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INVESTIGATING THE ROLE OF PARP-1 IN HIV-1 REPLICATION

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ABSTRACT

Poly (ADP-ribose) Polymerase 1 (PARP-1) is a cellular protein that that has multiple roles in the cell including DNA repair and transcription. PARP-1 has also proven by our laboratory to impair HIV-1 replication in T cells infected with HIV-1 strains entering via CD4/CXCR4. **The goal of my thesis is to increase our understanding of the mechanism implicated in the anti-HIV-1 activity of PARP-1.** Initially, I focused my research in deciphering the impact of PARP-1 on the replication of HIV-1 strains entering the cell through the alternative pathway CD4/CCR5. However, technical difficulties prevented answering this question. Nevertheless, aided by the technical training gained in the first project I investigated the implication of PARP-1 chromatin binding activity and subcellular distribution in its anti-HIV-1 activity. These studies were additionally relevant because the mechanism of chromatin binding of PARP-1 in cells is ill-defined, despite of the central role of this function in all PARP-1 cellular roles. Using cell fractionation and immunochemistry techniques I found that PARP-1 is tightly bound to chromatin under basal conditions. This binding required the N-terminal region of PARP-1 that was also sufficient. Importantly, the N- but not the C-terminus exhibited anti-HIV-1 activity, establishing correlation between anti-viral effects and chromatin binding. Mapping in further details N-terminal domains mediating chromatin binding, I found that deletion of the BRCT domain, nuclear localization signal, zinc fingers I or II did not affect PARP-1 chromatin binding. Zinc finger domains are essential for DNA binding therefore my data showed that PARP-1 in cells mainly interacts with chromatin through binding to chromatin-associated proteins rather than to DNA. To distinguish the implication of DNA-binding activity of PARP-1 in its anti-HIV-1 activity, we used a

PARP-1 inhibitor that ejects zinc from zinc fingers impairing DNA binding. This inhibitor blocked the anti-HIV-1 activity of PARP-1 but did not impair its chromatin binding, highlighting the relevance of DNA binding, but not association with chromatin-bound proteins, in the anti-HIV-1 mechanism. I also found no correlation between subcellular distribution, determined by immunofluorescence, and anti-HIV-1 activity. In summary, my results indicate that **the anti-HIV-1 activity of PARP-1 requires DNA binding rather than association to chromatin-bound proteins or nuclear residency**.

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INTRODUCTION

The HIV infection pandemics. HIV continues to be a major health threat worldwide. The latest statistics indicate that by the end of 2016, an estimated 36.7 million people were living with HIV. Most of them are in low income countries (1). This epidemic has caused a large negative impact on health and the economy globally (2). It has caused more than 35 million deaths so far (1).

Anti-retroviral treatment. Antiretroviral therapy (ART) has proved to provide HIV-infected a relatively good quality of life. Having some variabilities, depending on the time of diagnosis of HIV, time of initiation of ART, ART being incompletely adhered and lifestyle factors (3), patients with HIV who are treated appropriately, have early enrolment in antiretroviral therapy and have successfully reached viral suppression with a regular CD4+ count after the first year of beginning ART have a normal life expectancy (4) (5). ART has also reduced new HIV infections by 39% and HIV-related deaths have decreased by one third between 2000-2016 (1). Although ART has improved quality of life for people with HIV that receive proper treatment, in 2016 only 53% of people with HIV, including children, were receiving ART (1).

Despite the progresses made in anti-HIV treatment, there is no cure for HIV. In addition, ART is expensive, inaccessible to some patients, has important side effects, has no effect on the latent reservoir (6), and if not taken correctly leads to drug resistant strains (7) (8). Due to the limitations that ART has, the development of improved treatments for HIV is necessary.

Our lab believes that a better understanding of the mechanism of HIV-1 replication is fundamental for the discovery of more effective treatments against HIV. This is the overarching goal of my thesis.

HIV-1 life cycle. HIV-1 is an enveloped virus that expresses on the virion surface the viral Env glycoproteins gp120 and gp41. These proteins and the host receptor CD4 and a coreceptor, either CXCR4 or CCR5, are required for infection (9). The tropism for CD4/CXCR4 or CD4/CCR5 differs between HIV-1 strains, being denoted as X4 and R5 viruses, respectively.

Initially, the viral envelope glycoprotein gp120 binds to the CD4 receptor and the coreceptor of the host cell, which results in a series of conformational changes that end in the activation of the fusogenic gp41 subunit of Env causing the fusion between the virus and the host cell membranes (10) (11). In addition to mediate entry, gp120 binding to CD4 and the co-receptor triggers receptor signaling that enhances HIV-1 infection.

In our lab we have observed that the host protein Poly (ADP-ribose) Polymerase 1 (PARP-1) impairs HIV-1 replication of X4 strains and we are interested in determining whether viruses entering through CD4/CCR5 are also affected by this anti-viral mechanism. After the fusion of the membranes has occurred, the virion core enters the cell. This subviral particles consist in a capsid formed by the viral capsid (CA) protein p24 surrounding the viral RNA genome that forms a ribonucleoprotein complex which includes the viral reverse transcriptase (RT), integrase (IN), matrix (MA), viral protein R (Vpr), viral infectivity factor (Vif) (12) and ill-defined host proteins. The virion core is transported via microtubules to the nuclear envelope where uncoating occurs through a non-fully understood mechanism. During the cytoplasmic transport of the virion core,

cyclophylin A and polyadenylation specificity factor 6 adheres to CA, preventing its premature uncoating and remains attached until it reaches the nuclear pore complex. Following uncoating at the nuclear pore reverse transcriptase enzyme transcribes the single stranded RNA genome into a double-strand DNA copy. HIV-1 uses tRNA Lys3 as a primer for the synthesis of negative stop strand DNA by RT. The RNAse H activity of RT then cleaves the RNA strand, removing the tRNA primer and leaving the minus strand DNA exposed. A region called the ppt (polypurine tract) is resistant to the RNAse cleavage, which serves as a template for the positive strand DNA, also synthesized by RT (13). The HIV DNA copy forms a complex with integrase and host proteins poorly defined (the pre-integration complex, PIC) that is imported into the nucleus by interaction of integrase with transportin 3 (TNPO3) at the nuclear pore (14).

In addition, integrase catalyzes the removal of two invariant nucleotides at the 3' end of the viral termini in a process called 3' processing and in the nucleus, this viral protein interacts with LEDGF/p75 that tethers the PIC to the host chromatin. Then, integrase catalyzes the integration of viral DNA into the host cell genome in a reaction called strand transfer, making infection irreversible for the entire life span of the cell.

The integrated viral DNA, provirus, is used as a template for the production of mRNAs by the host RNA Pol II (15) (16). HIV-1 transcripts are alternatively spliced by the host cell machinery to make the mRNAs necessary to produce viral proteins. The completely spliced mRNA encode for Tat, Rev and Nef (17). Initially, only the completely spliced mRNAs are transported from the nucleus into the cytoplasm. Then sufficient Rev is made to transport the incompletely spliced mRNAs which encode for Env, Vpu, Vif, and

Vpr and unspliced mRNAs that encodes the precursor structural proteins Gag and Gag-Pol or is encapsidated within the new viral progeny (18).

The Env gene is translated into gp160 precursor protein at the rough endoplasmic reticulum, which is cleaved in the endoplasmic reticulum into gp120 and gp41 (19) and these proteins are transported to the cell surface where remains anchorage at membranes regions rich in cholesterol.

Gag and Gag-Pol are translated in the cytoplasm as polyproteins that contain the viral structural proteins Matrix (MA), Capsid (CA), nucleocapsid (NC), p6 and two spacer peptides SP1 and SP2 (Gag region) and the viral enzymes protease, reverse transcriptase, and integrase (Pol region). Gag and Gag-Pol proteins also migrate into the plasma membrane and assemble with Env recruiting the unspliced form of the proviral transcript (16). This complex assemblies into viral particles that then bud from the cells as immature virions. After budding the protease region of Gag-Pol cleaves Gag and Gag-Pol and the released structural viral proteins rearrange with the viral enzymes, the genome, and cellular proteins into a mature virions (15).

HIV-1 LTR Transcription. The viral genome is flanked by long terminal repeat (LTR) sequences. The LTR located at the 5' end of the HIV-1 provirus, contains the U3, R and U5 regions that are the cis-elements that regulate transcription of the viral genome by RNA polymerase II (RNAP II) (20). The LTR regulates transcription initiation via the binding of host transcription factors (21) and a TATA box, and transcription elongation through the transactivation response RNA element (TAR) within the R region. Transcription factor binding sites are scattered along the LTR region but the most

important are in the core promoter, core enhancer and modulatory region located within U3 region where the TATA box is also placed.

The HIV-1 core promoter sequence consists of three SP1 binding sites, a TATA element and an active initiator sequence (17). The TATA binding protein and other proteins within the RNAP II transcription complex binds to the TATA BOX. HIV-1 transcription is also dependent on an enhancer region, located upstream of the SP1 binding sites and the core promoter, contains two NF-kB binding motifs. The modulatory region, located upstream of the core promoter, contains binding sites for multiple factors that modulate the action performed by the core and enhancer region (22).

The integrated viral DNA, provirus, serves as a template for the production of mRNAs by the host RNAP II (15). RNAP II intiates transcription of the provirus but it stops early during elongation upon the transcription of the TAR sequence. The HIV-1 accessory protein Tat is essential to remove the pausing imposed by TAR. Tat complexed with a heterodimer formed by Cdk9 and cyclin T1 (P-TEFb) binds to TAR and the CDk9 suunit phosphorylates the C-terminal domain of paused RNAP II, and other host factors in the complex such as NELF and DSIF, promoting transcriptional elongation (23).

