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Characterizing the Role of the FACT Complex in HIV-1 Infection

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Dedication

Le dedico este trabajo a mis padres. El amor y el apoyo que me han brindado desde siempre hizo posible este logro.

(I dedicate this work to my parents. The love and support they have always bestowed on me has made this accomplishment possible.)

CHARACTERIZING THE ROLE OF THE FACT COMPLEX IN HIV-1 INFECTION

by

ANGELICA PATRICIA LOPEZ, B.A.

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Abstract

The lens epithelium-derived growth factor p75 (LEDGF/p75) is a chromatin-bound protein essential for efficient lentiviral integration. Knockdown of this protein severely affects HIV-1 integration levels and thus it's replication. Genome-wide studies have located LEDGF/p75 inside actively transcribed genes where it mediates lentiviral integration. Although this is clearly established, the molecular mechanisms of LEDGF/p75 in HIV-1 infection remain in part elusive.

Using protein-protein interaction assays, we demonstrated that LEDGF/p75 complexes with a chromatin remodeling complex, Facilitates Chromatin Transcription (FACT), a heterodimer of the structure specific recognition protein 1 (SSRP1) and the human homolog of suppressor of Ty 16 (hSpt16). The FACT complex is a histone chaperone that remodels nucleosomes in an ATP-independent fashion, providing Pol II access to the DNA during transcriptional elongation. Functional characterizations demonstrate a role for this novel complex in the regulation of HIV-1 replication. shRNA-mediated partial knockdown of SSRP1 reduces HIV-1, but not Murine Leukemia Virus (MLV), infection in human $CD4^+$ T cells. Similarly, SSRP1 knockdown affects infection by HIV-1-derived viruses that express genes from the viral LTR but not from an internal immediate-early CMV promoter, suggesting a role of SSRP1 in LTR-driven gene expression but not viral DNA integration. Detailed analysis of the interaction of LEDGF/p75 with the FACT complex indicates that LEDGF/p75 interacts with SSRP1 in an independent and direct manner as is shown by immunoprecipitation results of recombinant LEDGF and SSRP1 proteins. This interaction requires the PWWP domain of LEDGF/p75 and the HMG domain of SSRP1. Together, our data demonstrate for the

first time the association of LEDGF/p75 with the FACT complex and give further support to a role of SSRP1 in HIV-1 infection.

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CHAPTER 1: Introduction

1.1 HIV: Structure and Organization

The human immunodeficiency virus (HIV) belongs to the family *Retroviridae*, which consists of a large group of diverse viruses that can be found in all vertebrates. A main characteristic of this family, setting it apart from other virus families, is the ability of retroviruses to reverse transcribe their own genomic RNA into cDNA and integrate it into the host genome. This process gives rise to a provirus that allows retroviruses to maintain a persistent infection within the host. Within this family is a subfamily, *Orthoretroviridae*, which encompasses six different genera, one of which is the genus Lentivirus to which HIV belongs [1].

Lentiviruses are characterized by a long incubation period and a unique ability to infect non-dividing cells. The members of this family are similar in genetic composition, molecular mechanisms of replication, and biological interactions with their hosts. They are species-specific and can infect a wide range of different hosts that include domestic animals, non-human primates, and humans. These viruses have evolved different strategies, such as the circumvention of host defenses, in order to competently remain a persistent infection [2]. The genus *Lentivirus* consists of five serogroups, HIV-1 belongs to the primate group.

Like all retroviruses, HIV-1 is an enveloped, positive-sensed, RNA virus. Its envelope is composed of a lipid bilayer acquired upon budding from the host cell, in which two viral proteins, gp41 and gp120, and various host cell membrane proteins are embedded. Encased by the envelope is the matrix composed of the matrix protein, p17, which coats the inside of the viral membrane and surrounds a cone-shaped capsid protein shell, p24. The capsid encloses two identical positive, single RNA strands, each

encoding the full length HIV genome, protected by the formation of a complex with the nucleocapsid protein, p7. Also found within the capsid are several enzymes required for successful viral replication [1]. (Figure 1.1, National Institutes of Health)

The genome of HIV-1 consists of nine genes and spans approximately 9.7 kilobases (Kb) in length (Figure 1.2, . These genes encode, through the efficient utilization of all three reading frames of the genetic sequence, 15 proteins that can be classified into structural, catalytic, and accessory groups [3,4]. Also found within the genome of the HIV-1 provirus, similar to other lentiviruses, are two long terminal repeats (LTRs), one located on the 5' end of the sequence and the other on the 3' end; they contain important elements required for successful gene expression such as an enhancer, promoter, signals for transcription initiation (5' LTR), and transcription termination and polyadenylation (3'LTR) [5,6]. These features are recognized by the cellular transcription machinery, leading to the transcription of the HIV-1 genome along with its own. Both LTR's are identical in sequence but both have distinct functions [5,6] .

The LTR of HIV-1 is approximately 640 base pairs in length and is comprised of three regions, U3 (-454 - +1), R (+1 - +98), and U5 (+99 - +181) [5,7]. The U3 region is further subdivided into the modulatory, enhancer, and core/promoter regions; these regions contain binding elements for important cellular factors. A prime example of one of those important binding elements is the TATAA sequence which binds the TATA binding protein (TBP), as well as other proteins that comprise the RNA polymerase II transcription complex [6]. The R region contains two stem-loop structures that are conserved, the transactivator (TAR) and poly (A) hairpins. The TAR (trans-activation-responsive) is a unique regulatory element to which the viral protein Tat binds to induce

LTR activity and increase viral expression. Without the use of Tat, the host cell machinery is only capable of supporting low levels of basal transcription, which is insufficient to support productive replication [6]. The last region, U5, encodes several elements for the binding of transcription factors.

Three genes, *gag, pol, and env*, common to all retroviruses, define the way in which retroviruses replicate. The *gag* gene encodes the core proteins that package the viral genomic RNA; the *pol* gene encodes the proteins responsible for processing precursor proteins, reverse transcribing RNA into cDNA, and integrate the cDNA into the host genome; the *env* gene encodes the proteins which surround the virion and are responsible for binding and entry [8]. The translation products of these three primary genes are initially synthesized as polyprotein precursors that are consequently cleaved into mature, particle associated- proteins by viral or cellular proteases [1,8]. The precursor for Gag (p55) is 55-kDa in size and is cleaved by the viral protease into the matrix (MA), capsid (CA), nucleocapsid (NC), p6, and the spacer peptides, Sp1 and Sp2, during or after the release of the progeny virions. A 160-kDa Gag-Pol polyprotein is auto processed by HIV protease to give rise to the mature Gag proteins mentioned above and the Pol-encoded proteins protease (PR), the heterodimeric reverse transcriptase (RT), and integrase (IN). The 160-kDa Env precursor, however, is proteolytically digested and converted into the gp120 surface (SU), and gp41 transmembrane (TM) cleavage products by cellular enzymes such as the endoplasmic reticulum enzyme furin [1,9].

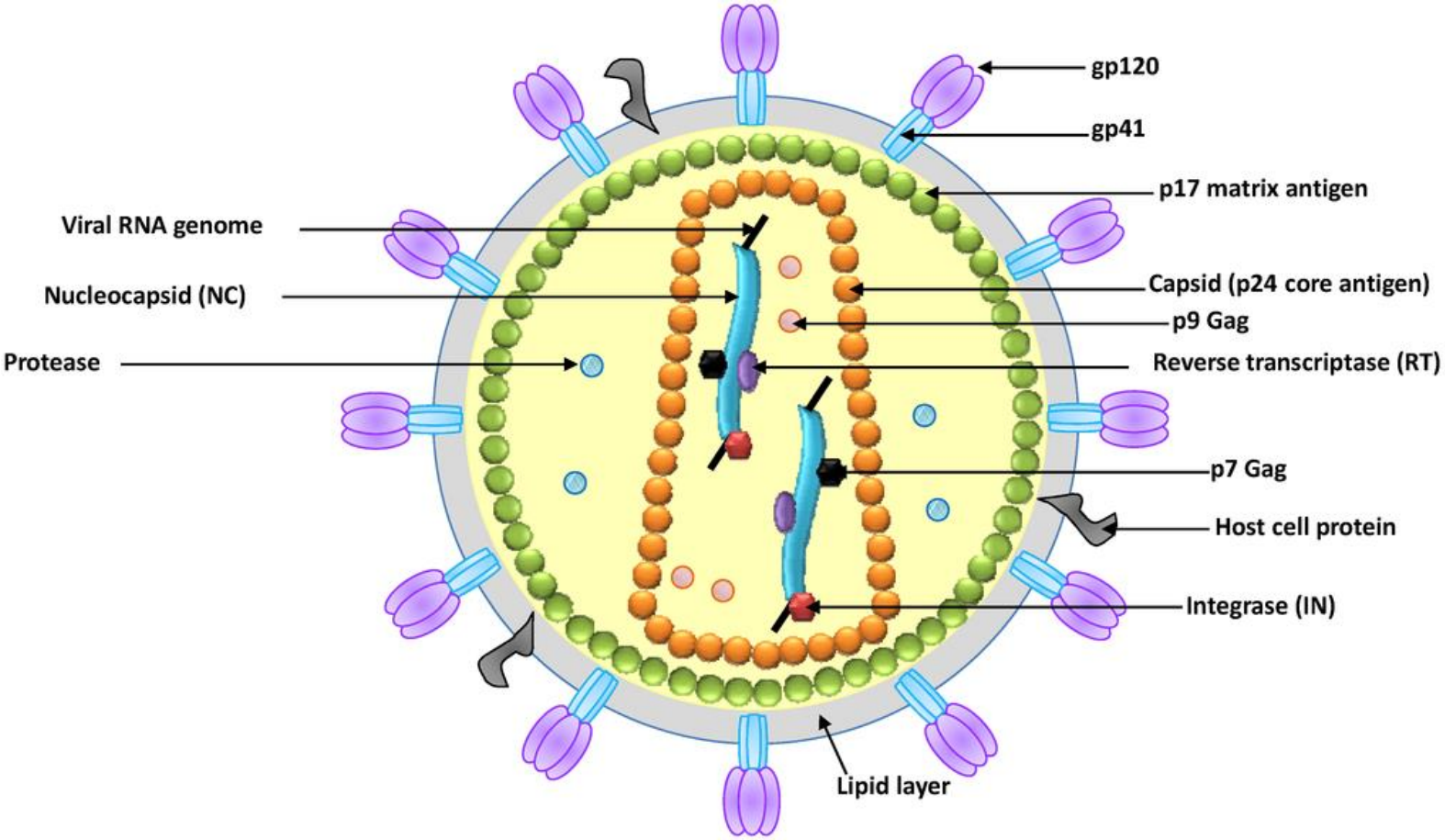


Figure 1.1 HIV-1 virion organization.

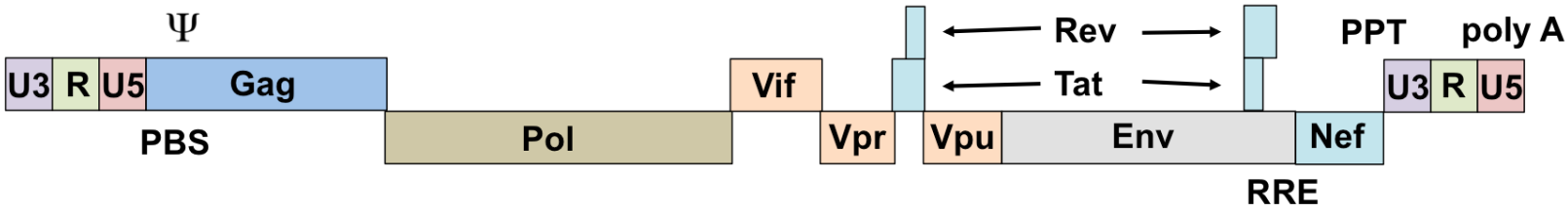


Figure 1.2 HIV-1 genome organization.

In addition to the structural and catalytic proteins described above, a group of accessory proteins, Vif, Vpr, Tat, Rev, Vpu, and Nef, result from the translation of singly or multiply spliced messenger RNAs (mRNAs) [1,9].

1.2 HIV: Replication Cycle

HIV can infect multiple types of cells in the host such as macrophages, dendritic cells, and other CD4 expressing cells [1,10,11]. However, the main target of HIV is the $CD4^+$ T cell or lymphocyte [1,12,13]. The replication cycle of HIV-1 begins when the gp120 glycoprotein in the virion's envelope binds to the CD4 surface receptor of the cell and undergoes a conformational change that allows it to bind to a chemokine co-receptor, predominantly CXCR4 or CCR5 [1,13]. This is an important step that has been thought to trigger membrane fusion and entry. Upon co-receptor binding, a conformational change occurs in gp41 leading to the exposure of its hydrophobic fusion peptide region, a process that results in a fusion-active state, which is then inserted into the host cell membrane [3,13,14]. This tethers the viral and host cell membranes and results in viral and host membrane fusion and subsequent release of the viral particle content into the cytoplasm [3,13,14].

Once in the cytoplasm, the virion's contents are still encased in a cone-shaped capsid that must be broken down in a process called uncoating. Currently, much about the mechanism of uncoating is still not well elucidated and there exists opposing models [15]. Until recently, it was thought that the capsid was unstable and lost soon after cell entry, however, the latest studies suggest that the capsid may stay associated with the virion for a longer period of time and may only fully uncoat until it has reached the nuclear pore complex. It has also been shown that the capsid may interact with various

cellular factors from the moment it enters the cytoplasm, enabling efficient uncoating and transport of the viral genome to the nucleus [15]. The most recent proposed model suggests that shortly after the viral core has entered the cytoplasm, cellular proteins are recruited to the core that suppress premature uncoating and help guide it to the nuclear pore complex and into the nucleus, where it is properly uncoated [16].

As previously mentioned, HIV is a positive-sensed RNA virus that, in order to replicate successfully, must integrate its genome into the host genome. HIV does this by converting its viral RNA into cDNA in a process called reverse transcription. Some studies propose that this process may occur simultaneously with the uncoating of the viral core [16]. Reverse transcription is carried out by a viral protein, reverse transcriptase (RT), that has DNA polymerase activity, which can copy an RNA or DNA template, and RNAse H activity, which will degrade RNA that is part of an RNA-DNA duplex [17]. RT uses the viral genomic RNA as a template and a host tRNA, Lys3, as a primer. When the tRNA binds to a primer binding site (PBS) on the viral RNA, the RT begins generating a single negative cDNA strand. As reverse transcription progresses, the RNAse H activity of RT degrades all the viral RNA except for a purine-rich region, ppt, which is resistant to RNAse H. The ppt is then used as the primer for the synthesis of the positive cDNA strand [17]. By the end of this process, a double-stranded viral cDNA genome has been generated.

Once the viral cDNA genome has been created, it associates with viral and cellular proteins to form a pre-integration complex (PIC) [18,19]. A specific viral protein from this complex, integrase (IN), is responsible for catalyzing the initial steps of integration. In the first step, IN binds to the 3’ end of each of the viral cDNA strands and

cleaves two nucleotides, called 3’ processing [18,19]. This is followed by the recruitment of the PIC to the host chromatin by interaction of IN with lens epithelium derived growth factor p75 (LEDGF/p75), a cellular protein. Subsequently, IN catalyzes a DNA-strand transfer step in which the two processed 3’ ends of the cDNA attack the phosphodiester bonds on the opposite strand of the target DNA and then are subsequently joined covalently to the host DNA. The last step of the integration process requires the repair of the 5’ overhangs and single strand gaps, which is hypothesized to be performed by the host cell DNA repair machinery. This whole process leads to the creation of a provirus within the host genome [18,20].

The integrated provirus now drives viral gene expression that is regulated at different transcriptional and posttranscriptional levels. At the beginning of infection, the provirus only produces short, completely spliced mRNAs that encode the regulatory viral proteins Tat, Rev and Nef [21]. This basal level of transcription is mediated by available cellular transcription factors [22]. However, once sufficient amount of Tat and Rev have been made, there is a substantial increase in the production of larger, incompletely spliced mRNAs, encoding Env and accessory proteins or full-length unspliced transcripts that serve as the genomic RNA for the virion, as well as the mRNA for the Gag and Gag-Pol polyproteins [21]. Binding of Tat to the TAR region of nascent viral mRNAs promotes elongation from the LTR promoter and increases viral transcription [21,22]. Rev shuttles the unspliced and incompletely spliced transcripts out of the nucleus and into the cytoplasm [22].

Once all the HIV proteins have been synthesized, the virion is assembled at the plasma membrane, a process that is coordinated by HIV Gag. In the cytoplasm, the viral

genomic RNA binds to Gag to form an RNA-protein complex that is then transported to the plasma membrane, along with the Gag-Pol polyprotein, where they are enriched in lipid rafts containing Env proteins [1,23,24]. Recruitment of the virion's envelope glycoproteins to the budding virion is promoted through their interactions with the MA subunit of the Gag polyprotein [23,24]. Cellular proteins are then recruited to the membrane where they mediate a scission between the cell and immature virion [23]. Once Gag and Gag-Pol multimerize, the viral protease is activated, which then processes Gag into its subunits. These subunits subsequently structurally rearrange themselves and give rise to a mature virion ready to infect the next cell [23].

1. 3 LEDGF/p75: The Protein

LEDGF is a ubiquitously expressed, chromatin-bound protein that exists in two variants, LEDGF/p75 and LEDGF/p52 [25–28]. These proteins are produced by the alternative splicing of the *PSIP1* (PC4- and SFRS-interacting protein 1) gene transcript. LEDGF proteins were first co-purified with the general transcriptional coactivator positive cofactor 4 (PC4) [25]. Both p75 and p52, like PC4, showed enhanced activity of the general transcription machinery in vitro and hence were termed transcriptional coactivators. Subsequently, p75 was found in a mouse lens epithelium library and was shown to protect mouse cells from oxidative damage [29]. This finding led to the coining of the name it is commonly referred to as today. Nonetheless, the term LEDGF is a misnomer as it is not found only in the lens epithelium and it has been demonstrated not to be a growth factor. LEDGF, however, is part of the hepatoma-derived growth factor (HDGF) family, which includes 5 other proteins [30].

LEDGF/p75 is comprised of conserved charged regions; AT-hook motifs; a nuclear localization signal (NLS); and two well-defined domains, the PWWP domain and the integrase-binding domain (IBD) (Figure 1.3). LEDGF/p75 and LEDGF/p52 share the same N-terminal (325 aa) but have different C-termini, in LEDGF/p52 there are an additional 8 aa and in LEDGF/p75 an additional 205 aa. LEDGF/p52 does not contain the IBD domain or the LEDGF/p75 C-terminal charged regions [28].

The PWWP is an evolutionarily conserved Pro-Trp-Trp-Pro motif-containing domain that has been identified in more than 60 chromatin-associated eukaryotic proteins involved in DNA repair, histone modification, transcriptional regulation, and DNA methylation [31–33]. Generally, the PWWP domain of proteins, including LEDGF/p75, interacts with the tri-methylated tail of histone H3 at the lysine in position 36 (H3K36me3) through a hydrophobic cavity formed by amino acids (aa) 14-15, 18, 21, 24, 43-45, and 47-51; while residues distributed across the basic surface of PWWP non-specifically bind to DNA [34,35]. The interaction of LEDGF/p75 with this specific histone modification is most likely the main contributing factor to the genome-wide enrichment of LEDGF/p75 inside actively transcribed genes, since H3K36me3 also accumulates at exons of highly expressed genes [36–38]. H3K36me3 has a regulatory role in the elongation phase of transcription. The functional implication of the genome-wide location of LEDGF/p75 inside actively transcribed genes is unknown but could suggest a role for LEDGF/p75 in transcriptional elongation.

Both LEDGF/p75 and LEDGF/p52 bind strongly to chromatin throughout all phases of the cell cycle. This strong interaction is mediated through the N-terminal region of both proteins, primarily through the PWWP domain, which binds to chromatin-

bound proteins and DNA, and the two AT-hook motifs that bind to the minor groove of AT-rich DNA regions [34,35]. These proteins are imported into the nucleus through the NLS and its deletion leads to trapping of LEDGF in the cytoplasm in non-dividing cells but in dividing cells the mutants can efficiently re-associate with chromatin [39]. Furthermore, LEDGF/p75 is not extracted from chromatin when cells are lysed in a buffer containing the detergent Triton X-100 [40]. However, inactivation of the PWWP domain and AT hook motifs yields complete impairment of Triton-resistant chromatin binding. Other regions of LEDGF/p75 have also been suggested to be implicated in chromatin binding but contribute in a lesser extent [40]. The ability of LEDGF/p75 to tightly bind chromatin is central for its cellular and virological roles [28,41,42].

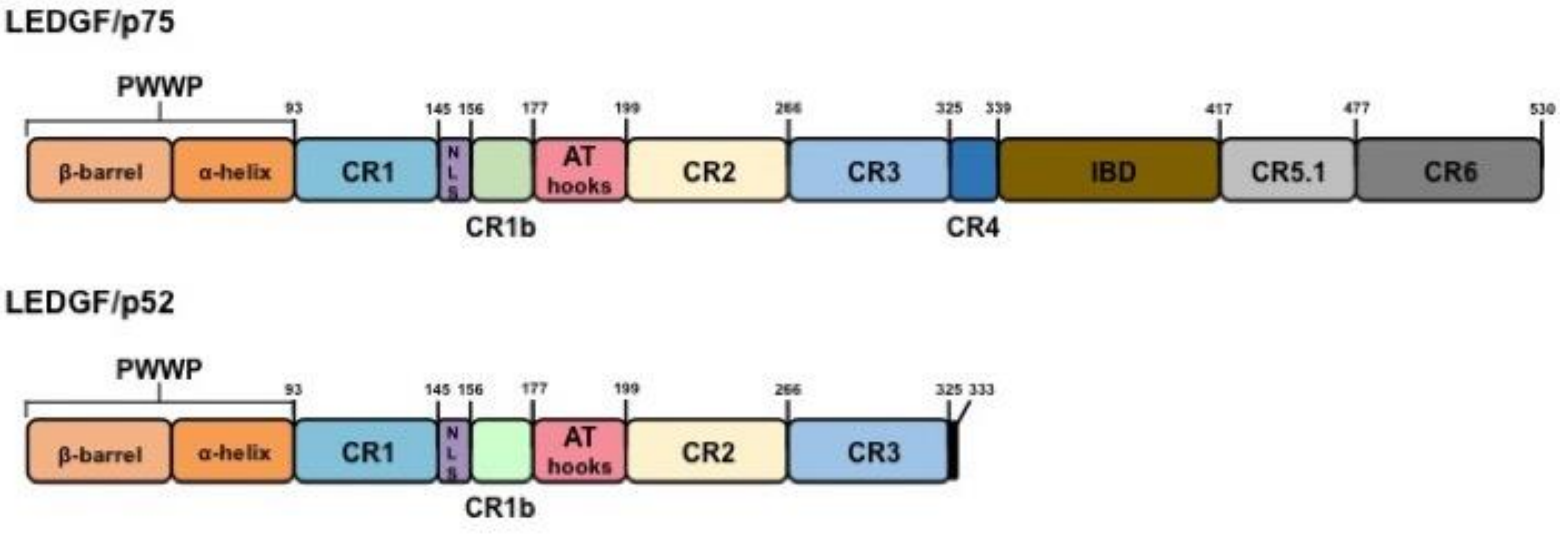


Figure 1.3 Domain architecture of LEDGF/p75 and LEDGF/p52.

