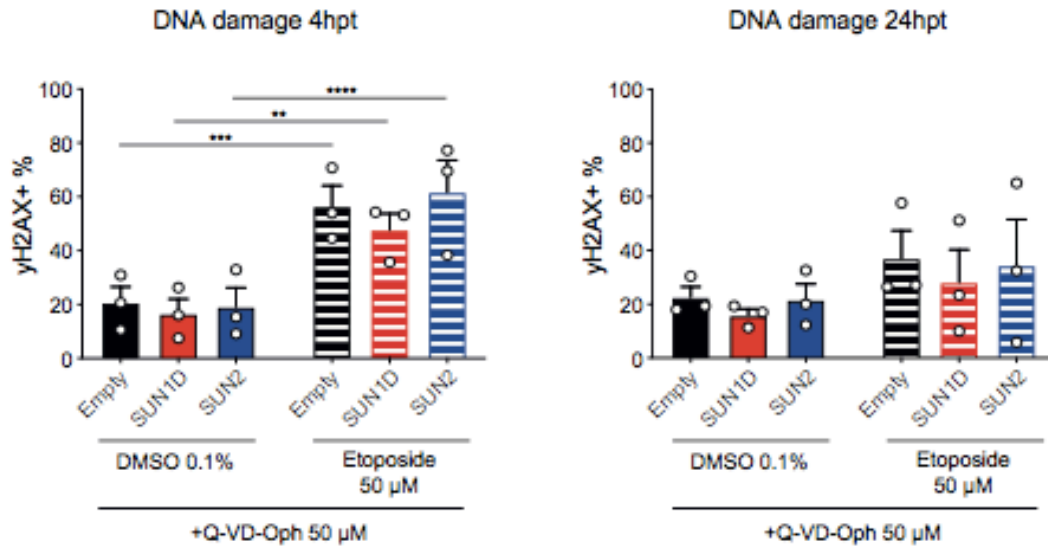
The key question at this point is understanding how SUN proteins, DNA damage and HIV-1 infection overlap with each other.

We therefore repeated the infection experiment, in the presence of etoposide treatment, on HeLa cell lines overexpressing SUN1, SUN2 or the empty vector as control. We measured  $\gamma$ H2AX signal, this time both at 4 and at 24 hours post treatment with 50  $\mu$ M etoposide and confirmed an ongoing DNA damage response at the time of infection (Figure 7A). Also in this case, the pan-caspase inhibitor Q-VD-Oph was added to cell culture media throughout the experiment. Furthermore, this time we decided to cross-compare two different HIV-1 strains: one containing and one lacking the accessory protein Vpr. This was done in light of the highly documented role Vpr plays in DNA damage, as described in section 2.4.4. In addition, Vpr has been reported to localize at the NE and induce herniations in the lipid bilayer [250], an aspect that could clearly be impacted in the context of NE protein manipulation. Lastly, Vpr has been shown to directly bind chromatin and induce structural changes that putatively lead to the activation of the DDR pathway [254]. For all the reasons mentioned above, we decided to investigate whether the effects we observe are dependent on the presence of Vpr. HIV-1 WT and HIV-1 Vpr- were produced and purified by ultracentrifugation on sucrose cushion to allow proper quantification of p24 by ELISA. This allowed us to infect the cells with p24 normalized, and therefore directly comparable, doses of the two viral strains.

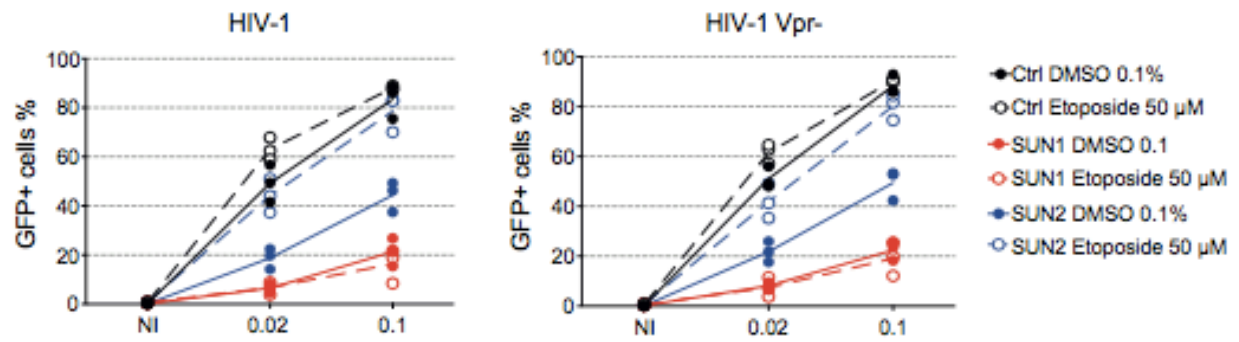
We confirmed that the overexpression of SUN1 and SUN2 was antiviral regardless of the presence or absence of Vpr (Figure 7A and 7B; we had previously observed this while comparing WT and P207S CA harboring full length HIV-1 strains, that contained all accessory proteins, data shown in Figure S1.4).

The striking result that was obtained from this experiment was that the etoposide-induced DNA damage managed to fully rescue infection in SUN2 overexpressing cells, in a manner that was independent of Vpr. This wasn't the case for SUN1: DNA damage failed to induce an increase in HIV-1 infectivity in these cells, suggesting that the effect of SUN1 overexpression is downstream of DNA damage. This result confirms in a more definitive manner that SUN1 and SUN2 regulate infection by HIV-1, at least to some extent, via distinct, non-redundant mechanisms.

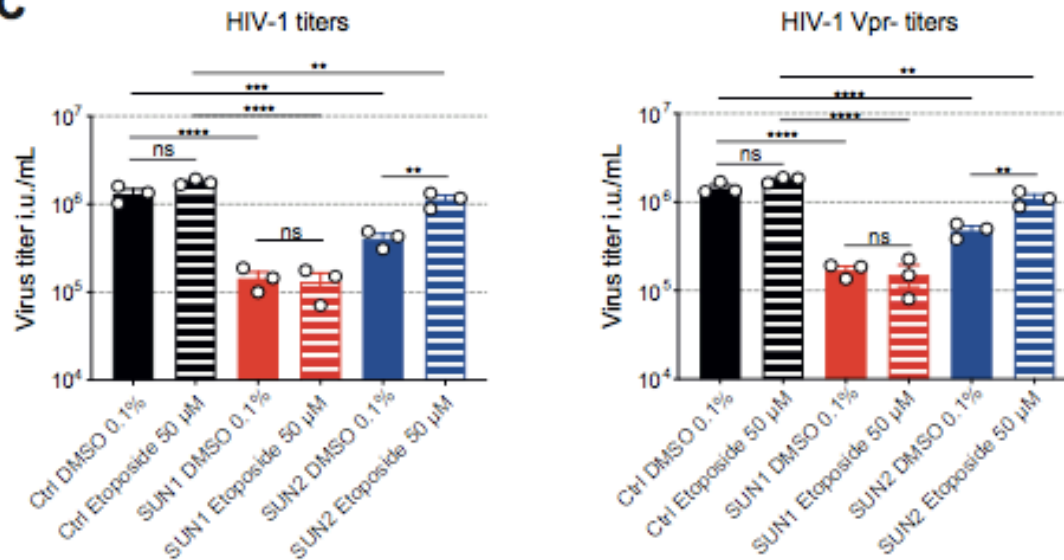
**A**



**B**



**C**

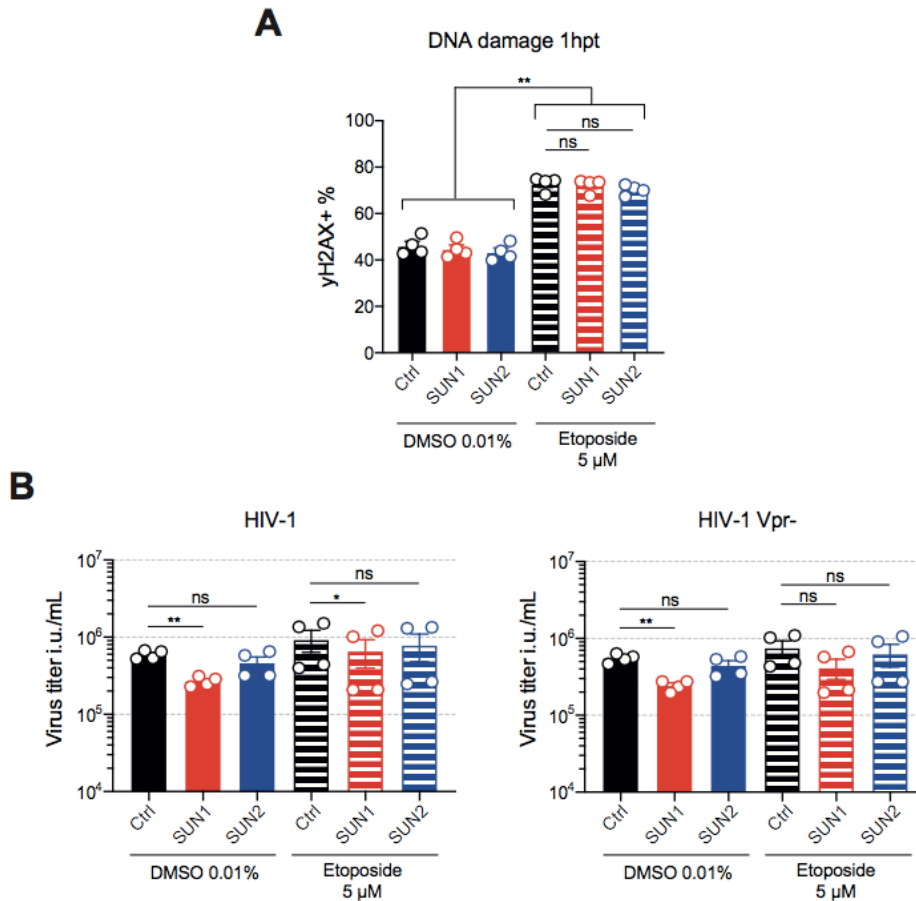


**Figure 7**

**Figure 7: The antiviral activity of SUN2 but not SUN1 is full rescued upon exogenous DNA damage induction**

- (A) Quantification of  $\gamma$ H2AX<sup>+</sup> cells across cell lines and conditions at 4 (left) and 24 (right) hpt (treatment duration: 4 hours; n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*p < 0.001, \*\*\*p < 0.0001; line at mean  $\pm$  SEM).
- (B) Percentage of GFP<sup>+</sup> HeLa cells 48 hrs after infection with p24-normalized dilutions of purified HIV-1 env-nef- or HIV-1 env-nef-vpr-, encoding GFP in the place of Nef, pseudotyped with VSV-G (n=3 independent experiments, v:v ratios are indicated on the X axis).
- (C) Viral titers based on percentages of GFP<sup>+</sup> cells (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*p < 0.001, \*\*\*p < 0.0001; ns, not statistically significant; line at mean  $\pm$  SEM).

To verify whether the results obtained in HeLa were also recapitulated in primary target cells of HIV-1, we transduced activated human CD4<sup>+</sup> T cells, isolated from peripheral blood, with lentivectors for overexpression of SUN1 and SUN2 (overexpression was confirmed by BFP expression via FACS, data not shown). Primary T cells were much more sensitive to etoposide treatment compared to cell lines so the dose was reduced to 5 $\mu$ M and treatment was carried out for only 1h prior to infection. Intranuclear staining at 1hpt confirmed induction of  $\gamma$ H2AX upon etoposide treatment (Figure 8A). However, no differences could be detected between SUN overexpressing cells compared to control cells in this system. Infection results confirmed the Vpr-independent antiviral activities of SUN1 and SUN2 overexpression (Figure 8B; although SUN2 overexpression didn't reach statistical significance with this method of titer computation, a dose-dependent trend was definitely observed). Interestingly, the induction of DNA damage by etoposide treatment didn't show any effect on HIV-1 infectivity. It must be kept in mind that these are highly metabolic and very actively cycling cells. Indeed, they show moderate levels of constitutive DNA damage at steady state, putatively due to replication stress. An etoposide-based damage induction may not be the best way to address the role of DDR in HIV-1 infection in this cell type, as shall be further discussed in Section 5.

**Figure 8**

**Figure 8: HIV-1 infection is independent of DNA damage induction by etoposide treatment in primary CD4<sup>+</sup> T cells but is sensitive to SUN1 overexpression**

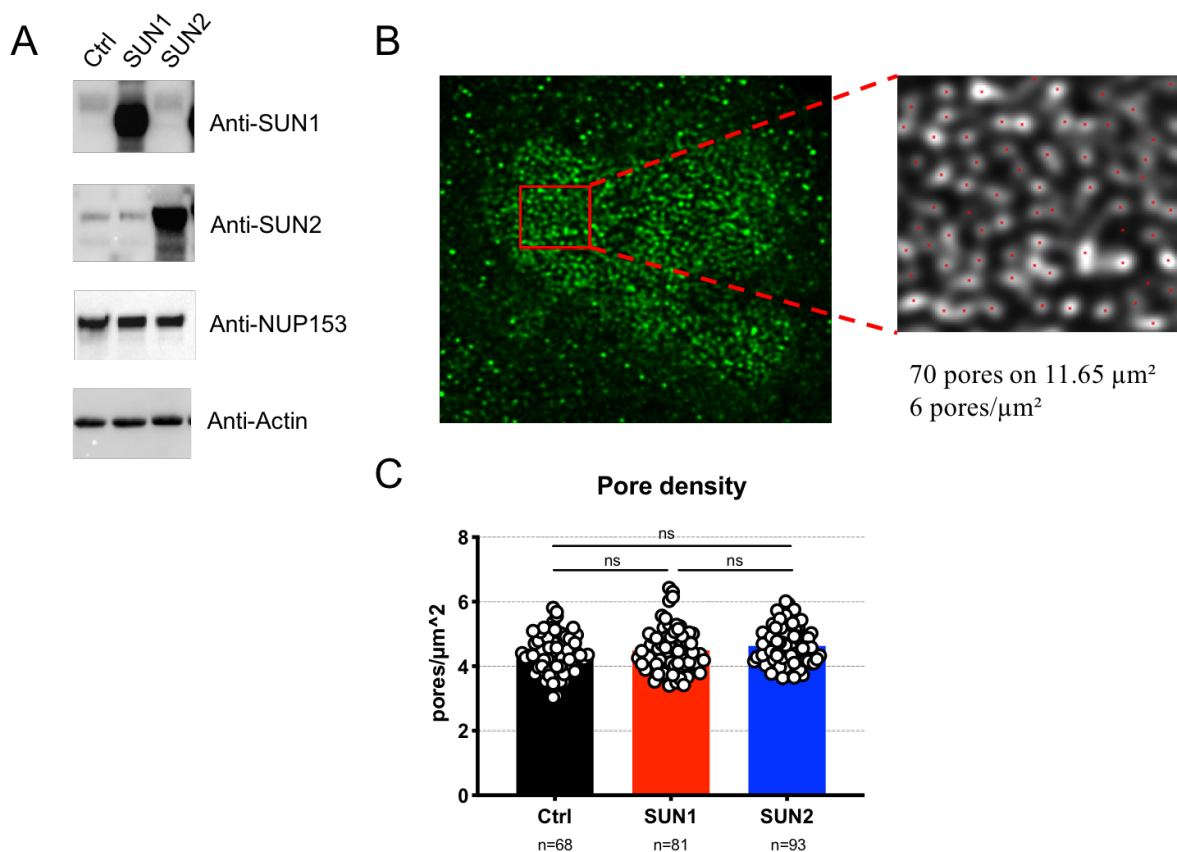
(A) Quantification of  $\gamma$ H2AX<sup>+</sup> signal across transduced CD4<sup>+</sup> T cell lines and conditions at 1 hpt (cells were pre-treated for 1 hour and drugs were washed away prior to infection; n=4 donors, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; line at mean  $\pm$  SEM).

(B) Viral titers based on percentages of GFP<sup>+</sup> cells, 48 hours after infection with p24-normalized doses of purified HIV-1 env-nef- or HIV-1 env-nef-vpr-, encoding GFP in the place of Nef, pseudotyped with VSV-G (n=4 donors, paired RM ANOVA one-way test + correction, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean  $\pm$  SEM).

## 4.2. Complementary Results

During the course of this PhD, other questions related to NE dynamics and nuclear import were also addressed. Some of the experiments were performed only once and not repeated. Considering they still produced somewhat informative results, I have decided to include a few of them in this section of the manuscript.

Keeping in mind the initial observation that SUN overexpression, especially SUN2, was impacting nuclear import of HIV-1 and HIV-2, we wanted to understand whether nuclear pore complexes themselves were being somehow impacted. We initially confirmed by Western Blotting in HeLa cells that neither SUN1 nor SUN2 overexpression were altering the expression levels of nucleoporin NUP153, deemed essential for HIV nuclear import (Figure 9A). Using confocal microscopy and immunofluorescence staining of NUP153, we further verified that the surface density and distribution of NPCs wasn't being altered upon SUN overexpression (Representative image and measurement strategy in Figure 9B; quantification in Figure 9C).