Role of Macrophages in mucosal transmission of HIV-1. Macrophages have been demonstrated to play an important role throughout the life cycle of HIV-1. They are one of the immune cells that face HIV-1 when transmitted from person to person through mucosal surfaces. Sexually transmitted HIV-1 uses CCR5 coreceptor for infection mostly, which implies that macrophages might be infected when transmitted sexually. HIV-1 variants that infect cells via the CCR5 coreceptor are prevalent during acute infection, which also implies that macrophages are involved in the acute phase of the

infection (24). A genetic mutation named CCR5Δ32 was discovered in 1996 which consists of a deletion of 32 base pairs within the CCR5 gene leading to the lack of expression of the receptor in the surface of the cell. People with this mutation are naturally protected from HIV-1 infection particularly when the virus is transmitted through mucosa (25) (26).

Due to the role of CCR5 in transmission of HIV-1 via mucosa we decided to investigate whether PARP-1 also impairs replication of R5 strains in addition to decrease infection of X4 strains.

Cellular roles of PARP-1. PARP-1, the most abundant protein in the PARP family that in humans contains 17 members. This protein is located in the nucleus where it participates in many cellular mechanisms including transcriptional regulation, regulation of chromatin structure, DNA repair among others essential biological processes (27). These multiple functions are mainly the result of PARP-1 enzymatic activity, although non-catalytic function of PARP-1 also has a role in some of these cellular processes.

PARP-1 is a NAD+ dependent enzyme that synthesizes polymers of ADP-ribose (PAR) and transfer them to particular amino acids within proteins or to PAR residues already attached to proteins, catalyzing protein PARylation. This post-translational modification modifies the conformation and function of target proteins (28). Although there is not a specific motif for PARylation, analysis of the amino acid modified by PARP-1 in thousands of substrates indicates that PARylation occurs on glutamic acid residues in sequences where the target glutamic acid is C-terminally followed by glutamic acid (EE) or proline (EP) or any amino acid and then a proline residue (EXP) (29).

As mentioned above, PARP-1 is implicated in DNA repair signaling, transcriptional regulation, and NAD+/ATP metabolism (27). Importantly regulation by PARP-1-mediated PARylation is highly redundant. Despite the multiple role of PARP-1 in processes essential for cell viability, PARP-1 KO cell lines can be establish in organisms such as Drosophila and chicken where PARP-1 is the only PARylating enzyme or in human and mouse where other PARP family members mediates PARylation although at significantly lower levels than PARP-1. For example, PARP-1 or PARP-2 KO mice are viable but the double KO is lethal. Therefore the regulatory role of PARylation seems to be substituted in the cell by other post-transcrptional modifications and by the activity of PARP-2 or PARP-3 in cells expressing them.

PARP-1 signals DNA repairing enzymes involved in DNA single- and double-strand breaks repair and in base excision repair pathways (30). PARP-1 detects single or double DNA strand breaks and transfers PAR mainly to itself utilizing NAD+. This transfer of PAR initiates the recruitment of DNA repair factors involved in these DNA repair pathways. When DNA damage is too severe, there is a large amount of PARP activation resulting in ATP and NAD+ reduction leading to cell death (31).

PARP-1 has also been also demonstrated to regulate transcription via different mechanism that involve the modification of the chromatin structure and the interaction of PARP-1 with transcription factors.

The regulatory role of PARP-1 transcriptional is better understood at the level of transcriptional initiation by mechanisms that require the enzymatic activity of this protein (27) (32) (33). Genome-wide analysis of PARylation sites indicate a robust enrichment of PARylation at promoters of transcriptionally active genes that are also enriched for

histone H3 lysine 4 trimethylation (29). The mechanisms implicated in PARP-1 transcriptional regulatory activity include the modification of chromatin and transcription factors, and the direct binding of PARP-1 to promoters and insulators (34) (35). For example, PARylation of nucleosomes has been shown to enhance transcription by its effect on chromatin decondensation. PARP-1 binds to histones H3 and H4 when nucleosomes are disassembled and PARylation is enhanced by H4. Histones H2B and H2A play an opposite role, inhibiting PARP-1 activity. PARP-1 also prevents demethylation of H3K4 by KDM5B demethylase, promoting transcription (35).

PARP-1 has been implied to act in conjunction with RNA Pol II transcription machinery, other transcriptional co-regulators, and DNA-binding transcription factors regulating transcription by either repressing or activating it (32). Generally, it is thought that PARP-1 is recruited by the DNA-binding factor to specific target promoters. In some cases PARP-1 enzymatic activity is necessary for its coregulatory activity, while in others, it is dispensable. (36). The transcription factor CTCF (CCCTC-binding factor) has to be PARylated in order for its role as an insulator. Therefore, the role of PARP-1 in transcriptional regulation is complex. PARP-1 can be acetylated at lysine residues and bind to NF-kB activating transcription. It can also be SUMOylated and prevent the histone acetyltransferase p300 and CREB-binding protein to acetylase PARP-1 leading to transcriptional repression (33). In addition to regulate transcriptional initiation, PARP-1 also promotes the elongation step by PARylation of the negative elongation factor that produce promoter-proximal pausing by RNA pol II.

Catalytic-independent regulation of transcription is ill-defined yet. The best know mechanism is the displacement of histone H1 by PARP-1 that triggers chromatin condensation and repressing transcription (37).

PARP-1 protein architecture. PARP-1 has an apparent molecular weight of 116 kDA protein and is organized in three domains, an N-terminal domain that binds to DNA, a central domain that is implicated in protein-protein interactions and the C-terminal domain that contains the catalytic activity (38). The N-terminal domain has a nuclear localization sequence and three zinc finger domains, that allow the binding of PARP-1 to DNA unspecifically (39). The first two zinc fingers are essential for the recognition of DNA strand breaks, for the binding of PARP-1 to the damaged DNA, and for activation of its enzymatic activity (40). The central domain or automodification domain is crucial for correct biological functioning, since the main PARylation target of PARP-1 is itself, and has about 15 PARylation sites. The C-terminal domain contains the catalytic site ofPARP-1 (41).

Chromatin-binding activity of PARP-1. The ability of PARP-1 to interact with chromatin is central to all its cellular functions. PARP-1 binds directly to DNA and to chromatin-associated proteins. DNA binding is mediated by the zinc finger domains located in the N-terminus; however, PARP-1 domains responsible for interaction with chromatin-bound proteins are ill-defined yet.

The interaction of PARP-1 with chromatin and free DNA has been studied mainly *in vitro* and their confirmation in cells is lacking. In my thesis I evaluated several of these *in vitro* evidences in the context of intact cells.

Chromatin interaction of PARP-1 is influenced by its PARylation status. *In vitro* automodification decreases affinity of PARP-1 for chromatin and stimulates a histone chaperone activity in PARP-1 that involves binding to histones and assembled nucleosomes. Using *in vitro* reconstituted chromatin it has been demonstrated that PARP-1 binds to nucleosomes through the N-terminal region (amino acids 1-464). This domain was demonstrated to be necessary and sufficient for this activity (42). In these studies, interaction of PARP-1 with histone H2B and H4 was demonstrated and binding to H4 was enhanced by DNA damage or an inhibitor of PARG (43). However, these *in vitro* evidence lack independent confirmation and do not clarify the mechanisms of chromatin interaction in any extent.

PARP-1 PARylates H1 displacing it from the promoters of immediate early genes. The eviction event is accompanied by binding of PARP-1 to these promoters (44). This reciprocal pattern of chromatin binding of H1 and PARP-1 has been demonstrated genome wide in cells. PARP-1 binding and eviction of H1 is associated with regulation of transcription (45). *In vitro* studies show that PARP-1 and H1 compete for binding to an overlapping site on nucleosomes (45). PARP-1 domains implicated in H1 competition for nucleosomes are unknown.

Effects of PARP-1 on HIV-1 infection. Several of the cellular functions of PARP-1 are exploited by viruses and in particular by HIV-1 (46). Multiple viruses activate DNA repair pathways recruiting proteins involved in DNA repair and utilize this cellular response for their own replication (47).

In particular PARP-1 has been reported to modulate HIV-1 infection at the transcriptional level. PARP-1 has been proven to decrease HIV-1 transcription initiation

by inhibiting NFκB activation (48) whereas it affect transcriptional elongation by blocking the recruitment of the Tat/P-TEFb complex to TAR (49).

In addition, work from our laboratory demonstrated that PARP-1 silences retroviral gene expression by mechanism known to alter chromatin structure. This effect is independently of its catalytic activity and occur before viral DNA integration takes place (47) (50). This PARP-1-mediated retroviral silencing requires the activity of histone deacetylases and DNA methylases, enzymes know to deposit heterochromatin on cellular genes.

More recently we have observed that PARP-1 also negatively affect the replication of HIV-1 in human CD4 T cells. The mechanism implicated is unknown but requires cellular entry through the interaction of viral gp120/gp41 with the host CD4/CXCR4 receptors. Replacement of the HIV-1 native envelope by the Vesicular Stomatitis Virus glycoprotein G (VSV-G pseudotyping) blocked the effect of PARP-1 on HIV-1. Therefore these data suggest that CD4/CXCR4 is implicated in the anti-HIV-1 PARP-1 activity. In addition, our lab has determined that HIV-1 gene expression is favored in cells lacking PARP-1 but not the number of proviruses per cell suggesting that PARP-1 affects HIV-1 transcription. In contrast, a recent report indicated that PARP-1 is a positive cellular cofactor in the replication of HIV-1 entering through CD4/CCR5 receptors. This study, however, was conducted in HeLa cells engineered to express CCR5 (51). These are epithelial cells that are not naturally infected by HIV-1. Nevertheless, PARP-1 could enhance HIV-1 transcription since this protein acts as a positive cofactor of NF-kB, NFAT, AP-1, and sp1 transcriptional function in inflammatory genes. As discussed above these transcription factors are relevant in HIV-1 transcription. However, it is

difficult to predict the outcome of the modulation of these transcription factors by PARP-1 on HIV-1 transcription. Promoter architecture importantly defines the transcriptional effect of the interacting transcription factors. Therefore, experimental evaluation of the effect of PARP-1 on HIV-1 transcription through these transcription factors is required. In addition, PARP-1 has been shown to PARylate NEFL inhibiting its inhibitory function of pTEFb. This mechanism could determine an enhancing effect of PARP-1 on HIV-1 transcription, although it has not been evidenced yet.

