BRCA1 FUNCTIONS AS A NOVEL TRANSCRIPTIONAL COFACTOR IN HIV-1 INFECTION

by

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DEDICATION

This work is humbly dedicated to my parents, Dr. Federico Güendel Umaña and Nydia Sánchez Boschini, who provided me with the support required to follow through and all the opportunities that have allowed me the freedom to choose my career. To my husband, Arron Brice, who has only shown me infinite supplies of patience, strength, and love throughout my daily science expeditions. To my brother, Federico Güendel Sánchez, who gave me alongside my parents, unwavering support throughout the years; and to my new sister, Dr. Anastasia Abramova-Güendel, for being able to talk science with me and see the world with wonder through the eyes of a scientist. To my host families, the Baines and Dunbars, you have been a lot more than guiding lights in the strange lands that led me to achieve this goal. Finally, I would like to dedicate the many years of effort and hard work to all the children who do not have the privilege of an education that I have been given.

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LIST OF ABBREVIATIONS

Adenosine triphosphate ..... ATP
Amino acid ..... aa
Arginine (residue number) ..... R(residue number)
Ataxia telangiectasia mutated ..... ATM
Ataxia telangiectasia and Rad3 related ..... ATM
Aurora A kinase ..... Aur A
Breast cancer 1 ..... BRCA1
Bovine serum albumin ..... BSA
Bromodomain-containing protein 4 ..... BRD4
C terminal domain ..... CTD
Capsid protein ..... CA
C-C chemokine receptor 5 ..... CCR_5
C-X-C chemokine receptor 4 ..... $CXCR_4$
Chromatin immunoprecipitation ..... ChIP
Cluster of differentiation 4 ..... CD4
Combined antiretroviral therapy ..... cART
Cyclin dependent kinase 2 ..... Cdk2
Cyclin dependent kinase 4 ..... Cdk4
Cyclin dependent kinase 9 ..... Cdk9
Deoxyribonucleic acid ..... DNA
Dithiothreitol ..... DTT
DNA damage response ..... DDR
DRB-sensitivity inducing complex ..... DSIF
Double strand break ..... DSB
Dulbecco's Modified Eagle's Medium ..... DMEM
Ethylenediaminetetraacetic acid ..... EDTA
Fetal bovine serum ..... FBS
Food and Drug Administration ..... FDA
Gap 1 ..... G_1
Gap 2 ..... G_2
Glutathione S-transferase ..... GST
Green fluorescent protein ..... GFP
Hexamethylene bisacetamide-inducible protein ..... Hexim
HIV-1 associated neurocognitive disorders ..... HAND
Horseradish peroxidase ..... HRP
Human Immunodeficiency Virus ..... HIV

Human Papilloma Virus....HPV
Human T-lymphotrophic Virus.... HIV
Hydrochloric acid....HCl
Immunoglobulin G.... IgG
Immunonoprecipitation....IP
Integrase.... IN
Kilodalton ....kDa
La ribonucleoprotein domain family, member 7 .... LARP7
Long terminal repeat....LTR
Luciferase.... Luc
Major histocompatibility complex.... MHC
Matrix protein ....MA
Messenger ribonucleic acid....mRNA
Methylphosphatase capping enzyme ....MePCE
Minute (s).... min
Mutant.... mut
National Institutes of Health .... NIH
Negative-acting elongation factor....NELF
Negative regulatory factor ....Nef
Nonyl phenoxypolyethoxylethanol.... NP40
Nuclear factor kappa-light-chain-enhancer of activated B cells.... NF-κB
Nucleocapsid protein ....NC
Nucleotide.... nt
Peripheral blood mononuclear cell .... PBMC
Phosphate buffered saline .... PBS
Phosphatidyl inositol 3' kinase-related kinases.... PIKK
Polyacrylamide gel electrophoresis ....PAGE
Polymerase chain reaction ....PCR
Polyvinylidine fluoride .... PVDF
Positive transcription elongation factor b ....pTEF-b
Protease....PR
Protein phosphatase 2 A.... PP2A
Quantitative Reverse Transcriptase PCR....qRT-PCR
Regulator of Virion....Rev
Repeat region ....R
Retinoblastoma ....Rb
Rev-response element....RRE
Reverse Transcriptase .... RT
Revolutions per minute....rpm
Ribonuclease H ....RNase H
Ribonucleic acid....RNA
Ribonucleic acid polymerase II .... RNAP II
Roswell Park Memorial Institute ....RPMI
Serine (residue number)....S(residue number)