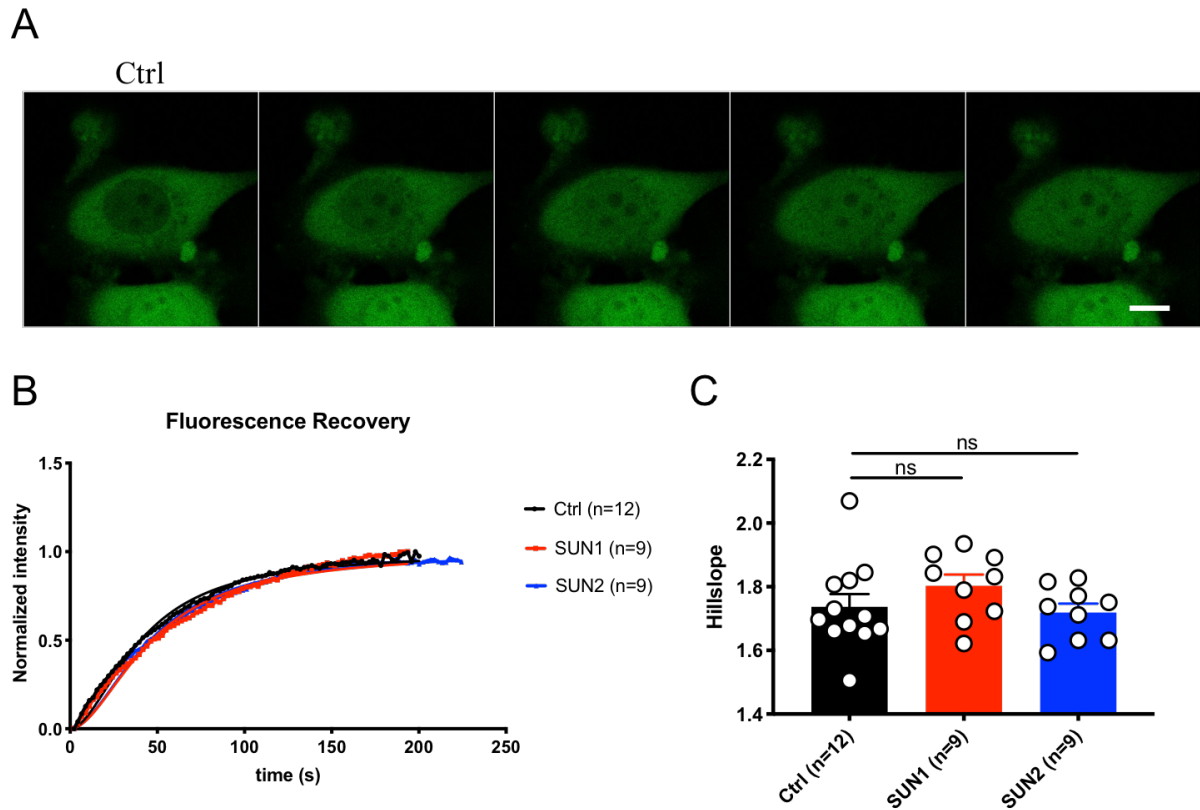
**Figure 9**

**Figure 9: SUN overexpression does not alter NUP153 distribution**

- (A) Western Blot against SUN1 and SUN2 confirming overexpression and against NUP153 showing no changes in expression level in HeLa cells. Actin was used as housekeeping control.
- (B) Representative image of NUP153 staining of a SUN2-overexpressing HeLa cell. The red box indicates a randomized area in which signal peak intensity was calculated using the “Find maxima” function of Image J, as a readout for nuclear pore density (zoomed image shown on right).
- (C) Quantification of nuclear pore density per  $\mu\text{m}^2$  in indicated number of nuclei from given HeLa cell lines (n=1 experiment; un-paired ANOVA one-way test, \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; ns, not statistically significant; line at mean).

Another question that we addressed was related to NPC functionality. SUN overexpression didn't alter NPC distribution or density at NE surface but perhaps the functionality of the pore itself might be compromised. We resorted to the Fluorescence Recovery After Photobleaching (FRAP) technique in order to understand whether passive diffusion to and from the nucleus is impacted upon SUN1 or SUN2 overexpression.

SUN-overexpressing HeLa cell lines were transduced with a GFP-encoding lentivector. GFP signal appeared distributed equally in the nucleus and in the cytoplasm of the cells, as assessed by live imaging via confocal microscopy. A high intensity laser was focused onto a small area within the nucleus of one cell at a time for 20 seconds in order to bleach the GFP signal within the nucleus. Immediately after bleaching, images of the whole cell were taken at the rate of one image every two seconds to observe and measure the rate of GFP recovery in the nucleus, as it diffused back in from the cytoplasm (a layout of the images over time from a Ctrl cell can be found in Figure 10A). The time required for the full recovery of GFP intensity in the nucleus was used as a readout to assess passive diffusion across the NE. Quantification results showed no important changes in diffusion in SUN overexpressing nuclei compared to Ctrl (Figure 10B). Although this experiment provides crucial insight on the biology of the NE upon overexpression SUN1 or SUN2, it is perhaps irrelevant to HIV biology, considering that the import of the PIC is not a diffusive process.

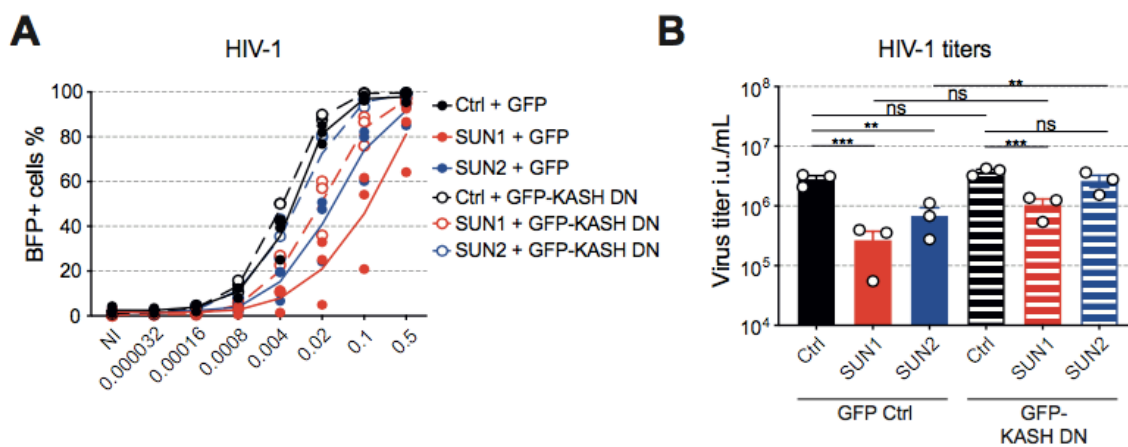


**Figure 10: SUN overexpression does not impact passive diffusion across the NE**

- (A) Representative serial layout of a photobleached Ctrl HeLa cell, ubiquitously expressing GFP. Imaging starts at  $t_0$  after high intensity laser exposure and goes up to  $t=120$  seconds, when fluorescence in the nucleus has been recovered (scale bar: 5  $\mu\text{m}$ )
- (B) Curves representing GFP intensity recovery over time (s). For each individual cell, the intensity was normalized to 0 at  $t_0$  after photobleaching while the max intensity reached was set to 1. Non-linear regression fits were calculated from mean intensity values from indicated number of cells per condition.
- (C) Hillslopes for each cell were calculated via non-linear regression fit ( $n=1$  experiment; un-paired ANOVA one-way test, \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; ns, not statistically significant; line at mean  $\pm$  SEM).



Our results, along with previous studies, make a point on how the antiviral activity of SUN proteins is linked to the two proteins' N-termini and is independent of their interaction with the cytoskeleton. Other than the use of truncated proteins, this was shown partially via nocodazole induced disruption of the microtubule network and by the use of SUN2 point mutants that are incapable of interacting with KASH proteins [127]. In both conditions, the antiviral activity of SUN2 was maintained. The first strategy was a highly indirect approach. We tried to answer the question of LINC complex involvement by exogenously overexpressing the KASH peptide (KASH-DN) in HeLa cells overexpressing SUN1 or SUN2. This peptide outcompetes endogenous nesprins and acts as a dominant negative for SUN-domain binding, therefore disrupting LINC complex formation. Expression of the KASH-DN was confirmed by Western Blot (data not shown). Upon infection with HIV-1, we observed a partial rescue of the antiviral activity of SUN1 and SUN2 in KASH-DN expressing cells compared to Ctrl (Figure 11A). The result was much more significant for SUN2 overexpressing cells in which a complete reversal of the phenotype was observed upon KASH-DN expression (Figure 11B). Once again, this shows that the antiviral activities of SUN proteins, in particular SUN1, are complex and multifactorial.



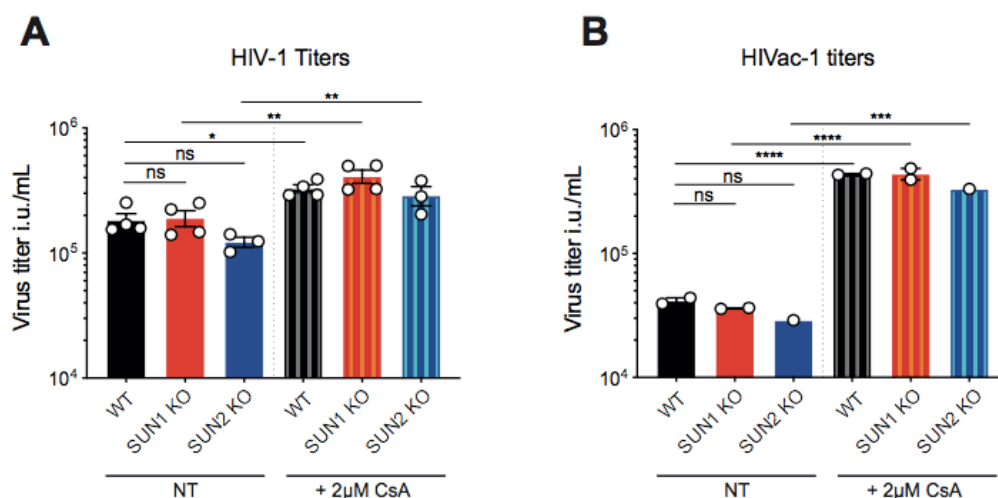
**Figure 11: The antiviral activity of SUN2 can be partially rescued upon disruption of LINC complex formation**

(A) Percentage of BFP<sup>+</sup> HeLa cells 48 hrs after infection with different dilutions of HIV-1 encoding BFP in the place of Nef, pseudotyped with VSV-G. The cell lines are co-transduced to express either nothing, SUN1 or SUN2, coupled to a RFP657 reporter and either GFP alone or GFP fused to KASH DN (n=3 independent experiments).

(B) Viral titers based on percentages of BFP<sup>+</sup> cells (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean ± SEM).

#### 4.2.1. Mouse data

The lab had previously reported that in mouse Bone-Marrow derived Dendritic Cells (BMDCs), infection by the HIV-1 CA mutant HIVac-1 was highly sensitive to the presence of CypA and could be rescued only upon CsA treatment [93]. In BMDCs from *Sun2*<sup>-/-</sup> mice, however, HIVac-1 was rescued even without the drug, suggesting that SUN2 is an important host factor for the CypA-mediated restriction of HIVac capsids in these cells. Having multiple mouse lineages available in the lab, we wanted to explore the role of endogenous SUN1 and SUN2 in HIV infection, in mouse immune cells. These experiments were performed in collaboration with lab post-doc Nilushi De Silva (ND). We initially investigated the role of endogenous SUN1 and SUN2 in mouse T lymphocytes isolated from spleen and lymph nodes of *wt*, *Sun1*<sup>-/-</sup> and *Sun2*<sup>-/-</sup> mice. We used the lentivector pTRIP-SFFV-TagBFP-2A, pseudo-typed with VSV-G as a surrogate for HIV-1 infection. The cells were activated and infected following the optimal conditions described in [270]. Results highlighted no apparent role of endogenous SUN proteins in mouse T cells in the context of HIV-1 infection (Figure 12A), in stark contrast to what was observed in human primary CD4<sup>+</sup> T cells [131]. Similarly, no differences in activation and/or proliferation were observed (data by ND, not shown). In this cell type, as reported in [270], treatment with CsA actually boosts levels of HIV-1 infection. In our hands, this occurred independently of the presence or absence of SUN proteins. We also asked whether the SUN2-mediated CypA sensitivity of the CA mutant HIVac-1, observed in BMDCs, was reproduced in mouse T cells. The lentivector pTRIP-SFFV-TagBFP-2A harboring a backbone with the V86I-IAP91LPA-M96L (HIVac-1) mutation was used to address this. No steady-state rescue of HIVac-1 infectivity was observed within SUN1 or SUN2 KO cells in the preliminary experiment (Figure 12B).



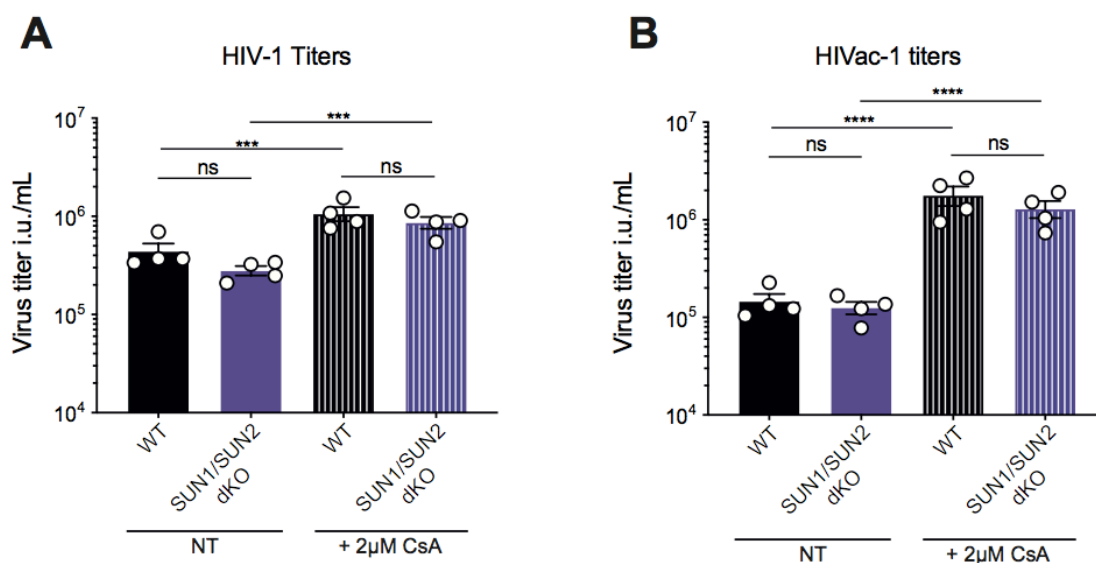
**Figure 12**

**Figure 12: Endogenous SUN1 and SUN2 are not required for infection by HIV-1 in mouse T cells and their loss does not rescue infectivity of HIVac-1**

(A) Viral titers based on percentages of BFP positivity in total activated mouse T cells, 48 hrs after infection with different dilutions of pTRIP-SFFV-TagBFP-2A, pseudotyped with VSV-G (n=4 mice per genotype, except n=3 mice for SUN2KO due to technical error; un-paired ANOVA one-way test on Log-transformed data, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean  $\pm$  SEM).

(B) Viral titers based on percentages of BFP<sup>+</sup> total activated mouse T cells, 48 hrs after infection with different dilutions of pTRIP-SFFV-TagBFP-2A, harboring the CA mutation V86I-IAP91LPA-M96L (HIVac-1), pseudotyped with VSV-G (n=2 mice per genotype, except n=1 mouse for SUN2KO due to technical error; un-paired ANOVA one-way test on Log-transformed data, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean  $\pm$  SEM).

Similar data was obtained from identical experiments performed on T cells, isolated from mice that are double knock-out for SUN1 and SUN2. Even in this case, absence of both SUN proteins did not have an impact on HIV-1 infection (Figure 13A) and treatment with CsA showed a proviral effect, regardless of the presence or absence of SUN proteins. As was the case for the single knock-outs, HIVac-1 infectivity was not rescued at steady state in the mutant, unless CsA was added during infection (Figure 13B).



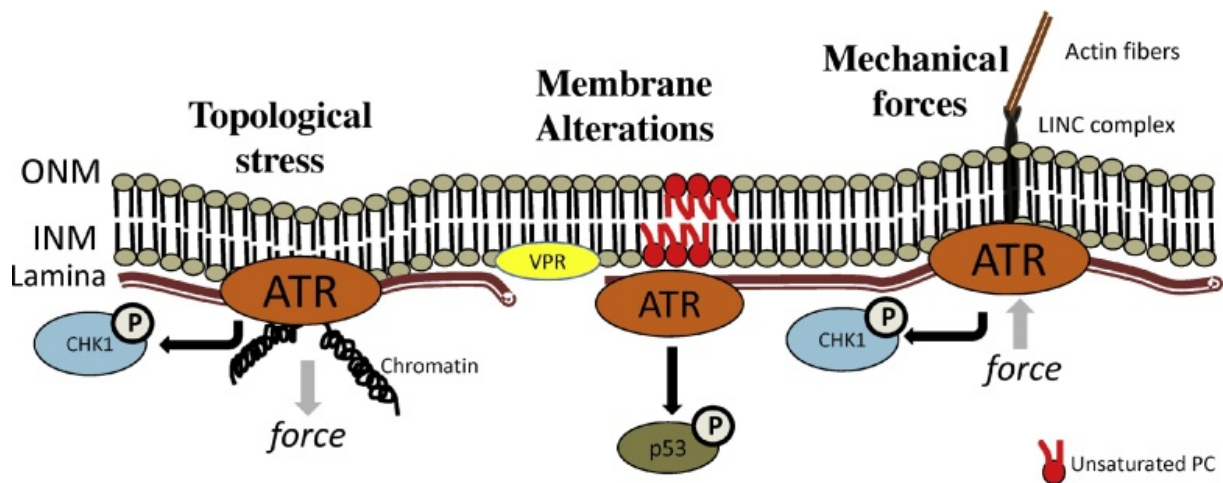
**Figure 13**

**Figure 13: Co-depletion of SUN1 and SUN2 doesn't impact infection by HIV-1 in mouse T cells and their loss does not rescue infectivity of HIVac-1**

- (A) Viral titers based on percentages of BFP positivity in total activated mouse T cells, 48 hrs after infection with different dilutions of pTRIP-SFFV-TagBFP-2A, pseudotyped with VSV-G (n=4 mice per genotype; un-paired ANOVA one-way test on Log-transformed data, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean ± SEM).
- (B) Viral titers based on percentages of BFP<sup>+</sup> total activated mouse T cells, 48 hrs after infection with different dilutions of pTRIP-SFFV-TagBFP-2A, harboring the CA mutation V86I-IAP91LPA-M96L (HIVac-1), pseudotyped with VSV-G (n=4 mice per genotype; un-paired ANOVA one-way test on Log-transformed data, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean ± SEM).