Therefore, I decided to clarify the role of PARP-1 on the replication of R5 HIV-1 strains using a more relevant cellular model. CD4+ T cells and macrophages express CD4/CCR5 receptors. Then, I will employ cells of myeloid linage (THP-1) and CD4+ T cells (SupT1 cells) in my studies. These are natural targets of HIV-1 *in vivo*.

HYPOTHESIS

Previous studies in my laboratory indicate that PARP-1 impairs replication of HIV-1 entering the cell via CD4/CXCR4 receptors; however, the mechanism implicated is unknown. **We hypothesize that PARP-1 will also decrease HIV-1 infection in cells entering through CD4/CCR5 receptors and that the chromatin-binding activity of PARP-1 will be required for its antiviral activity.**

SPECIFIC AIMS

1. **To generate a PARP-1 KO THP-1 cell line.**
   These cells will be useful to determine the role of PARP-1 in HIV-1 replication in myeloid cells.
2. **To determine the correlation between chromatin-binding activity, subcellular distribution and anti-HIV-1 activity of PARP-1.**
   A panel of PARP-1 mutants were characterized for their anti-HIV-1 activity, subcellular localization, and chromatin-biding activity.

The main goal of my thesis is to better define the mechanisms implicated in the anti-HIV-1 activity of PARP-1. Data from my laboratory indicate that PARP-1 affects the infection of HIV-1 strains entering via CD4/CXCR4 (X4 viruses). However, the role of PARP-1 on viruses using CD4/CCR5 (R5 viruses) for infection is unknown. THP-1 is a robust model to answer this question because these cells allows replication of both R5 and X4 HIV-1 strains reducing the variability introduced by the use of more than one cell line to match the HIV-1 tropism. PARP-1 is a protein that interacts with chromatin and DNA; however, the protein domains implicated in these activities are poorly defined. Most of the studies on the chromatin/DNA-binding activity of PARP-1 have been conducted with *in vitro* models in the

absence of cells. This gap in knowledge precludes establishing correlation between this function of PARP-1, which is essential for all its cellular functions, and its anti-HIV-1 activity. Using biochemistry and cell fractionation techniques I will define the impact of several mutations in PARP-1 on its chromatin-binding activity in cells, and this information will be correlated with the anti-HIV-1 activity of these mutants. Similarly, the subcellular localization of these PARP-1 mutants will be determined by immunofluorescence in cells and correlation with their anti-HIV-1 activity will be established.

MATERIALS AND METHODS

1. **Production of lentiviral vectors.** Cas9 and pTRIP SIN eGFP PARP-1 CRISPR lentiviral vectors were produced in HEK 293T cells. This was done by co-transfection with calcium-phosphate of 15ug of the plasmid of interest expressing either Cas9 or the PARP-1-specific sgRNA, 5ug of Vesicular Stomatitis Virus glycoprotein G (VSV-G) and 15 ug of Delta-R8.9, the packaging construct. The transfection medium was replaced 24 hrs later and 72 hours after transfection the cell supernatant containing the lentiviral vector was collected and concentrated by ultracentrifugation at 124,750 g for 2 hrs thorugh a 20% sucrose cushion. The viral pellet was resuspended in culture medium and used to transduce THP-1 cells.

2. **Generation of a stable cell line expressing pTRIP SIN eGFP PARP-1 CRISPR**

   To generate a THP-1-derived expressing a PARP-1-specific sgRNA cells were transduced with a lentiviral vector that encode this sgRNA genetically linked to the expression of eGFP. We plated 5,000 THP-1s per well in a 96-well plate and transduced them with different amounts of the viral vector (7, 2, 1.5, 1, 0.6, 0.5, 0.1 ul) and one culture was not transduced (control). The next day we removed the virus by centrifuging the cells, removing supernatant and replacing it with fresh RPMI media. Only the cells transduced with 0.5 and 0.1 ul survived and were expanded as

necessary. eGFP expression was confirmed in the survival cells by fluorescent microscopy examination to confirm transduction.

3. Generation of PARP-1 KO THP-1 cells

We plated in a 96 well plate 5,000 THP-1s expressing PARP-1-specific sgRNA and eGFP and transduced them with different amounts of a viral vector (7, 2, 1.5, 1, 0.6, 0.5, 0.1 ul) expressing Cas9 genetically linked to the puromycin resistant gene and cells were selected in the presence of Puromycin (0.2ug/ml). The cells transduced with 4, 2 and 1ul survived and were expanded as necessary.

Immunoblot analysis of THP1-derived cell lines. 1 million THP-1 cells transduced with PARP-1 SgRNA and Cas9, as mentioned previously, were lysed in 2X Laemmli sample buffer Cell lysates. These were resolved by 7% SDS-PAGE and left for an overnight transfer to a PDVF membrane at 100 Amp at 4°C. Membrane was then blocked for one hour in TBS (25 mM Tris·HCl, 150 mM NaCl, pH 7.6) with 10% milk and left incubating overnight at 4°C with anti-PARP-1Santa Cruz Biotechnology (sc-7150 Lot # D2115) at a 1/1000 dilution in TBS 5% ,o;l and 0.05% Tween 20. As a loading control alpha tubulin was detected with anti-alpha tubulin mAb (Clone B-5-1-2 Sigma) in a 1/4000 dilution for two hours at room temperature. Then membrane was washed in 0.1% Tween 20 and antibodies were detected with Sigma goat anti-mouse IgG-HRP at a 1/2000 dilution. Chemo-luminescence detection was done afterwards

Single cell cloning. Cell lines derived from THP-1 by transduction with lentiviral vectors expressing PARP-1-specific sgRNA and Cas9 were subjected to single cell cloning by plating serial dilutions of 3, 1 and 0.5 cells per well in 96 well plates. Clones derived from this experiment will be characterized for PARP-1 expression by immunoblot analysis.

4. **PMA differentiation of THP-1-derived cells.** To differentiate THP-1 cells with PMA we plated 0.1 million cells in 6 well plates. Some cultures were left without treatment, and the others were treated with 200 ng/mL of PMA. 72 hrs later the medium was replaced and the cells were used for FACS analysis and HIV-1 infection.
5. **FACS analysis of THP-1-derived cell lines.** THP-1 cells treated or not with PMA for 72 hrs were analyzed by FACS for the expression of CD4 (APC mouse anti-human CD4 clone RPA-T4, PRODUCER 561840), CXCR4 (APC mouse anti-human CD184, PRODUCER 560936) and CCR5 (APC-Cy7 mouse anti-human CD195, PRODUCER 557755). Cells were pelleted at 216g for 5 minutes and resuspended in ice-cold PBS containing specific antibodies (5ul / 100ul of PBS). Cells and antibodies were incubated for 20 min on ice and then washed by centrifugation at 216g for 5 mins. Stained cells were resuspended in 500ul of ice-cold PBS and analyzed by FACS.
6. **Chromatin-Binding Assay**. Previously described procedures (22) were followed with minor modifications. Figure 3 of reference (22) shows a validation experiment where multiple proteins located in different cellular compartments were evaluated. Briefly, $18x10^6$ T_{L3}-derived cells expressing different PARP-1 mutants were washed in PBS and distributed in three aliquots containing equal amount of cells. Two of the samples were

lysed for 15 mins on ice in 100µl of CSK I buffer (10mM Pipes pH6.8, 100mM NaCl, 1mM EDTA, 300mM sucrose, 1mM $MgCl_2$, 1mM DTT, 0.5% Triton X-100) containing protease inhibitors (final concentration: leupeptine 2µg/ml, aprotinin 5µg/µl, PMSF 1mM, pepstatin A 1µg/ml). The third sample was lysed for 15 mins on ice in 100µl CSK I buffer supplemented with 350mM NaCl and protease inhibitors, centrifuged at 22,000g for 3 mins at 4°C and supernatant saved for further analysis (total fraction, T). Cells lysed in CSK I buffer were centrifuged at 1000g for 6 mins at 4°C and the supernatant pooled (non-chromatin-bound fraction, S1). S1 supernatants were clarified further by centrifugation at 22,000g for 3 mins and supernatant transfer to a fresh tube while pellets were washed once in 200µl of CSK I buffer. One of these pellets was resuspended in CSK I 350mM NaCl buffer and incubated on ice for 15 mins followed by centrifugation at 22,000g for 3 mins at 4°C and the supernatant was collected for further analysis (chromatin-bound fraction, P1). The other pellet, obtained after cell lysis in CSK I, was resuspended in 100µl of CSK II buffer supplemented with protease inhibitors, 4 units of turbo DNase (Ambion) and 11µl of 10X turbo DNase reaction buffer. DNase treatment of this pellet was conducted at 37°C for 30 mins and then followed by extraction with $(NH_4)_2SO_4$ 250mM for 15 mins at 37°C. The DNase/ $(NH_4)_2SO_4$ treated sample was centrifuged at 22,000g for 3 mins and the supernatant saved for analysis (chromatin-bound fraction, S2). The resulting pellet was further extracted with CSK I 350mM NaCl for 15 mins on ice, centrifuged at 22,000g for 3 mins and the obtained supernatant (non-chromatin-bound fraction, P2) collected for analysis. A volume of 15.7µl of S1, P1, P2 and T and 20µl of S2 (amounts equivalent to $0.9x10^6$ cells) was evaluated by immunoblotting using an anti-FLAG Mab.