There are two well-defined domains and several protein regions that are evolutionarily conserved (CR). The PWWP, AT-hooks, and to an extent the NLS, mediate the interaction of LEDGF/p75 with chromatin. The integrase of HIV interacts with the IBD.

1.4 LEDGF/p75: Cellular Roles

LEDGF/p75 has been found to have cellular roles in transcriptional regulation, cancer pathogenesis, cell survival, DNA repair, and autoimmunity [28]. In their initial discovery, both LEDGF protein variants were found to have transcriptional coactivator activity in vitro and overexpression of LEDGF/p75 was shown to increase transcription of a set of stress-related genes through its binding to heat shock and stress-related elements in the promoters [25,28]. The IBD domain of LEDGF/p75 has also been found to recruit the menin/MLL complex to various genes, including the Hox genes, for their transcriptional regulation [43]. Hox gene regulation is necessary for the establishment of the vertebrate body plan during development. The dysregulation of these genes leads to homeotic skeletal transformations, such as the ones observed in LEDGF/p75 knockout mice [44]. LEDGF/p75 has also been implicated in regulating genes involved in pathogenesis of myeloid leukemias [43]. Furthermore, in patients with acute or chronic myeloid leukemia, studies have shown that during active disease a translocation occurs that gives rise to the expression of a fusion protein between LEDGF/p75 and nucleoporin, Nup98; an event common in these cancers [45–48].

Cells that overexpress LEDGF/p75 or have been treated with recombinant LEDGF/p75 have been rescued from death induced by different environmental insults such as serum starvation, oxidative damage, heat shock, and UVB irradiation [28]. This has been attributed mainly to the regulation of stress-responsive gene transcription. In recent studies, LEDGF/p75 has also been shown to recruit the C-terminal binding protein interacting protein (CtIP) to DNA double strand breaks, where CtIP-dependent DNA-end resection occurs [49]. Lastly, LEDGF/p75 has been shown to play a role, as

an autoantigen, in autoimmune disorders. In prostate cancer, LEDGF/p75 functions as a nuclear autoantigen that produces an autoantibody response. However, besides being present in patients with chronic inflammatory diseases and cancer, a high prevalence of LEDGF/p75 autoantibodies have also been reported in healthy individuals [50,51]. Thus, their pathogenic significance is not well known. These findings also highlight the fundamental influence of LEDGF/p75-interactors in the cellular function of LEDGF/p75.

1.5 LEDGF/p75: Role in HIV-1 Infection

Lentiviruses, but not other retroviruses, have also exploited the genome-wide location and chromatin-tethering ability of LEDGF/p75 for viral replication [52]. In several studies, LEDGF/p75 was shown to immunoprecipitate with HIV-1 IN and consequent characterization led to the discovery of the domains involved in this interaction and the essential role of LEDGF/p75 in HIV-1 integration [35,52–56]. Cells deficient in LEDGF/p75 resist infection by HIV-1 and feline immunodeficiency virus (FIV), however, they fully support infection by murine leukemia virus (MLV), a gammaretrovirus [52]. When LEDGF/p75 proteins lacking the chromatin-binding and integrase-binding domains are expressed in LEDGF/p75 deficient cells and cells are subsequently infected with HIV-1, infection is not rescued [52,55,56].

When IN is expressed in a cell, in the absence of other viral proteins, it is easily detected in the nucleus but upon LEDGF/p75 depletion it shifts to the cytoplasm. Additionally, not only is its localization affected but its stability as well. In LEDGF/p75 deficient cells, expressed IN is difficult to detect by confocal microscopy or immunoblotting, even though mRNA levels are unchanged [57]. IN was found to be

ubiquitinated and upon proteasome inhibition, these defective protein levels of IN are restored to normal [57,58]. Moreover, LEDGF/p75 deletion mutants lacking the chromatin-binding and nuclear localization regions shield IN from the proteasome; however, mutants lacking the integrase-binding domain do not, suggesting that LEDGF/p75 protects IN from proteasome-mediated degradation [57].

As mentioned above, in early infection, after the HIV genome is reverse transcribed, the resulting cDNA non-covalently associates with IN within the pre-integration complex. This complex is then transported into the nucleus where IN firmly binds to the IBD of chromatin-bound LEDGF/p75, tethering IN and the viral cDNA to the host chromatin. LEDGF/p75-mediated chromatin tethering allows IN to efficiently insert the HIV-1 cDNA into the host genome, an essential step in the retroviral life cycle [28,41,42]. In LEDGF/p75 deficient cells, HIV-1 integration is severely affected [28,52]. In addition, the interaction of LEDGF/p75 with IN favors integration of HIV-1 cDNA inside actively transcribed genes [19,59–61], where LEDGF/p75 is enriched [28].

1.6 The FACT Complex

The eukaryotic genomic DNA associates with numerous proteins that help package and organize it into the nucleus of the cell. This compacted form is called chromatin and is primarily composed of a repeating unit called the nucleosome [62]. A nucleosome is formed by a histone octamer that has ~145-147 bp of DNA wrapped around it and each nucleosome is connected to each other by short DNA segments called “linker DNA” [62–64]. These nucleosomes interact with other nucleosomes to form chromatin fibers and subsequently these fibers interact with each other to create a higher degree of compaction [62]. Chromatin, however, is an ever-changing, dynamic

structure that helps regulate the accessibility of DNA to the cell machineries involved in transcription, recombination, DNA repair, and DNA replication [62,65].

In order to properly arrange nucleosomes, a diverse group of proteins called histone chaperones exist that allow for proper ordered formation of nucleosomes [64]. These proteins “chaperone” the histones and prevent nonspecific interactions between the negatively charged DNA and the positively charged histones [63,64]. Histone chaperones, along with chromatin remodelers, enable the disassembly and reassembly of higher order chromatin to provide cell machinery access to the DNA template. One such histone chaperone is the facilitates chromatin transcription (FACT) complex.

The human FACT complex is a histone chaperone comprised of a heterodimer of hSpt16 and SSRP1 proteins (Figure 1.4). It has been found to be essential in DNA replication, transcriptional elongation, DNA damage repair [66–75], and the targeted disruption of the SSRP1 subunit is embryonic lethal in mice [76]. In any function, FACT works through nucleosome reorganization by disrupting core histone-histone and histone-DNA interactions [66,67,71,75]. Specifically, hSpt16 interacts with an H2A-H2B dimer, facilitating its displacement and allowing SSRP1 to interact with an H3-H4 tetramer and nucleosomal DNA. After destabilization, the FACT complex has been shown to reassemble the nucleosome [67].

Once the HIV genome is integrated, its gene expression is regulated by cellular transcription factors and the higher order chromatin structure of the HIV-1 proviral DNA [77,78]. Precisely positioned within the 5’ LTR of the HIV-1 provirus are five nucleosomes (nuc-0 to nuc-4), one containing the transcription start site (nuc-1) [77,78]. When infected cells are activated, nuc-1 is specifically remodeled and leads to an

increase in HIV-1 transcription [78]. By using a ChIP assay, it was demonstrated that in non-activated infected cells the FACT complex is present on nuc-1 at low levels, however, upon activation the complex becomes more apparent in this region [78]. In the same study, a Tat-mutant cell line was transfected with Tat and after performing a ChIP assay showed that the H2A histones were removed from nuc-1 but not nuc-0. In these cells, FACT was also recruited to the promoter region around nuc-1 [78].

The hSpt16 (suppresor of Ty 16) subunit is a 140kDa protein homolog of the yeast nuclear protein, ySpt16, which has been found to be essential for transcription in yeast [63]. All eukaryotic homologs of Spt16 are composed of three distinctly defined domains, the N-terminal domain (NTD), the dimerization domain (DD), and the middle domain (MD); and a negatively charged intrinsically disordered C-terminal region (CTD) [63]. Biophysical studies of ySpt16 have demonstrated a physical interaction between the NTD and the H3-H4 tetramer and deletion of the CTD disrupts histone chaperone function and prevents transcription through chromatin templates *in vitro* [63,79].

In a genetic yeast model, Spt16 has been shown to be involved in the repression of HIV-1 transcription and plays a role in maintaining basal levels of transcription from the 5’ LTR [80]. In this study, they constructed a chimeric yeast-HIV transcription unit containing a fragment of the HIV-1 transcribed region (+1/+671) placed under the transcriptional control of a Ty1 promoter, which drove a retroelement with low levels of basal transcription. Upon disruption of Spt16, the mRNA levels of the HIV transcription unit increased [80]. In other studies, also performed in yeast, they showed that mutants defective in Spt16 activate transcription of cryptic promoters [81].

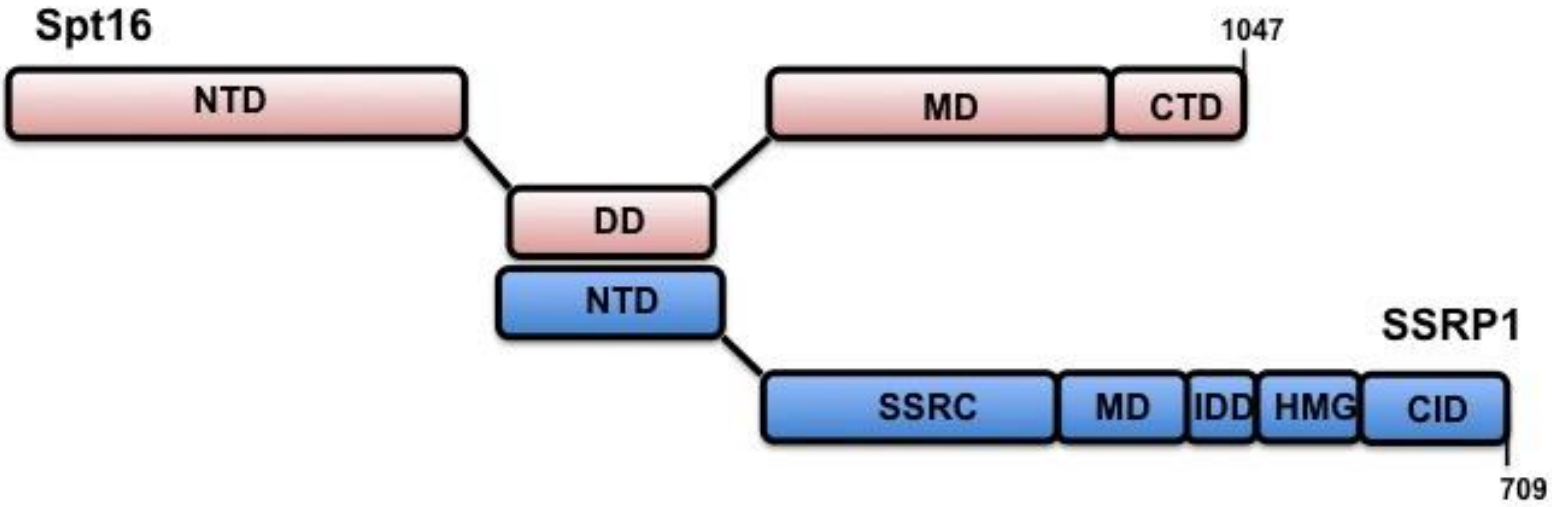


Figure 1.4 Domain architecture of the FACT complex.

The hFACT complex is composed of a heterodimer of hSpt16 (pink) and hSSRP1 (blue) subunits. The two subunits interact through the dimerization domain on hSpt16 and the N-terminal domain of SSRP1.

Furthermore, hSpt16 has been identified as an interactor of HIV-1 Tat [82,83]. Deficiency of hSpt16 in human primary cells led to a 3-fold increase in Gag mRNA levels but had no effect in MLV-infected cells [83]. Additionally, hSpt16 depletion was shown to modulate HIV-1 LTR activity and enhance the transcription of an LTR-driven luciferase reporter gene with expression of Tat and the presence of the TAR, suggesting that Spt16 has a role in the repression of HIV-1 provirus [83].

Two structural domains, a structure-specific recognition (SSRC) motif and an HMG-box domain, have been previously described in the 80kDa hSSRP1 (structure specific recognition protein 1) subunit, in addition to four evolutionarily conserved regions designated N-terminal domain (NTD), middle domain (MD), intrinsically disordered domain (IDD), and C-terminal domain (CID). The yeast homolog of SSRP1 is made up of Pob3 and an additional HMG-containing protein, Nhp-6 [63]. HMG domains are able to bind DNA, mediating the interaction of proteins with the minor groove of DNA, and proteins [84,85]. These interactions induce local changes in the structure of the target DNA that triggers different DNA-dependent functions [63]. HMG may aid FACT in the recognition, binding, and reorganization of chromatin. The dimerization between hSpt16 and SSRP1 occurs through the DD of hSPT16 and the NTD of SSRP1 [63,69].

It has been suggested that SSRP1 may have Spt16-independent functions such as in transcription regulation and DNA repair [86,87]. A microarray analysis conducted to evaluate the gene expression profile of human cells knocked down of SSRP1 or hSpt16 showed a set of genes regulated by SSRP1 independent of hSpt16 [87]. In other studies, SSRP1 has been shown to interact with key homologous recombination

proteins. Its knockdown increases HR events involving DNA double-strand breaks and its overexpression leads to a decrease in HR events in response to spontaneous damage [86].

1.7 Significance and Hypothesis

HIV-1 is the agent responsible for one of the most critical pandemics to have emerged in recent history, the AIDS pandemic. In 1983, HIV-1 was discovered to be the causative agent of the Acquired Immune Deficiency Syndrome (AIDS), a disease in which the body suffers a severe loss of its cellular immunity leading to a lower resistance to infections and, in most cases, eventually death [1]. Although at first this syndrome was on the rise amongst homosexual men, it has ultimately spread throughout the entire population, affecting all races, ages, and genders. In the last 30 years, there have been an estimated 75 million HIV-related deaths worldwide and more than 35.3 million people are currently infected with HIV.

In the mid-1990s, a breakthrough in the treatment of HIV-1 enabled infected persons to extend their life expectancies, better their quality of life, and reduce the risk of transmitting HIV to others. This breakthrough was the implementation of Highly Active Antiretroviral Therapy or HAART, a treatment that included the administration of a cocktail of three or more antiretroviral medications, each acting on different stages of the HIV life cycle. Within 10 years of its application, HAART led to a dramatic decrease in HIV related deaths by more than 50% [88]. However, HAART has been shown to have significant limitations. It is not a cure, it is expensive, not accessible to everyone, not 100% efficient, confers undesirable side effects, has no effect on the latent reservoir

within the host, and, if not consistently taken, leads to drug resistant strains of HIV-1 [89,90] .

There is a discernible need for the development of additional and better therapeutics against HIV. One relatively novel approach to finding new therapeutic targets is by identifying cellular cofactors that participate in HIV-1 infection but have redundant or non-essential roles in the cell. Due to the very low fidelity of reverse transcriptase, HIV-1 mutates very quickly, thus leading to drug resistance. Hence, targeting cellular proteins rather than viral proteins might prove to be a better alternative to ensure a more effective and long-lasting treatment.

In our lab, it is believed that HIV-1 will preferentially interact with cellular proteins that interact with an array of other cellular proteins whose roles could potentially be useful for successful viral replication. LEDGF/p75, a transcription factor in the cell, has been shown to be essential for HIV-1 genome integration into the host genome and proteins that interact with it could be implicated in HIV-1 infection. Therefore, our lab preformed a proteomic analysis to identify cellular binding partners of LEDGF/p75. The identified interactions were then verified through the use of co-immunoprecipitations of endogenous proteins (Figure 1.5). For the first time, these experiments generated results highlighting proteins that are involved in transcriptional elongation and DNA repair as LEDGF/p75-interactors. Some of these proteins included Ku86, SSRP1, hSpt16, CDK9, and PARP-1. SSRP1, however, specifically garnered our interest as a LEDGF/p75 interactor to further evaluate.

SSRP1, along with hSpt16, comprises the FACT complex. This complex is a histone chaperone that remodels chromatin to allow the RNA polymerase II access to

the host DNA during transcription. It is essential for DNA replication, transcriptional elongation, and DNA damage repair. More specifically, it is SSRP1 that has been shown to be involved in DNA repair. The function of this complex in chromatin remodeling and the specific role of SSRP1 in DNA repair made this complex an interesting candidate to evaluate. The objective of this dissertation is to characterize and evaluate the potential role that the FACT complex might have in HIV-1 infection.

The research of this dissertation focuses on elucidating a novel cellular cofactor in HIV-1 infection. Specifically, the work will determine if this complex has any role in HIV-1 infection and characterize the interaction between SSRP1 and LEDGF/p75. **We hypothesize that the FACT complex plays a positive role in HIV-1 infection, particularly by modulating the HIV-1 cofactor activity of LEDGF/p75.** The observed reported function of the FACT complex in chromatin remodeling [63,67,91], suggests that LEDGF/p75-recruited FACT could be implicated in HIV-1 integration or post-integration events. It is possible that FACT could facilitate the access of integrase to the host DNA by rearrangement of nucleosomes at integration sites in a similar fashion to which FACT allows cellular enzymes to access the chromatinized DNA template during DNA transcription, repair, or replication [67,68,70,71,73]. Similarly, FACT could contribute to post-integration DNA repair [68,73], through its SSRP1 subunit, and/or to the remodeling of nucleosomes on the promoter region of the provirus influencing HIV-1 gene expression. FACT is involved in transcriptional regulation and DNA repair, making it a prime candidate to act as a cellular cofactor in HIV-1 infection.

To address our hypothesis, we first evaluated if the FACT complex had any role in HIV-1 infection. Since the FACT complex is essential to the cell we developed a

transient knockdown system for SSRP1, one of the subunits, using shRNA-expressing, lentiviral vectors harboring a catalytically death integrase mutant. In these SSRP1 knockdown cells, the FACT complex was transiently disrupted. These cells were then challenged with HIV single-round or WT infection viruses, HIV-derived viruses that utilize a CMV or LTR promoter, and an MLV virus. Overall, we found that the knockdown of SSRP1 negatively affected HIV-1 infection but not MLV infection, suggesting it to be HIV-1 specific. Furthermore, we evaluated whether the chromatin binding of LEDGF/p75, the feature essential for its cofactor activity, was affected by the knockdown of SSRP1 and vice versa. We found that the chromatin binding strength of each protein, however, was not affected by the knockdown of the other.

Additionally, we wanted to characterize the interaction between the SSRP1 subunit of the FACT complex and the LEDGF/p75 protein. Further characterization of this interaction will be beneficial in further exploring the role of the LEDGF/p75 and SSRP1 interaction in HIV-1 infection. Through the generation of SSRP1 and LEDGF/p75 mutants lacking each domain of each protein and performing co-immunoprecipitations, the domains required and essential for the interaction were identified. Moreover, through the use of recombinant proteins, we further determined that the interaction between the two proteins is direct and does not require the presence of the other subunit of the FACT complex, hSpt16, or post-translational modifications.

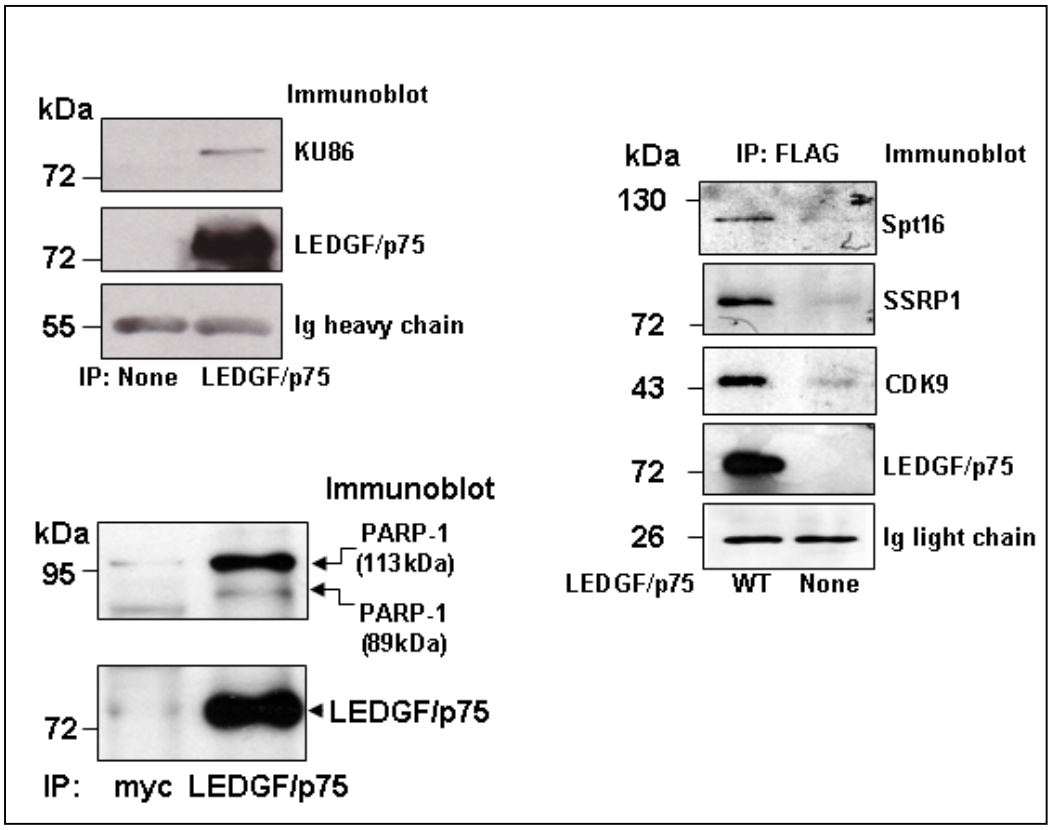


Figure 1.5 Newly identified LEDGF/p75-interacting proteins.
Co-immunoprecipitations of endogenous proteins of DNAse treated, nuclear extracts, using FLAG-tagged LEDGF/p75, were performed to verify results of a proteomic analysis. Specific antibodies were used to determine the presence of each protein.

CHAPTER 2: Evaluation of the FACT complex in HIV-1 infection

2.1 Introduction

The FACT complex catalyzes nucleosomal rearrangements allowing enzymes involved in DNA transcription, repair, and replication to access the chromatinized DNA template [66–75]. LEDGF/p75 tethers the HIV-1 pre-integration complex to the host chromatin allowing for efficient integrase-mediated viral cDNA integration into the host genome [28,41,42]. It could be possible that the chromatin remodeling activity of the LEDGF/p75-interacting FACT complex could contribute to HIV-1 infection by facilitating the access of integrase to the chromatinized DNA during integration, or at post-integration steps by influencing DNA repair, or transcription of the provirus. Implications of the nucleosome remodeling activity of the FACT complex in facilitating the access of cellular enzymes to the host DNA during DNA repair and transcription have been extensively reported. Furthermore, the complex could be implicated in the HIV-1 cofactor role of LEDGF/p75, as has been previously demonstrated for other LEDGF/p75 associated proteins [34,35]. In order to assess the role of the FACT complex in HIV-1 infection, we will knock down SSRP1, thus disrupting the FACT complex, in cells and evaluate their susceptibility to HIV-1 infection.