### 4.3. Side story: ATR

In parallel with our work on nuclear envelope proteins SUN1 and SUN2, we explored the potential role of DDR sensor ATR in HIV infection, due to its vast repertoire of implications across DNA damage response, Vpr-mediated regulation of HIV infection and modulation/sensing of NE deformation. In particular, I would like to stress the last point, which was the main reason behind our exploration of ATR inhibition as a tool to affect infection by HIV-1. It has been shown that ATR relocates constitutively to the NE during S phase and in the event of mechanical stress [274, 275]. This localization and subsequent activation are independent of the DDR pathway and have been found to be important for chromatin stability and release of topological tension on the NE itself (*Figure 8*).

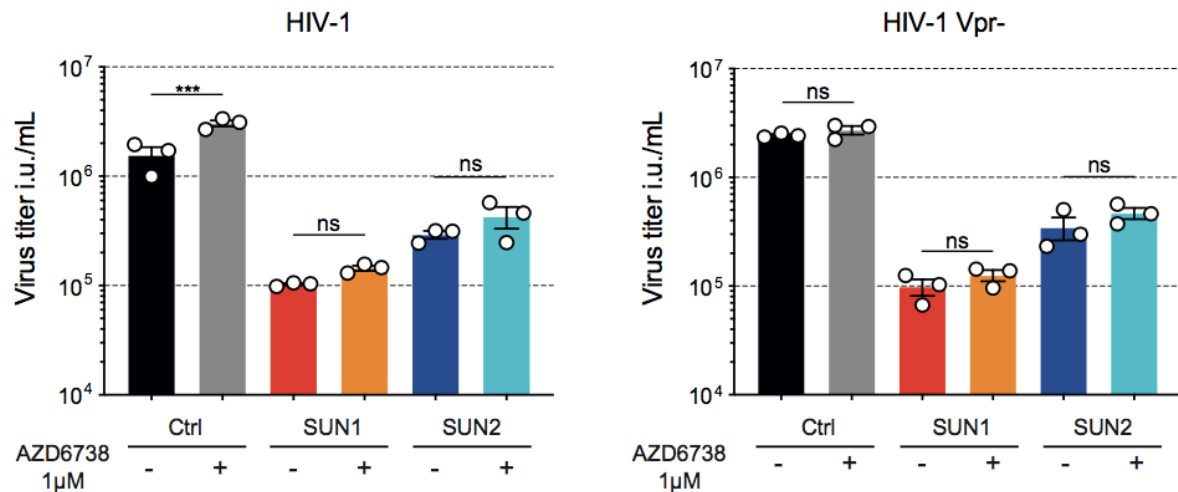


*Figure 9: Schematics of ATR recruitment and activation at the Nuclear Envelope in the event of mechanical stress (adopted from [276]).*

Inhibition or loss of ATR caused phenotypes that ranged from aberrant nuclear plasticity to nuclear rupture and death by mitotic catastrophe, in the event of osmotic or mechanical stress [275]. In particular, HeLa cells depleted of ATR showed invaginations of the NE that strongly reminded us of the deformations we observe upon SUN overexpression. We therefore wondered whether ATR at the NE could be playing a role in mediating the antiviral activities of SUN1 and SUN2 and whether these could be reversed upon ATR inhibition.

Unsynchronized HeLa cells are actively cycling cells. Therefore, ATR is active in this cell type and acts as a sensor for replicative stress. In order to inhibit ATR activity, we resorted to the potent and selective inhibitor AZD6738 [277]. HeLa cells overexpressing SUN1 or SUN2 were infected with p24 normalized doses of HIV-1 +/- Vpr with and without ATR inhibition (ATRi). Surprisingly, ATRi led to increased infection of Ctrl cells by the WT virus but not the Vpr- (Figure 14). As a matter of fact, it seems that the WT virus produced a slightly lower titer to begin with, compared to its Vpr-deficient counterpart. This reduction in infectivity was rescued

upon ATRi (compare the black bars between the two graphs in Figure 14). Furthermore, the presence of SUN1 or SUN2 overexpression was, yet again, dominant over this effect because no increase in infection upon ATRi was observed in cells overexpressing SUN1 and SUN2, for either viral strains.

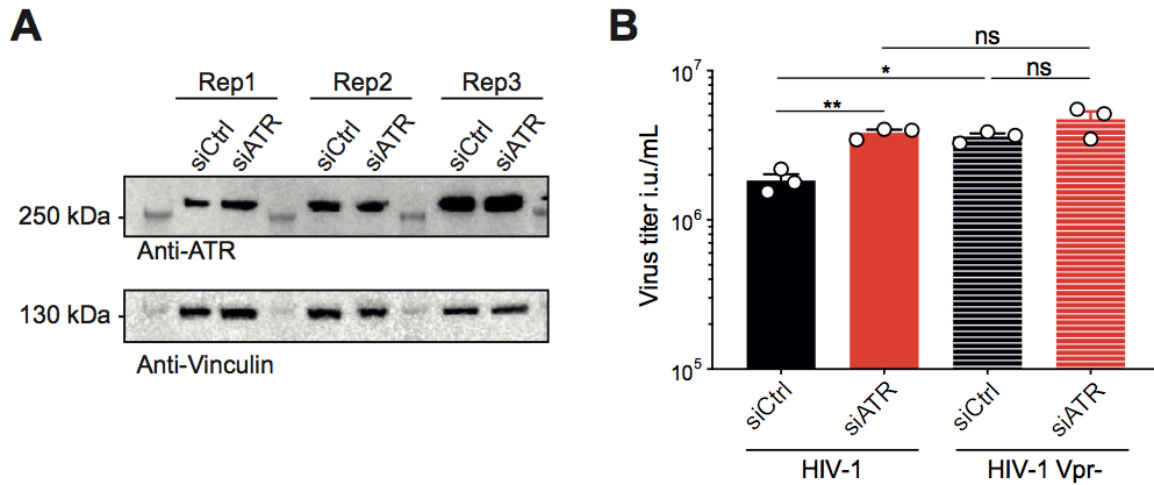


**Figure 14: Inhibition of ATR activity enhances infection by HIV-1 in a Vpr-dependent manner but fails to reverse the antiviral block imposed by SUN overexpression**

Viral titers based on percentages of GFP<sup>+</sup> HeLa cells from given cell lines, 48 hours after treatment with 1 µM AZD6738 and infection with p24 normalized doses of purified HIV-1 env-nef- or HIV-1 env-nef-vpr-, encoding GFP in the place of Nef, pseudotyped with VSV-G (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean ± SEM).

We also attempted to target endogenous ATR by various means of depletion. In HeLa cells, we tried to knock-down ATR via siRNA, via two consecutive rounds of transfection. Unfortunately, we were never able to prove efficient knock-down via Western Blot (Figure 15A) but I nevertheless decided to show the results of infection because of their consistency with what was observed upon chemical inhibition of ATR. This experiment was performed only on non-transduced HeLa, therefore without SUN overexpression. Upon putative ATR knock-down and infection by HIV-1 +/- Vpr, we observed a rescue of infection by the WT strain in cells lacking ATR compared to siCtrl cells. The Vpr- strain showed higher titers at steady state and ATR depletion didn't increase infection further, confirming what was observed and shown in Figure 14. The lack of observable knock-down by Western Blot may be linked to the limitation of this technique, that is incapable of picking up subtle fluctuations of expression

levels. A more quantitative approach like RT-qPCR may be required to pick up the slight decrease in ATR transcript levels that may, however, be sufficient to modulate infection.

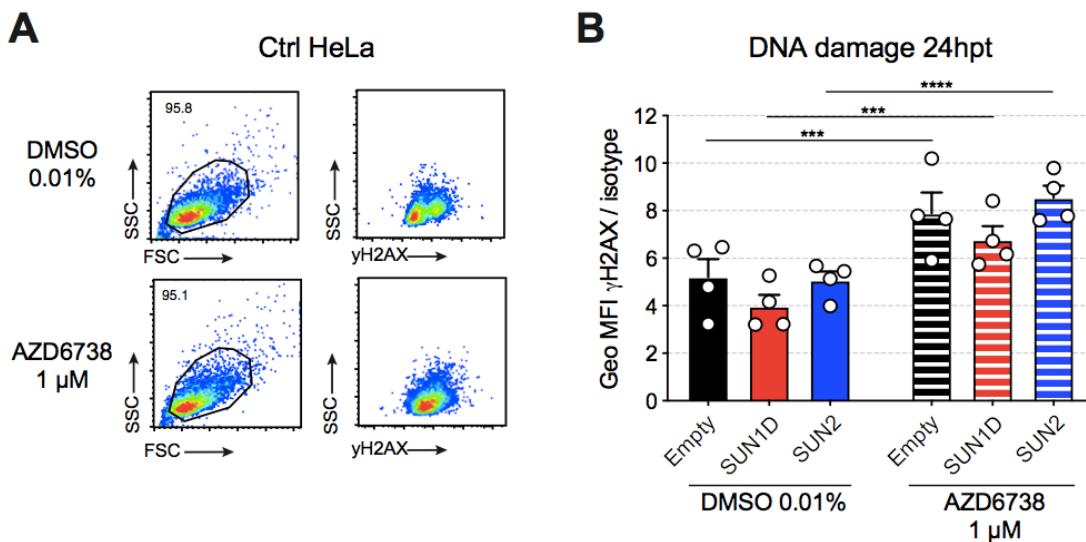


**Figure 15: Slight reduction in endogenous ATR expression level may enhance infection by HIV-1 in a Vpr-dependent manner**

- (A) Western Blot against ATR in an attempt to show protein knock-down in HeLa cells, transfected twice with siRNA targeting ATR mRNA. A non-targeting siRNA sequence was used as negative control (siCtrl). The three experimental replicates are shown side by side and indicated as Rep1, 2 and 3. Vinculin was used as housekeeping control.
- (B) Viral titers based on percentages of GFP<sup>+</sup> HeLa cells, 48 hours after infection with p24 normalized doses of purified HIV-1 env-nef- or HIV-1 env-nef-vpr-, encoding GFP in the place of Nef, pseudotyped with VSV-G (n=3, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean ± SEM).

We wanted to shed light on the role of endogenous ATR in HIV-1 infection in primary human CD4<sup>+</sup> T cells. We attempted to deplete the protein in activated T cells via two different strategies: transduction of lentivectors expressing shRNA sequences directed against ATR transcripts and nucleofection of a CRISPR/Cas9 system with guide RNAs, also targeting ATR. While we completely failed at obtaining any knock-out of ATR with CRISPR/Cas9 in our pilot experiments, we obtained varying degrees of protein knock-down with shRNA. However, due to donor to donor variability and high loss of viability observed with our most efficient shRNAs, it was hard to obtain any conclusive and reproducible data from the infection experiments (data not shown).

ATR is one of the key players of the DNA damage response. It is one of the first kinases to get activated and trigger the downstream events that lead to cell cycle arrest until efficient repair has been achieved. In light of this, one would expect that the chemical inhibition of its kinase activity, would lead to accumulation of DNA damage due to replicative stress in HeLa and an inability to efficiently resolve exogenously induced damage. We stained for  $\gamma$ H2AX foci in HeLa cell lines overexpressing the Ctrl BFP vector, SUN1 or SUN2 in the presence or absence of treatment with 1  $\mu$ M of AZD6738 for 24 hours. Although we didn't quite observe an increase in percentage of  $\gamma$ H2AX positive cells upon treatment with ATRi, we did see a population shift suggesting an increase in  $\gamma$ H2AX intensity, that can be correlated to an aggravated state of unresolved damage within cells (a representative flow cytometry plot for  $\gamma$ H2AX staining in Ctrl cells is shown in Figure 16A). The intensity of  $\gamma$ H2AX signal increased upon ATRi consistently in all three cell lines (Figure 16B). Thinking back to the infection data shown in Figure 14, we can once again hypothesize that a state of increased DNA damage may be beneficial for HIV-1 infection and that the antiviral activity of SUN overexpression (especially SUN1) is downstream and dominant over the DDR-mediated proviral effect.



**Figure 16**



**Figure 16: Inhibition of ATR leads to increase in  $\gamma$ H2AX intensity in HeLa cells**

- (A) Representative FACS plots from one experiment showing  $\gamma$ H2AX intra-nuclear staining in Ctrl HeLa cell line, 24h post treatment with either 1  $\mu$ M of AZD6738 or 0.01% DMSO as control.
- (B) Quantification of  $\gamma$ H2AX signal across cell lines and conditions as geometric mean fluorescence intensity (GeoMFI) of the PE signal divided by the GeoMFI of the isotype control (n=4, paired RM ANOVA one-way test, \*p < 0.05, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; ns, not statistically significant; line at mean  $\pm$  SEM).

## **DISCUSSION**

## 5. Discussion

The role of the Nuclear Envelope in the context of HIV infection has long been an object of mystery and curiosity in the field of HIV virology. The NE consists of a physical barrier that the virus crosses, to gain access to its final destination within the host cell: the nucleus. Indeed, HIV is a retrovirus that is somewhat unique in its ability to infect non-dividing cells [74]. This means that it doesn't require the nuclear envelope breakdown that occurs at cell division, in order to get past the NE. How the virus actually breaches this barrier, how it orchestrates its entry into the nuclear compartment and which are the key host factors it takes advantage of during the process, are only some of the questions that have been partially and non-exhaustively answered over the last decades.

The nuclear envelope itself is a complex structure. It consists of a lipid bilayer with highly selective permeability, whose purpose is to isolate and compartmentalize the cell's genomic material. It is interspersed with proteins that have either a structural function (LINC complex) or that of transport to and from the nucleus (NUPs). In the former category, we can find the SUN proteins, transmembrane proteins spanning the inner nuclear membrane. SUN proteins are one of the principal components of the LINC complex. Briefly, on the nucleoplasmic face, SUN proteins anchor themselves to the lamina meshwork underlying the NE [124]. In the perinuclear space, they interact with the KASH domains of giant spectrin-repeat proteins called nesprins [123, 124, 151]. These, in turn, span out into the cytoplasm and interact with the cytoskeleton. Overall, the LINC complex bridges the nucleoskeleton to the cytoskeleton, thus providing support to the nucleus for proper positioning, migration and signal transduction of mechanical cues [149].

SUN protein homologs SUN1 and SUN2 have been reported to be involved in the regulation of infection by HIV-1 and HIV-2 [93, 127-129, 131]. The modulation of their expression levels at the NE perturbs optimal productive infection by HIV in cell lines and primary cells. Drastic effects on nuclear morphology upon SUN2 overexpression have also been observed. However, the studies so far have been highly descriptive and it hasn't been possible to explain how exactly these NE proteins are perturbing infection by HIV.

The goal of this PhD project was to shed further light on the potential mechanisms of HIV infection regulation by the INM proteins SUN1 and SUN2. The project was supposed to take into account both the overall alteration of the nuclear architecture and the observed overlap with the CypA requirement by HIV-1. Taken altogether, results would contribute to better understand how the crucial steps of HIV nuclear entry and beyond actually occur.

Our results displayed and confirmed the antiviral activity of SUN protein overexpression. Single-round infection by both HIV-1 and HIV-2 was strongly inhibited upon overexpression of SUN1 and SUN2 in HeLa cell lines and primary monocyte-derived macrophages. Infection by HIV-1 was also shown to be inhibited by SUN1 (and partially by SUN2) overexpression in primary CD4<sup>+</sup> T cells. We showed that this antiviral activity was intrinsic to the cells actually overexpressing either SUN1 or SUN2 and it didn't extend to co-cultured cells *in trans*. SUN proteins also showed a functional overlap or downstream activity with respect to HIV-1's requirement of CypA. Indeed, disruption of the CypA-CA interaction led to decreased infection by HIV-1 in control cells but no further decrease was observed upon SUN1 or SUN2 overexpression in either HeLa or MDMs, suggesting the two effects are not additive, as was previously demonstrated for SUN2 alone [93]. The overlap between SUN proteins and CypA was not pursued further in a conclusive manner.

One of the key observations made throughout the PhD was that in every experimental setup, we observed differential trends for the two SUN proteins. SUN1 overexpression appeared to be much more efficient at inhibiting HIV-1 than SUN2. Meanwhile, SUN2 showed a preferential antiviral activity against HIV-2 in all analyzed cell types. This was the first of a series of indications suggesting that the proteins, though homologs, may be playing less redundant roles than initially thought. SUN1 and SUN2 may actually be carrying out their antiviral activities via different mechanisms. An analysis of the viral step being impacted by the two proteins also revealed a discrepancy between the two: while it was overall clear that SUN2 overexpression was blocking HIV nuclear import, for SUN1 things appeared to be hazier and certainly multifactorial.

By generating chimeras, we mapped the preferential antiviral activities to the N-termini of SUN proteins, confirming previous observations [127, 128]. The N-termini lie in the nucleoplasm and interact with underlying lamina and chromatin. However, we showed that the presence of endogenous lamins A/C and B2 is not required for the antiviral profiles of SUN1 and SUN2. Interestingly, loss of lamin A/C alone actually boosted infection by HIV-1 but this effect was cancelled out by SUN protein overexpression.

We also observed nuclear envelope deformation and invaginations upon overexpression of SUN1 and SUN2. This led us to hypothesize that alteration of NE architecture may be responsible for inefficient HIV infection. However, considering that lamin A/C depletion also rendered nuclei more deformed and yet led to an opposite effect on infectivity, we ruled out the fact that NE deformations alone are sufficient to bestow an antiviral effect against HIV.

We also ruled out that SUN overexpression may be leading to a strengthening of the NE by increasing nuclear rigidity or to a change in NPC content and distribution (in particular the FG-repeat protein NUP153, essential for HIV import [86-88]).

With hopes of better characterizing the morphological changes in nuclear architecture observed in HeLa cells overexpressing SUN1 and SUN2, we resorted to live imaging of chromatin staining in these cells. After all, the antiviral activity maps to the nucleoplasmic N-termini of SUN proteins and yet is independent of the presence of endogenous lamins. It is only reasonable to assume that the SUNs may be interfering directly with the chromatin in a way that is compromising optimal infection by HIV. It is also important to point out that a transcriptomic Affymetrix® analysis performed on these cells didn't indicate any significant changes in gene expression. One could therefore hypothesize that SUN overexpression is having a more direct, possibly mechanical, impact on chromatin organization that is hampering with HIV's ability to efficiently integrate in sites that are ideal for viral expression.

