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Determining The Role Of Poly [adp-Ribose] Polymerase 1 (parp-1) In Retroviral Infection

Luis Valdes Rodriguez

University of Texas at El Paso, lvaldesrodriguez@utep.edu

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DETERMINING THE ROLE OF POLY [ADP-RIBOSE] POLYMERASE 1 (PARP-1) IN RETROVIRAL INFECTION

LUIS VALDES

Doctoral Program in Biological Sciences

APPROVED:

Manuel Llano, M.D./ Ph.D., Chair

Robert A. Kirken, Ph.D.

German Rosas-Acosta, Ph.D.

Juan C. Noveron, Ph.D.

Kristine M. Garza, Ph.D.

Charles Ambler, Ph.D.
Dean of the Graduate School

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Dedication

To my loving and supportive family. Thank you for always being there for me.

DETERMINING THE ROLE OF POLY [ADP-RIBOSE] POLYMERASE 1 (PARP-1) IN RETROVIRAL INFECTION

by

LUIS VALDES, B.S.

DISSERTATION

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The University of Texas at El Paso
in Partial Fulfillment
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First and foremost I want to thank God for leading me in the right direction down many winding roads of my life. He has guided me in every way, He has provided me with a wonderful family and He has always surrounded me with the right people at the right moment. He is the reason for all things and I am deeply thankful.

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Abstract

Possible links between PARP-1 and HIV-1 have been previously reported but the role of this protein during viral infection has remained elusive. Based on the high evolutionary conservation of PARP-1, from *Drosophila* to humans, we have been able to counteract the difficulties related with the functional redundancy manifested in the PARP family by studying the influence of PARP-1 on the expression of an endogenous retrovirus using the chicken B lymphoblastoid cell line DT40, this cell line exhibits low PARP-1 functional redundancy and is viable after PARP-1 knockout. We have determined that the transcription level of the endogenous retrovirus RAV-1 is significantly higher in PARP-1 KO cells than in PARP-1 WT cells but the WT phenotype is restored when PARP-1 KO cells are engineered to re-express human PARP-1 (PARP-1 h-1 cells). Such results suggest that PARP-1 is a cell factor with a negative influence in retroviral transcription and led us to investigate the role of this protein in HIV replication within human cells.

Using PARP-1 KD and control human CD4⁺ T cell lines we have been able to demonstrate that PARP-1 deficiency enhances the replication of the HIV-1. The effect of PARP-1 deficiency in HIV-1 replication in human cells was observed upon pharmacological inhibition of PARP-1. Importantly, inhibitors targeting the zinc finger domains of PARP-1, implicated in DNA binding, but not those inhibiting its catalytic activity caused an enhancement of HIV replication. These results highlight the relevance of the DNA binding domain of PARP-1 in this effect on HIV-1 replication. Intriguingly, in contrast to its effect on HIV-1 replication, PARP-1 antagonism was not affected by a single-round of infection with an HIV-1 lentiviral vector. These replication incompetent viruses only

recapitulate the early events of the viral life cycle, leading us to suggest that PARP-1 does not play a role during the early stages of the HIV life cycle.

Considering that primary CD4⁺ T cells are the natural target of the HIV-1 virus, we finally decided to study the role of PARP-1 in HIV-1 replication in these cells. The use of PARP-1 inhibitors and a novel cell model to study HIV latency, allowed us to conclude that in primary cells the presence of PARP-1 negatively modulates HIV-1 replication. This finding implies that PARP-1 could be a restriction factor for HIV replication in human cells.

Our data indicates the possibility of using PARP-1 as a new point of therapeutic intervention against the HIV virus in humans.

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CHAPTER 1: Introduction

1.1. HIV Overview

Acquired Immune Deficiency Syndrome (AIDS) was first recognized as a new disease in 1981 when increasing numbers of young homosexual men succumbed to unusual opportunistic infections and rare malignancies[1]. A retrovirus, now termed human immunodeficiency virus (HIV), was subsequently identified as the causative agent of what has since become one of the most devastating infectious diseases to have emerged in recent history [2, 3]. Currently there are more than 35 million people living with HIV and nearly 39 million people with AIDS have died worldwide since the epidemic began.

Human immunodeficiency virus isolates are currently grouped into two types, HIV-type 1 (HIV-1) and HIV-type 2 (HIV-2). The worldwide main agent of AIDS is HIV-1, while HIV-2 is restricted to some regions of Western and Central Africa[4].

HIV is a genetically related member of the Lentivirus genus of the *Retroviridae* family. This family has a unique enzyme called reverse transcriptase (RT) that converts viral RNA into DNA upon viral entry into the cell. The genome of HIV-1 contains two identical copies of single stranded positive RNA molecules which encode nine open reading frames (Figure 1.1) that produce 15 proteins. Structural proteins ensemble with two copies of the viral RNA genome into an enveloped virus of 0.1 microns in diameter (Figure 1.2).



Figure 1.1 Depiction of the HIV nine open reading frames[5]

The *gag* gene encodes the structural proteins of the core (p24, p7, p6) and matrix (p17), the *env* gene encodes the viral envelope glycoproteins gp120 and gp41, which recognize cell surface receptors, and the *pol* gene encodes for enzymes crucial in viral replication, which are the reverse transcriptase that converts viral RNA into DNA, the integrase (IN) that incorporates the viral DNA into host chromosomal DNA and the protease (PR) that cleaves large Gag and Pol protein precursors into their components. HIV-1 encodes six additional proteins; Tat and Rev, two regulatory proteins, and four so called accessory proteins, Vif, Vpr, Vpu and Nef [6]. Tat protein is expressed very early after infection and promotes the expression of HIV genes by facilitating transcriptional elongation while Rev ensures the export from nucleus to cytoplasm of the correctly processed viral messengers and genomic RNA. The function of the other accessory HIV proteins is less well understood; it is believed that the Vpr protein is involved in the arrest of the cell cycle and counteraction of the HIV-1 restriction factor SAMHD1. This protein also enables the reverse transcribed DNA to gain access to the nucleus in non-dividing cells such as macrophages. Vpu is a protein necessary for the correct release of virus particle by counteracting the antiviral protein tetherin, whereas Vif enhances the infectiveness of progeny virus particles by triggering degradation of the antiviral protein

APOBEC3G. Finally, the Nef protein has multiple functions including cellular signal transduction and the down regulation of the CD4 receptor on the cell surface to allow virus budding in the late stages of the virus replication cycle.

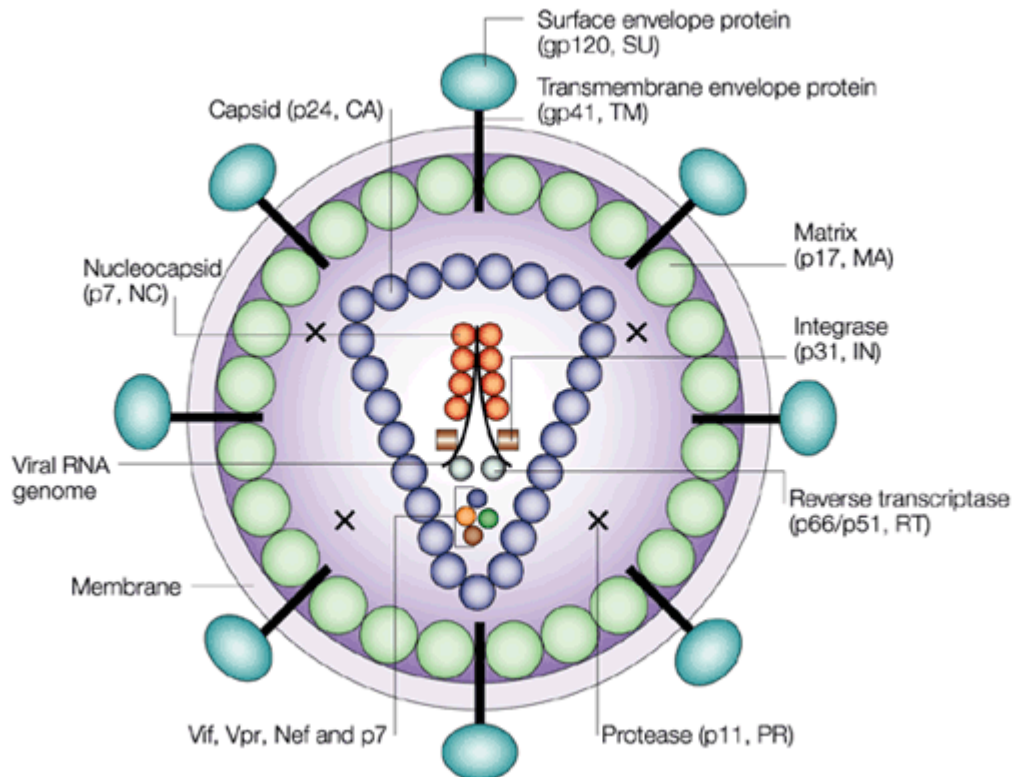


Figure 1.2 Schematic of HIV-1 [5]

HIV viral particles contain two copies of non-covalently linked, unspliced, positive-sense single-stranded RNA molecules associated with structural and accessory proteins. Structural proteins are encoded by long open reading frames: Gag (MA, CA, SP1, NC, SP2, and P6), Env (gp41, gp120), and Pol (PR, RT, IN), while accessory and regulatory proteins are encoded by smaller open reading frames: Vif, Vpr, Nef, Tat, Rev and Vpu.

1.2. HIV Life Cycle

The life cycle of HIV-1 can be divided into two phases: the early stage occurs between entry into the host cell and integration into its genome, and the late phase occurs from the state of integrated provirus to full viral replication (Figure 1.3).

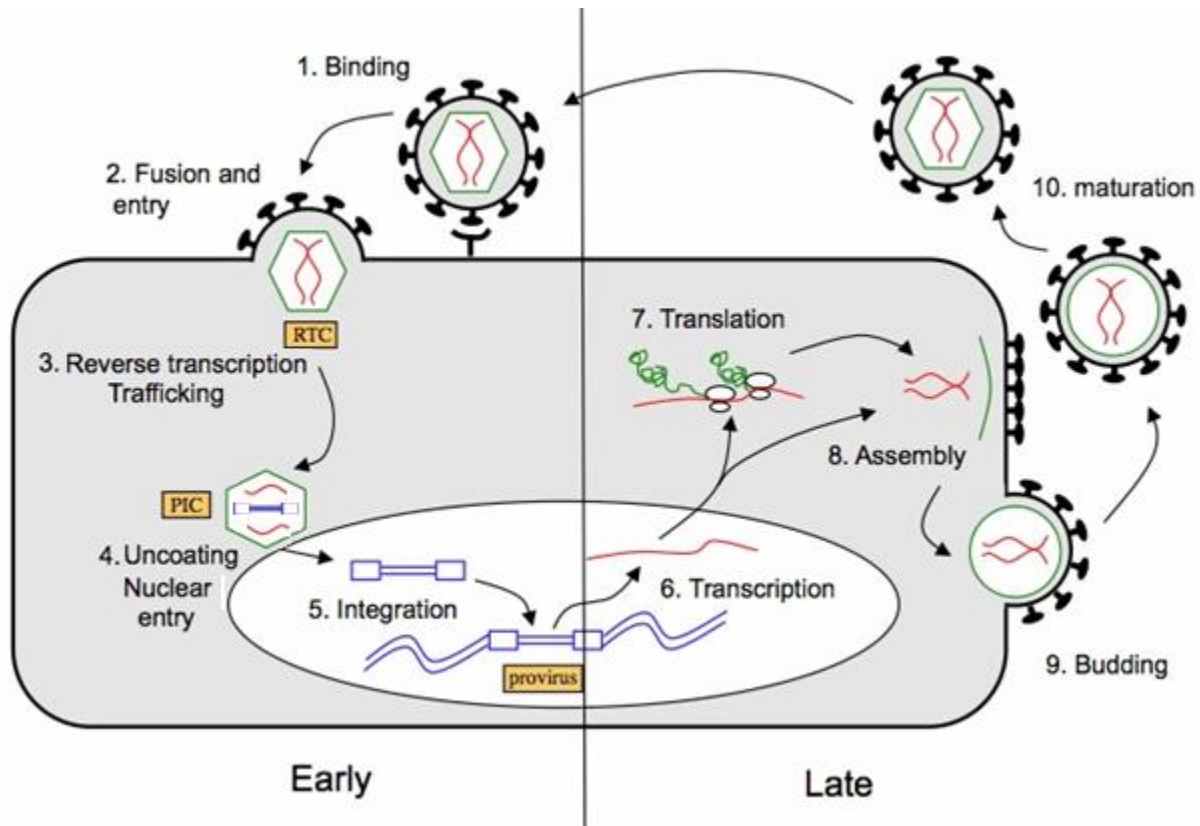


Figure 1.3 The HIV life cycle [7].

The early events of HIV-1 replication in infected cells include binding of the virus to a target cell, fusion and entry of the viral core into the cytosol, reverse transcription of the viral genome to produce a viral copy cDNA, uncoating of the viral core and nuclear entry, followed by integration of the viral genome into the host chromosome. The late phase of the cycle consists of transcription of the integrated provirus, transport of spliced and unspliced viral RNA molecules to the cytosol, translation of the viral RNA to produce viral proteins, assembly of these proteins with unspliced viral RNA molecules, budding of new immature viral particles from the cells and finally the maturation of these particles to produce replication competent viral particles.

Viral entry involves the fusion of viral and cellular membranes through successive interactions with CD4 and CXC chemokine receptor type 4 (CXCR4) or CC chemokine receptor type 5 (CCR5) [8]. These interactions induce conformational changes in gp120 and gp41 that lead to the exposure of the fusion peptide at the N-terminus of gp41. The fusion peptide insertion into the target membrane triggers the fusion of viral and host lipid bilayers and subsequent release of the viral core into the target cell cytoplasm. Once in the cell cytosol the virus is faced with the challenge of reverse transcribing the viral RNA with the concert of RT and transporting the proviral precursor to the nucleus for integration. In this process, the viral core is rearranged to form the reverse transcription complex (RTC). This results in changes to the viral protein composition of the original core and is facilitated by host cell protein involvement. Completion of RTC maturation and reverse transcription of the viral genome generates the pre-integration complex (PIC) containing a linear double-stranded cDNA (dscDNA) molecule with terminal direct repeats and blunt ends[9], which is integration competent.

HIV uncoating, defined as the loss of viral capsid that occurs at the nuclear envelope, is also an obligatory step that accompanies the transition between RTCs and PICs. The study of the nature and timing of HIV-1 uncoating has been paved with difficulties, particularly as a result of the vulnerability of the capsid assembly to experimental manipulation. Nevertheless, recent studies of capsid structure, retroviral restriction and mechanisms of nuclear import, as well as the recent expansion of technical advances in genome-wide studies and cell imagery approaches, have substantially changed our understanding of HIV uncoating. Although early work suggested that uncoating occurs immediately following viral entry in the cell, thus attributing a trivial role

for the capsid in infected cells, recent data suggest that uncoating occurs several hours later, in close proximity to the nuclear pore, and that capsid has an all-important role in the cell that it infects: for transport towards the nucleus, reverse transcription and nuclear import [10].