Conversely, determining the role of FACT in the HIV-1 cofactor activity of LEDGF/p75 or in HIV-1 infection is challenging because the chromatin remodeling activity of FACT is essential for cell survival. This indispensable function of FACT precludes drastic reduction of the cellular levels of this complex. To circumvent this problem, we analyzed the effects of transient partial knockdown of SSRP1 on HIV-1 infection. Knockdown was achieved by expressing an SSRP1-specific shRNA in the target cells using a non-integrative HIV-1-derived viral vector that harbors an integrase

catalytic death mutant, D64V. Non-integrative vectors transduce cells as efficiently as their integrative counterparts but express lower levels of the transgenes per cell. Therefore, at the same MOI, DNA integration-incompetent lentiviral vectors expressing shRNAs more likely produce a partial instead of a more stringent knockdown observed with integrative lentiviruses.

Moreover, the effect of SSRP1 deficiency on HIV-1 infection could be LEDGF/p75-dependent or -independent. For example, the requirement of the FACT complex for transcription of chromatinized genes in the HIV-1 provirus, a LEDGF/p75-independent process, could explain an effect of SSRP1 knockdown on HIV-1 infection. In order to evaluate these possible mechanisms and define whether this phenomenon is HIV-1 specific, we determined the effect of SSRP1 partial deficiency on infection by the gamma retrovirus Murine Leukemia Virus (MLV). Similar to HIV-1, the expression of chromatinized MLV provirus depends on FACT activity; however, MLV infection is LEDGF/p75-independent [52].

Chromatin tethering activity of LEDGF/p75 is central in the HIV-1 cofactor role of this protein [52]. However, several experimental evidences suggest that chromatin-tethering-independent mechanisms could also mediate the virological role of LEDGF/p75 [34,35]. Therefore, we also decided to investigate whether SSRP1 deficiency modifies the chromatin-binding strength of LEDGF/p75.

2.2 Materials and Methods

2.2.1 Generation of retroviral vector plasmids and shRNA expressing HIV-derived viral vector plasmids

The plasmids used to generate retroviral vectors were described previously [52]. HIV-1-derived vectors were produced using pHIV luc, pMD.G, pTRIP eGFP [92], pCMVΔR8.91, and pCMVΔR8.91 IN D64V (a gift of Che Serguera, INSERM MIRCen LMB). pHIVLuc was derived from pNL4-3.Luc.R–E– [93] by introducing a deletion in the env open reading frame. pMD.G encodes the Vesicular Stomatitis Virus glycoprotein G (VSV-G). pTRIP eGFP was derived from pTRIP by substituting the LacZ open reading by the eGFP cDNA [52]. eGFP is transcribed from an internal CMV promoter in pTRIP-derived vectors [92]. pCMVΔR8.91 and pCMVΔR8.91 IN D64V express the HIV-1 gag-pol polyprotein harboring a wild type or a D64V integrase mutant, respectively. Murine Leukemia Virus (MLV)-derived vectors were produced using pMLV luc [52] that was obtained by cloning firefly luciferase cDNA into pLPCX (Clontech) and the packaging plasmid pCS+mGP (a gift of M. Emerman. Fred Hutchinson Cancer Research Center).

pTRIP eGFP shRNA SSRP1 and pTRIP eGFP shRNA Scrambled express SSRP1-specific and scrambled sequences, respectively. They were constructed by cloning the U6 promoter and the respective shRNA sequences at a unique PpuMI site in pTRIP eGFP. The U6-shRNA expression cassettes were PCR amplified from pSilencer 2.1-U6 hygro Negative Control (Ambion) and pSilencer 2.1-U6 hygro SSRP1. The latter plasmid was generated by annealing oligonucleotides 5’-GATCCGCACCACAGTACTGCGTCTGTTTTCAAGAGAAACAGACGCAGTACTGTGGTGTTTTTTGGAAA-3’ and 5’-

AGCTTTTCCAAAAAACACCACAGTACTGCGTCTGTTTCTCTTGAAAACAGACGCAG TACTGTGGTGCG-3' [94] and cloning them into the pSilencer 2.1-U6 hygro (Ambion). This shRNA targets nucleotides 708-728 in SSRP1. In experiments in which the effect of SSRP1 knockdown on HIV-derived viruses expressing eGFP was evaluated, the shRNAs were delivered using a modified TRIP vector that lacks the eGFP cDNA. The identity of these constructs was verified by overlapping DNA sequencing of the entire cDNAs.

2.2.2 Cell lines and culture conditions

The LEDGF/p75-deficient human $CD4^+$ T cell lines studied were previously described [52,95]. T_{L3} and T_{C3} cell lines were derived from SupT1 cells by stable expression of a shRNA sequence targeting LEDGF/p75 or a control scrambled sequence, respectively. T_{L3} cells express 97% less LEDGF/p75 mRNA than T_{C3} cells as determined by qPCR. LEDGF/p75-deficient T_{L3} cells were later engineered to re-express a C-terminally FLAG-tagged LEDGF/p75 wild type (T_{L3} LEDGF/p75 WT). SupT1 cells and SupT1-derived cell lines were grown in RPMI 1640. Culture media was supplemented with 10% of heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin.

2.2.3 Generation of retroviruses

Procedures previously described [95] were followed. Briefly, $3x10^6$ HEK293T cells were plated in a T75 cm2 tissue culture flask and co-transfected the next day with the corresponding plasmids by the calcium-phosphate precipitation method. Eighteen hours later the transfection medium was replaced with fresh medium and the cells cultured for forty-eight hours until the viral supernatant was harvested and filtered.

Single-round infection viral vectors were further concentrated by ultracentrifugation at 124,750 g for two hours on a 20% sucrose cushion. Viral preparations were stored at -80°C until use.

VSV-G-pseudotyped HIV-derived reporter virus expressing firefly luciferase (HIVluc) was prepared by co-transfection of 15 µg of pHIV luc and 5 µg of pMD.G. VSV-G-pseudotyped HIV-1-derived viral vectors expressing shRNAs were produced by transfection of 15 µg of the corresponding pTRIP-derived plasmids, 15 µg of pCMVΔR8.91 D64V, and 5 µg of pMD.G. HIV-1_{NL4-3} was produced by transfection of 15 µg of pNL4-3 HIV-1 expression plasmid. VSV-G-pseudotyped MLV-derived vector was produced as described above. Cells were cotransfected with of 75 µg of pCS+mGP (packaging plasmid), 75 µg of pMLVLuc, and 15 µg of pMD.G. Expression of pCS+mGP into was intended to boost MLV vector production.

2.2.4 Generation and evaluation of SSRP1 knockdown cells

SupT1 cells (~$1x10^5$) were transduced with Scrambled and SSRP1 shRNA-expressing HIV-derived viral vectors at different MOIs and three days later were harvested. Lysates were made by the addition of 2X Laemmli sample buffer and subsequently samples were analyzed by western blot. Protein samples were resolved by SDS-PAGE and transferred overnight to PDVF membranes at 100 mAmp at 4°C. Membranes were blocked in TBS containing 10% skim milk for one hour and then incubated in the corresponding primary antibody diluted in TBS-5% skim milk-0.05% Tween 20 (antibody dilution buffer). SSRP1 was detected with anti-SSRP1 (1/500) and alpha tubulin was detected with anti-alpha tubulin mAb (1/4000). Membranes were incubated overnight at 4°C with anti-LEDGF or -SSRP1 antibodies, whereas anti-alpha

tubulin mAb and anti-beta actin were incubated for 2 hours at 25°C. Primary antibody-bound membranes were washed in TBS-0.1% Tween 20 and all bound antibodies detected with goat anti-mouse IgG-HRP (1/2000) followed by chemo-luminescence detection. Densitometry analysis of immunoblots was performed with the gel analysis software UN-SCAN-IT gel 6.1.

2.2.5 Evaluation of SSRP1 partially knockdown cells infected with HIVluc, MLVluc, and Trip Luc

SupT1 cells or T_{L3} and T_{C3} cells were plated at $1x10^5$ cells in 500µl of RPMI 1640 culture medium in 24-well plates and transduced with Scrambled or SSRP1 shRNA-expressing HIV-derived viral vectors at different MOIs. Three days later these cells were infected with HIVluc, MLVluc, or Trip Luc. T_{L3} and T_{C3} cells were only infected with HIVluc or Trip Luc. Three days post-infection, cells were collected by centrifugation at 1000 g for six minutes and the pellet re-suspended in 200 µl of PBS. Half of the sample was mixed with 100 µl of luciferase substrate and the other half with 100 µl of cell viability substrate. Cell lysates were incubated for 10 minutes at room temperature in the dark and then luminescence measured in triplicate in 50 µl-samples using a microplate luminometer reader.

2.2.6 Evaluation of HIV-$1_{NL4.3}$ virus infected, SSRP1 partially knockdown cells by HIV-1 p24 ELISA

SupT1 cells (~$1x10^5$) were transduced with Scrambled and SSRP1 shRNA-expressing HIV-derived viral vectors at MOI 1 in 500µl of RPMI 1640 culture medium in 24-well plates. Three days later, the cells were infected with HIV-1_{NL4-3} (2.1 ng of HIV-1

p24). Twenty-four hours after infection, the cells were washed three times by centrifugation in 10 mls of culture medium each time to remove the input virus. Cell supernatant was then collected three to four days after HIV-1_{NL4-3} infection, and used for HIV-1 p24 quantification by ELISA.

HIV-1 p24 levels were determined by a sandwich ELISA following manufacturer instructions. Briefly, 200 µl of the viral samples were diluted appropriately and incubated on the ELISA wells overnight at 37°C. Unbound proteins were removed by washing the wells six times with 200 µl of washing buffer, and bound HIV-1 p24 was detected by incubating each well with 100 µl of the anti-HIV-1 p24 secondary antibody for one hour. Unbound antibodies were removed by washing as described above and bound antibodies were detected by incubating each well with 100 µl of substrate buffer for 30 minutes at room temperature until the reaction was stopped by adding 100 µl of stop solution into each well. The absorbance of each well was determined at *450 nm* using a microplate reader.

2.2.7 Assessing SSRP1 partially knockdown cells infected with HeGFP and Trip eGFP

About $1x10^5$ cells were transduced with Scrambled and SSRP1 shRNA-expressing HIV-derived viral vectors. Three days later, they were infected with HeGFP and Trip eGFP. After seventy-two hours, the fluorescence of the cells was measured using flow cytometry.

2.2.6 Evaluation of chromatin binding in LEDGF/p75 or SSRP1-deficient cells by a salt extraction assay

Previously described procedures [95] were followed with minor modifications. Briefly, to evaluate SSRP1 chromatin binding in LEDGF/p75 knockdown cells, $3x10^6$ T_{L3} and T_{C3} cells were lysed in CSK I buffer CSKI buffer supplemented with NaCl to a final concentration of 175 mM and fractionated by centrifugation at 1000 g for 6 minutes to obtain a supernatant containing non-chromatin bound proteins (S1) and a pellet (P1) that represents the chromatin-bound fraction and triton-insoluble proteins. P1 was then re-suspended in 100 ul of 2X Laemmli sample buffer and S1 was mixed with 15 ul of 6X Laemmli sample buffer. A total fraction (T) was obtained by lysing $3x10^6$ cells in 100 µl of 2X Laemmli sample buffer. Samples were heated at 100°C for 10 minutes and then S1 (17.7 µl), P1 (15 µl of a 1/5 dilution), and T (15 µl of a 1/5 dilution) were analyzed.

To evaluate SSRP1 knockdown on LEDGF/p75 chromatin tethering, SupT1 cells (~$1x10^5$) were transduced with Scrambled and SSRP1 shRNA-expressing HIV-derived viral vectors. Three days later these cells were harvested for salt extraction. The CSK I buffer used in the extraction was supplemented with NaCl to a final concentration of 150 mM. The samples were then analyzed by Western Blot using anti-LEDGF/p75 (1/500), -SSRP1 (1/500), and -beta actin (1/4000) antibodies.

2.3 Results

2.3.1 Partial knockdown of SSRP1 specifically reduces HIV-1 infection

In order to evaluate the effect of FACT complex disruption on HIV-1 infection and to evade the problem of cell death, a partial knockdown of SSRP1 was achieved

through the use of a non-integrative lentiviral vector expressing an shRNA for SSRP1 and cells were consequently used for HIV-1 infection experiments. Cells of the human $CD4^+$ T cell line SupT1 were transduced at different MOIs with non-integrative HIV-derived viral vectors (TRIP D64V shRNA) expressing an SSRP1-specific or scrambled shRNA sequence, and three days after transduction the cells were infected with a VSV-G pseudotyped, single-round infection, HIV-derived virus expressing LTR-driven luciferase (Hluc). The partial knockdown was assessed by lysing cells infected with the shRNA-expressing lentivirus and performing a western blot analysis. Three days later the luciferase activity and ATP levels were measured. In addition, morphology and growth rate of the infected cells were evaluated by daily microscopy examination during the experiment.

Results in Figure 2.1a indicate a modest but reproducible decrease in the susceptibility of SSRP1-knockdown cells to HIV-1 infection at the highest MOIs evaluated. Importantly, under these conditions, cell viability was unremarkable as indicated by the cellular ATP content (Figure 2.1b), a very sensitive indicator of cell viability, as well as by cellular morphology and growth rate of the infected cells (data not shown).

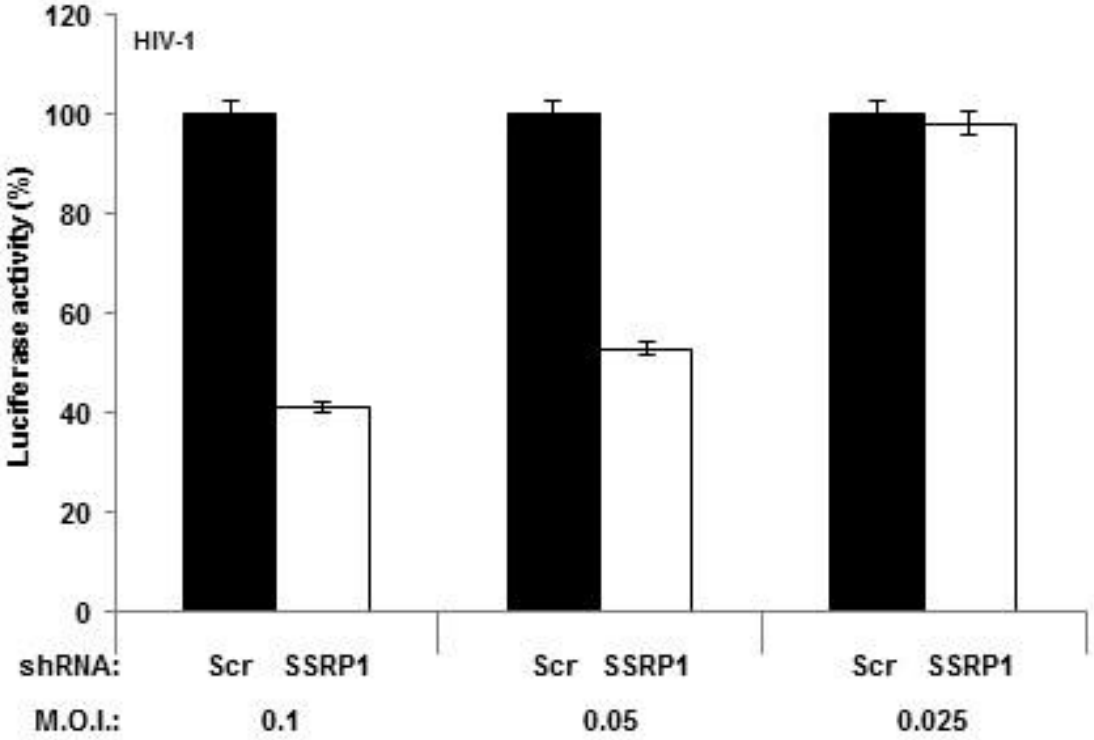
Furthermore, results in Figure 2.1a were additionally verified by multiple experiments performed on different days (Figure 2.1c) highlighting the reproducibility of this effect. In these experiments, SSRP1 in the knockdown cells was 57% of the levels found in control cells, as indicated by densitometry analysis of immunoblots (Figure 2.1d). This partial reduction likely correlates with the modest decrease in infectivity observed in the SSRP1-deficient cells. Therefore, findings represented in Figure 2.1

indicate that SSRP1 partial knockdown affects HIV-1 infection without altering cell viability.

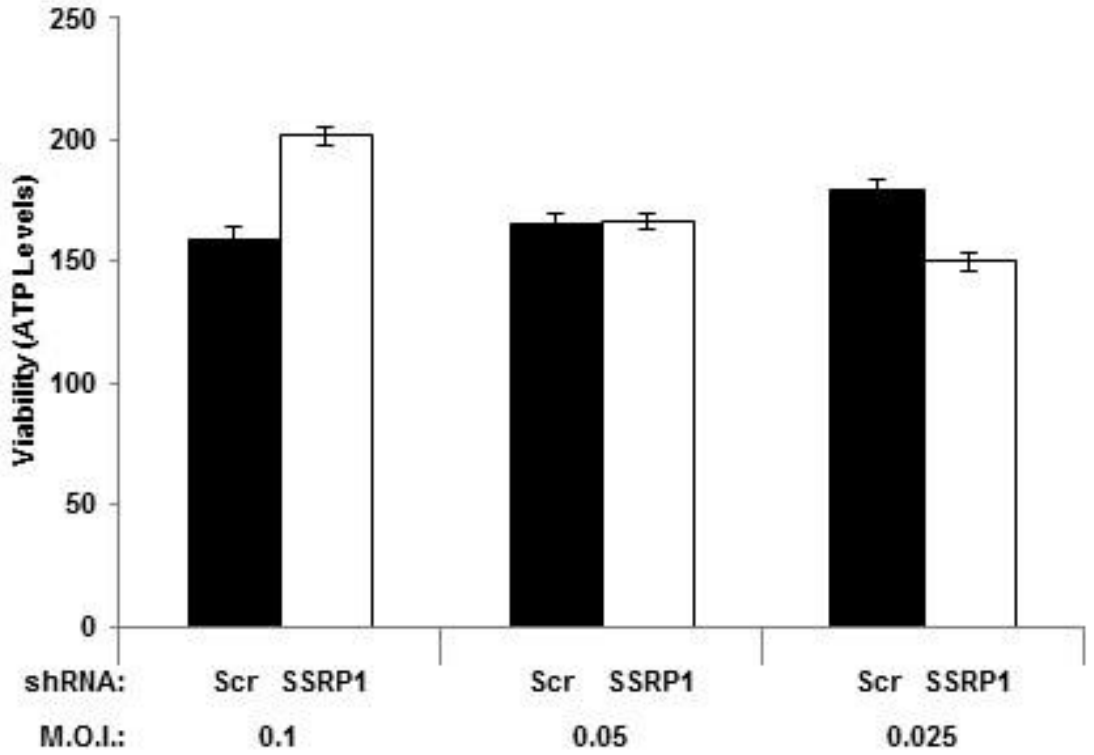
To further evaluate the effect of SSRP1 deficiency on HIV-1 infection, we transduced SupT1 cells at MOI 1 with the shRNA expressing retroviral vectors and on the third day post-transduction the cells were infected with HIV-1_{NL4-3}. Twenty-four hours later the infected cells were extensively washed to remove the input HIV-1_{NL4-3} and the viral supernatant was harvested 72 hours later for HIV-1 p24 quantification by ELISA. In three independent lentiviral transduction/HIV-1 infection experiments performed on different days we observed that partial knockdown of SSRP1 caused a modest (~ 40%) but reproducible reduction in HIV-1 p24 levels (Figure 2.2).

As aforementioned, the effect of SSRP1 deficiency on HIV-1 infection could be LEDGF/p75-dependent or –independent. This was evaluated by infection of SSRP1 partially deficient cells with MLV. Briefly, SupT1 cells were transduced with the TRIP D64V shRNA expressing either an SSRP1-specific or scrambled shRNA sequence and three days after transduction the cells were infected with a VSV-G pseudotyped, single-round infection, MLV-derived virus expressing luciferase (MLVluc). In these experiments, we observed that MLV infection was not affected (Figure 2.3) excluding the possibility that the effect of SSRP1 knockdown on HIV-1 infection is due to the role of the FACT complex in transcription or to unnoticed cellular toxicity. These data also indicate that the effect of SSRP1 on HIV-1 infection is specific and could be LEDGF/p75-dependent.

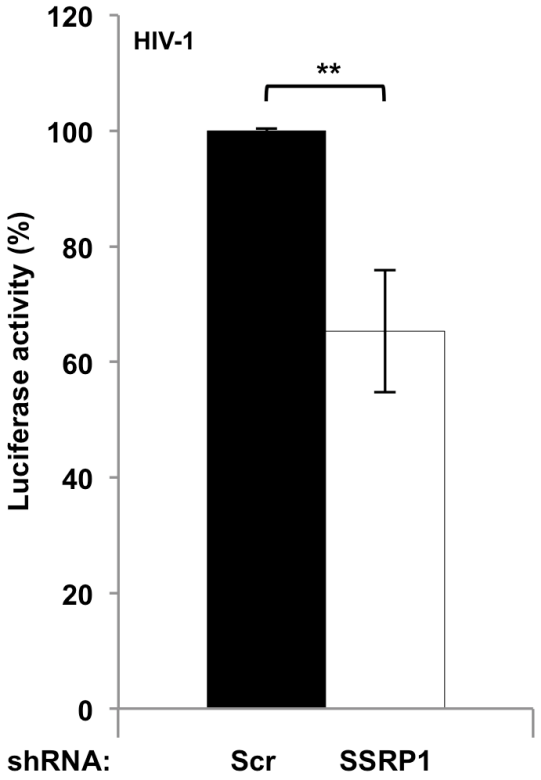
a


HIV-1
Luciferase activity (%)
120
100
80
60
40
20
0
shRNA:
Scr
SSRP1
Scr
SSRP1
Scr
SSRP1
M.O.I.:
0.1
0.05
0.025

b


Viability (ATP Levels)
250
200
150
100
50
0
shRNA:
Scr
SSRP1
Scr
SSRP1
Scr
SSRP1
M.O.I.:
0.1
0.05
0.025

c


HIV-1
**
120
100
80
60
40
20
0
Luciferase activity (%)
shRNA:
Scr
SSRP1

d

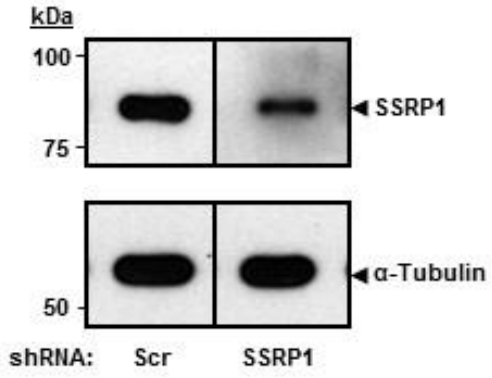

kDa
100
75
50
SSRP1
α-Tubulin
shRNA:
Scr
SSRP1

Figure 2.1 Effect of SSRP1 partial knockdown on single-round HIV-1 infection.