By live imaging the DNA in these cells, we noted a striking difference between SUN overexpressing nuclei and lamin A/C-depleted nuclei, that had been included as positive controls for nuclear deformation. Lamin A/C-depleted nuclei, though deformed, rapidly changed shape over time. Their DNA content also appeared to be flowing more freely within each nucleus and appeared more mobile. This is consistent with the fact that lamin A provides important support for chromatin anchoring and acts as a docking platform for many chromatin-associated proteins. This effect, that appeared very clear by eye, was quantified and confirmed using the Particle Image Velocimetry (PIV) plug-in tool from Image J.

In contrast, SUN overexpressing nuclei appeared quite stable over time: though deformed, their shape remained generally unaltered throughout the live imaging. What we observed within SUN1 overexpressing nuclei was, however, striking: the chromatin within these nuclei seemed to be particularly immobile, as though it had been locked in position. PIV analysis confirmed that very little to no DNA movement was detected in these nuclei between one time frame to another. We therefore conclude that SUN1, but not SUN2, overexpression leads to reduced chromatin mobility. This effect, though initially unexpected, is actually very much in line with the reported role of SUN1 in regulating chromatin mobility in specific physiological processes [233, 240]. In particular, SUN1 and chromatin mobility have been implicated as essential elements in optimal resolution of DNA damage.

Keeping in mind both the literature and the striking effect on chromatin mobility that we observe, we became curious as to whether DNA damage had anything to do with the SUN protein-mediated regulation of HIV infection. After all, a link between DNA damage and

infection by HIV-1 has been proposed multiple times, especially in relation to the accessory protein Vpr [246-248, 253].

Using the signal of H2AX, phosphorylated at position Ser139 ( $\gamma$ H2AX), as a readout for DNA damage [193], we evaluated the response of our SUN-overexpressing cell lines to etoposide-induced DNA damage. What we observed, once again, highlighted a difference between SUN1 and SUN2. Upon treatment with a high dose of etoposide, all cell lines responded by up-regulating their levels of  $\gamma$ H2AX. However, the upregulation of the signal was reduced in SUN1 overexpressing cells compared to controls and augmented upon SUN2 overexpression. This not only confirmed an interplay between SUN proteins and DDR but also showed that the two NE proteins may be regulating the damage response pathways differently.

This result is particularly interesting if we consider that SUN1 and SUN2 have behaved in non-redundant ways in our hands, under multiple circumstances. Let's think about HIV infection for example: SUN1 and SUN2 showed different antiviral profiles. Also, when it came to morphology, SUN2 overexpression deformed nuclei much more than SUN1 overexpression did, yet it is the latter that led to reduced chromatin mobility.

We observed in parallel that etoposide treatment boosted infection by HIV-1 in HeLa cells, the same was not true for HIV-2. This result clearly suggested that DNA damage induction has a pro-viral effect on HIV-1.

It was when we combined the etoposide treatment with SUN overexpression, that we observed the unexpected: SUN1 overexpression fully cancelled out the proviral effect of etoposide treatment on HIV-1 infection in HeLa cells. The antiviral activity of SUN2 was, however, almost entirely rescued.

Let me summarize the correlations so far, at least regarding SUN1, in HeLa cells:

- 1) SUN1 overexpression shows a strong anti-viral activity against HIV-1
- 2) Etoposide-induced DNA damage is beneficial for HIV-1 infection
- 3) SUN1 is limiting the formation of  $\gamma$ H2AX foci
- 4) SUN1 is cancelling the proviral effect of exogenous DNA damage

These results suggest that SUN1 activity lies downstream of DNA damage in the context of HIV-1 infection regulation. It also confirms, yet again, that the antiviral activities of SUN1 and SUN2 are, at least partially, non-redundant.

Interestingly, we observed a similar pattern with the inhibition of DDR sensor and effector ATR. When we chemically inhibited the kinase activity of ATR, we observed an increase in infection by HIV-1. By observing  $\gamma$ H2AX staining in parallel, we confirmed an increased DNA

damage signature in cells treated with the ATR inhibitor. Taken together, we found, yet again, evidence for a proviral effect of exogenous DNA damage.

This increase, however, occurred only upon infection with the wild type (WT) HIV-1, not the strain lacking accessory protein Vpr. This is in line with previous observations that have reported Vpr to directly engage and activate ATR and the subsequent DDR axis [253, 260].

However, in contrast to what has been reported so far, we observed baseline reduced infection levels for the WT virus compared to its dose-normalized counterpart lacking Vpr. As a matter of fact, it seemed that inhibition of ATR activity is actually what rescued the full infectivity of the WT virus. This went against previous studies that have reported a Vpr-mediated boost of LTR-driven viral transcription [251].

One explanation for this discrepancy lies in the fact that, in our experimental system, infection with a WT virus impacted cell viability much more than a Vpr- virus. Therefore, an overall healthier state of cells infected by a Vpr- virus can explain the slightly higher viral titers we observed with this strain, at baseline.

Another explanation can be found in the fact that we have only used single-round infection in asynchronous cells in our systems. It is known that Vpr induces cell cycle arrest at the G2 phase and it is through this arrest that it enhances productive infection. In order to observe the full extent of Vpr activity, multiple cycles of both viral replication and cell division may be required.

We observed that ATRi-mediated DNA damage enhanced infection by WT HIV-1 but not by the Vpr-. This was not the case for etoposide treatment, in which case infection by both strains was enhanced. This is probably due to the differential nature of the damage induced by etoposide and by ATRi. In the first scenario, we're directly causing DNA strand breaks, leading to a more severe phenotype of damage that could be bypassing the requirement for Vpr. In the other, we're interfering with optimal repair of replicative stress and inactivating a host factor that HIV-1 engages with, in a Vpr-dependent manner.

Either way, what was in common between the two is the downstream effect of SUN1 overexpression. Also in the case of ATR inhibition, while WT HIV-1 infection was enhanced in control cells, this was not the case for cells overexpressing SUN1, suggesting the antiviral activity of the NE protein is dominant over the proviral effect of ATRi. This time, the same could be held true also for SUN2 overexpression, as no enhancement of infection occurred upon ATRi.

While the picture is more consistent with SUN1, an open question about SUN2 remains: why is SUN2-mediated antiviral activity rescued by etoposide treatment but not by ATR inhibition?

The answer could lie, yet again, in the type and severity of the damage induced by etoposide vs ATRi. First of all, it should be kept in mind that etoposide treatment led to a higher  $\gamma$ H2AX signature in SUN2 overexpressing cells compared to Ctrl, suggesting these cells may be particularly more sensitive to this route of DNA damage induction. This was not really the case with ATR inhibition.

Secondly, the antiviral activity of SUN2 is not as efficient as that of SUN1 against HIV-1. Therefore, a strong DNA damage induction by etoposide may be sufficient to overcome this block.

Lastly, SUN2 overexpression may be carrying out its antiviral activity mainly via more upstream, NE-related, mechanisms such as direct impairment of viral nuclear import, as has been reported previously. We also confirmed this via two observations: 1) SUN2 overexpression reduces the formation of HIV 2-LTR circles (a hallmark of viral nuclear import) and 2) the overexpression of a dominant negative KASH peptide, that leads to disruption of the LINC complex, rescues the antiviral activity of SUN2, significantly more than SUN1.

Let us circle back to another striking observation that clearly sets SUN1 apart from SUN2: SUN1 overexpression caused a reduction in chromatin mobility, not SUN2. It is therefore tempting to think that SUN1 overexpression may be cancelling out the beneficial effect of DNA damage on HIV-1, by reducing chromatin displacement within nuclei. Chromatin displacement, especially that of damaged sites, is required for optimal execution of DDR [240, 241, 243]. We can therefore hypothesize that this process may also be required for productive infection by HIV-1. Indeed, chromatin mobility may be enhancing either optimal integration site selection or efficient viral transcription and expression, both processes that have been reported to be influenced by DNA damage [251, 278]. These processes might therefore be compromised in SUN1 overexpressing cells, due to its effect on chromatin mobility.

Let's not forget that HeLa cells depleted of lamin A/C showed increased chromatin mobility. Consistent with our hypothesis of chromatin mobility being required for efficient HIV-1 infection, we observed an increase in HIV-1 viral titers in these cells compared to controls. Intriguingly, we also later confirmed that  $\gamma$ H2AX signal was massively increased upon lamin A/C depletion (preliminary data, not shown), even when depletion was combined with SUN overexpression. This was, therefore, a drug-free route of DNA damage induction that recapitulated what we observed with etoposide and ATRi: DNA damage being proviral towards HIV-1 and overexpression of SUN proteins being downstream of it. This is a retrospective interpretation of the experiments showing that the antiviral activity of SUN overexpression is independent of endogenous lamins. Note that in the case of lamin A/C, infection was performed



with a Vpr- virus. The extent of damage (or absence of functional repair as measured by  $\gamma$ H2AX signal) was so high in these cells, that even this strain was impacted, as with etoposide treatment.

### **5.1. A model in HeLa cells**

If we attempt to summarize our results in HeLa cells in light of our interpretation, we see that three distinct strategies lead to an induction of DNA damage:

- 1) direct DNA damage induction by etoposide treatment
- 2) inhibition of ATR activity
- 3) genetic depletion of lamin A/C

In all three conditions, we observed an increase in infectivity by HIV-1 in control cells. In all three conditions, a simultaneous overexpression of SUN proteins proved to be antiviral and cancelled out the proviral effect of DNA damage. Results strongly suggest that SUN proteins lie downstream of DNA damage in the context of infection by HIV-1.

Only in the case of etoposide treatment, SUN2 overexpressing cells were particularly sensitive to this route of damage induction and HIV-1 infection was rescued.

SUN2 overexpression has been reported to be antiviral mainly at the level of viral nuclear import and we confirm this observation, in part. The interpretation for SUN1 on the other hand is less clear.

We observed a reduced level of chromatin mobility with SUN1 overexpression. We hypothesize, therefore, that SUN1 may be rendering HIV-1 incapable of using DNA damage to its advantage and that it may be doing so by reducing the overall mobility of chromatin.

Chromatin mobility is possibly advantageous for HIV-1 infection to access sites of damaged DNA and unlock the proviral effect that damage and DDR have on optimal infection. Whether this occurs in a Vpr-dependent manner or not, depends on the route and extent of the DNA damage itself.

Alternatively, chromatin mobility, triggered by DNA damage, could be necessary for HIV-1 to access sites of optimal integration. SUN1 overexpression may be leading to less productive integration of HIV-1, by blocking DNA movement.

Consistently with the fact that HIV-2 is insensitive to the proviral effect of DNA damage in our hands, SUN1 overexpression is much less efficient at blocking HIV-2 than HIV-1.

## **5.2. Validation in primary cells and limitations**

The validation of our experimental results in primary monocyte-derived macrophages has been quite limited for the moment. We confirmed the antiviral activities of SUN1 and SUN2 overexpression against HIV-1 and HIV-2 in these cells but did not further explore or analyze the implication of DNA damage. We found that ATR is not constitutively expressed in these cells, consistently with their non-cycling phenotype. It is definitely worth exploring whether treatment with etoposide can enhance HIV-1 infection in MDMs and whether SUN proteins can rescue it. Being adherent cells in culture, they are probably being governed by similar physical laws of tension and traction as HeLa cells. Therefore, based on this simple analogy, one could expect similar constraints on chromatin dynamics in these cells as well and hypothesize that HIV-1 may benefit from an increase in DNA mobility.

We attempted to go a bit further with primary CD4<sup>+</sup> T cells. We used anti-CD3/anti-CD28 activated T cells for our experiments [279]. When looking at  $\gamma$ H2AX staining, with or without etoposide treatment, in CD4<sup>+</sup> T cells, we immediately noticed the presence of a constitutive baseline level of DNA damage. This wasn't present in un-stimulated T cells from the same donors (data not shown). We assume this damage is caused by replicative stress at DNA level due to the high proliferation rate of activated and blasting T cells [280, 281]. Considering the highly metabolically active state of these cells,  $\gamma$ H2AX signal could also be linked to oxidative damage.

An interesting outlook on the story is that this baseline damage itself is boosting HIV-1 infection. This is, however, hard to determine considering we lack a control in our system, in which this constitutive damage is reversed. We had initially wondered whether the overexpression of SUN proteins in these cells could be able to restrict or reduce the baseline level of  $\gamma$ H2AX signal. This however proved not to be the case, as percentages of  $\gamma$ H2AX<sup>+</sup> cells were comparable across control and SUN-overexpressing CD4<sup>+</sup> T cells.

Though we observed an antiviral activity of SUN protein overexpression in CD4<sup>+</sup> T cells, we couldn't really recapitulate the proviral effect of etoposide treatment that we observed in HeLa. One major limitation came from the fact that primary cells are much more sensitive to exogenous DNA damage. In our experimental systems, it was hard to keep cells alive post-damage induction. We therefore had to resort to pretreating the cells with a low dose. We cannot, therefore, be sure that the treatment occurred in an optimal time-window for an impact on HIV-1 infection. Furthermore, harsh DNA damage in primary cells triggers a cascade of responses that can be much more deleterious than in cancerous cell lines. Among these, we can

mention DDR-mediated p53 activation and subsequent apoptosis [190]. Therefore, what is required is a more gentle route of DNA damage induction, that is constitutively present throughout infection and that can be correlated to infection level after 48 hours. At the time of drafting this manuscript, we had started analyzing CD4<sup>+</sup> T cells with shRNA-mediated depletion of lamin A/C. Preliminary results (not shown) showed us high donor to donor variability but a generalized increase of  $\gamma$ H2AX signal upon loss of lamin A/C as compared to control cells. In the donors where this increase was present, this correlated to a concomitant increase of HIV-1 infected cells, suggesting that DNA damage correlates to HIV-1 infection, also in this system.

This experiment has not been combined with SUN overexpression yet but our hypothesis and model suggest that SUN proteins should cancel any lamin A/C depletion-induced increase in HIV-1 infectivity.

### **5.3. Perspectives and implications**

Some unanswered questions emerge from the formulation of our hypothesis. Further studies are required to better characterize and confirm our model.

We haven't yet validated whether it is DNA damage *per se* or the impairment of functional repair that is upregulating HIV-1 infection. After all, H2AX phosphorylation can indicate both. A COMET assay performed on cells to evaluate the entity of the DNA damage itself, may be helpful in answering this question.

It needs to be understood whether DNA damage is directly beneficial to HIV-1 or it goes through the chromatin mobility route. In the case of the latter option, a PIV analysis on cells treated with etoposide or ATRi should theoretically also show an increase in mobility. Furthermore, one would expect SUN1 overexpression to be able to cancel this increased mobility.

A follow-up experiment that needs to be performed to validate the role of chromatin mobility in HIV-1 integration is Integration Site Selection sequencing. This would reveal whether SUN1 overexpression changes the pattern of where the HIV-1 provirus integrates. An alteration in integration site, perhaps caused by inefficient DDR pathway exploitation and/or by reduced chromatin mobility, at least in HeLa, could explain the reduced infection of HIV-1 in these cells.

As for primary CD4<sup>+</sup> T cells, it is intriguing to think that endogenous, post-activation damage at baseline may be enhancing HIV-1 infection. An interesting approach would be to find ways

to reverse/rescue this phenotype and assess whether levels of infection are reduced compared to donor-matched controls.

We currently have no knowledge on whether DNA damage or SUN protein levels influence the mobility of chromatin in CD4<sup>+</sup> T cells. It is hard to apply a live imaging + PIV approach in this cell type due to fact these are non-adherent cells in culture. A more sophisticated approach to study and quantify chromatin dynamics in these primary cells may be necessary to understand the implication of DNA mobility and NE proteins in HIV infection.

As stated before, CD4<sup>+</sup> T cells are cultured in suspension. Therefore, there are not necessarily placed under strict regimes of tension and pressure as adherent cells. Perhaps SUN proteins require a state of cell adhesion that exerts stronger forces on the nucleus, in order to efficiently impact chromatin dynamics. After all, in physiological conditions, T cells are obliged to migrate and proliferate within tissues and tight interstitial spaces. *Ex vivo* cell culture conditions should be able to mimic that. Unfortunately, forcing T cells to adhere is technically difficult and only transiently possible, not allowing enough time for drug or virus-based manipulation. A microfluidics based or, even better, a tissue-based imaging approach could perhaps be a way to address this problem.

Lastly, the role of endogenous SUN proteins requires further characterization in CD4<sup>+</sup> T cells. It has been shown that depletion of SUN2 in CD4<sup>+</sup> T cells leads to reduced cell proliferation, viability and activation [131]. It might be worth checking whether loss of SUN2 is impacting DDR in this cell type, perhaps leading to increased damage, therefore impacting HIV infection through this effect.

Overall, the results obtained during the course of this project highlight a deep interconnection between host NE proteins, underlying nuclear dynamics and modulation of HIV infection. They shed light on how alterations that are more mechanical and biophysical, rather than biochemical, can drive the course of productive infection. The information obtained here may, among other things, help in understanding why certain cell types offer the virus a more favorable nuclear environment than others. The project provides novel insight on the pathophysiology of a virus that has managed to remain elusive and mind-boggling under many aspects, for the last forty years. This could thus pave the way for the development of putative therapeutic or preventive strategies, that take into account these biophysical aspects of host-virus interaction.

## **RÉSUMÉS EN FRANÇAIS**

## **6. Résumé des chapitres**

### **6.1. Introduction**

Le virus de l'immunodéficience humaine (VIH) comprend deux espèces virales diploïdes distinctes, le VIH-1 et le VIH-2, qui sont classées comme lentivirus humains, appartenant à la famille des Retroviridae, sous-famille des Orthoretrovirinae. Ainsi, lors de son entrée dans la cellule hôte, son génome est rapidement transcrit en ADN par la transcriptase inverse virale [1]. Un complexe nucléoprotéique contenant l'ADN viral nouvellement synthétisé, connu sous le nom de complexe de pré-intégration (PIC), est ensuite transféré dans le noyau cellulaire, où l'intégrase virale intervient dans l'intégration réussie de l'ADN viral dans le génome de l'hôte [2].