Viral trafficking of the PIC is mediated through microtubules by retrograde transport and uses dynein [11]. At the nuclear pore complex, the PIC enables the transport of dscDNA through the pore, thereby allowing the infection of resting, non-dividing cells. Inside the nucleus, the viral dscDNA either integrates into the host cell chromosomes or remains in an unintegrated state, like linear cDNA or DNA circles[12]. Host factors such as LEDGF/p75 are key molecules that facilitate HIV-1 integration. LEDGF is a transcriptional co-activator that binds HIV IN and acts as a tethering factor to promote viral integration [13]. After integration, the LTR-flanked provirus behaves as a cellular gene: the 5' LTR operates like any eukaryotic promoter and the 3' LTR acts as the polyadenylation and termination site [14].

Activation of the T cell induces binding of the transcriptional pre-initiation complex to enhancer elements in the 5' LTR proximal promoter. This complex gathers essential host transcription factors, such as nuclear factor- κ B (NF- κ B)[15], nuclear factor of activated T cells (NFAT)[16], and specificity protein 1 (SP1)[17], among others. These enhancer proteins transmit activation signals to basal factors belonging to the general transcription machinery and promote binding of RNA polymerase II (RNAPII) to the TATA box to initiate mRNA transcription. A 59-nucleotide stem-loop structure termed the transactivation response element (TAR) is then formed at the 5' end of the nascent viral transcript, creating a binding site for the viral trans-activator Tat [18]. The Tat–TAR

interaction promotes efficient elongation of viral transcripts by recruiting cellular factors that increase the functional capacity of RNAPII, such as positive transcription elongation factor b (PTEFb), which is composed of cyclin-dependent kinase 9 (CDK9) and cyclin T1 [19, 20]. Efficient elongation of viral transcripts allows the synthesis of mRNA, which is further processed by the regulatory protein Rev. Rev is a viral RNA-binding factor that regulates the nucleo-cytosolic transport and splicing of viral mRNA species [21].

Once new viral proteins are synthesized in the cytosol of the infected cell, the last event in the HIV life cycle, called virion morphogenesis takes place. This essential step can be divided into three stages: assembly, wherein the virion is created and essential components are packaged; budding, wherein the virion crosses the plasma membrane and obtains its lipid envelope; and maturation, wherein the virion changes structure and becomes infectious [22].

HIV-1 virion assembly occurs at the plasma membrane, within specialized membrane microdomains. The HIV-1 Gag (and Gag-Pro-Pol) polyprotein itself mediates all of the essential events in virion assembly, including binding the plasma membrane, making the protein–protein interactions necessary to create spherical particles, concentrating the viral Env protein, and packaging the genomic RNA via direct interactions with the RNA packaging sequence (termed Ψ). These events all appear to occur simultaneously at the plasma membrane, where conformational change(s) within Gag couples membrane binding, virion assembly, and RNA packaging. Although Gag itself can bind membranes and assemble into spherical particles, the budding event that releases the virion from the plasma membrane is mediated by the host ESCRT (endosomal sorting complexes required for transport) machinery [22].

The virion acquires its lipid envelope and Env protein spikes as it buds from the plasma membrane. Unlike Gag, Env is an integral membrane protein. It is inserted co-translationally into ER membranes and then travels through the cellular secretory pathway where it is glycosylated, assembled into trimeric complexes, processed into the trans-membrane (TM; gp41) and surface (SU; gp120) subunits by the cellular protease furin, and delivered to the plasma membrane via vesicular transport [22].

The Gag polyprotein initially assembles into spherical immature particles, in which the membrane-bound Gag molecules project radially toward the virion interior. As the immature virion buds, PR is activated and cleaves Gag into its constituent MA, CA, NC, and p6 proteins, thereby also releasing the spacer peptides 1 (SP1) and SP2. Proteolysis is required for conversion of the immature virion into its mature infectious form. Like other retroviral proteases, HIV-1 PR is a dimeric aspartic protease that recognizes specific sites within Gag and cleaves them in an ordered fashion. Gag proteolysis triggers major changes which include condensing and stabilizing the dimeric RNA genome, assembling the conical capsid about the genomic RNA–nucleocapsid-enzyme complex, and preparing the virion to enter, replicate, and uncoat in the next host cell. Thus, viral maturation can be viewed as the switch that converts the virion from a particle that can assemble and bud from a producer cell into a particle that can enter and replicate in a new host cell [23].

1.3. PARP-1 Structure and Functions

ADP-ribosyltransferases (ARTs) comprise a family of structurally conserved enzymes that catalytically cleave NAD⁺ and transfer the ADP-ribose moiety to acceptor residues of target proteins [24]. Poly (ADP-ribosyl) polymerases (PARPs) are a subset of

the ART family that continue this reaction to create long chains of linear and/or branched poly (ADP-ribose) (PAR) (Figure 1.4) (Table . 1.1). Currently, only the first six members of this family (ARTs 1–6) are regarded as having poly ADP-ribosylation activity: PARP-1, PARP-2, PARP-3, PARP-4, PARP-5a, and PARP-5b (Figure 1.5). The remaining ARTs 7–17, although originally considered PARPs (PARPs 6–16) [25], are only capable of producing mono-ADP-ribose modifications and are referred to as mono-ARTs (MARTs) [26].

Poly (ADP-ribose) polymerases are multi-domain proteins that are related through their highly conserved ART domain. Outside of the ART domain, distinct domain architectures quickly differentiate the structure and function of each PARP. The catalytic domain crystal structures have been solved for all current PARPs except for PARP-4. The crystal structures of some non-catalytic domains of PARPs have been solved, although there is no crystallographic data on any full-length PARP [27].

PARP-1 and PARP-3 are the only PARPs for which structures of all domains are known. PARP-1 is a 116 kDa protein (1014 amino acids in mice and humans) with a modular domain architecture comprising three main domains: an N-terminal DNA-binding domain (DBD), a central automodification domain and a C-terminal catalytic domain. The DBD contains three zinc-fingers (Zn1, Zn2, and Zn3), which have different roles in DNA binding, interdomain cooperation, chromatin compaction and protein–protein interactions, and a nuclear localization signal (NLS). In the central automodification domain glutamate, aspartate and lysine residues serve as putative acceptors for auto (ADP-ribosyl)ation, a leucine zipper motif mediates homo- or heterodimerization and a breast cancer-associated protein C-terminal (BRCT) motif mediates protein–protein interactions.

Adjacent to the catalytic domain a tryptophan-, glycine-, and arginine-rich WGR domain has also been shown to be required for DNA-damage induced PAR synthesis. The catalytic domain contains the “PARP signature” sequence required for the catalysis of PAR synthesis (Figure 1.6) [28].

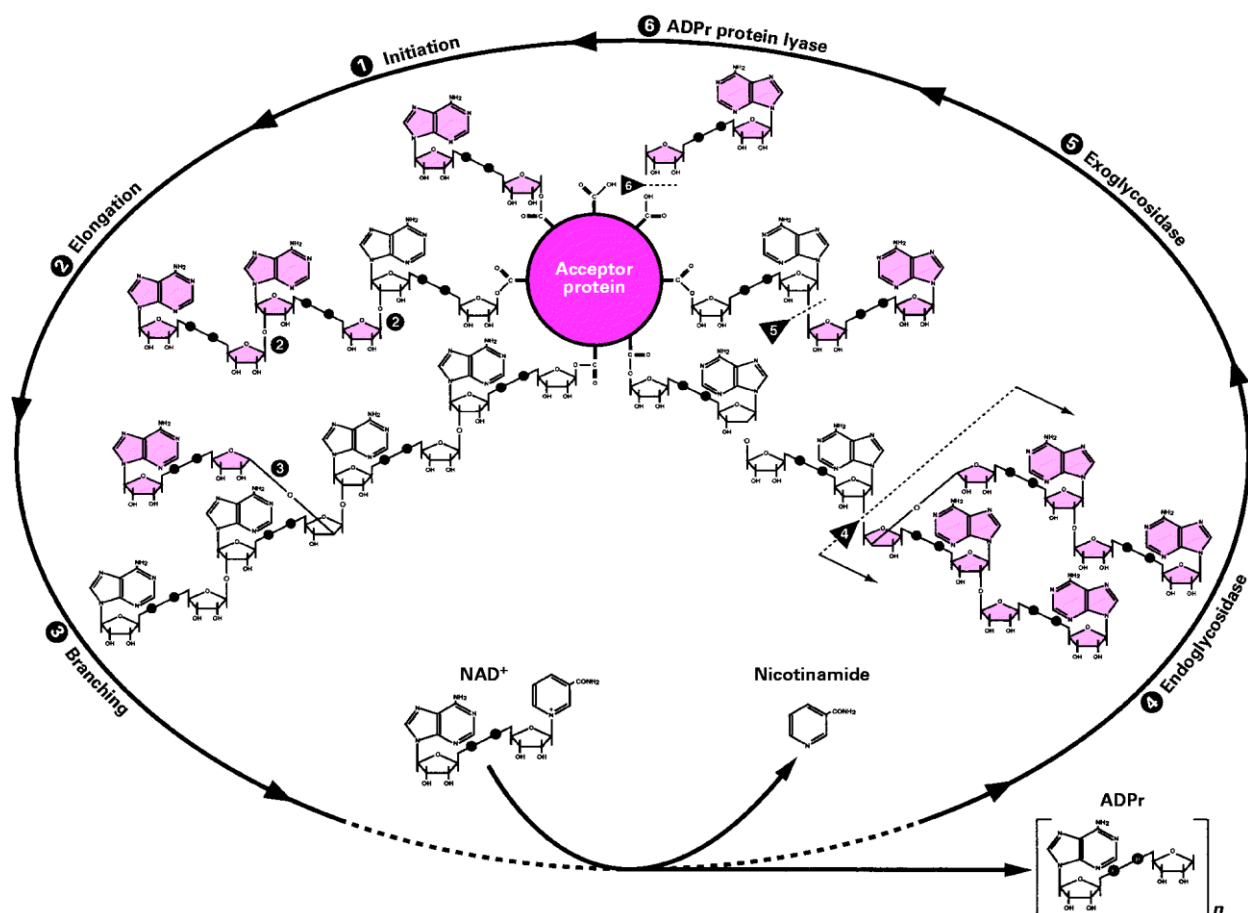


Figure 1.4 Poly (ADP) ribose (pADPr) metabolism [28]

PARP proteins use NAD⁺ as their substrate to modify acceptor proteins with adenosine diphosphate-ribose (ADPr) modifications while PARG is the major enzyme responsible for the catabolism of poly (ADP-ribose) polymers.

Table 1.1 PARP family member; enzymatic activities and functional domains [29]

The PARP family members differ not only in subcellular localization and structure but also in enzymatic activity since some of them are only capable of producing mono-ADP-ribose modifications (M) while others have poly ADP-ribosylation activity (P).

Member	Alternative name	Transferase name	Subcellular localization	Triad motif	Enzymatic activity	Functional domains
PARP1		ARTD1	nuclear	H-Y-E	P	Zinc-fingers, WGR, BRCT
PARP2		ARTD2	nuclear	H-Y-E	P	WGR
PARP3		ARTD3	nuclear	H-Y-E	P	WGR
PARP4	vPARP	ARTD4	cytosolic	H-Y-E	P	BRCT
PARP5A	Tankyrase 1	ARTD5	Nuclear cytosolic	H-Y-E	P	Ankyrin repeat, SAM
PARP5B	Tankyrase 2	ARTD6	Nuclear cytosolic	H-Y-E	P	Ankyrin repeat, SAM
PARP6		ARTD17	ND	H-Y-Y	M	
PARP7		ARTD14	ND	H-Y-I	M	Zinc-fingers, WWE
PARP8		ARTD16	ND	H-Y-I	M	
PARP9	BAL1	ARTD9	ND	Q-Y-T	M	Macrodomain
PARP10		ARTD10	Nuclear cytosolic	H-Y-I	M	
PARP11		ARTD11	ND	H-Y-I	M	WWE
PARP12		ARTD12	cytosolic	H-Y-I	M	Zinc-fingers
PARP13	ZAP1	ARTD13	cytosolic	H-Y-V	M	Zinc-fingers
PARP14	BAL2	ARTD8	cytosolic	H-Y-L	M	Macrodomian, WWE
PARP15	BAL3	ARTD7	cytosolic	H-Y-L	M	Macrodomian
PARP16		ARTD15	ND	H-Y-I	M	

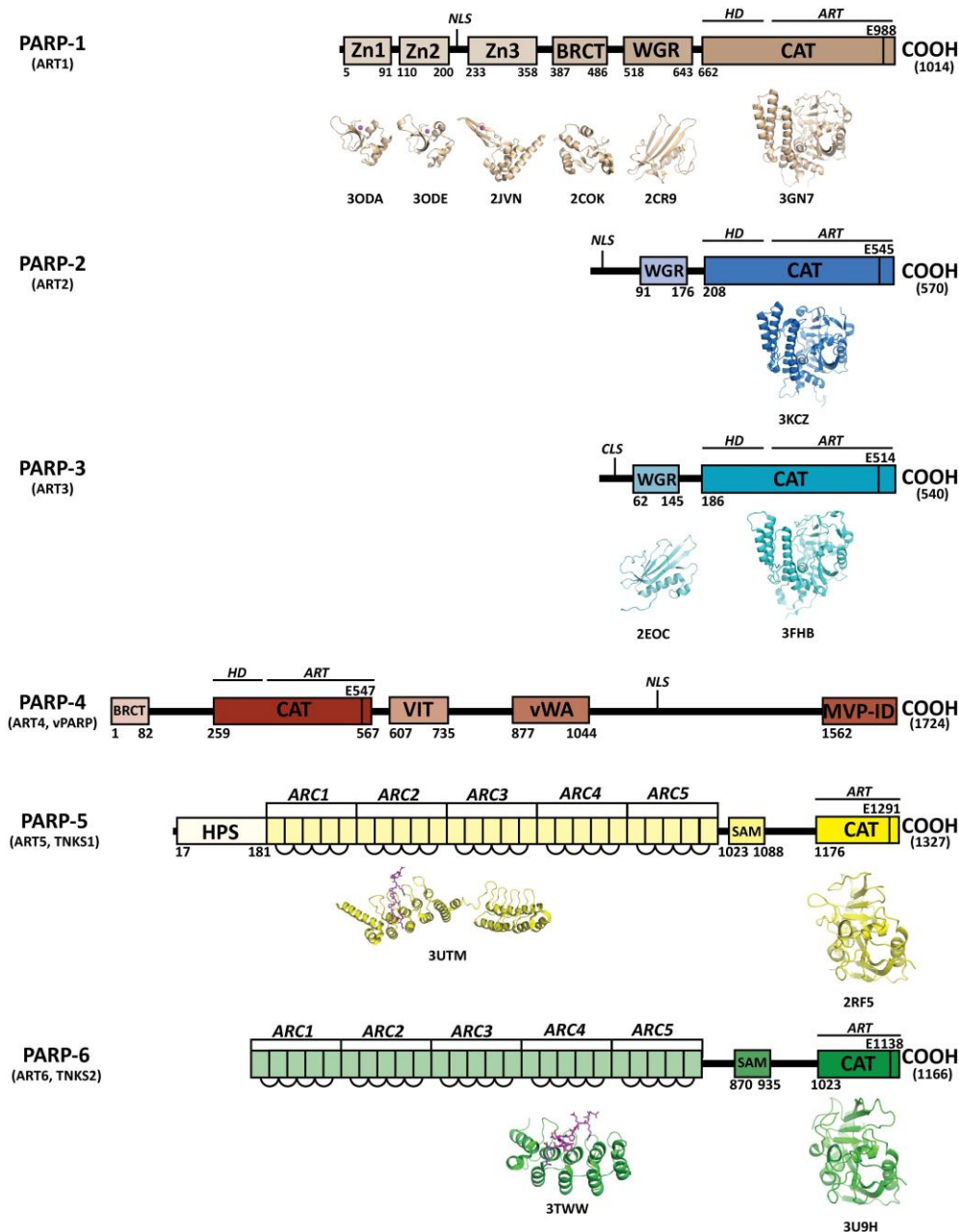


Figure 1.5 Domains of human PARPs [26]

PARP-1 domains have been crystalized as well as the catalytic and some non-catalytic domains of other PARPs except PARP-4. The ADP-ribosyltransferase domain (ART) is conserved among the family. PARPs 1–4 contain a helical domain (HD) that is implicated in allosteric regulation. PARPs 1–3 have a WGR domain related with DNA-dependent catalytic activation. Some other domains like the breast cancer susceptibility protein-1 C-terminus (BRCT) domain, found in DNA repair and checkpoint proteins, is present in PARP-1 and PARP-4. Zinc-fingers are part of the DNA binding domain of PARP-1. Zn1 and Zn2 of PARP-1 are important in binding DNA, while the third zinc-finger (Zn3) is important in DNA-dependent catalytic activation.

