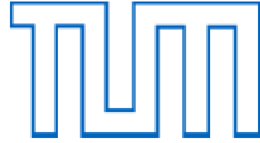


Fakultät für Medizin

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# The nuclear factor SPOC1 enhances HIV-1 integration but suppresses viral gene expression


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Vollständiger Abdruck der von der Fakultät für Medizin

der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

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Prüfer der Dissertation:

1. Univ.-Prof. Dr. U. Protzer
2. Priv.-Doz. Dr. Th. Floß


Die Dissertation wurde am 09.12.2014 bei der Technischen Universität München

eingereicht und durch die Fakultät für Medizin am 11.03.2015 angenommen.

# Contents

**Contents** ........................................................................................................ I
**Zusammenfassung** ........................................................................................................ IV
**Summary** ........................................................................................................ V
**1. Introduction** ........................................................................................................ 1
1.1 History and epidemiology of the human immunodeficiency virus Type 1 - HIV-1 ........ 1
1.2 Genome-Structure and morphology of HIV-1 ........................................................ 2
1.3 The replication cycle of HIV-1 ........................................................ 4
1.3.1 Adsorption and Entry ........................................................ 4
1.3.2 Uncoating and Reverse transcription ........................................................ 4
1.3.3 Nuclear Import and Integration ........................................................ 5
1.3.4 Repair mechanisms after HIV-1 integration ........................................................ 6
1.3.5 Viral biogenesis and egress ........................................................ 7
1.4 AIDS Progression ........................................................ 8
1.5 HIV-1 Vpr ........................................................ 10
1.6 SPOC1 ........................................................ 11
1.6.1 Possible functions of SPOC1 ........................................................ 13
1.7 Aim of this Study ........................................................ 16
**2 Materials & Methods** ........................................................ 17
2.1 Cell lines ........................................................ 17
2.2 Bacteria ........................................................ 17
2.3 Media ........................................................ 18
2.3.1 Media for cell culture ........................................................ 18
2.3.2 Media for bacteria ........................................................ 18
2.4 Nucleic acids ........................................................ 19
2.4.1 Oligonucleotides ........................................................ 19
2.4.2 Plasmids ........................................................ 19
2.4.3 Small interfering RNAs (siRNAs) ........................................................ 20
2.5 Enzymes, Markers & Dyes ........................................................ 20
2.5.1 Restriction endonucleases ........................................................ 20
2.6 Antibodies ........................................................ 20
2.6.1 Primary antibodies ........................................................ 20
2.6.2 Secondary antibodies ........................................................ 21
2.7 Reagents ........................................................ 21
2.7.1 Chemicals ........................................................ 21
2.7.2 Reagent systems (Kits) ........................................................ 22
2.7.3 Buffers and Solutions ........................................................ 23
2.7.4 Drugs & Inhibitors ........................................................ 24

2.8 Laboratory equipment ....24
2.9 Consumables ....26
2.10 Software and Databases ....27
**3 Methods ....28**
3.1 Microbiology Methods ....28
3.1.1 Cultivation of Bacteria ....28
3.1.2 Transformation ....28
3.1.3 Isolation of Plasmids ....28
3.1.3.1 Isolation of Plasmid-DNA via mini and midi preparation.... 28
3.1.3.2 Isolation of genomic DNA .... 29
3.1.3.3 Isolation of DNA from agarose gels .... 29
3.1.3.4 Measuring of nucleic acid concentrations .... 29
3.2 Molecular biology methods ....29
3.2.1 Polymerase Chain Reaction – PCR ....29
3.2.1.1 *Alu*-LTR Real-Time nested PCR .... 30
3.2.2 Gel electrophoretic separation of DNA and purification of DNA from agarose gels....31
3.2.3 Restriction of DNA and PCR Fragments ....32
3.2.4 DNA Fragment Ligation....32
3.2.5 DNA sequencing....32
3.3 Cell-Biological Methods ....32
3.3.1 Cultivation of Eukaryotic cells....32
3.3.1.1 Freezing of eukaryotic cells.... 32
3.3.1.2 Thawing of eurkaryotic cells .... 32
3.3.2 Isolation of mononuclear cells from peripheral blood ....33
3.3.3 Transfection of Eukaryotic cells....33
3.3.3.1 Transfection of cells via the calcium phosphate method .... 33
3.3.3.2 Transfection of cells via Lipofectamin™2000.... 34
3.3.3.3 Transfection of suspension cells via microporation .... 34
3.3.4 FACS-based FRET ....34
3.3.5 HIV-1 p24 Antigen capture assay - ELISA....35
3.4 Virological techniques ....35
3.4.1 Preparation of VSV-G pseudotyped virus stocks ....35
3.5 Biochemical Methods ....36
3.5.1 Cell lysis....36
3.5.2 Discontinuous SDS Polyacrylamide Gel Electrophorese (SDS-PAGE) ....36
3.5.3 Western Blot....36

**4 Results ............ 38**
4.1 Endogenous SPOC1 protein expression in HIV-1 target cells ............ 38
4.2 HIV-1 reduces SPOC1 steady-state levels ............ 39
4.3 SPOC1 interacts with HIV-1 protein Tat ............ 40
4.4 HIV-1 Vpr is important for SPOC1 reduction ............ 42
4.5 Transfection of single HIV-1 protein is not sufficient for SPOC1 reduction ............ 43
4.6 Transfection of full length HIV-1 does not lead to SPOC1 reduction ............ 44
4.7 Infection is essential for SPOC1 decrease ............ 45
4.8 Virion associated Vpr is able to reduce SPOC1 ............ 47
4.9 Integration of HIV-1 provirus is a central step for SPOC1 degradation ............ 48
4.10 SPOC1 is degraded via the proteasomal pathway ............ 49
4.11 GSK3-beta is important for SPOC1 degradation ............ 50
4.12 SPOC1 is reduced independently from the CUL4A ubiquitin ligase complex ............ 52
4.13 SPOC1 overexpression before infection enhances productive HIV-1 expression ............ 53
4.14 SPOC1 overexpression in human osteosarcoma cell line also enhances productive HIV-1 infection ............ 55
4.15 SPOC1 overexpression enhances HIV-1 integration ............ 58
4.16 Impact of SPOC1-knock down on HIV-1 infection ............ 59
4.17 SPOC1 overexpression post HIV-1 integration suppresses HIV-1 transcription ............ 60
4.18 SPOC1 depletion enhances HIV-1 gene expression in the context of infections with Vpr-defective HIV-1. ............ 62
**5 Discussion ............ 65**
5.1 Interplay between SPOC1 and HIV-1 ............ 65
5.2 Biological relevance of SPOC1 during the HIV-1 replication cycle ............ 67
**6 References ............ 72**
6.1 Internet References ............ 80
**Abbreviations ............ 81**
**Acknowledgement ............ 85**

# Zusammenfassung

Bis heute haben sich weltweit über 30 Millionen Menschen mit HIV-1 infiziert und sind an dem damit verbundenen erworbenen Immundefektsyndrom (AIDS) gestorben. Obwohl mehr als 25 spezifische antivirale Medikamente zur Verfügung stehen, ist bisher keine Heilung gegen eine Infektion möglich. Dazu leben die meisten Infizierten Menschen in Entwicklungsländern wo nur ein begrenzter oder überhaupt kein Zugang zu Therapien möglich ist. Hinzukommt, dass bis heute keine prophylaktische oder therapeutische Impfung zur Verfügung steht.

Die sehr Wirksamen und vielfältigen Mechanismen von HIV-1 um einer Immunantwort zu entgehen stellen den Hauptgrund einer bisher erfolglosen Impfung und das Anwenden von Therapien dar. Alternative Ansätze, zur Behandlung von HIV-1 könnten durch die Charakterisierung der Mechanismen von neu entdeckten Wirts- Restriktionsfaktoren, erhalten werden. Das kürzlich identifizierte Nukleoprotein SPOC1 (Survival time associated PHD finger in Ovarian Cancer 1), welches an Chromatin binden kann und wichtig für DNA Reparaturprozesse ist, könnte ein möglicher neuer antiviraler Restriktionsfaktor darstellen. Diese Studie befasst sich daher intensiv mit der Rolle von SPOC1 während des HIV-1 Replikationszyklus und charakterisiert seine potentielle antivirale Funktion.

Beachtenswerter weise führte eine SPOC1 Expression von HIV-1 Zielzellen vor Infektion zu einer verbesserten Infektionsraten als Folge einer höheren provirale Integration führt. Daher scheint SPOC1 ein Faktor zu sein, der die Integration des HIV-1 DNA in das Wirtsgenom positiv regulieren kann. Dennoch wurde SPOC1 von HIV-1 über das Proteasom in Abhängigkeit der GSK-3-ß Kinase Aktivität abgebaut. Bemerkenswert ist, dass dieser Abbau erst nach Integration des Provirus in das Wirtsgenom stattfindet und durch das akzessorische HIV-1 Vpr Protein vermittelt wird. Umfassende Versuche mit einer Überexpression oder siRNA vermittelten Reduzierung von SPOC1 in der späten Phase von HIV-1 induzieren, dass SPOC1 die Genexpression hemmend beeinflussen kann. Während SPOC1 die Integration in der frühen Phase positive regulieren kann, ist es für die späte virale Genexpression und die Neu-Synthese von HIV-1 Partikeln kontraproduktiv. Um diesen antiviralen Effekt entgegenzuwirken wird SPOC1 durch HIV-1 Vpr abgebaut.

Somit stellt SPOC1 keinen gewöhnlichen Restriktionsfaktor mit nur einer hemmenden Funktion dar. Vielmehr zeigt diese Arbeit zum ersten Mal ein Nukleoprotein welches eine Doppelfunktion auf unterschiedliche Schritte im HIV-1 Replikationszyklus hat. Dabei wendet sich diese Studie ein wenig gegen die vorherrschende Ansichtsweise von viralen Restriktionsfaktoren sowie Wirtszellfaktoren.

# Summary

HIV-1 infection of mankind is one of the most devastating diseases worldwide having caused over 30 million deaths due to the acquired immunodeficiency syndrome (AIDS). Although, up to date, more than 25 specific antiviral drugs are available there is no cure against the infection and the majority of infected people living in developing countries have no or only limited access to therapy. Furthermore, no prophylactic or therapeutic vaccination is available.

One major obstacle preventing the success of vaccination and leading to failure of the hosts' immune system to control the infection without therapy is the potent and diverse immune evasion exerted by HIV-1. Hence, it is crucial to identify and characterize novel mechanisms of host restriction that could ultimately help to engineer alternative approaches leading to effective HIV-1 control. Recently, a nuclear localized DNA binding protein termed SPOC1 (Survival time associated PHD finger in Ovarian Cancer 1) was identified and it was suggested that this protein which is capable to bind to chromatin and is further involved in DNA damage response could be a broad antiviral restriction factor. Hence, the task of this study was to characterize the role of the SPOC1 protein and its potential antiviral function for HIV-1 replication.

Of note, SPOC1 overexpression before HIV-1 infection of target cells resulted in enhanced infection rates as a consequence of higher proviral integration. Hence, SPOC1 seems to be a factor that positively regulates integration of the HIV-1 DNA into the host cell genome. Nevertheless, SPOC1 was degraded by HIV-1 through the proteasome dependent on the activity of nuclear kinase GSK3ß. This degradation occurred post integration of the provirus and was mediated by the accessory HIV-1 protein Vpr. Comprehensive experiments inducing SPOC1 overexpression or siRNA mediated knockdown post integration of the HIV-1 genome revealed that the presence of SPOC1 suppressed gene expression at this stage of viral replication. In conclusion, while SPOC1 positively regulates HIV-1 integration early post infection its presence is counterproductive for viral gene expression and de novo HIV-1 production at later stages. Therefore, HIV-1 Vpr degrades SPOC1 to counteract its antiviral activity.

Altogether, SPOC1 seems not to be a classical restriction factor exerting purely repressive effects throughout HIV-1 replication. In contrast, this work has identified for the first time a nuclear host cell factor with differential functions at various stages of viral replication. Thereby, the present doctoral thesis challenges the prevailing view and perception of antiviral restriction or beneficial host cell factors.

# 1. Introduction

## 1.1 History and epidemiology of the human immunodeficiency virus Type 1 - HIV-1

In the early 1980s the US Center for Disease Control and Prevention (CDC) reported a cluster of *Pneumocystis carinii* pneumonia (PCP) in five homosexual men for the first time [1; 2; 4]. The causative agent of the pneumonia is a yeast-like fungus, which particularly leads to lung illness in immunocompromised patients. Yet, the cause of immunosuppression in these patients was not clear, it was soon known as the acquired immunodeficiency syndrome (AIDS). Shortly after, the group of Luc Montagnier identified a new retrovirus which was isolated from human T-cells of AIDS patients. This new virus was then rapidly denoted as human immunodeficiency virus type 1 (HIV-1) [3; 12].

In 1983, the US Public Health Service released the first recommendations for AIDS prevention and 1985 the first enzyme linked immunosorbent assay (ELISA) test kit to screen for HIV antibodies was available [1]. Clevel and colleagues isolated the human immunodeficiency virus type 2 (HIV-2) from African AIDS patients in 1986 [5]. Also, Francoise Barré-Sinoussi and Luc Montagnier visualized the virus using electron microscopy technique and for this received the Nobel Prize in Medicine in 2008. However, the origin of HIV still puzzles scientist ever since. Today it is generally accepted that HIV has its roots in the simian immunodeficiency virus (SIV). In 1989 the group of Johnson *et al*. showed that HIV-2 corresponds to the SIV found in the sooty mangabey monkey, which is indigenous to western Africa [1; 6]. The more virulent, pandemic strain HIV-1 was not placed until 1999, when a captive member of the sub-group of SIV-chimpanzee (SIVcpz) known as *Pan troglodytes troglodytes* (P. t. troglodytes) was found which was almost identical to HIV-1 [7]. Geographic and phylogenetic analysis of HIV-1 and SIVcpz suggest that this zoonotic infection took place in central Africa in the early 20$^{th}$ century [1; 4; 7; 8].

Since beginning of the epidemic, around 75 million people have become infected with HIV and 36 million have died of AIDS-related illnesses, respectively. UNaids reports from 2013 showed that in 2012 35.3 million people lived with HIV, which makes 2.3 million newly infected patients [16; XI]. This is a yearly decrease of the infection rate by 33 % compared to 2001. The number of AIDS deaths also declined with 1.6 million in 2012, down from 2.3 million in 2005. Nevertheless, the rate of people living with HIV still rises (Figure 1).

Infection with HIV in the United States, Europe, central Africa and most other parts of the world are normally associated with taxa of HIV-1. This is because of its large variability categorized into four different groups: group M (Main) is the main causative agent of the AIDS pandemic and can be divided in the subtypes A-K. Whereby every subtype is special for a geographic location in the world. Group O (outlier) was discovered in 1990 and is much less prevalent than group M [9]. Only 13 cases of group N

(non-M, non-O) infection could be documented so far. This group is therefor even less prevalent than group O. It was originally discovered in 1998 in individuals from Cameroon [9; 10]. The fourth group was just recently discovered in 2009 in only 2 persons thus far [9].

Work in this thesis covers HIV-1 group M, since it is the predominant strain causing the AIDS pandemic. For simplification HIV-1 group M will be abbreviated as HIV-1.

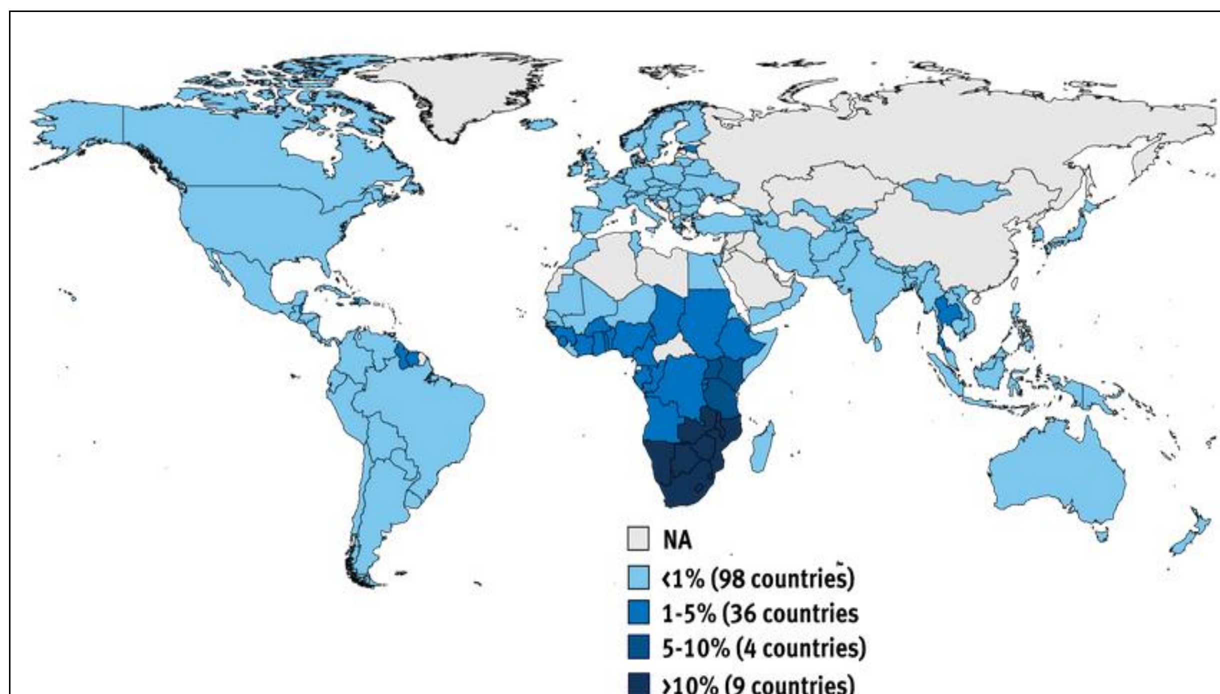


**Figure 1 Global adult HIV-1 prevalence rate in 2012**

The global HIV/AIDS prevalence rate is at 0.8 %. Prevalence rate include adults ages 15-49. Data are estimates. Color indicates prevalence of HIV/AIDS within the countries in %.
Figure modified from [16; XII].

## 1.2 Genome-Structure and morphology of HIV-1

HIV-1 belongs to the family of *Retroviridae* and to the genus *Lentivirus* and has a diameter of 100 - 120 nm. A single HIV-1 particle contains two copies of a single-stranded, positive-sense RNA, which is approximately 10 kb in length. The genome is at the 5'end modified with a Cap-group and at the 3'end it is polyadenylated. It comprises the primer binding (pb), the redundant (r), the unique U3' and U5', and at the 3'end a polypurin (pp) region. It encodes for 3 polyproteins Gag, Pol and Env, along with the proteins Tat, Rev, Vif, Vpr, and Vpu. The promoter for transcription, of the integrated provirus by the RNA-polymerase II, is located in the 5'LTR.

The capsid protein p24 builds the conical capsid and harbors the viral genome, the Pol subunits protease, integrase, reverse transcriptase and the accessory proteins Vif and Vpr. Furthermore, while the regulatory proteins Rev and Tat as well as the accessory protein Vpu are not packaged, the Gag subunit p6 is encapsulated by the virion.

The p17 matrix protein builds the inner coat of the virion, which is further enveloped by a cellular phospholipid bilayer in which the Gp120-Gp41 complexes are integrated [11].
The genomic region of the group specific antigens (gag) encodes for the matrix (MA-p17), capsid (CA-p24), nucleocapsid (NC-p7) and the p6 protein [11; 13]. Gag is able to form virus like particles by itself and is consequently the central structural protein of the virus.

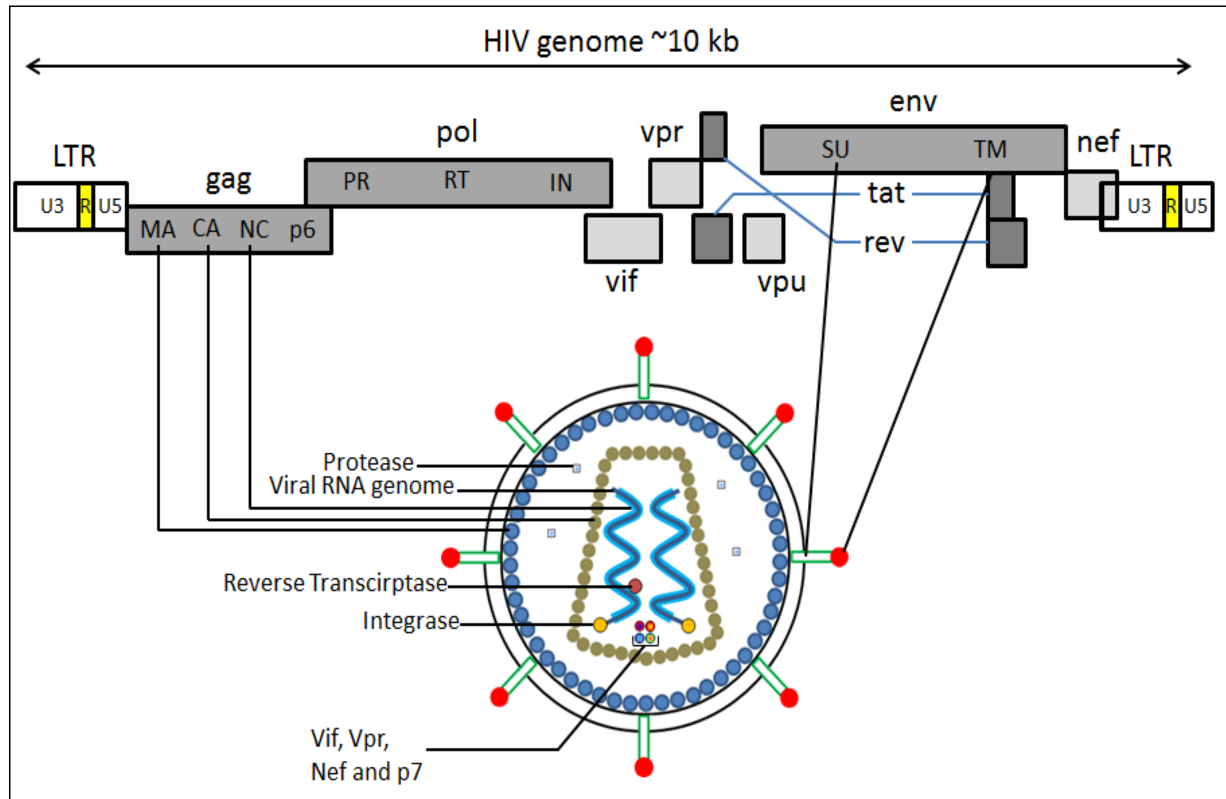


**Figure 2 Overview of the integrated provirus and a HIV-1 particle**

HIV-1 genome encodes nine open reading frames. These involve the viral structural proteins *gag, pol* and *env*; the essential regulatory elements *tat* and *rev*; and the accessory regulatory proteins *nef, vpr, vif, and vpu*. For more details see text.
Figure modified from [11].

The envelope (env) genes produce a precursor viral glycoprotein (gp160), which is processed into the surface (SU-gp120) and the transmembrane (TM-gp41) glycoprotein [11; 14]. Together these structural components make up the core of the virion and the outer membrane envelope.

Viral enzymes which are critical for viral replication are the three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN). These proteins provide essential enzymatic functions and are encapsulated within the virus particle. They are not functional in their monomeric forms and must therefore build either dimers (PR), heterodimers (RT) or tetramers (IN) to be catalytically active [11; 26].

Additionally to these genes, HIV-1 encodes for four proteins, termed as the accessory proteins. Three of them (virion infectivity factor [Vif], viral protein r [Vpr] and negative factor [Nef]) are found in the viral particle. Viral protein u (Vpu), which is only found in HIV-1 not in HIV-2, indirectly assists in assembly of the virion [28]. Vpr, Vpu and Vif are incomplete spliced products, and thus are expressed only during the late phase. The accessory proteins are not essential for viral replication *in vitro* systems, but are very important virulence factors *in vivo* [11; 27; XIII].

The transactivator of transcription (Tat) regulates mRNA transcription from the LTR and the regulator of expression of virion proteins (Rev) is important for nuclear mRNA export. In addition, these two proteins provide essential gene regulatory functions [11; 27; 28].

## 1.3 The replication cycle of HIV-1

### 1.3.1 Adsorption and Entry

HIV-1 targets different cell types within the body, including brain cells, but it mainly infects haematopoietic cells expressing the CD4 receptor and a co-receptor such as the C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4). This includes especially T-helper lymphocytes, macrophages, and dendritic cells [38]. For the binding process gp120 has to be present in its native conformation as a trimeric, glycosylated complex. Gp120 binds with high affinity to the CDR2 and CDR3 domains of the CD4 receptor. This triggers a structural change, which initiates a cascade of conformational changes leading to the fusion of the viral and cellular membrane. Finally, the viral core enters the cell's cytoplasm [29; 38].

### 1.3.2 Uncoating and Reverse transcription

A critical, but yet not fully understood step post entry stage of cellular entry, is the uncoating of the HIV capsid within the cytoplasm. The HIV-1 Core is composed of a polymer of capsid protein (CA), which encapsulates the RNA genome associated with NC, the enzymes RT and IN, as well as the proteins Vif, Vpr, Nef, and p7 (Fig. 3) [41; 11; 13].

The uncoating of the viral capsid requires several host cell proteins, trafficking pathways, and is a multistep process, which starts with loosening of capsid protein interactions or partial disruption of the capsid. Subsequently, the core strips of most or all CA monomers. The positive sense single-stranded RNA genome is then released into the cytoplasm where reverse transcription into double stranded DNA immediately starts. In 1970, Howard Temin and David Baltimore discovered and presented independently the enzyme reverse transcriptase (RT) which can generate DNA using RNA as template [42; 43]. The enzyme inherits a DNA polymerase activity that transcribes either RNA or DNA templates and RNase H activity that degrades RNA in a RNA-DNA duplex.

The reverse transcription starts with the annealing of the host primer tRNA, on the complementary sequence near the 5' end of the viral RNA called primer binding site (pbs) [44; 45].

The short RNA-DNA duplex serves as a substrate for RNase H, which removes the 5' end of the viral RNA, exposing the newly synthesized minus-strand DNA. This strand is then transferred to the 3' end of the viral RNA (template switching). Afterwards, synthesis of the full length minus-strand can continue. Simultaneously the RNase H degrades the RNA-DNA duplex. Initiated from the 3' polypurine tract (ppt), the RT generates a plus-strand DNA, next to the U3. After the first 18 nucleotides of the tRNA are copied, the RNase H removes the tRNA primer. This step leads to extension of the plus and minus strand which results in synthesis of the complete double stranded linear viral DNA and the long terminal repeats flanking the HIV-1 genome and serving as viral promotor [44]. One important step is the cleavage and the specificity of the RNase H at the end of the process, because it defines the end of the U3 LTR, which is used as a substrate for integration [44; 46].

### 1.3.3 Nuclear Import and Integration

In the late stage of the reverse transcription process, the reverse transcription complex builds up the preintegration complex (PIC), consisting of newly synthesized DNA, the Pol proteins RT and IN, the nucleocapsid- and matrix- protein, and the accessory protein Vpr. The HIV-1 Vpr proteins as well as the matrix protein p17, which is associated with the inner membrane of the virus, are involved in the transport of the PIC in the nucleus. The majority of retroviridae can only integrate their DNA into the nucleus during mitosis when the nuclear membrane resolves. However, the PIC of HIV-1 is actively transported into the nucleus also of non-dividing cells. The gap that occurs in the initiation and elongation process of the DNA secondary strand in the overlap region (flap) is necessary for the transport. A triple helix at the ppt interacts with proteins of the PIC and has to mediate the interaction with the components of the core pores by which PIC enters the nucleus. Next, the HIV-1 genome has to be integrated into the chromatin of the host cell. The HIV-1 enzyme Integrase is 288 amino acids in lengths and catalyzes the clipping of the host DNA and joining of the proviral genome to the clipped ends.

In a first step the enzyme acts on the ends of the viral DNA where it removes two nucleotides from the 3'-ends. It is possible that this step already occurs in the cytoplasm. In a second step, the two newly shaped 3'ends are linked to phosphor atoms in the backbones of the complementary strands of the host DNA [65].

### 1.3.4 Repair mechanisms after HIV-1 integration

One effect of this cleavage-ligation reaction is that the host cell DNA suffers a double strand break (DSB) at the ends of which are held together by single strand links to viral DNA. Post integration repair process is important for maintenance of host DNA integrity and for the genetic association of retroviral DNA with the host chromosomes. If the DNA is damaged and integrity and accessibility of essential information in the genome is lost, the cell functions are restricted. The cell has a variety of repair strategies, but most types of damage are repaired by lesion-specific pathways. To ensure that no damage escape correction may cause delaying of replication forks during S-phase the cell encodes robust system to sense and repair such damage [65].