SupT1 cells were transduced at different MOIs with a lentiviral vector expressing a Scrambled or SSRP1-specific shRNA. Seventy-two hours later the transduced cells were infected with a single-round HIV-1 virus expressing luciferase. (a) Luciferase and (b) ATP levels were measured three days post-infection. Luciferase was normalized to ATP content in the same samples. Standard deviations in (a) represent the variability in luciferase readings of single experiments and in (c) the variability of three independent experiments at MOI 0.1, (**) represents a p value < 0.01. (d) SSRP1 levels in one of the experiments represented in (c) were determined by immunoblot analysis. α-tubulin was measured as a loading control. The level of knockdown achieved is representative of the other two infection experiments. The vertical line separating the lanes indicates that the samples were ran in the same electrophoresis gel and immunoblot membrane but not in adjacent positions.

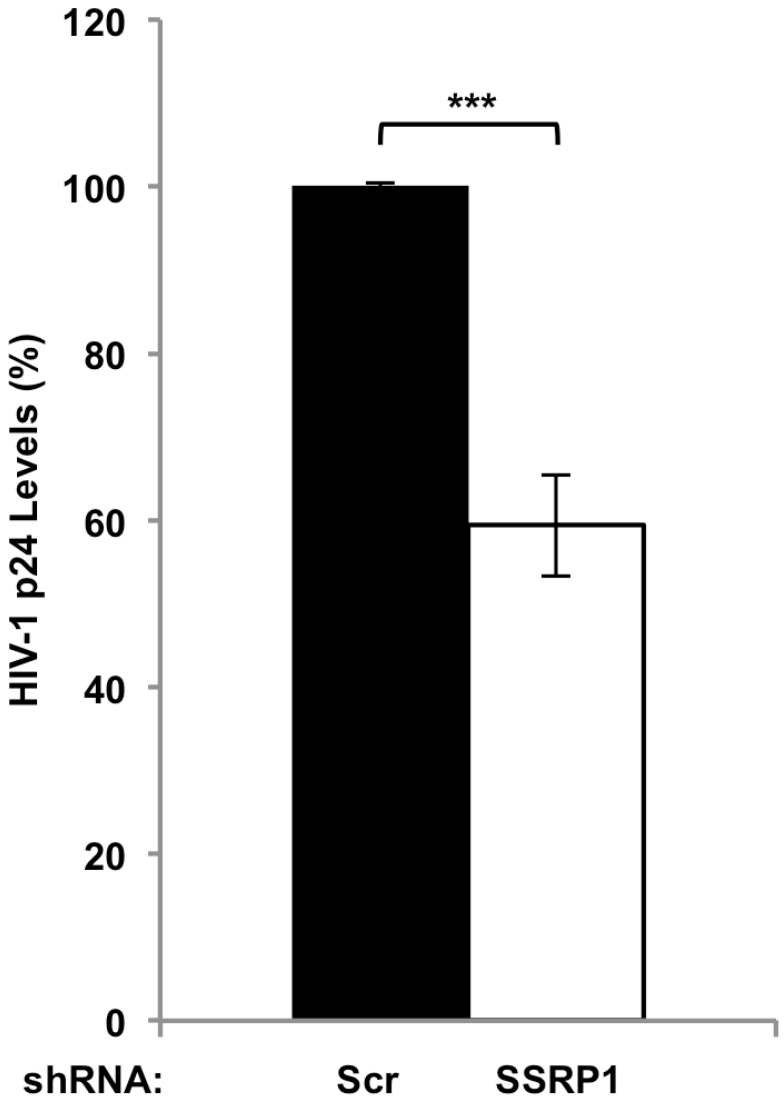


Figure 2.2 Effect of SSRP1 partial knockdown on wild type HIV-1 infection.
SupT1 cells were transduced with either the scrambled or SSRP1-specific shRNA expressing lentiviral virus. Seventy-two hours later the transduced cells were infected with HIV-1_{NL4-3}. After 24 hours, infected cells were extensively washed to remove input HIV-1_{NL4-3} virus and 96 hours post-infection the supernatant was harvested and HIV-1 p24 quantified by ELISA. Standard deviations represent data from three different experiments, (***) represents a p value < 0.001.

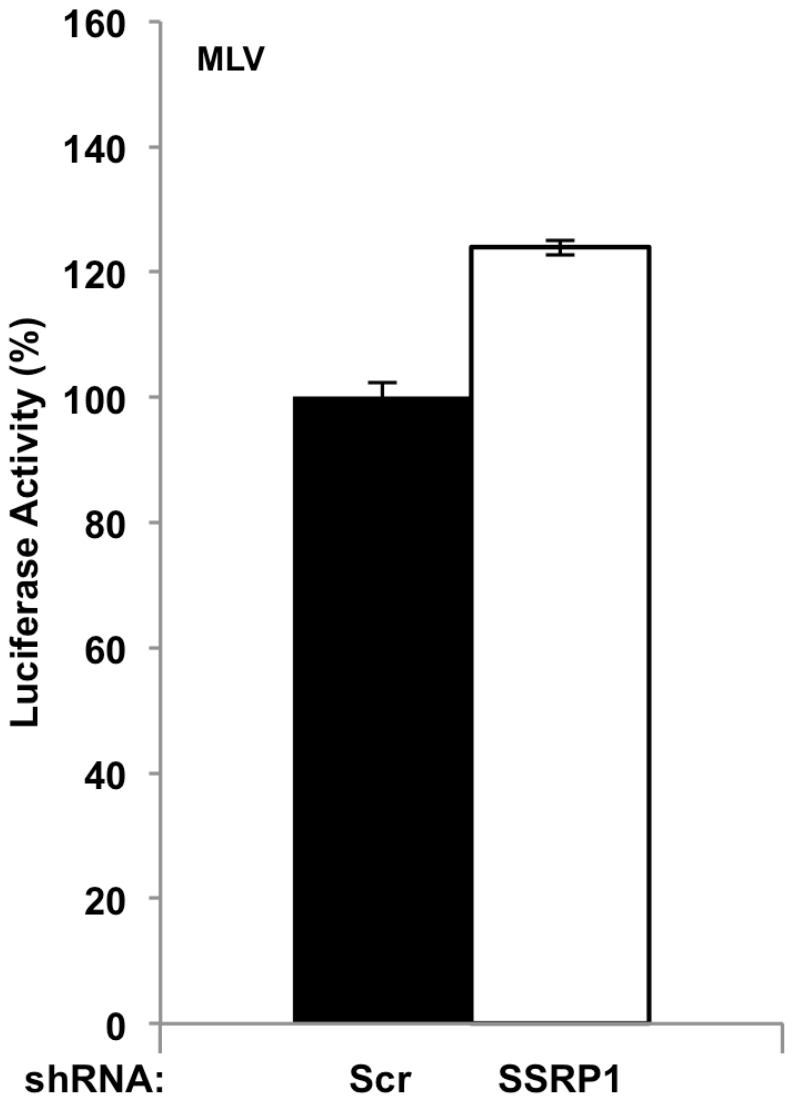


Figure 2.3 Influence of partial knockdown of SSRP1 on MLV infection.
SupT1 cells were transduced with a lentiviral vector encoding a Scrambled or SSRP1-specific shRNA, three days later the cells were infected with an MLV-derived virus expressing luciferase, and seventy-two hours post-infection, luciferase was measured. Standard deviations represent the variability of luciferase activity readings from single experiments, p value > 0.05.

Although the SupT1 target cells were transduced at the same MOI with the same lentiviral vector preps in the MLV and HIV-1 infection experiments described above, small variations in SSRP1 knockdown levels in the target cells could account for the differential effect of the deficiency of this protein on the infection by these retroviruses. To control for this potential confounding factor, SupT1 cells were transduced with the shRNA-expressing lentiviruses at MOI 1 and three days later the cells were used either for infection with HIV-1_{NL4-3} or with MLVluc, or for immunoblotting analysis of SSRP1 expression. Three days after infection, cellular ATP levels and MLV-encoded luciferase activity were measured in the MLV-infected cells, and HIV-1 p24 quantified in the supernatant of HIV-1NL4-3 infected cells.

Densitometry analysis of the immunoblot in Figure 2.4a indicates that these knockdown cells expressed 52% of the SSRP1 levels found in control cells. In these cells, HIV-1 replication was impaired (Figure 2.4c) to levels comparable to those achieved in experiments reported earlier, which correlate with the partial SSRP1 knockdown obtained in the target cells (Figure 2.4a). In contrast to HIV-1, MLV infection was not impaired in these cells (Figures 2.4b), corroborating previous observations (Figure 2.3). As expected, cell viability was not affected in the SSRP1-knockdown cells studied (Figure 2.4d). Data in Figures 2.1-2.4 highlight the reproducibility of our results and indicate a specific role for SSRP1 in HIV-1 infection that seems to be LEDGF/p75-dependent.

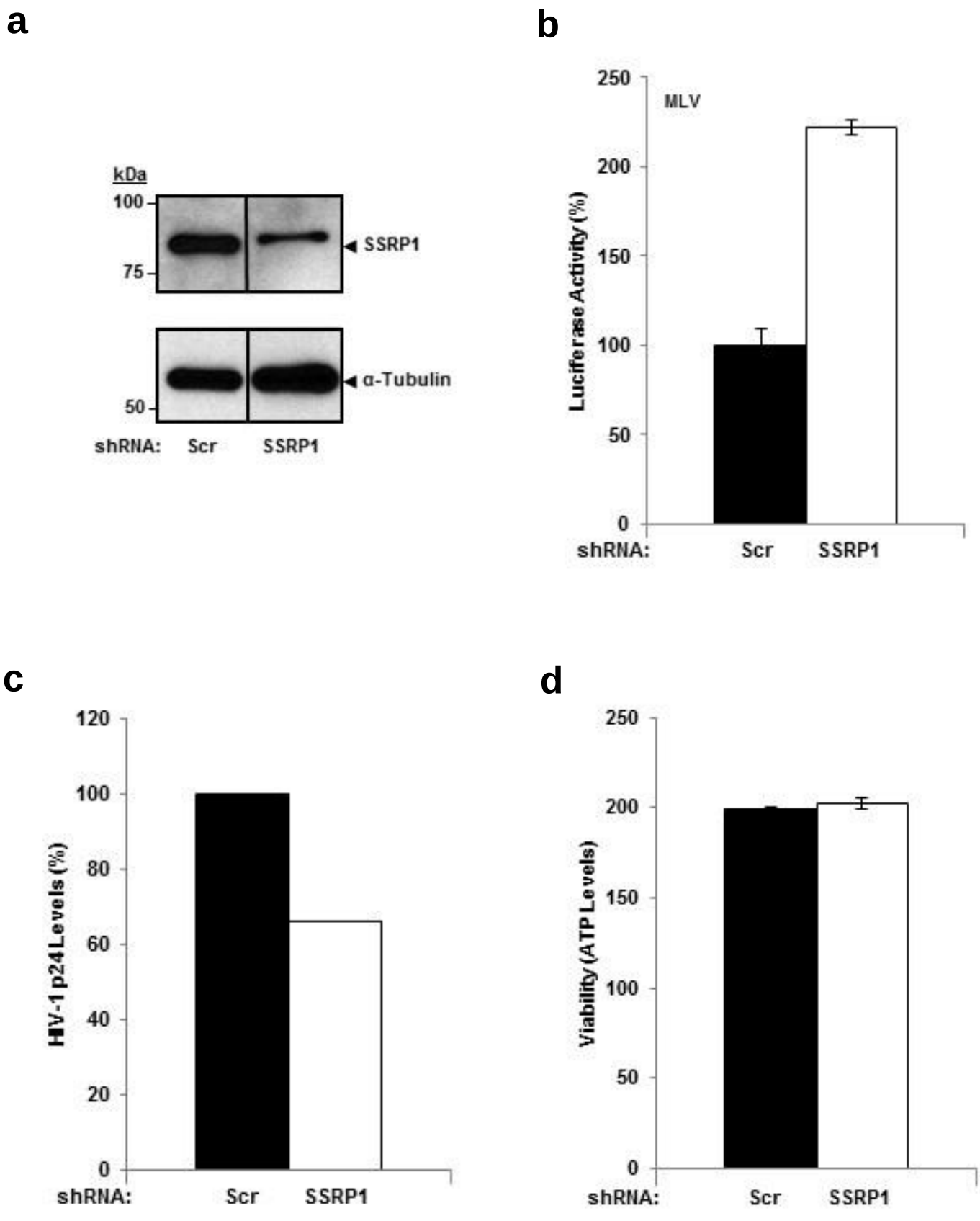

a
kDa
100
75
50
SSRP1
α-Tubulin
shRNA: Scr SSRP1
b
MLV
Luciferase Activity (%)
250
200
150
100
50
0
shRNA: Scr SSRP1
c
HV-1 p24 Levels (%)
120
100
80
60
40
20
0
shRNA: Scr SSRP1
d
Viability (ATP Levels)
250
200
150
100
50
0
shRNA: Scr SSRP1

Figure 2.4 Parallel MLV and HIV-1 infections of SSRP1 partial knockdown cells.
(a-d) SupT1 cells were transduced at MOI 1 with a lentiviral vector encoding a Scrambled or SSRP1-specific shRNA. Seventy-two hours later one-third of the transduced cells were analyzed for SSRP1 expression by immunoblot (a) and the remaining cells were infected with MLV (b) or HIV-1_{NL4-3} (c). Three days after infection, luciferase activity was measured in the MLV-infected cells (b) and HIV-1 replication was determined by quantifying HIV-1 p24 in the supernatant of the HIV-1_{NL4-3} infected cells by ELISA (c). Total ATP content (viability) (d) was also measured for these cells. Standard deviation in (b and d) represents the variability of luciferase activity and ATP content readings from single experiments. Data in (c) represents a single experiment. The vertical line separating the lanes in (a) indicates that the samples, although in the same electrophoresis gel and immunoblot membrane, were not in adjacent positions.

As previously discussed, SSRP1 deficiency could affect HIV-1 infection by reducing LTR-driven transcription or viral DNA integration. In order to distinguish between these potential mechanisms we compared the effect of SSRP1 deficiency on infection by HIV-1-derived viruses that, although replicate through viral DNA integration, use different promoters for transgene expression. TRIP viruses [92] express their transgenes from an internal immediate-early CMV promoter in contrast to HeGFP or Hluc viruses (used in Figures 2.1-2.4) in which the transgenes are expressed from the HIV-1 LTR. As described above, SupT1 cells were transduced with shRNA retroviral vectors expressing a control or an SSRP1 shRNA and three days later were infected with TRIP eGFP, TRIP luc, HeGFP, or Hluc. In these experiments the shRNAs were delivered using a retroviral vector lacking the eGFP reporter. As shown in Figure 2.5a and 2.5b, SSRP1 knockdown diminished infection of viruses expressing the transgene from the LTR but not from the CMV promoter, suggesting that SSRP1 deficiency affected LTR-driven transgene expression rather than viral DNA integration.

Data in Figures 2.3-2.5b combined also suggest that LEDGF/p75 could affect HIV-1 infection by modulating LTR-driven gene expression through its interaction with SSRP1, in addition to the previously reported role in HIV-1 DNA integration [52,55]. In order to evaluate further the role of LEDGF/p75 on HIV-1 LTR-dependent gene expression, we compared the effect of LEDGF/p75-deficiency on infection by TRIPluc and Hluc. shRNA-mediated LEDGF/p75-deficient SupT1 cells (T_{L3}) and control cells expressing a scrambled sequence shRNA (T_{C3},) [52] were infected with TRIPluc or Hluc and three days later luciferase activity was determined. In two independent experiments (Figure 2.5c), it was observed that Hluc was 3-6 fold more sensitive to LEDGF/p75

deficiency than TRIPluc, further suggesting the existence of LTR-dependent and –independent mechanisms in the HIV-1 cofactor activity of LEDGF/p75. We further evaluated the effect of SSRP1 knockdown on infectivity of Hluc and TRIPluc in T_{L3} and T_{C3} cells. These cells were transduced with control or SSRP1 shRNA expressing retroviral vectors, and three days later were infected with Hluc or TRIPluc, and analyzed for luciferase expression three days after infection (Figure 2.5d). As expected, SSRP1 knockdown reduced infectivity of Hluc but not TRIPluc viruses in both LEDGF/p75-deficient and control cells to a similar extent. No differences in the effect of SSRP1 deficiency on HIV-1 infection between T_{L3} and T_{C3} cells are expected considering that HIV-1 infection in T_{L3} cells, although greatly diminished as compared to control cells, is still LEDGF/75-dependent. For example, expression of a LEDGF/p75 dominant mutant in T_{L3} cells decreased by two logs their susceptibility to HIV-1 infection [96].

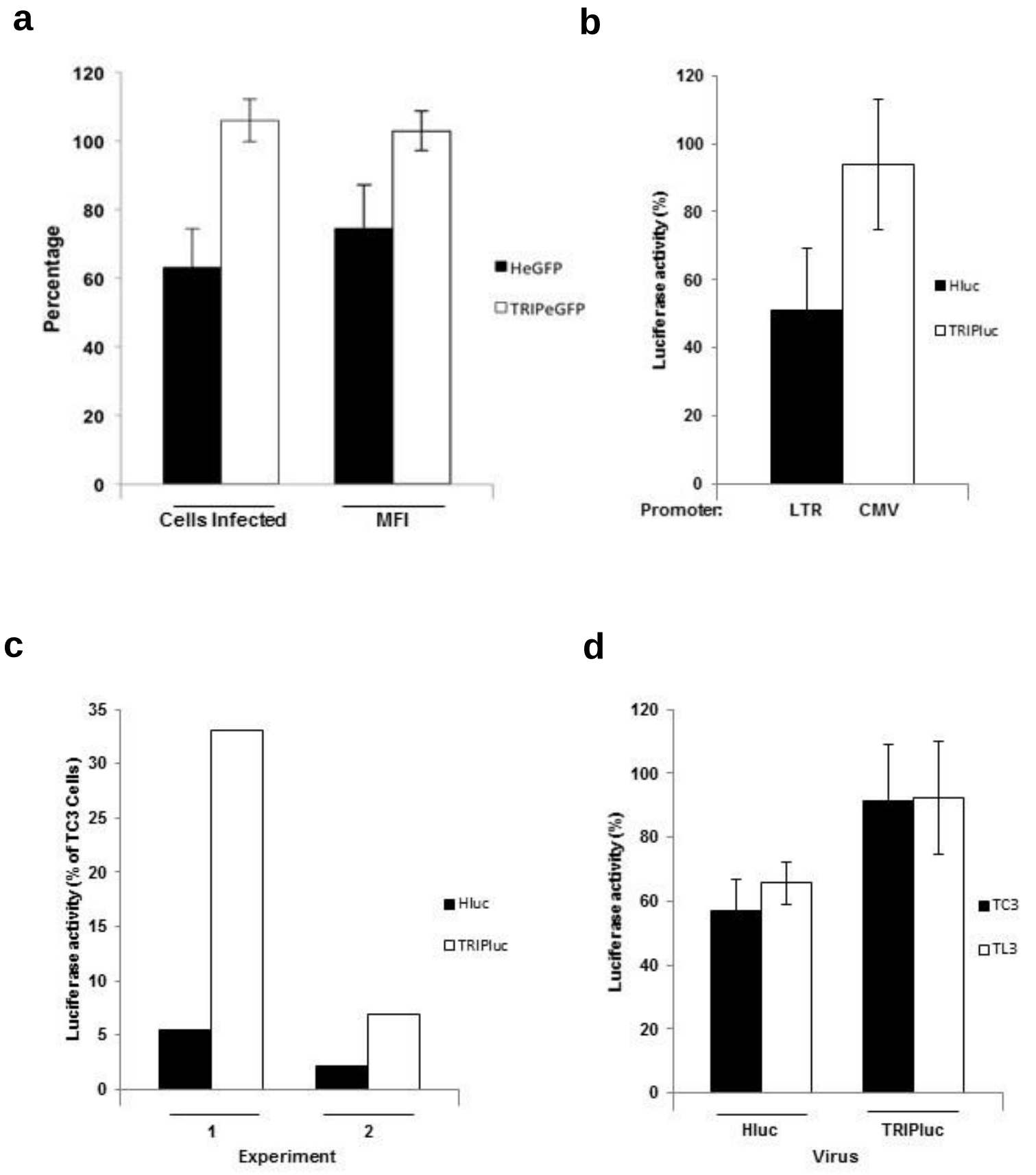

a
120
100
80
60
40
20
0
Percentage
HeGFP
TRIPeGFP
Cells Infected
MFI
b
120
100
80
60
40
20
0
Luciferase activity (%)
Hluc
TRIPluc
Promoter:
LTR
CMV
c
35
30
25
20
15
10
5
0
Luciferase activity (% of TC3 Cells)
Hluc
TRIPluc
1
2
Experiment
d
120
100
80
60
40
20
0
Luciferase activity (%)
TC3
TL3
Hluc
TRIPluc
Virus

Figure 2.5 Influence of partial knockdown of SSRP1 on HIV-1 infection carrying different promoters.

(a and b) SupT1 cells were transduced with a lentiviral vector encoding a Scrambled or SSRP1-specific shRNA, three days later the cells were infected with HIV-derived viruses expressing eGFP or luciferase from the HIV-1 LTR (HeGFP/Hluc) or from an internal immediate early CMV promoter (TRIPeGFP/TRIPluc). Seventy-two hours post-infection, luciferase or eGFP were measured. Standard deviations represent the variability of 4 independent experiments. (c) LEDGF/p75-deficient (T_{L3}) and control cells (T_{C3}) were infected with Hluc or TRIP and luciferase was measured three days later. Two independent experiments are represented. (d) T_{L3} and T_{C3} cells were transduced with a lentiviral vector encoding a Scrambled or SSRP1-specific shRNA, three days later the cells were infected with Hluc or TRIP luc and seventy-two hours post-infection, luciferase or eGFP were measured. Luciferase activity measured in the cells transduced with control shRNAs was considered 100% values. Standard deviations represent the variability of 4 independent experiments.

2.3.2 Chromatin binding of LEDGF/p75 and SSRP1 occur independently of each other

To determine if chromatin binding was affected for LEDGF/p75 in SSRP1 partially deficient cells or vice versa, cells deficient in the respective proteins were analyzed by a salt extraction assay. SupT1 cells were transduced with the shRNA-expressing viral vector at an MOI 2.5-folds higher than the MOI used in the HIV-1 and MLV infection experiments described above, and three days later these cells were used to determine the chromatin binding strength of LEDGF/p75 by the salt extraction method [95]. In this assay, the effect of salts on the chromatin binding strength of LEDGF/p75 is evaluated. Briefly, the transduced cells were lysed in CSKI buffer supplemented with NaCl to a final concentration of 175 mM, under these conditions LEDGF/p75 is only partially extracted from chromatin. Then, the cell lysates were fractionated by centrifugation into a soluble salt-extracted fraction, which contains chromatin non-bound proteins (S1), and in an insoluble salt-resistant fraction (P1), which includes chromatin bound proteins and other Triton X-100 insoluble proteins. Subsequently, these cellular fractions were evaluated by immunoblotting for the presence of LEDGF/p75 and SSRP1. As expected, at the higher MOI used in these experiments, a more stringent SSRP1 knockdown than in the retroviral infection experiments reported above, was reached (compare total fraction in Figure 2.6a with Figures 2.1d and 2.4a). Densitometry analysis of immunoblot in Figure 2.6a indicated that knockdown cells express only 13% of the SSRP1 levels found in the control cells. Nevertheless, the salt extraction pattern of LEDGF/p75 in these more stringent SSRP1 knockdown cells was identical to the pattern observed in control cells (Figure 2.6a)

which indicates that cellular levels of SSRP1 lower than those that affect HIV-1 infection do not affect the chromatin binding strength of LEDGF/p75. These results suggest that LEDGF/p75 binds to chromatin independently of SSRP1 and that the effect of SSRP1 partial deficiency on HIV-1 infection, although likely LEDGF/p75-dependent, does not seem to involve the chromatin-binding activity of LEDGF/p75.