PARP-1, the best studied protein in the PARP family, is a protein that is found in most eukaryotes except for yeast [30]. After the histones, it is the most abundant nuclear protein (one molecule of enzyme per 1000 bp of DNA [31]), and its activity accounts for 90% of PAR production in the cell[32]. The pool of freely diffusible nucleoplasmic PARP-1 is very small, with most of the PARP1 protein bound to chromatin and accumulated in nucleoli [33-37]. The distribution of PARP1 in chromatin is nonrandom, occurring in characteristic profiles specific for distinct cell types[38].

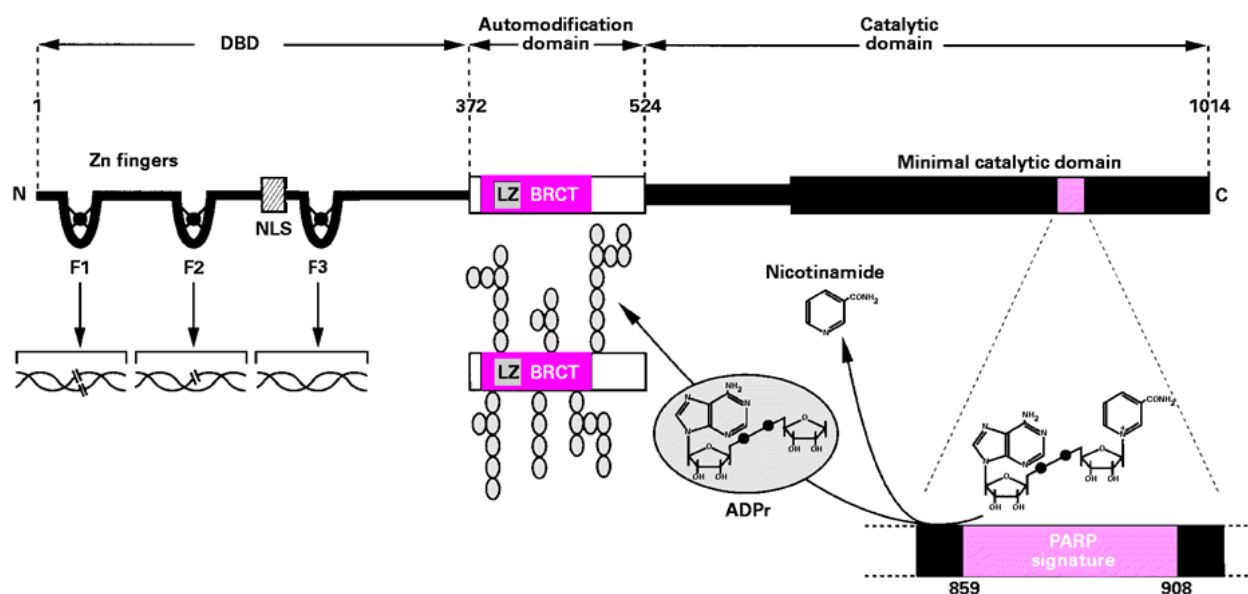


Figure 1.6 Molecular structure of PARP-1 [28]

The numbers relate to the boundaries of the domains in human PARP. NLS, nuclear localization signal; HTH, helix–turn–helix motif; LZ, leucine-zipper motif.

PARP-1 plays essential roles in the cell, including DNA repair, translation, transcription, telomere maintenance, and chromatin remodeling [38]. It regulates a broad range of essential nuclear events, including two complementary processes (1) regulation of protein–nucleic acid interactions by means of protein shuttling and (2) utilizing poly-ADP-ribose as an anionic matrix for trapping, recruiting, and scaffolding proteins [38].

PARP-1 is active as a dimer [39, 40]. ADP-ribose modification of its dimerization domain through automodification causes PARP-1 dimers to dissociate from both each other and active chromatin while losing enzymatic activity.

PARYlation carries out a plethora of cellular functions, which explains the high abundance of PARP-1 molecules in the cells; only a small fraction of them are present in their PARYlated form. The presence of numerous inactive PARP molecules guarantees an immediate response to cell signaling or DNA damage [41, 42]. In fact, when cells are adapting to stress, the level of long ADP-ribose polymers (pADPr) is dramatically increased; however, they are quickly degraded as their half-life goes down from 6–7 min to a few seconds [43].

DNA strand breaks remarkably increase basal activity of PARP-1 (up to 500 times) [44]. Compelling evidence suggest that PARP-1, through its physical association with or by poly (ADP-ribosyl)ation of partner proteins, regulates chromatin structure, DNA metabolism and gene expression.

Poly(ADP-ribose) polymerases acts in a wide variety of situations by locally derepressing gene transcription or other chromosomal processes that are blocked by the tight binding of histones and other inhibitory chromatin proteins. Two mechanisms by which PARP1 may loosen chromatin have been suggested by biochemical studies. First, PARP1 may catalyze the transfer of ADP-ribose moieties from NAD to abundant nuclear proteins, such as histones. Indeed, in vitro modification by pADPr destabilizes the interaction of chromatin components with DNA including histones [45, 46]. Second, the formation of long pADPr residues on PARP1 itself may contribute to loosening [36, 47]. Many chromatin proteins exhibit high affinity for pADPr and may relocate from chromatin and bind to automodified PARP1 at the sites of activation. Alternatively, the network of pADPr may generate an environment conducive to the formation of active loci, organizing the processes of transcribing, packing, and processing RNA. The observation that nucleoli disintegrate following PARP1 inhibition favors the latter model.

PARP1 can modify proteins both covalently and noncovalently; that is to say, a protein's function or localization can be dramatically changed, either by the covalent attachment of poly-ADP-ribose or noncovalent interaction with it. Approximately thirty proteins have been identified, both in vivo and in vitro, as covalent targets of pADPr[28]. These proteins include a broad range of components and regulators in key nuclear processes: modulating chromatin structure, DNA synthesis, DNA repair, transcription, cell cycle, and additional miscellaneous targets. Many proteins have been identified as having strong noncovalent affinity for pADPr proteins, including histones, hnRNPs and various others involved in DNA repair and checkpoints [48, 49]

PARP1 has also been found to poly-ADP-ribosylate TATA-binding protein (TBP), Yin Yang 1, NFkappaB, Sp1, and CREB, resulting in their inability to bind DNA [50]

1.4. PARP-1 DNA binding domain (DBD)

The N-terminal DBD extends from the initiator methionine to threonine 373 in human PARP-1[51]. This domain has a molecular mass of approximately 42 kDa and contains three zinc fingers (ZnF1, residues 6-91; ZnF2, residues 105-202 and ZnF3, residues 224-360) and a bipartite nuclear localization signal (NLS, residues 207-226). PARP zinc fingers are structurally and functionally unique, since they coordinate zinc molecules with a Cys-Cys-His-Cys motif, they contain more residues than other zinc fingers usually consisting on 12-13 residues, and they recognize altered structures in DNA rather than particular sequences[52].

Although it has been suggested that ZnF1 recognizes double strand DNA breaks (DSB) and that ZnF2 has a binding preference for single strand DNA breaks (SSB), both fingers are required for full activation of PARP-1. It is, however, not well known how ZnF1

and ZnF2 recognize diverse types of DNA structures. ZnF2 is linked to ZnF3 by a 26-residue peptide. This zinc finger was identified in recent years as being involved in PARP-1-PARP-1 homodimer formation. Zn3 mutants are not activated by DNA breaks, indicating that functional homodimerization of PARP-1 through ZnF3 is required for efficient activation of PARP-1 [53].

Mutational and deletion analysis of the ZnF1 and ZnF2 domains indicates a pivotal role for the ZnF1 domain in PARP-1 DNA-dependent activity in vitro, whereas the ZnF2 domain is not essential, but perhaps plays a role in PARP-1 binding to particular damaged DNA structures. A combination of mutations that simultaneously disrupts both the ZnF1 and ZnF2 domains interferes with PARP-1 interaction with chromatin in the absence of DNA damage. This further highlight that PARP-1 interaction with DNA through the ZnF1 and ZnF2 domains can adapt to a range of DNA structures, including nucleosome-bound conformations of continuous, undamaged DNA. The mode of interaction with DNA for the ZnF1 and ZnF2 domains has been a notable deficiency in our understanding of PARP-1, and the mechanism by which the ZnF1 domain specifically contributes to DNA-dependent activation of PARP-1 is unknown[54].

In addition to the ZnF1 domain, the ZnF3 domain is also required to support DNA-dependent PAR synthesis activity of the catalytic domain (Figure 1.7) [54]. The ZnF3 domain structure consists of a unique type of zinc-ribbon fold and a α -helical N-terminal region. Importantly, the structure is entirely unrelated to the ZnF1 and ZnF2 domain structures and is therefore expected to have a distinct function. Zinc-ribbon folds frequently mediate protein-protein interactions; therefore, this is a likely role for the zinc-ribbon fold of the ZnF3 domain. In the crystal structure, the C-terminal tail of the ZnF3

domain forms an extensive interface between two ZnF3 monomers that are related by 2-fold crystallographic symmetry. Although the isolated ZnF3 domain is a monomer in solution, conserved residues are involved in forming the ZnF3 homodimer interface suggesting that the ZnF3 domain might exist as a dimer in the DNA-activated state of PARP-1.

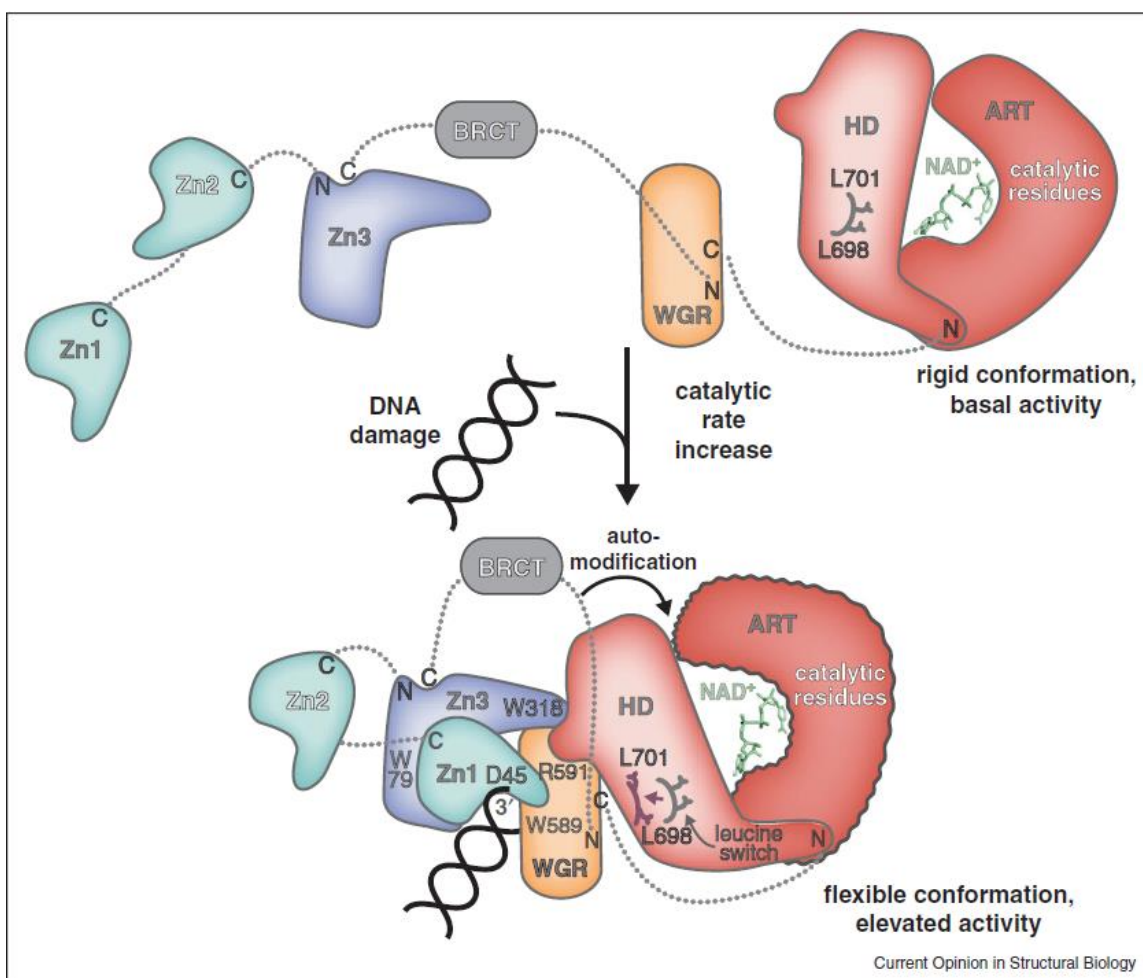


Figure 1.7 Model for DNA damage-dependent activation of PARP-1 [54]

In the absence of DNA damage PARP-1 domains exist in an extended, 'beads-on-a-string' conformation. The HD serves as a modulator of PARP-1 activity, holding the ART in a rigid conformation. Upon detecting DNA damage the Zn1, Zn3, and WGR domains collapse together, forming a network of interdomain contacts that perturb the structure of the HD, displacing a 'leucine switch' that decreases the stability of the catalytic domain and increases the catalytic activity. A more flexible, dynamic ART conformation is more efficient to perform the multi-step synthesis of poly (ADP-ribose). The collapsed conformation positions the automodification region adjacent to the catalytic domain, providing substrate specificity and contributing to an enhanced rate of poly (ADP-ribose) production.