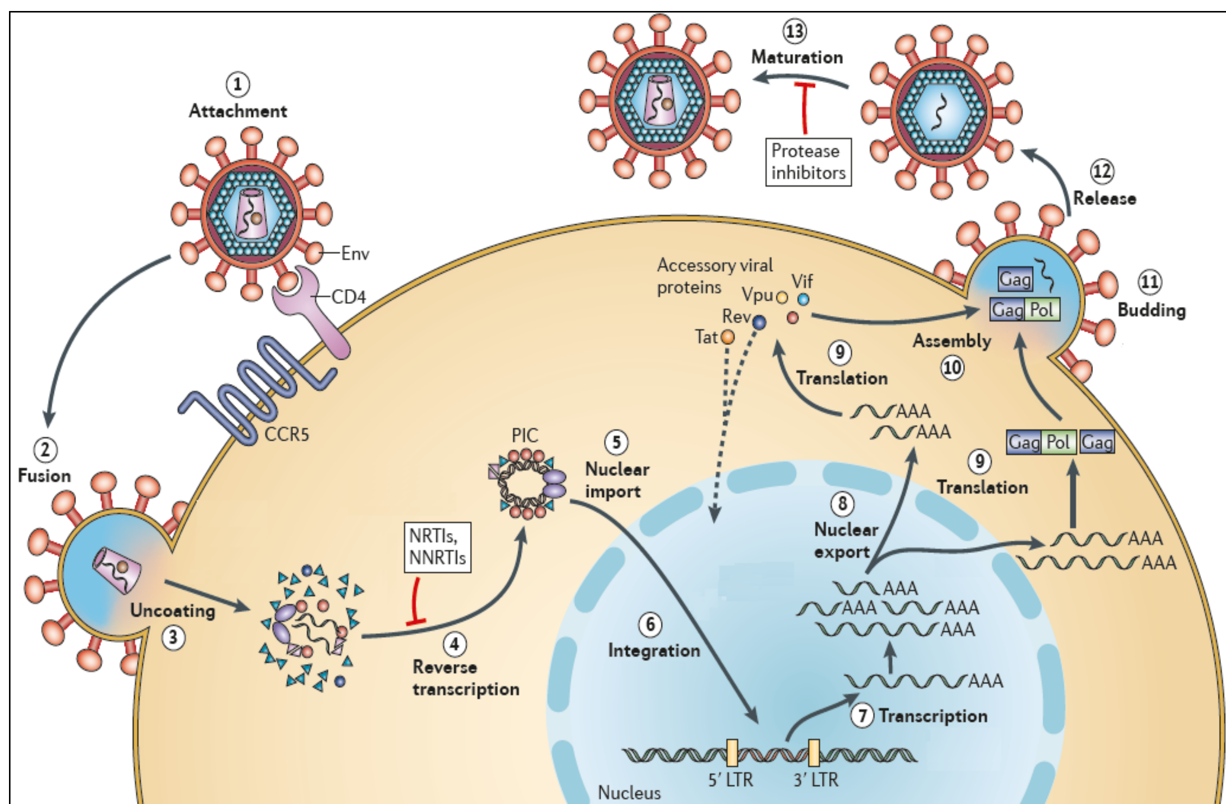


**Figure 3 Illustration of the main steps in the HIV-1 replication cycle**

(1) Binding to the CD4 receptor and co-receptor, followed by (2) the fusion with the host cell membrane and (3) uncoating of the viral capsid. After the HIV-RNA and proteins are released into the cytoplasm the reverse transcription takes place (4). Next, formation and translocation of the pre-integration complex (PIC) into the nucleus (5). Once in the nucleus the viral DNA is integrated into the host DNA (6) and subsequently transcribed and translated to form new viral particles (7-9). Virions are assembled and released (10-12). During maturation, the protease cleaves the structural polyprotein to form mature Gag proteins, resulting in the production of newly infectious virions (13).
Figure modified from [29].

The highly conserved PI-3K-related protein kinases, ataxia-telangiectasia mutated (Atm) and ATm-Radrelated (Atr) play a pivotal role [66]. Atm function appears to be most important for repair of DSBs, whereas Atr is critical for repairing DNA damage caused by UV or replication fork stalling. At this point it is unclear how these protein kinases are activated by DNA damage. Phosphorylation of target effector proteins by Atm and Atr is required for cell-cycle checkpoint response and recruits repair proteins. The induced DSB by HIV integration leads to the two major pathways for DSB repair in mammalian cells, which is the non-homologous end joining (NHEJ) and homologous recombination repair (HRR). Which of these pathways are favored after HIV integration is a subject of intense investigation [63; 64; 66].

In the NHEJ pathway, broken DNA ends are joined without a homologues chromosome as a template. A DNA-dependent protein kinase (DNA-PK) most likely holds the DNA ends together and the DNA Ligase IV, which forms a complex with the cofactor Xrcc4, joins the two ends. During this process of repair many proteins are phosphorylated, which is essential for their activation. NHEJ is important before the cell has replicated its DNA and can therefore take place from $G_1$- through $G_2$-phases of the cell cycle [62-64].

In the HRR pathway, broken ends are repaired by invading and copying sequences from a sister chromatid or a second intact DNA duplex. It mostly occurs when new DNA is replicated, which is often the case in $G_2$- and S-phases of the cell cycle. Repair promoted by HRR restores genetic content with high fidelity. The broken ends are processed and resected prior to loading of a Recombinase (Rad51). Strand invasion by the Recombinase filament produces a four-way “Holliday” junction which is migrated and processed to repair damage [63].

### 1.3.5 Viral biogenesis and egress

Successful integration of the HIV genome into the host cell chromosome is the basic requirement for HIV transcription. HIV has established some strategies to regulate the transcription and processing of its primary transcript. Many cellular factors operate and interact with the HIV LTR region which serves as HIV-1 promoter. Two Sp1 binding motifs and two nuclear factor NF-kB binding sites within the LTR regulate basal HIV transcription [53]. The LTR also includes binding elements for positive nuclear factor of activated T cells, AP-1, and negative, YY1 and LSF, transcriptional regulators. Some cytokines, such as tumor necrosis factor-α (TNF-α), can induce NF-kb and thereby activate HIV transcription in infected cells [54]. Epigenetic modifications at the LTR like acetylation or methylation can furthermore regulate HIV transcription.

The integrated proviral genome is transcribed by the host transcription machinery into a single 9.2kb pre mRNA from a complex promoter located within the 5’ long terminal repeat (LTR). The pre mRNA is alternatively spliced in mRNA coding for Tat, Rev and Nef. Tat and Rev translocate into the nucleus to support the transcription process. Tat binds to the Trans-activation response (TAR)-sequence of the mRNA and recruits the positive transcription elongation factor (P-TEFb). P-TEFb is a cyclin dependent kinase which contains Cdk9 and one of several cyclin subunits, cyclin T1, T2, and K. Among other things, it phosphorylates the carboxyl terminal domain of the large subunit of RNA polymerase II (PolII), which leads to efficient elongation of viral mRNA.

Next, Rev binds to the Rev-Response-Element (RRE), recruits the CRM1/Exportin1 to the mRNA and guides mRNA export out of the nucleus [55]. Translation of the HIV proteins and enzymes starts, producing a Gag precursor polyprotein, Pr55Gag. The Env-precursor protein gp160 is glycosylated in the endoplasmatic reticulum, from where it is further translocated and processed in the Golgi network by furin proteases to gp120 and gp41. After generation of the viral structural proteins and the enzymes of the RNA genome, the new virus is assembled at the cell membrane.

The unprocessed Gag precursor is targeted to the plasma membrane and promotes Gag-Gag interactions, encapsidates the viral RNA genome, associates with the viral Env glycoprotein, and stimulates budding from the cell. After release of the immature viral particle from the cell, the Gag-polyprotein is catalytically cleaved by the protease enzyme to produce the capsid protein p24, the matrix protein p17, the nucleocapsid protein p7, and the protein p6. At the end, by rearrangement of the Gag-subunits, the p24-capsid get its final conical capsid shape, the newly formed virus particle matures and becomes infectious [56; 57].

## 1.4 AIDS Progression

Although the risk factors vary, most of the newly acquired HIV infections are transmitted heterosexually. Injection drug users (IDUs), homosexuals, especially men who have sex with men and prostitutes are special risk groups. The immunopathogenic mechanisms of HIV infection and progression to AIDS are well-documented. AIDS progression can be segmented into three different stages: first the acute infection, second the clinical latency stage, and finally AIDS. Once the Virus enters the body, it infects primarily cluster of differentiated CD4+ T cells where it replicates rapidly.
After primary infection, most people develop an acute retroviral syndrome. Patient often suffer from flu-like illness, which is often misdiagnosed for acute infectious mononucleosis or viral hepatitis [18]. In this first, acute infection stage, the number of HIV-RNA copies/ml is very high in the plasma which is also called viremia. Simultaneously, the number of CD4+ T cells declines dramatically [17; 18; 20; 21].

After initial viremia, the host immune system is able to control viral infection with CD8+ positive T cells and B cell produced antibodies [19; 20]. While HIV levels in the blood are now strongly reduced, simultaneously the CD4+ T cell counts recover.

During the clinical latency stage, a person infected with HIV may remain free of HIV-related symptoms for several years despite the fact that HIV continues to replicate in the lymphoid organs where it initially seeded. Synchronously there is a steady decline in the number of CD4+ T cells lymphocytes [20].

As the CD4+ T cells count drops to <200 cells/mm³ plasma, the patient reaches the stage of AIDS and becomes particularly vulnerable to serious opportunistic infections and cancer. These opportunistic infections cause the death of the patient within months to few years [4; 20; 21].

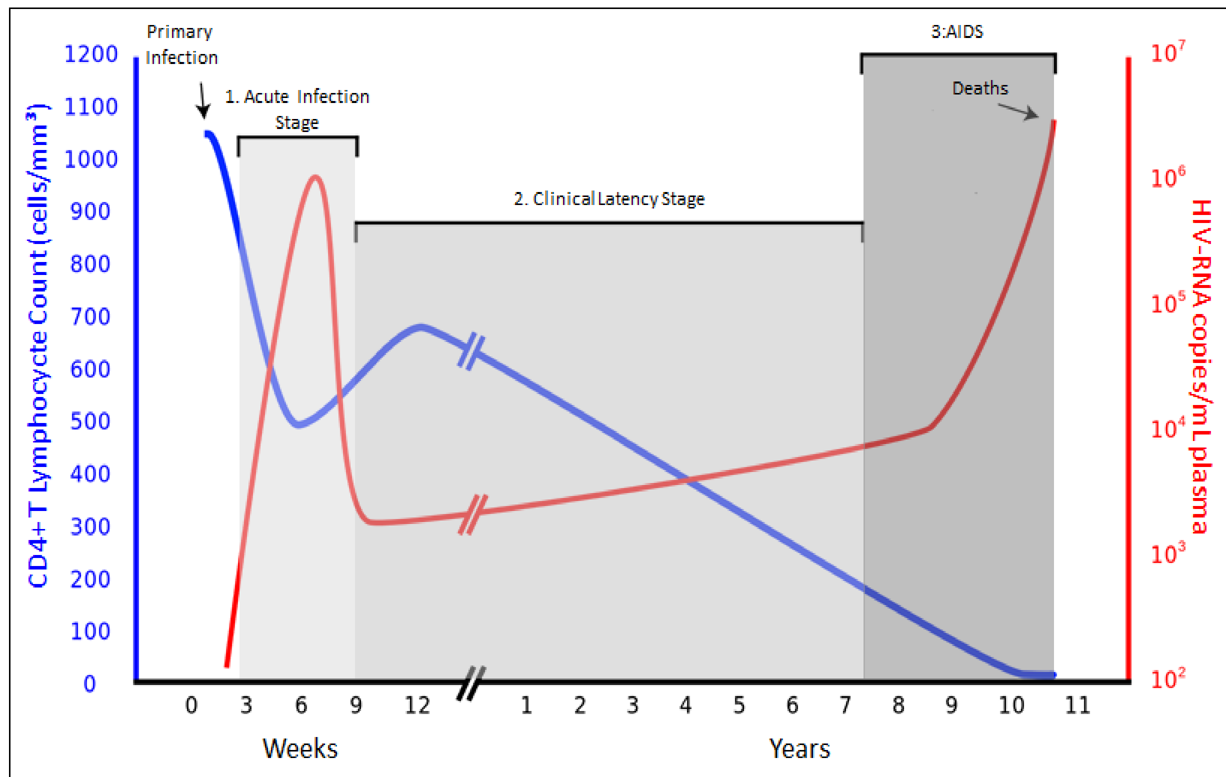


**Figure 4 Clinical progression of HIV-1**

Graphs shows the association between the levels of viral load and CD4+ T cell counts over the average course of untreated HIV infection. The course of HIV infection is divided in 3 stages. First stage is the acute infection, followed by the clinical latency stage and finally the AIDS stage. For more information see text.
Figure modified from [21].

In 2012, around 9.7 million of the infected people had access to antiretroviral therapy (ART) as the only possible treatment against this deadly course of disease progression [16; XI]. Highly active antiretroviral therapy (HAART), which is a potent combination of three or more antiretroviral drugs (nucleosidic-and non-nucleosidic reverse transcriptase inhibitors, protease inhibitors, entry inhibitors, and integrase inhibitors) belonging to at least two different antiretroviral drug classes, can efficiently lower the viral load and viral set point for those infected with HIV. With this treatment it is possible to expand the chronic phase theoretically to the natural end of patient's life. Nevertheless, it is not possible to eradicate HIV within the body, because of latently infected cells and slowly replicating HIV in persistently infected cells.

## 1.5 HIV-1 Vpr

The HIV-1 Vpr protein consists of 96 amino acids, has a size of 14 kDa and is highly conserved among HIV-1 and HIV-2. This rather small protein has multiple effects and many interactions of Vpr and host cell factors are described but not characterized in detail yet [95; 96]. It was shown that Vpr is packaged in significant quantities (appr. 300 molecules) into the virion by direct interaction of Vpr with the C-terminal p6 region of the Gag- precursor protein [95]. This observation suggests that many of its functions are important during the early phase of the infection. Vpr molecular functions include nuclear import of viral preintegration complex (PIC), influence of the reverse transcription of HIV-1, induction of $G_2$ cell cycle arrest, modulation of T-cell apoptosis, transcriptional coactivation of viral and host genes, and regulation of nuclear factor kappa B (NF-kB) activity [96; 97]

Several studies showed that Vpr co-localizes with the viral nucleic acids and IN with purified HIV-1 reverse transcription complex (RTC) and remains associated with the viral DNA within 4-16 h after infection [96]. In addition to a potential role in the initiation step of the reverse transcription process, it has been shown that Vpr modulates the in vivo mutation rate of HIV-1 by influencing the accuracy of the reverse transcription. Mutation rate was 4-fold higher in the absence of Vpr expression when measured in dividing cells [96].

The viral genome is reversely transcribed in full length viral double-strand DNA which is associated with viral and host cell proteins into the pre-integration complex (PIC). HIV-1 PIC can enter the nucleus by an active process without causing structural damage to the nuclear envelope. It was shown that Vpr is important for PIC assembly, localization of the PIC to the nucleus, and the nuclear import of the viral genome [96; 97]. Thus, HIV-1 is able to infect resting cells, such as macrophages [99]. Since Vpr has no NLS domain, Vpr most likely supports PIC translocation into the nucleus by an unknown mechanism which does not depend on the classical NLS- or M9 pathway.

Furthermore, Vpr has the ability to induce $G_2$ cell cycle arrest by targeting a general cellular mechanism that controls progression from $G_2$ to mitosis. First studies showed that Vpr leads to the accumulation of the hyperphosphorylated form of the cyclin-dependent kinase CDC2. This inactive form of the complex would be able to block the cell cycle before mitosis [96; 98]. Hence, arrest of cells within the $G_2$ phase could be a viral strategy to improve HIV-1 replication and protein expression, and even to reactivate the virus through an epigenetic control of the LTR promotor. Accordingly, the HIV-1 LTR seems to be more active in the $G_2$ phase.

Vpr does not only induce expression of viral, but also cellular proteins. A function of Vpr could be related to the modulation of viral gene expression in the transactivation of the HIV-1 LTR (long terminal repeat). The LTR region is modulated by both the p53 and transcription factors Spl. The transcription factor Spl is important for transactivation by Vpr [100], whereas p53 inhibits the activation by Vpr.

Furthermore, Vpr could enhance HIV-1 LTR activation by interaction with the transcriptional cofactors p300 / CBP. Transcriptional cofactors of the p300 / CBP family increase gene expression by at least three independent signaling pathways. The chromatin structure is decondensed by the histone acetyltransferase activity. Moreover, the host transcription machinery can be recruited to the LTR and act as adapter proteins. Lastly, they bind to Vpr and can be directly guided to the HIV-1 LTR [101].

Altogether, Vpr is a multifunctional HIV-1 accessory protein which associates with the viral PIC, modulates transcriptional activation and generally has positive effects in the early phase of infection. From a mechanistic point of view, Vpr is an adaptor protein that interacts with a Cul4A ubiquitin ligase complex. It is conceivable, that by this strategy Vpr might target cellular genes for proteasomal degradation and/or influences their stability and signaling. However, the biological significance of this interaction and the cellular targets remain elusive.

## 1.6 SPOC1

Survival time associated PHD finger in Ovarian Cancer 1 (SPOC1) is a human gene identified in 2005 by Mohrmann *et.al.* [22]. It is described to be located on chromosomal region 1p36.3, a region which is implicated in tumor development and progression [22]. The SPOC1 protein is 300aa in length, has a predicted molecular mass of 34.4 kDa and is conserved across higher eukaryotes. It contains a conserved centrally located bipartite nuclear localization signal (NLS) from residue 110-127, two PEST domains at amino acid residues 52-88 and 141-190, a conserved C-terminal Plant-Homeo-Domain zinc finger (PHD domain) from amino acid 232-280 and a conserved N-terminal domain from residue 20-70 of unknown function, but which can also be found in the related PHF23 protein [15] (Figure 5).

It was shown by Northern blot hybridization assay that SPOC1 RNA can be detected in most human tissues. The highest expression of SPOC1 was found in the testis, where it has been only found in spermatogonia. Apart from that, SPOC1 RNA expression is weak in spleen, thymus, prostate, ovary, small intestine, colon, and peripheral blood [22].

The NLS within proteins are amino acid sequences which mark a protein for import into the cell nucleus by nuclear transport. It was shown that the NLS sequence of the SPOC1 protein is at least partly responsible for its nuclear localization [15].

PEST-domains are associated with a short intracellular half-life of proteins. They have been identified in transcriptional regulators, key metabolic enzymes and cell cycle regulating proteins. PEST sequences are rich in proline (P), glutamic acid (E), serine (S), threonine (T), and a lesser extent aspartic acid [15; 40]. Structural proteins which are considered to be stable and fulfill their functions in the nucleus generally lack a PEST sequence. In contrary to that, regulatory proteins with a specific and temporary function in the nucleus possess at least one PEST sequence [61]. Indeed, Kinkley *et al*. showed that SPOC1 ΔPEST1 or ΔPEST2 resulted in a markedly stabilized protein [15].

Eight glycogen synthase kinase 3 beta (GSK-3-β) phosphorylation motifs (SxxxS) are predicted to exist within the SPOC1 amino acid sequence, six of them directly overlap with the two PEST domains [15]. It has been reported that GSK-3-β phosphorylation sites are important for regulation of protein stability for a variety of labile proteins and in some cases by directly cooperating with the ubiquitin proteasome system [67]. GSK-3-β is a serine/threonine kinase which belongs to the glycogen synthase kinase family. The enzyme phosphorylates the hydroxyl group of serine or threonine substrates which have to be pre-phosphorylated by an additional priming kinase [15; 67].

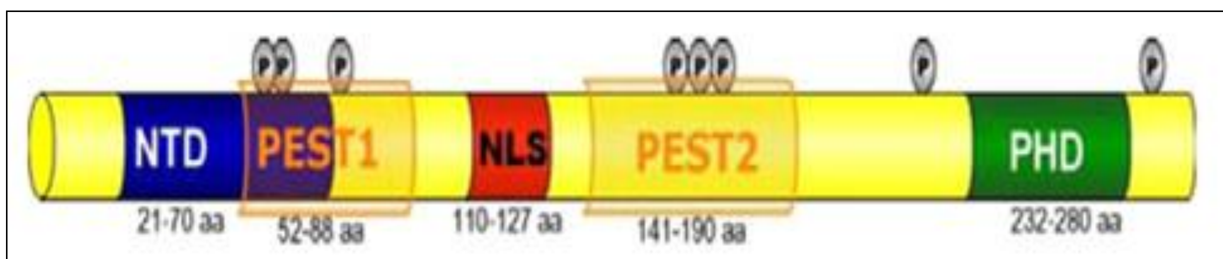


**Figure 5 Schematic illustration of SPOC1**

The SPOC1 protein is 300aa in length and contains a centrally located bipartite NLS, two PEST domains, a C-terminal PHD and a conserved N-terminal domain of unknown function.
Figure based from [15].

Characteristic for the PHD domain is the short sequence motif of about 50-80 aa and a unique $Cys_4$-His-$Cys_3$ pattern. Also, PHD domains are found in less than 150 mammalian proteins and their functions are ranging from transcriptional activators, repressors to chromatin remodeling enzymes HATs (Histone acetyltransferases), HDACs (Histone-Deacetylases), demethylases and methyltransferases. Many of the PHD proteins are chromatin-associated and can interact with N-terminal methylated histones and act therefore as epigenetic readers, capable of modulating and regulating chromatin structure and function [22; 39]. Thus the predicted C-terminal PHD domain of SPOC1 implicates a chromatin related function.

### 1.6.1 Possible functions of SPOC1

The first study concerning the protein expression and function of SPOC1 showed that endogenous soluble SPOC1 is susceptible to degradation and therefore has a very short half life time [15]. In contrast to that, SPOC1 is strongly stabilized when it is associated with chromatin. One reason for the short half-life time of the protein is the presence of two PEST-domains, which can confer susceptibility to degradation. Exogenous SPOC1 was markedly stabilized if one of the PEST domains is deleted [15]. In line with that, Kinkley *et al*. showed that the GSK-3-ß motifs, which lay within the PEST domains, are also important for the regulation of SPOC1 and its degradation. This was presented by inhibitor-assays for GSK-3-ß, which resulted in stabilized SPOC1 expression in comparison to the appropriate control. Moreover, it was shown that SPOC1 is degraded through the proteasomal pathway [15].

SPOC1 is a nuclear protein and has the ability to bind to H3K4me2/3 (Histone 3 lysine 4 methylation 2/3) and to regulate chromatin specific interactions through its PHD domain [15; 23]. Additionally, SPOC1 interacts with the KRAB-associated protein 1 (KAP-1, also known as TRIM28) and H3K9 methyltransferases (KMTs), inhibits KAP-1 phosphorylation and enhances H3K9 trimethylation [23]. Mund *et.al* described SPOC1 to be able to transform H3K4me2/3 containing chromatin into a more condensed form, by chromatin mediated increase of H3K9 KMTs and H3K9me3 [23].

H3K4me3 is found on promoters of actively transcribed genes, particularly shortly downstream the transcription start site. In contrast, H3K9 trimethylation (H3K9me3) is highly correlated with repression of gene expression by promoting the formation of heterochromatin, where DNA is inaccessible to transcription factors [59]. Previous work about SPOC1 revealed that overexpression of wild/type SPOC1 leads to condensed and less accessible chromatin, whereas siRNA mediated reduction of SPOC1 resulted in less condensed and a more accessible genome architecture. The correlation between SPOC1 protein levels and H3K9me3, as well as expression of several H3K9 KMTs, implicates that SPOC1 is involved in chromatin condensation and DDR as well [22]. KAP-1 is a nuclear co-repressor for KRAB domain-containing zinc finger proteins (KRAB-ZFPs) [68].

It is described to mediate gene silencing by recruiting the nucleosome remodeling and deacetylation (NuRD) histone complex (HDAC), the histone methyltransferase SETDB1, and the heterochromatin-associated protein HP-1 to the promoter regions on target promoters [69; 70; 71]. KAP-1 belongs to the tripartite motif (TRIM) protein family which displays antiviral properties (Figure 6) [71].

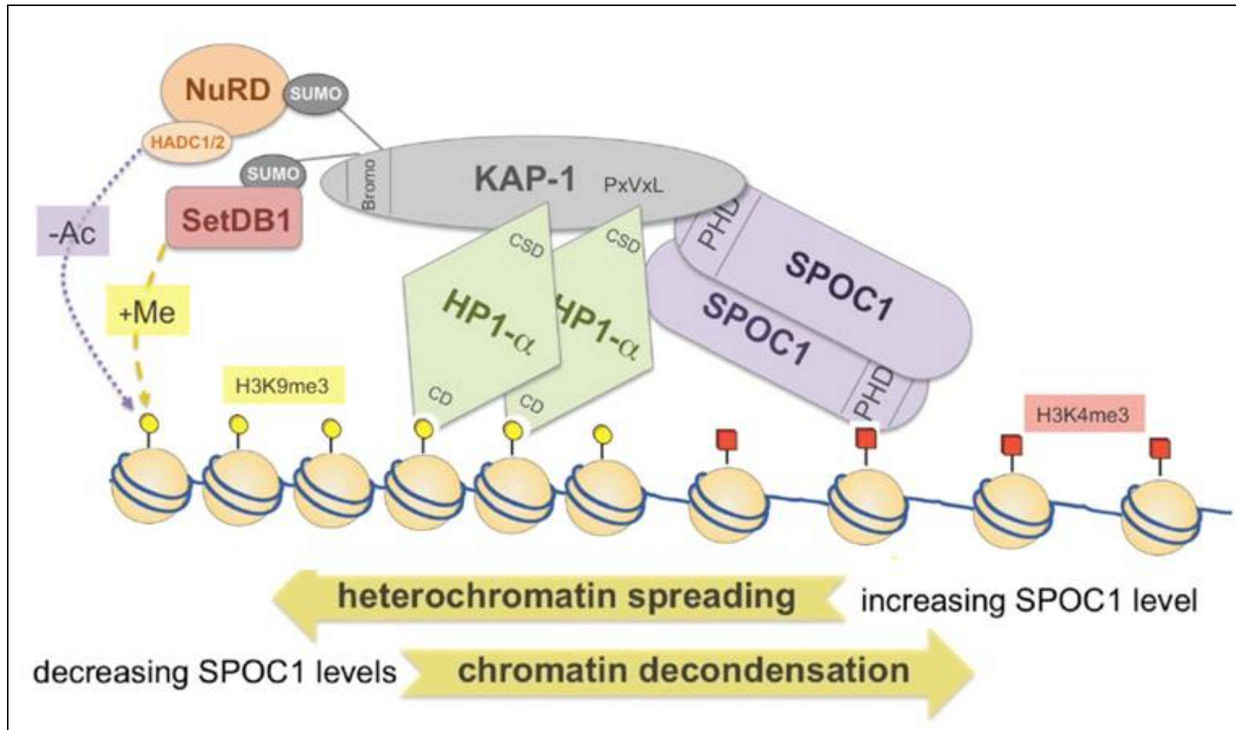


**Figure 6 SPOC1 binding and interaction partners**

SPOC1 binds to H3K4me3 through its PHD-domain and recruits KAP-1 and HP1-α, which leads to the deacetylation and methylation of the chromosome and consequently to heterochromatin spreading. In contrary, decreasing SPOC1 levels leads to chromatin decondensation. Figure kindly provided by Andreas Mund.

It was also shown that SPOC1 modulates chromatin structure and is important for chromosome condensation during mitosis. Particularly during prophase and anaphase SPOC1 associates with mitotic chromosomes [15]. It is dynamically regulated during the cell cycle and mitosis. Whereas in early $G_1$-S phase transition as well as early S phase SPOC1 expression levels are significantly decreased, SPOC1 levels are increased during late $G_2$ and M phases. This indicates a potential functional role of SPOC1 during both late $G_2$ and mitosis. In line with this, it was shown that the expression level and localization of SPOC1 are very dynamically regulated during the cell cycle [15].

It is known that SPOC1 is cooperating with chromatin modifiers and DDR (DNA damage respone) regulators. Cells undergo a coordinated cascade of events after DNA damage (compare with 1.3.4.). After gamma-irradiation, SPOC1 is recruited to DSBs and influences the DDR [23]. These events maintain genome stability and are critical for preventing tumorigenesis.

If the DNA is damaged, the DDR is activated, which in turn induces cell cycle arrest, and ensures DNA repair or apoptosis. Such a DNA damage site and the recruited DDR proteins can be visualized as repair foci. It was demonstrated that SPOC1 is recruited and accumulates to I-SceI-induced DSBs in an ATM-dependent manner, as well as to OPT (Oct-1, PTF, transcription) domains that arise at sites of DNA damage [23]. The minority of radiation-induced DSBs are repaired with slower kinetics through a process that requires ATM and accumulates at ionizing radiation induced foci (IRIF) [72]. The Histone variant H2AX is phosphorylated by ATM kinase in response to DSBs. Next, H2AX associates with the MRN complex, leading to further chromatin alterations and focal recruitment of additional DDR mediators, including 53BP1. Both H2AX and 53BP1 play definite roles in DDR initiation and DNA repair of heterochromatin. SPOC1 also accumulates at radiomimetic drug and gamma-IR induced repair foci with slow repair kinetics, providing the idea that SPOC1 contributes to structural changes in chromatin required of DSBs in heterochromatin [23].

SPOC1 has the ability to inversely affect NHEJ and HR activity (see also 1.3.3). It was shown that, after SPOC1 knockdown, NHEJ were approximately 20 % more active but there was no change in HR activity. Otherwise, SPOC1 overexpression decreased NHEJ activity by 40 %. Those results from Mund *et al.* showed that SPOC1 can shift the balance between NHEJ and HR [23].

The SPOC1 gene is located in chromosomal region 1p36.23, a region with frequent heterozygous deletions implicated in tumor development and progression [73]. Consistent with this, elevated SPOC1 RNA levels in primary and recurrent epithelial ovarian cancers have been associated with decreased survival rates in patients [22]. Moreover, SPOC1 RNA can be detected in most human tissues, with the highest levels in the testis, where it has been exclusively detected in spermatogonia [22; 24].

Most recent work by Schreiner *et al*. identified SPOC1 as a novel host restriction factor which is targeted by human adenovirus type 5 (Ad) during viral infection. SPOC1 repressed viral gene expression and was depleted after infection with Ad. Schreiner and Coworkers could demonstrate that SPOC1 is degraded by the proteasomal pathway mediated by the E1B-55K/E4orf6 E3 ligase complex of Ad. SPOC1 overexpression leaded to decreased adenovirus yield, viral DNA synthesis and viral protein synthesis. Additionally it was shown that SPOC1 repressed gene expression at the level of transcription. Of note, interaction of the adenovirus structural viral core protein pVII initially stabilized SPOC1 protein levels before expression of any viral early proteins [25]. Interestingly, the group presented data that infection with other human pathogenic viruses (HSV-1, HSV-2, HIV-1, and HCV) also led to a reduced SPOC1 expression.

Overall, SPOC1 is a multifunctional chromatin-associated protein, associated with DNA repair and chromatin organization. It is recruited to DSBs and regulates the kinetics of DSB repair. Further functions are related with stem cell differentiation and oncogenesis. In line with this, high SPOC1 RNA levels in primary and recurrent epithelial ovarian cancers have been associated with decreased survival

rates in patients. Additionally, SPOC1 is described to be a novel host restriction factor in a variety of different human viruses. It repressed viral gene expression and was depleted after infection with Ad and other human pathogenic virus.

## 1.7 Aim of this Study

SPOC1 (Survival time associated PHD finger in Ovarian Cancer 1) is a novel multifunctional nuclear localized protein capable of modulating chromatin architecture and double-strand break repair (DSB) mechanisms [15]. SPOC1 could have a variety of potential roles during HIV-1 replication since chromatin structure is important for HIV-1 Integrase activity and DSB pathways are important for integration of viral DNA into the host cell‘s genome. SPOC1 was previously described as a possible novel host restriction factor for human adenovirus type 5 and HIV-1 also depleted SPOC1 in infected PM-1 cells [25]. In line with this, pre-tests confirmed HIV-1 mediated SPOC1 degradation in a concentration dependent manner. However, the mechanisms of SPOC1 degradation by HIV-1 or the biological relevance of SPOC1 expression for the HIV-1 replication cycle remains unclear.