Une fois intégré, il peut exister indéfiniment sous forme de provirus, constituant ainsi un réservoir viral latent dans un petit pourcentage de cellules infectées [3]. L'existence d'un tel réservoir est l'une des principales raisons pour lesquelles il a été impossible, après près de 40 ans de recherche scientifique, de développer une cure définitive contre l'infection par le VIH.

Le VIH est un agent pathogène qui cible les cellules du système immunitaire, en particulier les cellules CD4+. En absence de traitement, l'infection peut évoluer vers le syndrome d'immunodéficience acquise (SIDA), une maladie dans laquelle les infections opportunistes se développent et prennent le dessus, en l'absence de réponse immunitaire fonctionnelle de l'hôte. Aucun traitement définitif de l'infection par le VIH n'a été mis au point à ce jour : le virus peut être maintenu sous contrôle avec un traitement antirétroviral (TAR) mais ne peut pas être entièrement éradiqué de l'hôte. Deux virus différents ont été identifiés dans les années 1980 : VIH-1 et VIH-2. Même si bien que les deux virus partagent des similitudes structurelles et fonctionnelles, le VIH-2 est nettement moins pathogène que le VIH-1 [10] le pronostic de la maladie varie considérablement.

Les génomes du VIH-1 et du VIH-2 partagent l'organisation globale et présentent de multiples similitudes. Les deux codent pour des protéines structurales, des enzymes, des protéines régulatrices et accessoires.

Le cycle de réplication virale est caractérisé par la reconnaissance des récepteurs de surface, la fusion dans la cellule, la transcription inverse et le transport vers le noyau. L'opinion générale et actuelle est que le PIC pénètre dans le noyau par le complexe de pores nucléaires (NPC), bien qu'une compréhension détaillée du mécanisme ne soit toujours pas disponible.

Une fois à l'intérieur, le PIC se dirige vers l'ADN hôte et l'intégrase virale catalyse l'intégration appropriée de l'ADNc viral dans le génome. À ce stade, le virus peut soit rester latent en tant que provirus intégré, soit entrer dans une étape de transcription active après laquelle l'ARNm viral est exporté vers le cytoplasme puis traduit dans les protéines structurales du VIH, qui sont ensuite assemblées avec le génome viral en une nouvelle particule virale.

Dans le cadre de ce projet, on se focalise sur l'étape de l'import nucléaire du virus et sur les protéines de l'hôte qui sont impliquées. En particulier, on va se concentrer sur les protéines structurales de la membrane nucléaire interne SUN1 et SUN2.

SUN1 et SUN2 [122], sont des protéines transmembranaires intégrales de type II présentes dans la membrane nucléaire interne (INM) de l'enveloppe nucléaire (EN). Ils possèdent un domaine SUN carboxy-terminal conservé qui s'étend dans l'espace périnucléaire dans lequel il lie le domaine Klarsicht-ANC1-Syne-homology (KASH) de protéines appelées Nesprins, protéines trans-membranaires de la membrane nucléaire externe qui se ramifient du noyau dans le cytoplasme pour lier des éléments du cytosquelette tels que l'actine, les microtubules et les filaments intermédiaires [123]. Ce complexe protéique est connu sous le nom de complexe Linker of Nucleoskeleton and Cytoskeleton (LINC). Dans le nucléoplasme, les protéines SUN interagissent avec le maillage de Lamin sous-jacent à l'EN via son domaine N-terminal [124, 125].

Il a été démontré que ces deux protéines ont une forte activité antivirale contre le VIH-1 lorsqu'elles sont surexprimées dans une vaste sélection de types de cellules [93, 127-129]. Cet effet serait lié à un défaut d'import nucléaire du virus.

De manière frappante, la surexpression de SUN2, bien que n'ayant pas d'impact sur la viabilité cellulaire, a conduit à un phénotype de noyaux moins circulaires et déformés dans plusieurs types cellulaires.

D'autre part, la manipulation des niveaux d'expression endogènes de SUN2 a également conduit à une activité antivirale envers le VIH d'une manière dépendante du type cellulaire [93, 127, 128]

Dans l'ensemble, SUN1 et SUN2 apparaissent comme des régulateurs critiques de l'infection du VIH, à la fois lors de la surexpression et au niveau endogène. Cela peut être dû à leur position stratégique et à leur fonction dans l'EN. La question de savoir si les modifications morphologiques ultérieures de l'EN, causées par la modulation du niveau des protéines SUN, sont directement ou indirectement impliquées dans l'effet antiviral est, en partie, l'objet de cette étude.

Globalement, la fonction des protéines SUN et leur positionnement stratégique dans l'EN sont à l'origine de leur implication dans de multiples processus nucléaires. De nombreuses études au fil du temps ont également mis en évidence les rôles différentiels de SUN1 par rapport à SUN2, contestant ainsi l'idée que les deux homologues soient redondants. Il a été démontré que les protéines SUN sont requises de manière différentielle dans de nombreux processus cellulaires tels que la division cellulaire, la régulation de l'exportation de l'ARNm et la réparation des dommages à l'ADN [160-167].

Ce dernier processus en particulier est fondamentale dans le cycle de vie d'une cellule mais aussi dans le cadre d'une infection par le VIH.

Afin de préserver l'intégrité génomique, les cellules sont équipées d'un réseau sophistiqué appelé DNA damage response (DDR). Il s'agit de multiples voies de réparation, chacune dépendant de la catégorie de la lésion, et de plusieurs points de contrôle et mécanismes de transduction du signal qui forment ensemble un système de maintenance qui décide du sort de la cellule: survie, sénescence répllicative ou mort [190].

Le DDR n'est pas un processus isolé. Il déclenche une réponse concertée de multiples voies de signalisation et points de contrôle, qui conduisent à l'arrêt du cycle cellulaire. Le blocus n'est idéalement pas levé tant que les dégâts n'ont pas été résolus. Dans le cas de dommages à l'ADN, les voies de réparation sont déclenchées par l'action de deux kinases principales: ATM et ATR. L'ATR en particulier est également un capteur de stress réplcatif qui se produit lors de la réplication de l'ADN pendant la phase S du cycle cellulaire.

Comme mentionné, le complexe LINC et ses composants se sont avérés impliqués dans la régulation du DDR de multiples manières, en particulier SUN1 et SUN2 [233-239]. Cela n'est pas du tout surprenant compte tenu du rôle que jouent les protéines de l'EN dans la fixation des chromosomes et l'architecture nucléaire en général. De plus en plus de preuves montrent que le complexe LINC est directement impliqué dans la relocalisation optimale des sites endommagés [240, 241] et donc, dans la mobilité de la chromatine, afin de permettre une réparation efficace. L'une des principales caractéristiques du DDR est la régulation du cycle cellulaire. On a longtemps observé que plusieurs virus pouvaient détourner le mécanisme de régulation des points de contrôle d'une cellule pour arrêter la prolifération [245]. Il en est de même pour le VIH, connu pour arrêter les cellules à la fin de G2 ou au début de la phase M [246, 247]. Il est également clair que la protéine accessoire Vpr du VIH est nécessaire et suffisante pour induire ce bloc G2 [246]. De multiples hypothèses ont été avancées pour tenter d'expliquer pourquoi un arrêt G2 serait bénéfique pour le virus. Une de ces hypothèses est que Vpr arrête les cellules au stade où l'expression virale est la plus forte [251].



Le lien solide et direct entre Vpr et DDR réside cependant dans l'implication directe par Vpr d'ATR. La déplétion ou l'inhibition pharmacologique de l'ATR abroge le bloc induit par Vpr dans G2 dans plusieurs types cellulaires. En plus,  $\gamma$ H2AX, une autre cible de ATR et un indicateur de la signalisation des dommages à l'ADN, est augmenté en présence de Vpr. Une étude plus récente a même révélé que Vpr provoque directement le déroulement de l'ADNdb, modifiant ainsi la structure de la chromatine, conduisant à la DDR en conséquence [258].

Dans l'ensemble, ces résultats créent un parallélisme évident entre l'activité Vpr et la régulation induite par des dommages à l'ADN de la transition G2 / M. Dans tous les cas, la question de savoir si Vpr lui-même induit des dommages à l'ADN ou les imite simplement pour déclencher la signalisation, a été une question bien débattue.

### **6.1.1. Objectif de la thèse**

Des travaux antérieurs du laboratoire avaient déjà mis en évidence l'implication de la protéine SUN2 dans le contexte d'une infection par VIH-1 et VIH-2 [93]. Considérant qu'il s'agit d'une protéine structurale de l'enveloppe nucléaire et que le bloc antiviral se produit au moment de ou avant l'entrée nucléaire virale, l'attention s'est concentrée sur ce que pourrait être le rôle possible de l'EN lui-même, ses éléments constitutifs et l'architecture nucléaire en général dans la régulation de l'infection par le VIH.

Ma thèse s'est d'abord concentrée sur la compréhension de la façon dont SUN2, son homologue SUN1 et d'autres protéines de l'EN régulent l'infection par le VIH. Le projet visait à comparer la régulation médiée par SUN du VIH-1 et du VIH-2 et à disséquer les besoins de la Cyclophilin A dans ce processus dans des lignées cellulaires et dans des cellules cibles primaires du VIH. De plus, il visait à établir s'il existe un lien entre la morphologie / architecture de l'EN et l'infection par le VIH, en étudiant les effets de la surexpression du SUN sur les propriétés biophysiques des noyaux et en comprenant leur impact sur la modulation de l'infection par le VIH. En effet, la surexpression des protéines SUN a un fort impact sur la forme nucléaire. Nous avons donc cherché à comprendre si ce phénotype morphologique a une conséquence mécanique sur le noyau qui perturbe les conditions d'une infection productive optimale.

Au cours de la thèse, les résultats ont montré que la modulation de l'infection médiée par les protéines SUN est médiée par les N-terminus nucléoplasmiques des protéines. Cela a permis au projet de se concentrer sur l'impact que les protéines SUN pourraient avoir sur l'organisation nucléaire elle-même, en particulier sur la mobilité de la chromatine et les dommages à l'ADN.

Compte tenu du rôle que joue le complexe LINC dans le maintien de l'intégrité du génome, nous avons cherché à déterminer s'il existe une régulation médiée par le SUN de la dynamique de la chromatine, qui pourrait avoir un impact sur l'infectivité du VIH. En effet, cela s'est avéré être le cas. Nous avons essayé d'explorer quelles voies ont été modifiées, quelles étapes de l'infection à VIH ont été impactées et, enfin, si les mêmes résultats pouvaient être récapitulés dans les cellules primaires qui sont des cibles naturelles du VIH.

## **6.2. Résultats et Discussion**

Le rôle de l'enveloppe nucléaire dans le contexte de l'infection par le VIH a longtemps été un objet de mystère et de curiosité dans le domaine de la virologie du VIH. L'EN consiste en une barrière physique que le virus traverse, pour accéder à sa destination finale au sein de la cellule hôte: le noyau. Comment le virus franchit réellement cette barrière, comment il orchestre son entrée dans le compartiment nucléaire et quels sont les principaux facteurs hôtes impliqués dans le processus, ne sont que quelques-unes des questions auxquelles on a répondu partiellement et de manière non exhaustive au cours des dernières décennies.

Il a été rapporté que les homologues SUN1 et SUN2 sont impliqués dans la régulation de l'infection par le VIH-1 et le VIH-2 [93, 127-129, 131]. La modulation de leurs niveaux d'expression à l'EN perturbe l'infection productive optimale par le VIH dans les lignées cellulaires et les cellules primaires. Des effets drastiques sur la morphologie nucléaire lors de la surexpression de SUN2 ont également été observés. Cependant, les études jusqu'à présent ont été très descriptives et il n'a pas été possible d'expliquer exactement comment ces protéines de l'EN perturbent l'infection par le VIH.

Le but de ce projet de doctorat était d'éclairer davantage les mécanismes potentiels de régulation de l'infection à VIH par les protéines de l'INM, SUN1 et SUN2. Nos résultats ont montré et confirmé l'activité antivirale de la surexpression de la protéine SUN. L'infection en un seul cycle par le VIH-1 et le VIH-2 a été fortement inhibée lors de la surexpression de SUN1 et SUN2 dans les lignées cellulaires HeLa et les macrophages primaires dérivés de monocytes. L'infection par le VIH-1 s'est également révélée inhibée par la surexpression de SUN1 (et partiellement par SUN2) dans les cellules T CD4 + primaires. On a montré que l'activité antivirale est intrinsèque à la cellule et n'est pas lié à une production et sécrétion des facteurs antiviraux, avec l'aide d'une analyse d'expression génique.

L'une des principales observations faites tout au long de la thèse était que dans chaque configuration expérimentale, la surexpression de SUN1 semble être beaucoup plus efficace

pour inhiber le VIH-1 que SUN2 alors que SUN2 a montré une activité antivirale préférentielle contre le VIH-2 dans tous les types de cellules analysées. C'était la première d'une série d'indications suggérant que les protéines, bien qu'homologues, pourraient jouer des rôles moins redondants qu'on ne le pensait initialement. Avec le développement des protéines chimères entre SUN1 et SUN2, nous avons montré que l'activité antivirale spécifique est liée au domaine N-terminale des deux protéines, qui se trouve dans le noyau.

Nous avons également observé une déformation de l'enveloppe nucléaire et des invaginations lors de la surexpression de SUN1 et SUN2. Cela nous a conduit à émettre l'hypothèse que l'altération de l'architecture de l'EN peut être responsable d'une infection à VIH inefficace. Nous avons cependant écarté le fait que les déformations suffisent à conférer un effet antiviral contre le VIH, vu que les noyaux privés de lamin A/C étaient aussi déformés mais montraient un effet proviral.

On pourrait donc émettre l'hypothèse que la surexpression de SUN a un impact plus direct, peut-être mécanique, sur l'organisation de la chromatine, ce qui entrave la capacité du VIH à s'intégrer efficacement dans des sites idéaux pour l'expression virale.

En imagerie en direct de l'ADN dans ces cellules, nous avons noté une différence frappante entre les noyaux surexprimant SUN et les noyaux privés de lamin A/C, qui avaient été inclus comme témoins positifs de la déformation nucléaire. Les noyaux appauvris en Lamin A/C, bien que déformés, changeaient rapidement de forme avec le temps. En revanche, les noyaux surexprimant SUN semblaient assez stables dans le temps: bien que déformés, leur forme restait généralement inchangée tout au long de l'imagerie en direct, en particulier SUN1. Nous concluons donc que la surexpression de SUN1, mais pas de SUN2, conduit à une mobilité réduite de la chromatine.

Vu ces effets sur la chromatine et la littérature, nous nous sommes demandé si les dommages à l'ADN avaient quelque chose à voir avec la régulation de l'infection par le VIH médiée par les protéines SUN.

En utilisant le signal de H2AX, phosphorylé à la position Ser139 ( $\gamma$ H2AX), comme lecture des dommages à l'ADN [193], nous avons évalué la réponse de nos lignées cellulaires surexprimant le SUN aux dommages à l'ADN induits par l'étoposide. Dans les cellules HeLa, nous avons observés que:

- 1) Les dommages à l'ADN induits par l'étoposide sont bénéfiques pour l'infection par le VIH-1
- 2) SUN1 limite la formation de foyers  $\gamma$ H2AX alors qu'ils sont favorisés par SUN2
- 3) SUN1 annule l'effet proviral des dommages exogènes à l'ADN
- 4) L'effet antiviral de SUN2 est annulé par l'étoposide

Ces résultats suggèrent que l'activité de SUN1 se situe en aval des dommages à l'ADN dans le contexte de la régulation de l'infection par le VIH-1. Il confirme également, une fois encore, que les activités antivirales de SUN1 et SUN2 sont, au moins partiellement, non redondantes.

Nous avons observé un effet similaire avec l'inhibition de ATR. Lorsque nous avons inhibé chimiquement l'activité kinase de l'ATR, nous avons observé une augmentation de l'infection par le VIH-1, mais seulement quand Vpr était présent. En observant le signal de  $\gamma$ H2AX en parallèle, nous avons confirmé une augmentation de la signature des dommages à l'ADN dans les cellules traitées avec l'inhibiteur de ATR.

Également dans le cas de l'inhibition de l'ATR, alors que l'infection par le VIH-1 WT était augmenté dans les cellules contrôles, ce n'était pas le cas pour les cellules surexprimant SUN1 ou SUN2, ce qui suggère que l'activité antivirale des protéines de l'EN est dominante sur l'effet proviral d'ATRi. Donc l'effet antiviral de SUN2 est annulé par l'étoposide mais pas par l'inhibition de ATR. Plusieurs explications sont possibles : la réponse pourrait résider dans le type et la gravité des dommages induits par l'étoposide vs ATRi. Il faut aussi se rappeler que le traitement à l'étoposide a conduit à une signature  $\gamma$ H2AX plus élevée dans les cellules surexprimant SUN2 par rapport au contrôle, ce qui suggère que ces cellules pourraient être particulièrement plus sensibles à cette voie d'induction de dommages à l'ADN.

Deuxièmement, l'activité antivirale de SUN2 n'est pas aussi efficace que celle de SUN1 contre le VIH-1. Par conséquent, une forte induction de dommages à l'ADN par l'étoposide peut être suffisante pour surmonter ce blocage.

Enfin, la surexpression de SUN2 peut exercer son activité antivirale principalement via des mécanismes plus en amont tels que l'altération directe de l'import nucléaire virale, comme rapporté précédemment. Enfin, la surexpression de SUN1 a provoqué une réduction de la mobilité de la chromatine, pas SUN2.

N'oublions pas que les cellules HeLa dépourvues de lamin A/C ont montré une mobilité accrue de la chromatine. Conformément à notre hypothèse selon laquelle la mobilité de la chromatine est nécessaire pour une infection efficace par le VIH-1, nous avons observé une augmentation des titres viraux du VIH-1 dans ces cellules par rapport aux contrôles. Curieusement, nous avons également confirmé plus tard que le signal  $\gamma$ H2AX était massivement augmenté en absence de Lamin A/C.