Current models indicate that PARP-1 functions as a catalytic dimer; therefore, it has been speculated that the ZnF3 dimer might participate in assembling two molecules of PARP-1 for activation. There are several possible mechanisms by which the ZnF3 interdomain contact might regulate PARP-1 activity. The ZnF3 domain could bridge an interaction between two PARP-1 domains, bringing them into the appropriate orientation for an association necessary for PAR synthesis. Another possibility is that the Zn3 interdomain contact acts to orient the automodified region of PARP-1 for efficient addition of PAR [27].

The ZnF3 domain is also an important element required for chromatin compaction that leads to maximal repression of transcription by PARP-1. A potential mechanism is homodimerization of two adjacent nucleosome-bound PARP-1 molecules. Several studies show that the ZnF1-ZnF2 domains are necessary and sufficient for the nucleosome binding property of PARP-1. The ZnF3 dimer observed in the crystal structure is a possible candidate for mediating the homodimerization of PARP-1 on chromatin. The fact that mutation of residues located at the ZnF3 dimer interface compromises chromatin compaction is consistent with this hypothesis. However, earlier studies indicated that the ZnF1-ZnF2-ZnF3 domains could not compact chromatin, but when linked to the catalytic domain (CAT) the chromatin compaction capability of PARP-1 was restored. These data underscore the possibility that both the ZnF3 domain and the CAT domain are intricately associated in the homodimerization of PARP-1. An appropriate model would be that ZnF1-ZnF2 domains are necessary for the binding of PARP-1 to chromatin, whereas the ZnF3 and CAT domains are collectively required for the dimerization of PARP-1. Rather than mediating homodimerization of chromatin bound

PARP-1, the ZnF3 domain alternatively could be important for positioning other PARP-1 domains for full compaction of chromatin. The DNA binding domain and the CAT domain of PARP-1 collaborate to induce chromatin compaction; therefore, a potential function of the ZnF3 domain is to coordinate the activities of the ZnF1-ZnF2 domains and the CAT domain [55].

The crystal structures for the individual human PARP-1 zinc fingers in complex with blunt-ended duplex DNA, a potent stimulator of PARP-1 activity has been previously studied. The ZnF1-DNA and ZnF2-DNA structures demonstrate a consistent mode of interaction with DNA that is distinct from other known DNA binding factors. The ZnF1 and ZnF2 domains bind to an uninterrupted segment of the phosphate backbone using a region that have been termed the phosphate backbone grip, and they engage the exposed nucleotide bases of DNA through a second region termed the base stacking loop. Biochemical analysis demonstrates that the ZnF1 domain has relatively weak DNA binding affinity, but this activity is required for activation of PARP-1. In contrast, the ZnF2 domain binds to DNA with much higher affinity yet is not essential for DNA-dependent PARP-1 activation in vitro or in vivo[56]. The ZnF1-DNA complex structure combined with mutational and structural analysis indicate that a specialized loop region of the ZnF1 domain is repositioned upon binding to DNA, and this situates key residues that contribute to the activation of PAR synthesis in a DNA-dependent manner [57].

The ZnF1-DNA and ZnF2-DNA crystal structures provide the first views of PARP-like zinc fingers bound to DNA, revealing a bipartite mode of DNA interaction that contacts sequence-independent features of the DNA structure: the sugar-phosphate backbone and exposed nucleotide bases. PARP-1 binds to DNA structures containing damage such

as single and double strand breaks; yet, it is interesting to note that the ZnF1 and ZnF2 domains do not contact the DNA at 3' or 5' terminus. Rather, the ZnF1 and ZnF2 domains bind to exposed nucleotide bases that are indeed present in DNA structures containing breaks, but would also be present in the undamaged, abnormal DNA structures that PARP-1 binds, such as hairpin and cruciform DNA. Thus, the ZnF1-DNA and ZnF2-DNA structures provide insights into how PARP-1 zinc fingers could interact with a variety of DNA structures. The phosphate backbone grip as a rigid component of the DNA interaction will engage uninterrupted 3-nucleotide segments of DNA backbone in a consistent manner when engaging all types of DNA structure. The base stacking loop will likely serve as a flexible component of the DNA interaction that will allow the zinc fingers to adapt to variability in the DNA structures, with a common element being the interaction between hydrophobic protein side chains and exposed DNA bases. Therefore the base stacking loop of the ZnF1 domain is a critical and specific factor that regulates DNA-dependent PARP-1 automodification activity [58].

It has been proposed that the base stacking loop interaction with DNA positions specific ZnF1 residues that will form key contacts with other essential domains of PARP-1, and these DNA-induced contacts contribute to PARP-1 DNA-dependent activity. Importantly, the ZnF1 residues that are important for mediating PARP-1 activation are not conserved in the ZnF2 domain, providing a molecular basis for ZnF1 specificity in regulating PARP-1 activity. Consistent with this model, previous biochemical analysis of PARP-1 DNA-dependent activity has demonstrated that the composition of duplex DNA influences the level of PARP-1 activation. These variations in DNA structure influence the positioning of the base stacking loop on DNA, and thus affect ZnF1 ability to efficiently

form interdomain contacts that support DNA-dependent PARP-1 activity. Interdomain contacts will ultimately impose structural changes in the PARP-1 catalytic domain that increase enzymatic activity, or will promote an arrangement of PARP-1 domain architecture that increases access to substrates [59].

The ZnF2 domain alone has substantial DNA binding affinity on its own, 100-fold higher than that of the ZnF1 domain alone. The robust DNA binding affinity of the ZnF2 domain could be important for the rapid localization of PARP-1 to sites of DNA damage, or the persistence of PARP-1 at damage sites, even though the ZnF2 domain is not strictly required for PAR synthesis activity at sites of damage [60].

The relatively weak DNA binding affinity of the ZnF1 domain could be an important feature for regulation of PARP-1 DNA dependent activity. A proteomic analysis of PARP-1 phosphorylation sites identified Ser-41 of the ZnF1 domain as a site of modification, and the phosphomimic S41E decreased PARP-1 recruitment and persistence at a microirradiated region of the nucleus containing DNA damage, presumably by disrupting ZnF1 interaction with DNA. Ser-41 is located on the base stacking loop adjacent to the exposed DNA bases, and therefore should not be readily accessible to kinase activity when the ZnF1 domain is bound to DNA. The weak DNA binding affinity of the ZnF1 domain would allow this surface to be accessible for phosphorylation a portion of the time, supporting modification at this site as a mode of regulating PARP-1 function [61].

The analysis of ZnF1 and ZnF2 specific functions indicates that the ZnF2 domain does not play a pivotal role in DNA-dependent activation of PARP-1. PARP-1 has important functions outside of the DNA damage response, contributing to transcription as both a general and specific regulator of gene expression. DNA-dependent PAR synthesis

activity is not required for all of PARP-1 transcriptional activities. The ZnF2 domain could serve specific roles in regards to PARP-1 function(s) in transcription. In this regard, the higher DNA binding affinity of the ZnF2 domain might play a more important role.

Individual mutation of the ZnF1 and ZnF2 domains of human PARP-1 might reveal specific functions for the zinc fingers in regulating PARP-1 transcriptional activities or interaction with chromatin.

PARP-1 binds to chromatin and influences chromatin structure, and thus functions as a DNA architectural protein. Interestingly, the manner in which PARP-1 zinc fingers engage DNA bases using hydrophobic protein side chains is reminiscent of other DNA architectural proteins that bind to distorted DNA structures, or induce DNA distortions upon binding, such as high-mobility group protein HMG1 and TATA-binding protein. Although the ZnF1 and ZnF2 domains are structurally distinct compared with both of these DNA architectural proteins, there are likely to be common elements to how the PARP-1 zinc fingers will engage exposed DNA bases in distorted DNA structures.

A critical distinction between the ZnF1 and ZnF2 domains and DNA architectural proteins with known structures is that the hydrophobic residues of the ZnF1 and ZnF2 domains insert into the major groove of the DNA, rather than the minor groove. This is best visualized by aligning a continuous B-form DNA helix to the duplex DNA contained in the ZnF1-DNA complex. Due to the positioning of the base stacking loop in the major groove of the DNA, it is expected that both the ZnF1 and ZnF2 domains will bind to DNA distortions that expose nucleotide bases in the major groove, and therefore bend DNA toward the minor groove. Interestingly, the structure of a tetramer of nucleosomes demonstrates this type of major groove distortion in the linker DNA connecting

nucleosomes, where PARP-1 is known to bind. Rigid body positioning of a PARP-1 zinc finger on the nucleosomal linker DNA suggests that this type of major groove distortion can better accommodate the base stacking loop in the major groove. This hypothetical model provides insight into how PARP-1 zinc fingers are capable of interacting with continuous, undamaged DNA structures [27, 62].

1.5. PARP-1 in retroviral infection

Possible links between PARP-1 and retroviral infection have been reported previously. In 1991, for example, Yamagoe et al. [63] observed a promotion of UV irradiation-induced HIV-1 gene expression by PARP-1 inhibitors in HeLa cells at the posttranscriptional level, although it is not yet known whether PARP-1 per se is indeed involved in the promotion. Ha et al. [64] also reported in 2000 a role for PARP-1 in HIV-1 integration, after observing an abolishment of infection in PARP-1 knockout mice fibroblasts, although Siva and Bushman [65] later tested the same model and concluded that PARP-1 is not strictly required for retroviral infection because replication steps, including integration, can proceed efficiently in its absence. More recently, Parent, M., et al reported that PARP-1 is a host cellular factor that negatively regulates HIV-1 transcription through competitive binding to TAR RNA with Tat positive transcription elongation factor b (p-TEFb) complex [66]. All these studies, flagged with the limitation of using HIV non-replicating virus, highlight the actual uncertainty about the role of PARP-1 in retroviral infection.

1.6. Significance and Hypothesis

HIV-1, in common with all viruses, requires the concerted contributions of numerous positively acting cellular factors and pathways to achieve efficient replication [67]. Recent studies have revealed that the number of host proteins involved in the response to viral infection exceeds 1000 for HIV-1 [68, 69]. Antiviral drugs, in addition to target viral proteins, could be directed to inhibit or enhance positive or negative, respectively, cellular regulators of viral replication. Drugs targeting cellular factors instead of viral proteins are expected to encounter less viral resistance mechanisms because of the higher genetic stability of host proteins as compared to viral proteins. It is well established that the high rate of mutation of HIV-1 notoriously render this viruses resistance to treatments. Then the strategy of targeting cellular cofactors of HIV-1 replication as therapeutic strategies is particularly promising.

My work is devoted to the functional characterization of PARP-1 in HIV-1 infection. Our laboratory has identified the interaction of PARP-1 with LEDGF/p75 in two independent proteomic experiments, LEDGF/p75 is an important cellular cofactor of HIV-1 replication, and also has demonstrated that PARP-1 represses the expression of retroviral vectors derived from Murine Leukemia Virus and HIV-1 in chicken cells [70]. This repressive function of PARP-1 requires the activity of Histone Deacetylases and DNA methylases. Similarly, in *Drosophila melanogaster* PARP-1 represses the expression of endogenous retrotransposons by promoting chromatin compactation at the genome of these retroelements [71, 72]. However, the repressive role of PARP-1 on retroviral gene expression has been demonstrated only in non-replicating retroviruses in non-human cells. This work will increase our knowledge further by evaluating the

implication of PARP-1 in HIV-1 replication in human CD4 T cells. I expect that after the conclusion of my research we will definitively define the role of PARP-1 as a cellular cofactor for HIV-1 replication.

The implication of PARP-1 in several biological processes that are important for HIV-1 replication, including chromatin organization, transcriptional regulation, and DNA repair, suggests that this enzyme could impact the HIV-1 life cycle at different steps.

In order to expand our knowledge on the potential role of PARP-1 in the retroviral life cycle we have exploited two PARP-1 KO cellular models. We have determined the effect of PARP-1 deficiency on the expression of Avian Leukemia Virus (ALV) by using PARP-1 KO chicken B lymphoblastoid cells and we have also investigated the role of this protein on HIV-1 replication, by using PARP-1 KO (and knockdown) Human CD4+ T lymphoblastoid cells. Using these approaches, we have been able to support our hypothesis that PARP-1 is implicated in the expression and replication of retroviruses. More relevant yet, we have also been able to test and corroborate this hypothesis by using PARP-1 inhibitors during the infection of human CD4+ T cell lines and human primary CD4+ T cells.

CHAPTER 2: Evaluating the effect of PARP-1 deficiency on Avian Leukemia Virus expression in chicken cells

2.1. Introduction

As previously mentioned, at the beginning of this work the role of PARP-1 in HIV-1 infection was not established. We hypothesized that one reason for this limitation was due to the functional redundancy of PARP-1 found in mammalian cells [73, 74]. Therefore, we thought that the study of PARP-1 functions in evolutionary simpler organisms that lack this functional redundancy will be advantageous as previously shown in other PARP-1 functions [37, 71, 72]. In addition, PARP-1 functions identified in simpler organisms are relevant to more complex organisms due to the high evolutionary conservation of PARP-1 from *Drosophila* to humans[70].

In this study we took advantage of the functional and structural evolutionary conservation of PARP-1 and we studied the role of the enzyme in the expression of an endogenous retrovirus using the chicken B lymphoblastoid cell line DT40. These cells exhibit low PARP-1 functional redundancy and are viable after PARP-1 knockout. DT40 cells naturally lack the PARP-2 gene, the closest PARP-1 paralog, and PARylation is completely abrogated after PARP-1 knockout, indicating that the enzyme is the principal, if not the only, PARP protein with enzymatic activity in these cells [75].

DT40 cells produce infectious Rous sarcoma virus (RAV-1), the retrovirus used to generate the cell line [76] which is closely related to HIV since they belong to the same family. Therefore, we decided to evaluate the effect of PARP-1 on RAV-1 gene expression by determining the production of the virus by DT40 cells.

2.2. Materials and Methods

2.2.1. Cell Lines

DT40-derived wild-type (WT) and KO cell lines and a PARP-1 knockout engineered to express human PARP-1 (KO h-1) were previously described[75] and were kindly provided by Shunichi Takeda (Crest Laboratory, Department of Radiation Genetics, Faculty of Medicine, Kyoto University, Kyoto, Japan). DT40-derived cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 6% heat-inactivated chicken serum, 2 mM L-glutamine, and 1% penicillin/streptomycin.