Similarly, the chromatin binding strength of SSRP1 in LEDGF/p75-deficient cells was evaluated using the salt extraction method described above. SupT1-derived LEDGF/p75-knockdown (T_{L3}) and control cells (T_{C3}) were subjected to salt extraction with CSKI buffer supplemented with NaCl to a final concentration of 150 mM. Then, the distribution of SSRP1 and LEDGF/p75 was determined in the salt-extracted and -resistant fractions by immunoblotting. As represented in (Figure 2.6b), the SSRP1 salt extraction pattern was similar in control and LEDGF/p75-deficient cells indicating that LEDGF/p75 does not influence the binding strength of SSRP1 to chromatin.

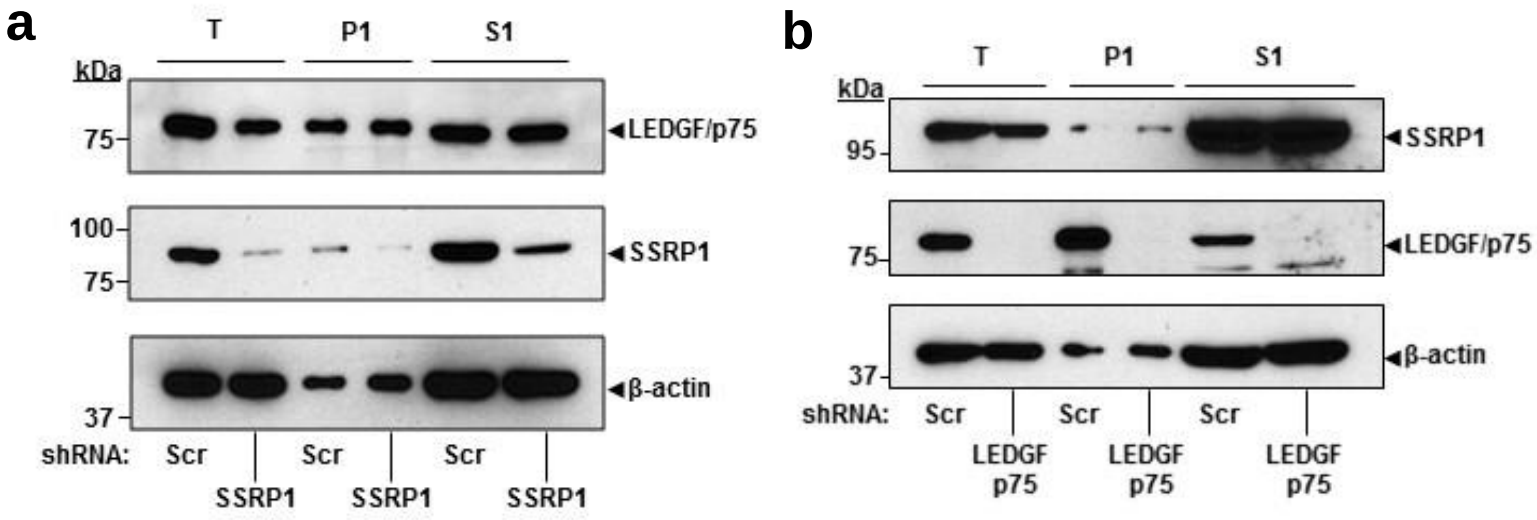


Figure 2.6 Analysis of the chromatin binding strength of SSRP1 and LEDGF/p75 in LEDGF/p75 and SSRP1-deficient cells by immunoblotting.

Chromatin-bound (P1) and non-bound fractions (S1) were obtained from SupT1 cells expressing control (Scr) or SSRP1- (a) or LEDGF/p75- (b) specific shRNAs. A total cell lysate (T) was obtained by lysing the aforementioned cells in 2X Laemmli sample buffer. The presence of SSRP1 and LEDGF/p75 proteins was detected in these fractions by immunoblot analysis. β-actin was detected as a loading control. To avoid saturation of the immunoblot signals, the amount of P1 fractions loaded was 1/5 of the total and S1 fractions.

CHAPTER 3: Characterization of the interaction between the FACT complex subunit SSRP1 and LEDGF/p75

3.1 Introduction

LEDGF/p75 tethers the pre-integration complex, through integrase, to the host chromatin where viral genome integration takes place. This co-factor activity of LEDGF/p75 is essential for the replication of the virus. Our laboratory postulates that other cellular proteins, with which LEDGF/p75 interacts, may prove beneficial to HIV-1 replication possibly through the regulation of the cofactor activity of LEDGF/p75.

As previously mentioned, once the FACT complex and LEDGF/75 were found to interact through a proteomic analysis, our lab verified the protein interactions through the use of co-immunoprecipitations of endogenous proteins (Figure 1.5). This positive verification led us to perform the infection experiments in Chapter 2 to investigate whether SSRP1 even had a role in HIV-1 infection. Once we found that the partial knockdown of SSRP1 had an effect on HIV-1 infection, we decided to characterize the interaction between LEDGF/p75 and SSRP1. Finding what domains are essential for the interaction in each protein can allow us to create and express mutants lacking these domains, leading to the disruption of the interaction between the two proteins, and then we can subsequently knockout each of the endogenous proteins. This would allow us to evaluate the importance of the interaction of these two proteins in HIV-1 infection. Interruption of this interaction may affect the HIV cofactor activity of LEDGF/p75 and could possibly be responsible for the effects in HIV-1 infection that we see in SSRP1 partially knockdown cells.

In order to evaluate the functional implication of the interaction of these proteins, it is important to identify the regions within SSRP1 and LEDGF/p75 that mediate their interaction. Once identified, these regions can be mutated and the impact of these

mutations on the virological roles of the proteins evaluated. This is particularly relevant for proteins like SSRP1 that due to their essential role in cells their expression cannot be effectively decreased to evaluate effects on viral infection. To investigate which region(s) or domain(s) are essential in the interaction between LEDGF/p75 and SSRP1, mutants of each protein, lacking a region or domain, were generated and evaluated by immunoprecipitation for loss of interaction. LEDGF/p52 and a LEDGF/p75 mutant containing only the C-terminal (aa 326-530) were evaluated for interaction with SSRP1 WT. Furthermore, LEDGF/p75 mutants lacking the following domains or regions were assessed: PWWP, CR1, NLS, CR1b, CR2, CR3, CR4, IBD, CR5.1, and CR6. SSRP1 mutants were also evaluated using the same method. The following domains or regions were evaluated: NTD, SSRC, MD, IDD, HMG, and CID.

Furthermore, upon defining the essential domain required for each protein in the interaction, we investigated whether each domain was also sufficient to mediate the interaction. If there were to be domains that could, by themselves, immunoprecipitate the other protein target then these could be used as dominant mutants to evaluate the virological role of the protein. For example, these domains would be expressed in the cell in abundance so that they could bind to endogenous target proteins, be it SSRP1 or LEDGF/p75, and disrupt the interaction between the two proteins. These cells could then be infected with HIV-1 and MLV and subsequently the infection levels would be measured.

Lastly, the interaction of LEDGF/p75 and SSRP1 was analyzed using purified recombinant proteins produced in I *E. coli* to determine whether post-translational modifications in SSRP1 or LEDGF proteins are implicated in their interaction and to

define whether these proteins interact directly.

3.2 Materials and Methods

3.2.1 Generation of expression constructs

LEDGF/p75 expression plasmids. pFLAG LEDGF/p75 and pFLAG LEDGF/p52 previously described [95,97] were used in transient expression experiments and to generate the LEDGF mutants described in this work.

SSRP1 expression plasmids. pSSRP1-Myc or pXpress-SSRP1 were generated by cloning the SSRP1 cDNA into pCMV, which contains the promoter and intron A, the immediate early gene promoter of the human Cytomegalovirus (CMV promoter) [98], with a C-terminal Myc or Xpress tag. SSRP1 cDNA was PCR amplified from a human lymph node cDNA library. These SSRP1 expression plasmids were further used to generate a panel of SSRP1 mutants.

Recombinant protein expression plasmids. Recombinant proteins His_6-SSRP1, GST-LEDGF/p52-FLAG, and GST-LEDGF/p75 C-terminal-FLAG were expressed in *E. coli* using pET-DEST42-SSRP1, pGEX-6P-1-LEDGF/p52-FLAG, and pGEX-6P-1-LEDGF/p75 C-terminal-FLAG expression plasmids, respectively.

All the mutants characterized in this work were generated using the Phusion™ Site-Directed Mutagenesis Kit. The sequences of the primers used are available upon request. The identity of these mutants was verified by overlapping DNA sequencing of the entire cDNAs.

3.2.2 Cell lines, culture, and transfection conditions

HEK293T and si1340/1428 cells were used for transient transfection experiments. si1340/1428 cells (a gift of E. Poeschla. University of Colorado Denver) are derived from HEK293T cells by stable expression of two LEDGF/p75-specific siRNAs, targeting sequences at nt 1340 and 1428 [98]. HEK293T and HEK293T-derived cell lines were grown in DMEM. All culture media were supplemented with 10% of heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin. LEDGF and SSRP1 expression plasmids were transfected in HEK293T or si1340/1428 cells using the calcium-phosphate co-transfection method. Briefly, cells were plated at 0.45 X 10^6 cells / well in a six-well plate and transfected with 2 µg of DNA of the corresponding plasmids. Transfection medium was replaced 18 hours later with fresh culture medium and cells were cultured for an additional 18 hours until they were harvested for analysis.

3.2.3 Co-immunoprecipitations of LEDGF/p75 or SSRP1 mutants lacking different domains or regions

si1340/1428 (LEDGF-deficient) and human embryonic kidney 293T (HEK293T) cells were used for co-immunoprecipitation experiments. FLAG-tagged LEDGF mutants and Myc-tagged SSRP1 WT expression plasmids were transfected into si1340/1428 cells; and Myc-tagged SSRP1 mutants and FLAG-tagged LEDGF WT expression plasmids were transfected in HEK293T cells, both using the calcium-phosphate co-transfection method.

Transfected cells (~ $3x10^6$) were harvested and lysed for 15 minutes on ice in 300 µl of CSK I buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 1 mM EDTA, 300 mM

sucrose, 1 mM $MgCl_2$, 1 mM DTT, 0.5% Triton X-100) containing protease inhibitors (final concentration: leupeptine 2 µg/ml, aprotinin 5 µg/µl, PMSF 1 mM, pepstatin A 1 µg/ml). Cellular lysates were centrifuged at 1000 g for 6 minutes at 4°C and separated into the supernatant fraction (S1), that contains the Triton X-100-soluble chromatin non-bound proteins, and a pellet (P1), that contains Triton X-100-insoluble proteins and chromatin-bound proteins. P1 was further treated with DNAse to solubilize the chromatin-bound proteins. To this end, P1 was re-suspended in 20 µl of CSK II buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 6 mM $MgCl_2$, 1 mM DTT) supplemented with protease inhibitors, 16 units of turbo DNase, 3.4 µl of $(NH_4)_2SO_4$, and 3.1 µl of 10X turbo DNase reaction buffer. DNase treatment was conducted at 37°C for 30 minutes. After incubation, 300 µl of CSK I buffer was added to the DNase/$(NH_4)_2SO_4$ treated sample to dilute the $(NH_4)_2SO_4$ and centrifuged at 22,000 g for 3 minutes. Then the supernatant (S2 fraction) was pre-cleared twice to remove any unspecific binding by incubation under rotation for 30 minutes at 4ºC with 150 µl of goat anti-mouse IgG-coated magnetic beads, referred hereafter as beads. From the pre-cleared S2 fraction 50 µl of sample were obtained and mixed with 10 µl of 6X Laemmli sample buffer, and saved as input. Beads (150 µl), that were previously loaded for 3 hours at 4ºC with 30 µg of anti-FLAG mAb diluted in TBS-5% milk-0.05% Tween 20, were magnetically separated from the unbound antibodies, mixed with the remaining pre-cleared S2 fraction (note that S2 contains DNAse), and rotated for 2 hours at 4°C. After this incubation, beads were washed five times for 5 minutes each in CSK I buffer and bound proteins were the eluted by boiling in 60 µl of 2X Laemmli sample buffer.

Immunoprecipitated proteins were analyzed by Western Blot. The samples were

detected with the following antibodies: FLAG-tagged LEDGF/p75 was detected with anti-FLAG mAb (1/500), non-tagged LEDGF/p75 was detected with an anti-LEDGF/p75 (1/500), Myc-tagged SSRP1 mutants were detected with anti-Myc mAb (1/500), and SSRP1 was detected with anti-SSRP1 (1/500).

In some experiments, cells were sub-fractionated into S1 and S2 fractions but then these fractions were mixed (S1/S2), pre-cleared, and used in immunoprecipitation experiments as described above (note that DNAse was also present during these experiments).

Densitometry analysis of immunoblots was performed with the gel analysis software UN-SCAN-IT gel 6.1 (Silkscientific).

3.2.4 Transfection and co-immunoprecipitation of LEDGF/p75 or SSRP1 essential interacting domains

FLAG-tagged PWWP domain was co-transfected along with Myc-tagged SSRP1 WT in si1340/1428 cells; and the Myc-tagged HMG domain was co-transfected along with FLAG-tagged LEDGF WT in HEK293T. Subsequently, the lysates of these cells were evaluated by immunoprecipitation, as previously described. In both cases, the S1 and S2 fractions were mixed and used for the immunoprecipitation.

The immunoprecipitated samples were analyzed by resolving them in an SDS-PAGE and successively performing a Western Blot. The samples were detected with the following antibodies: FLAG-tagged PWWP domain and LEDGF/p75 WT were detected with anti-FLAG mAb (1/500) and Myc-tagged HMG domain and SSRP1 WT were detected with anti-Myc mAb (1/500).

3.2.5 Expression, purification, and co-immunoprecipitation of recombinant proteins

GST- and His-tag fusion recombinant proteins were produced in *E. coli* Rossetta 2DE3 pLacI. Shake-flask cultures of bacteria containing GST-LEDGF/p75 C-terminal-FLAG and His6-SSRP1 were grown to an optical density of 0.4-0.6 at 600 nm and were induced for 2 hours by addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside at 37 °C. Shake-flask culture of bacteria containing GST-LEDGF/p52-FLAG was grown to an optical density of 0.4-0.6 at 600 nm was and induced for 4 hours by addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside at 25 °C. Pelleted bacterial cells were disrupted by sonication in 20 mM Tris-Cl, 150 mM NaCl, 0.5 mM DTT, 1 mM EDTA, and Protease Inhibitor Cocktail tablet. The crude lysates were pre-cleared by centrifugation at 16,000 g for 10 minutes and the supernatants were collected for protein purification. Recombinant protein containing GST-LEDGF/p52-FLAG and GST-LEDGF C-terminal-FLAG were purified using a 5 ml GSTrapTM HP column. His6-SSRP1 was purified using a 5 ml HisTrapTM HP column. Bound GST-tagged proteins were eluted with a linear reduced glutathione gradient from 0-15 mM reduced glutathione in 20 mM Tris-Cl, 150 mM NaCl, 2 mM beta-mercaptoethanol, and 10% glycerol. GST-LEDGF/p75 C-terminal-FLAG and GST-LEDGF/p52-FLAG were eluted at 10 mM and 5 mM reduced glutathione, respectively. Bound His6-SSRP1 was eluted with a linear imidazole gradient from 0-100 mM imidazole in 20 mM Tris-Cl, 150 mM NaCl, 2 mM beta-mercaptoethanol, and 10% glycerol. His6-SSRP1 eluted at 100 mM imidazole.

Purified recombinant His6-SSRP1 (30 µg) was mixed or not with purified recombinant GST-LEDGF/p52-FLAG (45 µg) or GST-LEDGF/p75 C-terminal-FLAG (45

µg) in CSK II buffer and incubated for 30 minutes at 4ºC. After this incubation, 40µl of the mixture were obtained and mixed with 8µl of 6X Laemmli sample buffer, and saved as input. Goat anti-mouse IgG-coated magnetic beads (150µl), which were previously loaded for 3 hours at 4°C with 30µg of anti-FLAG mAb diluted in TBS-5% milk-0.05% Tween 20, were magnetically separated from the unbound antibodies, the remaining mixture of recombinant proteins was added to these beads and rotated for 2 hours at 4°C. Then, beads were washed five times for 10 minutes each with CSK I buffer and bound proteins were eluted by boiling in 50µl of 2X Laemmli sample buffer.

The immunoprecipitated samples were analyzed by resolving them in an SDS-PAGE and successively performing a Western Blot. The samples were detected with the following antibodies: FLAG-tagged LEDGF/p52 and LEDGF/p75 C-terminal were detected with anti-FLAG mAb (1/500) and SSRP1 was detected with anti-SSRP1 (1/500).

3.3 Results

3.3.1 SSRP1 and LEDGF proteins interact directly and independently of hSpt16

Earlier, it was evaluated whether or not LEDGF/p75 interacts with the chromatin remodeling factor: FACT complex, a heterodimer composed of hSpt16 and SSRP1 proteins [63] (Figure 1.5). The FACT complex remodels nucleosomes in an ATP-independent fashion allowing RNA polymerase II to access the DNA during transcriptional elongation. Approximately fifty percent of the cellular FACT complex is associated to chromatin, making this fraction the most likely to participate in chromatin remodeling [63,91].

SSRP1, however, not hSpt16, was identified as a LEDGF/p75-interactor in the proteomic analysis mentioned above, suggesting that LEDGF/p75 could interact with the FACT complex by binding primarily to the SSRP1 subunit. To evaluate further whether LEDGF/p75 and SSRP1 bind directly and independently of hSpt16, and to determine what region of LEDGF/p75 was implicated in the binding, we studied the interaction of LEDGF proteins with SSRP1 using purified recombinant proteins produced in *E. coli*. In these experiments, we took advantage that LEDGF/p52 is a LEDGF splicing product that contains the entire N-terminal region of LEDGF/p75 (aa 1-325) and that, like the C-terminal region of LEDGF/p75 (aa 326-530), is more stable than LEDGF/p75 as recombinant proteins. Purified recombinant His_6-tagged SSRP1 and GST fused FLAG-tagged LEDGF/p52 or C-terminal region of LEDGF/p75 proteins were mixed and then subjected to immunoprecipitation with an anti-FLAG mAb, followed by anti-SSRP1 immunoblotting. Results in Figure 3.1 indicate a robust interaction of SSRP1 with LEDGF/p52 but not with the C-terminal region of LEDGF/p75. These results clearly show that the shared N-terminal region of LEDGF proteins interacts directly with SSRP1 in an hSpt16-independent manner and that binding does not require cellular chromatin or post-translational modifications of LEDGF or SSRP1 proteins.

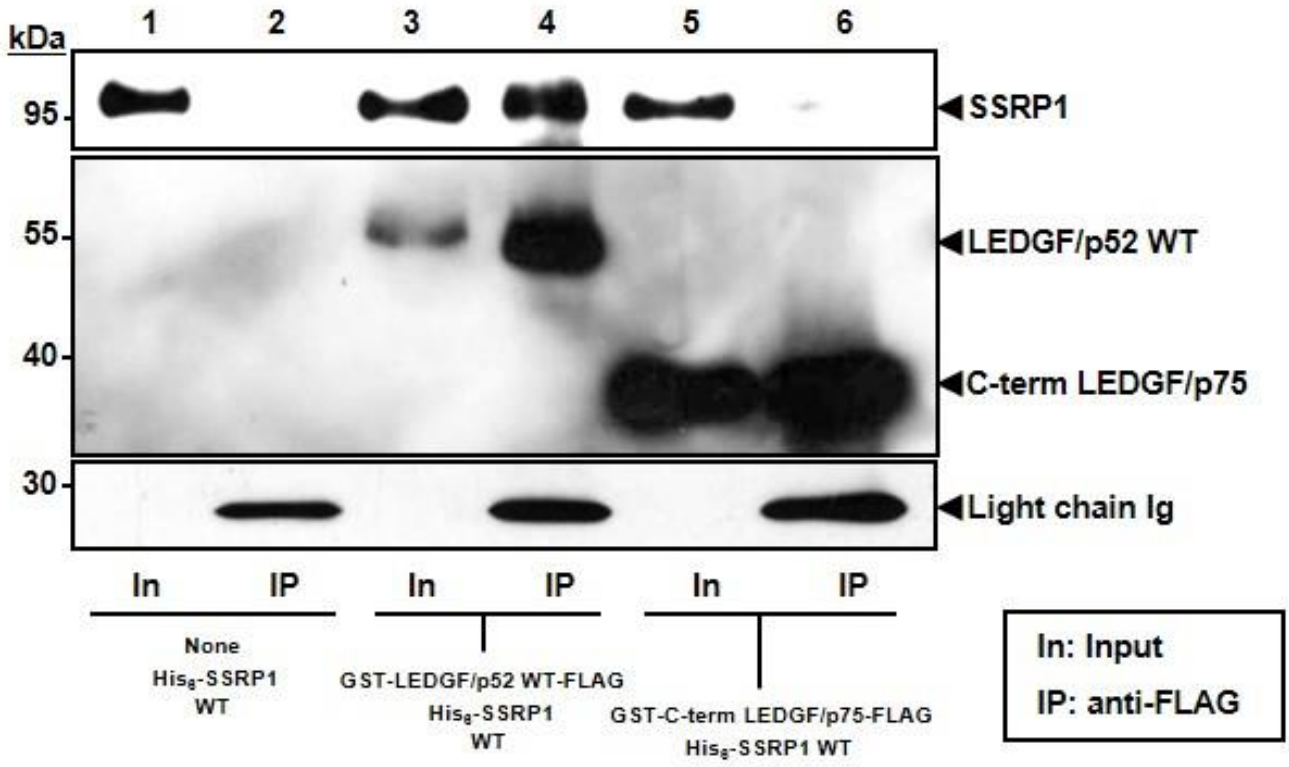


Figure 3.1 Interaction of recombinant LEDGF/p52 and LEDGF/p75 C-terminal proteins with recombinant SSRP1 protein

Purified His_6-SSRP1 WT alone (lanes 1 and 2) or mixed with purified GST-LEDGF/p52-FLAG (lanes 3 and 4), or with GST-LEDGF/p75 C-terminal-FLAG (lanes 5 and 6) were co-immunoprecipitated with an anti-FLAG mAb. The presence of SSRP1 and LEDGF proteins were evaluated by immunoblotting with tag-specific antibodies.

3.3.2 SSRP1 interacts with the N-terminal region of LEDGF proteins

Our data in Figure 3.1, using purified recombinant proteins, indicate that the N-terminal region of LEDGF proteins is implicated in hSpt16-independent SSRP1 binding. To characterize further this interaction in cells, we determined whether LEDGF/p75 binds or not to an SSRP1 mutant that lacks the N-terminal domain (NTD) of SSRP1 required for interaction with hSpt16 [63]. Myc-tagged SSRP1 wild type or mutant were co-expressed with FLAG-tagged LEDGF/p75 in HEK293T cells and immunoprecipitated with an anti-FLAG antibody. The presence of SSRP1 proteins in the immunoprecipitated fraction was then determined by immunoblot analysis with an anti-Myc antibody. Findings in Figure 3.2 indicate that LEDGF/p75 strongly bind to the SSRP1ΔNTD mutant confirming the hSpt16-independent interaction of SSRP1 and LEDGF/p75 in cells.