7. **Salt extraction assay.** $36x10^6$ T_{L3} cells or T_{L3} cells expressing PARP-1 mutants were washed with PBS and distributed in six samples, each containing an equal amount of cells. One of the samples was resuspended in 100µl of Laemmli sample buffer, boiled for 9 mins and centrifuged at 22,000g for 3 mins and the resulting supernatant was further analyzed (total fraction, T). The other five cellular aliquots were lysed on ice for 15 mins in 100µl of CSK I containing protease inhibitors and supplemented with increasing concentrations of NaCl (final concentration: 100, 150, 200, 350 and 500mM). Lysed cells were centrifuged at 22,000g for 3 mins at 4°C and the supernatant (salt-extracted fraction) collected for analysis. The obtained pellets (salt-resistant fraction) were subsequently boiled in 100µl of Laemmli sample buffer, centrifuged at 22,000g for 3 mins at 4°C and the resulting supernatant collected. The presence of PARP-1 in the total, salt-extracted or salt-resistant fractions was analyzed by immunoblotting with an anti-FLAG Mab using 20µl of cell lysate, an equivalent amount of $6x10^4$ cells.
8. **Immunofluorescence assays.** $2x10^5$ 293T PARP-1 KO cells were plated in LabTeck containers. 24 hours after cells were plated, transfections were done with calcium chloride with pTRIP PARP-1, ΔC, Zn I, Zn III, ΔNLS and ΔN individually. For each LabTeck, 6.76ul of CaCl was mixed with 1ug of DNA and 67.5ul of HBS. The CaCl/DNA/HBS mix was added to each container after a 15 minute incubation. The day after transfection, transfection media was removed and replaced with fresh DMEM. 72 hours post-transfection, cells were washed with PBS, then fixed with 4% paraformaldehyde in PBS for 10 minutes. Second fixation was done with 95% ethanol for 2 minutes. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and washed with PBS. After washing, cells were incubated in 5% BSA/PBS

at room temperature for 30 minutes. PARP-1 anti-body (EPR18461 Alexa Fluor 488, abcam) was used for staining Zn I, Zn III, ΔNLS and ΔN transfected cells and anti-myc (M4439, Sigma) was used for staining ΔC transfected cells. Antibodies were diluted in 1% BSA in PBS for 1 hour and cells stained with anti-myc were followed by a wash with PBS then an incubation with goat anti-mouse IgG-FITC (sc-2010, Santa Cruz Biotechnology) for 30 minutes. Cells were again washed and cell nuclei was stained with DAPI for 5 minutes.

RESULTS

Specific aim 1. To generate a PARP-1 KO THP-1 cell line.

Effect of PMA differentiation of THP-1 cells on the expression of CD4, CXCR4, and CCR5. We decided to evaluate the expression of CD4, CXCR4, CCR5 receptors in THP-1 cells under basal conditions and upon PMA differentiation. It has been reported that PMA differentiation of THP-1 decrease the density of CD4. This receptor is thought to interact stronger with CCR5 than CXCR4, then the lower levels of CD4 in THP-1 cells has been linked to an increase in their susceptibility to infection by R5 strains(9). Therefore, in order to evaluate the suitability of THP-1 cells for infection with R5 strains we determined first the effect of PMA-induced differentiation on the expression levels of these receptors.

Non differentiated THP-1 cells expressed CD4 (91.95 %), CXCR4 (92.77 %), and CCR5 (62.45%) (Fig.1a). Importantly, upon PMA-differentiation CD4 expression drastically dropped (8.70%) while CXCR4 decreased by more than 50% (42.53 %) and CCR5 slightly decreased (58.73%) (Fig.1b). These findings were in agreement with previously reported results (9).

In addition, these results suggested that THP-1 could be a suitable model for replication of both X4 and R5 strains.

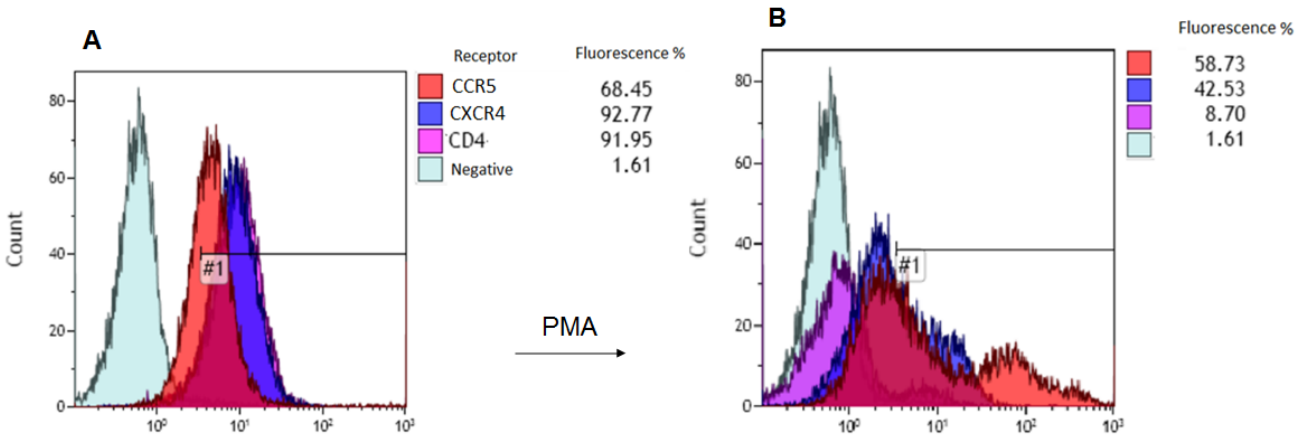

Figure 1 A and B. Expression of CD4, CXCR4, CCR5 on THP-1 cells under basal and PMA-activated conditions. Cells were treated or not with PMA for 24 hrs and the cell surface receptors detected by FACS analysis with specific antibodies. Negative indicate unstained cells used as control. The percentage of cells expressing each receptor is indicated.

Generation of PARP-1 KO THP-1 cells. Next we decided to generate PARP-1 KO THP-1 cell line using lentiviral-delivered CRISPR/Cas9. THP-1 cells were transduced with lentiviruses expressing a PARP-1-specific sgRNA and Cas9. Transduced cells were expanded in culture and a whole cell lysate was obtained from the established cell line. Then, PARP-1 expression levels were determined by immunoblot (Fig. 2), detection of α-tubulin as a loading control.

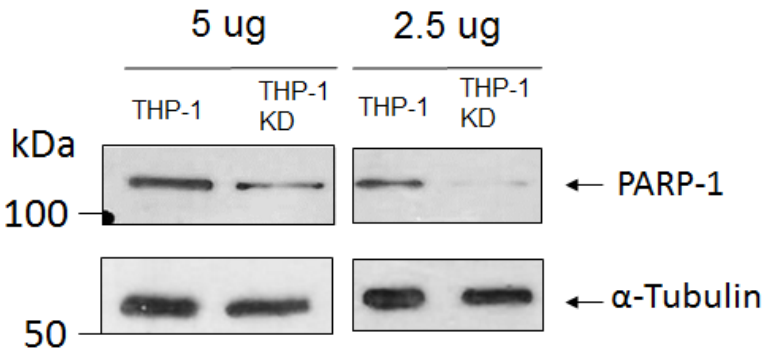

Figure 2. THP-1 PARP-1 knockdown cell line. THP-1 cells were co-transduced with lentiviruses expressing PARP-1-specific sgRNA and Cas9. A lysate was obtained from the cell line generated and PARP-1 and α-Tubulin expression was determined by Western Blot Analysis.

Levels of PARP-1 decreased by two-fold in the transduced cells as compared with control THP-1 cells. These, findings could indicate the existence of a mixed cell population containing KO and wild type cells or that all the cells of the culture were partially KO. To distinguish between these two possibilities we attempted separation of individual cells by single cell cloning, expecting to obtain THP-1 PARP-1 KO cells. Single cell cloning was attempted in 96-well plates with 0.5, 1, 3, 10, 50 and 100 cells per well. One month after cells were plated, no cell growth was observed.

We also tried to obtain single-cell clones using culture medium supplement with 20% fetal calf serum plus 50% fresh THP-1-conditioned medium and the results were the same. Additionally, we took advantage that expression of the PARP-1 sgSHRNA was genetically linked to eGFP and sorted the population of eGFP positive cells but the cells never re-grow after sorting.

Unfortunately, failure to obtain single-cell clones precluded the generation of PARP-1 KO THP-1 cells.

CONCLUSIONS

These results demonstrated that although THP-1 cells could be a suitable cell line to evaluate replication of X4 and R5 viruses. However, these cells do not grow at low cell densities impeding generation of single-cell clones hence the isolation of KO cells.

Future direction: Most of the studies characterizing replication of R5 strains use THP-1 cells. Therefore, the use of other human myeloid cell lines is disadvantageous because of the lack of characterization of HIV-1 replication in them.

Primary myeloid cells have also been employed routinely in HIV-1 replication studies, but these cells are notoriously difficult to transduce with lentiviruses making very difficult the generation of PARP-1 KO cells.

As collaboration we obtained SupT1 cells engineered to express low and high levels of CCR5 receptors (Fig. 3). SupT1 cells naturally express CD4 and CXCR4, therefore the engineered cells are suitable for replication of X4 and R 5 viruses. We will use these cells to generate PARP-1 KO cell lines. Our laboratory has expertise in generating PARP-1 KO cells in SupT1 cells. This model will allow illuminating the role of CCR5 in the effect of PARP-1 on the replication of R5 strains.

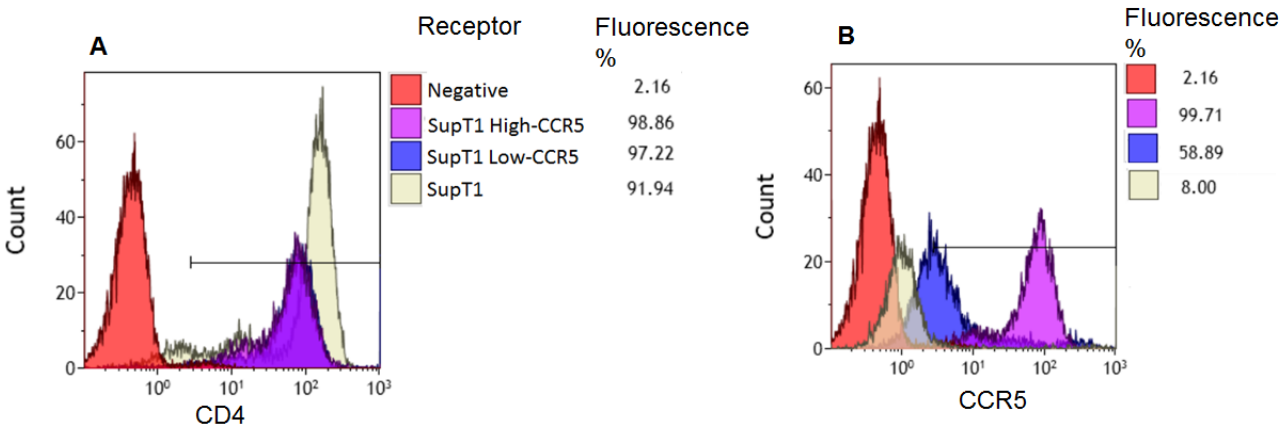

Figure 3 A and B. Expression of CD4 and CCR5 as detected by FACS analysis of SupT1 cells engineered or not to express CCR5. Negative indicate unstained cells used as control. The percentage of cells expressing each receptor is indicated.