Single stranded ..... ss
Small interfering ribonucleic acid ..... siRNA
Small nuclear ribonucleic acid ..... snRNA
Small nuclear ribonucleic acid particle ..... snRNP
Sodium chloride ..... NaCl
Sodium dodecyl sulfate ..... SDS
Small Ubiquitin-like Modifier ..... SUMO
Super Elongation Complex ..... SEC
Surface glycoprotein 120 ..... SU
SWItch/Sucrose NonFermentable ..... SWI/SNF
Synthesis ..... S
Tat-associated kinase ..... TAK
Threonine (residue number) ..... T(residue number)
Trans-acting responsive element ..... TAR
Trans-activator of transcription ..... Tat
Transcription Factor II B ..... TFIIB
Transcription factor II D ..... TFIID
Transcription factor II H ..... TFIIH
Transmembrane protein 41 ..... TM
Unique 3' U3
Unique 5' U5
Viral protein R ..... Vpr
Viral protein U ..... Vpu
Virion infectivity factor ..... Vif
Wild type ..... wt

ABSTRACT

BRCA1 FUNCTIONS AS A NOVEL TRANSCRIPTIONAL COFACTOR IN HIV-1 INFECTION

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George Mason University, 2014

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Viruses have naturally evolved elegant strategies to manipulate host cellular machinery, including ways to hijack cellular DNA repair proteins to aid in their own replication. Retroviruses induce DNA damage through integration of their genome into the host DNA. DNA damage signaling proteins including ATR, ATM and BRCA1 contribute to multiple steps in the HIV-1 life cycle, including integration and Vpr-induced G_2/M arrest. However, there have been no studies to date regarding the role of BRCA1 on HIV-1 transcription. Size-exclusion chromatography experiments showed that BRCA1 eluted in the same fraction as other cellular proteins involved in HIV-1 transcription, including Cyclin T1 and Cdk9. BRCA1 was found to be important for viral transcription as cells that lack BRCA1 displayed severely reduced HIV-1 Tat-dependent transcription, and overexpression or selective depletion resulted in enhanced or decreased transcription. Moreover, small molecule inhibition of BRCA1 phosphorylation effector

kinases, ATR and ATM, decreased Tat-dependent transcription, whereas a Chk2 inhibitor showed no effect. Furthermore, treatment with curcumin and an ATM inhibitor decreased BRCA1 LTR occupancy. In addition, Tat was found to associate with BRCA1. These results suggest that HIV-1 infection upregulates expression of BRCA1 and may utilize its transcriptional cofactor function for efficient transcription. The presence of BRCA1 at the HIV-1 promoter highlights a novel function of the multifaceted protein in HIV-1 infection. The BRCA1 pathway or enzymes that phosphorylate BRCA1 could potentially be used as complementary host-based treatment for combined antiretroviral therapy, as there are multiple potent ATM inhibitors in development as chemotherapeutics.

CHAPTER ONE
STATEMENT OF PROBLEM

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of the acquired immunodeficiency syndrome (AIDS). Currently approved combined antiretroviral therapy (cART) relies on viral-based inhibitors, and present research efforts focus in finding new non-essential host targets and pathway modulation that can provide viral inhibition without creating drug resistance selective pressure on the virus. Therefore understanding the involvement of host cell factors might present a way to design better approaches for complementary treatment and expand upon therapy considerations when multiple host co-infections or HIV-associated malignancies are present.

CHAPTER TWO
SPECIFIC AIMS

Breast cancer protein 1 (BRCA1) is a tumor suppressor protein that has implications in processes such as cell cycle, transcription, DNA damage response, RNAi pathway and chromatin remodeling. BRCA1 exerts its many effects through modulating transcription of various factors. In the context of viral infection, BRCA1 has been poorly studied. Mainly, it has been linked to the human papilloma virus (HPV) and human T-lymphotropic virus (HTLV) oncogenic viruses through the Ataxia telangiectasia mutated kinase, a serine/threonine protein kinase best known for its role in modulating DNA repair, cell cycle, and cell death. In the case of HIV-1, BRCA1 has been linked to the accessory protein Vpr and the viral integrase.