SSRP1 interacts with nucleosomes tharough hSpt16 [63,66,67,91], and as expected is fully recuperated (Figure 3.3a) in the Triton-soluble chromatin unbound fraction of cell lysates [52]. The binding of LEDGF/p75 to SSRP1ΔNTD confirms that this interaction is independent of nucleosomal proteins (Figure 3.3b). Therefore, data in Figures 3.1, 3.2, and 3.3a-3.3.b indicate that LEDGF/p75 binds to SSRP1 directly in an hSpt16- and chromatin-independent manner.

Next, we defined what region of LEDGF/p75 was responsible for SSRP1 recognition in cells. The interaction of Myc-tagged SSRP1 with FLAG-tagged LEDGF/p75 or LEDGF/p52 wild type proteins, or a LEDGF/p75 mutant containing only the C-terminal region was evaluated by co-immunoprecipitation experiments. HEK293T-derived LEDGF/p75-deficient cells (si1340/1428 cells) were co-transfected with

expression plasmids encoding these proteins, and then two days later S2 fractions were obtained and subjected to immunoprecipitation with an anti-FLAG mAb. The immunoprecipitated proteins were analyzed by immunoblotting with tag-specific antibodies. Results in Figure 3.4 indicate that LEDGF/p75 and p52 interact with SSRP1 with similar efficiency; however, C-terminal LEDGF/p75 does not bind to SSRP1. These results corroborated our findings in Figure 3.1, demonstrating that the shared N-terminal region of the LEDGF proteins interacts with SSRP1 in the absence of DNA in cells.

To formally exclude any role of the C-terminal region of LEDGF/p75 in SSRP1 binding, the interaction of SSRP1 with a panel of LEDGF/p75 mutants lacking each of the regions described in the C-terminus of this protein [95] was evaluated by co-immunoprecipitation using S2 fractions, as described above. Co-immunoprecipitated SSRP1 was detected by immunoblotting and quantified by densitometry analysis (Figure 3.5a). In further support of our conclusions that the C-terminal region of LEDGF/p75 is dispensable for SSRP1 binding, FLAG-tagged LEDGF/p75 deletion mutants lacking IBD (aa 340-417), CR5.1 (aa 443-477), or CR6 (aa 478-530) efficiently interacted with SSRP1, whereas the interaction of a LEDGF/p75 mutant lacking CR4 (aa 326-339) was only slightly reduced (Figure 3.5a).

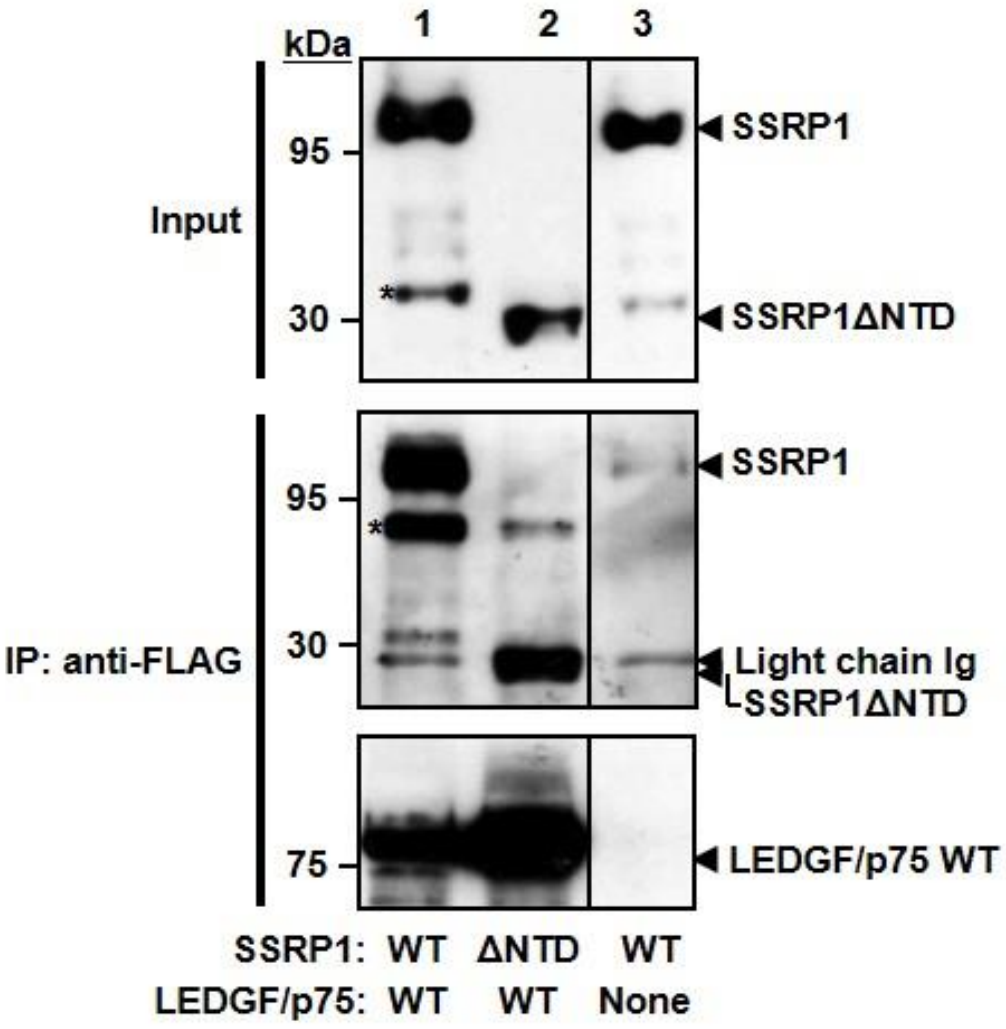


Figure 3.2 Immunoprecipitation analysis of the dependence of SSRP1 on hSpt16 in the interaction with LEDGF/p75

HEK293T cells were co-transfected with plasmids expressing: FLAG-tagged LEDGF/p75 and Myc-tagged SSRP1 WT (lane 1), FLAG-tagged LEDGF/p75 and Myc-tagged SSRP1ΔNTD (lane 2), or Myc-tagged SSRP1 WT and an empty plasmid (lane 3). Samples were analyzed in the same gel, the line dividing lanes 2 and 3 indicate that lanes in between were removed for comparison in this figure.

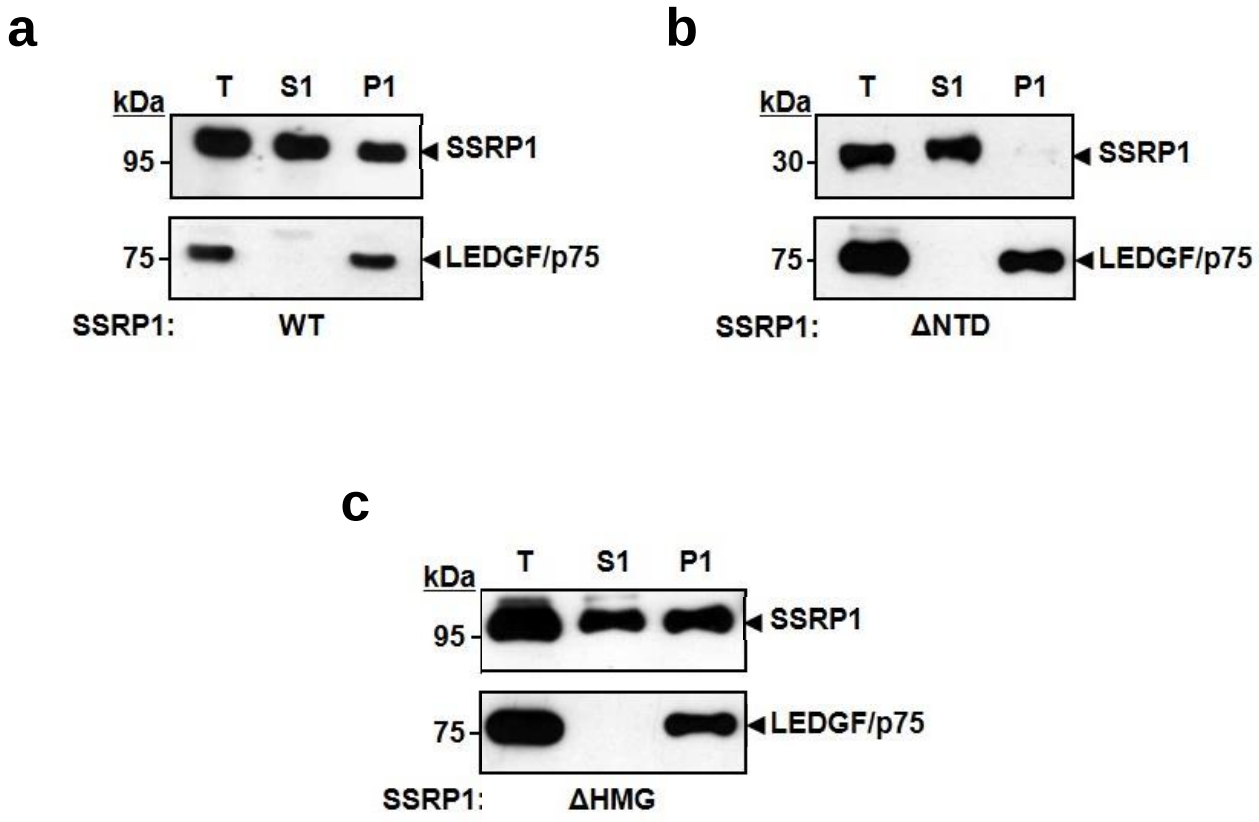


Figure 3.3 Subcellular distribution of SSRP1 mutants

LEDGF/p75-deficient HEK 293T cells were transiently co-transfected with FLAG-tagged LEDGF/p75 wild type (WT) and (a) Myc-tagged SSRP1 WT or mutants lacking the (b) N-terminal (NTD) or the (c) HMG domains. Thirty-six hours after transfection total cell lysates (T), chromatin non-bound (S1), and chromatin bound (P1) fractions were obtained from these cells and analyzed by immunoblot with anti-FLAG or anti-Myc antibodies.

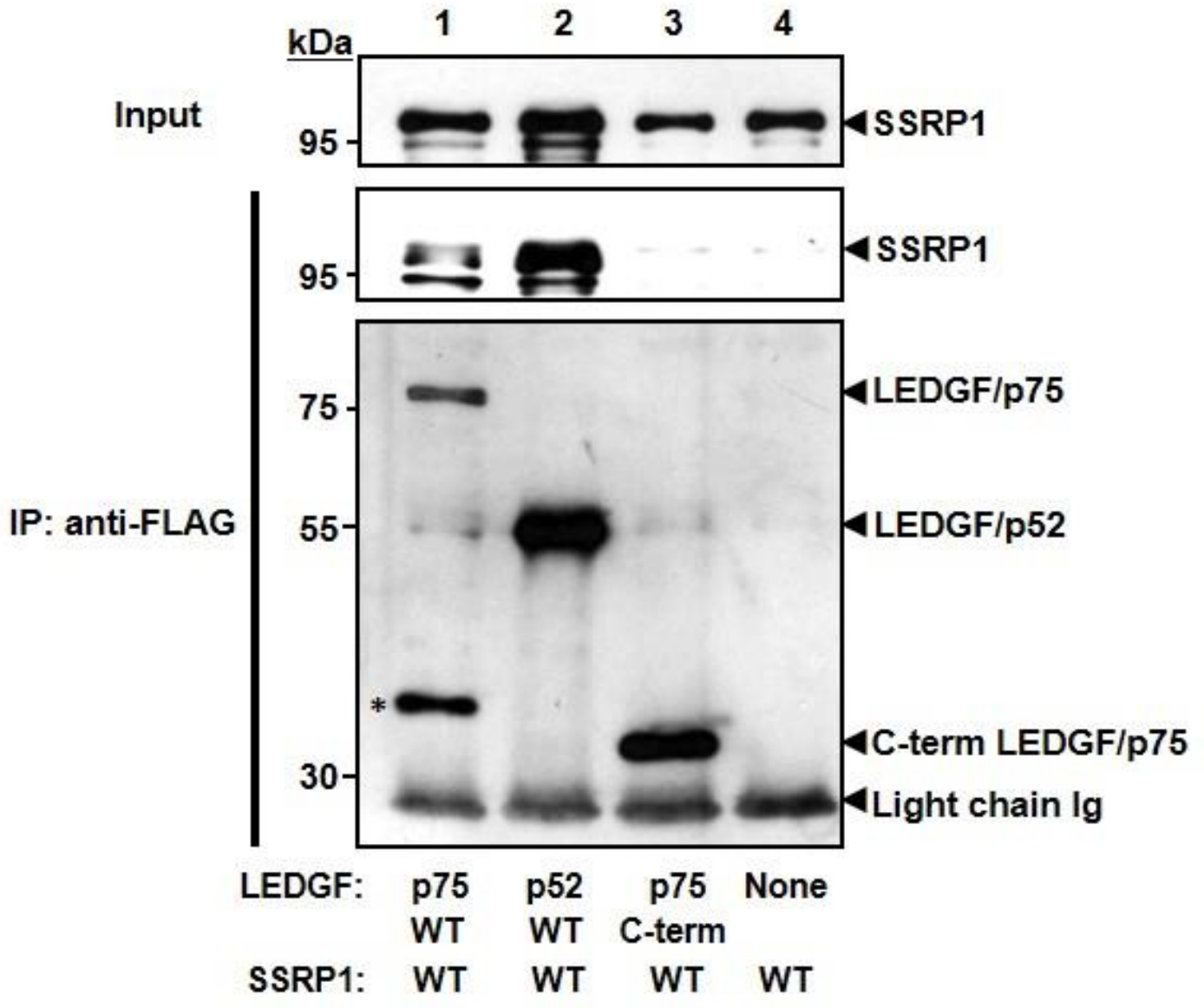
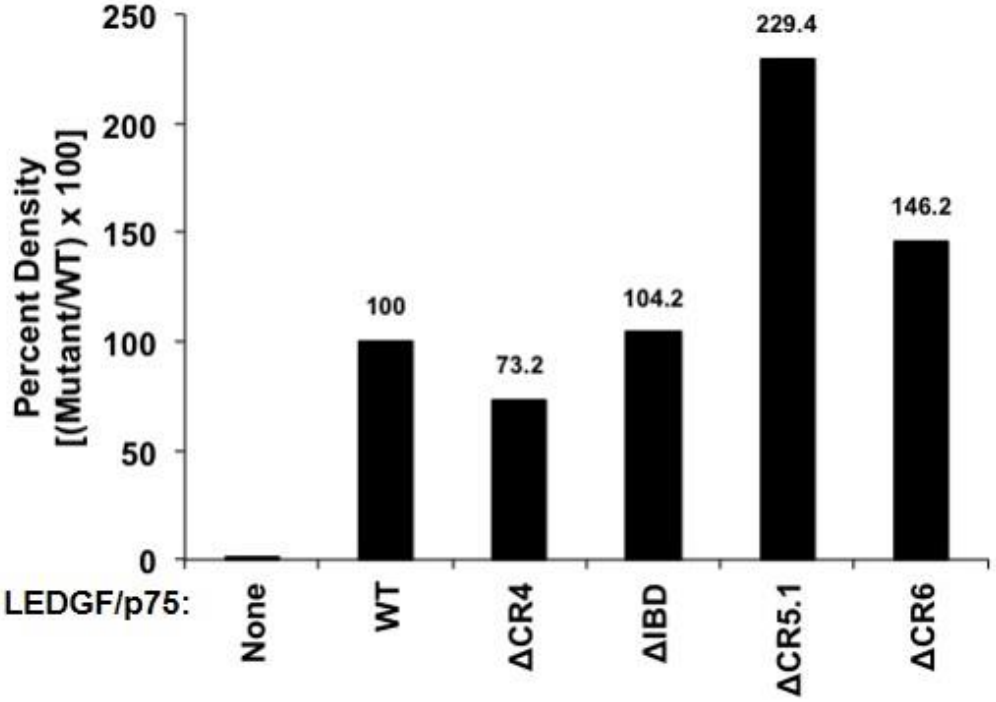


Figure 3.4 Mapping the LEDGF regions implicated in SSRP1 binding

si1340/1428 cells were co-transfected with plasmids encoding Myc-SSRP1 and either LEDGF/p75-FLAG (lane 1), LEDGF/p52-FLAG (lane 2), C-terminal LEDGF/p75-FLAG (lane 3), or an empty expression plasmid (lane 4). Immunoprecipitation analyses were performed as previously described. (*) Marks degradation products of LEDGF/p52-FLAG.

a


Percent Density
[(Mutant/WT) x 100]
250
200
150
100
50
0
100
73.2
104.2
229.4
146.2
LEDGF/p75:
None
WT
ΔCR4
ΔIBD
ΔCR5.1
ΔCR6

b

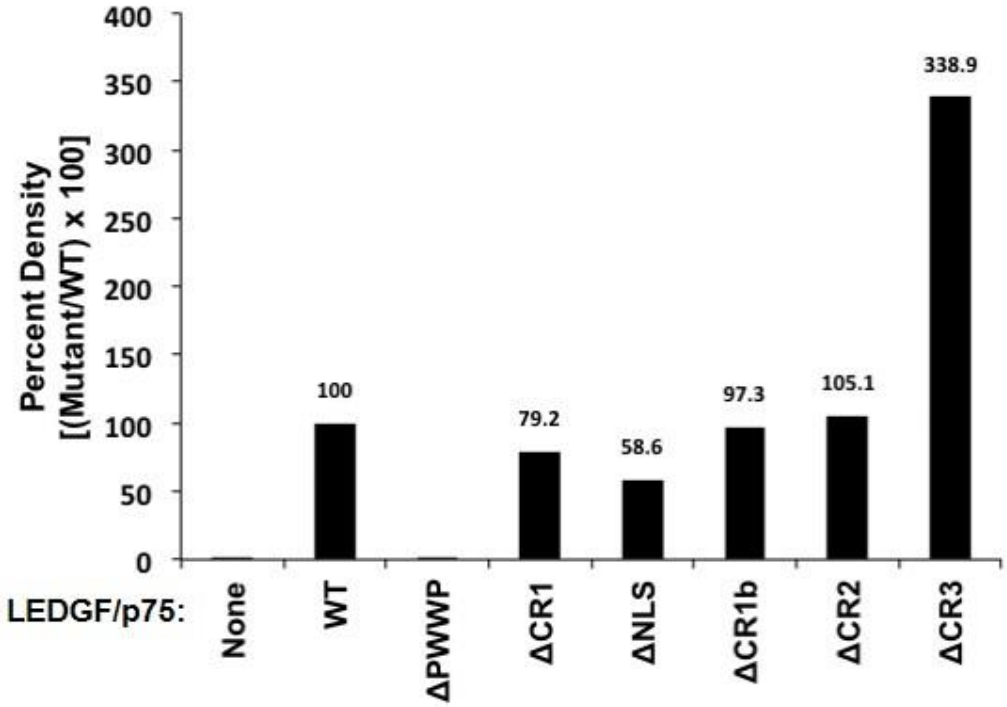

Percent Density
[(Mutant/WT) x 100]
400
350
300
250
200
150
100
50
0
100
79.2
58.6
97.3
105.1
338.9
LEDGF/p75:
None
WT
ΔPWWP
ΔCR1
ΔNLS
ΔCR1b
ΔCR2
ΔCR3

Figure 3.5 Evaluating each region of LEDGF/p75 for its interaction with

SSRP1

Densitometry analysis of the interaction of SSRP1 with LEDGF/p75 wild type (WT) or (a) mutants lacking C-terminus protein regions or (b) mutants lacking the N-terminus protein regions. Bars represent the densitometry quantification of the level of SSRP1 detected in the different immunoprecipitation reactions of representative immunoblots. Immunoprecipitations were performed as described previously using S2 fractions obtained from si1340/1428 cells expressing Myc-SSRP1 WT alone (none) or together with different FLAG-tagged LEDGF/p75 proteins.

3.3.3 The PWWP domain of LEDGF proteins is necessary and sufficient for SSRP1 interaction

We further mapped the SSRP1-binding domain of LEDGF proteins by co-immunoprecipitation experiments, as described above. FLAG-tagged LEDGF/p75 mutants lacking different regions of the N-terminus [95] were co-transfected with Myc-SSRP1 in si1340/1428 cells and the S2 fractions obtained from these cells subjected to immunoprecipitation with an anti-FLAG mAb, the presence of SSRP1 in the immunoprecipitated proteins was then evaluated by immunoblotting with an anti-SSRP1 antibody. Data in (Figure 3.5b) indicate that deletion of CR1b (aa 157-177), CR2 (aa 199-266), or CR3 (aa 267-325) of LEDGF/p75 did not impair its binding to SSRP1. LEDGF/p75 mutants lacking CR1 (aa 94-145) and the nuclear localization signal (aa 146-156) were only partially defective in the interaction but the binding to SSRP1 was unambiguously evidenced. However, the LEDGF/p75 mutant lacking the PWWP domain (aa 1-93) was severely impaired in SSRP1 binding (Figure 3.5b), indicating that this domain is required for this protein interaction.

To demonstrate further that the PWWP domain is the SSRP1-binding domain of LEDGF proteins, we determined by immunoprecipitation experiments the effect of deleting the PWWP on the ability of LEDGF/p52 to interact with SSRP1. The same strategy described above was followed. LEDGF/p52ΔPWWP is mostly distributed to the S1 fraction and the positive control LEDGFp52 to the S2 fraction whereas SSRP1 is distributed to both S1 and S2. Therefore, in order to have similar levels of all of these proteins and an identical buffer composition in the immunoprecipitation input, this experiment was performed with S1 and S2 fractions combined (S1/S2). As expected, in

these experiments (Figure 3.6a) we also observed that the deletion of the PWWP domain completely abrogated the interaction of LEDGF/p52 with SSRP1 demonstrating the importance of this domain in the interaction between LEDGF proteins and SSRP1.

Next, we determined whether the PWWP domain alone was sufficient for SSRP1 binding. For the reasons discussed above, immunoprecipitations in this experiment were performed with S1/S2 fractions obtained from si1340/1428 cells co-expressing Myc-SSRP1 and FLAG-tagged LEDGF/p75 WT or PWWP domain using an anti-FLAG mAb, followed by immunoblotting analysis of the pulled-down proteins with an anti-SSRP1 antibody. Findings in Figure 3.6b clearly demonstrate that the PWWP domain alone firmly interacts with SSRP1, indicating that this domain is sufficient for specific binding to SSRP1.