Si nous tentons de résumer nos résultats dans les cellules HeLa à la lumière de notre interprétation, nous voyons que trois stratégies distinctes conduisent à une induction de dommages à l'ADN:

- 1) Induction directe de dommages à l'ADN par traitement à l'étoposide
- 2) inhibition de l'activité de ATR
- 3) déplétion génétique de lamin A/C

Dans les trois conditions, nous avons observé une augmentation de l'infectiosité par le VIH-1 dans les cellules contrôles. Dans les trois conditions, une surexpression simultanée des protéines SUN s'est avérée antivirale et a annulé l'effet proviral des dommages à l'ADN. Les résultats suggèrent fortement que les protéines SUN se trouvent en aval des dommages à l'ADN dans le contexte d'une infection par le VIH-1.

Nous avons observé un niveau réduit de mobilité de la chromatine avec la surexpression de SUN1. Nous émettons donc l'hypothèse que SUN1 pourrait rendre le VIH-1 incapable d'utiliser les dommages à l'ADN à son avantage et qu'il pourrait le faire en réduisant la mobilité globale de la chromatine. La mobilité de la chromatine est peut-être avantageuse pour l'infection par le VIH-1 pour accéder aux sites d'ADN endommagé et débloquent l'effet proviral que les dommages et le DDR ont sur une infection optimale. Le fait que cela se produise ou non de manière dépendante de Vpr dépend de la voie et de l'étendue des dommages à l'ADN.

Alternativement, la mobilité de la chromatine, déclenchée par des dommages à l'ADN, pourrait être nécessaire pour le VIH-1 pour accéder aux sites d'intégration optimale. La surexpression de SUN1 peut conduire à une intégration moins productive du VIH-1, en bloquant le mouvement de l'ADN.

La validation de nos résultats expérimentaux dans les cellules primaires a été assez limitée pour le moment. Nous avons confirmé les activités antivirales des protéines SUN dans les macrophages mais n'avons pas exploré les dommages à l'ADN.

En regardant le signal de  $\gamma$ H2AX, avec ou sans traitement à l'étoposide, dans les cellules T CD4+, nous avons immédiatement remarqué la présence d'un niveau de base constitutif de dommages à l'ADN et les pourcentages de cellules  $\gamma$ H2AX+ étaient comparables entre les cellules T CD4+ contrôles et celles qui surexprimaient SUN. Bien que nous ayons observé une activité antivirale de surexpression de la protéine SUN dans les cellules T CD4+, nous n'avons pas pu vraiment récapituler l'effet proviral du traitement par l'étoposide que nous avons observé dans les HeLa. Une limitation majeure vient du fait que les cellules primaires sont beaucoup

plus sensibles aux dommages exogènes à l'ADN. Des dommages sévères à l'ADN dans les cellules primaires déclenchent une cascade de réponses qui peuvent être beaucoup plus délétères que dans les lignées cellulaires cancéreuses.

Certaines questions sans réponse émergent de la formulation de notre hypothèse. Des études complémentaires sont nécessaires pour mieux caractériser et confirmer notre modèle. Les expériences futures peuvent inclure des COMET assays, la visualisation de l'effet de l'étoposide ou ATRi sur la mobilité de la chromatine et le séquençage de la sélection des sites d'intégration du VIH-1 dans les cellules qui surexpriment SUN.

Idéalement, ces questions doivent également être abordées dans les cellules T CD4+. Ces cellules se développent en suspension et prolifèrent à un taux élevé, ce qui pose des défis importants par rapport à les HeLa. Plusieurs approches expérimentales sont discutées.

Dans l'ensemble, les résultats obtenus au cours de ce projet mettent en évidence une interconnexion profonde entre les protéines de l'EN de l'hôte, la dynamique nucléaire sous-jacente et la modulation de l'infection par le VIH. Ils mettent en lumière comment des altérations qui sont plus mécaniques et biophysiques que biochimiques peuvent conduire à une infection productive. Les informations obtenues ici peuvent, entre autres, aider à comprendre pourquoi certains types de cellules offrent au virus un environnement nucléaire plus favorable que d'autres. Le projet fournit un nouvel aperçu sur la physiopathologie d'un virus qui a réussi à rester insaisissable sous de nombreux aspects, au cours des quarante dernières années. Cela pourrait ainsi ouvrir la voie au développement de stratégies thérapeutiques ou préventives qui prennent en compte aussi ces aspects biophysiques de l'interaction hôte-virus.

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## **APPENDIX**

## 8. Appendix

Cytokine and Growth Factor Reviews 40 (2018) 59–67



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## Let me in: Control of HIV nuclear entry at the nuclear envelope

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### ABSTRACT

The nuclear envelope is a physical barrier that isolates the cellular DNA from the rest of the cell, thereby limiting pathogen invasion. The Human Immunodeficiency Virus (HIV) has a remarkable ability to enter the nucleus of non-dividing target cells such as lymphocytes, macrophages and dendritic cells. While this step is critical for replication of the virus, it remains one of the less understood aspects of HIV infection. Here, we review the viral and host factors that favor or inhibit HIV entry into the nucleus, including the viral capsid, integrase, the central viral DNA flap, and the host proteins CPSF6, TNPO3, Nucleoporins, SUN1, SUN2, Cyclophilin A and MX2. We review recent perspectives on the mechanism of action of these factors, and formulate fundamental questions that remain. Overall, these findings deepen our understanding of HIV nuclear import and strengthen the favorable position of nuclear HIV entry for antiviral targeting.

### 1. Introduction

One of the remarkable properties of the Human Immunodeficiency Virus (HIV) is its ability to infect non-dividing cells. In non-dividing cells, the nuclear envelope functions as a physical barrier to separate the nuclear content from the cytoplasm. The nuclear envelope is interspersed with nuclear pore complexes (NPCs), which are assembled at the end of mitosis and mediate both passive and highly regulated active transport through the nuclear envelope. The current view of HIV nuclear entry sees the virus passing through the NPC to reach the nuclear interior and to integrate into the host DNA, a view that is supported by a vast series of genetic, biochemical and imaging studies.

Despite the highly protective barrier that is the nuclear envelope, and the highly gated nature of NPCs, HIV is remarkable in its ability to thwart these natural defenses. Nonetheless, this ability comes at a cost: the virus has a narrow range of mutations that it can tolerate, in particular in the viral capsid (CA), without losing this precious capability, and it depends in turn on multiple cellular factors to successfully enter the nucleus, such as Cyclophilin A, NUP153, NUP358, TNPO3, CPSF6 and SUN2. As part of their innate defenses, cells can also express type I interferon-inducible antiviral effectors that may limit HIV nuclear entry, such as MX2. Here, we review some of the latest contributions that focus on HIV entry into the nucleus (Fig. 1).

### 2. Role of viral determinants in nuclear import

The ability of HIV to enter the nucleus of non-dividing cells is not a characteristic shared by all retroviruses: Murine Leukemia Virus (MLV) requires mitosis to productively infect target cells [1]. To identify the elements specific to HIV that allow infection of non-dividing cells, chimeric viruses between HIV-1 and MLV were generated and it was observed that only viral particles containing HIV CA protein retained the ability to infect aphidicolin-mediated cell-arrested MAGI cells or terminally differentiated macrophages [2]. The inability of chimaeras lacking HIV-1 CA to infect non-dividing cells was linked with nuclear entry defects: while levels of viral reverse-transcripts detected by Real Time quantitative PCR (RT-qPCR) were comparable among chimaeras in dividing and growth-arrested HeLa cells, levels of 2-Long Terminal Repeats (2-LTR) circles, recognized hallmarks of nuclear entry, were reduced by more than 10-fold in aphidicolin-treated cells compared to controls for viruses lacking HIV CA. A later study also identified HIV-1 CA mutants that are defective in their ability to infect non-dividing cells; they also showed that sensitivities of these different mutants vary from one cell type to another [3]. Mutants T54A and N57A were consistent in their reduced ability to infect non-dividing and arrested cells [3]. In this case however, reductions of 2-LTR circles were not sufficient to explain the drastic reduction in infectivity in non-dividing cells. This suggests that block in infection by these mutants may not strictly be occurring at the nuclear entry step but rather at a later stage or be a result of disruption of multiple factors. Overall, this data suggests that

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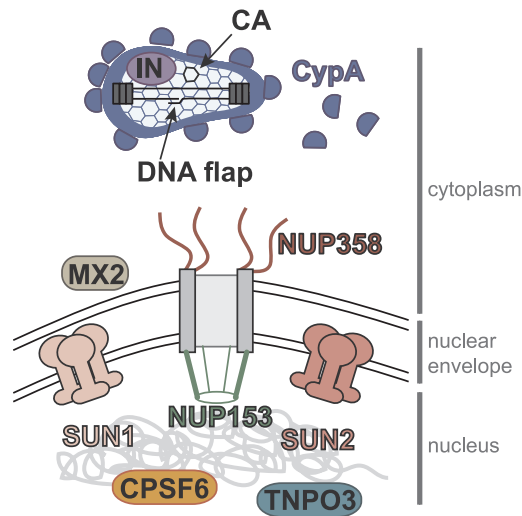


Fig. 1. Actors at the wall: main viral and host factors implicated in the control of HIV-1 nuclear import. Note that a single localization of each protein was selected, which may not reflect the dynamic localization events occurring in live cells.

CA plays a key role either during or after nuclear entry upon infection of non-dividing cells.

In addition to CA, integrase (IN) also plays a role in nuclear import. A non-canonical Nuclear Localization Signal (NLS) located on the IN has been identified by protein fusion and translocation assays in HeLa cells and shown to be key in the nuclear import of Pre-Integration Complexes (PICs) [4]. Indeed, viruses with integrases harboring non-functional NLS (IN mutants V165A and R166A) were inefficient at infecting a variety of dividing (peripheral blood mononuclear cells (PBMCs) and the T cell lines CEM-SS/CCR5 and C8166/CCR5) and non-dividing (monocyte-derived macrophages (MDMs) and microglia) cells [4]. RT-qPCR on integrated provirus species showed a 1000-fold reduction of integrated provirus with the mutated IN compared to wild-type controls while levels of RT cDNA were comparable among the two. The accumulation of 2-LTR circles was also reduced in IN mutant viruses. This suggests that IN NLS is required at a step prior to integration. These IN NLS mutants are, however, not catalytically inactive as one may expect from this result because they were able to rescue integration of viruses harboring catalytically dead IN (with the D64A mutation) in CEM-SS/CCR5 cells when present in trans as fusion proteins with Vpr. Thus, the NLS of IN likely contributes to nuclear import of HIV, before integration.

Another determinant that plays a role at the level of nuclear import is the DNA Flap, a plus strand overlap of around 99 nucleotides produced by a strand displacement within the central poly-purine tract (cPPT) of unintegrated linear HIV DNA, during reverse transcription. While rates of viral DNA synthesis are not affected in cPPT mutants lacking this central DNA flap, 90% of the viral cDNA accumulates as unintegrated linear DNA, instead of progressing to viral integration [5]. Fluorescent In-Situ Hybridization of full length viral DNA followed by confocal immunofluorescence imaging (P4 HeLa cells) or Transmission Electron Microscopy (TEM, in MT4 cells) showed that while signal from wild-type virus at the nuclear envelope is lost between 12 and 24 hours post-infection (hpi), concomitant with appearance of integrated viral DNA, signal from the Flap-defective mutant accumulates and persists at the cytosolic face of the nuclear envelope up to and beyond 72 hpi [5,6].

The requirement for the DNA flap in infection of non-dividing cells

was confirmed and appeared even more striking in primary cells including peripheral blood lymphocytes (PBLs), MDMs and monocyte-derived dendritic cells (MDDCs) [7,8]. Interestingly, in viruses with combined cPPT and IN mutations, it was observed that re-introduction of the cPPT could partially rescue infectivity, suggesting that it is sufficient for nuclear translocation [8].

### 3. HIV and NUPs

NPCs are large multi-component protein structures that span the nuclear membrane and are responsible for active and passive nucleocytoplasmic trafficking [9]. Nucleoporins (Nups) constitute a group of proteins that collectively compose the NPCs. Several Nups contain phenylalanine-glycine (FG) repeats that branch out towards both the nucleoplasmic and cytoplasmic regions creating a gelatinous meshwork that serves as a docking platform for receptor-cargo complexes and confers dynamic selectivity to the pore [10]. NUP153, initially identified by three genome wide screens aiming to identify host factors implicated in optimal HIV-1 infection, is now well established to play an essential role in HIV-1 infection across cell types [11–13]. Other NPC related proteins NUP358 (also known as RANBP2), NUP98 and NUP214 have also been proposed to participate to HIV nuclear entry, but their requirement is less firmly established.

NUP153 directly binds CA through its FG repeats [14]. shRNA-mediated knockdown of NUP153 led to a reduction of HIV-1 infectivity up to 2-log in P4-CCR5 cells and 8-fold in HeLa cells [15,16]. While no change was observed in levels of viral Late RT products by RT-qPCR compared to control cells, levels of 2-LTR circles and integrated provirus were significantly lower (2.3-fold and up to 7-fold respectively) upon NUP153 knockdown [15–17], suggesting that endogenous NUP153 plays a role in viral nuclear entry. This hypothesis was confirmed using an immunofluorescence based visualization of HIV-1 viral DNA [18]. This technique alongside co-staining of viral CA showed that CA enters the nucleus with vDNA in HeLa cells and in primary MDMs. Upon infection of HeLa T4 cells with NUP153 depletion, a 5-fold decrease of vDNA and CA nuclear entry was observed compared to control cells at 12 hpi.

Depletion of NUP358 by siRNA or shRNA led to a 6 to 8-fold reduction of HIV-1 infection in HeLa cells and P4-CCR5 cells [15,19,20] and RT-qPCR analysis on viral transcripts in infected SVGA, Magi or standard HeLa cells showed that the anti-viral effect is linked to nuclear entry considering that levels of 2-LTR circles were dramatically reduced upon knock-down of NUP358 compared to controls whereas levels of Late RT transcripts remained unaltered. NUP358 contains a Cyclophilin domain that can bind to the HIV-1 CA, however, this domain is not required in NUP358 for infection [21].

Interestingly, the knockdown of NUPs 98 and 214 leads to decreased HIV-1 infectivity but only depletion of NUP358 and NUP153 affects nuclear entry, as assessed by measuring 2-LTR circles in infected cells [15]. Immunofluorescence analysis based on p24 staining at 6 hpi performed in HeLa cells showed that depletion of NUP358 also leads to loss of signal at the nuclear rim suggesting that this nucleoporin may be involved in the optimal docking of viral CA at the nuclear envelope [15]. This hypothesis is consistent with the fact that NUP358 is the main cytoplasmically-oriented component of the NPC. The same results were confirmed by immunoblotting p24 in nuclear fractions after cell fractionation of infected cells with knock-down of NUPs 98, 214, 358 and 153 at 6 hpi (at this time point, p24 signal detected within the nuclear fraction corresponds to viral complexes docked at the nuclear envelope and not to intranuclear p24 [15,22]): nuclear p24 signal was distinctly low only in fractions of NUP358 knocked-down cells and not in those of control cells and knocked-down cells of the other nucleoporins. Overall, it can be hypothesized that both NUP358 and NUP153 play distinct roles in HIV-1 nuclear entry. While NUP358 may be required for docking of the viral complex at the nuclear envelope, NUP153, which is nucleoplasmically-oriented unlike NUP358, may play

an active role in viral nuclear entry.

An unexpected finding from these studies is that several Nups appear to participate in HIV-1 nuclear entry and very few single Nups appear to be strictly essential for entry itself. An interesting possibility is that FG-motif binding pockets in the HIV-1 CA [14] and perhaps in the HIV-1 integrase as well [17], play a collective role to enable entry of the HIV-1 PIC through the NPC, which could explain the discrepant requirement for Nups in the literature. The Nups required by HIV-2 for its nuclear entry are not yet extensively defined: loss of infectivity by a laboratory-adapted strain of HIV-2 was observed upon knockdown of NUP153 in HOS cells [14] and upon knockdown of NUP358 and NUP153 in PMA treated THP-1 cells [23]. Furthermore, knock-down of NUP153 and NUP358 in Jurkat and THP-1 cells led to reduced infectivity by HIV-2 primary isolates, with results showing patient to patient variability [24].

While the role of Nups in HIV-1 infection has been extensively studied in cancer cell lines, data in relevant primary CD4+ target cells is sparse. It is now well recognized that the NPCs are heavily remodeled in cancer [25]. It is thus critical that the role of Nups in HIV-1 infection be investigated in relevant primary immune cells.

#### 4. TNPO3 and CPSF6

Two other factors that have been shown to be required for HIV-1 infection and whose requirement has been mapped to the CA protein are the importin- $\beta$ -like karyopherin TNPO3 that promotes the nuclear import of serine/arginine-rich splicing factors (SR proteins) and the Cleavage and polyadenylation specific factor 6 (CPSF6) that is a component of the RNA maturation machinery [13,26–28].

In the case of TNPO3 knock-down, infection by wild-type HIV-1 is compromised in HeLa, Jurkat and primary CD4+ cells and the inhibitory effect has been correlated to the reduction of 2-LTR circle formation [29]. However, a decrease in integrated provirus that correlated to decreased infectivity was not consistently detected [29,30]. These results suggest that TNPO3 may not directly regulate nuclear import of the virus.

CPSF6 was found to be involved in HIV-1 infection through a cDNA expression screen for host restriction factors [26]. The screen identified a splice variant of CPSF6 that was truncated at the C-terminus, mCPSF6-358, capable of restricting infection by HIV-1 when expressed in NIH3T3.hCycT1 cells and primary CD4+ T cells. Truncation of the protein at its C-terminus beyond residue 526 is enough for loss of nuclear localization and induction of its antiviral activity. Intriguingly, upon CPSF6 depletion, infection levels were either unaffected or marginally affected in HeLa cells and THP-1 cells, suggesting that endogenous CPSF6 is not fully essential for HIV infection in the cell types tested [23,26]. The effect of mCPSF6-358 was mapped to HIV-1 CA. The viral mutant N74D is, however, capable of replicating efficiently even in the presence of stable mCPSF6-358 expression.

Interestingly, CA mutant N74D is also capable of escaping the antiviral activities of TNPO3 knock-down, NUP358 knock-down and NUP153 knock-down in HeLa cells [16,26,31], suggesting that this mutant is perhaps transported to the nucleus using a different pathway than wild-type HIV-1 and thus interacts with different host partners.