To date there is no knowledge of the involvement of BRCA1 in HIV-1 transcription. Our studies are indicative of increased BRCA1 expression and phosphorylation in infection, similar to previous reports. In addition, our findings also suggest that it may play a role in Tat-dependent viral transcription. The **long term goal** of our research is to develop the understanding of BRCA1 in HIV-1 infection and to further elucidate host signaling pathways modulated by HIV-1. Recent studies have linked BRCA1 to participate in chromatin remodeling and transcriptional initiation and elongation. Moreover, various members of BRCA1-containing or BRCA1-interacting functional protein complexes have been shown to play an important function in HIV-1

transcription. Our **short term goal** is to focus on the characterization of BRCA1-containing complexes that are unique to HIV-1. The **objective** is to elucidate the functional participation of BRCA1 in viral transcription. We **hypothesize** that HIV-1 alters host protein dynamics that change host factor complexes in favor of the viral life cycle. As a transcriptional repressive or activating cofactor, BRCA1 may function in HIV-1 Tat-dependent transcription independently of its reported role in integration and G_2/M arrest during active HIV-1 infection. There is a **gap of knowledge** regarding the involvement of BRCA1 in HIV-1 transcription. Our **rationale** for the current proposal is based on preliminary data showing (1) HIV-1 specific upregulation of BRCA1, and (2) decreased Tat-dependent promoter transcription upon selective BRCA1 depletion. The information generated from these studies may aid in the identification of new host factors that are required for optimal viral transcription. Specifically, we will:

2.1 AIM I

Characterize BRCA1 expression in HIV-1 infection.

BRCA1 expression was characterized in established HIV-1 uninfected and infected T cell lines, Jurkat and J1.1, respectively. Importantly, we confirmed our results in primary blood mononuclear cells that were infected with the 89.6 dual-tropic HIV-1 strain. In addition, the correlation between infection and the phospho-status of BRCA1 was studied in TZM-bl cells. Specifically, the distribution of BRCA1 in low, medium, or high molecular weight complexes was measured by size-exclusion chromatography whole cell lysate fractions.

2.2 AIM II

Characterize BRCA1-containing macromolecular complexes in HIV-1 infection.

Based on literature annotation, we have confirmed that BRCA1 may be associated with pTEF-b and Tat in a high molecular weight multimeric complex by western blot. We have showed that this association exists first by performing GST pull-down-based assays, and consequently confirmed by immunoprecipitation. Preliminary mass spectrometry results indicate that BRCA1 may be in association with proteins involved in mRNA splicing and processing, as well as actin polymerization components.

2.3 AIM III

Determine the functional consequence of BRCA1 in HIV-1 Tat-dependent transcription.

We have determined increased BRCA1 expression and activation by phosphorylation in HIV-1 infected TZM-bl cells. We have demonstrated that BRCA1 functions as an enhancer of Tat-dependent LTR transcription and possibly, as a mediator of integration in new infection. We have shown that treatment with curcumin and ATR/ATM inhibitors targeting BRCA1 expression or upstream effector kinases, respectively, decrease Tat promoter transactivation and BRCA1 occupancy of the HIV-1 LTR promoter.

CHAPTER THREE
HUMAN IMMUNODEFICIENCY VIRUS TYPE 1: A BRIEF BACKGROUND

3.1 THE AIDS EPIDEMIC

It has been more than twenty years since HIV was identified as the etiological agent of AIDS [1,2]. AIDS is majorly characterized by its progressive degeneration of the human immune system [3]. Worldwide, it is estimated that 32.2-38.8 million people were living with HIV in 2012. Although that number has increased due to the use of antiretroviral therapy, the number of new infections has declined by 33% since 2001 [4]. Of these, ~1.1 million people reside in the United States, and almost 1 in 6 are unaware of their HIV-positive status [5,6].

cART has transformed HIV infection into a manageable chronic condition. However, multiple challenges exist that affect effectiveness of cART including long-term toxicity, regimen adherence, and non-opportunistic chronic comorbidities [7].

HIV-1 is the major cause of AIDS in the human population and the most commonly used model in research [8]. Therefore, this dissertation primarily focuses on the study of the HIV-1 promoter, with use of the dual tropic 89.6 (T cells and macrophages) and single tropic NL4.3 (T cells) strains.

3.2 GENOME AND STRUCTURE OF HIV-1

3.2.1 Genome

The HIV- 1 genetic material is transmitted as single stranded, positive sense RNA, but is incorporated and exists within an infected cell as an integrated double stranded DNA provirus. The structure of the HIV-1 genome and composite promoter proximal region are shown in Figure 1.