The PWWP domain is organized into two independent subdomains. The N-terminal region of the PWWP domain is composed of a five-stranded beta-barrel core (aa 1-60) and the C-terminal is comprised of an alpha-helix bundle (aa 61-93). The beta-barrel is solvent exposed and contains a hydrophobic cavity that harbors a tryptophan (aa 21), which is essential for the interaction of PWWP with H3K36me3 [34]. To further characterize the surface of interaction of the PWWP domain, S2 fractions from si1340/1428 cells transiently co-expressing Xpress-SSRP1 and either FLAG-tagged LEDGF/p75 WT, LEDGF/p75ΔPWWPβ-barrel (Δaa1-60), LEDGF/p75ΔPWWPα-helix (Δaa61-93), or LEDGF/p75W21A were immunoprecipitated with an anti-FLAG mAb, and the pulled-down proteins evaluated by immunoblot for the presence of SSRP1 or LEDGF/p75 with specific antibodies. Data in Figure 3.7a-3.7c indicate that none of the mutations introduced into the PWWP domain affected the

interaction between LEDGF/p75 proteins and SSRP1. These results suggest that both sub-domains of PWWP importantly contribute to SSRP1 binding and that interaction of LEDGF/p75 with the nucleosomal protein H3K36me3 is not a pre-condition for SSRP1 interaction.

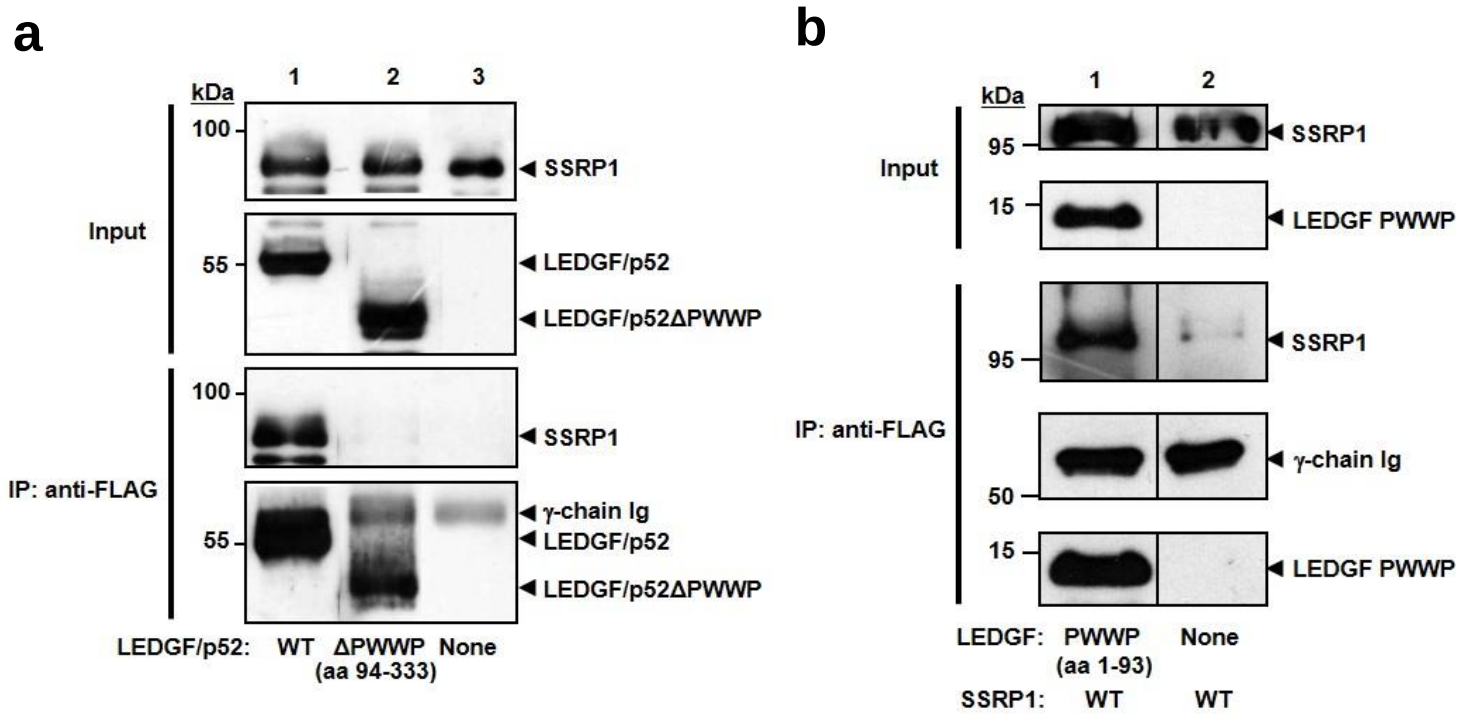


Figure 3.6 Analysis of the PWWP domain on the interaction with SSRP1

(a) Implication of PWWP domain in the interaction of LEDGF/p52 with SSRP1. Cell lysates were obtained from si1340/1428 cells co-transfected with plasmids expressing Myc-SSRP1 and either LEDGF/p52-FLAG (lane 1), LEDGF/p52ΔPWWP-FLAG (lane 2), or an empty plasmid (lane 3). Immunoprecipitations were performed as previously described. (b) si1340/1428 cells were co-transfected with plasmids expressing Myc-SSRP1 and PWWP-FLAG (lane 1) or an empty plasmid (lane 2). Immunoprecipitations were performed as previously described. Results in (b) are representative of two independent experiments. The line separating lanes 1 and 2 indicate that lanes of the gel in between contained irrelevant samples that were removed. In 3.6a, (*) indicates degradation products of SSRP1.

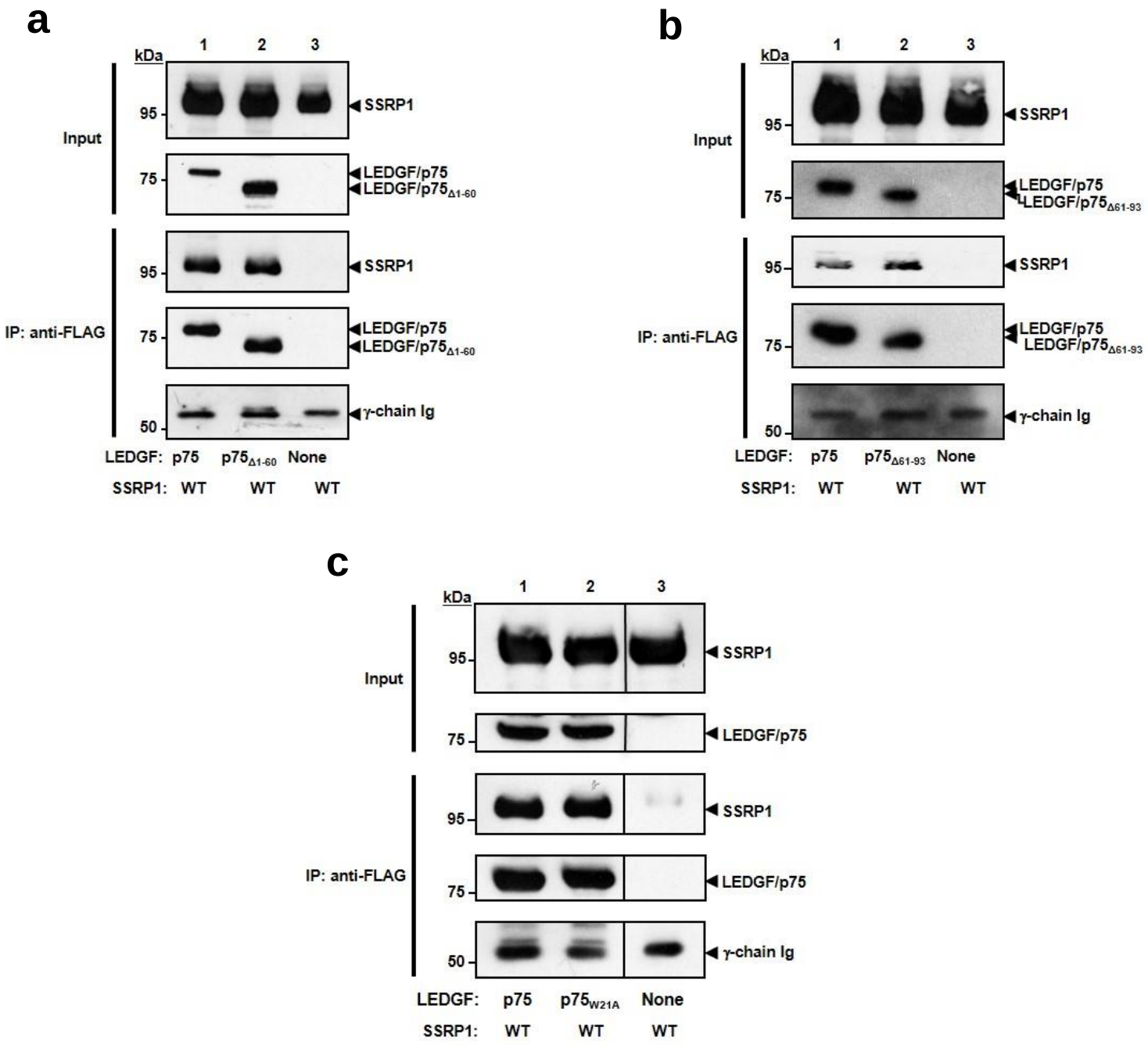


Figure 3.7 Defining the role of the PWWP subdomains in the SSRP1 interaction

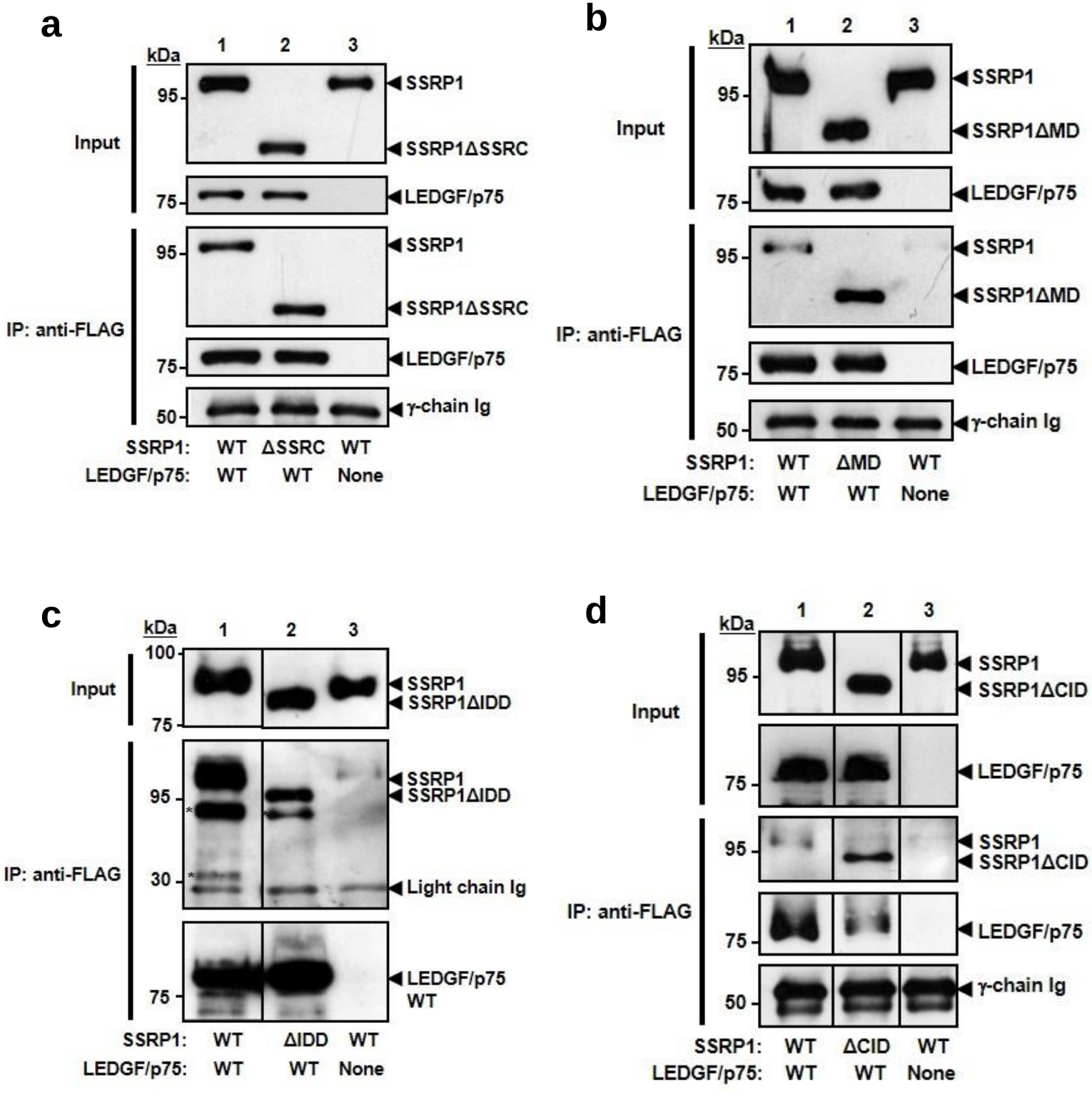
(a-c) Analysis of the binding of LEDGF/p75 PWWP mutants to SSRP1 by immunoprecipitation. si1340/1428 cells were co-transfected with plasmids expressing Myc-SSRP1 WT and FLAG-tagged LEDGF/p75 WT (lanes 1) or PWWP mutants (lanes 2), or an empty plasmid (lanes 3). In these experiments the following mutants were analyzed: (a) LEDGF/p75ΔPWWPβ-barrel, (b) FLAG-LEDGF/p75ΔPWWPα-helix, and (c) LEDGF/p75W21A. Immunoprecipitations were performed as described previously.

3.3.4 The HMG domain of SSRP1 is required but not sufficient for the interaction with LEDGF/p75

We next defined the LEDGF-binding domain of SSRP1. Two structural domains, a structure-specific recognition (SSRC) motif (aa 187-437) and an HMG-box domain (aa 548-613), have been previously described in SSRP1, in addition to four evolutionarily conserved regions designated N- terminal domain (NTD, aa 1-186), middle domain (MD, aa 438-514), intrinsically disordered domain (IDD, 515-547), and C-terminal domain (CID, aa 614-709) [91]. A panel of Myc-tagged SSRP1 mutants lacking each of these domains and the WT protein were co-expressed with FLAG-tagged LEDGF/p75 in HEK293T cells and S2 fractions obtained were subjected to immunoprecipitation with an anti-FLAG antibody. The presence of SSRP1 proteins in the immunoprecipitated fraction was then determined by immunoblot analysis with an anti-Myc antibody. Data in Figure 3.8 show that the interaction between LEDGF/p75 and the SSRP1 mutants lacking the SSRC (Figure 3.8a), MD (Figure 3.8b), IDD (Figure 3.8c), and CID (Figure 3.8d) domains were not impaired. Similarly, we showed that NTD is also dispensable for LEDGF/p75 binding (Figure 3.2). Conversely, the interaction with the mutant lacking the HMG domain was severely defective (Figure 3.9a), suggesting the implication of this SSRP1 region in the LEDGF/p75 binding. The lack of HMG domain did not alter the distribution of SSRP1 in the chromatin bound or unbound fractions (Figure 3.3c), indicating that the defective interaction was not due to a change in the subcellular distribution of the SSRP1 mutant protein.

In order to clarify further the role of HMG SSRP1 in LEDGF/p75 binding, we determined whether this domain is sufficient for the interaction of these proteins. As

described above, HEK293T cells were transiently co-transfected with FLAG-tagged LEDGF/p75 WT and Myc-tagged SSRP1 HMG domain expression plasmids. Their interaction was evaluated by immunoprecipitation from S1/S2 fractions with an anti-FLAG antibody followed by anti-Myc immunoblot analysis. Results in Figure 3.9b revealed that the HMG domain alone failed to bind to LEDGF/p75, indicating that, although required, this domain is not sufficient for binding. These findings suggest that the surface of interaction of SSRP1 with LEDGF/p75 is complex and other regions of SSRP1 may contribute to the binding.


a
kDa
1
2
3
Input
95
SSRP1
SSRP1ΔSSRC
75
LEDGF/p75
IP: anti-FLAG
95
SSRP1
SSRP1ΔSSRC
75
LEDGF/p75
50
γ-chain Ig
SSRP1: WT ΔSSRC WT
LEDGF/p75: WT WT None
b
kDa
1
2
3
Input
95
SSRP1
SSRP1ΔMD
75
LEDGF/p75
IP: anti-FLAG
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SSRP1ΔMD
75
LEDGF/p75
50
γ-chain Ig
SSRP1: WT ΔMD WT
LEDGF/p75: WT WT None
c
kDa
1
2
3
Input
100
75
SSRP1
SSRP1ΔIDD
IP: anti-FLAG
95
30
SSRP1
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75
LEDGF/p75
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d
kDa
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95
SSRP1
SSRP1ΔCID
75
LEDGF/p75
50
γ-chain Ig
SSRP1: WT ΔCID WT
LEDGF/p75: WT WT None

Figure 3.8 Analyzing the function of SSRP1 regions in the interaction with LEDGF/p75

HEK293T cells were co-transfected as described in figure legend 3. The Myc-tagged SSRP1 mutants analyzed were: (a) SSRP1ΔSSRC, (b) SSRP1ΔMD, (c) SSRP1ΔIDD, and (d) SSRP1ΔCID. S2 fractions were obtained from transfected cells and subjected to immunoprecipitation as described above.

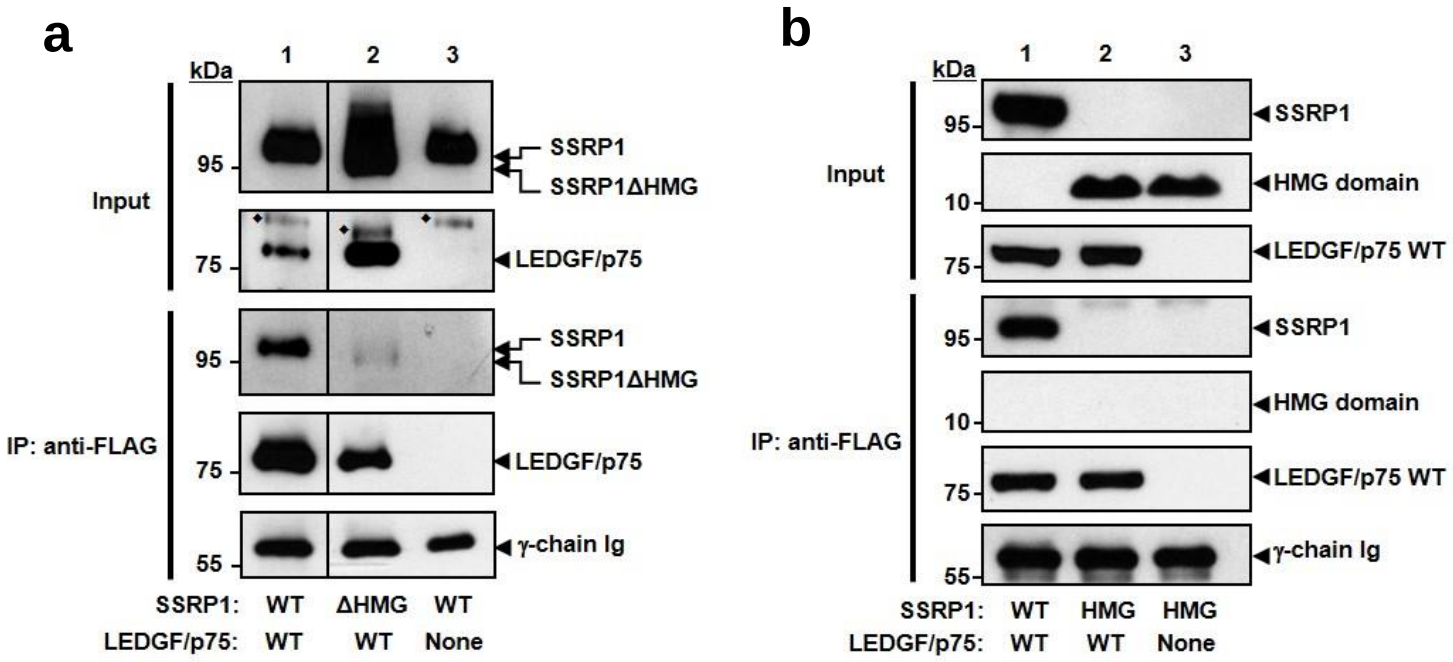

Figure 3.9 Immunoprecipitation analyses of the interaction of SSRP1 mutants with LEDGF/p75

(a) HEK293T cells were co-transfected with plasmids expressing: FLAG-tagged LEDGF/p75 and Myc-tagged SSRP1 WT (lane 1), FLAG-tagged LEDGF/p75 and Myc-tagged SSRP1ΔHMG (lane 2), or Myc-tagged SSRP1 WT and an empty plasmid (lane 3). Results in (a) are representative of three independent experiments. The vertical lines separating lanes 1 and 2 indicate that samples were in the same gel but not in adjacent positions. (*) Denotes residual SSRP1 resulting from partial antibody stripping. (b) HEK293T cells were co-transfected with plasmids encoding LEDGF/p75-FLAG and SSRP1 WT-Myc (lane 1) or HMG-Myc (lane 2). Lane 3 was co-transfected with SSRP1 WT-Myc and an empty plasmid. S1 and S2 fractions were obtained from the transfected cells and mixed. Mixed fractions were immunoprecipitated. Results are representative of two independent experiments.

CHAPTER 4: Discussion

A role of LEDGF proteins in transcriptional regulation at promoter regions has been described [25,28], yet the participation of LEDGF/p75 in other steps of transcriptional regulation has not been evaluated. A genome-wide study indicates that LEDGF/p75 is enriched downstream of the transcription start sites of actively transcribed genes [36] suggesting a potential role of this regulator in transcriptional elongation. However, the mechanism associated with this phenomenon remains unknown. The data presented in this study demonstrate for the first time that LEDGF/p75 associates with the FACT complex, suggesting a role for LEDGF/p75 in transcriptional elongation. We demonstrated that LEDGF/p75 specifically co-immunoprecipitates with the chromatin-bound molecular form of the FACT complex that is likely engaged in transcriptional activity. These interactions occur *in vivo* in a DNA- and nucleosome-independent fashion. These findings correlate with the global effects of LEDGF/p75 on transcription [60] and with the high degree of co-localization of LEDGF/p75 with SSRP1 under basal conditions. Our findings also suggest that the location of LEDGF/p75 in transcriptional elongation complexes could determine its role in targeting HIV-1 into actively transcribed genes [59].

In this study we also characterized in detail the interaction of LEDGF/p75 with the FACT complex. The FACT complex is a histone chaperone comprised of a heterodimer of hSpt16 and SSRP1 proteins. It has been found to be essential in DNA replication, transcriptional elongation, and DNA damage repair [63,66–75,91] and the targeted disruption of one of its components, SSRP1, is embryonic lethal [76]. In any function, FACT works through nucleosome reorganization by disrupting core histone-histone and histone-DNA interactions [63,66–75,91]. Specifically, the FACT complex attaches to the

nucleosome through the binding of the hSpt16 subunit to the H2A-H2B dimer, facilitating its displacement and allowing SSRP1 to interact with an H3-H4 tetramer and nucleosomal DNA. After destabilization, the FACT complex has been shown to reassemble the nucleosome [67].