Initially, the goal was to measure the endogenous expression pattern of the SPOC1 protein in HIV-1 target cells as well as SPOC1 degradation by HIV-1 in CD4+ relevant T cell lines. Furthermore, one aim was to assess the effects of SPOC1 knock down or overexpression in T-cells on productive HIV infection. Since HIV-1 induces SPOC1 degradation, it was followed up on the hypothesis that viral proteins might directly interact with SPOC1. Furthermore, to narrow down which viral determinants are necessary for SPOC1 degradation, HIV-1 variants carrying inactivating mutations in different genes were used to infect T cells and monitor SPOC1 levels.

Since SPOC1 is associated with DNA repair, regulates the kinetics of DSB repair and leads in increasing amounts to heterochromatin spreading, another aim of this work was to exam a possible role of SPOC1 during the integration process of HIV-1. Finally, following up on previous findings that SPOC1 represses gene expression of human adenovirus, similar effects were investigated in the context of HIV-1.

Altogether, the aim of this thesis is to elucidate the biological relevance of the chromatin remodelling factor SPOC1 for HIV-1 replication and the underlying mechanism leading to its degradation.

# 2 Materials & Methods

## 2.1 Cell lines

| Name | Description | Reference |
|---|---|---|
| U2OS | Human osteosarcoma cells | [15] |
| U2OS- Clone 5 | U2OS cells stably expressing SPOC1 under the control of a tetracycline promoter | [15] |
| HEK 293T | Human embryonic kidney cell line, transformed with Adenovirus Typ 5, expresses the SV40 (simian virus 40) large T-Antigen. | [30] |
| Jurkat | Human leukemic T cell line | [32] |
| Jurkat-TAg | Human leukemic Jurkat T-lymphocytes stably transfected with the adenovirus large-T antigen | [32] |
| J-Lat | J-Lat 8.4 is a clonal cell line of Jurkat T cells latently infected with the Env-defective molecular HIV-1 clone HIV-R7/E-/GFP encoding for a GFP open reading frame in place of the nef gene | [74] |
| PBMC,<br>Macrophage,<br>CD4+ | Peripheral blood mononuclear cells isolated from blood including CD4+ T-lymphocytes, CD8+ T-lymphocytes, B-lymphocytes, NK-lymphocytes, monocytes and macrophages | |
| SupT1 | Human lymphoblastic T cell line | [31] |

## 2.2 Bacteria

| Name | Description | Company |
|---|---|---|
| One Shot® Top10 | Chemic competent Escherichia coli F- mcrA Δ(mrr-hsdRMS-mcrBC)ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL(StrR) endA1 nupG | Invitrogen, Karlsruhe, Germany |
| XL10-Gold Ultracompetent Cells | Tet$^{r}$ Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte* [F´ *proAB lacI*$^{q}$ZΔM15 Tn*10 (Tet*$^{r}$*) Amy Cam*$^{r}$*].* | Agilent Technologies, Inc., USA |

## 2.3 Media

### 2.3.1 Media for cell culture

| Cellline | Media | Supplementary |
|---|---|---|
| **Adherent** | | |
| HEK 293T | DMEM | 10 % heat inactivated FBS,<br>Penicillin/Streptomycin antibiotic-mix (120 µg/ml) and<br>350 µg/ml L-glutamine |
| U2OS<br>U2OS-Clone 5 | | + HEPES buffer solution (100mM/ml) |
| **Suspension** | | |
| Jurkat<br>Jurkat-TAg<br>J-Lat<br>PBMC | RPMI | supplemented with 10 % heat inactivated FBS,<br>Penicillin/Streptomycin antibiotic-mix (120µg/ml),<br>350µg/ml L-glutamine and<br>1mM Sodium Pyruvate |
| Macrophages | RPMI | supplemented with 4 % (v/v) human AB-serum,<br>the antibiotics penicillin [120 µg/ml] and<br>streptomycin [120 µg/ml],<br>MEM non-essential amino acids solution 10 mM,<br>MEM sodium pyruvate,<br>MEM vitamins and 350 µg/ml L-glutamine |
| Freezing Media | | 10 % (v/v) DMSO, 50 % Media, 40 % (v/v) FBS |

### 2.3.2 Media for bacteria

| Name | Components | | |
|---|---|---|---|
| Luria-Bertani-Medium (LB- media) | 10.0 | g/l | bacto trypton |
| | 5.0 | g/l | yeast extract |
| | 8.0 | g/l | NaCl |
| | 1.0 | g/l | glucose |
| | The pH was set to 7.2 with NaOH<br>Addition of 100 mg/l ampicillin or kanamycin | | |
| LB- agar plates | 15 | g | agar ad 1l LB media |
| | 1 | mg/ml | ampicillin or kanamycin |
| SOC- medium | 20.0 | g/l | bacto trypton |
| | 5.0 | g/l | yeast extract |
| | 2.5 | mM | NaCl |
| | 10.0 | mM | MgCl2 |
| | 10.0 | mM | MgSO4 |
| | 20.0 | mM | glucose |

## 2.4 Nucleic acids

### 2.4.1 Oligonucleotides

| Name | Restriction site | Sequence (5'-3' direction) |
|---|---|---|
| SPOC1-pCG-For | XbaI | CG*TCTAGA*ATATGGACTCTGACTCTTGCG |
| SPOC1-pCG-Rev | MluI | CT*ACGCGT*TCAGTCCAGGAACAGCTTCCGG |
| SPOC1-pE_C-term For | NheI | CCG*GCTAGC*ATGGACTCTGACTCTTGCGCC |
| SPOC1-pE_C-term Rev | AgeI | TCG*ACCGGT*GCACCTGCTCCGTCCAGGAACAGCTT |
| SPOC1-pE_N-term-For | EcoRI | CGC*GAATTC*ATGGACTCTGACTCTTGC |
| SPOC1-pE_N-term-Rev | BamHI | T*GGATCC*GGTCAGTCCAGGAACAGCTTCCG |
| LTR-3’ | | TGCTAGAGATTTTCCACACTGACTAAAAGGG |
| LM677-opti | | ATGCCACGTAAGCGAAACTCTGGCTAGCTAGGGAACCCACT |
| Alu-1 | | TCCCAGCTACTGGGGAGGCTGAGG |
| LambdaT | | ATGCCACGTAAGCGAAACT |
| β-globin-for | | ACACAACTGTGTTCACTAGC |
| β-globin-rev | | CAACTTCATCCACGTTCACC |

### 2.4.2 Plasmids

| Name | Description | Reference |
|---|---|---|
| pBR-NL4-3 IRES-eGFP | pBr322 backbone, encoding HIV-1-NL4-3-IRES GFP HIV-1 NL43 provirus with an eGFP under the control of an IRES | [49] |
| pBR-NL4-3 env* | pBr322 backbone, that has the HIV-1 NL43ΔEnv (GP160 is deleted) | [52] |
| pBR-NL4-3 IRES-eGFP ΔVpr | pBr322 backbone, with the HIV-1 NL43 provirus and a HIV-Vpr deletion | [51] |
| pBR-NL4-3 IRES-eGFP ΔNef | pBr322 backbone, with the HIV-1 NL43 provirus and a HIV-Nef deletion | [51] |
| pBR-NL4-3 IRES-eGFP ΔVpu | pBr322 backbone, with the HIV-1 NL43 provirus and a HIV-Vpu deletion | [51] |
| pEYFP-C1/N1 | Clontech YFP expression vectors | [49] |
| pECFP-C1/N1 | Clontech CFP expression vectors | [49] |
| pEYFP-CFP | FRET positive control, YFP tagged CFP | [49] |
| pHIT-G | *Vesicular Stomatitis Virus* (VSV-G) expression vector | [50] |

### 2.4.3 Small interfering RNAs (siRNAs)

| Name | Target Sequence (5'-3' direction)/description | Refernce |
|---|---|---|
| siSPOC1 | UCA CCU GUC CUG UGC GAA A | Riboxx |
| Control siRNA | iBONi siRNA Negative Control-N2 | Riboxx |

## 2.5 Enzymes, Markers & Dyes

| Dyes | Company |
|---|---|
| Ethidiumbromide | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Gel Loading Dye, Blue (6x) for DNA | New England Biolabs (Ipswich, USA) |
| Bromphenole blue | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |

| Enzymes | Company |
|---|---|
| GoTaq® Green Master Mix | Promega (Madison, USA ) |
| Pfu Turbo DNA Polymerase | Stratagene ( La Jolla, USA) |
| Phusion DNA Polymerase | New England Biolabs (Ipswich, USA) |
| FastDigest Green buffer | Thermo Fisher Scientific (Waltham, USA) |
| LightCycler480 SYBR Green I Master | Roche (Mannheim, Germany) |
| 0.05 % EDTA-Trypsin | Invitrogen/Gibco (Karlsruhe, Germany) |
| Alkalic Phosphatase | Roche (Mannheim, Germany) |
| T4-DNA-Ligase | Promega GmbH (Mannheim, Germany) |

| Markers | Company |
|---|---|
| Page Ruler™ Prestained Protein Ladder | Fermentas (Burlington, Canada) |
| GeneRuler™ DNA 1 kb DNA Ladder | Thermo Fisher Scientific (Waltham, USA) |

### 2.5.1 Restriction endonucleases

Restriction endonucleases were ordered from New England Biolabs GmbH (Frankfurt, Germany) or Fermentas GmbH (St. Leon-Rot, Germany) and used with the buffer systems approved by the manufacturer.

## 2.6 Antibodies

### 2.6.1 Primary antibodies

| Antigen | Origin | Dilution | Manufacturer/Reference |
|---|---|---|---|
| HIV-1 p24 | Mouse | 1:5000 | Abcam |
| SPOC1 | Rat | 1:50 | Kinkley et al. [15] |
| HIV-1 Vpr | Rabbit | 1:2000 | Votteler et al. 2007 [58] |
| α-Tubulin | Mouse | 1:10000 | Sigma |

### 2.6.2 Secondary antibodies

| Name | Dilution | Manufacturer |
|---|---|---|
| Peroxidase-AffiniPure Goat Anti-Mouse IgG + IgM (H+L) | 1:10000 | Jackson ImmunoResearch |
| Peroxidase-AffiniPure Goat Anti-Rat IgG (H+L) | 1:10000 | Jackson ImmunoResearch |
| Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) | 1:10000 | Dianova |

## 2.7 Reagents

### 2.7.1 Chemicals

| Chemicals | Company |
|---|---|
| 2-Propanol (99,95 %) | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| 30 % Acrylamide-bis solution (37.5:1) | Merck KGaA (Darmstadt, Germany) |
| HEPES | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Agar | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Agarose NEEO Ultra Quality | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Ammonium persulfate (APS) | Sigma Aldrich (St. Louis, USA) |
| Ampicillin | Ratiopharm GmbH (Ulm, Germany) |
| Bacto-Trypton | BD Biosciences Pharmingen (San Diego, USA) |
| Bovine Serum Albumine (BSA) | Sigma Aldrich (St. Louis, USA) |
| $CaCl_2$ | Sigma (Munich, Germany) |
| Dimethylsulfoxid (DMSO) | Sigma Aldrich (St. Louis, USA) |
| Doxycycline | Sigma Aldrich (St. Louis, USA) |
| Dithiothreitol (DTT) | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Dulbecco's Modified Eagle Medium (DMEM) (1x) + GlutaMAX™-I | Gibco (Darmstadt, Germany) |
| Ethanol | Merck KGaA (Darmstadt, Germany) |
| Ethylenediaminetetraacetic acid (EDTA) | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Geneticin® | Gibco (Darmstadt, Germany) |
| Glycerin | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Glycine | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| HCl | AppliChem GmbH (Darmstadt, Germany) |
| Heat Inactivated Fetal bovine serum (FBS) | Gibco (Darmstadt, Germany) |
| Human AB-serum | Sigma (Munich, Germany) |
| Interleukine-2 | Sigma (Munich, Germany) |
| Kanamycin | Ratiopharm GmbH (Ulm, Germany) |
| L-glutamine | PAA Laboratories GmbH (Cölbe, Germany) |
| MgCl2 | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| MgSO4 | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| MEM vitamins | Biochrom (Berlin, Germany) |

| | |
|---|---|
| Methanol | Sigma Aldrich (St. Louis, USA) |
| Tetramethylethylenediamine (TEMED) | Sigma (Munich, Germany) |
| Natrium chloride | Carl Roth® GmbH+Co.KG (Karlsruhe) |
| Natriumdesoxycholate | Sigma (Munich, Germany) |
| Normal goat serum | Gibco (Darmstadt, Germany) |
| Normal mouse serum | Sigma (Munich, Germany) |
| Opti-MEM® | GIBCO/Invitrogen (Carlsbad, USA) |
| Paraformaldehyde (PFA) | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Penicillin/Streptomycin (100x) | PAA Laboratories GmbH (Cölbe, Germany) |
| Ponceau S | AppliChem GmbH (Darmstadt, Germany) |
| Powdered milk (blotting grade) | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| RNase A | Macherey-Nagel (Düren, Germany) |
| Roswell Park Memorial Institute (RPMI) Medium 1640(1x) | Gibco (Darmstadt, Germany) |
| Sodium dodecyl sulfate (SDS) Ultrapure (≥ 99 %) | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Sodium Pyruvate (100mM) | Gibco (Darmstadt, Germany) |
| Sure blue peroxidase substrat | KPL (Gaithersburg, USA) |
| Tris PUFFERAN® (≥ 99.9 %) | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Triton-X-100 | Carl Roth® GmbH & Co.KG (Karlsruhe, Germany) |
| Trypsin-EDTA (0,05 %) | Gibco (Darmstadt, Germany) |
| Yeast extract | BD Biosciences Pharmingen (San Diego, USA) |

### 2.7.2 Reagent systems (Kits)

| Kits | Company |
|---|---|
| GeneJet Genomic DNA purification Kit | Thermo Fisher Scientific (Waltham, USA) |
| Pure Yield™ Plasmid Midiprep System | Promega GmbH (Mannheim, Germany) |
| QIAamp® DNA Micro Kit (50) | Qiagen (Valencia, USA) |
| Qiaprep Spin Miniprep Kit | Qiagen (Valencia, USA) |
| T4 DNA ligase | New England Biolabs (Ipswich, USA) |
| T4 polynucleotide kinase | New England Biolabs (Ipswich, USA) |
| UltraClean™ 15 DNA Purification Kit | MO BIO Laboratories, Inc. (Carlsbad, USA) |

### 2.7.3 Buffers and Solutions

| Buffers | Company |
|---|---|
| ELISA wash solution | KPL (Gaithersburg, USA) |
| Lysis Buffer P2 | Qiagen (Valencia, USA) |
| Neutralization Buffer P3 | Qiagen (Valencia, USA) |
| PBS | PAA Laboratories (Pasching, Austria) |
| Resuspension Buffer P1 | Qiagen (Valencia, USA) |
| SuperSignal West Pico Chemiluminescent | Thermo Fisher Scientific (Waltham, USA) |

| 10x HBS | | | |
|---|---|---|---|
| NaCl | | 8.18 | g |
| HEPES | | 5.94 | g |
| Na2HPO4x2H2O | | 0.25 | g |
| $H_20$ | ad | 100 | ml |

Dilute to 2xHBS by adding $H_20$ and set pH to 7.23

| 5x SDS loading Buffer | Stock | | |
|---|---|---|---|
| 10 % (w/v) SDS | 2 M | 10.0 | g |
| 30 % Glycerol | | 30.0 | ml |
| 250mM TrisHCl (pH 6.8) | | 12.5 | ml |
| 0.02 % Bromophenol Blue | 0.04 % | 52.0 | ml |
| 5 % β-Mercaptoethanol | | 05.0 | ml |
| | Total | 100.0 | ml |

| 10x SDS running Buffer | | | |
|---|---|---|---|
| 0.25 M Tris-Base | | 30.28 | g |
| 1.92 Glycine | | 144.13 | g |
| 1 % (w/v) SDS | | 10.00 | g |
| $H_20$ | ad | 1.00 | l |

| 10x Salt | | | |
|---|---|---|---|
| 0.23 M Tris-Base | | 27.86 | g |
| 1.92 Glycine | | 144.13 | g |
| $H_20$ | ad | 1.00 | l |

| 20x TBST | | | |
|---|---|---|---|
| 1 M Tris-Base | | 121.14 | g |
| 18 % (w/v) NaCl | | 180.00 | g |
| 1 % Tween20 | | 10 | ml |
| Set pH to 7.6 | | | |
| $H_20$ | ad | 1.00 | l |

| Blocking Buffer | | | |
|---|---|---|---|
| 10 % (w/v) milk powder | | 5 | g |
| TBST | ad | 50 | ml |

| 10x TAE | | | |
|---|---|---|---|
| Tris | | 242 | g |
| Glacial acetic acid | | 57,1 | ml |
| 0.5 M EDTA 8.0 | | 100 | ml |
| $H_20$ | ad | 1.0 | l |

| RIPA-Buffer | Stock | | |
|---|---|---|---|
| 50 mM Tris-HCl 7.4 | 1 M | 50.0 | ml |
| 150 mM NaCl | 4 M | 37.5 | ml |
| 2 mM EDTA | 0.5 M | 4.0 | ml |
| 1 % NP-40 | | 10.0 | ml |
| 0.1 % SDS | | 1 | g |
| $H_20$ | ad | 1.0 | l |

| Transfer-Buffer | | | |
|---|---|---|---|
| 1x Salt | | 100 | ml |
| 20 % Methanol | | 200 | ml |
| $H_20$ | | 700 | ml |
| | Total | 1 | l |

| ECL-A | | | |
|---|---|---|---|
| 1M Tris-Hcl ph 8.6 | | 100 | ml |
| Luminol Sodium | | 250 | mg |
| $H_20$ | | 900 | ml |
| | Total | 1 | l |

| ECL-B | | |
|---|---|---|
| DMSO | 100 | ml |
| p-Coumaric acid | 125 | mg |

### 2.7.4 Drugs & Inhibitors

HIV drugs which were used, includes both approved and experimental substances. Inhibitors of early HIV replication phase were the inhibitor of reverse transcriptase (RT) efavirenz (EFV; non-nucleoside RT inhibitor, NNRTI), raltegravir (RAL; approved integrase inhibitor), and flavopiridol (FLV; transcription inhibitor; kinase inhibitor). For the late phase the peptidic protease inhibitor (PPI) Saquinavir (SQV) were selected [XVIII].

MG132 is a cell-permeable proteasome inhibitor. It reduces the degradation of ubiquitin-conjugated proteins in mammalian cells.

Lactacystin is an organic compound which binds and inhibits specific catalytic subunits of the proteasome.

SB-216763 is a small molecule that competes with ATP and potently inhibits the activity α and β isozymes of GSK-3 [XVII].

E3 ubiquitin ligases are regulated by the covalent attachment of the ubiquitin-like protein Nedd8 to the cullin subunit. Neddylation can be prevented by MLN4924, a drug that inhibits the nedd8-activating enzyme. With this small molecule it is possible to block the CUL4a complex [77].

| Name | Final concentration | | Company |
|---|---|---|---|
| Efavirenz | 250 | nM | NIH AIDS Reagent Program |
| Raltegravir | 50 | nM | NIH AIDS Reagent Program |
| Flavopiridol | 50 | nM | NIH AIDS Reagent Program |
| Saquinavir | 50 | nM | NIH AIDS Reagent Program) |
| MG132 | 10 | µM | Merck (Darmstadt, Germany) |
| Lactacystin | 10 | µM | Sigma (Munich, Germany) |
| Insulin | 100 | nM | Sigma (Munich, Germany) |
| SB216763 | 10,40,100 | µM | Sigma (Munich, Germany) |
| MLN4924 | 0,1-1 | µM | BostonBiochem (Cambridge,UK) |

## 2.8 Laboratory equipment

| Centrifuges | Company |
|---|---|
| Heraeus Biofuge Pice | Kendro (Hanau, Germany) |
| HeraeusTM Fresco 17+21 TM | Thermo Fisher Scientific (Waltham, USA) |
| HeraeusTM MegafugeTM 40 | Thermo Fisher Scientific (Waltham, USA) |
| HeraeusTM PicoTM 21 Microcentrifuge | Thermo Fisher Scientific (Waltham, USA) |
| Multifuge X3R | Thermo Fisher Scientific (Waltham, USA) |
| Rotina 420 R | Hettich (Tuttlingen,Germany) |
| SIGMA 3-16 Centrifuge | Sigma (Newton, UK) |
| Sprout®Mini-Centrifuge | Heathrow Scientific (Illinois, USA) |

| Gel electrophoresis equipment | Company |
|---|---|
| Horizontal electrophoresis system | Thermo Fisher Scientific (Waltham, USA) |
| Power Supply, PowerPacTM Basic | Bio-Rad Laboratories (Hertfordshire, UK) |
| Power Supply, Standard Power Pack P25 | Biometra (Göttingen, Germany) |
| Vertical electrophoresis system | Biometra (Göttingen, Germany) |
| Wet blotting system | Bio-Rad Laboratories (Hertfordshire, UK) |

| Incubators, mixers &shakers | Company |
|---|---|
| CO2 incubator, Heracell 150i | Thermo Fisher Scientific (Waltham, USA) |
| Hotplate stirrer, Variomag | Thermo Fisher Scientific (Waltham, USA) |
| Incubater V30 | Memmert (Schwachbach, Germany) |
| Incubation shaker | Multitron Infors (Bottmingen/Basel, Switzerland) |
| Rocking and rotating mixer, RM 5-V | Ingenieurbüro CAT (Staufen,Germany) |
| Small shaker, MS3 basic | IKA®-Werke (Staufen, Germany) |
| Thermomixer | Eppendorf (Hamburg,Germany) |
| Vortex Mixer, Vortex Genie®2 T | neoLab Migge Laborbedarf (Heidelberg,Germany) |
| Waving platform shaker, Polymax 1040 | Heidolph Instruments (Schwabach, Germany) |

| Microscope | Company |
|---|---|
| Axiovert 25 | Carl Zeiss Microscopy (Jena,Germany) |
| Inverted microscope, Cell Observer® | Carl Zeiss Microscopy (Jena,Germany) |
| Inverted microscope, Primo Vert | Carl Zeiss Microscopy (Jena,Germany) |

| Pipettes | Company |
|---|---|
| Manual Dispenser, Multipette® plus | Eppendorf (Hamburg, Germany) |
| Pipette controller, PIPETBOY | INTEGRA Bioscience (Zizers, Switzerland) |
| Pipette controller, Pipetus® | Hirschmann Laborgeräte (Eberstadt, Germany) |
| Serological pipettes | Sarstedt (Nümbrecht, Germany) |
| Single Channel Pipettes (10-1000 µl) | Eppendorf (Hamburg, Germany) |
| Single Channel Pipettes, PIPETMAN classicTM (10-1000 µl) | Gilson (Middleton, USA) |

| additional equipment | Company |
|---|---|
| Analytical balance ML104 | Mettler Toledo (Gießen, Germany) |
| Aspiration system | VACUSAFE IBS, INTEGRA Bioscience (Zizers, Switzerland) |
| Flow cytometer, BD FACSCanto | BD, Becton Dickinson (San José, USA) |
| Flow cytometer, BD FACSCanto II | BD, Becton Dickinson (San José, USA) |
| Fusion FX7™ Multi-Imagingsystem | Peqlab (Erlangen, Germany) |
| Imaging system, Gel DocTM XR+ | Bio-Rad Laboratories (Hertfordshire, UK) System |
| LightCycler®480 System | Roche (Basel, Switzerland) |
| Microplate reader, Infinite® M200 | Tecan (Männedorf, Switzerland) |
| Microwave 9029GD | Privileg (Stuttgart, Germany) |
| Molecular Imager® Gel Doc™ XR System | Biorad (Munich, Germany) |
| Neon® Transfection System | Invitrogen (Carlsbad, USA) |
| PCR Cycler, Mastercycler® | Eppendorf (Hamburg, Germany) |
| PCR gradient Cycler, Mastercycler® | Eppendorf (Hamburg, Germany) |
| PCR SPRINT Thermal Cycler | Thermo Fisher Scientific (Waltham, USA) |
| Portable PT600 Weight | Sartorius AG (Göttingen, Germany) |
| UV Transilluminator | Bachofer (Reutlingen, Germany) |
| UV-Vis Spectrophotometer 2000c | Thermo Fisher Scientific (Waltham, USA) |
| Workbench Z100 | Schulz Lufttechnik (Sprockhövel, Germany) |

## 2.9 Consumables

| Consumables | Company |
|---|---|
| Cell culture flasks (25-175 cm²) | Sarstedt (Nümbrecht, Germany) |
| Conical tubes, BD FalconTM (15-50 ml) | BD, Becton Dickinson (San José, USA) |
| Filter pipette tips (10-1000 µl) | Sarstedt (Nümbrecht, Germany) |
| Pipette tips (10-1000µl) | Sarstedt (Nümbrecht, Germany) |
| Reaction tubes (0.5-2 ml) | Sarstedt (Nümbrecht, Germany) |
| Multiwell culture plates, CELLSTAR® | Greiner Bio-One GmbH (Frickenhausen, Germany) |
| Nitrocellulose blotting membrane | Whatman (Maidstone, UK) |
| WhatmanTM filter papers | Whatman (Maidstone, UK) |
| FACSClean ™ | BD, Becton Dickinson (San José, USA) |
| FACSFlow™ | BD, Becton Dickinson (San José, USA) |
| FACSRinse™ | BD, Becton Dickinson (San José, USA) |
| Serological pipettes | Sarstedt AG & Co (Numbrecht, Germany) |

## 2.10 Software and Databases

**Website**

http://multalin.toulouse.inra.fr/multalin/

http://web.expasy.org/translate/

http://www.bioinformatics.org/sms/rev_comp.html

http://www.graphpad.com/quickcalcs/index.cfm

http://www.lifetechnologies.com/de/de/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

http://www.metabion.com/biocalc/index.html

http://www.ncbi.nlm.nih.gov/pubmed/

http://www.uniprot.org/

**Software**

| | |
|---|---|
| Epson Scan | Epson, Long Beach |
| Microsoft Office 2010 Professional | Microsoft Corporation, Redmond |
| Serial Cloner 2.5 | Serial Basics, France |
| Chromas Lite Version 2.01 | Technelysium Pty Ltd, South Brisbane |
| Quantity one® | Bio-Rad Laboratories GmbH, Munich |
| Photoshop CS5 | Adobe System, San Jose |
| Fusion FX7 | Vilber, Eberhardzell |
| FACS Diva Software | B&D, Becton Dickinson, San Jose |
| i-control™ Software | Tecan, Männedorf |
| nanoDrop 2000c | Thermo Fisher Scientific, Waltham |
| ImageJ 1.46R | NIH, USA |

# 3 Methods

## 3.1 Microbiology Methods

### 3.1.1 Cultivation of Bacteria

Bacteria cells were cultivated in LB-medium in a shaking incubator at 200 rpm, 37 °C ; in a rotating drum or were grown overnight (16-18 h) on agar plates with appropriate antibiotics.

### 3.1.2 Transformation

Transformation of bacteria, fungi and yeast with recombinant DNA to reproduce specific genes represents one of the oldest biotechnologies. In order to perform a successful transformation, cells have to be able to uptake, incorporate and express genetic material, cells which are capable of being transformed are called competent. *E.coli* represent a gram-negative bacteria, which is normally not competent, however there are several ways to cause the competence of the cells by chemical, heat or electro treatment.

Transformation of One Shot® Top10 chemically competent and XL10-Gold Ultracompentent *E.coli* cells was performed according to the manufacturer's transformation protocol [I] from lifetechnologies and agilent technologies [II].

### 3.1.3 Isolation of Plasmids

Plasmid DNA was isolated from overnight cultures using two different methods. Whereas on the mini-preparation is a fast, small-scale method, with the midi-preparation it is possible to reach plasmid concentrations of 500 – 800 µg/µl.

#### 3.1.3.1 Isolation of Plasmid-DNA via mini and midi preparation

For mini-preparation of plasmid DNA 5 ml of LB Media was inoculated with the appropriate single colony and cultured overnight. Bacteria were harvested by centrifugation at 3200 x g for 10 min. The pellet was resuspended in 300 µl Qiagen resuspension buffer (P1), supplemented with RNase and subsequently lysed with 300 µl Qiagen lysis buffer (P2). By adding 300 µl Qiagen neutralization buffer (P3) the lysis was stopped. The suspension was then centrifuged at 20 000 x g for 25 min to spin down denatured proteins. Followed by precipitated of Plasmid DNA in the supernatant with 500 µl Isopropanol for 10 min and again centrifuged at 20 000 x g for 30 min. After discarding the supernatant, the DNA-pellet was washed with 70 % ethanol and centrifugated at 20 000 x g for 5 min. At the end, plasmid DNA was dried and dissolved in 50 µl $ddH_2O$.

For Midi-preparation 500 ml LB media were inoculated with a single colony and incubated overnight. Midi-preparation was performed using the PureYield™ Plasmid Midiprep kit from Promega. All preparation steps were performed according to the manual instruction [III]. The purified DNA was eluted from the spin column in either $ddH_2O$ or elution Buffer (EB Buffer) provided with the kit.

#### 3.1.3.2 Isolation of genomic DNA

Genomic DNA from various cell cultures was purified with the Thermo Scientific GeneJET Genomic DNA Purification Kit. All preparation steps were performed according to the manual instruction [XV].

#### 3.1.3.3 Isolation of DNA from agarose gels

If needed DNA-fragments were excised from the agarose gel, extraction and purification of the DNA was then performed using UltraClean™ 15 DNA Purification Kit, following the instructions of the manufacturer [IV]. The DNA was eluted from the spin column using $ddH_2O$.

#### 3.1.3.4 Measuring of nucleic acid concentrations

The DNA concentrations were determined by measuring the DNA absorbance with a NanoDrop 2000c (Thermo Scientific) by absorbance spectrophotometry at 260nm. With the ration of 260nm/280nm it is possible to calculate the purity of the DNA, while a ratio of 1.8 is generally accepted as pure. The purified DNA solutions were stored at 4 °C (short-term) or -20 °C (long-term).