2.2.2. Immunoblotting

Cells (3×10^6) were lysed in 100 μ l of Laemmli sample buffer (12mM Tris-Cl, pH 6.8, 0.4% SDS, 2% glycerol, 1% β -mercaptoethanol, 0.002% bromophenol blue), and 15 μ l of the sample was resolved in 10% Acrylamide by SDS-PAGE and transferred overnight to polyvinylidene difluoride (PVDF) membranes at 100 mA at 4 °C. The membranes were blocked in Tris-buffered saline (TBS) containing 10% milk for 1 h and then incubated with the corresponding primary antibody diluted in TBS-5% milk-0.05% Tween 20 (antibody dilution buffer). PARP-1 was detected with MAb 2-C-10 [77] (diluted 1/1,000), and PARP-2 with a rabbit antibody (Santa Cruz sc-133886, diluted 1/500). As a loading control, anti- α -tubulin MAb (clone B-5-1-2; Sigma) was used at a 1/4,000 dilution. Membranes were incubated overnight at 4 °C with anti-PARP-1 or -2, whereas anti- α tubulin MAb was incubated for 2 h at 25 °C. Primary-antibody-bound membranes were washed in TBS-0.1% Tween 20, and bound antibodies were detected with goat anti-mouse Ig-

horseradish peroxidase (HRP) (Sigma) diluted 1/2,000 in antibody dilution buffer, followed by chemiluminescence detection.

2.2.3. PAR ELISA

Protein-coupled or -uncoupled PAR was measured in cell lysates by ELISA following the manufacturer instructions (Catalog # 4520-096-K; Trevigen). DT40 cells were collected and washed in 1 ml of ice-cold 1X PBS by centrifugation at 1000 x g for 6 mins. The pellet was resuspended in 100 µl of cell lysis buffer supplemented with protease inhibitors and incubated on ice for 15 minutes with periodic vortexing. Then, SDS was added to the samples to achieve a final concentration of 1% and incubated at 100°C for 5 minutes. Once the cell extracts cooled to room temperature, 0.01 volume of Magnesium Cation (catalog # 4520-096-12; Trevigen) and 2 µl of DNase I (2 Units/µl, catalog # 4520-096-07; Trevigen) were added, followed by incubation at 37°C for 90 minutes. To remove cellular debris, cell extracts were centrifuged 10,000 x g for 10 minutes at room temperature and then, without disturbing the pellet, the supernatants (about 90 µl) were collected in a fresh tube for ELISA analysis.

2.2.4. Analysis of the production of RAV-1

DT40 cells are known to continuously produce and release infectious RAV-1 into the supernatant [76]. Then we evaluated the amount of RAV-1 produced by DT40-derived cells lacking or expressing PARP-1.

2.3. Results

2.3.1. Characterization of DT40 WT, KO and KO h-1 cell lines

DT40 B lymphoblastoid cell lines were characterized by determining the expression of PARP-1 and PARP-2 proteins following an immunoblotting procedure. As represented in Figure 2.1, PARP-1 protein is present in DT40 WT cells and DT40 KO h-1 cells but not in DT40 KO cells. PARP-2 was not detected in any of the cell lines studied. α -tubulin protein was immunoblotted as a loading control.

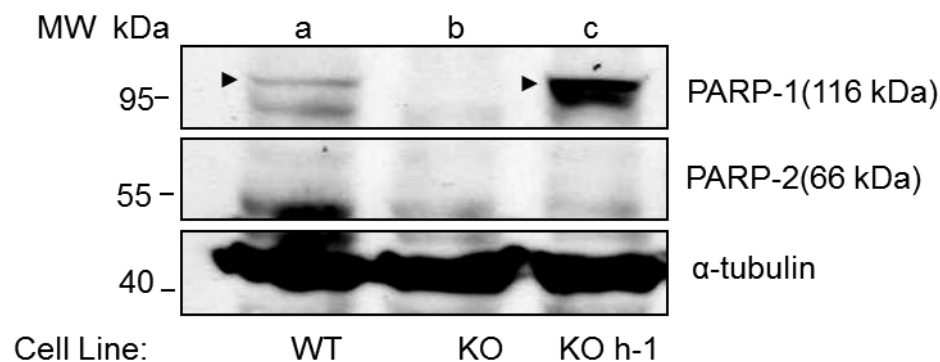


Figure 2.1 Immunoblotting of PARP-1 and PARP-2 in DT40 WT (lane a), KO (lane b), KO h-1 (lane c) PARP-1 and PARP-2 proteins were detected in lysates from DT40 WT, KO and KO h-1 chicken cell lines using human α -PARP-1 or human α -PARP-2 antibodies respectively. α -tubulin expression was used as a loading control.

These results, as expected, show that PARP-1 is in fact the only PARP protein expressed in chicken cells, at least by judging the deficiency of PARP-2 which is usually the second most common PARP protein, and also corroborate the successfully generation of a PARP-1 KO cell line. Human PARP-1 has also been efficiently expressed in the DT40 KO cells.

The expression levels of a protein do not always correlates with its enzymatic activity. Since we were able to verify the deficiency of PARP-1 protein in DT40 KO cells and the expression of human PARP-1 in DT40 KO h-1 cell lines we decided to investigate the intracellular PARylation activity in those cells, considering that PARP-1 accounts for most of PAR production in the cell, as we previously mentioned.

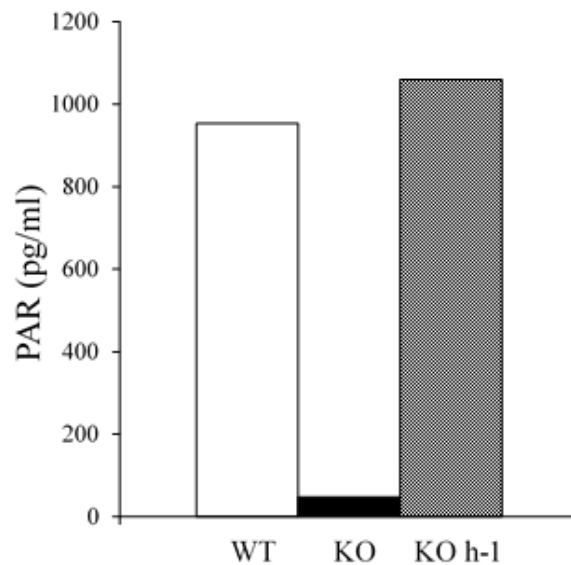


Figure 2.2 PARylation assay in DT40 WT, KO and KO h-1 cells

The amount of intracellular PAR units, expressed in pg/ml, was quantified in lysates from chicken DT40 WT, KO and KO h-1 cell lines.

Intracellular PAR levels, expressed in pg/ml of PAR, detected in our experiment (Figure 2.2) show that the deficiency of PARP-1 in DT40 KO correlates with a drastically decreased amount of PAR units in the cell. The amount of PAR detected in KO cells was almost 20 times lower than in WT cells and the expression of human PARP-1 restored the amount of synthesized PAR to comparable levels in WT cells.

2.3.2. The production of RAV-1 is affected by the levels of PARP-1

DT40 cell lines were generated by infecting a chicken with RAV-1 [76]. These cells in culture are known to continuously produce and release infectious avian Rous-associated virus type 1 (RAV-1) into the supernatant.

We reasoned that if PARP-1 is implicated in the expression of an integrated retrovirus, the difference in PARP-1 expression between these cell lines would implicate a difference in RAV-1 viral expression measured by the levels of viral reverse transcriptase activity as an indicator of infectious viral particles.

The data in Figure 2.3 clearly indicate that the production of RAV-1 was affected by the levels of PARP-1 in the producer cells. RAV-1 production was 2.5-fold and 12.5-fold higher in PARP-1-null cells (KO cells) than in WT or KO h-1 cells, respectively. The reason for a higher repression in viral expression when human PARP-1 was re-expressed in previously PARP-1 KO chicken cells when compared to the repression of viral expression observed in chicken WT cells could be due to human PARP-1 being more restrictive than chicken PARP-1 or the fact that the human PARP-1 expression achieved during the generation of the KO h-1 cell line was even higher than the observed in chicken WT cells as depicted in the figure 2.1. These data demonstrated that PARP-1 modulates the expression from the LTR promoter of an endogenous retrovirus.

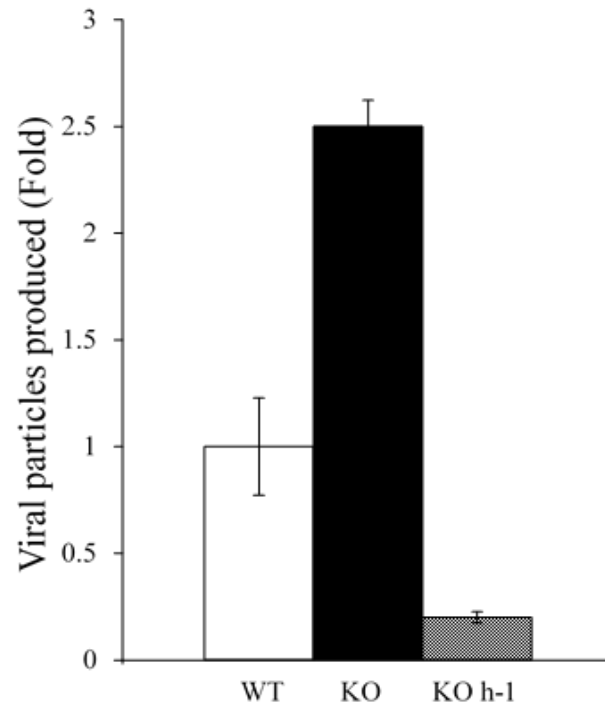


Figure 2.3 Analysis of RAV-1 retrovirus production in DT40 WT, KO, and KO h-1 cells
Reverse transcriptase levels were measured in DT40 WT, KO, and KO h-1 cells as an indicator of infectious viral particles and expressed in fold increase.

CHAPTER 3: Determining the role of PARP-1 in HIV-1 replication in human cell lines

3.1. Introduction

One of the most exciting experimental approaches used to investigate the influence of a particular host protein in viral replication is the generation of stable knockdown or knockout cell lines with low expression or protein deficiency, respectively. If a phenotype is observed in these cell lines with respect to the wild type, after infection, then it could be inferred that the protein in question influences the life cycle of the virus. The specificity of the protein in the observed effect can also be addressed by back-complementing or re-expressing it in the deficient cell lines, in which case the wild type phenotype should be restored.

We envisioned that the generation of PARP-1 KD and KO CD4⁺ T cell lines would allow us to determine the influence of this protein in HIV replication by comparing the infectivity of the virus in these cells with the infectivity in the PARP-1 back-complemented counterpart. The use of specific PARP-1 inhibitors could also mimic or reproduce any previously observed results in KD and KO cell lines after viral infection.

In order to address the possible mechanism linking PARP-1 with HIV replication we decided to investigate the effect of PARP-1 deficiency on the early phase of the HIV life cycle by infection of target cells with an HIV single round vector, VSV-G pseudotyped virus. It is important to notice that these viruses enter the cell independently of CD4/chemokine receptor and recapitulates all the steps of the HIV life cycle but do not produce infectious particles because of the lack of HIV-1 envelop proteins, limiting the infection to one round.

We generated a human PARP-1 knockdown (KD) and PARP-1 knockout (KO) cell line in SupT1 cells, a CD4⁺ T-lymphoblastoid cell line that supports optimal HIV

replication, then we proceeded to re-express the protein by using lentiviral vectors generating the corresponding back-complemented lines. This exogenous PARP-1 cDNA expressed in KD cells is engineered to have several silent point mutations allowing it to escape the previously introduced shRNA but producing a wild type protein. In KO cells PARP-1 was re-expressed with the wild type cDNA sequence. Similar levels of CD4 and CXCR4 were verified by FACS analysis in the resulting PARP-1 deficient and back-complemented cell lines. These cells were then used in HIV-1 infection experiments using replication competent and incompetent viruses.

Considering the commercial availability of several PARP inhibitors that target different domains of the protein, we also evaluated their effect on HIV-1 infection.

3.2. Materials and Methods

3.2.1. Cell Lines

Human PARP-1 KD and control cells were generated by transduction of SupT1 cells with HIV-derived vectors produced with plasmids pTRIP EGFP shRNA PARP-1 and pTRIP EGFP shRNA control. These plasmids were constructed by cloning shRNA expression cassettes that contain PARP-1-specific or scrambled shRNA sequences at a unique PpuMI site in pTRIP EGFP. The PARP-1-specific shRNA expression cassette was generated by annealing oligonucleotides 5'-GatcccgAAGTATCCCAAAAAGTTCTtcaa gagaAGAACTTTTTGGGATACTTtttttgaaa-3' and 5'-agcttttccaaaaaaAAGTATCCCAA AAAGTTCTtctcttgaaAGAACTTTTTGGGATACTTcgG-3' and cloning them into the pSilencer 2.1-U6 hygro expression plasmid (Ambion). Then, the U6 promoter and the shRNA sequences were amplified by PCR with primers EE5 (sense; 5'-TATAGGG

ACCCGT AAAACGACGGCCA GTGCC-3') and EE6 (antisense; 5'-TATAGGGTC CCGAATTCCCCAG TGGAAAGACG-3') and cloned into pTRIP EGFP. Generated PARP-1 KD cell lines were sorted for 10% highest eGFP expression and characterized by western blot and FACS (CD4/CXCR4).

Human PARP-1 knockout (KO) cell lines were generated by transduction of SupT1 cells with HIV-derived vectors encoding a zinc finger nuclease (ZFN) expressed from a CMV promoter, which specifically targets PARP-1. A CompoZr Knockout Zinc Finger Nuclease kit specific for human PARP-1 was purchased from Sigma-Aldrich and the included plasmid pZFN (4129bp) was digested with the restriction enzymes NheI (697) and XhoI (1917) to generate a fragment containing the PARP-1 specific zinc finger domain and the nuclease domain of FokI. This fragment was cloned into pTrip eGFP used to generate lentiviral vectors and target cells were infected followed by single cell cloning. Generated PARP-1 KO cell lines were characterized by Western blot and FACS(CD4/CXCR4)

For generation of PARP-1 BC cell lines with previously PARP-1 KD cells, the latter cells were infected with a retroviral expression vector carrying a PARP-1 cDNA cassette with several silent mutations to escape shRNA recognition. The escape mutations were introduced by phusion PCR using the Thermo Scientific Phusion Site-Directed Mutagenesis kit (Cat: F-541). Infection with a retroviral expression vector carrying PARP-1 cDNA wild type was used to generate PARP-1 BC cell lines with previously PARP-1 KO cells. In both cases an MLV retroviral vector (pJZ308) was used, so the gene expression was driven from an MLV promoter. Cell lines were characterized by Immunoblotting and FACS as previously described.

SupT1 and HEK293T cells were grown in RPMI 1640 and DMEM, respectively. All culture mediums were supplemented with 10% of heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin.

3.2.2. Retroviral Vectors

Previously described procedures [78] were followed for the production of the different retroviruses used in this study. Briefly, 293T cells were co-transfected by calcium-phosphate with 15 µg of the expression plasmids and 5 µg of the Vesicular Stomatitis Virus glycoprotein G expression plasmid, pMD.G. At 48 h post-transfection, the viral supernatants were harvested and concentrated by ultracentrifugation at 124,750 x g for 2 h on a 20% sucrose cushion. Viral aliquots were stored at -80 °C until use.

HIVpNL4-3 wild type virus was produced by transfection of 15 µg of the corresponding expression plasmids in HEK293T cells, as previously described.