The roles of TNPO3 and CPSF6 are now considered linked: TNPO3 is a karyopherin that imports proteins with SR domains and the truncated mCPSF6-358 lacks precisely that. When TNPO3 is depleted, endogenous CPSF6 fails to localize at the nuclear envelope and is mostly cytoplasmic, like its truncated mutant [29]. When ectopically-expressed CPSF6 is retargeted to the nuclear envelope in TNPO3-depleted TZM-bl HeLa cells, infectivity of HIV-1 is rescued. This suggests that infectivity decrease in TNPO3-depleted cells might actually be a consequence of cytoplasmic re-localization of endogenous CPSF6.

Using imaging as read-out, individual loss of TNPO3 and CPSF6 leads to decreased nuclear entry of viral DNA and CA in HeLa-T4 cells but when depletions of the two proteins are combined, the decrease is

comparable to that of CPSF6 depletion alone [18]. As viral nuclear entry is reduced and not entirely abolished, it is presumable that other host partners besides NUPs, CPSF6 and thus TNPO3 may be involved in nuclear import. This is consistent with the fact that CA mutants N74D is able to enter the nucleus independently of these factors, as mentioned above.

#### 5. HIV and CypA

Cyclophilin A (CypA) is a cytoplasmic peptidyl-prolyl isomerase known to interact with HIV-1 CA protein [32] and be incorporated into nascent viral particles during virion assembly [33,34]. Interaction with CypA maps to HIV-1 CA residues Gly89 and Pro90 that are found within a proline-rich loop. Disruption of the CypA-CA interaction, using multiple experimental techniques including treatment with the competitive inhibitor immunosuppressive drug cyclosporine A (CsA), knockout or knockdown of endogenous CypA, use of CA mutants defective for CypA binding such as G89A and P90V, has consistently shown that CypA plays a key role in promoting HIV-1 infection in certain cell types but not others: CypA is required for optimal HIV-1 infection in Jurkat T cells, 293T, MT4, CEM, HOS, TE671 and human primary PBMCs, CD4+ T cells and MDM [35–42]. However, infection is CypA independent in other target cells such as HeLa and H9 T cells, for treatment with CsA does not significantly impact infection levels of wild-type HIV-1 compared to untreated cells [38,43,44]. Intriguingly, the positive modulation of HIV-1 infection is a consequence of the presence of CypA in the target cells rather than the CypA incorporated in the virion [37,39].

Repeated passages of infection in CD4+ HeLa cells treated with CsA selected viral mutants that are dependent on CypA for replication in this cell type [45]. Two such mutants are A92E and G94D, which are sensitive to CypA in HeLa and H9 cells and require CsA to replicate more efficiently (De Iaco et al. showed that addition of 5  $\mu$ M CsA increased replication of mutant A92E up to 11-fold in HeLa cells). In other cell types however, they are CsA-resistant because they can replicate even in presence of the drug, unlike wild-type HIV-1 [37,43,45]. Interestingly, CA mutants N74D and A105T are sensitive to CsA treatment also in HeLa and H9 cells, unlike wild-type HIV-1. When encoded in cis, they are not only capable of rescuing A92E's infectivity in HeLa cells in absence of CsA, they also render the virus sensitive towards CsA treatment, thus exercising a dominant effect [43,46,47].

Several attempts at understanding the mechanism behind CypA-mediated promotion of HIV-1 infection have been made but no unambiguous model has been identified, perhaps owing to the complexity of the cell type-specific phenotypes observed. Multiple studies have correlated the effect of CypA to an early phase of infection and to enhancement of reverse transcription, possibly through stabilization of HIV-1 CA cores as observed in Jurkat T cells [35,48]. However, a later study that included a panel of 27 cell lines concluded that the effects of CypA on viral core stability correlate better with nuclear entry rather than with reverse transcription [43].

Regarding nuclear import, viral mutants that are defective in binding CypA (G89V and P90A) are also insensitive towards NUP358 depletion but remain sensitive towards depletion of TNPO3 in HeLa cells [19]. Treatment with aphidicolin that leads to cell-cycle arrest doesn't further inhibit replication of G89V and P90A mutants, suggesting that the CypA-binding mutants may perhaps use a NUP358-independent route of entry in the nucleus. Furthermore, pyrosequencing analysis on HIV-1 integration sites showed that wild-type virus treated with CsA, or G89V and P90A mutations, directed integration towards sites with increased density of transcription units, defined as regions enriched in genes and associated features such as CpG islands, DNAaseI hypersensitive sites and high GC content [19] compared to wild-type HIV-1. It is thus inferred that disruption of CypA-CA interactions (and possibly NUP358-CA interactions) guides viral nuclear entry through a route that enhances integration in regions with high transcription unit density. However, it must be kept in mind that these

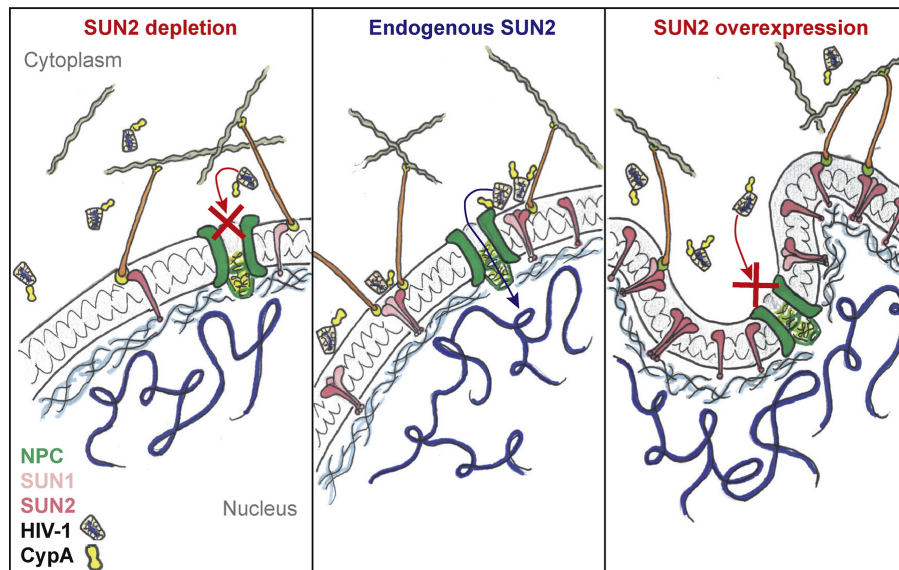


Fig. 2. A sweet-spot of SUN: HIV-1 depends on optimal levels of SUN2 for infection. At endogenous SUN2 levels (middle), HIV-1 enters the nucleus (depicted as viral particles docked at the NPC), leading to viral integration. Upon SUN2 depletion (left) or SUN2 overexpression (right), HIV-1 infection is impaired. In the case of SUN2 over-expression, the nuclear envelope is deformed (depicted as ruffles). Note that virus docking to the NPC at endogenous levels of SUN2 and the multimerization state of SUN1 and SUN2 have only an illustrative purpose and are not meant to indicate actual mechanisms.

mutants show reduced infectivity in most cell types, as described above: it could be speculated that since these mutants show reduced infectivity, as measured by viral expression, in most cell types, inefficient viral gene expression from these altered sites of integration could also contribute to the reduction.

Importantly, addition of CsA on infected HeLa cells rescued the infectivity defect of wild-type HIV-1 induced by shRNA-mediated depletion of NUP358 [19]. This suggested that the activities of CypA on HIV-1 guide the virus to use NUP358 for faithful nuclear entry and integration. It must be pointed out that these results were obtained in HeLa cells, in which CsA treatment has no inhibitory effects on wild-type viral replication. In primary cells such as MDM, CsA prevents replication of wild-type HIV-1 [19,41], suggesting that productive infection of a relevant target cell type relies on the presence of CypA. If the idea suggesting that CypA inhibition drives the virus to use alternative pathways of nuclear entry stands true, it must mean that these alternative pathways are more efficient in HeLa cells – even when they are cell-cycle arrested – rather than primary cells in which HIV-1 has naturally evolved to use co-factors like NUP358/NUP153 and TNPO3 for productive infection.

Recently, CA mutant viruses were identified that showed a consistent CsA-dependency in all cell types tested, including primary CD4<sup>+</sup> target cells of human and macaques: P86HA in HIV-2 (short HIVac-2; HIV CypA affinity-enhanced CA) and V86I-IAP91LPA-M96L in HIV-1 (short HIVac-1) [23]. These mutated viruses, and most dramatically HIVac-2, show an enhanced affinity for CypA, enhanced viral incorporation of CypA, and exhibit a profound defect in infection that is consistently rescued by inhibiting or disrupting CypA across cell types and species. For HIV-2, the mutated HIVac-2 had no defect in reverse transcription, but showed reduced levels of integrated DNA and 2-LTR circles in non-cycling CD4<sup>+</sup> dendritic cells. In contrast, CypA promoted the reverse transcription step of HIV-1 in primary CD4<sup>+</sup> T cells. Overall, results suggest that CypA can promote both reverse transcription and nuclear entry, in a context- and strain-specific manner.

Interestingly, when combined with second-site mutation N74D (N73D in HIVac-2), infectivities of HIVac-1 and -2 were restored and were rendered insensitive towards CsA in HeLa, GHOST and MDDCs. N74D and N73D are thus second-site mutations capable of escaping CypA's antiviral activity. Intriguingly, the rescue mutation did not reduce CypA incorporation in viral particles, suggesting that other host factors might be implicated in the process.

Altogether, there are two main possibilities to explain the diverse activities of CypA on HIV infection: either CypA has multiple and truly independent effects on HIV infection at various steps of the replication cycle (uncoating, reverse-transcription, nuclear import, integration, etc), or CypA regulates a singular molecular mechanism of HIV, that manifests itself variably according to context. Considering this latter possibility, it was proposed that reverse transcription and uncoating of HIV-1 is likely delayed until docking to the nuclear envelope in true primary cells [49]. In various cell lines, considering that these are cycling cancer cells, the kinetics and modalities of reverse transcription and nuclear import could be imperfect and misleading representations of the behavior of the virus in primary cells, thus resulting in inconsistent manifestations of contributions by CypA to HIV-1 infection. This strongly suggests that grasping the true nature of CypA activities on HIV infection will eventually demand a rationalized model that is relevant for primary target cells, instead of cycling cancer cells.

## 6. HIV and SUN

SUN1 and SUN2 (Sad and UNC domain containing 1 and 2), initially identified as UNC84A and UNC84B [50], are type II integral transmembrane proteins present in the Inner Nuclear Membrane (INM) of the nuclear envelope. They possess a conserved coxy-terminal SUN domain that stretches out in the perinuclear space in which it binds the Klarsicht-ANCL-Syne-homology (KASH) domain of proteins known as Nesprins, trans-membrane proteins of the Outer Nuclear Membrane (ONM) that branch out of the nucleus into the cytoplasm to bind

elements of the cytoskeleton such as actin, microtubules and intermediate filaments [51]. This protein complex is known as the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. In the nucleoplasm, SUN proteins interact with the Lamin meshwork underlying the nuclear envelope through its N-terminal domain [52,53] (Fig. 2).

SUN2 was initially included in a screen for interferon-stimulated genes (ISGs) as a result of its expression in a gene-expression study [54]. SUN2 remarkably turned up as a positive hit exhibiting selective antiviral activity against HIV-1 infection, without any significant effect on infection by other viruses tested (HCV, Yellow Fever Virus, West Nile Virus, Venezuelan Equine Encephalitis Virus and Chikungunya Virus).

However, further study showed that SUN2 expression is minimally induced by IFN- $\alpha$  in CHME microglial cells and MT4C5 lymphoid cells and is not induced by IFN- $\alpha$  in THP-1 cells, primary PBMCs and MDDCs or by IFN- $\alpha$ , - $\beta$ , or - $\gamma$  in primary CD4+ T cells [23,55]. Thus, SUN2 should not be considered an ISG. The expression of SUN2 across various tissues and cell types, in particular in primary CD4+ target cells, has not yet been studied in extensive detail.

The antiviral activity of ectopically expressed SUN2 was confirmed [23,55], considering that overexpression of SUN2 indeed led to reduced infection by HIV-1 and HIV-2 in HeLa cells, by HIV-1 in CHME cells (with up to a 95-fold inhibition of infection compared to control for a 2 ng/mL p24 viral dose and a 9-fold decrease for a higher, 72 ng/mL p24 dose) and in primary MDDCs (in which a 5-fold decrease of HIV-1 infection could be observed in SUN2 over-expressing cells compared to controls [55]). Overexpression of the homolog SUN1 also led to a 20-fold decrease in HIV-1 infection in U87 MG CD4<sup>+</sup>CXCR4<sup>+</sup> cells [56]. The infection block is independent of the route of virus entry considering that infection by both Env and vesicular stomatitis virus envelope glycoprotein (VSV-G)-pseudotyped viruses was blocked by SUN1 and SUN2. The antiviral effect of SUN2 on HIV-1 did not require viral integration [55]. RT-qPCR performed at 28 hpi on the various viral DNA species produced during a single round of infection of CHME cells showed that levels of viral reverse-transcription were unaffected with SUN2 overexpression. However, levels of 2-LTR circles were decreased by almost 3-fold in SUN2-overexpressing cells compared to controls, suggesting that SUN2 blocks the virus before or at the level of nuclear entry [55]. Levels of integrated provirus were also accordingly reduced. Similarly, RT-qPCR performed on control and SUN1 overexpressing U87 MG CD4<sup>+</sup>CXCR4<sup>+</sup> cells infected with HIV-1 GFP lentivirus showed that while levels of GFP transcripts were comparable amongst conditions, there was a 10-fold reduction of 2-LTR circles in SUN1 over-expressing cells compared to controls, suggesting that even the SUN1-mediated block of viral infection in this cell type occurs at or just prior to nuclear entry [56].

Donahue et al. identified an HIV-1 CA mutant in position 207 that is resistant to the antiviral activity of SUN2 towards NL4-3: when infected with this P207S mutant, SUN2 over-expressing CHME cells show < 2-fold reduction of infection as opposed to the 8-fold reduction observed with NL4-3 wild-type [55]. This particular mutant had already been identified previously to be an escape-mutant towards MX2-mediated restriction [57], suggesting that the two anti-viral activities may be partially overlapping. Intriguingly, the BRU strain of HIV-1 and several transmitter-founder viruses were also naturally insensitive to SUN2 antiviral activity in this study.

Strikingly, SUN2 over-expression, though not impacting cell viability, led to a less circular, ruffled nuclei phenotype in CHME and HeLa cells, up to the point of reaching a lobulated, flower-like nuclear shape in CHME cells [23,55]. No changes in nuclear morphology upon SUN1 overexpression have been reported so far.

Both changes in nuclear morphology and antiviral activity mapped to the N-terminal domain: SUN2 deletion mutants lacking the entire SUN domain retained their antiviral activity and the capacity to induce flower-like nuclei when over-expressed. On the contrary, the lamin-binding N-terminus of SUN2 is required both for the changes in nuclear shape and for the antiviral activity upon over-expression: indeed,

overexpression of deletion mutants lacking the N-terminus but retaining the trans-membrane domain (SUN2  $\Delta$ 1-158) led to cells with round nuclei and no observed antiviral activity [55]. However, the truncated SUN2 did not properly localize to the nucleus, suggesting that the antiviral effect and the impact on the nuclear envelope shape likely require proper localization of the protein. In the case of SUN1, deletion of the first 90 amino acids in the N-terminal domain abrogated all antiviral activity, while deletion of the carboxy-terminal SUN domain did not have an impact on reduction of infectivity [56]. Here, the N-terminal truncated SUN1 retains its transmembrane domain and nuclear localization and it can be concluded that the domain is truly required for antiviral activity in the nucleus. This kind of experiment suggested that the anti-viral activity of the over-expressed protein is independent of its interaction with KASH-proteins and thus with the cytoskeleton. This assumption was further validated by testing SUN2 point mutants S641E and Y707A in which interaction with KASH-domains is abrogated or by pre-treating control and SUN2 overexpressing cells with the microtubule-disrupting agent nocodazole [55]. A reduction in the antiviral block imposed by the overexpression of SUN2 compared to control cells could not be observed in any of the above-mentioned conditions proving that SUN2's antiviral activity is independent of the LINC complex – at least in over-expression conditions.

On the flip side, manipulation of endogenous expression levels of the protein also leads to an antiviral activity towards HIV in a cell-type dependent manner [23,55,56]. Endogenous levels of SUN2 don't seem to have an impact on infection in CHME, 293T and HeLa cell lines because shRNA-mediated depletion didn't lead to altered infectivity upon infection with HIV-1 [55]. However, when SUN2 was depleted in primary cells such as MDDCs and CD4+ T cells, infection levels were reduced for both single-round and replication-competent HIV-1 and HIV-2 viruses [23]. Furthermore, disruption of SUN2 in CD4+ T cells led to reduced viability, cell proliferation and activation compared to control cells [58]. This was striking because SUN2 knockout mice are viable, and this could suggest that the requirement for SUN2 in HIV infection may be intimately linked with an essential process of lymphocyte physiology. Interestingly, infection levels of CA mutant P207S are reduced to the same extent as wild-type CA in the case of SUN2 depletion suggesting that P207S mutant is not an escape mutant of the antiviral activity of SUN2 depletion as is the case for SUN2 over-expression [55,58]. SUN2 gene-disruption by CRISPR/Cas9 in THP-1 cells also led to decreased infection by HIV-1 [56]. Contrary to SUN2, CRISPR/Cas9 mediated SUN1 gene disruption in THP-1 cells didn't show any changes in infectivity, pointing to a SUN2-specific role on HIV infection when it comes to the endogenous protein.