## 3.2 Molecular biology methods

### 3.2.1 Polymerase Chain Reaction – PCR

The Polymerase chain reaction (PCR) is used to directly amplify specific DNA sequences. In today’s research there are numerous applications and methods of the PCR. A typical PCR is preceded by a thermostable DNA Polymerase which duplicates the desired DNA sequence in the presence of dNTP’s and two specific oligonucleotides. The oligonucleotides are complementary to the ends of the desired DNA sequence and are present in great excess to the DNA fragment. PCR reactions were normally carried out in a Volume of 50 µl and proceed in a PCR 96 well thermal cycler (Eppendorf).

A typical PCR process started with an initial denaturation step, 25-35 denaturation, annealing and elongation steps and followed with a final elongation step. Thereby the times and temperatures of every step could be changed, depending on the Polymerase and Primers used, as can be seen in the following Tables [V; VI]

| **Phusion** | | | **DreamTaq™** | | |
|---|---|---|---|---|---|
| Component | Volume | | Component | Volume | |
| Phusion Polymerase (0.5-2 U/µl) | 0.5 | µl | Taq DNA Polymerase (1,25 U) | 0.5 | µl |
| 5x Phusion HF Buffer | 10.0 | µl | 10x Taq Buffer | 5.0 | µl |
| dNTPs (2 mM each) | 200.0 | µM | dNTPs (2 mM each) | 200.0 | µM |
| Primer 1 | 0.5 | µM | Primer 1 | 0.5 | µM |
| Primer 2 | 0.5 | µM | Primer 2 | 0.5 | µM |
| DNA-Template (10 pg- 1 µg) | 2.0 | µl | DNA-Template (10 pg- 1 µg) | 2.0 | µl |
| Nuclease-free water | ad 50.0 | µl | Nuclease-free water | ad 50.0 | µl |
| | 50.0 | µl | | 50.0 | µl |

| Cycle Step | Temperature | Time (Phusion//DreamTaq™) | Number of Cycles |
|---|---|---|---|
| Initial denaturation | 95 °C | 30 sec. // 1-3 min | 1 |
| Denaturation | 95 °C | 10 sec. // 30 sec. | 25-35 |
| Annealing | Depending on primer | 30 sec. // 30 sec. | |
| Elongation | 72 °C | 30 sec./kb // 1 min/kb | |
| Final Elongation | 72 °C | 10 min. // 5-15 min. | 1 |

#### 3.2.1.1 *Alu*-LTR Real-Time nested PCR

The *Alu*-long terminal repeat (LTR)-based real-time nested PCR is a method to quantify the integrated HIV-1 provirus DNA in infected cells [35]. This method operates with the fact that every 3-4 kb per haploid genome an *Alu*-Element, approximately 900.000 in total, can be found [36; 47]. In a first round of PCR, integrated HIV-1 sequences were amplified with outward-facing *Alu* primers that anneal within conserved regions of *Alu* sequences together with an HIV-1 LTR specific primer (L-M667, Alu1, Alu2). The L-M667 Primer produces a DNA overhang containing a specific sequence originating from the lambda T phage. In a second PCR which uses the product of the first-round PCR as template the HIV-1 LTR sequences can specifically be amplified with a specific lambda primer (Lambda T) and LTR Primer (LTR 3'). In this cycle the PCR product is generated and measured in the Light Cycler LC 480 from Roche. Therefore a mastermix is used where the dsDNS dye SYBR Green is included. This specific dye binds to all double-stranded (ds) DNA in the PCR, causing fluorescence of the dye. The fluorescence intensity is increasing with an increasing DNA amount. The fluorescence is then measured with the LC480 of Roche at each cycle, allowing quantifying the DNA concentration. By determining the Cp-Values (crossing-point)/ct-values which indicate the time point were the fluorescence signal crosses a certain threshold level. With these values relative amounts of proviral DNA can be calculated with the $2^{-\Delta\Delta CT}$ method of Pfaffl [48; XIV].

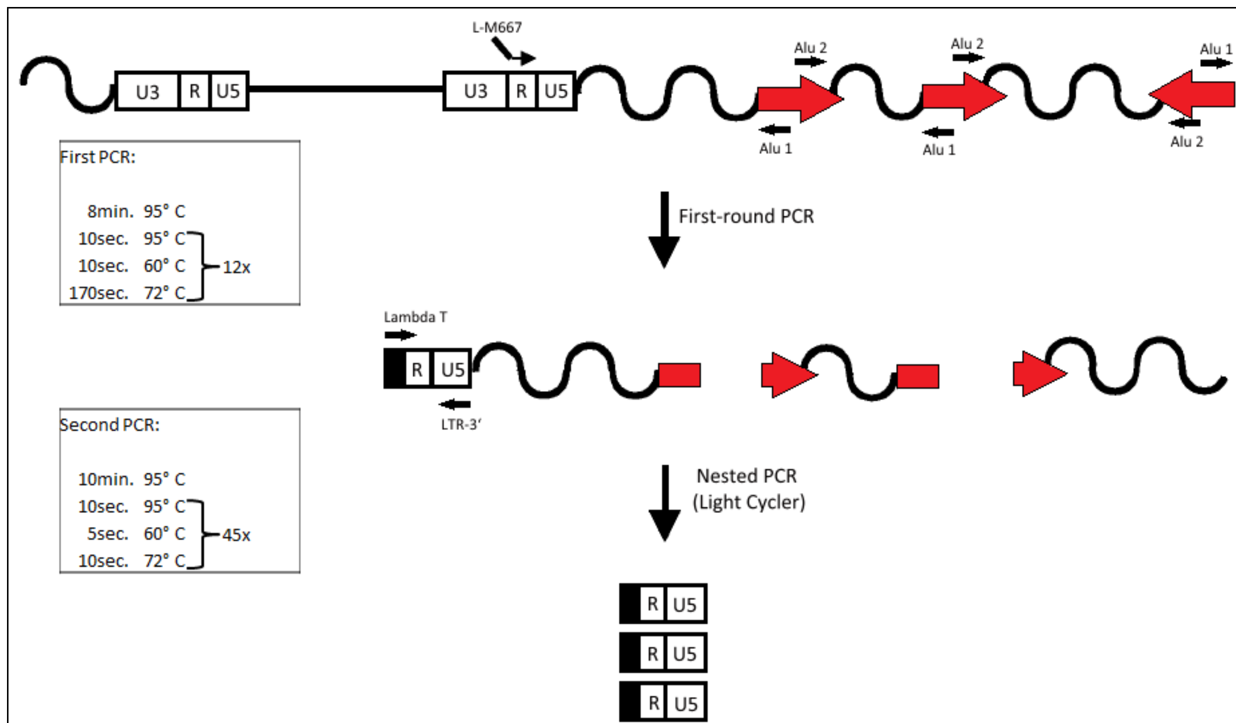


**Figure 7 Overview of the *Alu*-LTR Real-time nested PCR**

Real-time PCR strategies and location of primers and probes for the quantification of integrated HIV-1 DNA.
Figure based from [47].

### 3.2.2 Gel electrophoretic separation of DNA and purification of DNA from agarose gels

Gel electrophoresis is a molecular technique for the separation and characterization nucleic acids. In this technique negatively charged DNA migrates in an applied electric field and is separated according to their length. Short DNA fragments are passing the gel matrix faster than longer fragments. For analytical purposes of nucleic acids, DNA fragments were separated by gel electrophoresis in a 0.8-1.5 % agarose gels, depending on size of the DNA-fragment [34].

For this purpose, agarose (1-3 % w/v) was melted in 1x TAE-Buffer by heating and after cooling down to approximately 60 °C, 1-3 µl ethidium bromide was added. Ethidium bromide is intercalating in the DNA and visible under ultraviolet light. DNA was mixed with 6x loading dye to prepare the samples for loading on the gel and separated by electrophoresis for 0.5 h in 1x TAE-Buffer with an applied voltage of 110 V. The Molecular Imager® Gel Doc™ XR System by Biorad was used to visualize the DNA. The GeneRuler™ DNA 1 kb DNA Ladder (Thermo Scientific) consisting of a mixture of DNA fragments of known size was used to quantify the size of analyzed DNA fragments.

### 3.2.3 Restriction of DNA and PCR Fragments

DNA was incubated with specific restriction enzymes, which cut double-stranded or single stranded DNA at specific recognition sequences. Enzymatic restrictions of plasmids or PCR products were performed with the appropriate restriction enzymes at an average enzyme concentration of 5-10 U/µl using the buffer conditions and temperatures according to the manufacturer's recommendations.

### 3.2.4 DNA Fragment Ligation

Ligation of DNA Fragments was performed using either the TaKaRa DNA Ligation Kit Ver.2.1 [VII] or the T4 DNA Ligase Kit [VIII] by Promega and was done as recommended by the manufacturer.

### 3.2.5 DNA sequencing

DNA sequencing was performed by MWG-Biotech AG. For an optimal sequencing result 15 µl (50-100 ng/µl) of plasmid or PCR product along with 15 µl (2 pmol/µl) of primer solutions were submitted.

## 3.3 Cell-Biological Methods

### 3.3.1 Cultivation of Eukaryotic cells

All used cell lines were cultivated at 37 °C and 5 % $CO_2$. DMEM Media for adherent cells was supplemented with 10 % fetal calf serum (FCS; Invitrogen), an antibiotic penicillin/streptomycin mix (120 µg/ml) and 1 % L-glutamin (PAA). Some adherent cells needed special Media condition (details see 2.3.1). Cells were passaged twice a week at a ratio of 1:20. Suspension cell lines were cultivated in RPMI with the supplements 10 % fetal calf serum (FCS; Invitrogen an antibiotic penicillin/streptomycin mix (120 µg/ml) and 1 % L-glutamin (PAA). Some suspension cell lines needed special Media condition (details see 2.3.1). Cells were passaged twice a week in ratio 1:10.

#### 3.3.1.1 Freezing of eukaryotic cells

For long-term storage adherent cells or suspension cells were washed with 1x PBS and suspended in a cell count range of approximately 1-5 x $10^6$ cells/ml (adherent) or approximately 1-10 x $10^6$ cells/ml (suspension cells) in freezing media containing 10 % DMSO. Cells were transferred to liquid nitrogen after 24 h at -80 °C.

#### 3.3.1.2 Thawing of eurkaryotic cells

Freezed cells were thawed on ice and diluted in 5 ml pre-warmed media. Subsequent centrifugation (1,500 x g, 5 min) supernatant was discarded and cells were resuspended in 10 ml fresh medium followed by cultivation (3.3.1).

### 3.3.2 Isolation of mononuclear cells from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from CMV negative Buffy Coats (Concentrated Lymphocyts from 500 ml human blood, provided by the Bavarian red cross) App. 50 ml from the Buffy Coat was diluted with 50 ml PBS. 25 ml was shifted on top of 30 ml Ficoll (Biochrom) and centrifuged with 800 x g for 35 min with lowest acceleration and without break. The interphase containing PBMCs was carefully harvested and washed twice with PBS. PBMCs were then cultivated at a density of 2.5 x $10^6$ PBMC per ml in RPMI Media containing 1 µl/ml IL-2. For stimulation of PMBCs 1 µl/ml PHA was added to the media.

For isolation of monocyte derived macrophages (MDM), PBMCs were seeded in 100 x 15 mm petri dishes with vent (Greiner bio-one) at a density of 1.5 x $10^6$ PBMC per ml in MDM medium. After adhesion of monocytes (app. 4 days) suspension cells (mainly lymphocytes), were washed away with PBS. Monocytes were cultivated for 3 additional days in MDM medium for differentiation to macrophages.

### 3.3.3 Transfection of Eukaryotic cells

Transfection is a method to deliberately introduce nucleic acids, such as DNA or RNA into eukaryotic cells. Transfection can be done by the calcium phosphate method, by electroporation or by cationic lipid transfection.

#### 3.3.3.1 Transfection of cells via the calcium phosphate method

Transfection of cells using the calcium phosphate method is one of the cheapest methods to introduce nucleic acids into cells. It was originally invented by F.L.Graham [37]. It is based on the principle that when combining the positive charged calcium and the negatively charged phosphate fine precipitates are formed which bind the DNA on its surface. For transfection, 1.75 x $10^5$ cells were seeded one day before transfection in 1 ml DMEM. The cells should be approximately 60-70 % confluent on the day of the transfection. In the next step 6,5 µl of 2M calcium chloride and 2,5 µg plasmid DNA are mixed together and adjusted to a total volume of 50 µl with deionized $H_20$. Then 50 µl 2x HBS is added and the mixture is vortexed vigorously. The solution is incubated for 15 min at room temperature before it was added to the cells dropwise and incubated for at least 6 hours at 37 °C and 5 % $CO_2$. Media was then replaced and cells were analyzed for expression of transfected plasmid 24-48 hours post transfection.

#### 3.3.3.2 Transfection of cells via Lipofectamin™2000

Another method uses lipofectamine, which is a cationic liposome that complexes with the Plasmid DNA and allows it to overcome the electrostatic repulsion of the cell membrane. Transfection of adherent cells using the Lipofectamin™2000 kit from Lifetechnologies was performed as recommended by the manufacturer [IX].

#### 3.3.3.3 Transfection of suspension cells via microporation

Suspension cells were normally transfected via electroporation using the Neon® Transfection System [X]. In this method cells are permeabilized by establishing an electric field, which is generated by a rapidly discharging capacitor. Thereby the conformation of the membrane components change and the cells are permeabilized to uptake nucleic acids. 1 x $10^6$ cells were washed with 1x PBS, centrifuged at 400 x g for 5 min and the supernatant was discard. The cell pellet was resuspended in the appropriate solution (Buffer R or T) containing 5 µg plasmid DNA or 100 nM siRNA and electroporated as recommended in the manufacterers protocol [X], e.g. Jurkat-TAg cells were electroporated with the delivery of three electric pulses (1350 V, 10 msec). Instantly, cells were transferred in pre-warmed RPMI 1640 Media without antibiotics and cultivated for 24-48 hours at 37 °C, 5 % $CO_2$ to yield appropriate levels of protein expression.

### 3.3.4 FACS-based FRET

This assay serves to measure direct interaction of two proteins of interest (POI) based on the well-known energy transfer from an excited donor fluorophore (POI-CFP) to an acceptor fluorophore (POI-YFP), which overlap in their emission and excitation spectrum and are simultaneously expressed in living cells. The Method and gating strategy is described in detail in the work of Banning et al., 2010 [49]. Important for this assays is, that an adequate amount of the two proteins is collocating within a range ≤ 10nm. In this case, a FRET signal can be emitted that is quantitatively measured by flow cytometry as the percentage of CFP+/YFP+ cells that show a YFP- specific emission (529|24 BP filter) as they pass the CFP-exciting laser (λ = 405nm). Specificity of the assay was controlled by co-expression of e.g. CFP-only with POI-YFP. In this thesis, 1,5 x $10^5$ HEK293T cells were seeded in a 12 well plate 12 hours before transfection. Plasmids were used were genes of interest are expressed as fusion proteins with YFP or CFP (as indicated) and co-transfected via Calcium phosphate transfection as described above (3.3.3.1). Cells were harvested 24 hours after transfection, washed once with PBS and resuspended in PBS containing 1 % FCS. Analysis of cells was carried out with a FACS CantoII (BD Bioscience).

### 3.3.5 HIV-1 p24 Antigen capture assay - ELISA

ELISA (enzyme-linked immunosorbent assay) is a common method that uses antibodies and color change to identify a substance. It has several applications, but mostly it is used to detect HIV-1 p24 antigen in a sample. Nowadays there are many different types of ELISA-assays but in general they all work in the same way. First an antigen from a sample is bound to a specific surface. In a next step, a precise enzyme-linked-antibody is applied over the surface so it can bind to the antigen. In a final step, a substance containing the enzyme's substrate is added. Subsequently the reaction produces a detectable signal, most commonly a color change in the substrate. The HIV-1 p24 antigen capture assay is a double antibody sandwich enzyme immunoassay that is used to calculate the concentration of HIV-1 p24 in different samples. Virus stocks or cell supernatants were lysed with Triton X-100 (Sigma) at 4 °C for 12 hours. In parallel, a 1:2 dilution series of a p24 standard (40 ng/ml- to 0,6125ng/ml) was prepared. HIV-1 p24 Antigen Capture Assay Kit (ABL inc.) was used to measure the amount of the capsid protein p24 as recommended by the manufacturer [XVI]. Unbound material was removed by several washing steps. Addition of Peroxidase Substrat (KPL) leads to a color change of the solution, which was stopped by adding 100 µl Stop solution. Absorbance was measured at 450nm in an Infinite® M200 (Tecan). Absolute p24 values [ng/ml] were calculated using a standard curve derived from the standard p24 dilution series.

## 3.4 Virological techniques

### 3.4.1 Preparation of VSV-G pseudotyped virus stocks

To produce VSV-G pseudotyped HIV-1 stocks, 3,5 x $10^6$ HEK 293T cells were seeded 18 hours before transfection in 100x 15 mm petri dishes to reach 60-70 % confluent at the time point of transfection. Cells were transfected with 28,8 µg of HIV-1 NL4-3 and 1,2 µg VSV-G plasmid DNA by the calcium phosphate method. Media was changed 6 hours post transfection and cells were incubated for 24 hours. Supernatants were collected after 24 hours and cell debris removed by centrifugation for 5 min 400 x g. Cells were cultured for additional 24 hours with fresh Media before supernatant was collected again. Virus stocks were stored up to two weeks at 4 °C.

## 3.5 Biochemical Methods

### 3.5.1 Cell lysis

Cells were harvested, centrifuged at 400 x g, 5 min and the supernatant was discarded. Cells were washed once with 1x PBS and centrifuged again at 400 x g, 5 min. The supernatant was removed and the cell pellet resuspended with 50 µl RIPA lysis buffer containing protease inhibitor (cOmplete protease inhibitor cocktail tablets- Roche) and subsequently incubated for 10 min at 4 °C. After incubation, the samples were centrifuged at 10000 x g, 10 min to remove cell debris from the total solubilized proteins present in the supernatant. To disrupt the native conformation and apply negative charges to the proteins, samples were mixed with 5x SDS sample buffer and boiled for 5 min at 95 °C. Denatured protein samples were stored at -20 °C.

### 3.5.2 Discontinuous SDS Polyacrylamide Gel Electrophorese (SDS-PAGE)

SDS-PAGE is a technique in which proteins can be separated according to their size and electrophoretic mobility. This technique is based on the ability of linearized and negatively charged proteins to migrate through a polyacrylamide gel upon application of an electric field. While small proteins migrate relatively fast towards the positive electrode, large proteins migrate slower. SDS-PAGE was performed in vertical 12 % polyacrylamide gel units with an upper stacking gel and a lower resolving gel. 30 % acrylamide mix, 1.5M Tris (pH 8.8), 10 % SDS, 10 % APS, TEMED and deionized $H_2O$ were mixed for the lower resolving gel. The upper stacking gel were prepared using 30 % acrylamide mix, 1.0M Tris (pH 6.8), 10 % SDS, 10 % APS, TEMED and deionized $H_2O$.

After Polymerization, the PAA gel was inserted into the SDS-PAGE electrode chamber and filled up with 1x SDS running buffer. 5-20 µl of the denatured protein samples were loaded onto the gel and electrophoresis was conducted for 20 min at 80V (V=const.) and for further 70 min at 160V (V=const.).

### 3.5.3 Western Blot

Following separation of proteins by SDS-PAGE, proteins in the gel were transferred to a Protran BA83 (0.2 µm) nitrocellulose membrane by an electric field using a PROTEAN® wet blotting system (BIO-RAD). For this step, the nitrocellulose membrane was pre-incubated in Transfer buffer and placed in with the PAA Gel between two Whatman filter papers into the blotting system. The gel stack was then transferred into the cassette of the electroblotting chamber and the tank was completely filled with Transfer buffer. The tank was connected conducted for 70 min at 100V (V=const.). Finally, unspecific binding sites are blocked by incubation in 10 % [w/v] milk powder in TBS-T for 1 hour at RT with shaking. This step is important to avoid strong background signals caused by unspecific antibody binding on the nitrocellulose membranes. With the use of specific antibodies it is possible to visualize the proteins on the membrane. Primary antibodies bind specific to the proteins of interest, whereby secondary antibodies bind to the primary antibody in a species-specific manner.

All secondary antibodies used in this thesis were linked to horseradish peroxidase, allowing a chemiluminescent-based detection. Incubation with primary antibody (diluted in 5 % [w/v] milk powder in TBS-T) was performed O/N at 4 °C with constant inversion. After three sequential washing steps with TBS-T, each for 10 min at RT with shaking, incubation with secondary antibody (diluted in 5 % [w/v] milk powder in TBS-T) was carried out for 3 hours at RT with shaking. The membrane was washed again three times as before, incubated for 30 sec. in enhanced chemiluminescence (ECL) solution and developed by using the Fusion FX7™ Multi-Imagingsystem (Peqlab).

# 4 Results

## 4.1 Endogenous SPOC1 protein expression in HIV-1 target cells

Experiments displaying the endogenous protein level of SPOC1 in many cells types and especially in HIV-1 target cells are still lacking. Hence, it was crucial to examine the endogenous SPOC1 protein expression in HIV-1 target, standard laboratory, and HIV-1 latently infected cells lines. U2OS-Clone 5 (U2OS-Cl.5), which is a doxycycline inducible SPOC1 expression human osteosarcoma cell line, described by Kinkley et al. [15], was used as reference cell line for the protein expression of SPOC1. SupT1 and Jurkat-TAg are well-established CD4+ expressing T-lymphocyte laboratory cell lines. Together with Macrophages, PBMC$^{+/-}$ and, CD4+ cells which were isolated from whole blood (3.3.2), they represent appropriate model cell lines for HIV-1 target cells. The J-Lat cell line is a clonal cell line of Jurkat T cells latently infected with an Env-defective HIV-1 [74]. In addition, to allow the conduction of further experiments in well-established cell culture cell lines, SPOC1 abundance was also determined in HEK 293T cells. Total-cell extracts of all cells were prepared as described in (3.5.1), equal amounts of protein were loaded on a 12 % SDS/PAA gel and endogenous SPOC1 expression was analyzed by immunoblotting (Figure 8). SPOC1 is predicted to be a 34 kDa large protein but it shows aberrant electrophoretic mobility with an apparent molecular mass of 43 kDa [15]. As expected, SPOC1 expression in U2OS-Cl.5 cells is low without doxycycline. Whereas no endogenous SPOC1 protein was detected in mature macrophages, all other tested cell lines including HIV-1 target cells showed a rather high endogenous SPOC1 expression.

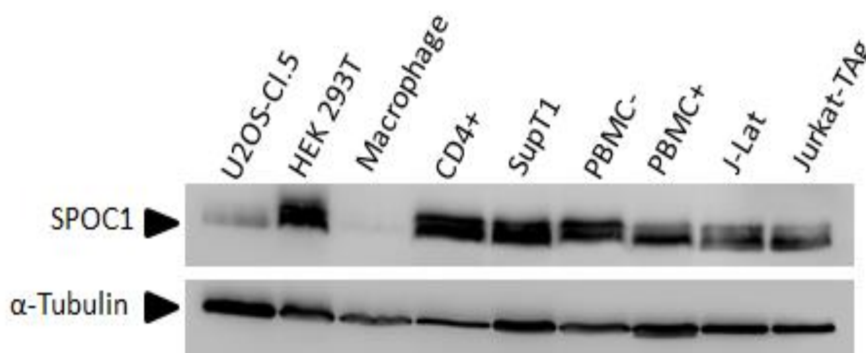


**Figure 8 Endogenous SPOC1 expression**

Total cell extracts were subjected to 12 % SDS-PAGE and immunoblotting using rat monoclonal antibody SPOC1 (43 kDa) was performed. Detection of alpha-Tubulin served as loading control.

## 4.2 HIV-1 reduces SPOC1 steady-state levels

The human adenovirus is able to degrade SPOC1 in human H1299 cells after infection and it was shown that other human pathogenic viruses significantly reduce SPOC1 protein levels as well [25]. To determine whether HIV-1 could also lead to SPOC1 reduction, SupT1 and Jurkat-TAg cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3-IRES eGFP (HIV-1 wt) particles for 48 hours and SPOC1 protein levels were determined by Western Blot. To evaluate efficient infection, viral capsid protein levels of HIV-1 p24 were monitored in parallel. Indeed, upon HIV-1 infection SPOC1 levels are reduced in human immune T-cells compared to uninfected cells (Figure 9).

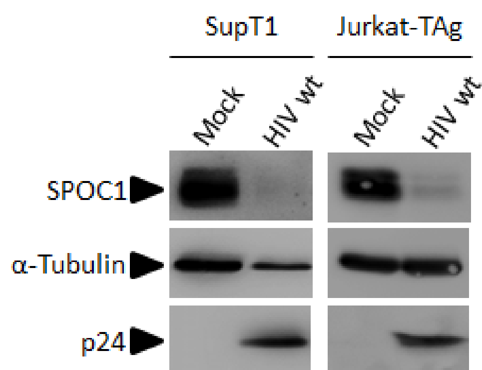


**Figure 9 SPOC1 is efficiently reduced during HIV-1 infection**

HIV-1 target cells were infected with wild/type HIV-1 or left uninfected (Mock). 48h post infection, total-cell extracts were prepared, separated by 12 % SDS-PAGE, followed by immunoblotting with rat monoclonal antibody SPOC1, mouse monoclonal antibody p24, and mouse monoclonal α-Tubulin as a loading control. Detection of p24 served as control of infection efficiency.

Based on these data, expression levels of SPOC1 in HIV-1 infected cells were analyzed over time. Jurkat–TAg cells were inoculated with HIV-1 wt particles or were left uninfected (mock). Total-cell extracts were prepared at indicated time points after infection (6-48 hpi), separated by SDS-PAGE and analyzed by immunoblotting.

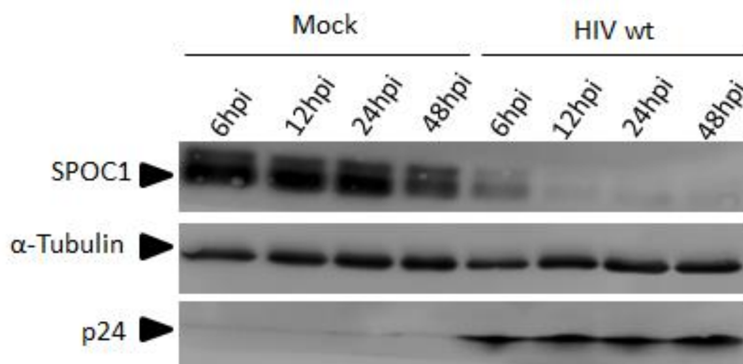


**Figure 10 Upon HIV-1 infection SPOC1 levels decreases over time**

Jurkat-TAg cells were infected with wild/type HIV-1 or left uninfected (Mock). Cells were harvested after indicated time points post infection, total-cell extract were prepared, separated by 12 % SDS-PAGE and subjected to immunoblotting. Detection was done by using rat monoclonal antibody SPOC1, mouse monoclonal antibody p24, and mouse monoclonal α-Tubulin as a loading control.

In uninfected cells (mock) SPOC1 expression levels are relative stable over time. A slight reduction of endogenous protein is visible at the latest time point (48 hpi). If cells are infected with HIV-1 wt, already, 6 hpi a significant reduction of SPOC1 compared to the uninfected control is detectable (Figure 10). This is even more pronounced at 12 hpi. Detection level of SPOC1 24 hpi and 48 hpi are comparable with the 12 hpi sample. These results indicate that HIV-1 infection leads to substantial reduction of SPOC1 at a rather early time point after infection and stays low over time.

## 4.3 SPOC1 interacts with HIV-1 protein Tat

Previous experiments have shown that infection leads to decrease of SPOC1. Since HIV-1 is known to be able to specifically degrade host proteins by interaction with viral proteins, it is possible that SPOC1 is directly degraded by interaction with one of the HIV-1 proteins. To test this and to see in general if SPOC1 interacts with one of the HIV-1 proteins, the well-established FACS-based FRET assay was used. The FACS based FRET assay serves to measure direct interaction of two proteins of interest (POI) based on the well-known energy transfer from an excited donor fluorophore (POI-CFP) to an acceptor fluorophore (POI-YFP) [49]. Here, different HIV-1 NL4-3 proteins, N-terminally fused to YFP, were used as acceptor fluorophores. Tested HIV-1 proteins included the accessory proteins Vpu, Nef, and Vpr; the transactivator of transcription Tat; the regulatory protein Rev; the structural protein Gag and the enzyme Integrase. SPOC1 was N-terminal fused to CFP and served as donor protein. In order to identify potential interactions between SPOC1 and HIV-1 proteins by using the FACS-based FRET assay, SPOC1 and one of the HIV-1 proteins were co-expressed in HEK 293T cells. Following 24 hours of cultivation, the cells were subjected to flow cytometry for FRET measurement. Cells expressing a CFP-YFP fusion protein served as positive control and almost 98 % (± 0,57 %) of these cells emitted FRET-signals (data not shown). In contrast, co-expression of CFP-only and YFP-only served as negative control (0,65 % ± 0,64 %). The two chromatin association proteins KAP1 (KRAB-associated protein 1) and HP1 (Heterochromatin-Protein 1) served as additional controls. Both proteins were also N-terminal fused to CFP and were tested for FRET with HIV-1 proteins (Figure 11). As shown in Figure 11 transfection of cells with Tat-YFP and SPOC1-CFP led to a FACS-FRET signal in about 17,5 % of cells (± 7,9 %) indicating a direct interaction of SPOC1 and Tat. Only weak or no FRET signals were detectable for Vpr (3,25 % ± 1,34 %); Integrase (1,80 % ± 0,57 %); Vpu (0,65 % ± 0,64 %); Nef (0,40 % ± 0,42 %); Gag (0,05 % ± 0,07 %); and Rev (0,25 % ± 0,07 %) respectively.