3.2.3. Immunoblotting

3x10⁶ cells were lysed in 100 µl of Laemmli sample buffer (12 mM Tris-Cl pH 6.8, 0.4% SDS, 2% glycerol, 1% β-mercaptoethanol, 0.002% bromophenol blue), and 15 µl of the sample resolved by SDS-PAGE, and transferred overnight to PVDF membranes at 100 mA at 4 °C. Membranes were blocked in TBS containing 10% milk for one hour and then incubated with the corresponding primary antibody diluted in 1X TBS, 5% milk, 0.05% Tween 20 (antibody dilution buffer). PARP-1 protein was detected with a mouse anti-PARP-1(C2-10) antibody (Santa Cruz Biotechnology, Cat: sc-53643). Membranes were incubated overnight at 4 °C with previously described primary antibody, then

washed in 1X TBS, 0.1% Tween 20 and bound antibodies detected with goat anti-mouse Igs-HRP.

3.2.4. Cell sorting and FACS analysis

eGFP expressing SupT1 cells were sorted for 10% highest expression at the Texas Tech University Health Science Center molecular biology core facility using a BD FACSJazz cell sorter. The expression of CD4/CXCR4 receptors on PARP-1 KD and KO cells was analyzed by flow cytometry after surface staining of the cells with purchased antibodies from BD Pharmigen (APC mouse Anti-Human CD184 Cat:560936, APC mouse Anti-Human CD4 Cat:561840).

3.2.5. HIV-1 replication assays

PARP-1 KD/Scramble/BC and KO/BC SupT1 cells (0.25×10^6 cells in 2 ml RPMI 1640) were infected with HIVpNL4-3 virus. Twenty-four hours after infection, the cells were washed three times by centrifugation in 10 ml of culture medium to remove the input virus and fresh media was added. Cell supernatant was then collected at different days post-infection and used for HIV-1 p24 quantification by ELISA.

3.2.6. HIV-1 extracellular p24 ELISA

HIV-1 p24 levels were determined by a sandwich ELISA (ZeptoMetrix Corporation, catalog number 0801008) following the manufacturer instructions. Briefly, 200 μ l 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 μ l of washing buffer, and bound HIV-1 p24 was detected by incubating each well with 100 μ l

of the anti-HIV-1 p24 secondary antibody for one hr. Unbound antibodies were removed by washing as described above and bound antibodies were detected by incubating each well with 100 μ l of substrate buffer for 30 minutes at room temperature until the reaction was stopped by adding 100 μ l of stop solution into each well. The absorbance of each well was determined at 450 nm using a microplate reader.

3.2.7. PAR ELISA

Protein-coupled or uncoupled PAR was measured in cell lysates by ELISA following the manufacturer instructions (Catalog # 4520-096-K; Trevigen). SupT1 cells were collected and washed in 1 ml of ice-cold 1X PBS by centrifugation at 1,000 x g for 6 mins. The pellet was resuspended in 100 μ l of cell lysis buffer supplemented with protease inhibitors and incubated on ice for 15 minutes with periodic vortexing. Then, SDS was added to the samples to achieve a final concentration of 1% and incubated at 100°C for 5 minutes. Once the cell extracts cooled to room temperature, 0.01 volume of Magnesium Cation (catalog # 4520-096-12; Trevigen) and 2 μ l of DNase I (2 Units/ μ l, catalog # 4520-096-07; Trevigen) were added, followed by incubation at 37°C for 90 minutes. To remove cellular debris, cell extracts were centrifuged 10,000 x g for 10 minutes at room temperature and then, without disturbing the pellet, the supernatants (about 90 μ l) were collected in a fresh tube for ELISA analysis.

In experiments that evaluated the effect of PARP inhibitors, the cells were collected by centrifugation at 1,000 x g for 6 minutes and the pellet was resuspended in 1 ml of ice-cold 1X PBS. A fraction (100 μ l) of the cell suspension was used to measure ATP levels (Cell Titer-Glo Assay; Promega) to determine cell viability. The remaining cells (900 μ l) were used for PAR quantification as indicated above.

3.2.8. Pharmacological inhibition of PARP

SupT1 cells were treated with several PARP inhibitors (selection based on enzymatic inhibitory activity) for 24 hours and then challenged with HIVpNL4-3, a replication competent virus. Viral input and drugs were washed 24 hours later. The supernatant from infected cells was collected for several days and then HIV-1 replication (p24 ELISA) and cell viability (ATP levels) were measured as described above. The PARP inhibitors set (Cat No. 528820, EMD) included 3-Aminobenzamide (ABA), 5-Iodo-6-amino-1, 2-benzopyrone (II), and 1, 5-Isoquinolinediol (NU).

3.2.9. HIV single round infectivity assay

PARP-1 KO/BC cells were plated at 1×10^5 cells in 500 μ l of RPMI1640 culture medium in 24-well plates and infected with an HIV single round infection vector H Δ Eluc, expressing a luciferase reporter gene from the viral LTR. Four days post-infection, cells were collected by centrifugation at 1000g for six minutes and the pellet resuspended in 200 μ l of PBS. Half of the sample was mixed with 100 μ l of luciferase substrate (Bright-Glow™ Luciferase Assay System, Promega) and the other half with 100 μ l of cell viability substrate (CellTiter-Glo® Assay, Promega). Cell lysates were incubated for 10 minutes at room temperature in the dark and then luminescence measured in triplicate in 50 μ l-samples using a microplate luminometer reader.

3.3. Results

3.3.1. Generation of PARP-1 KD, Ctrl, BC and KO cell lines

As depicted in Figure 3.1, we have been able to generate a PARP-1 KD cell line by targeting this protein with a specific shRNA cloned into a lentiviral vector and a PARP-1 KO cell line by using a zinc finger nuclease. Notice the low level of PARP-1(116 kDa) expression in KD cells and the lack of expression in KO cells when compared with the expression in Supt1 cells. These two characterized cell lines are just a representation of our results since several cell lines have been generated. α -tubulin was used in this blot as a loading control and as expected the bands are similar in both cell lines. The expression of CD4/CXCR4 surface markers was analyzed in these cell lines by flow cytometry and we did not find any difference (data not shown).

The Western blot illustrated in Figure 3.2 shows the expression of PARP-1 in SupT1 control cells, KD, KO and BC cell lines. The BC cell line generated by re-expression of PARP-1 in KD cells displays an approximately 30% restoration of PARP-1 levels as determined by a gel density analysis software. Ultimate efforts to generate a higher PARP-1 expressing BC cell line have failed for unknown reasons. The BC cell lines generated with previously PARP-1 KO cells show comparable expression of the protein with respect to the expression in control cells. Tubulin was used as a loading control and it is evident that loading differences are not significant. Cell lines were characterized by CD4 and CXCR4 expression and no differences were observed.

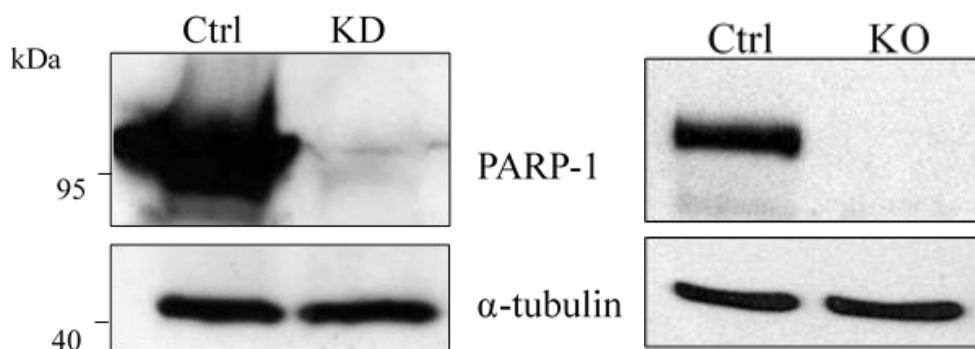


Figure 3.1 Immunoblotting of PARP-1 in Ctrl, KD and KO cells.

Human PARP-1 was detected in cell lysates from PARP-1 Ctrl, KD and KO cells lines by using an α -human PARP-1 antibody. α -tubulin was used as a loading control.

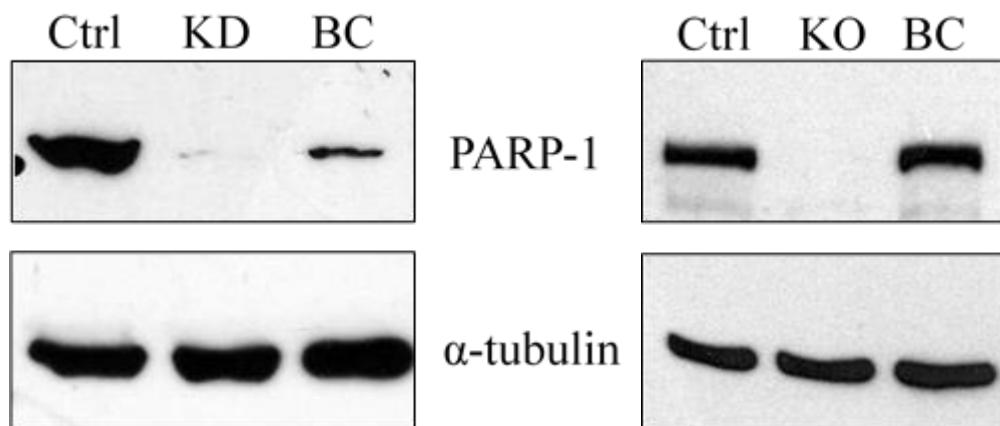


Figure 3.2 Immunoblotting of PARP-1 in Ctrl, KD, KO and BC cells

Human PARP-1 was detected in cell lysates from PARP-1 Ctrl, KD, KO, and the respective BC cell lines by using an α -human PARP-1 antibody. α -tubulin was used as a loading control.

3.3.2. The deficiency of PARP-1 enhances HIV-1 replication in CD4⁺ T cell lines

Figure 3.3 represents a replication curve of the HIV virus in PARP-1 KD, BC and control cells. Supernatant from infected cells was taken at different time points and the amount of p24 present was analyzed by ELISA. The results show that the deficiency of PARP-1 in PARP-1 KD cells lines enhances the permissivity of these cells to HIV viral infection when compared to control cells. Notice that the HIV p24 values have been expressed in a logarithmic scale, which means the actual differences in p24 levels are very dramatic. Contrary to this finding, by re-expressing PARP-1 in KD cell lines we were able to partially restore the phenotype observed in control cells, consisting in a reduction of the viral replication rate. We hypothesize that the BC cell lines do not behave as control cells because of the low PARP-1 protein expression (30% of wild type cells) achieved in KD cells (Fig. 3.2). The difference in viral replication between the KD and BC cell lines persists during almost the entire collection period but after 16 days the p24 levels become similar which might be explained by the fact that the target cells are dying due to the cytopathic effect of HIV-1 replication. Similar to the results observed in PARP-1 KD and BC cell lines, in Figure 3.4 a higher replication rate in PARP-1 KO cell lines was detected compared to the replication in PARP-1 BC cell lines. Also notice the p24 values were plotted using a logarithmic scale.

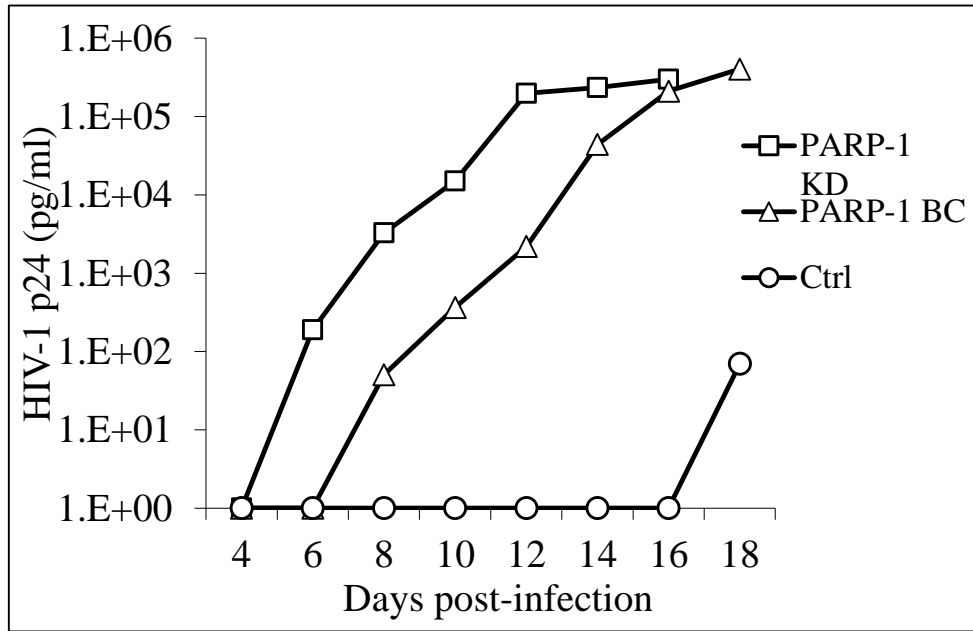


Figure 3.3 HIV replication assay in PARP-1 Ctrl, KD, and BC cells

Infectivity was measured by determining the levels of HIV p24 protein in supernatant of infected cells at different time points. Only one representative experiment has been presented.

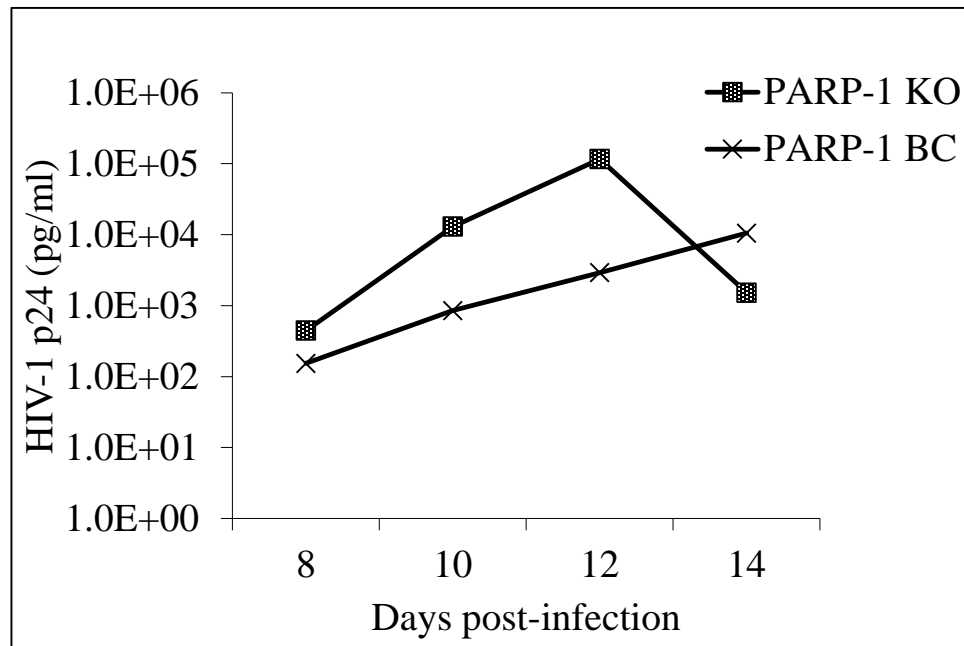


Figure 3.4 HIV replication assay in PARP-1 KO and BC cells

Infectivity was measured by determining the levels of HIV p24 protein in supernatant of infected cells at different time points. Only one representative experiment has been presented.