Specific aim 2. To determine the correlation between chromatin-binding activity, subcellular distribution and anti-HIV-1 activity of PARP-1.

Chromatin Binding activity of PARP-1

Chromatin-binding of PARP-1 has been extensively characterized *in vitro* using purify proteins and artificial substrates. However, studies with cells have been very limited; therefore, I evaluated the chromatin-binding activity of a panel of PARP-1 mutants expressed in PARP-1 KO CD4 T cells (SupT1 cells).

First, we determined for the first time the characteristics of chromatin binding of PARP-1 in cells. SupT1 cells were lysed in the presence of Triton X-100, this buffer efficiently extract chromatin non-bound proteins located both in the cytoplasm or nucleus of the cell. On the contrary, chromatin-bound proteins persist attached to this structure and therefore remain insoluble. Due to this differential partition, upon centrifugation of the cell lysate two fractions are obtained. A supernatant enriched in chromatin non-bound proteins and a pellet that contains, among other Triton-insoluble proteins, chromatin bound proteins.

Using this procedure we evaluated effect of different ionic strengths on the binding of PARP-1 to chromatin (Fig. 4). At physiological ionic strength (100 mM NaCl) PARP-1 almost exclusively resides in the chromatin-bound fraction (Triton insoluble pellet). However, at increasing salt concentrations the protein is efficiently extracted and solubilized into the supernatant.

In these experiments we detected in the same membranes LEDGF/p75 and α-tubulin as control of the cell fractionation efficiency (Fig. 4). LEDGF/p75 tightly binds to chromatin

whereas α-tubulin is a chromatin non-bound protein. Comparison of PARP-1 and LEDGF/p75 chromatin binding strengths indicated that PARP-1 is a weaker binder.

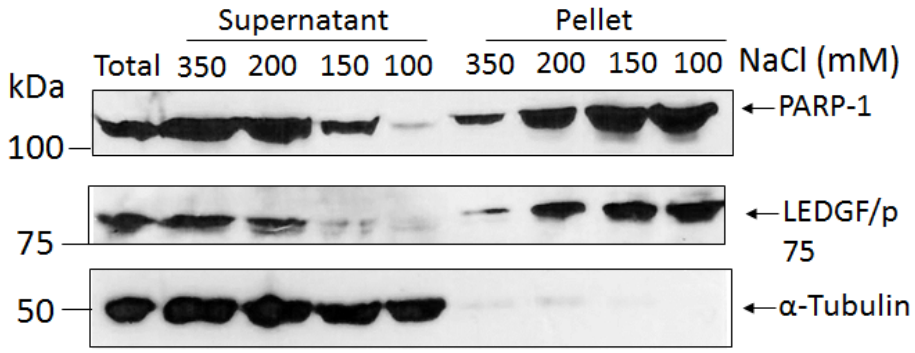

Figure 4. Chromatin-binding activity of PARP-1. SupT1 cells were lysed in a buffer containing 1% of Triton X-100 supplemented with different amounts of NaCl. Cell lysates were centrifuged and a pellet (chromatin bound proteins) and a supernatant (chromatin non-bound proteins) fractions were obtained. A whole cell lysate (Total) was obtained by lysing cells in Laemli buffer. PARP-1, LEDGF and α-tubulin were detected in the different samples by immunobloting.

Densitometry analysis of the PARP-1 levels in the different fractions and normalization of these values to total levels obtained in whole cell lysates, indicate that at physiological ionic strength only 2% of PARP-1 is not chromatin bound (Fig. 5). That fraction increases to 50% at 150 mM and the protein was 3-fold enriched in soluble fractions obtained with ionic strengths above 200 mM NaCl.

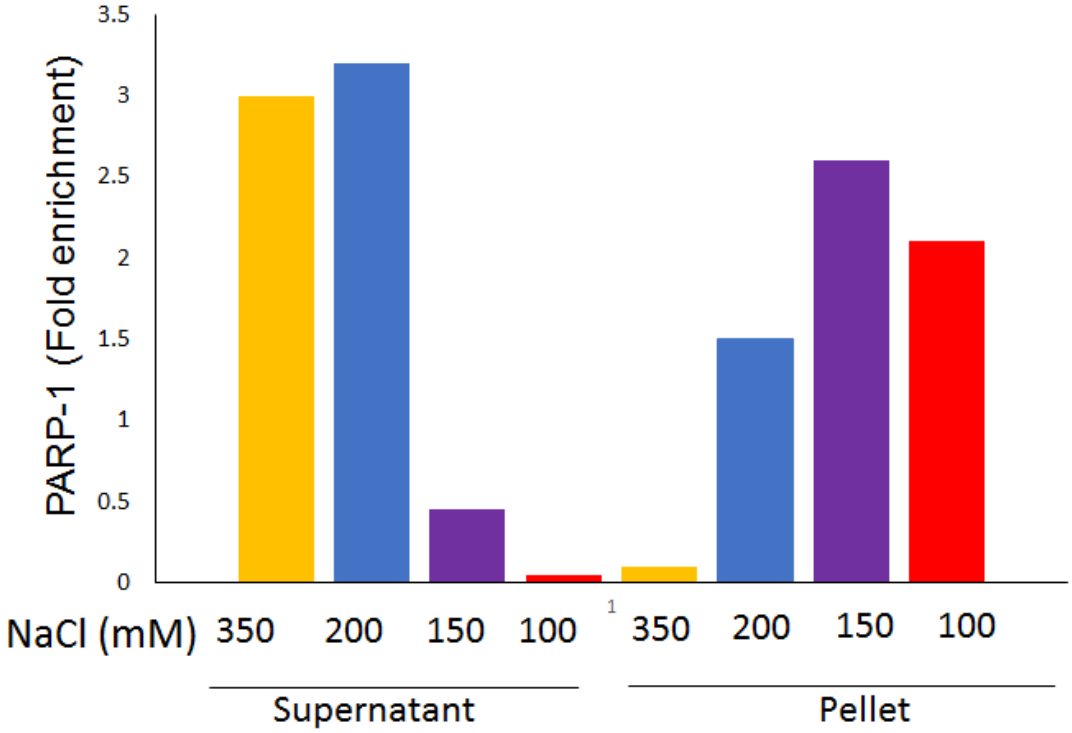

Figure 5. Chromatin-binding activity of PARP-1. Densitometry analysis of PARP-1 immunoblots showing the amounts of PARP-1 present in chromatin-bound (pellet) and unbound (supernatant) fractions obtained in the presence of Triton X-100 and different ionic strengths. PARP-1 values in each fraction was normalized to the levels found in a whole cell lysate.

Therefore, to evaluate the effect of different mutations and PARP-1 inhibitors on the chromatin binding activity of this protein we considered the presence of PARP-1 only in chromatin non-bound fractions obtained at physiological ionic strength (100 mM). Nevertheless, experiments using a wider range of ionic strengths were also performed and are reported here.

Effect of mutations on PARP-1 chromatin-binding activity. We evaluated the chromatin-binding activity of different deletion mutants (Fig. 6). PARP-1 KO SupT1 cells engineered to express these mutants were lysed in a buffer containing 1% Triton X-100 and 100 mM NaCl. As explained above cellular proteins solubilized with this buffer are considered chromatin non-bound. Conversely, proteins that are not solubilized and are enriched in the resulting pellet are classified as chromatin-bound proteins. LEDGF/p75 and α-tubulin were detected as controls of the efficiency of the cell fractionation procedure (Fig. 6).

In these experiments we found that full length PARP-1 and the N-terminal domain were present in the pellet fraction obtained at physiological ionic strength (100mM NaCl). On the other hand, C-terminal domain was present in the supernatant fraction under the same conditions (Fig. 6). These data indicated that N-terminus of PARP-1 is necessary and sufficient for chromatin-binding, whereas the C-terminus is dispensable.