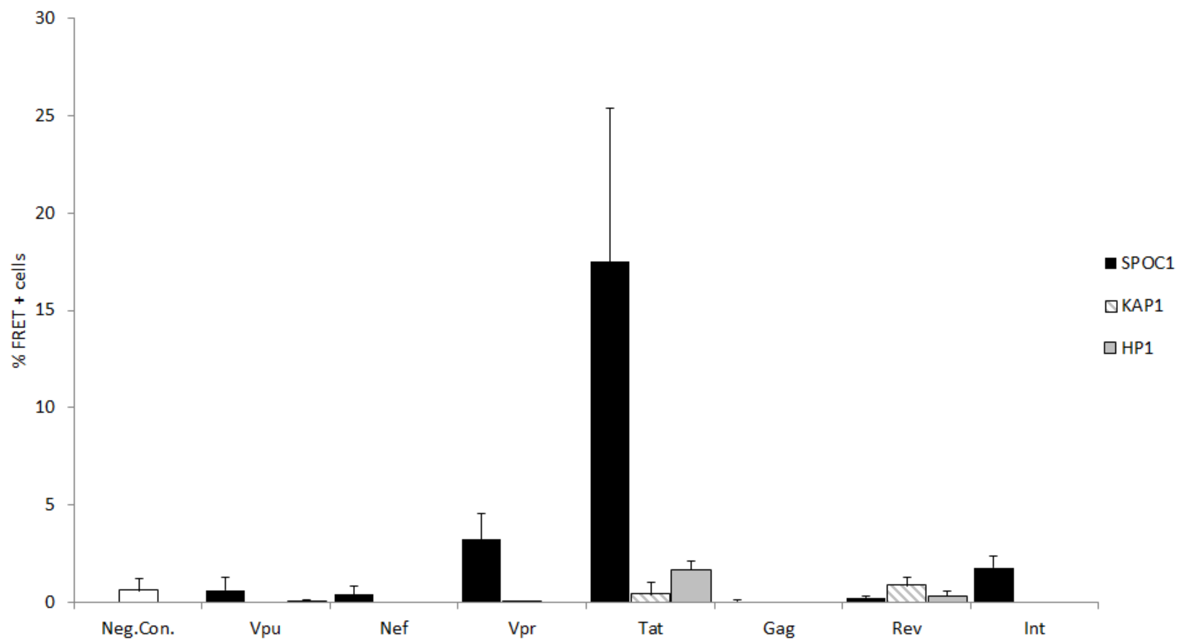


**Figure 11 SPOC1 interaction with single HIV-1 proteins in a FACS based FRET assay**

FACS-based FRET assay of fusion protein SPOC1-CFP against different HIV-1 NL4-3 proteins fused to YFP. HEK 293T cells were transfected with corresponding plasmids and 24 h later analyzed by flow cytometry. Values correspond to the mean of three independent experiments and error bars indicate the standard deviation.

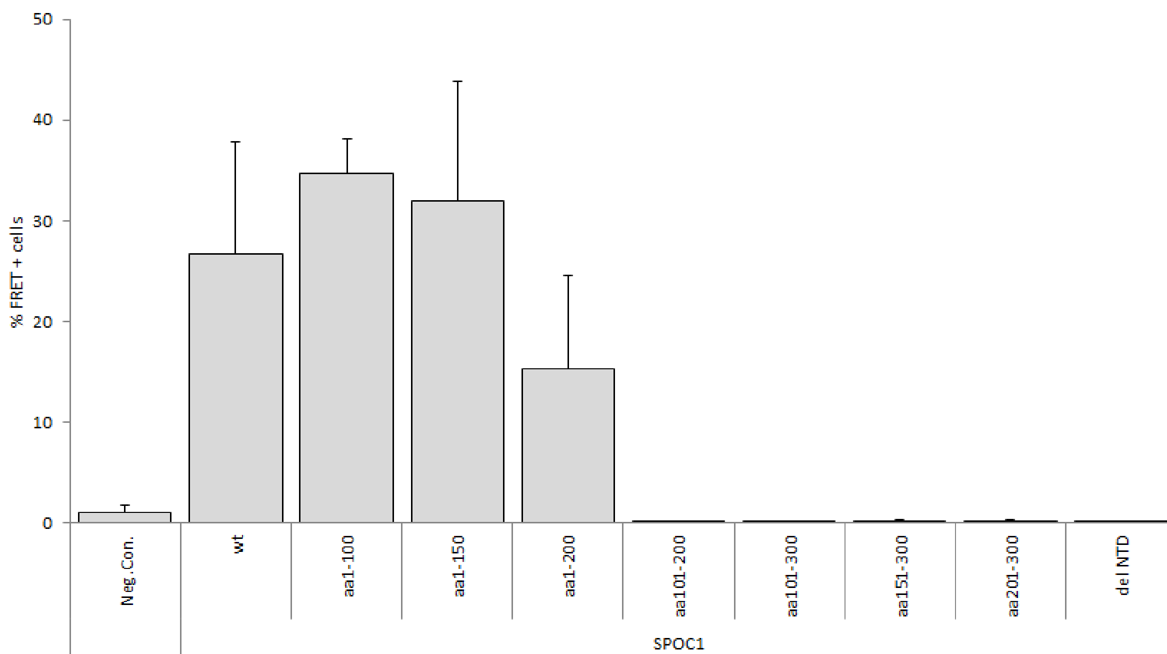


**Figure 12 Interaction of truncated SPOC1 with HIV-1 Tat in a FACS based FRET assay**

FACS-based FRET assay of truncated fusion protein SPOC1-CFP against HIV-1 NL4-3 Tat protein fused to YFP. HEK 293T cells were transfected with corresponding proteins and 24 h later analyzed by flow cytometry. Values correspond to the mean of three independent experiments and error bars indicate the standard deviation.

Since SPOC1 and HIV-1 Tat showed the highest interaction rate, additional measurements by FACS-based FRET with Tat fused to YFP and truncated SPOC1 mutant proteins N-terminal fused to CFP were used to identify domains necessary for interaction (kindly provided by Hans Will). FRET signal was measured when only the first 100aa of SPOC1 were present (34,67 % ± 3,4 %). With the first 150aa the signal was slightly reduced (31,97 % ± 11,8 %) and even further diminished when only the first 200aa were present (15,3 % ± 9,2 %). If wild type SPOC1 was coexpressed with HIV-1 Tat, a FRET signal that was in the range as in previous tests (26,67 % ± 11,1 %) was observed. Intriguingly, if the first 100aa were completely deleted, no FASC-FRET signal could be measured at all (Figure 12). Also, if the N-terminal domain (from aa 21-70) is deleted, no FRET signal was detectable. Thus, this data reveals that the first 200aa are essential for the interaction of SPOC1 with HIV-1 Tat (Figure 12).

## 4.4 HIV-1 Vpr is important for SPOC1 reduction

Next, it was investigated which viral determinants are important for SPOC1 reduction. Since the HIV-1 accessory proteins generally serve as adaptors, linking cellular factors to degradation pathways, SPOC1 degradation upon infection with HIV-1 mutants lacking one of the three accessory proteins were used. VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (HIV-1 wt), NL4-3 IRES eGFP ΔVpr, NL4-3 IRES eGFP ΔNef and NL4-3 IRES eGFP ΔVpu viruses were generated by transfection of producer cells (HEK 293T) with the mutant HIV-1 genomes and a plasmid expressing the VSV-G surface protein. Infection with VSV-G pseudotyped viruses allows a more efficient infection which is also independent from the CD4 or chemokine coreceptor. SupT1 cells were infected with GFP expressing lentiviral particles and 48 hpi successful and equal infection of cells was measured by the GFP signal via flow cytometry. Uninfected cells served as control and showed no GFP signal (**Error! Reference source not found.** A). Infection rates were 78,3 % for HIV-1 wt, 65,5 % (HIV-1 ΔVpr), 73,9 % (HIV-1 ΔNef) and 74,8 % for HIV-1 ΔVpu. In parallel, total-cell extracts were prepared, proteins separated by SDS-PAGE and subjected to immunoblotting to determine SPOC1, p24 and Vpr expression levels.

As anticipated, SPOC1 expression was dramatically reduced in cells infected with HIV-1 wt (**Error! Reference source not found.** B). Comparable to wild/type, SPOC1 was also strongly reduced in cells infected with the HIV-1 ΔNef and ΔVpu mutants. Interestingly, upon infection with the HIV-1 ΔVpr mutant, no decrease of SPOC1 levels was observed. These results strongly indicate that the HIV-1 accessory protein Vpr is involved and necessary for the observed SPOC1 decrease.

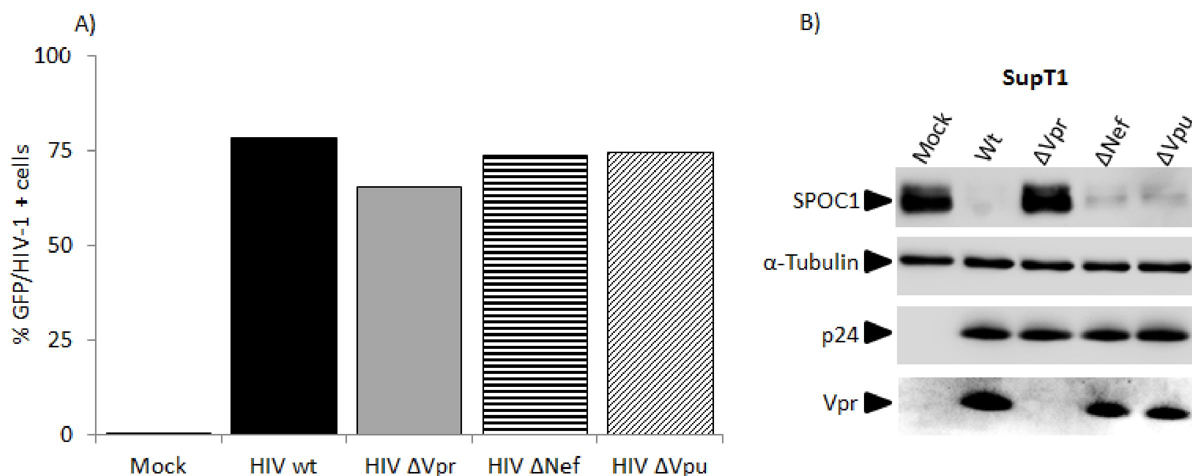


**Figure 13 HIV-1 Vpr is important for SPOC1 reduction**

SupT1 cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt), NL4-3 IRES eGFP ΔVpr, NL4-3 IRES eGFP ΔNef, NL4-3 IRES eGFP ΔVpu, or were left uninfected (Mock). Cells were analyzed 48 hpi by flow cytometry and cell lysates were subjected to SDS-PAGE and immunoblot

A) Diagram showing the % of HIV-1 infected (GFP positive) cells measured by FACS
B) Immunoblot with rat monoclonal antibody SPOC1, mouse monoclonal antibody p24, mouse monoclonal α-Tubulin (loading control) and rabbit monoclonal antibody Vpr (15kDa)

## 4.5 Transfection of single HIV-1 protein is not sufficient for SPOC1 reduction

In the previous experiment it was established that Vpr is important for the degradation of SPOC1 and Tat interacts with SPOC1. Hence it should be tested whether expression of the single Vpr protein alone or in conjunction with Tat could lead to degradation of SPOC1. Therefore, HEK 293T cells were transfected with plasmids expressing GFP only, NL4-3 Tat IRES eGFP (HIV-1 Tat), NL4-3 Vpr IRES eGFP (HIV-1 Vpr), NL4-3 nef* without GFP (No Tag), and a combination of Tat+Vpr. After confirming efficient transfection by FACS (Figure 14 A), total-cell extracts were prepared, separated by SDS-PAGE and SPOC1 expression analyzed by immunoblotting (Figure 14 B). As expected, transfection with control plasmids or a GFP-plasmid did not change SPOC1 expression levels. Intriguingly, SPOC1 was also not depleted in cells transfected with plasmids encoding for HIV-1 Tat, Vpr or a combination of Tat and Vpr (Figure 14 B). This indicates that transfection of single proteins is not sufficient to reduce SPOC1 and additional viral proteins could be necessary for the degradation of SPOC1.

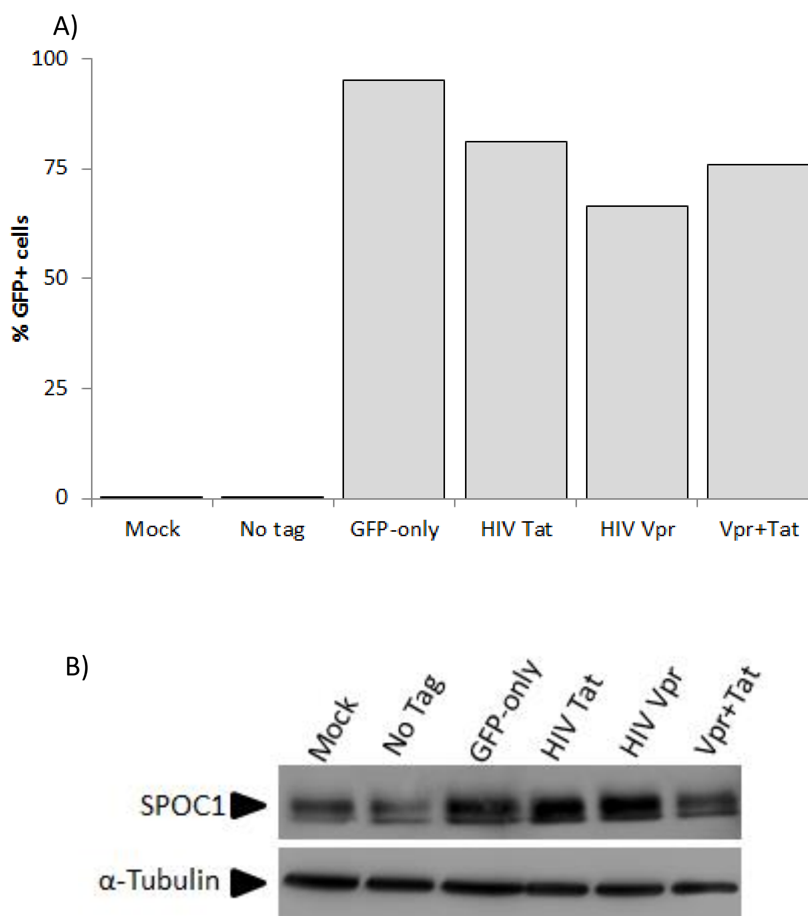


**Figure 14 Transfection of HEK 293T cells with single HIV-1 protein does not reduce SPOC1**

HEK 293T cells were transfected with plasmid expressing GFP only, NL4-3 Tat IRES eGFP (HIV-1 Tat), NL4-3 Vpr IRES eGFP (HIV-1 Vpr), NL4-3 nef* without GFP (No Tag), and a combination of Tat+Vpr. 24 h post transfection cells were analyzed by flow cytometry and total cell lysates were subjected to SDS-PAGE and immunoblot

A) Diagram showing the % of GFP positive cells measured by FACS
B) Immunoblot using rat monoclonal antibody SPOC1 and mouse monoclonal α-Tubulin as a loading control.

## 4.6 Transfection of full length HIV-1 does not lead to SPOC1 reduction

While transfection of single HIV-1 proteins did not result in a reduction of SPOC1 it was tested if transfection of full length wild/type HIV-1 or different mutants would lead to SPOC1 decrease. HEK 293T cells were transfected with plasmids encoding for wild/type HIV-1 NL4-3 IRES eGFP or Vpr, Nef, Vpu or Env defective NL4-3 IRES eGFP. Transfection of a plasmid encoding for GFP only served as control. Transfection efficiencies were measured by FACS-analysis and confirmed that transfection rates of all proviral plasmids were in a similar range between 50 and 60 % (Figure 15 A). Again, total-cell extracts were separated by SDS-PAGE and subjected to immunoblotting. No reduction of SPOC1 was detectable in untransfected cells (mock) and GFP-only transfected cells. Surprisingly, neither transfection with the HIV-1 wt nor with one of the HIV-1 deletion mutants resulted in a decrease of SPOC1 (Figure 15 B), implying that transfection of cells with full length HIV-1 is also not sufficient for SPOC1 degradation.

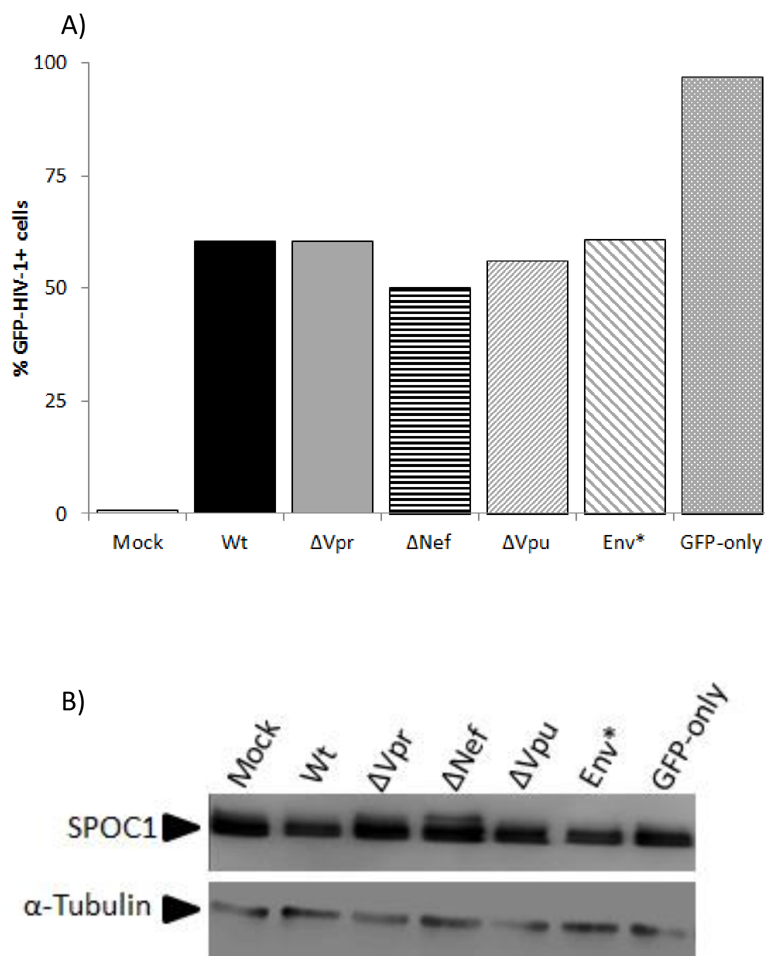


**Figure 15 Transfection of wild/type HIV-1 and single defective mutants is not sufficient for SPOC1 reduction**

HEK 293T cells were transfected with wild/type HIV-1 NL4-3 IRES eGFP (wt), NL4-3 IRES eGFP ΔVpr, NL4-3 IRES eGFP ΔNef and NL4-3 IRES eGFP ΔVpu, NL4-3 IRES eGFP Env*, and a plasmid expressing GFP only. 24 h post transfection cells were analyzed by flow cytometry and total cell lysates were subjected to SDS-PAGE and immunoblot

A) Diagram showing the % of HIV-1 infected (GFP positive) cells measured by FACS
B) Immunoblot using rat monoclonal antibody SPOC1 and mouse monoclonal α-Tubulin as a loading control.

## 4.7 Infection is essential for SPOC1 decrease

Since previous data have shown that neither transfection of Vpr alone nor with the full length HIV-1 genome is sufficient for SPOC1 reduction, it was tested if in contrast infection is required for SPOC1 reduction in direct comparison to transfected cells. HEK 293T cells were either transfected or infected with wild/type HIV-1 NL4-3 or HIV-1 ΔVpr NL4-3 IRES eGFP. For infection of HEK 293T cells, viruses were VSV-G pseudotyped, to allow HIV-1 infection of cells independent of the CD4 or chemokine co-receptor.

36 h after treatment, cells were monitored by FACS for GFP expression and total-cell extracts were prepared, separated by SDS-PAGE and subjected to immunoblotting.

Uninfected and not transfected cells served as control and showed no GFP signal. Transfection of HIV-1 wt resulted in 92,5 % GFP-positive cells, HIV-1 ΔVpr in 79,1 % (Figure 16 A). Furthermore, infection of HEK 293T resulted in 96,1 % GFP-positive cells for HIV-1 wt and 98,1 % for HIV-1 ΔVpr (Figure 16 A).

As expected, non-transfected and uninfected cells showed no reduction of SPOC1. Similar, in transfected or HIV-1 ΔVpr infected cells, no decrease of SPOC1 was observed. This confirms the importance of Vpr in the reduction of SPOC1. Intriguingly, while transfection of HIV-1 wt did not lead to a reduction of SPOC1, HIV-1 wt infection led to a significant reduction of SPOC1 (Figure 16 B). According to this, the full HIV-1 replication cycle is necessary for reduction of SPOC1. Conversely, transfection of cells, even with full length wild type HIV-1 is not sufficient for SPOC1 decrease. This already implies, that the early events in HIV-1 infection, i.e. entry or integration are mediators of Vpr-mediated SPOC1 reduction.

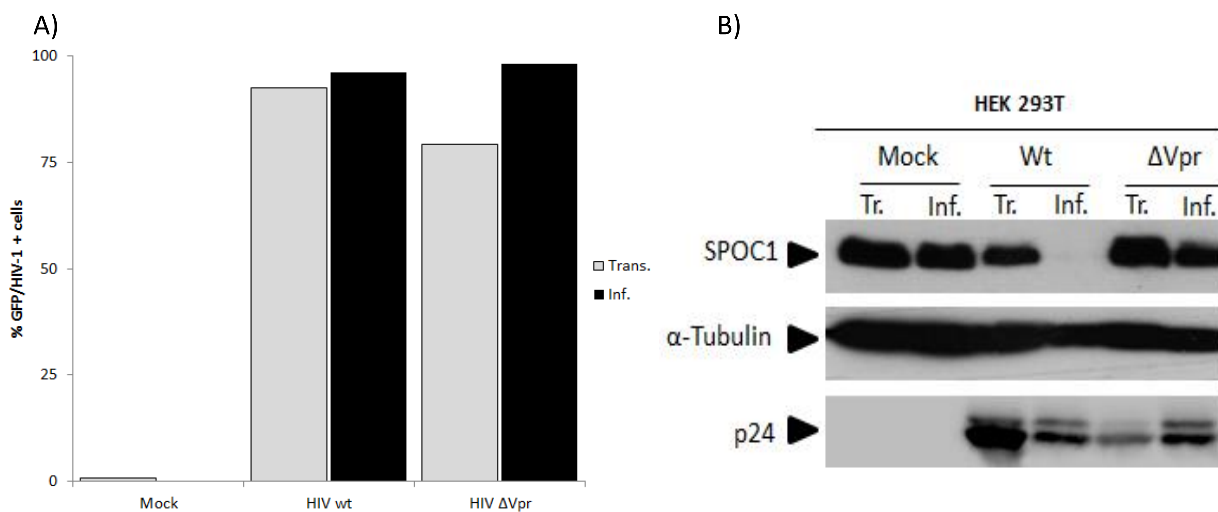


**Figure 16 SPOC1 levels are reduced upon HIV-1 infection**

HEK 293T cells were either infected or transfected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt), NL4-3 IRES eGFP ΔVpr or were left uninfected/not transfected (Mock). 36 h after treatment cells were analyzed by flow cytometry cell lysates were subjected to SDS-PAGE and immunoblot.

A) Diagram showing the % of HIV-1 infected (GFP positive) cells measured by FACS (grey bars= Transfection; black bars= infection)
B) Immunoblot with rat monoclonal antibody SPOC1, mouse monoclonal antibody p24, mouse monoclonal α-Tubulin (loading control)

## 4.8 Virion associated Vpr is able to reduce SPOC1

Previous experiments have shown that Vpr is essential for SPOC-1 degradation. In line with this, HIV-1 ΔVpr mutant virus infection did not lead to SPOC1 degradation. Thus we wanted to test if we can recover SPOC1 degradation by HIV-1 ΔVpr virus by transcomplementation with Vpr in the producer cell. Therefore, HIV-1 ΔVpr NL4-3 IRES eGFP were co-transfected with a plasmid encoding for Vpr. During virus budding, exogenously expressed Vpr is incorporated into virus particles through interaction with p6 [43; 44]. Efficiency of Vpr incorporation in the particles was previously confirmed in the context of another PhD thesis by Kristin Höhne [102].

To allow efficient infection of target cells, viral particles were VSV-G pseudotyped. Viral supernatants of HIV-1 wt, Vpr-transcomplemented Virus and HIV-1 ΔVpr were used to inoculate SupT1 cells for 48 h. Infection efficiencies were determined by FACS analyzes of GFP expression. As can be seen in Fig.17A) infection of cells was in a similar range between 58 and 66 %.

Western blot analysis of total cell extracts showed no reduction of SPOC1 in mock sample. As expected, infection with HIV-1 wt resulted in a complete reduction of SPOC1. In contrast, if SupT1 cells are infected with HIV-1 lacking Vpr, no reduction of SPOC1 was observed (Figure 17 B). However, if Vpr is transcomplemented into the viral particle, a reduction of SPOC1 protein level can be detected again. Hence, virion delivered Vpr is sufficient for HIV-1 mediated SPOC1 degradation. This is in line with previous experiments, already implying that SPOC1 is degraded during the early steps of HIV-1 infection and denovo synthesis of Vpr e.g. by transfection of plasmids or from the proviral backbone is dispensable for this effect.

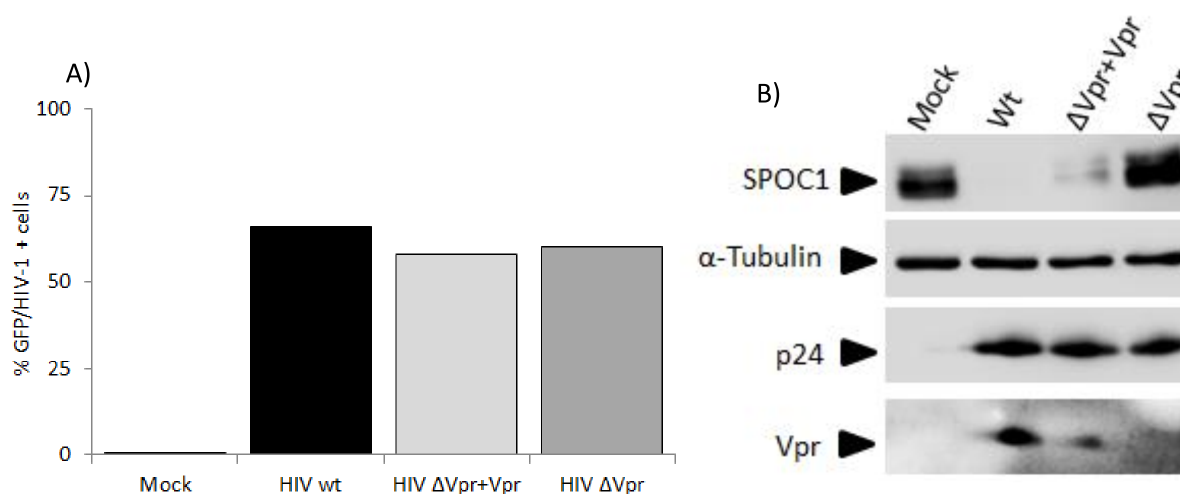


**Figure 17 SPOC1 levels are reduced upon HIV-1 infection**

SupT1 cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt), HIV-1 NL4-3 IRES eGFP ΔVpr, transcomplemented NL4-3 IRES eGFP ΔVpr+Vpr, or were left uninfected (Mock). 48 h after infection, cells were analyzed by flow cytometry and total cell extracts were prepared, separated by SDS-PAGE and analyzed by immunoblot.

A) Diagram showing the % of HIV-1 infected (GFP positive) cells measured by FACS
B) Immunoblot with rat monoclonal antibody SPOC1, mouse monoclonal antibody p24, mouse monoclonal α-Tubulin as a loading control, and rabbit monoclonal antibody Vpr

## 4.9 Integration of HIV-1 provirus is a central step for SPOC1 degradation

To gain better understanding which step of the replication cycle is important for the degradation of SPOC1, HIV-1 inhibitors that block different steps of the replication cycle, were used. Cells were infected with HIV-1 wt virus and simultaneously incubated with the different inhibitors (see 2.7.4). 48h post infection, cells were analyzed via FACS measurements for GFP expression, cell extracts separated by SDS-PAGE and subjected to immunoblotting. Untreated cells (n.t.) and DMSO treated cells served as controls and showed the highest infection rate at around 77 %.

As expected, samples treated with raltegravir and efavirenz showed the lowest GFP-signal with fewer than 10 % and treatment with saquinavir and flavopiridol resulted in GFP positive cells in the range of 50-67 % (Figure 18).

No reduction of SPOC1 was visible in all uninfected samples as determined by immunoblotting. Infection of cells with HIV-1 wt resulted in reduction of SPOC1 in untreated and DMSO treated cells.

Additionally, SPOC1 reduction was observed in samples treated with saquinavir and flavopiridol. On the contrary, the use of raltegravir and efavirenz resulted in stable SPOC1 levels. Still, these samples showed the highest p24 signal, confirming efficient entry of the virus (Figure 19). Whereas saquinavir and flavopiridol target late steps of viral replication after integration, raltegravir and efavirenz inhibit steps prior to integration. Thus our data indicates that SPOC1 is degrated by Vpr early post integration, before viral gene expression occurs.

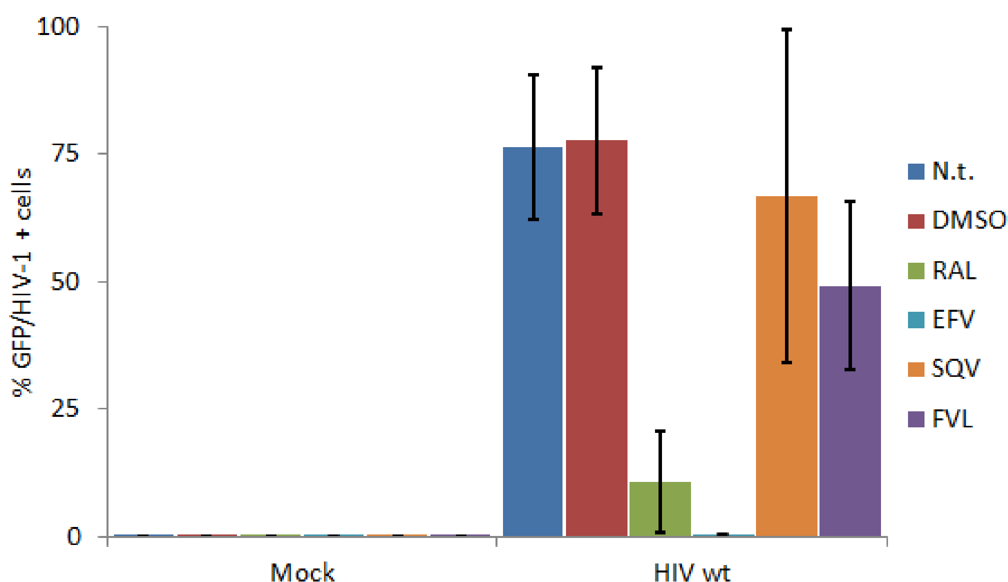


**Figure 18 Impact of HIV-1 inhibitor on SPOC1 degradation**

SupT1 cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt) or left uninfected (Mock), simultaneously cells were incubated with different inhibitors. 48 h after infection, cells were analyzed by flow cytometry and cell cell lysates were subjected to SDS-PAGE and immunoblot

Diagram showing the % of HIV-1 infected (GFP positive) cells measured by FACS. Values correspond to the mean of three independent experiments and error bars indicate the standard deviation.