We demonstrated that LEDGF proteins bind directly to SSRP1 independently of hSpt16, chromatin, or SSRP1 post-translational modifications. Through mapping studies of the protein domains implicated in the LEDGF/SSRP1 binding we demonstrated, for the first time, the interaction between HMG and PWWP domains. Intriguingly, the fragment of TOX4 that interacts with PWWP LEDGF/p75 contains an HMG domain, however, data demonstrating the interaction of TOX4 HMG with the LEDGF/p75 PWWP domain are missing [99]. PWWP and HMG domains are present in multiple proteins involved in transcriptional regulation, DNA repair, and epigenetics [32,84,100–103]. PWWP is a nucleosomal-binding domain that interacts specifically with H3K36me3 and H4K20me histone codes and non-specifically with DNA, whereas HMG domain binds to DNA and several proteins implicated in transcriptional regulation. HMG binds to the minor groove of DNA inducing local changes in the DNA structure that triggers different DNA-dependent functions [84,85]. In addition, the HMG domain of Sox proteins have been demonstrated to bind to different protein domains present in transcription factors, including homeodomain, paired domain, POU domains, zinc finger, basic helix-loop-helix and leucine zipper. These HMG-interacting domains mediate the binding of transcription factors to DNA, modulating their transcriptional activity [104–107]. Therefore, our findings expand the diversity of protein domains that interact with HMG domains.

Similar to the interaction of HMG Sox with other protein domains [106], the interaction of HMG SSRP1 with LEDGF/p75 seems to be strengthened by other SSRP1 protein regions since HMG is required but not sufficient for LEDGF/p75 binding. In contrast, the PWWP domain is required and sufficient for LEDGF binding to SSRP1. A potential mechanism for this phenomenon is the direct binding of other regions of SSRP1 to PWWP LEDGF/p75. Alternatively, other protein regions in SSRP1 could stabilize a particular conformation of HMG required for PWWP association. In support of the latter possibility, it has been described the binding of HMG in SSRP1 of *Drosophila melanogaster* to nucleosomal DNA is modulated by intramolecular interactions between the Middle (aa 438-514), the Intrinsically Disordered (aa 515-547), and the HMG (aa 548-613) domains of SSRP1 [108]. Therefore, it is possible that a similar intramolecular crosstalk between HMG and other regions of SSRP1 could influence the binding to PWWP in LEDGF. The contribution of other SSRP1 regions to the interaction of HMG with PWWP domain also suggests that the binding of these two protein domains will not be universal but protein-specific. Further studies of the binding of other HMG and PWWP domains could potentially provide an insight into the functional implications of these interactions.

A few PWWP domain interactors are known [109]. Different PWWP domains have been found to bind to methylated histones, in particular H3K36me3 and H4K20me3 [109–115], and the PWWP in DNA (cytosine-5-)-methyltransferase 3 alpha and beta have been demonstrated to bind to a region of SALL3 that contains a zinc finger domain [116] and to SUMO-1 [117], respectively. Despite the relevance of the PWWP domain in the cellular and virological roles of LEDGF/p75 [28,41,42], no direct

interactors, other than H3K36me3, have been previously demonstrated [109]. Therefore, SSRP1 is the first known non-histone protein to interact with LEDGF PWWP domain in a physiological setting. Other proteins, such as TOX4 and NOVA1, have been proposed to bind to PWWP in LEDGF/p75; however, these interactions were not demonstrated to occur with endogenous proteins and were found to require the presence of DNA or a polynucleosome, suggesting that they may be indirect, requiring DNA as a mediator [99]. Importantly, LEDGF/p75 is also the first protein reported to bind to the HMG domain of SSRP1.

The association of LEDGF/p75 with SSRP1 is predicted to have important functional implications. LEDGF/p75 has a role in the repair of DNA double-strand breaks by the homologous recombination pathway [49] as well as in transcriptional regulation [28], processes that requires the chromatin-remodeling function of the FACT complex. Therefore, the interaction of LEDGF/p75 and SSRP1 could allow the simultaneous recruitment of both proteins to areas of the chromatin engaged in DNA repair or active transcription. Similarly, it is expected that this protein interaction could affect the HIV-1 co-factor activity of LEDGF/p75 [28,41,42]. In support of the latter hypothesis, we have observed a modest, reproducible, and specific defect in HIV-1 infection in cells partially knocked down for SSRP1. This effect seems to be LEDGF/p75 dependent since infection by the gamma retrovirus MLV, a LEDGF/p75-independent retrovirus [52], was not affected by SSRP1 deficiency.

LEDGF/p75 acts as a molecular tether that forms a linkage between HIV-1 integrase and chromatin, favoring viral integration into actively transcribed genes where LEDGF/p75 is enriched [19,28,41,42,60,61]. In addition to this chromatin-tethering

mechanism, a role for regions of LEDGF/p75 or interacting proteins not involved in chromatin or integrase binding have been reported to influence its HIV-1 cofactor activity [95,99]. Our data add further support to the existence of these chromatin-tethering independent mechanisms in the role of LEDGF/p75 in HIV-1 replication. Even at cellular levels of SSRP1 lower than those that affect HIV-1 infection, the chromatin binding strength of LEDGF/p75 was not impaired, suggesting that SSRP1 deficiency does not affect HIV-1 infection by altering the LEDGF/p75 chromatin-tethering function. In addition, SSRP1 deficiency affected the expression of proviral transgenes transcribed from the HIV-1 LTR but not from an internal immediate-early CMV promoter, suggesting that LTR activity rather than integration was the target. In further support of this mechanism LEDGF/p75-deficiency in human $CD4^+$ T cells more severely impaired infection by LTR- than by CMV promoter-driven HIV-1.

Comparison of their relative contribution indicates that the chromatin-tethering dependent mechanisms are more relevant than the independent ones in the HIV-1 cofactor activity of LEDGF/p75. Mutations affecting the chromatin-tethering activity of LEDGF/p75 more severely affect its HIV-1 cofactor activity [52,55] than interventions that affect the independent mechanisms, such as overexpression of the LEDGF/p75-interactors TOX4 and NOVA1 [99], mutation of specific residues not implicated in the binding of LEDGF/p75 to chromatin [95], or knockdown of SSRP1 (Figures 2.1-2.5). Alternatively, the modest effect of SSRP1 deficiency on HIV-1 replication observed could be due to the high levels of residual SSRP1 in the knockdown cells as detected by immunoblot analyses. It was previously demonstrated that minute amounts of LEDGF/p75 are sufficient to support wild type HIV-1 infection [52,57]. Unfortunately, the

essential role of SSRP1 and, more specifically, of the SSRP1 LEDGF/p75-interacting domain, HMG, preclude analyses of the susceptibility to HIV-1 infection of cells exhibiting significantly low levels of SSRP1 or only expressing SSRP1 mutants unable to bind to LEDGF/p75 [76,118].

Our data in correspondence with previous observations [78] indicate a role of SSRP1 in HIV-1 LTR-driven expression. It has been demonstrated that TNF-α-induced activation of HIV-1 LTR-mediated transcription increased FACT occupancy at nucleosome 1 of the LTR and nucleosomes in the Env region, and removal of H2A/H2B dimers from these locations [78]. This role of SSRP1 is expected considering the reported function of this protein in chromatin remodeling [63,67,91] and the fact that HIV-1 proviral gene expression is subjected to the same mechanisms governing transcriptional regulation of host genes [119–121]. In addition, the FACT subunit hSpt16 has been reported to interact with Tat [82,83,122]. In apparent contradiction with our findings, down regulation of SSRP1 and/or hSpt16 has been reported to modestly increase HIV-1 expression [83,122]. The inhibitory role of hSpt16 on HIV-1 expression could illustrate FACT-independent hSpt16 functions previously described [87]. Alternatively, down regulation of FACT levels in the cell could globally alter the chromatin structure, leading to transcriptional initiation from cryptic initiation sites within the coding region of genes, including HIV-1, as previously reported in yeast [70,123].

Finally, our data suggest a model that expands the role of LEDGF/p75 in HIV-1 replication beyond viral DNA integration [52,55]. We envision that LEDGF/p75 recruits the FACT complex to the integrated provirus at a Tat-independent stage of the life cycle promoting HIV-1 gene expression and replication.

Furthermore, our findings may provide the foundation for the exploration of another venue with which to treat HIV. We have identified, in this work, a cellular protein that plays a role in HIV-1 infection possibly through its interaction with an already known HIV-1 cellular cofactor. If further experiments, namely ones listed in future directions, show that the complete disruption of the interaction between these two proteins leads to a greater decrease in HIV-1 infection, this could precede the development of a new therapeutic for HIV-1. As previously mentioned, HIV-1 has the ability to evolve resistance to current antiretroviral treatments. These drugs mainly target viral proteins and because the fidelity of HIV-1 reverse transcriptase is so poor the virus is eventually able to escape the effect of these drugs. Hence, a bigger effort should be made to focus on finding a therapeutic target amongst cellular proteins rather than viral ones. With the findings obtained through this work, as well as through further characterization of the HIV-1 effect seen by the depletion of SSRP1, we may in the future be able to develop small molecules that can engage the binding site between LEDGF/p75 and SSRP1 and abrogate the interaction between the two proteins. Moreover, if we can see the same or greater effect on HIV-1 infection in cells treated with these new molecules as we see with the deletion of SSRP1, we could theoretically begin to develop a therapeutic drug for HIV from these molecules.

CHAPTER 5: Future Directions

5.1 Introduction

The results obtained in this work indicate a role for SSRP1 in HIV-1 infection given that it modestly and specifically reduces HIV-1 infection when it is partially knockdown. Furthermore, experimental evidence suggests that this role of SSRP1 in HIV-1 infection is LEDGF/p75 dependent. Detailed exploration of this phenotype, however, is at the moment difficult to pursue due to the essentiality of SSRP1 for cell viability. From the results obtained in Chapter 3 we were hoping to be able to identify domains and/or regions of each protein that were essential for the interaction between the two proteins yet did not contribute to the overall function of the protein. However, this was not the case. The results from Chapter 3 indicate that the PWWP domain is the domain that is essential and also sufficient to mediate the interaction between LEDGF/p75 and SSRP1 and that for the SSRP1 protein, the HMG domain was the one found to be necessary but not sufficient for this interaction. Due to the importance of the HMG domain in the function of SSRP1, we cannot express a mutant lacking this domain in cells and evaluate the effect on HIV-1 infection without affecting the protein's normal function. Additionally, the PWWP domain is important for LEDGF/p75 cofactor activity in HIV-1 infection and its removal would impair HIV-1 infection independently of its interaction with SSRP1. Therefore, new strategies for future work on this investigation should be explored.

5.2 Proposed Work

5.2.1 Swapping of the hSSRP1 HMG domain with Drosophila HMG domain to maintain protein functionality but lose interaction with LEDGF/p75

To disrupt the interaction between LEDGF/p75 and SSRP1, without affecting the function of either protein, the HMG domain of SSRP1 could be replaced with a similar, yet LEDGF/p75 non-interacting, HMG domain from another protein homolog. In this way, the new HMG domain will expectantly render SSRP1 a functional protein in the cell, hopefully avoiding any cell viability problems, while simultaneously interrupting the interaction between the two proteins. This new SSRP1 protein could then be expressed stably in a cell line and the endogenous SSRP1 protein subsequently knocked down using a CRISPR/Cas9 system. CRISPR/CAS9 is a recently developed tool that can knockdown a protein efficiently and effectively. These knockdown cells would then be infected with HIV-1 and evaluated.

A conserved HMG domain-containing SSRP1 has been identified for various mammals, ranging from mouse to chicken, plants, and *Drosophila melanogaster.* The family of HMG-containing proteins can be classified into three distinct groups: HMG-I, HMG 14/17, and HMG -1/2 [124]. The SSRP1 family forms a separate subgroup within the HMG -1/2 class, which is divided into two subfamilies according to the number of HMG domains present in the protein [124,125]. The HMG domain of the human SSRP1 proteins enables SSRP1 to interact with DNA and mediates various protein interactions. SSRP1 was initially identified by its specific binding to V-(D)-J recombination signals [126] and cisplatin-modified DNA [127]. Most studies report that SSRP1 HMG binds to non-sequence specifically but recognizes certain DNA structures [127–129], however,

there are contrasting studies that show that SSRP1 binds to DNA with a modest affinity but with clear sequence specificity [126,130]. In particular, *Drosophila* SSRP1, similarly to its human counterpart, binds to cisplatin-damaged DNA, an HMG-mediated function, with a high affinity [131]. However, the degree of functional conservation existent between SSRP1 HMG domains from different species is still unclear.

I have already begun working on this strategy. A comparison between human and *Drosophila* SSRP1 using NCBI BLAST showed 48% identity conservation for the entire protein (not shown) and only 42% at the HMG domain (Figure 5.1a), suggesting the possibility that Drosophila HMG will not mediate LEDGF/p75 binding. We have swapped the HMG domain in human SSRP1 with the homologous domain from *Drosophila* SSRP1. Using this construct we demonstrated by LEDGF/p75 co-immunoprecipitation that the chimeric protein does not interact with LEDGF/p75. Briefly, HEK293T cells were plated at 0.45 X 10^6 cells per well in a six-well plate and transfected with 2 µg of DNA of the corresponding plasmids, FLAG-tagged LEDGF/p75 and myc-tagged SSRP1 with *Drosophila* HMG domain. Transfection medium was replaced 18 hours later with fresh culture medium and cells were cultured for an additional 18 hours until they were harvested and analyzed by Western Blot. Interestingly, data in Figure 5.1b show that the replacement of the human HMG domain of SSRP1 with the *Drosophila* HMG domain abrogated the interaction between LEDGF/p75 and SSRP1.

In order to efficiently express this chimeric protein in relevant HIV-1 target cells, I cloned this cDNA into an HIV-derived expression system and presently I am generating stable cell lines in SupT1 cells expressing this protein.

Currently, our lab is working on cloning the U6 promoter and a CRISPR sequence specific for SSRP1 that I generated into a lentiviral vector. In parallel, our lab is also cloning the CAS9 protein into a separate lentiviral vector for efficiently expression. In the future, these two vectors will be used to transduce SupT1 cells expressing the chimeric swapped SSRP1 protein to knockout endogenous SSRP1. Once transduced, human SSRP1 knockout cells expressing the chimeric SSRP1 protein will be isolated by single cell cloning. The generation of this cell line with the chimeric protein might not supply the essential functions of SSRP1 wild type and affect the viability of these cells, however, if successful, the lab will perform infection experiments on this newly generated cell line.

The results obtained by performing these experiments could bring to light a new role for LEDGF/p75 in HIV-1 infection. The data presented in this work indicate that knockdown of SSRP1 affects LTR dependent HIV-1 expression and that SSRP1 interacts with LEDGF/p75. If these proposed experiments were to find that there is a relationship between the interaction of LEDGF/p75 with SSRP1 and HIV-1 infection then this could suggest that LEDGF/p75 might have a role downstream of integration.

a

Sequence ID: lcl|Query_40799 Length: 66 Number of Matches: 1

Range 1: 1 to 66 Graphics ▼ Next Match ▲ Previous M

Score	Expect	Method	Identities	Positives	Gaps
58.9 bits(141)	5e-18	Compositional matrix adjust.	28/66(42%)	45/66(68%)	2/66(3%)

```
Query  9   PKRATTAFMLWLNDTRESIKRENPGIKVTEIAKKGGEMWKEL--KDKSKWEDAAAKDKQR  66
           PKR  +A+MLWLN +RE IK ++PGI +T+++KK GE+WK +  + K +W+  A   ++
Sbjct  1   PKRPMSAYMLWLNASREKIKSDHPGISITDLSKKAGEIWKGMSKEKKEEWDRKAEDARRD  60

Query  67  YHDEMR  72
           Y   M+
Sbjct  61  YEKAMK  66
```

b

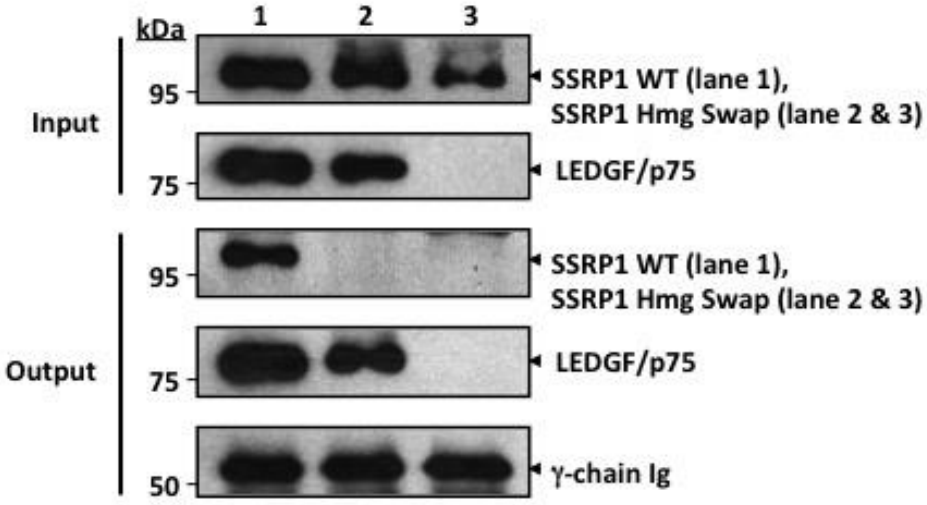

Figure 5.1 Analysis of *Drosophila melanogaster* SSRP1 HMG domain

(a) NCBI BLAST amino acid sequence alignment between the HMG domain of *Drosophila melanogaster* (Query) and the HMG domain of human SSRP1 (Sbjct). (b) HEK293T cells were co-transfected with plasmids expressing: FLAG-tagged LEDGF/p75 and Myc-tagged SSRP1 WT (lane 1), FLAG-tagged LEDGF/p75 and Myc-tagged SSRP1 with Dm HMG (lane 2), or Myc-tagged SSRP1 with Dm HMG and an empty plasmid (lane 3). Immunopreciptiations were performed and samples analyzed by Western Blot. Results in (b) are representative of two independent experiments.

5.2.2 Minimal mutagenesis of the SSRP1 HMG domain to interrupt the interaction with LEDGF/p75

Preliminary data indicate that the strategy described above might have the potential to definitively answer the question of whether the interaction between LEDGF/p75 and SSRP1 is important for the effect seen in SSRP1 partial knockdown cells. However, there is still the possibility that once the cell line is generated the cells are still not viable and therefore cannot be used in infection experiments. If this were the case, another strategy that can be pursued would be to mutate specific amino acids in the HMG domain that are not shared between the human SSRP1 protein and the *Drosophila* homolog. Since we already know that the swapping of these domains disrupts the interaction between LEDGF/p75 and SSRP1, this shortens the list of amino acids within that domain that might be important for the interaction. Using the BLAST analysis that I performed (Figure 5.1b), we can focus on mutating the amino acids that are not conserved between the human and the Drosophila domains and that are exposed on the surface of the HMG domain, accordingly with the reported structure for this domain [132]. The effect of these mutations on the interaction of SSRP1 with LEDGF/p75 could be evaluated by co-immunoprecipitations and minimal mutants disrupting the interaction could be expressed in SupT1 cell lines following the strategy described above.

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Abbreviations

AIDS – Acquired immune deficiency syndrome

ATP – Adenosine triphosphate

BLAST – Basic local alignment search tool

CA – Capsid

Cas9 – CRISPR associated protein 9

CCR5 – C-C chemokine receptor type 5

$CD4^+$ - Cluster of differentiation, member 4 activated

cDNA – Complementary deoxyribonucleic acid

ChIP – Chromatin immunoprecipitation

CMV – Cytomegalovirus

CRISPR – Clustered regularly-interspaced short palindromic repeats

CTD – C-terminal region

CtIP – C-terminal binding protein interacting protein

CXCR4 – C-X-C chemokine receptor type 4

DD – Dimerization domain

Dm – *Drosophila melanogaster*

DNA – Deoxyribonucleic acid

eGFP – Enhanced green fluorescent protein

ELISA – Enzyme-linked immunosorbent assay

FACT – Facilitates chromatin transcription

FIV – Feline immunodeficiency virus

H3K36me3 – Tri-methylated histone H3 at the lysine in position 36

H4K20me – Methylated histon H4 at the lysine in position 20

H4K20me3 – Tri-methylated histone H4 at the lysine in position 20

HAART – Highly active antiretroviral therapy

HDGF – Hepatoma-derived growth factor

HEK293T – Human embryonic kidney cells 293 T

HIV-1 – Human immunodeficiency virus type 1

HMG – High-mobility group

HR – Homologous recombination

hSpt16 – Suppressor of Ty 16

IBD – Integrase binding domain

IDD – Intrinsically disordered domain

IN – Integrase

IP – Immunoprecipitation

Kb – Kilobases

kDa – Kilodaltons

LEDGF/p75 – Lens epithelium derived growth factor/p75

LTR – Long terminal repeat

MA – Matrix

mAb – Monoclonal antibody

MD – Middle domain

MLV – Murine leukemia virus

MOI – Multiplicity of infection

mRNA – Messenger RNA

NC – Nucleocapsid

NCBI – National Center for Biotechnology Information

NLS – Nuclear localization signal

NTD – N-terminal domain

NOVA1 – Neuro-oncological ventral antigen 1

PBS – Primer binding site

PC4 – Positive cofactor 4

PCR – Polymerase chain reaction

PIC – Pre-integration complex

Pol II – RNA polymerase II

PR – Protease

PSIPI – PC4- and SFRS-interacting protein 1

PWWP – Proline-tryptophan-tryptophan-proline

RNA – Ribonucleic acid

RT – Reverse transcriptase

RT PCR – Real-time polymerase chain reaction

SALL3 – Sal-like protein 3

SDS-PAGE – Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis

shRNA – Small hairpin RNA

SSRC – Structure-specific recognition motif

SSRP1 – Structure specific recognition protein 1

SUMO-1 – Small ubiquitin-like modifier 1

TAT – Trans-activator of transcription

TAR – Trans-activation-responsive

TBP – TATA binding protein

TBS – Tris-buffered saline

TOX4 – TOX high mobility group box family member 4

tRNA – Transfer RNA

VSV-G – Vesicular stomatitis virus glycoprotein G

Vita

Angelica Patricia Lopez was born in El Paso, Texas on November 1, 1988 to parents Patricia D. Lopez and Frank Lopez. She earned her Bachelor of Arts degree in Biology with a concentration in Microbiology from Cornell University in the spring of 2010. In the fall of that same year she joined the Master's program at The University of Texas at El Paso in the Department of Biological Sciences. Two years later, in 2012, she transferred into the doctoral program. In her first and a half years in the graduate program, Angelica worked as a teaching assistant for the Molecular Cell Biology and General Biology laboratories.

While pursuing her degree, Angelica was awarded the Bridge to the Doctorate Fellowship in the spring of 2012. Later, in the fall of 2012 she was admitted into the Research Initiatives for Scientific Enhancement (RISE) Graduate Scholars Program to fund her studies. In the summer of 2015, she participated in the Department of Homeland Security Summer Internship Program and interned at the U.S. Army Engineer Research and Development Center - Construction Engineering Research Laboratory. In her last year of her doctorate, she was awarded the Dodson research grant to help her fund the completion of her dissertation. Before graduating, Angelica submitted a manuscript to the Journal of Molecular Biology entitled, "Association of the Structure Specific Recognition Protein 1 with the Lens Epithelium-Derived Growth Factor/p75 modulates HIV-1 replication".

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