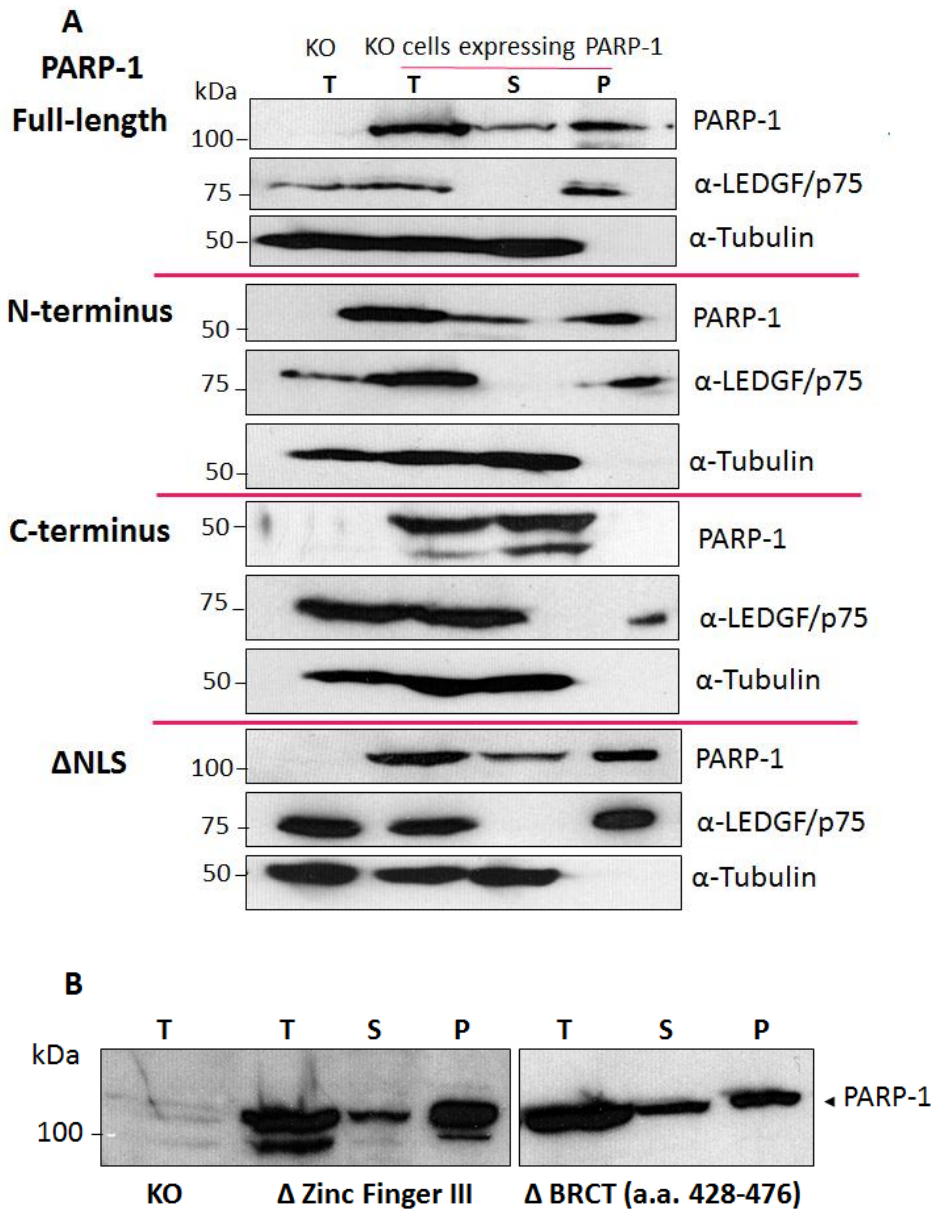

Figure 6. PARP-1 KO cells engineered or not to express PARP-1 proteins were lysed in Laemli buffer to generate a total cell lysate (T). KO cells engineered to express different PARP-1 proteins were lysed in a buffer containing Triton X100 and 100 mM NaCl and then two fractions were obtained by centrifugation. A chromatin non-bound fraction (S, supernatant) and a chromatin bound (P, pellet) fractions were analyzed by immunoblot.

In order to map the implication of different functional domains present in the N-terminus,

I analyzed the chromatin-binding activity of PARP-1 mutants lacking individual domains.

Importantly, deletion of zinc finger I, III, nuclear localization signal (NLS) and the BRCT domain did not affect the chromatin-binding activity of PARP-1 (Fig. 6). I did not evaluate the contribution of zinc finger II because our laboratory is still generating cell lines stably expressing this mutant.

The lack of relevance of zinc fingers I and III in the chromatin binding activity suggest that chromatin interaction is not importantly influenced by DNA binding of PARP-1, since these domains are required for DNA interaction. In addition, my data suggest that more than one domain in the N-terminus mediates chromatin binding of PARP-1. Importantly, our data indicating the relevance of the N-terminus in chromatin binding in cells corroborate the *in vitro* findings previously reported (42).

Analysis of the anti-HIV-1 and the chromatin-binding activities of PARP-1 indicates a strictly correlation. Findings from our laboratory showed that C-terminus PARP-1 lacks anti-HIV-1 activity in contrast with N-terminus PARP-1 and deletion mutants lacking the NLS or the BRCT domain. Similarly, C-terminus PARP-1 did not bind to chromatin in contrast with N-terminus PARP-1 and the two deletion mutants mentioned above. Therefore these results indicate that chromatin-binding is required for PARP-1 to impair HIV-1 replication. Analysis of the anti-HIV-1 activity of mutants lacking zinc finger domains I and III is still in progress.

PARP-1 interacts with similar affinity with DNA and nucleosomes, according to *in vitro* studies. In our experiments we were unable to evaluate the contribution of all the zinc finger domains to chromatin binding and to the anti-HIV-1 activity of PARP-1, this work is still in progress. To overcome this limitation we evaluate the effect of inhibitor II on the chromatin-binding activity of PARP-1. This inhibitor ejects zinc from the zinc fingers

destroying the ability of PARP-1 to interact with DNA. Moreover, data from our laboratory indicate that inhibitor II blocks the anti-HIV-1 activity of PARP-1.

SupT1 cells were treated with inhibitor II or DMSO (vehicle) and then PARP-1 chromatin-binding activity was evaluated. In these studies we observed that inhibitor II increased chromatin-binding of PARP-1, indicating that DNA interaction did not significantly contribute to chromatin binding (Fig. 7a and b).

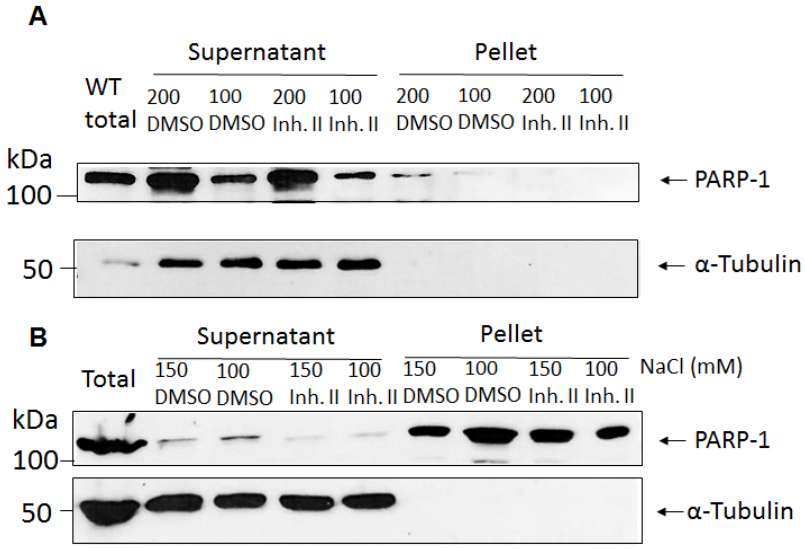

Figure 7 A and B. Effect of PARP-1 inhibition on its chromatin-binding activity. SupT1 cells were treated with DMSO or Inhibitor II for 30 min and then lysed in a buffer containing 1% of Triton X-100 supplemented with different amounts of NaCl. Cell lysates were centrifuged and a pellet (chromatin bound proteins) and a supernatant (chromatin non-bound proteins) fractions were obtained. A whole cell lysate (Total) was obtained by lysing the cells in Laemli buffer. PARP-1 and α-tubulin were detected in the different samples by immunobloting. Results from two independent experiments are displayed.

Densitometry analysis from the results of two independent experiments indicated a decrease of chromatin non-bound PARP-1 of 2.3 and 16 folds, respectively, as

compared to the amounts detected in cells treated with DMSO (Fig. 8a and b). These findings are also in agreement with the deletion mutagenesis analysis described above. Furthermore, my data indicate that DNA binding, rather than interaction of PARP-1 with chromatin-associated proteins, is relevant for its anti-HIV-1 activity.

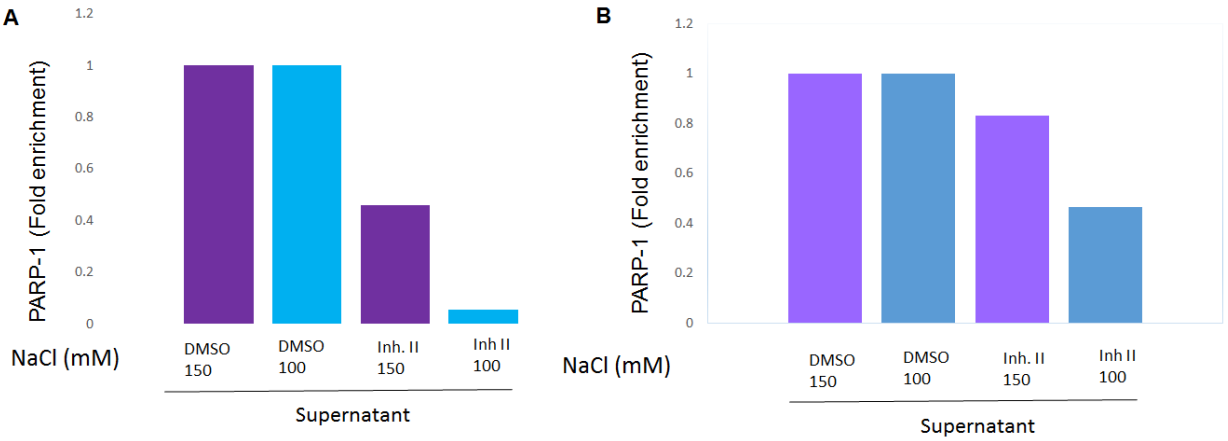

Figure 8 A and B. Effect of PARP-1 inhibition on the chromatin-binding activity of PARP-1. Densitometry analysis of PARP-1 immunoblots showing the amounts of PARP-1 present in chromatin-bound (pellet) and unbound (supernatant) fractions obtained in the presence of Triton X-100 and different ionic strengths. PARP-1 values in each fraction was normalized to the levels found in a whole cell lysate.

Inhibitor II impairs the activity of PARP-1 by an alloesteric mechanism. DNA binding enhances by 100 fold the catalytic activity of PARP-1 by inducing a conformational change in the protein. Inhibitor II ejects zinc from the zinc finger domains removing the ability of PARP1 to bind to DNA and therefore the allosteric stimulation of the catalytic activity. This effect results in inhibition of the enzymatic activity of PARP-1. To exclude an effect of PARP-1 enzymatic inhibition on its ability to interact with chromatin we evaluated the effect of Oleparib on PARP-1 chromatin binding. This compound also inhibits the catalytic activity of PARP-1 without affecting the function of zinc fingers. In contrast to inhibitor II, Oleparib competes with NAD for binding to the catalytic site of

PARP-1. Importantly, our laboratory as evidenced that Oleparib does not affect the anti-HIV-1 activity of PARP-1.