Untreated cells (n.t.); Raltegravir=RAL; Efavirenz=EFV; Saquinavir =SQV; Flavopiridol =FVL

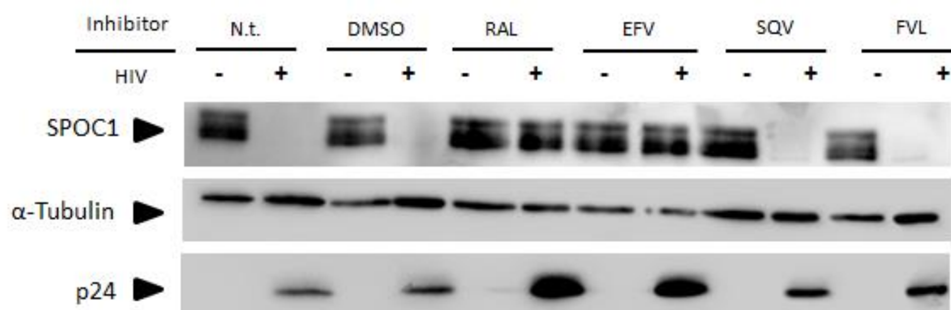


**Figure 19 Impact of HIV-1 inhibitor on SPOC1 degradation**

SupT1 cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt) or left uninfected (Mock), simultaneously cells were incubated with different inhibitors. 48 h after infection, cells were analyzed by flow cytometry and cell cell lysates were subjected to SDS-PAGE and immunoblot.
Immunoblot with rat monoclonal antibody SPOC1, mouse monoclonal antibody p24, mouse monoclonal α-Tubulin (loading control). Untreated cells (n.t.); Raltegravir=RAL; Efavirenz=EFV; Saquinavir =SQV; Flavopiridol =FVL

## 4.10 SPOC1 is degraded via the proteasomal pathway

Previous published studies showed that in general the proteasomal pathway is involved in regulation the stability and degradation of SPOC1 [15; 25]. Hence, reduction of SPOC1 steady-state protein levels post HIV-1 infection might also involve proteasomal degradation. To address this question, two proteasomal inhibitors were used in further experiments. HEK 293T cells were infected with HIV-1 wt and 12 h post infection cells were treated with proteasome inhibitors MG 132 and Lactacystin for additional 36 h. Afterwards, cells were analyzed by FACS for GFP expression and total-cell extracts were separated by SDS-PAGE and subjected to immunoblotting. Flow cytometry measurements showed an infection of cells of around 80 % in untreated and lactacystin treated cells. Treatment with MG132 resulted in reduction of the GFP signal to only 43 % (Figure 20 A).
Western blot examination of uninfected samples displayed no reduction of SPOC1. In contrast, HIV-1 infection in untreated cells led to reduction of SPOC1. Lactacystin slightly inhibited SPOC1 reduction after HIV-1 infection. Strikingly, MG132 completely inhibited the reduction of SPOC1 (Figure 20 B), suggesting that SPOC1 most likely is degraded by the proteasome.

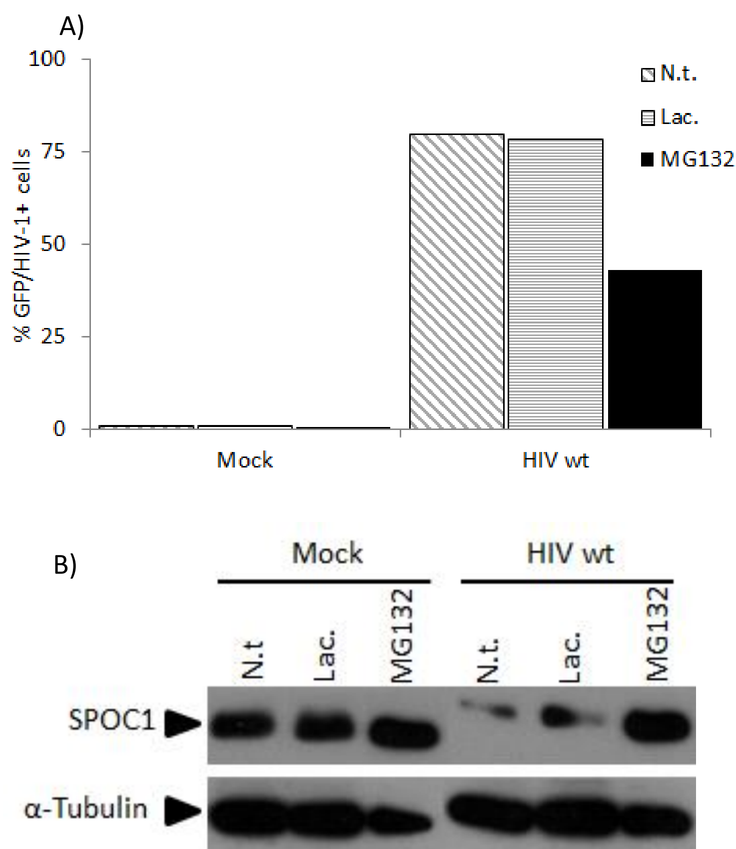


**Figure 20 Analyses of proteasomal inhibitors in context of HIV-1 related SPOC1 degradation**

HEK 293T cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt) or left uninfected (Mock). 12 hpi cells were treated with the proteasome inhibitor Lactacystin (Lac, Konz) and MG123 (Konz) for additional 36h. Cells were then analyzed by flow cytometry and total cell extracts were subjected to SDS-PAGE and protein expression analyszed by immunoblot

A) Diagram showing the % of HIV-1 infected (GFP positive) cells measured by FACS.
B) Immunoblot using rat monoclonal SPOC1 antibody and mouse monoclonal α-Tubulin antibody as a loading control.
N.t.= no inhibitor

## 4.11 GSK3-beta is important for SPOC1 degradation

GSK-3-β phosphorylation motifs are important for SPOC1 stability [15]. To test if GSK-3-β is involved in HIV-1 mediated SPOC1 degradation, activity of GSK-3-β was blocked by treating HEK 293T cells with either SB216763 in different concentrations or with insulin, two know GSK-3-ß inhibitors (see 2.7.4.). After a 6 hour treatment with the inhibitors, cells were infected with HIV-1 wt and incubated for additional 48 h. Subsequently, cells were analyzed by FACS measurements for expression of GFP and total-cell extracts were separated by SDS-PAGE and subjected to immunoblotting. FACS-analyses revealed that the inhibitor SB216763 led to GFP-autofluorescence of about 37 % in the highest concentration in uninfected cells (Figure 21 A). Infection with HIV-1 wt of all samples resulted in the same infection range of about 85-93 % GFP positive cells (Figure 21 A). Western blot analysis confirmed SPOC1 reduction by HIV-1 wt infection in untreated cells. Treatment of cells with the inhibitor SB216763 resulted in a reduction of SPOC1 in uninfected cells, but stabilized SPOC1 at higher concentrations. Reduction of SPOC1 in SB216763 treated and infected cells were more pronounced compared to uninfected cells.

Likewise, SPOC1 levels were stabilized more significantly at higher inhibitor doses in HIV-1 wt infected cells (Figure 21 B). Cells cultured with insulin showed no SPOC1 reduction in infected cells compared to uninfected cells. These results indicate that HIV-1 mediated SPOC1 depletion can be inhibited by suppression of GSK-3-β activity and suggest that GSK-3-ß induced phosphorylation of SPOC1 might be the mechanism of SPOC1 degradation.

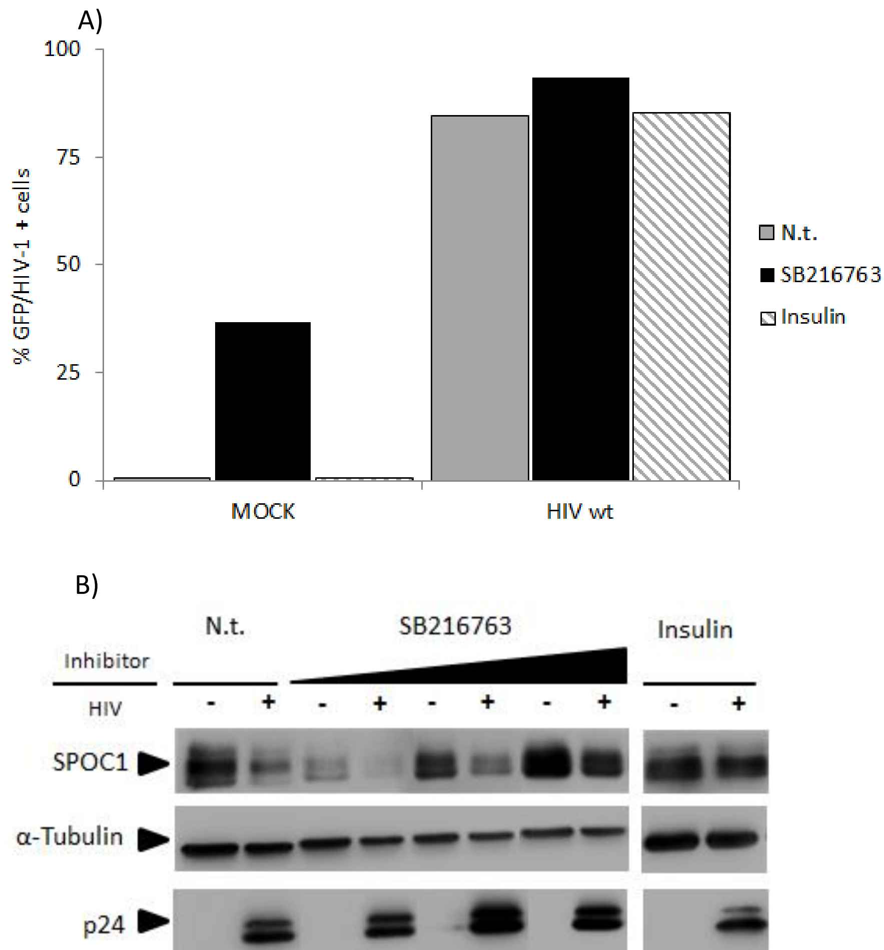


**Figure 21 GSK-3-β is important for SPOC1 reduction**

HEK 293T cells were treated with GSK-3-ß inhibitor for 6 hours, followed by infection with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt) or left uninfected (Mock). 48 h after infection, cells were analyzed by flow cytometry and western blot.

A) Shown is a diagram presenting the HIV-1 infected (GFP positive) cells of not treated (n.t.), SB216763 100 µM, and Insulin 100nM samples measured by FACS analysis.

B) Total cell lysates were subjected to SDS-PAGE and immunoblot. The immunoblot was done by using rat monoclonal SPOC1 antibody, mouse monoclonal α-Tubulin antibody as a loading control, and mouse monoclonal p24 antibody.
Untreated cells (n.t.); plus= Infection with HIV-1; minus= No infection ; SB216763 conc.= 10, 40, 100 µM ; Insulin = 100nM

## 4.12 SPOC1 is reduced independently from the CUL4A ubiquitin ligase complex

HIV-1 Vpr is known to use the CUL4A ubiquitin ligase complex to degrade proteins [78; 79]. Consequently CUL4As role for reduction of SPOC1 was analyzed, by inhibiting the complex with the small molecule MLN4924 (see 2.7.4). SupT1 cells were incubated for 3 hours with the inhibitor before they were infected with HIV-1 wt. 48 hpi, GFP expression of the cells was monitored by FACS and total-cell extracts were separated by SDS-PAGE and subjected to immunoblotting. No GFP expression was measurable in uninfected, untreated or DMSO treated cells.

As shown in Figure 22A, infection resulted in 78 % GFP-positive cells in untreated or DMSO treated cells. All cells treated with MLN4924 showed a similar GFP signal in the range between 54-57 % (Figure 22 A). Western blot analysis confirmed SPOC1 expression in uninfected cells and SPOC1 reduction in untreated or DMSO treated cells upon HIV-1 wt infection. Interestingly, independent from inhibitor concentrations, SPOC1 was reduced in all approaches compared to uninfected controls (Figure 22 B). This data implies that SPOC1 is reduced independent from the CUL4A complex.

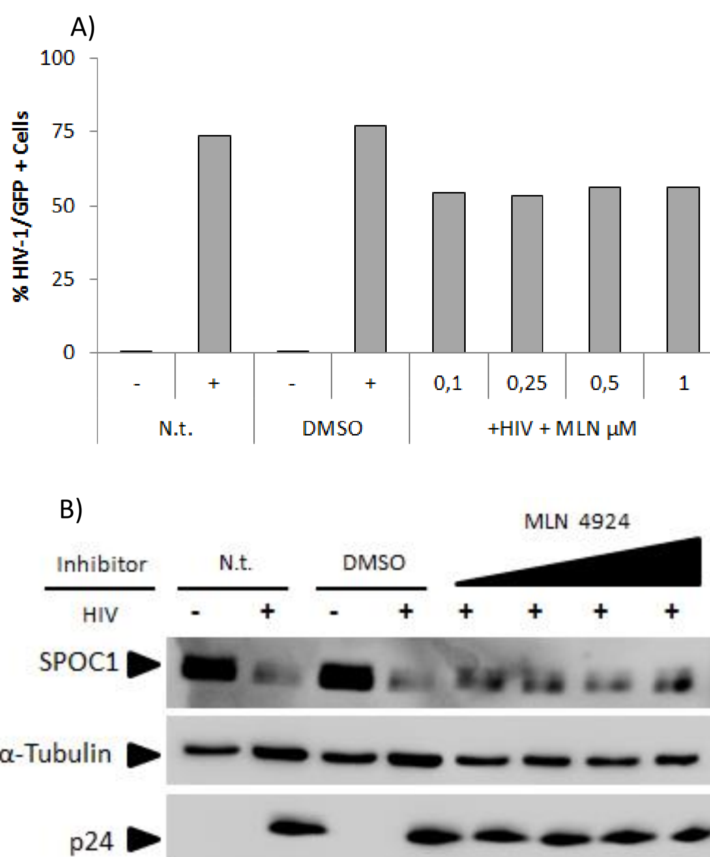


**Figure 22 CUL4A complex has no impact on SPOC1 reduction**

SupT1 cells were incubated with MLN4924, DMSO or were left untreated for 3 h. Subsequently cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP (wt) or left uninfected (Mock). 48 hpi, cells were analyzed by flow cytometry and cell lysates were subjected to SDS-PAGE and immunoblot

A) Diagram showing the % of HIV-1 infected (GFP positive) cells measured by FACS.
B) Total cell extracts were subjected to 12% SDS-PAGE and immunoblotting using rat monoclonal SPOC1 antibody, mouse monoclonal α-Tubulin antibody as a loading control, and mouse monoclonal p24 antibody.
Untreated cells (n.t.); plus indicates infection with HIV-1 wt VSV-G; minus indicates no infection; MLN4924 conc.= 0,1; 0,25; 0,5; 1 µM

# 4.13 SPOC1 overexpression before infection enhances productive HIV-1 expression

HIV-1 degrades SPOC1 post infection and previously it was demonstrated, that SPOC1 is an antiviral factor inhibiting Adenoviral replication. Hence the next step was to investigate the consequence of SPOC1 overexpression or siRNA mediated knock-down for HIV-1 replication.

In a first attempt, microporation was conducted to deliver the pCG-SPOC1-IRES-mtagBFP expression constructs into Jurkat-TAg cells to express exogenous SPOC1 (see 3.3.3.3). Another subset of cells was electroporated with pCG-IRES-mtagBFP only and served as negative control. 24 h post microporation total-cell extracts was separated by SDS-PAGE and subjected to immunoblotting. As expected, SPOC1 was highly overexpressed in Jurkat-TAg cells which were microprated with SPOC1 vector. Cell extracts from untransfected and BFP only transfected cells showed a comparable weak SPOC1 expression pattern (Figure 23).

Next, 24 h post microporation, cells were infected with HIV-1 wt and 24 hours later, cells were analyzed for BFP and GFP expression by flow cytometry. Detection of BFP-positive cells referred to SPOC1 expression, whereas GFP-positive cells indicated productive HIV-1 infection. Surprisingly, FACS analyses indicated that SPOC1 overexpression resulted in an almost 2,5 fold increase (225,18 % ± 28,04 %) of double positive cells compared to the BFP only sample (Figure 24 A). Furthermore, the mean fluorescence intensity (MFI) as measurement for gene expression and LTR activity were also determined and were simultaneously increased by SPOC1 overexpression (220,72 % ± 25,56 %)(Figure 24 B). These results suggest that SPOC1 mediates an increase of HIV-1 infection rates in Jurkat-TAg cells.

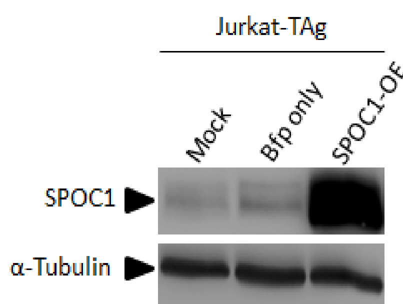


**Figure 23 Exogenous SPOC1 expression in Jurkat-TAg cells after microporation**

Jurkat-TAg cells were microporated with SPOC1 or BFP only expression plasmid and SPOC1 expression was analyzed 24 hours post microporation. Total-cell extracts were subjected to 12 % SDS-PAGE and immunoblotting was performed using rat monoclonal SPOC1 antibody and mouse monoclonal α-Tubulin antibody as a loading control.

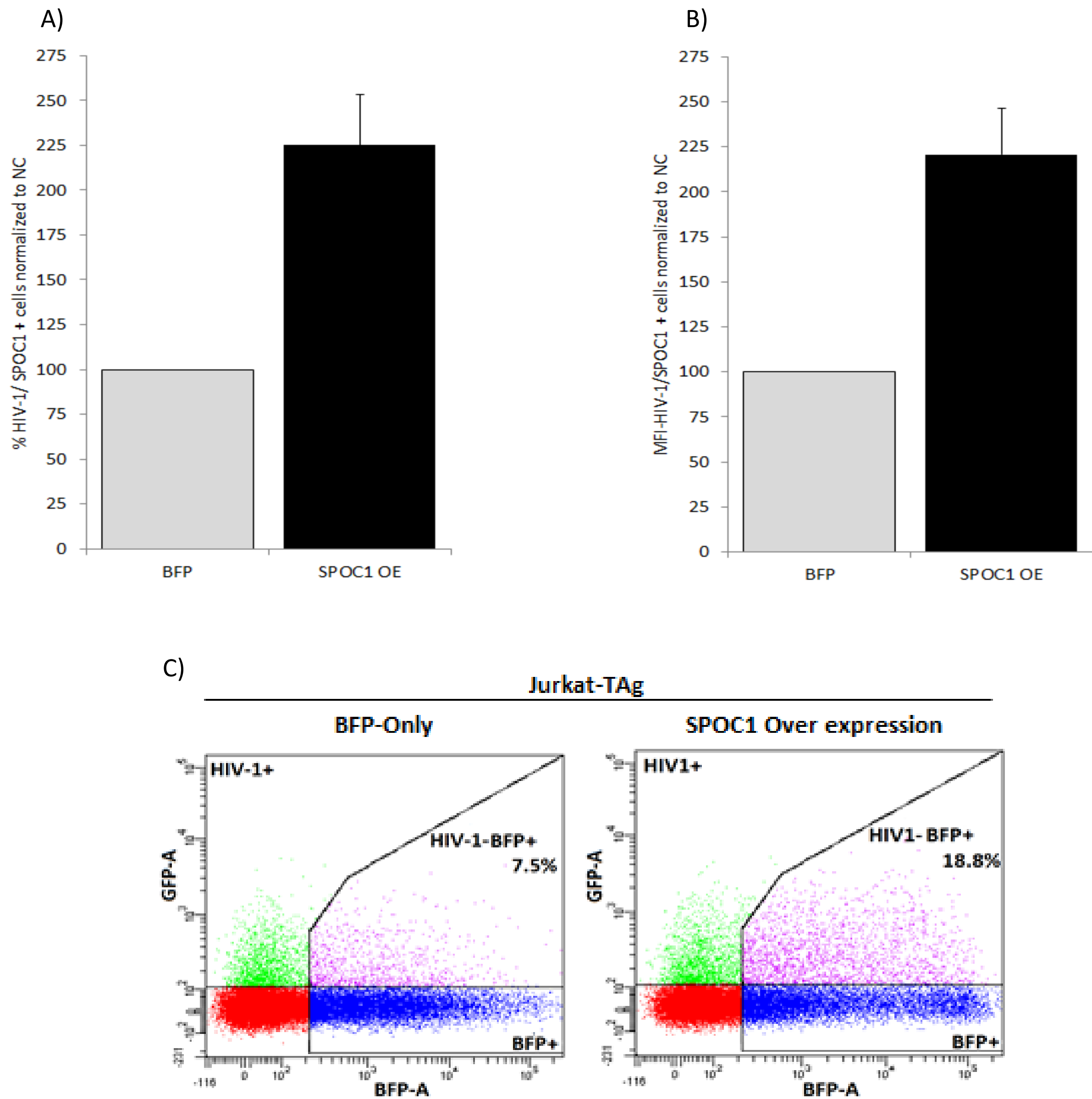


**Figure 24 Impact of SPOC1 overexpression on HIV-1 infection in Jurkat-TAg cells**

Jurkat-TAg cells were microporated with SPOC1-BFP or BFP only expression plasmid and infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP 24 hours later. 24 hpi cells were analyzed by flow cytometry.

A) The mean percentage of BFP-GFP double positive cells of three independent experiments was calculated and the resulting data were subsequently normalized to the control which in turn was adjusted to 100 %.
B) Diagram presenting the MFI values measured by FACS, calculated as the mean percentage of three independent approaches normalized to the control which was adjusted to 100 %. Error bars indicate the standard deviation.
C) Representative FACS dot-plots showing GFP- and BFP-positive cells and the percentages of BFP/GFP-double-positive cells.

## 4.14 SPOC1 overexpression in human osteosarcoma cell line also enhances productive HIV-1 infection

To confirm the observation that SPOC1 overexpression leads to a higher HIV-1 infection rate and to understand the role of SPOC1 during HIV-1 infection, experiments in a SPOC1-inducible human osteosarcoma cell line (U2OS-Cl.5), which expresses exogenous SPOC1 after doxycyclin (dox) treatment, were performed. Prior to the analysis, SPOC1 protein expression in the absence and presence of doxycycline was monitored by immunoblotting. Indeed, 24 hours after treatment with doxycycline, extracts from U2OS-Cl.5 cells showed a high SPOC1 protein expression compared to uninduced cells (Figure 25). 24 hours after dox treatment, cells were infected with HIV-1 wt stocks and incubated for additional 24 hours. Subsequently, GFP expression and MFI values were monitored by flow cytometry. As seen in Fig.26 A, SPOC1 overexpression led to an increase of GFP positive cells (174 % ± 13 %) compared to untreated cells. Additionally, the MFI was monitored and displayed a similar increase (178,63 % ± 31,08 %) (Figure 26 B). In agreement with the experiments performed in SPOC1 expressing Jurkat T cells, SPOC1 overexpression prior to infection led to enhanced HIV-1 infection, suggesting that SPOC1 has a positive effect on an early step during viral replication.

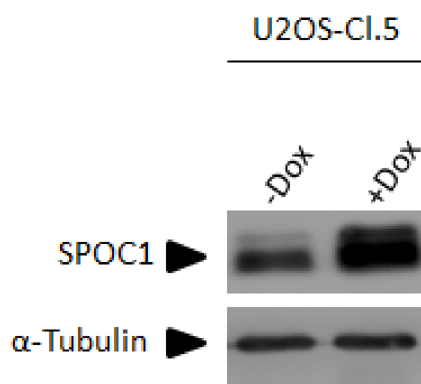


**Figure 25 Exogenous SPOC1 expression in U2OS-Cl.5 cells after Doxycycline treatment**

24 h after doxycycline treatment, total-cell extracts were subjected to 12 % SDS-PAGE and immunoblotting was performed using rat monoclonal SPOC1 antibody and mouse monoclonal α-Tubulin antibody as a loading control.

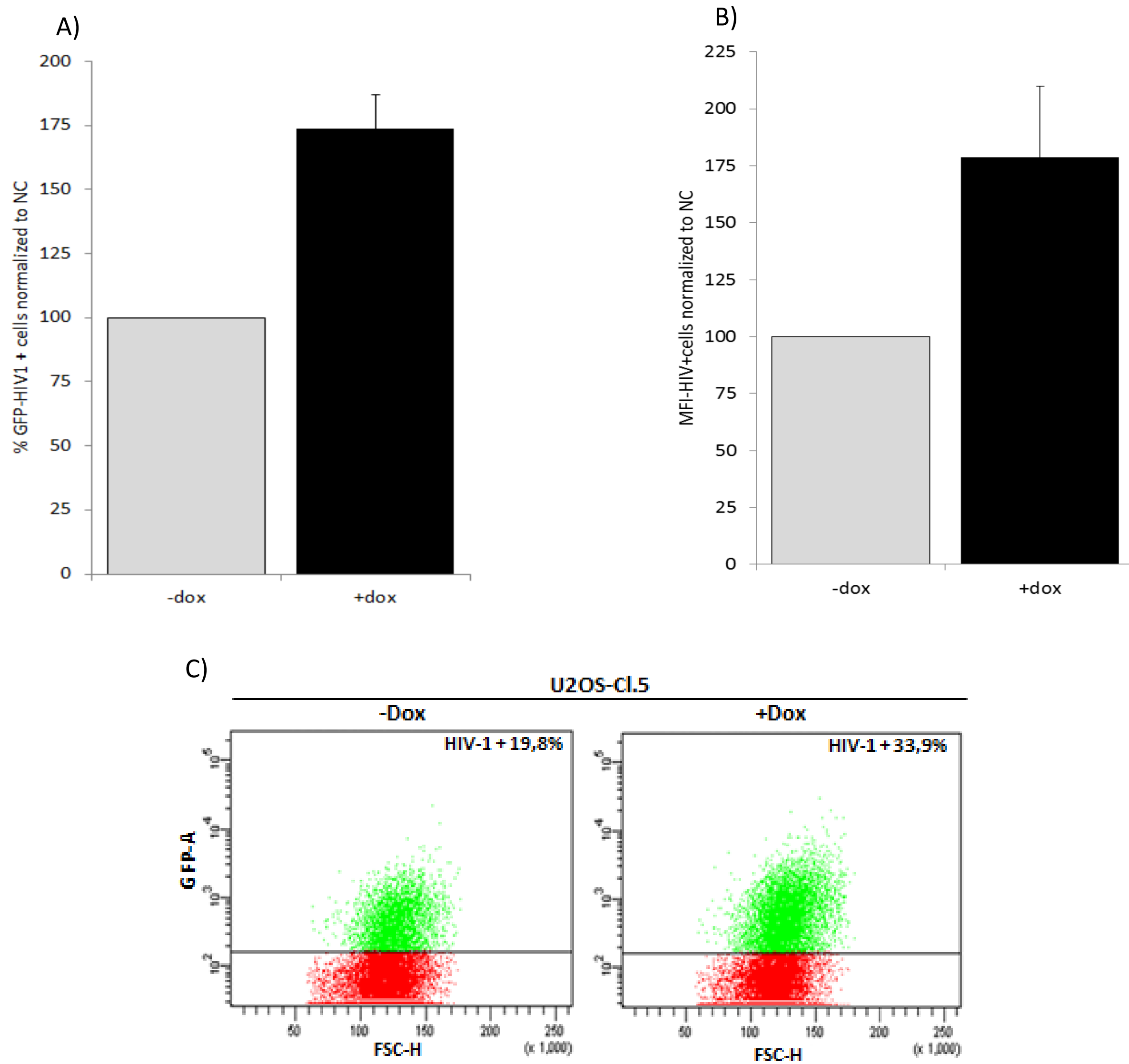


**Figure 26 SPOC1 overexpression in U2OS-Cl.5 cells leads to enhanced HIV-1 infection**

U2OS-Cl.5 cell were treated with doxycycline or left untreated for 24 hours before cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP. 24hpi cells were analyzed by flow cytometry.

A) The mean percentage of GFP positive cells of three independent experiments was calculated and the resulting data was subsequently normalized to the untreated cells, which was adjusted to 100 %.

B) Diagram presenting the MFI values measured by FACS, calculated as the mean percentage of three independent approaches normalized to the untreated cells, which was adjusted to 100 %. Error bars indicate the standard deviation.

C) Representative FACS dot-plots showing GFP-positive cells and percentages of GFP-positive cells

To exclude the possibility that the observed effect is a result of the used Tet-On system in the U2OS-Cl.5 and the dox treatment, the same experiment was performed with the native human osteosarcoma cell line (U2OS). As shown in Figure 27, treatment of U2OS cells with doxycycline had no effect on HIV-1 infection (GFP positive cells; 99,62 % ± 6,03 %) compared to untreated cells. Moreover, the MFI was equal in both approaches (dox=105,27 % ± 24,73 % - see Figure 27 B). Conclusively, these results verify previous results and indicate a specific positive SPOC1 influence on the early phase of virus infection.