3.3.3. Inhibitors targeting the PARP-1 DNA binding domain impair HIV replication

In Figure 3.5 we have represented the parylation activity of SupT1 cells in the presence of several PARP inhibitors, by detecting the amount of PAR in the cell. ABA, 5-Iodo-6-amino-1, 2-benzopyrone (II) and NU have a similar inhibitory effect and therefore we selected them as candidates for future HIV replication assays.

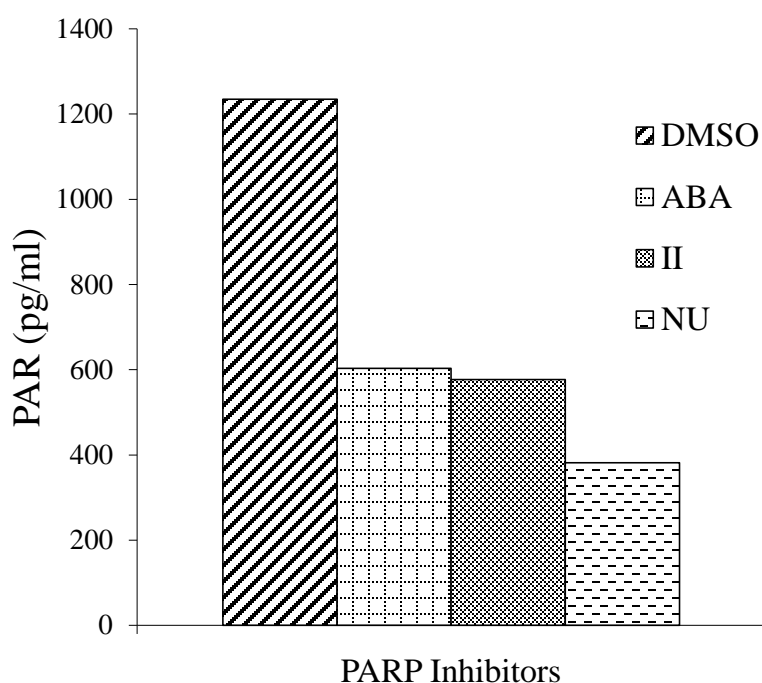


Figure 3.5 Parylation assay in SupT1 cells in the presence of PARP inhibitors

Several PARP inhibitors were tested on their ability to inhibit PARP by detecting a reduction in parylation activity. The amount of PAR was expressed in pg/ml.

Cells were infected in the presence of these inhibitors or DMSO, and 24 hrs later the compounds and virus were removed and replication was followed for several days. Importantly, inhibitor II caused a significant increase in HIV replication rate when compared to the replication in the presence of the vehicle DMSO or in cells treated with the other two PARP-1 inhibitors (Figure 3.6). This enhancement in replication is significant considering that the levels of p24 are expressed using a logarithmic scale.

Most of PARP inhibitors, like ABA and NU, block the activity of the PARP enzymes by mimicking the nicotinamide moiety of nicotinamide adenine dinucleotide (NAD) and binding to the PARP catalytic site, which either directly blocks PARP enzymatic activity or causes PARP to accumulate on DNA (known as PARP trapping). One downside to this mechanism of action is that they are broad spectrum and target not just PARP-1 and PARP-2, but other members of the PARP family[79].

Inhibitor II, on the other side, is specific for PARP-1 and impairs its DNA binding activity. It belongs to the family of coumarins, and in the cell undergoes rapid oxidation at the 6-amino position to form 5-iodo-6-nitroso-1, 2 benzopyrone which is the reactive species. The latter binds with high affinity to zinc-fingers of enzyme PARP-1 and by oxidizing the SH groups of these zinc fingers to -S-S groups thus eliminates or ejects zinc from PARP-1. Zinc ejection inactivates PARP-1 since the enzymatic process is dependent on DNA, [80].

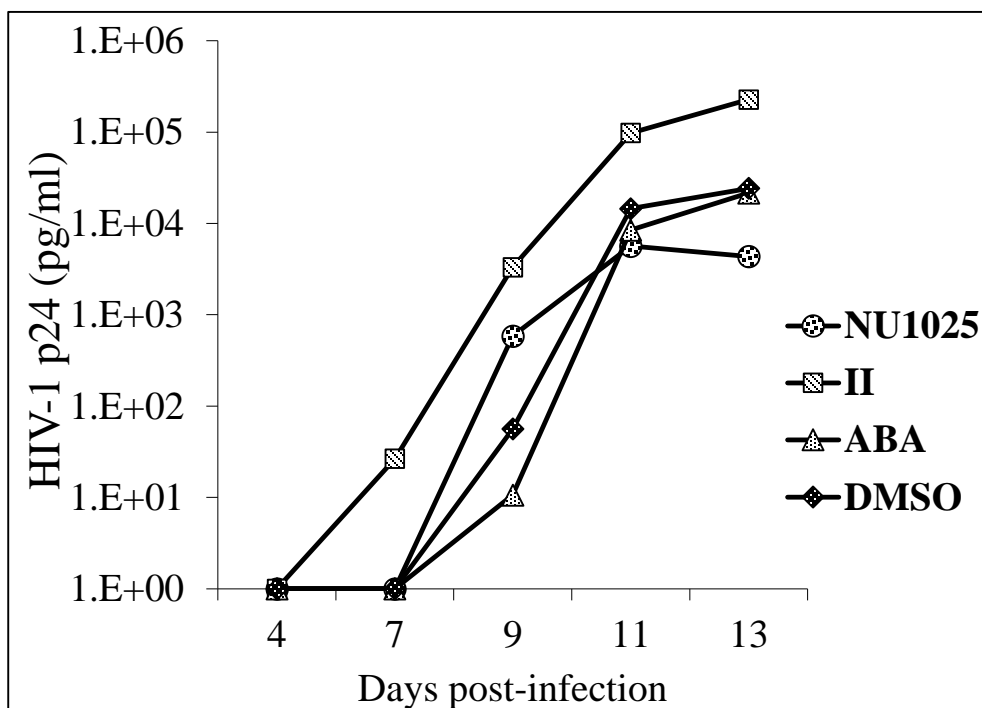


Figure 3.6 HIV replication assay in SupT1 cells in the presence of PARP-1 inhibitors

Infectivity was measured by determining the levels of HIV p24 protein in supernatant of infected cells at different time points. Despite observing similar PARP-1 inhibitors effect on HIV replication in four independent experiments performed only one of them has been presented in this figure because we have not been able to consistently detect the inhibitors effect after the same number of days post-infection. Several independent experiments have been planned in order overcome this issue.

3.3.4. PARP-1 does not affect the infection of single-round HIV-1 virus

To determine the step of the viral life cycle affected by PARP-1 we characterized the susceptibility of PARP-1 KO and BC cells to single-round infection HIV-1 expressing luciferase from the viral promoter. Figure 3.7 shows the amount of intracellular reporter protein luciferase, expressed from the HIV LTR promoter, quantified after four days of infection. Only two independent experiments are represented but the infection was performed with different clones and several replicates. Surprisingly, no significant difference in the infection of KO and BC cells was observed in these experiments (Figure 3.7), which is in marked contrast with the effect of PARP-1 deficiency HIV-1 replication

that we previously observed. Importantly, similar results were obtained with PARP-a KD and BC cells and with SupT1 cells treated with inhibitor II.

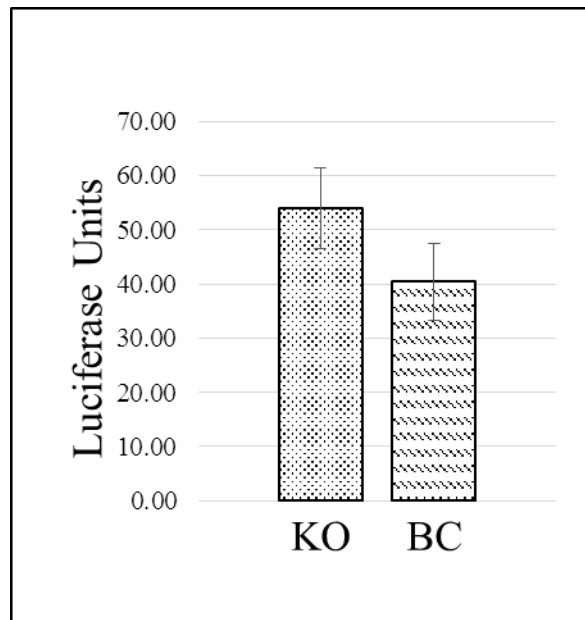


Figure 3.7 HIV single round infection of PARP-1 KO and BC SupT1 cells. A single round HIV virus carrying a luciferase reporter gene was used. Luciferase was measured 4 days after infection.

Since single-round infection HIV-1, contrary to wild type HIV-1, bypasses CD4/CXCR4-mediated entry and does not undergoes multiple rounds of infection, these results strongly suggest that PARP-1 deficiency enhances HIV-1 replication by modulating events related to CD4/CXCR4-mediated entry, or the infectivity of the produced viruses.

CHAPTER 4: Determining the role of PARP-1 on HIV-1 replication in human primary central memory T cells

4.1. Introduction

Our previous data is indicative that PARP-1 deficiency promotes HIV-1 replication in human CD4⁺ T cell lines. Therefore, we hypothesized that PARP-1 inhibition could stimulate viral replication in cells that promote HIV-1 latent infection. To evaluate this hypothesis we used determine the role of PARP-1 in central memory T cells that represent one of the major latency compartments in infected individuals. The cells were generated *in vitro* following a well-established *in vitro* paradigm of viral latency [60]. Briefly, naïve CD4⁺ T cells were purified from peripheral blood, activated *in vitro* with CD3/CD28 stimulation in the presence of IL2, and then infected with HIV-1. Subsequently, infected cells were expanded with IL2 in the absence of CD3/CD28 stimulation to allow the cells to return to a quiescence state. During quiescence reentry HIV-1 latency is established [60]. We adapted this model to evaluate the role of PARP-1 in HIV-1 latency by determining the effect of inhibitor II on the replication of HIV-1 during the quiescence re-entry period. We expected that if PARP-1 is implicated in latency establishment, HIV-1 will replicate better in the cells treated with the inhibitor than in the DMSO-treated cells.

4.2. Materials and Methods

4.2.1. Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected from unidentified, healthy donors in heparin containing collection tubes and later transferred to 50 ml conical tubes. An equal volume of phosphate buffer saline (DPBS: Dulbecco's Phosphate-Buffered Saline, 1X without calcium and magnesium; Mediatech-Cellgro -21-031-CV) was used to wash the collection

tubes and then mixed with the previous blood sample. Half the total volume per tube of Lymphocyte separation media (LSM) (Fisher #MT25072CV) was then quickly added with a pipet to the bottom of each 50 ml tube. Once all tubes have pipets with LSM in them, carefully put pipet back onto the pipet-aid without removing it from the tube and slowly expel the remaining LSM; carefully remove the pipet from the tube and discard. Centrifuge at 400 x g for 30 minutes at 18-20 °C (brake off). Aspirate down to the lymphocyte layer leaving enough liquid so as not to disturb the layer (approximately 5-7 ml above the layer should be plenty). Use a 5-ml pipet to collect all the mononuclear cells from the interface in a circular motion and transfer to a new 50ml tube. Add 2X volume of PBS, mix well and centrifuge at 250 x g for 10 minutes at room temperature. Aspirate the supernatant, being careful not to disrupt the pellet. Wash and combine the tubes to one tube using PBS. Centrifuge at 250 x g for 10 minutes at room temperature. Resuspend in 5-10 ml of complete media.

4.2.2. Isolation of Naïve CD4+ T cells from PBMC

This step was performed by using the Naïve CD4+ T cell Isolation kit II from Miltenyi Biotec (Order no. 130-094-131). Basically we count 1×10^8 PMBC, centrifuge the cell suspension at 300 x g for 10 minutes and then aspirate the supernatant completely. We then resuspend the cell pellet in 40 μ L of buffer per 10^7 total cells and add 10 μ L of Naive CD4+ T Cell Biotin-Antibody Cocktail II per 10^7 total cells. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C). After incubation we add 30 μ L of buffer per 10^7 total cells and 20 μ L of Naive CD4+ T Cell MicroBead Cocktail II per 10^7 total cells. Mix well and incubate for an additional 10 minutes in the refrigerator (2–8 °C). Finally we proceed to magnetic separation by using the Miltenyi Biotec LS columns provided with the kit and

we collect the flow-through containing unlabeled cells, representing the enriched naïve T cells.

4.2.3. Activation of Naïve CD4+ T cells

After Isolation, naïve CD4+ T cells were activated as previously described by Bosque and Planelles[81]. 5×10^5 cells were stimulated with 5×10^5 beads coated with α CD3 and α CD28 antibodies (Dynabeads CD3/CD28 T cell Expander, Dynal/Invitrogen) in 1 ml of complete medium. To ensure homogenous activation of the cells, 100 μ l of the cell mixture, together with beads were plated in a 96-well, round bottom plate (BD Falcon, Bedford, MA) and incubated for 3 days at 37 °C. After activation, cells were resuspended to remove clumps and Dynabeads were removed using a magnet. Activated T cells were re-plated at 1×10^6 cells/ml in complete medium with 30 IU/ml of rIL-2 (AIDS Research and Reference Reagent Program) for 4 days at 37 °C. Medium and rIL-2 were replaced every 2 days.

4.2.4. Infection of activated T cells with HIV wild type in the presence of PARP-1 inhibitors

Activated T cells (7 days after stimulation with α CD3/CD28) were infected by spinoculation (spinoculation dramatically increases the adsorption of HIV-1 to T cells) with HIV wild type virus in the presence of DMSO or inhibitor II (INH₂BP). Spinoculation was performed in a Sorvall Super T21 using an ST-H750 rotor at 2900 rpm (1,741g) during 2h at 37 °C. The virus and DMSO/II containing supernatant was removed and cells kept in culture at 1×10^6 cells/ml in complete medium with 30 IU/ml of rIL-2 at 37 °C during an additional 7-day period in the absence of stimulatory beads to allow activated cells to

return to a quiescence state. At days 3, 5 and 7 post-infection, media and rIL-2 were replaced and cells were kept at a density of 1×10^6 cells/ml. Supernatant was collected at different time points and used to measure extracellular p24 levels by ELISA.

4.2.5. HIV-1 extracellular p24 by ELISA

The levels of HIV-1 extracellular p24 were measured by using the ZeptoMetrix p24 ELISA kit as previously described in section 3.2.6

4.3. Results

4.3.1. The deficiency of PARP-1 enhances HIV-1 replication in primary human CD4 T cells

We have previously demonstrated that the deficiency of PARP-1 enhances the expression of an integrated retrovirus in chicken cells. In human CD4 T cell lines we also showed that HIV replication is improved when PARP-1 is not present. Here we show for the first time that in primary human CDT cells, the main target for HIV replication, the use of inhibitors targeting the DNA binding domain of PARP-1, but not the catalytic domain, enhances HIV replication.