Strikingly, SUN2 plays a role in the CypA-mediated regulation of HIV-1. In the context of SUN2 overexpression in CHME cells, the antiviral effects of SUN2 overexpression and CypA inhibition (either by CsA treatment or by protein knock-down) on HIV-1 infection weren't additive [55]. This was also observed in HeLa cells in which the CypA-dependent CA mutant N74D was tested: infectivity of mutant N74D decreased in the presence of SUN2 overexpression but treatment with CsA didn't decrease infectivity further [23]. Intriguingly, the G89V CA mutant was less sensitive to SUN2 overexpression than the wild-type, but remained nonetheless partially sensitive [55]: while SUN2 decreased infection by wild-type virus by 7.6 fold in CHME cells, the mutant was blocked only 2.5 fold, regardless of the presence or absence of CsA, further suggesting that CypA is involved in the infection block of SUN2 overexpression but also that additional activities of CypA, unrelated to its interaction with the viral CA, may be at play.

The endogenous levels of SUN2 also contribute to CypA's activities. In primary CD4+ T cells, as mentioned before, depletion of endogenous SUN2 led to decreased infectivity by HIV-1 and HIV-2 [23,58]. To address the role of CypA in this context, a non-immunosuppressive analog of CsA was used, CBS1 [59]. Indeed, CsA also targets the Calcineurin pathway, and in primary CD4+ T cells this results in immunosuppression and a strong inhibition of proliferation following T

cell receptor activation [60]. Consistent with what observed upon inhibition of CypA in primary CD4+ T cells, it was found that CBS1 decreased infectivity by HIV-1. Strikingly, the combination of SUN2 depletion with CypA inhibition did not decrease HIV-1 infectivity further, confirming that SUN2 mediates the positive effects of CypA on HIV-1 replication in CD4+ T cells. A second study challenged this conclusion on the basis of experiments performed with CsA [58]. The choice of using CsA as a CypA inhibitor in primary T cells is questionable considering its strong anti-proliferative and inhibitory effect on T cell activation [60]. The results were also analyzed in terms of linear fold changes of percentages of infected cells, which is not ideal to question functional interactions, because viral infection is a non-linear process by definition. In contrast, the computation of linear viral titers, from titrated infections, revealed the non-additive effect of SUN2 depletion and CypA inhibition [58]. Thus, it is our view that based on this data, it cannot be concluded that effects of SUN2 silencing impair HIV-1 infection independently of Cyclophilin A. It remains intriguing that SUN2 depletion impaired proliferation of T cells and not other cells [58], which we believe may point to the relevant pathway for understanding CypA activities on HIV infection in primary CD4+ T cells, rather than invalidating the link between CypA activities and endogenous SUN2.

Importantly, the role of endogenous SUN2 in mediating CypA activities was further confirmed in mouse cells by infection by HIVac-1 in BMDCs derived from *Sun2*<sup>-/-</sup> mice [23]. In wild-type control cells, infectivity of HIVac-1 was impaired compared to wild-type HIV-1 and could be rescued by CsA treatment. In *Sun2*<sup>-/-</sup> BMDCs, infection by HIVac-1 was largely rescued even without CsA treatment, proving that SUN2 is a cofactor for CypA mediated restriction of the HIVac CA in mouse primary cells. Intriguingly, transient depletion of SUN2 in human MDDCs, CD4+ T cells and THP-1 cells is not sufficient to rescue HIVac-1 infectivity [23,56] suggesting that a different regulation may be at play in these cells compared to mouse primary cells.

Overall, SUN1 and SUN2 emerge as critical regulators of HIV infection, both upon over-expression and at the endogenous level (Fig. 2). SUN2 and CypA are in functional interaction during HIV-1 infection in multiple, but not all, conditions of cell types and virus strains (Table 1). Importantly, endogenous SUN2 has the unique ability to promote CypA activities in HIV-1 infection in primary target cells. Finally, whether or not morphological changes of the nuclear envelope are directly implicated in the antiviral effect requires further study.

7. MX2 and HIV

The *Myxovirus resistance (Mx)* genes, initially identified in mice as type I IFN $\alpha$  induced influenza virus resistance genes (reviewed in [61]), encode conserved high-molecular-weight guanosine triphosphatases (GTPases) belonging to the dynamin superfamily. Humans encode two different Mx proteins, MX1 and MX2 that differ in localization and activity. While MX1 has been shown to have broad antiviral activity against RNA and DNA viruses [61,62], screens have identified MX2 to be restrictive only towards certain viruses such as VSV, mouse herpes virus type 68 (MHV-68), and HIV-1 [54,63].

Similar to SUN2, MX2 was first identified as an antiviral gene in an ISGs screen [54]. Later studies showed that MX2 plays an important role in the IFN $\alpha$  induced antiviral activity towards HIV-1 infection and is an HIV-1 inhibitor per se when over-expressed in cancer cell lines, including U87 MG CD4<sup>+</sup>CXCR4<sup>+</sup>, K562, SupT1 cells [64–66]. As was the case for SUN2, the effect of MX2 was independent of the route of viral entry.

Most studies reported that the inhibitory action of MX2 occurs prior to or at the level of nuclear import considering that in RT-qPCR assays on HIV DNA products, performed in U87 MG CD4<sup>+</sup>CXCR4<sup>+</sup> cells and HOS cells with overexpressed MX2, a decrease in levels of 2-LTR circles was observed [64,65]. However, defects in nuclear import do not fully explain the MX2-induced reduction in HIV-1 infectivity, suggesting that

Table 1 Interplay between SUN1/2 and Cyclophilin A across cell types upon HIV-1 infection.

HIV-1 Capsid (NLA-3)	Modulation of SUN expression	Targeting of Cyclophilin A	Cell Type	SUN-CypA functional interaction	Description	Reference
WT	SUN2 overexpression	CsA treatment	CHME	YES	Inhibition by CsA is not additive with SUN2 overexpression	Domahue et al. [55]
WT	SUN2 overexpression	CypA knock-down	CHME	YES	Inhibition by CsA is not additive with SUN2 overexpression	Domahue et al. [55]
N/4D	SUN2 overexpression	CsA treatment	HeLa	YES	Inhibition by CsA is not additive with SUN2 overexpression	Lahaye et al. [23]
WT	SUN2 knock-down	CBS1 treatment	Primary CD4+ T cells	YES	Inhibition by CBS1 is not additive with SUN2 knockdown	Lahaye et al. [23]
WT	SUN2 knock-down	CsA treatment	THP-1	YES	Inhibition by CsA is not additive with SUN2 knockdown	Lahaye et al. [23]
HIVac-1	SUN2 knock-out	CsA treatment	<i>Sun2</i> <sup>-/-</sup> Mouse BMDCs	YES	<i>Sun2</i> <sup>-/-</sup> phenocopies CsA treatment for rescue of HIVac-1 infection	Lahaye et al. [23]
WT	SUN2 knock-down	CsA treatment	Primary CD4+ T cells	YES	Inhibition by CsA is not additive with SUN2 knockdown	Domahue et al. [58]
HIVac-1	SUN2 knock-down	CsA treatment	MDDCs, Primary CD4+ T cells	NO	CsA, but not SUN2 knockdown, rescues HIVac-1 infection	Lahaye et al. [23]
HIVac-1	SUN2 knock-out	CsA treatment	THP-1	NO	CsA, but not SUN2 knock-out, rescues HIVac-1 infection	Schaller et al. [56]
HIVac-1	SUN1 knock-out	CsA treatment	THP-1	NO	CsA, but not SUN1 knock-out, rescues HIVac-1 infection	Schaller et al. [56]



the block could be occurring at multiple steps in a cell-type specific manner [57]. Of note, MX2 localization is likely regulated: it can accumulate at the cytoplasmic front of NPCs [67,68] and species-specific factors contribute to localization [57].

Overexpression experiments with various MX2 mutants that were deficient for GTP binding and hydrolysis showed that MX2's antiviral activity against HIV-1 is retained and is independent of its GTPase domain [64,65]. This is in contrast to what was observed for MX1, which requires GTPase activity for an efficient antiviral response against Influenza A virus [69].

The antiviral effect maps to the first 29 amino acids in the N-terminal region of MX2 [57]. Studies with chimaeric proteins generated from human MX2 and the canine MX2 (which doesn't possess any antiviral activity against HIV-1) showed that only chimaeras retaining the human N-terminus retain both nuclear localization at the level of NPCs and antiviral activity initially suggesting that localization of MX2 at the nuclear pore might be required for the protein's inhibitory action on HIV-1. Later studies however showed that there is no strict correlation between the two features: while antiviral activity does map to the N-terminal domain of MX2, nuclear localization is not necessary [70]. Using fusion-proteins based assays, the same study also mapped the nuclear localization signal of MX2 to residue K20 and showed that its disruption had no effect on antiviral activity. However, authors do not exclude the presence of another NLS outside residues 1–25 of MX2 that may play a role in HIV-1 restriction.

The N-terminal region of MX2 is required as a CA binding motif [70,71]; as a matter of fact, a triple-arginine motif in the first 25 residues of the N-terminus of MX2 is required for interaction with the CA and subsequent restriction [70,72].

Liu et al. attempted to identify HIV-1 mutants that were resistant to MX2 restriction using repeated viral passaging in the presence of MX2. They discovered that the CA mutation in position A88 allows the virus to completely resist the antiviral activity of overexpressed MX2. The fact that this mutation occurs within the CypA binding loop suggested that CypA may play a role in viral sensitivity towards MX2. In fact, disrupting HIV-1-CypA interaction using Csa or by targeting CypA using shRNA abrogated the antiviral activity of MX2 in SupT1 cells, implying that MX2 depends on CypA for restriction. In support of this notion, other CypA binding deficient mutants, G89V and P90A were also found to be insensitive towards MX2 activity [64,65]. Interestingly, co-immunoprecipitation experiments showed that CypA interacts with MX2 but not with MX1 [66]. However, a later study showed that MX2 does not require CypA for antiviral activity in all cell types as the same set of experiments performed in a different CD4+ cell line produced entirely different results [57].

Further studies by Busnadiago et al. led to the identification of many other MX2 resistant CA mutations lying both within and outside the CypA binding loop; amongst the latter the most notable are P207S, G208R and T210K. P207S is particularly interesting due to a species-specific MX2 resistance/sensitivity profile.

Moreover, the N74D CA mutant, which is incapable of binding CPSF6 and NUP153 [26], was also reported to have reduced sensitivity towards MX2 in cell lines [64,65]. It can be concluded that HIV-1 CA governs the sensitivity towards MX2 and co-factors that interact with CA at the level of nuclear import may impact, though not exclusively control, MX2-mediated restriction of HIV-1.

Based on the evidence that higher order structures and oligomerization are required elements for antiviral activity of MX1 [73,74] and based on existing structural data on MX2 [75], site-directed mutagenesis was performed on putative oligomerization sites in MX2 to understand whether formation of higher order structures is required for antiviral activity [76]. Over-expression of mutant proteins in U87 MG CD4+ CXCR4+ cells followed by HIV-1 infection showed that dimer interface mutants M574D, Y651D and F647D completely lost all antiviral activity while this was only lost partially in the case of mutant V578D. Chemical crosslinking experiments confirmed that M574D,

Y651D and F647D failed to assemble as high order oligomers and existed only as monomers while mutant V578D (with partial antiviral activity against HIV-1) inefficiently formed lower order oligomers. Intriguingly, fusion of MX2 N-terminal domain to unrelated dimerization domains also confers antiviral activity [72]. These results point to a clear requirement for the oligomerization of MX2 for antiviral activity against HIV-1.

Overall, MX2 is a potent inhibitor of HIV infection that seems to operate at the level of nuclear import in cell lines. However, whether the antiviral effect of MX2 topologically occurs at the nuclear envelope remains unresolved. Furthermore, to what extent MX2 is relevant for IFN-induced antiviral activities in primary CD4+ target cells has not been reported.

## 8. Conclusions

In conclusions, HIV is dependent upon a high number of cellular factors at the nuclear envelope for its replication, and human cells are well equipped with antiviral factors that can inhibit nuclear import as part of their innate immune defenses, such as MX2. In the case of SUN2, the virus appears to depend on a set level of the endogenous protein: its infectivity is impaired by either decreased or increased expression – a phenomenon referred to as hormesis. This highlights an Achilles' heel in the viral replication cycle and suggests that nuclear entry is an excellent target for therapeutic intervention. However, some fundamental questions remain and an unambiguous molecular model to explain how HIV enters the nucleus is still lacking. First, it is generally considered that the size of the HIV pre-integration complex is too large to pass through the NPCs [77], which could imply that the NPC must be remodeled to enable nuclear entry. Second, the nuclear entry of an infectious viral particle has never been directly observed. Third, despite its widely recognized implication in HIV infection of non-dividing cells, the mechanism by which CypA promotes HIV infection remains elusive. Fourth, the nuclear envelope can transiently rupture during migration in vivo of primary immune cells [78,79], revealing a dynamic nature of the nuclear envelope in non-dividing cells and challenging the view that the NPC is the only path that enables cytoplasmic material to enter the nucleus. Altogether, this reveals that our current outlook on HIV nuclear entry is insufficient to grasp its true *modus operandi*. This could suggest that a fundamental paradigm shift from the current view of HIV nuclear entry will be required. Deciphering how the nuclear envelope functions during HIV nuclear entry, in the light of host dependency factors and antiviral effects, is thus a pressing question, even more so now than ever, for our understanding of HIV.

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A. Bhargava et al.

Cytokine and Growth Factor Reviews 40 (2018) 59–67

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*In stemus itaque et perseveremus.  
Plus, quam profligavimus, restat,  
Sed magna pars est profectus, velle proficere.*

*Therefore, let us press on and persevere.  
There remains much more of the road than we have put behind us;  
But the greater part of progress is the desire to progress itself.*

***Epistulae morales ad Lucilium***  
Lucio Anneo Seneca

## RÉSUMÉ

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Lors d'une infection par le virus de l'immunodéficience humaine (VIH), le virus traverse l'enveloppe nucléaire (EN) pour s'intégrer dans le génome de l'hôte. Le VIH accède au noyau à travers les complexes des pores nucléaires (CPN). Cependant, le mécanisme sous-jacent n'est pas entièrement compris. Il a été récemment décrit par plusieurs groupes dont le nôtre, que SUN1 et SUN2, protéines structurales de la membrane nucléaire interne, jouent un rôle dans l'import nucléaire du VIH. Ces travaux ont montré que la modulation des niveaux d'expression de SUN1/2 inhibe l'infection par le VIH, révélant que le virus dépend d'un taux optimal de protéines SUN dans les cellules. La surexpression de SUN dans divers types cellulaires n'a pas d'impact sur la viabilité cellulaire, mais provoque une déformation du noyau et des invaginations de l'EN.

Nous observons que la surexpression de SUN1 et SUN2 conduit à une réduction de l'infection par le VIH-1 et le VIH-2 dans les cellules HeLa, ainsi que dans des cellules cibles du VIH, les macrophages primaires dérivés de monocytes et les cellules T CD4+. Nous avons validé aussi que les protéines SUN et la Cyclophiline A (CypA) interagissent fonctionnellement dans l'infection par le VIH.

Une spécificité a été observée : SUN1 réprime plus fortement le VIH-1 tandis que SUN2 inhibe préférentiellement le VIH-2. Ces activités spécifiques antivirales sont orientées par les domaines N-terminaux des SUN, qui interagissent avec les lamines. Toutefois, les lamines endogènes ne sont pas nécessaires à l'activité antivirale médiée par SUN.

En utilisant les cellules knock down de lamine A/C comme contrôle positif de la déformation nucléaire, aucune corrélation entre la déformation et l'infection n'a été trouvée. L'absence de lamine A/C, contrairement à la surexpression de SUN1/2, ne montre aucun effet antiviral.

De plus la surexpression de SUN1 a révélé des propriétés uniques des noyaux : une mobilité réduite de la chromatine et une signature de réparation de l'ADN réduite. L'induction exogène de dommage à l'ADN est bénéfique pour l'infection par le VIH-1 (mais pas le VIH-2). Néanmoins, cette induction dans les cellules qui surexpriment SUN1 n'influence pas l'infection du VIH-1.

Ainsi nos résultats suggèrent un rôle de SUN1 dans la modulation de la dynamique nucléaire, en lien étroit avec les voies de réparation de l'ADN, qui conduit à la régulation de l'infection productive du VIH-1.

## MOTS CLÉS

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VIH ; Enveloppe ; Noyau ; Infection

## ABSTRACT

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During infection by Human Immunodeficiency Virus (HIV), the virus crosses the nuclear envelope (NE) to invade the host genome. HIV gets access to the nucleus by passing through the nuclear pore complex (NPC). However, the underlying mechanism is not fully understood. It was recently described that SUN1 and SUN2, structural proteins of the inner nuclear membrane, play a role in the nuclear import of HIV. Modulating the levels of SUN1 or SUN2 inhibits HIV infection, revealing that the virus depends on a sweet-spot of SUN protein levels in cells. Intriguingly, increasing SUN protein levels in various cell types doesn't impact cell viability but causes deformation of the nucleus and ruffling of the NE.

We observed that overexpression of SUN1 and SUN2 led to reduced infection by both HIV-1 and HIV-2 in HeLa cells, primary monocyte-derived macrophages and CD4+ T cells, with the last two being physiologically relevant HIV target cells. We further validated that SUN proteins and Cyclophilin A (CypA) functionally interact in HIV infection.

A strain-specific selectivity was observed in the fact that SUN1 shows stronger restriction of HIV-1 while SUN2 preferentially inhibits HIV-2. These preferential antiviral activities were mapped to the N-terminal, lamin-binding domains of SUN proteins. However, endogenous lamins are not required for SUN-mediated antiviral activity.

By using lamin A/C knock down cells as a positive control of nuclear deformation, no simplistic correlation between deformation and infection was found: The absence of lamin A/C, unlike SUN1/2 overexpression, showed no anti-viral activity. Instead, we identified properties that were unique to SUN1 overexpressing nuclei: reduced chromatin mobility and a reduced DNA damage signature. We find that induction of exogenous DNA damage is beneficial for HIV-1 infection (but not HIV-2) in cells. This is not the case for SUN1 overexpressing cells where additional damage does not lead to increased infection, suggesting that SUN1 modulates HIV infection downstream of the DNA damage events.

Overall our results suggest a role of SUN1 in modulation of nuclear dynamics, with subsequent interplay with the DNA damage pathway, that leads to control of productive HIV-1 infection.

## KEYWORDS

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HIV ; Envelope ; Nucleus ; Infection