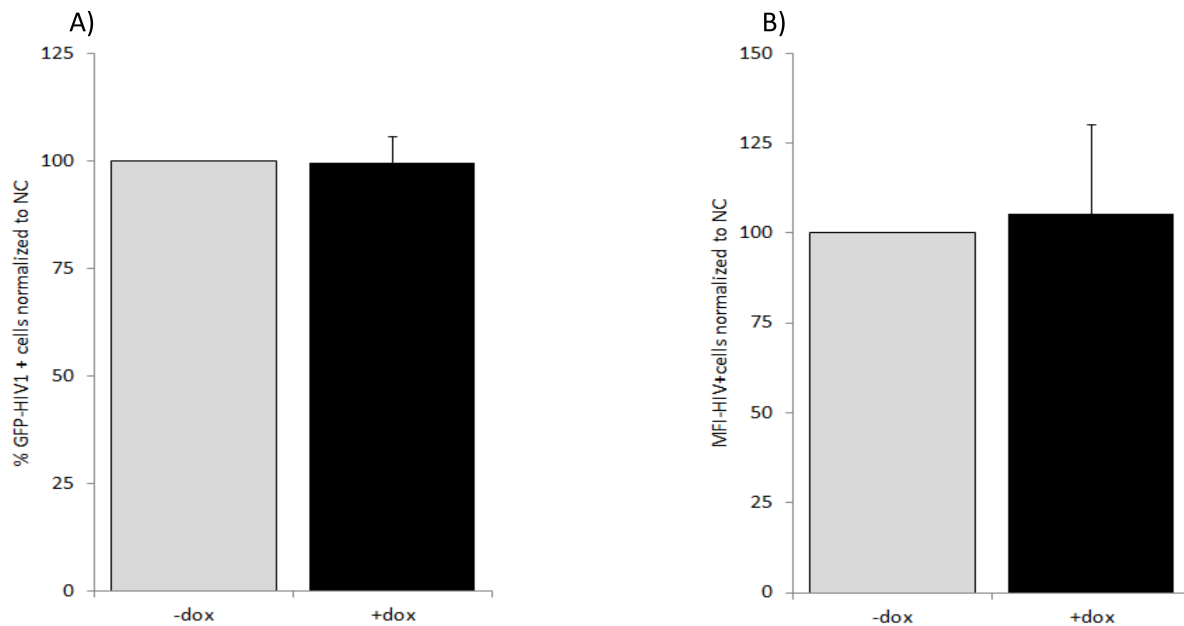


**Figure 27 Doxycycline treatment has no influence on HIV-1 wt infection in U2OS cells**

Native U2OS cell were treated with doxycycline or left untreated for 24 hours before infection with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP was performed. 24hpi cells were analyzed by flow cytometry.

A) The Mean percentage of GFP positive cells was calculated and the resulting data were subsequently normalized to untreated cells, which was adjusted to 100 % (n=3).
B) Diagram presenting the MFI values measured by FACS, calculated as the mean percentage of three independent approaches normalized to untreated cells, which was adjusted to 100 %. Error bars indicate the standard deviation.

## 4.15 SPOC1 overexpression enhances HIV-1 integration

As SPOC1 is associated with DNA repair, is recruited to DSBs and regulates the kinetics of DSB repair [15; 22], it is possible that SPOC1 has an influence on the integration process of HIV-1. Therefore the following experiments should elucidate if SPOC1 overexpression would lead to changes in HIV-1 integration.

Jurkat-TAg and U2OS-Cl.5 cells were treated similar to the previous experiments (4.13 & 4.14). Briefly, 24 h post SPOC1 overexpression cells were infected with HIV-1 wt for 6 h. Cells were washed to remove inoculum and incubated for additional 18 h, before genomic DNA was isolated and *Alu*-PCR was performed (see 3.1.3.2 & 3.2.1.1).

Another subset of U2OS-Cl.5 cells was additionally treated with the integrase inhibitor Raltegravir, serving as negative control. SPOC1 overexpression in Jurkat-TAg cells increased integrated HIV-1 provirus by seven fold (7,75 +/- 4,18) compared to the control. Similar effects were observed when monitoring dox treated U2OS-Cl.5. Here SPOC1 overexpression also enhanced HIV-1 proviral integration by almost three fold (2,82 +/- 0,55) compared to untreated cells (Figure 28). In samples treated with the integrase inhibitor no measurable integrated HIV-1 provirus was detected. Together, these results indicate that SPOC1 expression prior to infection with HIV-1 positively influences/enhances the integration process of HIV-1.

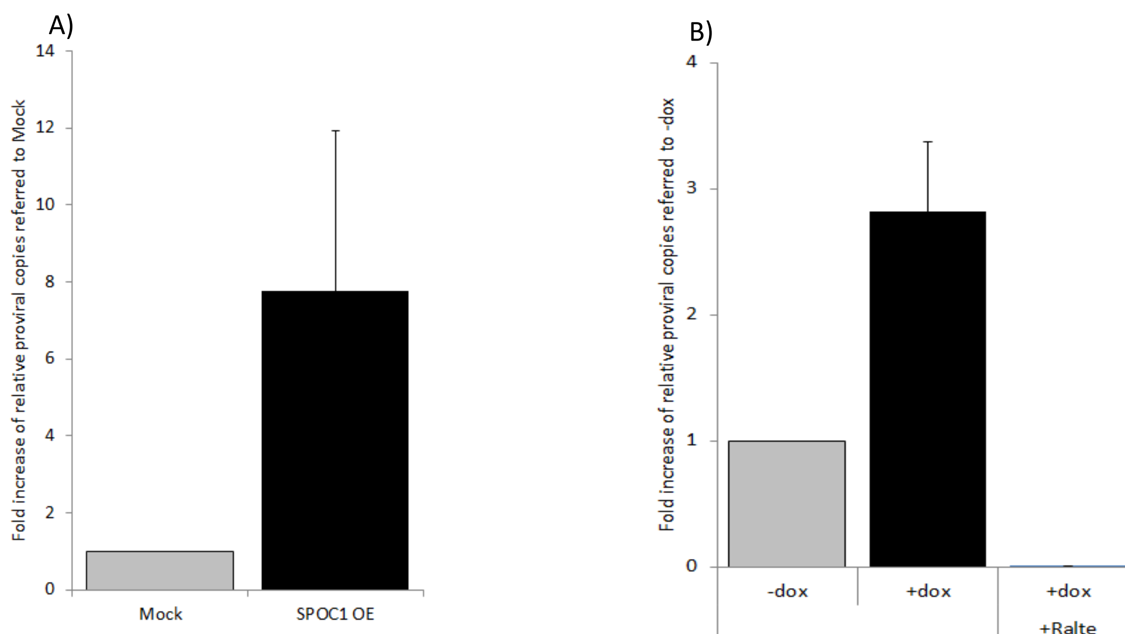


**Figure 28 Early SPOC1 overexpression leads to increase in HIV-1 integration**

SPOC1 was overexpressed in Jurkat-TAG (A) and U2OS-Cl.5 (B) cells for 24 hours before cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP. 6hpi cells were washed and incubated for additional 18h. Subsequently, genomic DNA was isolated and analyzed via *Alu*-PCR for integrated HIV-1 provirus. Mean fold of relative proviral copies of two independent approaches in Jurkat-TAg cells (A) and three in U2OS-CL.5 cells (B). The mean was calculated and the resulting data was subsequently normalized to the control which was adjusted to 1. Error bars indicate the standard deviation.

## 4.16 Impact of SPOC1-knock down on HIV-1 infection

To further investigate the impact of SPOC1 on early steps of the replication cycle, SPOC1 expression was depleted by transient knock-down with specific siRNA (see 2.4.3).

SPOC1 siRNA was delivered into Jurkat-TAg cells by microporation and in U2OS-Cl.5 cells by transfection with lipofectamin. 36 h post transfection, total-cell extracts was separated by SDS-PAGE and subjected to immunoblotting to confirm knock-down of SPOC1 expression. As expected, SPOC1 was depleted in Jurkat-TAg and U2OS-CL.5 cells upon siRNA transfection. Extracts from non-transfected cells and cells transfected with scrambled siRNA showed no change in SPOC1 expression (Figure 29 A).

Next, 36 h after transfection with siRNA, Jurkat-Tag and U2OS-Cl.5 cells were infected with HIV-1 wt. 24 hours later, cells were analyzed by flow cytometry for GFP expression representing efficient HIV-1 infection. Surprisingly, SPOC1 knock down had neither an effect in Jurkat-TAg cells nor in U2OS-Cl.5 cells as measured by flow cytometry. Samples infected with HIV-1 wt showed similar infection rates in a range between 88 and 100 % (Figure 29 B). These data imply that SPOC1 knock down prior to HIV-1 infection does not interfere with HIV-1 infection.

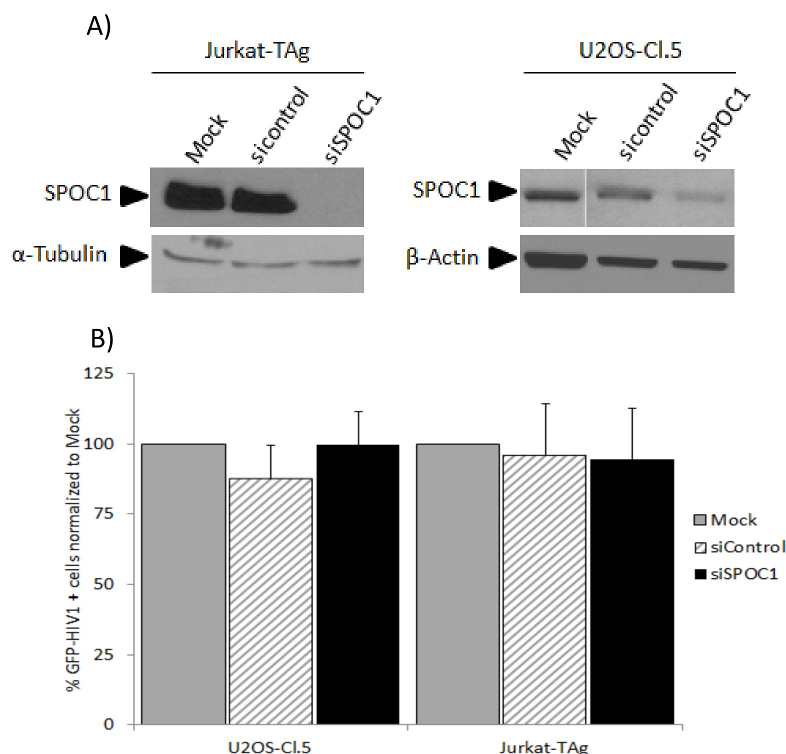


**Figure 29 SPOC1 knock down in Jurkat-TAg and U2OS-Cl.5 cells shows no effect**

Jurkat-TAg and U2OS-Cl.5 cells were transfected with siRNA constructs directed against SPOC1 (siSPOC1 5' UCA CCU GUC CUG UGC GAA A 3'), control siRNA (siRNA Negative Control-N2 from Riboxx), or left untreated (Mock). 36 hours later cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP for 24h and analyzed by flow cytometry.

A) Validation of SPOC1 knock down 36h after transfection with specific siRNA in Jurkat-TAg (left panel) and U2OS-Cl.5 Cells (right panel). Total-cell extracts were subjected to 12 % SDS-PAGE and immunoblotting using rat monoclonal SPOC1 antibody and mouse monoclonal α-Tubulin/beta-actin antibody as a loading control was performed.
B) FACS diagram displaying the relative mean percentage of GFP positive cells (n=3). Mean was calculated and the resulting data were subsequently normalized to Mock which was set as 100 %. Error bars indicate the standard deviation.

## 4.17 SPOC1 overexpression post HIV-1 integration suppresses HIV-1 transcription

The data thus far indicates that SPOC1 has a positive influence on HIV-1 integration and is degraded early upon completion of this process by HIV-1 Vpr protein. Hence, in contrast to its positive effect on integration, SPOC1 expression might negatively influence late steps of the HIV-1 replication cycle. To gain a better understanding of the role of SPOC1 in the late phase of HIV-1 replication, U2OS-Cl.5 cells were infected with HIV-1 wt for 6 h. Cells were then washed and incubated for additional 18 h. Subsequently, cells were treated with doxycyline to induce SPOC1 expression or left untreated for 24h, before the percentage of GFP+ (HIV-1 infected) cells and the GFP MFI (HIV-1 LTR activity) was determined by FACS. Flow cytometry revealed that untreated (86,27 % ± 3,78 %) as well as dox treated cells (77,75 % ± 2,62 %) showed equal infection efficiencies (Figure 30 A). However, in cells overexpressing SPOC1 the MFI decreased by nearly 70 % compared to the untreated sample (Figure 30 B), suggesting a negative impact of SPOC1 on the late phase of the HIV-1 replication cycle, in particular LTR activity and viral gene expression.

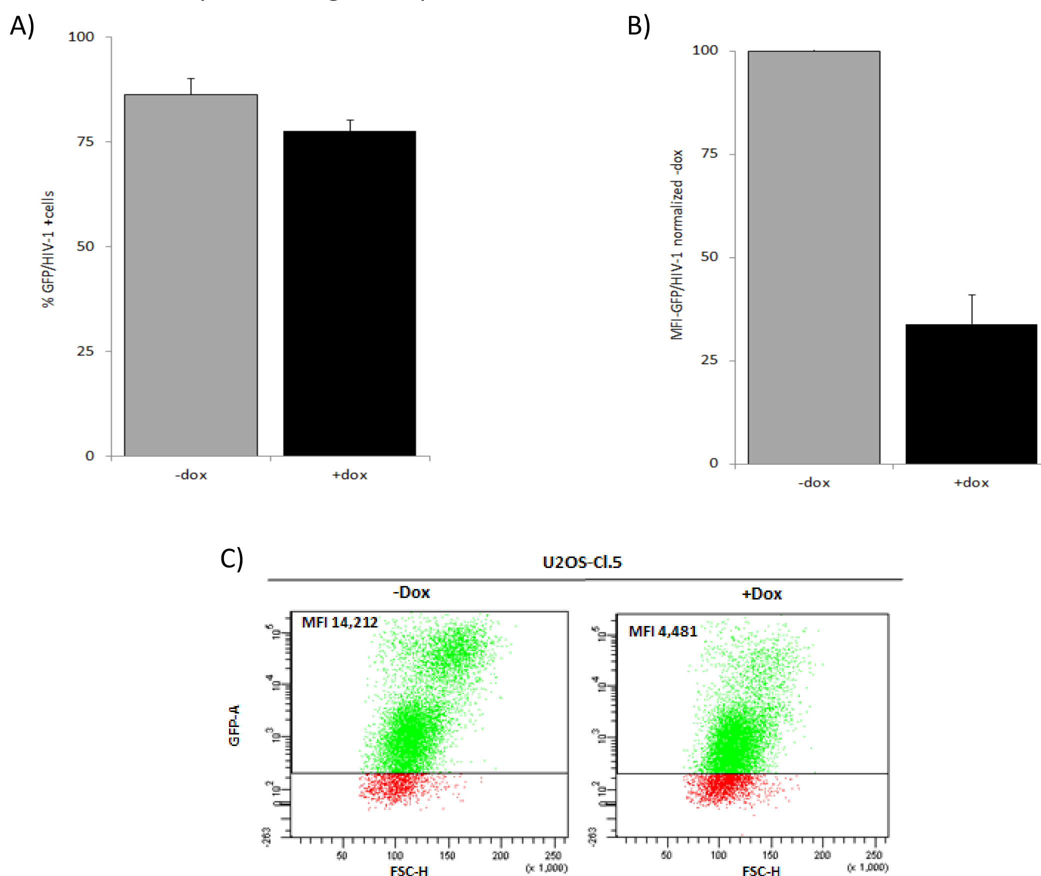


**Figure 30 Overexpression of SPOC1 in U2OS-Cl.5 cells represses HIV-1 gene expression**

U2OS-Cl.5 cell were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP for 24 h. Thereafter, cells were treated with doxycycline or left untreated to induce SPOC1 expression for 24 hours before cells were analyzed by flow cytometry.

A) Diagram presenting the relative mean percentage and standard deviation of HIV-1-infected (GFP-positive) cells, which was calculated of three independent approaches and subsequently normalized to the control which was set as 100 %. Error bars indicate the standard deviation.
B) Diagram displaying the MFI of GFP+ cells normalized to the untreated control as before.
C) Representative FACS dot-plot showing GFP-positive cell. The GFP-MFI is indicated in the corner.

To verify if induction of SPOC1 overexpression leads to a reduction in HIV-1 LTR transactivation and viral gene expression in late steps during viral replication, supernatants were analyzed for released HIV-1 particles in parallel. Therefore, supernatants of cells were analyzed by ELISA for HIV-1 p24 CA antigen for detection of virus production and release. Additionally, SupT1 cells were inoculated with the same supernatants and incubated for 48h. Next, GFP expression of cells was monitored by flow cytometry. As expected, SPOC1 overexpression in the late phase of HIV-1 replication resulted not only in decreased LTR-activity but also in a decrease of p24 production of about 50 % (Figure 31 A). As shown in Figure 31 B), this reduction on p24 production results in less infectious viral particles, since infection of SupT1 with supernatants of SPOC1 overexpressing cells was reduced accordingly (12,7 % ± 2,12 %) compared to SupT1 cells infected with supernatants from untreated U2OS.Cl5 cells (22,05 % ± 1,34 %) (Figure 31 B). These results further indicate a repressive effect of SPOC1 during HIV-1 viral gene expression.

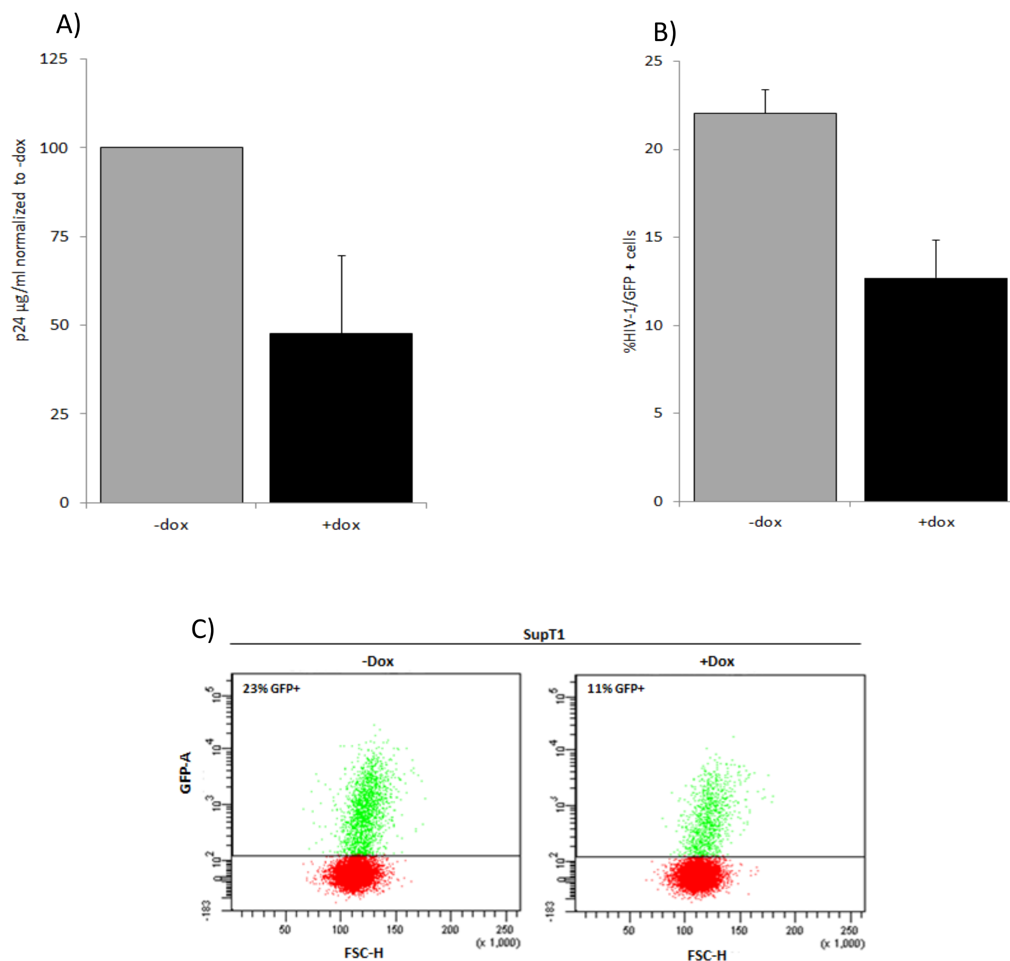


**Figure 31 Overexpression of SPOC1 at later stages of viral replication leads to reduced infectious virus particle release**

Supernatants of previous experiments (4.17) were analyzed for p24 content by ELISA and were used to inoculate SupT1 cells. 24hpi cells were analyzed by flow cytometry.

A) Diagram presenting mean p24 antigen amounts in the supernatant normalized to the control which was set as 100 %.
B) FACS diagram displaying the relative mean percentage and standard deviation of HIV-1-positive (GFP) cells of newly infected SupT1 cells. Error bars indicate the standard deviation.
C) Representative FACS dot-plots showing GFP-positive cells in SupT1 cells after infection with supernatant of previous experiments

To exclude unspecific impact of the Tet-On system and the doxycycline treatment in the U2OS-Cl.5, the experiment was repeated again with the native human osteosarcoma cell line (U2OS). As shown in Figure 32, no differences in GFP expression as well as in MFI were detected in untreated compared to doxycycline treated cells. Thus, these results confirm a specific negative SPOC1 impact independent from doxycycline induction on HIV-1 gene expression.

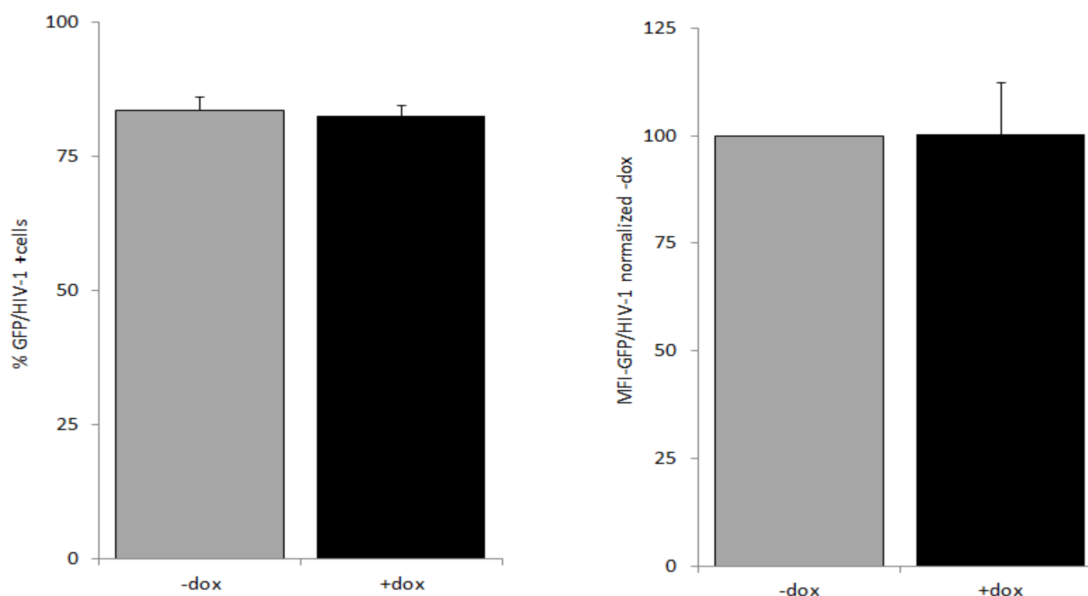


**Figure 32 Treatment of U2OS with Doxycycline after infection with HIV-1**

Native U2OS cells were infected with VSV-G pseudotyped wild/type HIV-1 NL4-3 IRES eGFP for 24 h. Thereafter, cells were treated with doxycycline or left untreated for 24 hours before cells were analyzed by flow cytometry.

A) FACS diagram of GFP positive cells in untreated (-dox) and treated cells (+dox). Mean of two independent approaches were calculated and the resulting data were subsequently normalized to the control which was set as 100 %. Error bars indicate the standard deviation.

B) MFI as read out for HIV-1 gene expression of GFP+ cells

## 4.18 SPOC1 depletion enhances HIV-1 gene expression in the context of infections with Vpr-defective HIV-1

As shown before, HIV-1 Vpr is important for reduction of SPOC1 during the replication cycle. Furthermore, SPOC1 overexpression in the late phase of the viral replication cycle reduces HIV-1 gene expression and virus release. Therefore the impact of SPOC1 on viral gene expression in the context of HIV-1 ΔVpr mutants was assessed in following experiments. U2OS-Cl.5 cells were transfected with siRNA targeting SPOC1, scrambled siRNA or were left untransfected. 24h post transfection, cells were infected with VSV-G pseudotyped HIV-1 ΔVpr NL4-3 IRES-eGFP for 6 h. Cells were washed to remove inoculum and incubated for additional 18 h. Untransfected cells were treated with doxycycline to overexpress SPOC1. 24 hours later, the percentage of GFP positive (HIV-1 infected) cells as well as the MFI were determined by FACS. Furthermore, supernatants were analyzed by ELISA to determine the amount of HIV-1 p24.

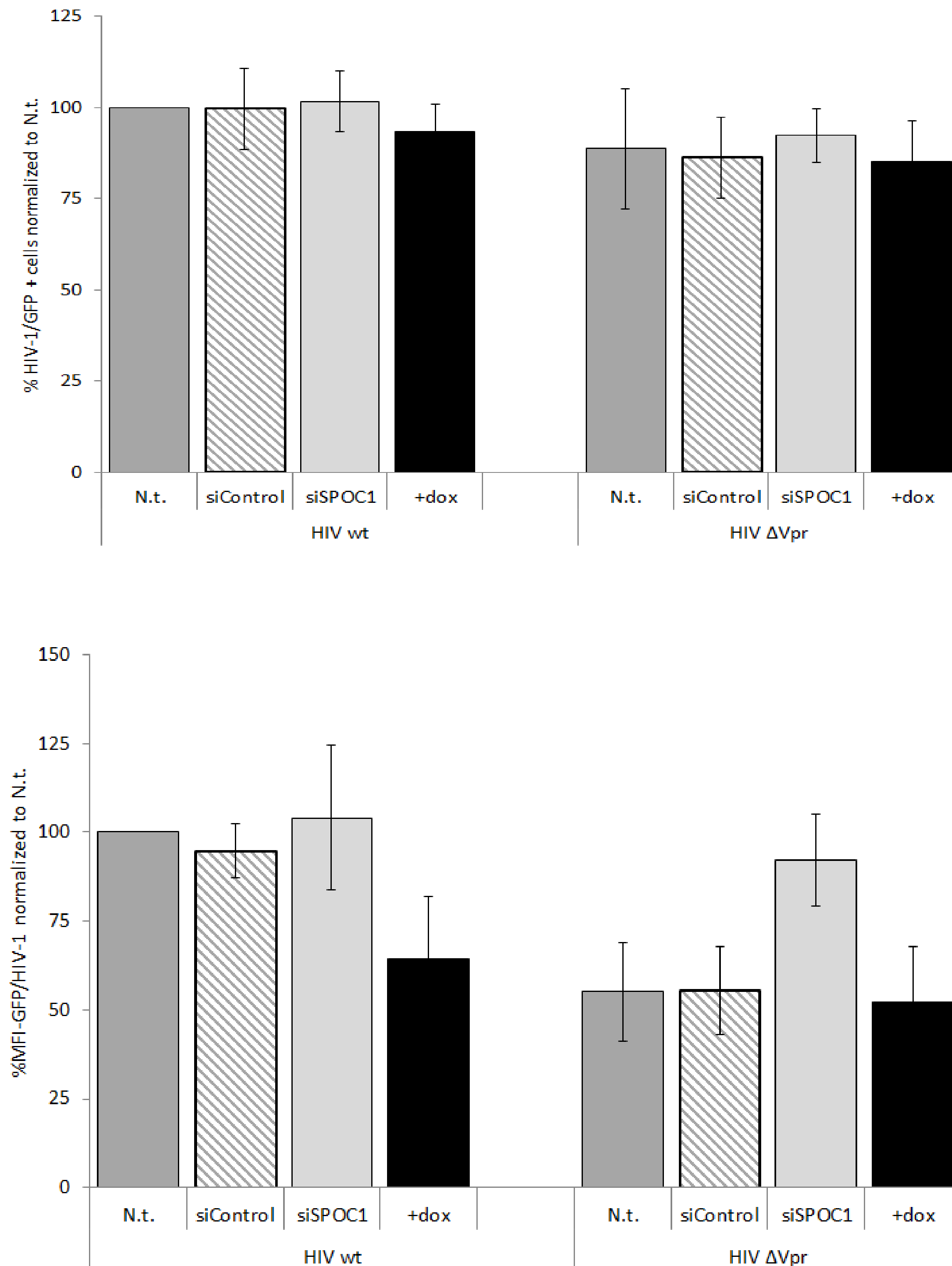


**Figure 33 SPOC1 knock down leads to higher LTR activity in HIV-1 ΔVpr mutants**

U2OS-Cl.5 cells were transfected with siRNA constructs directed against SPOC1, control siRNA, or left untreated (N.t.). 24 hours later cells were infected with VSV-G pseudotyped HIV-1 NL4-3 ΔVpr IRES eGFP for 6h, washed and incubated for additional 18 h. Finally, 24h later cells were analyzed by flow cytometry and p24 antigen load in supernatants was determined by ELISA.

A) FACS diagram displaying the relative mean percentage of HIV-1-positive (GFP) cells
B) FACS diagram displaying the MFI of HIV-1-positive (GFP) cells
   Diagram were calculated of three independent approaches and subsequently normalized to the HIV-1 wt control which in turn was adjusted to 100 %. Error bars indicate the standard deviation.

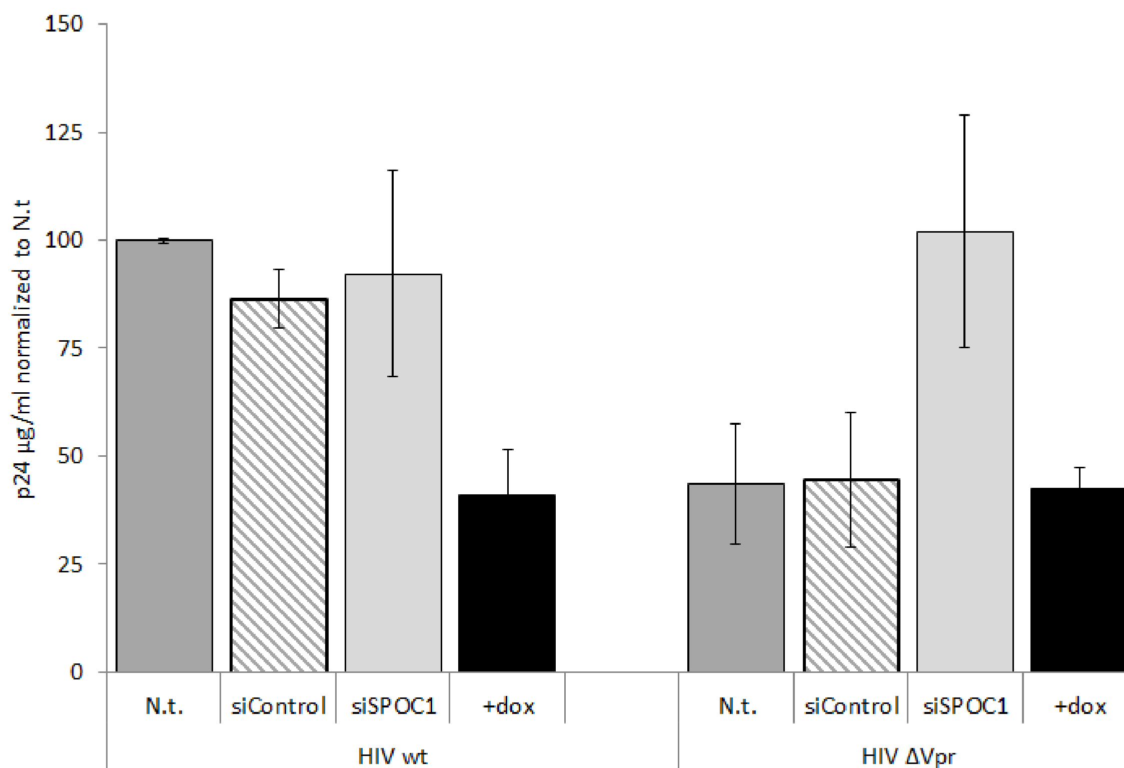


**Figure 34 SPOC1 knock down leads to higher LTR activity in HIV-1 ΔVpr mutants**

U2OS-Cl.5 cells were transfected with siRNA constructs directed against SPOC1, control siRNA, or left untreated (N.t.). 24 hours later cells were infected with VSV-G pseudotyped HIV-1 NL4-3 ΔVpr IRES eGFP for 6h, washed and incubated for additional 18 h. Finally, 24h later cells were analyzed by flow cytometry and p24 antigen load in supernatants was determined by ELISA.