As previously mentioned, it has been proposed that CD4 primary T cells can be used as model to study HIV latency. CD4 T cells must be activated *in vitro* with CD3/CD28 stimulation in the presence of IL2, and then infected with HIV-1 while expanded with IL2 but in the absence of CD3/CD28 stimulation. Under these conditions infected cells return to a quiescence state where HIV-1 latency is established.

We rationalized that if inhibitors targeting the DNA binding domain of PARP-1 were able to enhance HIV viral replication in human CD4 T cell lines it would be possible that the use of the same inhibitors during the infection of previously activated primary human

CD4 T cells under conditions that normally promote latency now would cause an enhancement in HIV replication.

Data in Figure 3.8 indicates that inhibitor II enhances HIV-1 replication in primary CD4+ memory T cells and that the effect is observed as early as two day post-infection but it is still observed after five days after infection. Viral replication was followed for several days but there was a dramatic decay in p24 values after five days post-infection associated with cell death due to viral replication. Since these cells are prone to establish a latent viral reservoir, the data also suggest an implication of PARP-1 in viral latency establishment.

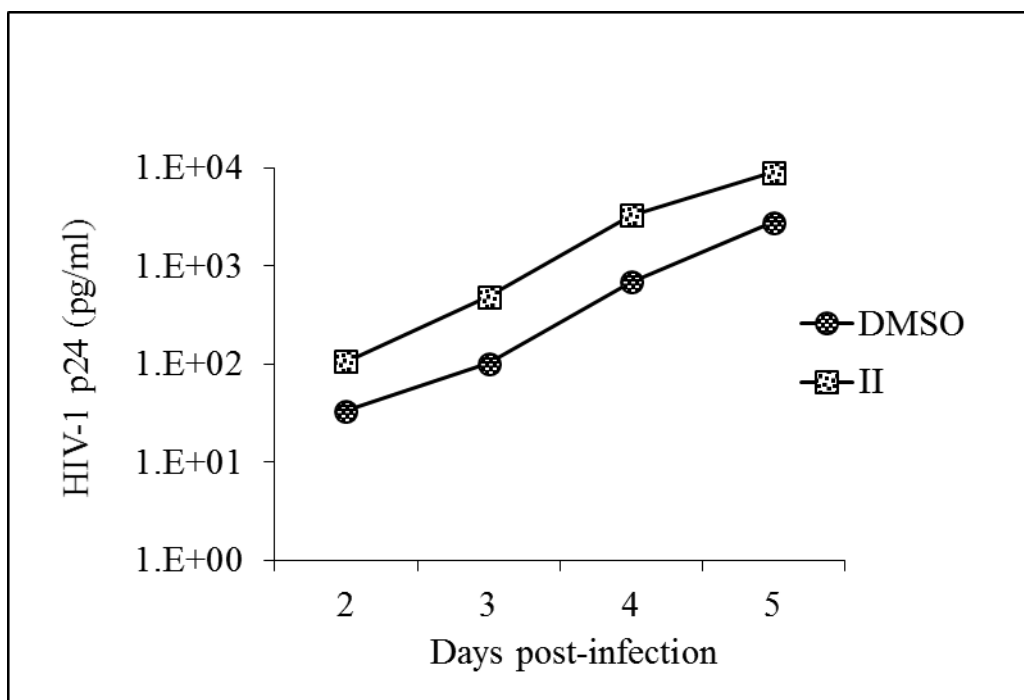


Figure 3.8 HIV replication assay in human primary CD4 T cells

The assay was performed in the presence of either DMSO or inhibitor II. Infectivity was measured by determining the levels of HIV p24 protein in supernatant of infected cells at different time points. Results correspond to one donor and are similar to the results obtained with a second donor.

CHAPTER 5: Discussion

A key pharmacological strategy for treating individuals living with HIV has been to simultaneously target multiple virus-encoded enzymes required for replication to overcome emergence of drug resistance. A parallel therapeutic strategy is targeting host factors required for the HIV life cycle. Such host proteins represent therapeutic targets that are not plagued by the twin problems of viral diversity and escape mutation that interfere with the effectiveness of conventional antiretroviral drugs. We anticipate that HIV would be hard-pressed to evolve resistance to drugs targeting cellular proteins, because it would have to evolve a new capability, not simply mutate a drug-binding site. Therefore, the identification of host factors implicated in HIV-1 infection (the focus of this work) is important.

Previous reports addressing the role of PARP-1 in HIV infection have resulted in a confusing interpretation of the actual requirement of this protein for completion of the viral life cycle. As we mentioned before, it could be attributed to the PARP functional redundancy in mammalian cells, highlighting the necessity to exploit simpler study models. Our findings in chicken cells[70], demonstrate that the expression of an endogenous retrovirus(RAV-1) is higher in cells lacking PARP-1(KO) than in wild type cells, and this phenotype can be reverted by expressing human PARP-1 in the KO cells. Thus, PARP-1 protein in chicken cells has an inhibitory effect on the expression of this endogenous retrovirus. Though this result is not directly related to HIV, it is compelling evidence since both viruses belong to the same family; in fact it determined our decision to investigate the effect of PARP-1 in HIV infection of human cells. It is also worth to mention that the transcription of RAV-1 in the cell takes place from an LTR promoter, similarly to the natural transcription of HIV in infected targets. Despite the differences

between the LTR promoters between both viruses, it is more probable to expect that the mechanisms governing the PARP-1 effects would be similar in HIV infection.

We have also demonstrated that PARP-1 is a host protein implicated in HIV replication in human cells. This conclusion has been supported by analyzing the effect of PARP-1 deficiency or inhibition of its DNA binding properties in viral replication in human cells. Our results clearly establish that PARP-1 has an inhibitory effect on the HIV-1 replication.

By investigating single-round infection HIV-1, we have determined that PARP-1 affects an event linked to CD4/CXCR4-mediated entry, or the infectivity of the viruses produced. These data also demonstrated that the main reason why the important role of PARP-1 in HIV-1 biology has been overlooked is because all the previous attempts (including us) of defining a role of this protein in HIV-1 infection have been done by analysis of single-round infection HIV-1.

PARP-1 inhibitors are an emerging class of agents that have the potential to play an important role in the treatment of a variety of cancers. Several of them are on trial for future use in humans but it turns out very interesting that little is known about the effect that they could have on HIV-1 infection. The majority of PARP-1 inhibitors target the catalytic activity of the protein, and the development of inhibitors affecting its DNA binding function has been largely neglected. Since inhibitors targeting the catalytic domain of PARP-1 did not influence the infectivity of HIV but the presence of inhibitor II during the infection caused enhancement, it is safe to conclude that the relation between PARP-1 and HIV is independent of the catalytic activity of this protein.

In regards to determining the role of PARP-1 on HIV-1 replication in primary central memory T cells, it is worth to mention that no study of the implication of PARP-1 in the replication of HIV-1 wild type in primary human CD4⁺ T cells have been reported so far; therefore our work is the first to address this question. Importantly, our data suggest that PARP-1 is implicated in latency establishment in central memory CD4 T cells.

CHAPTER 6: Future Directions

The main line of investigation we will follow in the future should be to investigate the mechanisms of action implicated in the influence of PARP-1 during HIV-1 replication.

Knowing that the PARP-1 DNA binding domain, but not the catalytic domain, determines the influence of this protein in viral replication is a key piece of information we should exploit by site specific directed mutagenesis since the generation of mutants would allow us to determine particular regions in this domain that are more important. This investigation will show us if the protein primary structure is determining the viral restriction activity or if it relies on protein conformation rather than protein sequence.

The analysis of PARP-1 influence during the late phase of the HIV life cycle should be addressed by studying the virus produced after transfection of HEK293 cells lacking this protein. As previously mentioned, this process recapitulates the late events of the viral cycle. We will generate PARP-1 KO HEK293 cells and transfect them with plasmids capable to generate a replication competent virus. We will measure viral production and infectivity of the virus produced from HEK293 KO and HEK293 WT. If PARP-1 is influencing the late phase of the HIV life cycle we should observe a higher viral production from cells lacking PARP-1. Less viral infectivity could be also observed when the virus produced from HEK293 KO is used to infect other cells. This information will be very useful considering this approach has not been exploited in any PARP-1 published research concerning HIV.

Another hypothesis to be tested is that PARP-1 could be affecting the infectivity of viral particles produced after a round of infection. This effect would imply a difference in the overall viral replication rate when a replication competent virus is used for infection. PARP-1 could affect the infectivity of viral particles by different manners, including the modification of viral proteins, or the packaging of other cellular factors into the virion, among others. We are planning on determining the infectivity of single-round and multiple-round infection viruses produced in PARP-1 deficient cells.

Also an important goal is to determine if the reason behind a difference in infectivity is a differential viral evolution depending on the presence or absence of PARP-1. It is well recognized that the human immunodeficiency virus is one of the fastest evolving entities known and that both cellular and viral factors influence this process. We propose to sequence the main viral structural proteins Gag, Pol and Env in viruses collected from PARP-1 KO and PARP-1 BC cells and determine the presence of possible viral advantageous mutations in those strains produced in the absence of PARP-1. Accessory and regulatory proteins could also be compared in the future.

The interplay between HIV-1 and plasma membrane signaling has been previously addressed and it has been proposed that it could probably have a higher impact that we think in terms of viral fitness and pathogenicity. In the future we would like to investigate if PARP-1 influences HIV replication by means of interfering CD4/CXCR4 signaling that regulates HIV gene expression. We plan to determine whether PARP-1 can regulate CD4/CXCR4-induced modulation of transcription factors like NF- κ B and SP1 by performing Western blot with lysates from PARP-1 KO and PARP-1 BC cells infected with an HIV replication competent virus.

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Abbreviations

ABA – 3-Aminobenzamide	CMV – Cytomegalovirus
ADP – Adenosine diphosphate	CTD – C-terminal domain
AIDS – Acquired immune deficiency syndrome	Ctrl – Control
ARC – Ankyrin repeat cluster	CXCR4 – C-X-C chemokine receptor type 4
ART – ADP-ribosyl transpherase	DBD – DNA binding domain
ATP – Adenosine triphosphate	DMSO – Dimethyl sulfoxide
ALV – Avian Leukemia Virus	DNA – Deoxyribonucleic acid
BAL – B-aggressive lymphoma protein	DPBS – Dulbecco's Phosphate-Buffered Saline
BC – Back-complemented	DSB – Double strand break
bp – base pairs	dscDNA – Double-stranded complementary deoxyribonucleic acid
BRCT – Breast cancer-associated protein C-terminal	eGFP – Enhanced green fluorescent protein
CA – Capsid	ELISA – Enzyme-linked immunosorbent assay
CCR5 – C-C chemokine receptor type 5	ESCRT – Endosomal sorting complex required for transport
CD4 ⁺ – Cluster of differentiation, member 4 activated	FACS – Fluorescence-activated cell sorting
CDK9 – Cyclin-dependent kinase 9	
cDNA – Complementary deoxyribonucleic acid	
CLS – Centriole-localization signal	

FDA – Food and Drug Administration

HAART – Highly active antiretroviral therapy

HD – Helical subdomain

HEK293T – Human embryonic kidney cells 293 T

HIV-1 – Human immunodeficiency virus type 1

HPS – His-Pro-Ser region

HR – Homologous recombination

HRP – Horseradish peroxidase

HTH – Helix-turn-helix motif

IBD – Integrase binding domain

IN – Integrase

INH2BP – PARP inhibitor II

Kb – Kilobases

KD – Knockdown

kDa – Kilodaltons

KO – Knockout

LEDGF/p75 – Lens epithelium derived growth factor/p75

LSM – Lymphocyte separation media

LTR – Long terminal repeat

LZ – Leucine zipper

MA – Matrix

MAB – Monoclonal antibody

MARP – Mono (ADP-ribosyl) transpherase

MLV – Murine leukemia virus

mRNA – Messenger RNA

MVP-ID – Major vault particle interaction domain

NAD⁺ – Nicotinamide adenine dinucleotide

NC – Nucleocapsid

NLS – Nuclear localization signal

pADPr – Poly(ADP-ribose)

PAR – Poly (ADP-ribose)

PARP – Poly (ADP-ribose) polymerase

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate buffer saline

PIC – Pre-integration complex

PNAI – Protein-nucleic acid interaction

PPI – Protein-protein interaction

PR – Protease

pTEFb – Positive transcription elongation factor b	VLP – Virus-like particles
PVDF – Polyvinylidene difluoride	vPARP – Vault Poly(ADP-ribose) polymerase 1
RAV-1 – Rous sarcoma virus 1	VSV-G – Vesicular stomatitis virus glycoprotein G
RNA – Ribonucleic acid	vWA – Von Willebrand type A protein
RNAP II – RNA polymerase II	WGR – tryptophan-glycine-arginine-rich domain
RT – Reverse transcriptase	WT – Wild type
RTC – Reverse transcriptase complex	ZAP1 – Zinc-finger antiviral protein 1
SAM – Sterile alpha motive	ZFN – Zinc finger
SDS-PAGE – Sodium dodecyl sulfate – polyacrylamide gel electrophoresis	
shRNA – Small hairpin RNA	
SSB – Single strand break	
SU – superficial	
TAR – Trans-activation-response element	
TAT – Trans-activator of transcription	
TBP – TATA binding protein	
TBS – Tris-buffered saline	
TM – Transmembrane	
TNKS – Tankyrase	
UV – Ultraviolet	
VIT – Vault protein inter-alpha-trypsin	

Vita

Luis Valdes was born in Villa Clara, Cuba on November 19, 1975 to parents Lidia Rodriguez and Luis Valdes Sr. He graduated with honors from the University of Havana, Cuba with a Bachelor of Science in Microbiology in 1999. Two years later he was assigned to the Autonomous University of Ciudad Juarez, Mexico where he would spend the next seven years lecturing different subjects like Microbiology, Biochemistry and Immunology, and several research projects. He would later join the MD program at the same university but the degree was not completed. He moved to the United States in 2009 and in 2010 he was admitted into the doctoral program at The University of Texas at El Paso in the Department of Biological Sciences. Since being enrolled in the Ph.D. program at UTEP Luis has worked as a teaching assistant for the Molecular Cell Biology, Microbiology and Immunology laboratories.

In 2013 Luis was one of the first students awarded the Dr. Keelung Hong Graduate Research Fellowship. His research has been presented at the 2013 UTEP College of Science Advisory Board Meeting, the 1st Annual Interdisciplinary Symposium: 21st Century Developments in Health Promoting Fields 2013, and at the Cold Spring Harbor Retroviruses Meeting 2013. Luis has authored a manuscript in Journal of Virology, 2013; "Poly (ADP-ribose) polymerase 1 promotes transcriptional repression of integrated retroviruses", and more recently in Journal of General Virology; "Poly (ADP-ribose) polymerase-1 silences retroviruses independently of viral DNA integration or heterochromatin formation".

Contact Information: lvaldesrodriguez@utep.edu

This thesis/dissertation was typed by Luis Valdes.