SupT1 cells were treated with Oleparib or DMSO and then PARP-1 chromatin-binding activity was determined (Fig. 9a and b).

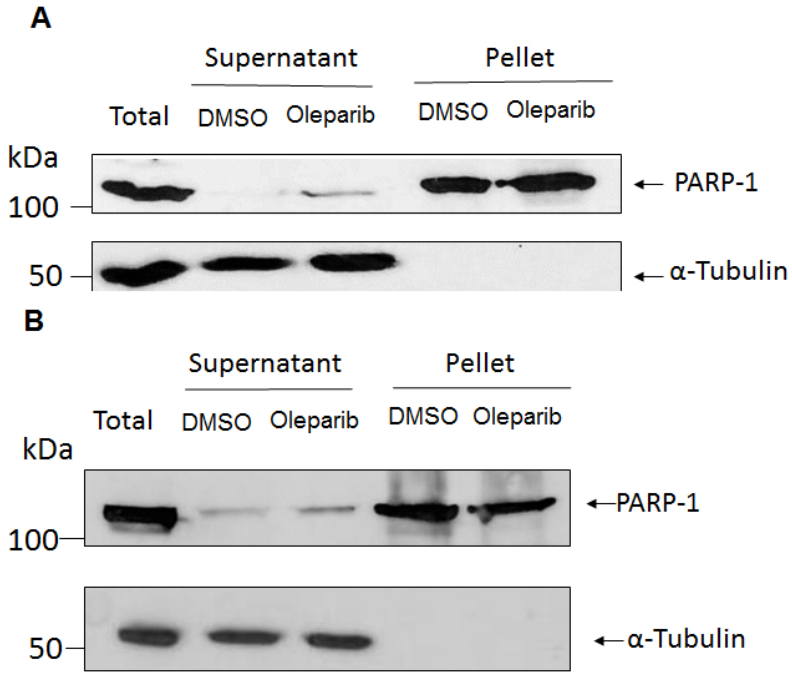

Figure 9 A and B. Effect of PARP-1 inhibition on its chromatin-binding activity. SupT1 cells were treated with DMSO or Oleparib (PARP-1 inhibitor) for 30 min and then lysed in a buffer containing 1% of Triton X-100. Cell lysates were centrifuged and a pellet (chromatin bound proteins) and a supernatant (chromatin non-bound proteins) fractions were obtained. A whole cell lysate (Total) was obtained by lysing the cells in Laemli buffer. PARP-1 and α-tubulin were detected in the different samples by immunobloting. Results from two independent experiments are represented.

In these experiments we observed that Oleparib decreased the chromatin-binding of PARP-1, suggesting that the effect of inhibitor II was independent of its effect on the catalytic activity of PARP-1. Densitometry analysis of results from two independent experiments indicate an increase of chromatin non-bound PARP-1 of 7.2 and 1.5 folds,

respectively, as compared to the amounts detected in DMSO-treated cells (Fig. 10). Our results with Oleparib-tretaed cells are in contradiction with *in vitro* experiments indicating that PARylation of PARP-1 detach this protein from nucleosomes.

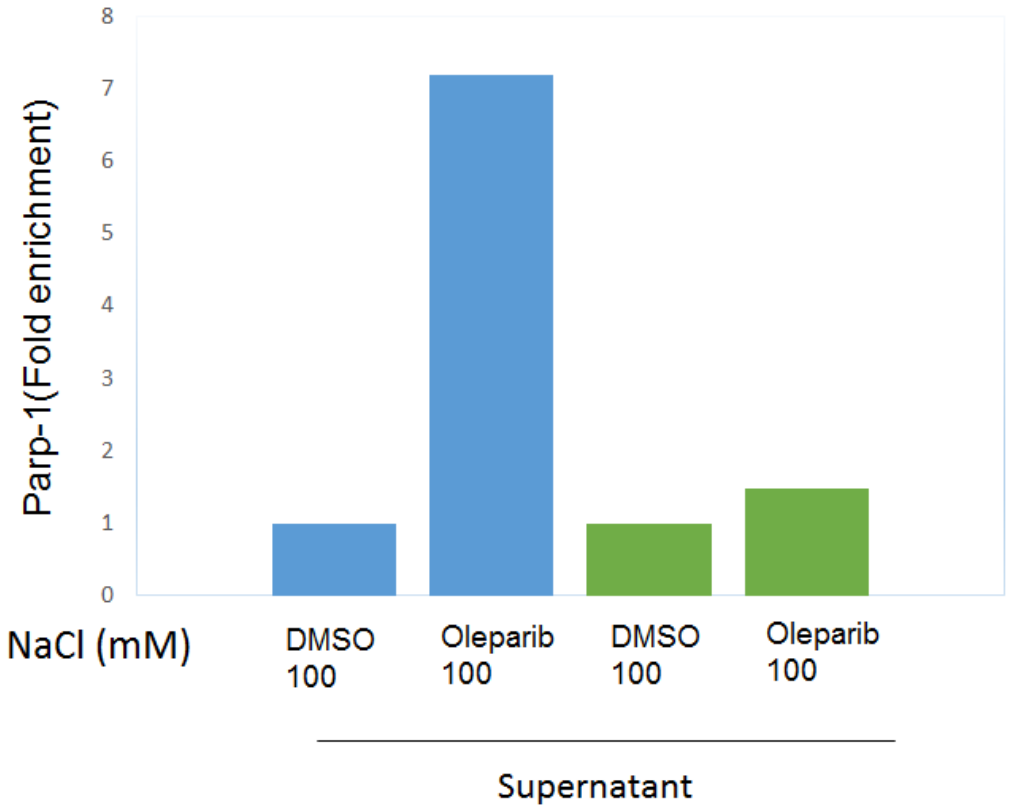

Figure 10. Effect of PARP-1 inhibition on its chromatin-binding activity. Densitometry analysis of immunoblots detecting PARP-1 in the chromatin non-bound fraction of cells treated with Oleparib or DMSO (vehicle). Two independent experiments are represented.

Effect of mutations on PARP-1 subcellular distribution. To better define the cellular localization of PARP-1 and to evaluate whether the subcellular distribution correlates with its anti-HIV-1 activity, we determined the localization of PARP-1 full-length, N-terminus, C-terminus, and mutants lacking zinc fingers I and III, and NLS of the protein.

We transiently expressed this panel of PARP-1 proteins in PARP-1 KO HEK293T cells and determined their subcellular distribution by indirect immunofluorescence assay combined with confocal microscopy analysis (Fig. 11). These experiments indicated that all these proteins, except for the mutant lacking the NLS, were exclusively localized in the nucleus. Destruction of the NLS, however, determined complete cytoplasm localization of the protein, as expected (Fig. 11g).

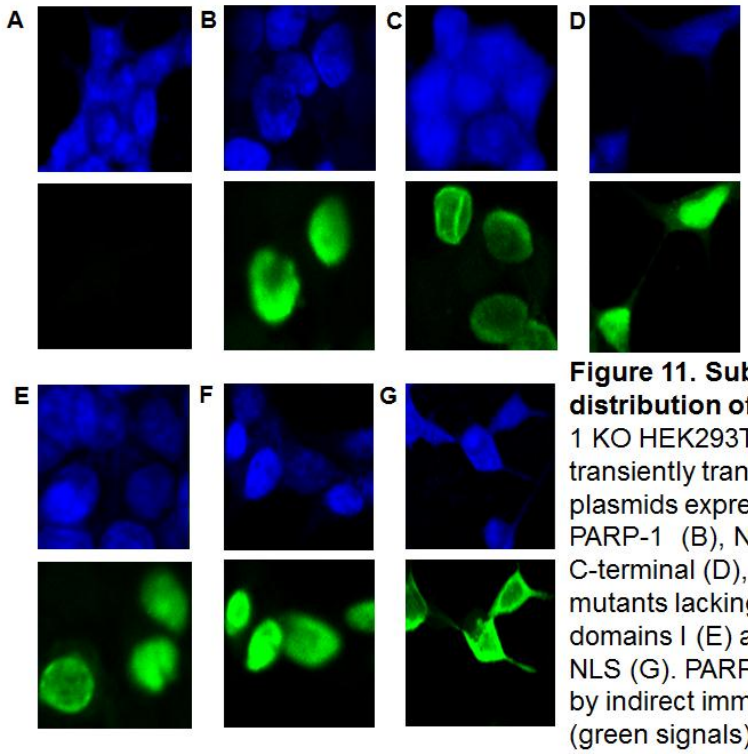

Figure 11. Subcellular distribution of PARP-1. PARP-1 KO HEK293T cells were transiently transfected with plasmids expressing full length PARP-1 (B), N-terminal (C), C-terminal (D), and deletion mutants lacking Zinc finger domains I (E) and III (F), and NLS (G). PARP-1 was detected by indirect immunofluorescence (green signals). Nuclei were stained with DAPI (Blue signals).

Surprisingly, our chromatin-binding activity assay indicated that PARP-1 ΔNLS was tightly bound to chromatin, similarly to the full-length protein, highlighting a contradiction with the subcellular distribution of this mutant. Potentially, the apparent discrepancy between chromatin-binding and subcellular distribution is due to the experimental model

used. Cells used for chromatin-binding assay express the PARP-1 mutant stably whereas the subcellular localization experiments were done with cells transiently expressing the mutant proteins. In stably expressing cells PARP-1 ΔNLS could interact with chromatin during the mix of cytoplasm and nuclear contents that occur during mitosis. This interaction could determine sequestration of the PARP-1 ΔNLS to the nucleus via chromatin interaction. A similar mechanism of chromatin abduction has been described for chromatin-bound protein LEDGF/p75 mutants lacking the NLS (52). However, this process of chromatin-mediated sequestration will not have occurred efficiently in cells transiently expressing the mutant because of insufficient amount of time. In the immunofluorescence experiments cells were analyzed 72 hrs after transfection. Previous observations from our lab (unpublished data) using plasmids expressing fluorescent proteins indicated that expression of the fluorescence reporters occur after 24 hrs of transfection. Furthermore, the doubling time for HEK293T has been reported to be 20 hrs. Then, these data suggest that in our experiments there was not sufficient amount of time for the cells to express the protein and go through rounds of cell division leading to the nuclear accumulation of the mutant. In summary, these studies indicate no correlation between the subcellular distribution of PARP-1 and its anti-HIV-1 activity demonstrating that nuclear residence is not sufficient for the anti-viral effect.