C) Diagram presenting mean p24 antigen amounts in the supernatant.
Diagram were calculated of three independent approaches and subsequently normalized to the HIV-1 wt control which in turn was adjusted to 100 %. Error bars indicate the standard deviation.

As can be seen in Figure 33A, percentage of GFP expressing cells and MFI were equal under all experimental setups, confirming comparable HIV-1 infection rates and gene expression around 100 %. Intriguingly, knock down of SPOC1 expression in HIV-1 ΔVpr infected cells led to an increase of the MFI by almost two fold (Figure 33 B). p24 ELISA revealed that untreated cells, cells transfected with control siRNA and cells overexpressing SPOC1 released comparable levels of p24. In contrast, SPOC1 knock down resulted in a two fold increase of released p24 (Figure 34). This result implies that HIV-1 Vpr counteracts the SPOC1-mediated repressive impact on HIV-1 gene expression.

In sum, it was shown that SPOC1 is degraded by the proteasome during HIV-1 infection in a Vpr and GSK3-beta dependant manner. Moreover, only in the context of viral infection and therefore HIV-1 proviral integration decrease of SPOC1 was observed. SPOC1 overexpression enhanced infection at early stages by increasing HIV-1 integration. In contrast, overexpression of SPOC1 at late stages of HIV-1 replication represses viral gene expression. Accordingly, knock down of SPOC1 during late stages enhances gene expression and virus release in the context of infection with HIV-1 ΔVpr mutants.

# 5 Discussion

In this thesis the expression and biological function of SPOC1 during HIV-1 infection was examined. SPOC1 was identified originally in highly proliferating ovarian cancer cells and was described as a possible novel restriction factor for the human adenovirus [25]. Therefore SPOC1 might also be a potent inhibitor of HIV-1 replication as it could possibly interfere with many steps of the HIV-1 replication cycle.

## 5.1 Interplay between SPOC1 and HIV-1

To date, only little information has been reported on SPOC1 protein expression in different cells and therefore initially endogenous protein levels of SPOC1 in HIV-1 target cells was examined. Endogenous SPOC1 was found in primary stimulated and non-stimulated T cells, immortalized T cell lines and some standard laboratory cell lines, i.e. 293T, U2OS and HeLa. In contrast, SPOC1 protein was not detectable in terminally differentiated, non-dividing macrophages. In line with this, previous reports showed that SPOC1 expression is high in rapidly proliferating cells [22], but low in mature, nonproliferating sperm cells, indicating a role of SPOC1 in proper mitotic chromosome condensation and cell division [15]. SPOC1 is tightly regulated in both expression and localization during the cell cycle and enhanced expression could be associated with proliferation [15]. Nevertheless, SPOC1 is expressed in HIV-1 target cell lines and primary T cells and could thus play a role in HIV-1 replication.

As SPOC1 might inhibit certain HIV-1 replications steps, it was speculated that SPOC1 protein levels could be altered upon HIV-1 infection. To test this theory, SPOC1 protein expression was examined after HIV-1 wild/type infection in different cell types. It was shown that HIV-1 infection led to a reduction of SPOC1 protein levels. Interestingly, further experiments showed that transfection of the HIV-1 genome is not sufficient but rather infection with HIV-1 viral particles was necessary to decrease SPOC1 levels. Transfected proviral DNA or plasmid expressing HIV-1 proteins reside mainly in the nucleus as an episome and are not integrated into the host genome. Further, transfected proviral DNA is not reversely-transcribed, formation and transport of the PIC into the nucleus does also not occur. On the other hand, infection of cells with HIV-1 virus stock induces all steps of the viral infectious cycle. Thus, transfection of viral DNA is a rather artificial approach that does not reflect transcription from fully integrated HIV-1 genomes and misses certain important steps of the HIV-1 replication cycle. Hence it was speculated that events early during the HIV-1 life cycle, more specifically those differing from transfection of proviral plasmid are important for the induction of SPOC1 degradation. Indeed, inhibition of the reverse transcription or integration step by approved drugs did abrogate degradation of SPOC1 by HIV-1. In contrast, inhibition of HIV-1 gene expression or particle maturation, all of which events occurring post integration, could not prevent SPOC1 degradation by HIV-1. This demonstrated that integration of the HIV-1 provirus is associated with SPOC1 depletion by HIV-1.

In infection experiments with HIV-1 Vpr deficient mutants no degradation of SPOC1 was detected, whereas infection with a full-length HIV-1 or Nef and Vpu deficient mutants still led to a reduction of SPOC1. This implies that HIV-1 Vpr is crucial for SPOC1 decrease. Additionally, virus associated Vpr was sufficient to reduce SPOC1 upon HIV-1 infection. This further implies, that not only integration but also the presence of Vpr within the nucleus is needed for reduction of SPOC1 and supports the previous notion that de novo synthesis of HIV-1 proteins is dispensable for SPOC1 degradation. HIV-1 Vpr has many functions and is important during the early phase of infection. This includes positive effects of Vpr on the nuclear import of the viral PIC, enhancement of HIV-1 reverse transcription, and induction of the $G_2$ cell cycle arrest [94; 95; 96]. Vpr can cause epigenetic disruption of heterochromatin by inducing displacement of heterochromatin protein 1-α (HP1-α) through acetylation of histone H3 [96]. HP1-α is an important complex partner of SPOC1 [15; 23] and acetylation of histone H3 could lead to the release of SPOC1 from the chromatin and consequently to the degradation of SPOC1. Moreover, Uracil-DNA glycosylase 2 (UNG2, also known as CCNO) and Single-strand selective monofunctional uracil DNA glycosylase (SMUG1) are known to be degraded by Vpr through the CRL4 ubiquitin ligase complex. Vpr uses the host ubiquitination machinery via the recruitment of an E3 ubiquitin ligase complex composed of VPRBP (viral protein R binding protein, also known as DCAF1), DNA damage binding protein 1 (DDB1), and cullin 4A (CUL4A). Therefore, it could also be possible, that Vpr mediates SPOC1 degradation by hijacking the ubiquitin proteasome pathway and subsequent targeting of SPOC1 for proteasomal degradation.

While the Vpr associated CUL4A ubiquitin ligase is not necessarily involved in SPOC1 degradation the proteasomal pathway in general and specifically GSK-3-ß are involved in Vpr mediated SPOC1 depletion in the context of an HIV-1 infection. This observation was intriguing, since GSK-3-β phosphorylation has been previously reported to play a role in the regulation of protein stability for a variety of labile proteins and in some cases by directly cooperating with the ubiquitin proteasome system [15; 94]. In the canonical Wnt signaling pathway, GSK3 phosphorylation mediates proteasomal targeting and degradation of β-catenin [67]. GSK-3-ß require its substrates to be pre-phosphorylated by additional priming kinase before it physically binds and phosphorylates its substrates [15]. Hence, it is possible that HIV-1 Vpr pre-phosphorylates SPOC1 directly or more likely by using an unknown intermediate player. It has also been shown that Vpr can directly bind and activate Wee1 kinase, following phosphorylation of $p34^{cdc2}$ leading to $G_2$ cell cycle arrest [94; 109].

Identifying possible interaction partners of SPOC1 can be essential for understanding its function. For example, the direct interaction of HIV-1 Vpu with neosynthesized CD4 or Vif that interacts with the cytidine deaminases APOBEC3F, and APOBEC3G leads to their degradation [81; 86; 87]. The used FACS-based FRET assay is a non-invasive technique to analyze protein-protein interactions in living cells established by Banning et.al. [49]. No interaction between HIV-1 Vpr and SPOC1 could be detected,

what can have multiple explanations. While SPOC1 is a nuclear localized protein with an active NLS sequence, most HIV-1 proteins localize in the cytoplasm and therefore a close proximity which is needed for a FRET positive signal, i.e. interaction is not given. Another aspect could be that HIV-1 Vpr and SPOC1 just transiently interact with each other or that a possible interaction is not measurable due to rapid degradation of SPOC1 by Vpr. Vpr is described to degrade other proteins like UNG2 and SMUG1 through the CRL4 ubiquitin ligase complex without directly interacting with the target proteins. Thus, for mediating SPOC1 degradation a direct interaction of Vpr with SPOC1 is not an essential prerequisite. On the other hand, it is of interest that SPOC1 interacted with HIV-1 Tat. This interaction could be an aspect of the suppressing effect of SPOC1 on HIV-1 transcription, which will be discussed in the next chapter.

In addition, to promote efficient viral replication and to disrupt elements of innate or acquired immunity, viruses have evolved ways to modulate the host cellular environment. As described for other viruses, HIV-1 influences the expression and activity of specific cellular factors, by modulating the ubiquitin-proteasome system. By proteasomal degradation of the MHC components (major histocompatibility complex) and the CD4 receptor, the human cytomegalovirus (HCMV) and HIV-1 can escape the cellular immune response [103; 104; 105]. Another specific example is HIV-1 Vpu, which has been shown to bind to newly synthesized CD4 molecules in the endoplasmic reticulum (ER) and subsequently induces their proteasomal degradation. It is supposed that Vpu thereby prevents binding of the viral envelope to CD4 molecules in the ER allowing appropriate virus assembly [106]. Usually, the destabilization of anti-viral proteins is achieved by virus-mediated ubiquitination [107]. It was shown in previous published work that SPOC1 was degraded by the E1B-55K/E4orf6 ubiqutin ligase complex, which uses also the proteasomal pathway [25]. In contrast in the context of HIV-1 infection, it seems as if HIV-1 Vpr induces degradation of SPOC1 via recruitment or activation of GSK-3-ß which leads to SPOC1 phosphorylation and subsequent proteasomal degradation.

## 5.2 Biological relevance of SPOC1 during the HIV-1 replication cycle

One important aspect of this thesis was to elucidate the relevance of SPOC1 for HIV-1 replication. A classical approach to analyze the function of a certain protein in living cells is to overexpress or to knock down the gene of interest and subsequently monitor phenotypic changes to get information about the proteins function [92]. SPOC1 overexpression and depletion were examined in Jurkat-TAg cells representing a HIV-1 relevant cell model and in human osteosarcoma cell line which overexpress SPOC1 upon the treatment of doxycycline (U2OS-Cl.5). Intriguingly, SPOC1 overexpression in Jurkat-TAg and U2OS-Cl.5 cells before infection with HIV-1 resulted in an increase of HIV-1 positive cells and increasing proviral integration. Strikingly, these experiments were confirmed multiple times with vectors expressing untagged SPOC1 expressed along with BFP via an IRES from a bicistronic mRNA and

in U2OS-Cl.5 cells expressing untagged SPOC1 after doxycycline treatment. With this setup it can be excluded that protein tags might affect proper protein folding, function and localization.

HIV-1 integration induces DNA double-strand breaks (DSBs) into the genome and the two major pathways for DSB repair in mammalian cells, the non-homologous end joining (NHEJ) or homologous recombination repair (HR) are activated [63; 108]. Previous data indicates that SPOC1 is recruited to DSBs in an ATM dependent manner and depletion of SPOC1 enhances non-homologous end-joining (NHEJ) repair activity but impairs homologous recombination (HR) [23]. Further, it was shown that SPOC1 overexpression delays γH2AX expansion and reduces NHEJ repair activity [25]. Interestingly, the group of Nakai-murakami showed that Vpr was found to stimulate the focus formation of Rad51 and BRCA1, which are involved in repair of DNA double-strand breaks by homologous recombination. A phenotype that could be associated with depletion of SPOC1 by Vpr. Overall, SPOC1 overexpression could lead to a faster repair by HR and consequently to an increase of HIV-1 integration events thereby increasing numbers of HIV-1 infected cells. Of note, since SPOC1 overexpression leads to increased integrated proviruses this supports the importance for HR as the essential DNA repair pathway involved in HIV-1 integration. In contrast to NHEJ.

Another aspect is that HIV-1 preferentially integrates in chromatin localized at the periphery of the nuclei, as shown by Single-Cell imaging of HIV-1 provirus (SCIP) in the study of Di Primio and colleagues in 2013 [93]. Heterochromatin is usually localized to the periphery of the nucleus and is formed by SPOC1 overexpression in increasing levels. This could favour HIV-1 integration.

In addition, the TRIM family protein KAP1 inhibits HIV-1 integration by binding to acetylated integrase and induces its deacetylation trough the formation of a protein complex which induces the deacetylation of HDAC1 [71]. SPOC1 mediates dose-dependent changes in chromatin association of DNA compaction factors KAP-1 and interacts with KAP-1 [23]. Accordingly, SPOC1 could sequester KAP-1 and interfere with KAP-1 mediated inhibition of HIV-1 integration. All these different functions associated with SPOC1 expression are in line with the findings presented here, demonstrating that SPOC1 overexpression enhances integration of proviral DNA.

Accordingly, with SPOC1 knock-down impairment of HIV-1 integration might be expected [91; 92]. In contrast to the overexpression experiments, siRNA mediated SPOC1 knock down in Jurkat-TAg and U2OS-Cl.5 before infection of cells did not lead to any differences in HIV-1 infection rates compared to the control.

This could be explained by the fact that SPOC1 mediated knock-down and monitoring of HIV-1 integration was conducted in cycling, progressively dividing cell lines. In such a setting, SPOC1 depletion might not represent an obstacle towards efficient integration, since upon cell division the proviral DNA can be easily integrated. Unfortunately, despite extensive experimentation, SPOC1 knock-down in primary T cells was not feasible. However, given the fact that macrophages naturally

express low levels of SPOC1 and are generally hard to infect by retroviruses including HIV-1 further supports that SPOC1 expression levels might be a determinant of efficient HIV-1 integration.

It was observed that SPOC1 overexpression post integration of the HIV-1 provirus and upon HIV-1 gene expression leads to a reduction of HIV-1 LTR transactivation and viral gene expression. SPOC1 is a nuclear protein and has the ability to inhibit KAP-1 phosphorylation and enhances H3K9 trimethylation (H3K9me3) [23]. Mund *et.al* described SPOC1 to be able to transform H3K4me2/3 containing chromatin into a more condensed form, by chromatin mediated increase of H3K9 KMTs and H3K9me3 [23]. H3K9me3 is highly correlated with repression of gene expression by promoting the formation of heterochromatin [59]. For proper transcriptional activation of the viral genome, DNA has to be in a decondensed form to allow access of certain transcription factors. Once the viral genome becomes transcriptionally activated, new viral proteins are synthesized. It can be assumed that SPOC1 represses the transcription of HIV-1 proteins by condensation of the chromatin and forming H3K9me3. These results are in accordance with the recent observations that SPOC1 expression leads to decrease of gene expression in human adenovirus [25].

SPOC1 expression could also lead to enhancement of the cell cycle, resulting in a decrease of HIV-1 LTR activation and transcription. It was shown that the expression level and localization of SPOC1 are very dynamically regulated during the cell cycle and might play a role for cell division [15]. In early $G_1$-S, as well as early S phase SPOC1 expression levels are significantly decreased but during late $G_2$ and M phases SPOC1 levels are increased. This indicates a potential functional role of SPOC1 during both late $G_2$ and mitosis. It was shown that the activation of the HIV-1 LTR seems to be more active in the $G_2$ phase, implying that the $G_2$ arrest may confer a favorable cellular environment for efficient transcription of HIV-1. Hence, SPOC1 expression post integration could enhance cell cycle progression and therefore would decrease HIV-1 LTR activation. This assumption is further supported by the data that SPOC1 is degraded by HIV-1 Vpr, which is known to induce $G_2$ cell cycle arrest. At present, Vpr-induced $G_2$ arrest is still not completely understood and many studies try to identify the potential target of Vpr which is degraded by the proteasome machinery. Several studies showed that Vpr binds the Cul4-DDB1-DCAF1 E3 ligase to trigger the degradation of a putative protein responsible for $G_2$ arrest [996]. New studies are proposing that Vpr is present specifically inside nuclear foci where it is associated with VprBP and the DDB1-CUL4a-E3 ubiquitin ligase. These foci colocalize with DNA repair foci containing proteins such as γH2AX and RPA2. This association may lead to the recruitment and the degradation of a chromatin-bound substrate, like SPOC1, which then leads to $G_2$ arrest. Thus, it is highly tempting to speculate that SPOC1 might be a target of the Vpr associated degradation complex and directly involved in induction of $G_2$ arrest.

It also not of surprise, that knockdown of SPOC1 post integration resulted in no difference to the control sample in wild/type HIV-1 infection. This phenomenon is explained by the fact that wild/type HIV-1 actively degrades endogenous SPOC1 and therefore HIV-1 transcription can be as active as in SPOC1 knock down cells.

Further, SPOC1 knock down in a Vpr deficient mutant led to HIV-1 LTR transactivation, protein expression and virus release compared to WT HIV-1 infections. This implies, that Vpr indeed degrades SPOC1 to allow efficient LTR transactivation. In addition, the effect can be saturated, since additional overexpression of SPOC1 in HIV-1 Vpr deficient infections has no further suppressing effect or does not completely block HIV-1 transcription. Altogether, these results support the hypothesis that Vpr degrades SPOC1 after proviral integration to achieve higher LTR-activation and transcription of HIV-1 and counteract SPOC1 mediated suppression of viral gene expression. Moreover it is likely, that by degradation of SPOC1 HIV-1 might prevent superinfection of the nucleus post successful integration.

Considering the cumulated results of this study, SPOC1 has opposing effects within the HIV-1 replication cycle. These are summarized in the final scheme (Figure 35). Prior to integration, SPOC1 is an important co-factor for the DNA DSB repair machinery and could therefore positively influence HR repair of DNA and the proviral genome. This increases and positively influences HIV-1 integration. Post integration, SPOC1 expression is dispensable or even counterproductive due to chromatin condensation, induction of cell cycle progression or a possible interference with Tat activity. All these effects could negatively influence LTR transcription. Altogether, SPOC1 seems to have antiviral functions but is not reminiscent of a “typical” restriction factor. To the contrary, SPOC1 might exert important functions necessary to achieve completion of the initial steps of the lentiviral replication cycle. On the other hand, this functionality might interfere with cellular features beneficial during productive gene expression. Fascinatingly, the virus seems to have evolved a sophisticated and highly regulated mechanism to cope with this “two-faced” role of SPOC1 and finally achieves optimal replication of its genome.

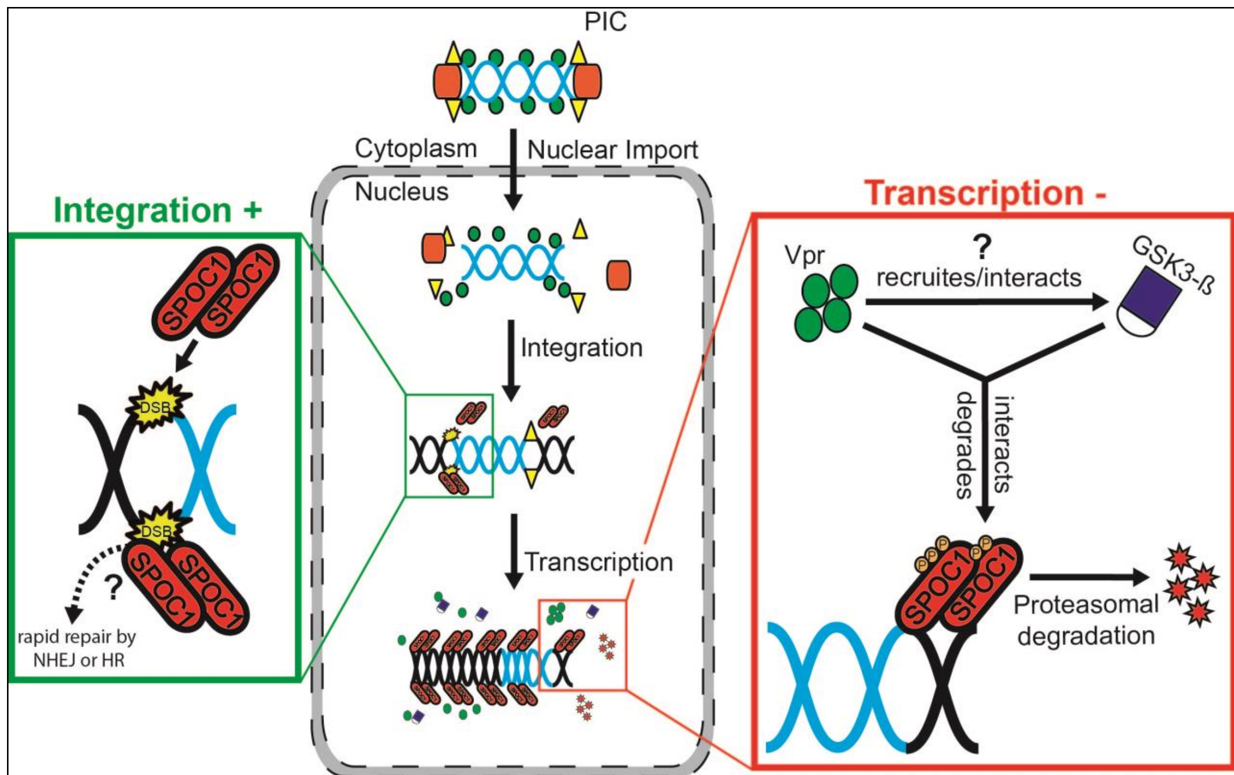


**Figure 35 Summary of SPOC1 impact during the HIV-1 replication cycle**

Immediately post entry into the nucleus the HIV-1 DNA genome is integrated into the host genome. As a consequence, the host cell DNA suffers a double strand break (DSB). SPOC1 is recruited DSBs regulates the DNA DSB repair machinery and positively influences HIV-1 integration. Post integration SPOC1 is degraded by HIV-1 Vpr and GSK-3-ß mediated proteasomal degradation to counteract SPOC1 mediated repression of HIV-1 LTR transcription.

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# Abbreviations

| A | | |
|---|---|---|
| aa | - | amino acid |
| AB | - | antibody |
| Ad | - | human adenovirus type 5 |
| AIDS | - | acquired immunodeficiency syndrome |
| ampC | - | ampicillin |
| APS | - | Ammonium persulfate |
| ART | - | antiretroviral therapies |
| Atm | - | ataxia-telangiectasia mutated |
| **B** | | |
| BFP | - | Blue fluorescent protein |
| bp | - | base pair |
| BSA | - | Bovine serum albumin |
| **C** | | |
| °C | - | degree(s) Celsius |
| CA/p24 | - | HIV-1 capsid protein |
| CCR5/ CXCR4 | - | chemokine receptor type 5 / 4 |
| CD (4) | - | cluster of differentiation (4) |
| CDC | - | Center of Desease Control and Prevention |
| CFP | - | cyan fluorescent protein |
| **D** | | |
| $ddH_2O$ | - | double distilled water |
| DMEM | - | Dulbecco's modified Eagle Medium |
| DMSO | - | Dimethyl sulfoxide |
| DNA | - | Deoxyribonucleic acid |
| dNTP | - | deoxynucleoside triphosphates |
| dpi | - | days post infection |
| DSBs | - | double strand break |
| DTT | - | Dithiothreitol |
| **E** | | |
| E. coli | - | Escherichia coli |
| e.g. | - | exempli gratia |
| EDTA | - | ethylenediamine-tetra acetic acid |
| ELISA | - | enzyme linked immunosorbent assay |
| Env/ Gp160 | - | HIV-1 envelope |
| et al. | - | et alii |
| **F** | | |
| FACS | - | Fluorescence-activated cell sorting |
| FBS | - | Fetal Bovine Serum |
| FCS | - | Fetal calf serum |
| FITC | - | fluorescein isothiocyanate |
| FRET | - | Förster resonance energy transfer |

**G**

| | | |
|---|---|---|
| G, mg,µg, ng, pg | - | gram, milligram, microgram, nanogram, picogram |
| Gag | - | HIV-1 group specific antigen |
| GFP | - | green fluorescent protein |

**H**

| | | |
|---|---|---|
| h | - | hour(s) |
| $H_2O$ | - | water |
| HAART | - | Highly active antiretroviral therapy |
| HATs | - | Histone acetyltransferases |
| HBS | - | HEPES buffered saline |
| HCl | - | hydrochlorid acid |
| HDACs | - | Histone-Deacetylases |
| HEK | - | human kidney epithelial cell |
| HEPES | - | N-(2-hydroxyethyl)-piperazine-N'-2-ethan Sulfonic acid |
| HIV-1 | - | Human immunodeficiency virus -1 |
| hpi | - | hours post infection |
| HRP | - | horseradish peroxidase |
| HRR | - | homologous recombination repair |

**I**

| | | |
|---|---|---|
| Ig | - | immunoglobulin |
| IN/ pol3 | - | HIV-1 integrase |

**K**

| | | |
|---|---|---|
| kDa | - | kilo Dalton |
| kan | - | kanamycin |
| KAP-1 | - | KRAB-associated protein 1 |
| Kb | - | kilo bases |
| kV | - | kilovolt |

**L**

| | | |
|---|---|---|
| L, ml, µl | - | liter, milliliter, microliter |
| LB | - | Luria Bertani |
| LTR | - | HIV-1 long terminal repeat |
| m, cm, mm, µm, nm | - | meter, centimeter, millimeter, micrometer, nanometer |

**M**

| | | |
|---|---|---|
| M, mM, µM, nM, pM | - | molar,millimolar, micromolar, nanomolar, picomolar |
| mA | - | milli ampere |
| MA/ p17 | - | HIV-1 matrix protein |
| MgCl2 | - | Magnesium chloride |
| MgSO4 | - | Magnesium sulfate |
| min | - | minute(s) |

**N**

| | | |
|---|---|---|
| n.d. | - | not determined |
| NaCl | - | sodium chloride |
| NaOH | - | sodium hydroxide |
| NC/ p7 | - | HIV-1 nuclear capsid protein |
| Nef | - | HIV-1 negative factor |
| NHEJ | - | non-homologous end joining |

| | | |
|---|---|---|
| NLS | - | nuclear localization signal |
| NS | - | not significant |

**O**

| | | |
|---|---|---|
| O/N | - | over night |
| OE, oe | - | over expression |

**P**

| | | |
|---|---|---|
| PBMC | - | peripheral blood mononuclear cells |
| PBS | - | Phosphate buffered saline |
| pbs | - | primer binding site |
| PCR | - | Polymerase Chain Reaction |
| PFA | - | Paraformaldehyde |
| PHA-E | - | Phytohaemagglutinine-E |
| PIC | - | preintegration complex |
| PIC | - | preintegration complex |
| POI | - | protein of interest |
| ppt | - | polypurine tract |
| PR/ pol 1 | - | HIV-1 protease |

**R/S**

| | | |
|---|---|---|
| Rev | - | HIV-1 regulator of expression of virion proteins |
| RPMI | - | Roswell Park Memorial Institute |
| RT | - | HIV-1 reverse transcriptase |
| SAM-HD1 | - | sterile alpha motif and HD domain 1 |
| SDS | - | Sodium dodecyl sulfate |
| SDS PAGE | - | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sec/ s | - | second |
| SIV | - | Simian Immunodeficiency Virus |
| SIVcpz | - | Simian Immunodeficiency Virus of chimpanzees |
| SOC | - | Super Optimal broth with Catabolite repression |
| SPOC1 | - | Survival time-associated plant homeodomain (PHD) finger protein in Ovarian Cancer 1 |
| SU/ Gp120 | - | HIV-1 surface protein |

**T**

| | | |
|---|---|---|
| t | - | time |
| TAR | - | trans-activation response |
| Tat | - | transactivator of transcription |
| TEMED | - | N,N,N’,N’-Tetramethyl-ethylenediamine |
| TM/ Gp41 | - | HIV-1 transmembrane protein |
| TNF-α | - | tumor necrosis factor-α |
| TRIS | - | Tris(hydroxymethyl)-aminomethan |

**V**

| | | |
|---|---|---|
| v/v | - | volume per volume |
| Vif | - | HIV-1 viral infectivity factor |
| Vpr | - | HIV-1 viral protein rapid |
| Vpu | - | HIV-1 viral protein out |
| VSV-G | - | glycoprotein G of Vesicular Stomatitis Virus |

**W**

| | | |
|---|---|---|
| w/v | - | mass per volume |
| Wt | - | wild/type |
| X4 | - | CXCR4 tropic |

**Y**

| | | |
|---|---|---|
| YFP | - | yellow fluorescent protein |

# Acknowledgement

First of all I would like to thank Prof. Dr. Michael Schindler for giving me the opportunity to work on this project and in his lab.

Prof. Dr. Ulrike Protzer deserves also special thanks for being my dissertation advisor and PD Dr. Thomas Floss for being my second examiner. Thank you both for the kind advice and for supervising my thesis.

I'm very grateful for the kind help of Dr. Martha Schneider! I really appreciated your help, for giving me always good advices and helping me with a lot of problems during my thesis.

For helping with all kinds of problems in the lab and giving me always good suggestions I'd like to thank the whole Ag76 and the Virology department at the HMGU. I'm very thankful for all your kind and calm advice that helped me in various stressful moments.

Special thanks go to Arthur Eichengrün, Stewart Adams, John Nicholson, Colin Burrows, Dietrich Mateschitz, H to usky- Husky, and Jack Daniels!

**Non describable thanks go to my whole family and friends for the incredible support through all difficult situations!**