CONCLUSIONS

Results in specific aim 2 demonstrated that DNA binding of PARP-1 but not its interaction with chromatin-associated proteins or its nuclear residency directly correlate with the anti-HIV-1 activity of PARP-1. Furthermore, I have demonstrated that chromatin binding of PARP-1 is not importantly influenced by its DNA binding activity. The inability of the deletion of several individual domains located in the N-terminus to affect chromatin-binding activity of PARP-1 suggests that more than one domain could be implicated in this PARP-1 function.

Future direction: Characterization of the contribution of zinc finger II to the chromatin-binding activity of PARP-1 and of the three zinc finger domains to the anti-HIV-1 activity of PARP-1 should be accomplished. Data from our laboratory indicating that inhibitor II impairs the anti-HIV-1 activity of PARP-1 suggest that zinc fingers will be required for the anti-viral activity of this protein.

DISCUSSION

Previous studies from our lab have proved that PARP-1 plays a negative role in HIV-1 replication when the virus enters via CD4/CXCR4 receptor. This anti-HIV-1 activity however, has not been studied in HIV-1 strains that enter through the CD4/CCR5 pathway. Initially my thesis was going to focus on determining the role PARP-1 in the replication of HIV-1 strains utilizing this alternative entry pathway. We selected a THP-1 for these studies. Unfortunately, THP-1 did not grow at low cell density conditions. This characteristic determined that we were unable to separate clones fully KO from partially KO and WT clones. Due to these technical difficulties I never was able to generate a PARP-1 KO in THP-1 and then this question was unable to be answered.

Nevertheless, as a result of the research conducted, I learned different laboratory techniques that I applied to answer a different research question that became the new thesis focus. I determined the potential correlation of PARP-1 chromatin binding activity and subcellular localization with the anti-HIV-1 activity of this protein. Although some studies have been conducted to determine the role that PARP-1 plays in chromatin and DNA binding, these have been performed with *in vitro* methodologies using purify proteins and DNA. However, the relevance of the mechanisms discovered in intact cells is not very well established. Therefore, I defined the role of different PARP-1 functional domains in its chromatin binding activity and subcellular localization in cells. I used a cell fractionation methodology that allows the isolation of fractions enriched in chromatin-bound and non-bound proteins. Cells lysed in a buffer containing Triton X-100 and salts at a physiological ionic strength (i.e. 100 mM NaCl) can be separated by low speed centrifugation into a pellet enriched in chromatin-bound proteins and a

supernatant that contain proteins that under physiological conditions are non-bound to chromatin. Using this methodology I defined that the N-terminus of PARP-1 is sufficient and required for chromatin binding but the C-terminus was dispensable. Subcellular distribution analysis of these mutants, however, indicates that these proteins were present in the nucleus of the cell. Detailed mutagenesis evaluation indicated that deletion of the individual domains in the N-terminus, including zinc fingers I and III, the BRCT region, and the NLS did not impair chromatin binding. Localization of these mutants was nuclear except for the protein lacking the NLS. This mutant was almost exclusively found in the cytoplasm of cells transiently transfected. Apparently, there is a contradiction between the chromatin-binding and cytoplasmic localization for this mutant. However, this contradiction is resolved by explaining that in cells stably expressing the protein during the mixing of the nuclear and cytoplasmic content that occurs during mitosis, the cytoplasmic PARP-1 binds to chromosomes and is actively sequester into the nucleus. A similar mechanism of nuclear trapping was described for a LEDGF/p75 lacking the NLS. LEDGF/p75 is a tightly bound chromatin protein. The C-terminus, although lacking a NLS, had the ability to locate inside the nucleus. Although the exact mechanism in which this domain of the protein has the ability to do so, we believe that by lacking its entire N-terminal domain, its folding allows it to act as a completely different protein and piggybacks with other proteins that have a NLS.

More importantly, my data indicate that chromatin-binding but not merely nuclear residence is required for PARP-1 to affect HIV-1 replication. PARP-1 mutants that interact with chromatin, such as N-terminal domain and mutants lacking zinc fingers I and III, the BRCT region, and the NLS, were able to impair HIV-1 replication similarly to

wild type PARP-1. In contrast the C-terminus of PARP-1 that was localized to the nucleus but not bound to chromatin failed to reduce HIV-1 replication. In conclusion, chromatin binding of PARP-1 is required and sufficient for its negative role in HIV-1 replication.

Evaluation of the effect of the zinc ejector inhibitor II that specifically impair the DNA binding activity of PARP-1 indicated that DNA binding does not significantly contribute to its interaction with chromatin-bound proteins. The fact that inhibitor II impairs the anti-HIV-1 activity of PARP-1 indicates that DNA binding rather than interaction with chromatin-associated proteins is required for the anti-HIV-1 activity of PARP-1. Moreover, the N-terminal region, that lacks catalytic activity, impaired HIV-1 replication. Then, these data indicated that the enzymatic activity of PARP-1 is dispensable and therefore the effect of inhibitor II on HIV-1 replication seems to be catalytic independent. It has been demonstrated in in vitro studies that PARP-1 binds to TAR competing with Tat for binding to this HIV-1 RNA regulatory element. Tat overcomes PARP-1 competition by forming a complex with P-TEFb that increases the affinity of Tat for TAR by 10-fold. Therefore, it is possible that this mechanism is operating in the effect of PARP-1 on HIV-1 replication.

Based on my and results from our laboratory and other laboratories, I propose the following mechanism of action: Interaction of HIV-1 Env with the CD4/co-receptor during viral entry triggers a signaling event that enhances the ability of PARP-1 to interact with HIV-1 TAR competing with Tat. This effect impairs HIV-1 transcription elongation early during infection delaying HIV-1 replication. HIV-1 can still replicate in the presence of PARP-1 potentially due to the increase in the amount of Tat over time.

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SUPPLEMENTAL DATA

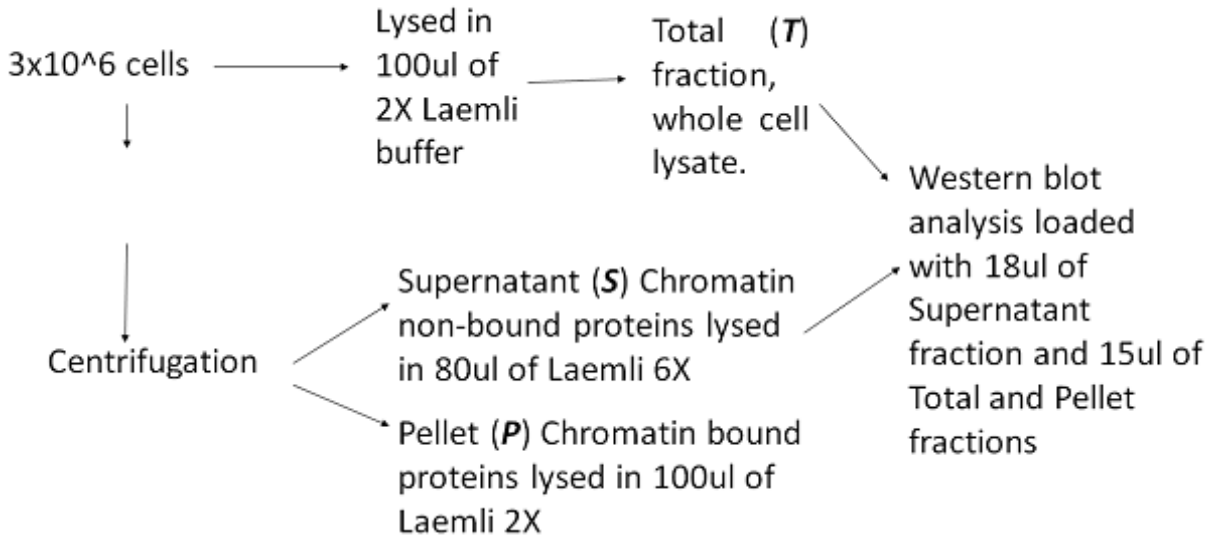

Figure 12. Cell Fractionation. 3X106^ cells were lysed to obtain either Total or Supernatant and Pellet fraction. These were analyzed by Western Blot, gel was loaded with 18ul of Supernatant fraction, since they were lysed in a smaller volume of lysis buffer, and 15ul of Pellet and Total fraction.

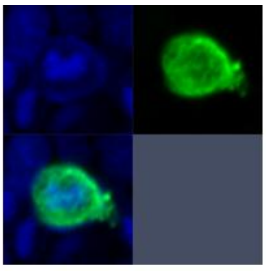

Figure 13. ΔNLS mutant co-localizing with DAPI inside the nucleus, as well as cytoplasmic.

VITA

My name is Sara Raquel Garcia and was born in El Paso, Tx. I am currently enrolled in a Master of Science program in Biological Sciences at the University of Texas of El Paso. I initiated this program in June of 2016 and completed my thesis titled "Investigating the role of PARP-1 in HIV-1 replication. During my Master's program, I worked from August 2016 to December 2017 as a Teaching Assistant teaching Anatomy and Physiology I Lab. My duties included preparing the class, teaching the class and grading the students. From June 2016- present I have been a Graduate Research Assistant in which my duties included assisting in laboratory experiments to investigate host factors involved in HIV-1 replication. I also performed a lot of Tissue and Cell Culture work, measured viral replication by ELISA, concentrated virus by ultracentrifugation, performed Western Blots, Polymerase Chain Reaction assays, Immunofluorescence measurement by Flow Cytometry and Confocal microscopy, Agarose gel electrophoresis, and other laboratory techniques

My previous degree is a Bachelor's of Science in Veterinary Medicine and Animal Husbandry from the Universidad Autonoma de Cd. Juarez at Cd. Juarez, Chihuahua, Mexico. I completed this program in December of 2012.