The HIV-1 long terminal repeat (LTR) flanks the coding region and is present on both ends of the integrated viral genome [9,10]. The 5' region contains regulatory elements needed for transcriptional initiation and elongation, while the 3' region functions in transcription termination and polyadenylation [11]. The 5' LTR is divided into the U3, R, and U5 regions that contain four functional elements regulating transcription. The U3 region contains a basal or core promoter (nt -78 to -1), core enhancer (nt -105 to -79), and a modulatory region (nt -454 to 104) [9]. The modulatory region has been suggested to contain a negative regulatory element (NRE) at nucleotides -340 to -184 given that deletions in this region increased viral transcription and replication [9,12,13]. Additionally, the core enhancer region contains three Sp1 binding sites and two NF-κB motifs that are key LTR-driven transcriptional regulatory elements, and are important for Tat-independent basal transcription [9]. The R region contains the transactivation response element (TAR) (nt +1 to +57) which binds Tat and is responsible for the recruitment of viral and cellular components to initiate and elongate transcription [9,14]. Lastly, the U5 region contains other important motifs including but not limited to the *gag* leading sequence (GLS) and three AP-1 binding sites [15-17].

The HIV-1 proviral genome consists of approximately nine kilobases [18]. Genes *gag*, *pol*, and *env* encode the structural viral proteins common to all retroviruses [19]. *Gag* and *pol* are further processed into Matrix (MA), Capsid (CA), Nucleocapsid (NC), Protease (PR), Reverse transcriptase (RT), and Integrase (IN) proteins. *Env* encodes the surface and transmembrane proteins gp41 and gp120. The regulatory genes *tat* and *rev* code for proteins that assist in viral transactivation and manipulation of cellular splicing events [18]. The accessory genes *vif*, *vpr*, *vpu*, and *nef* are involved in and budding of mature virions, immunosuppression, and survival [18].

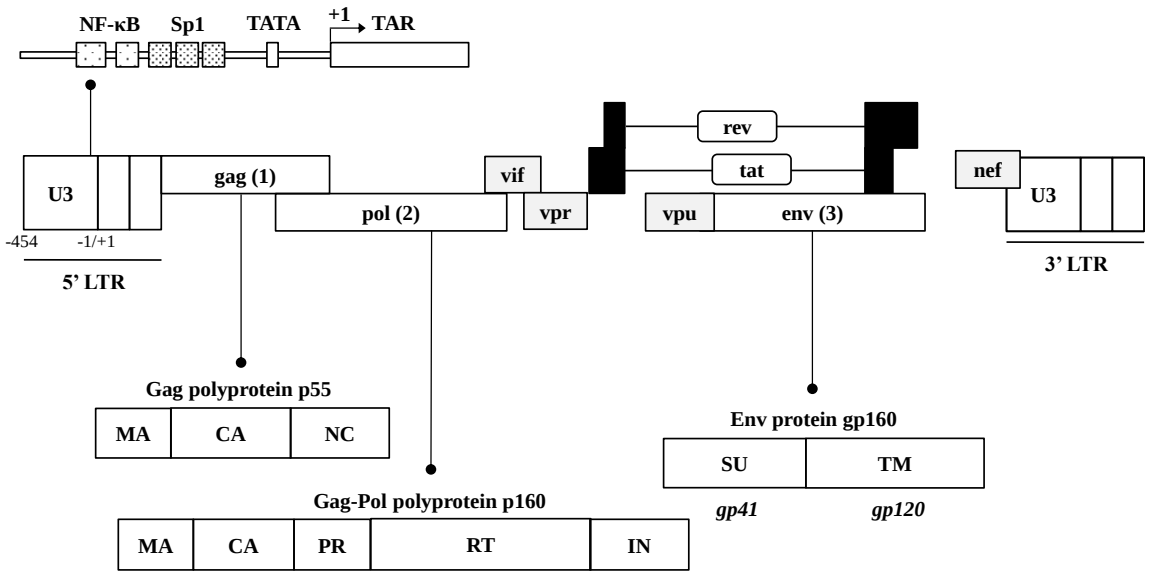

Figure 1 HIV-1 genomic organization and LTR region.

HIV-1 viral genes are shown from left to right in the 5' to 3' direction. Two long terminal repeats (LTR) span both ends of the genome and contain regulatory and structural elements. Genes common to all retroviruses include the structural protein-coding *gag*, *pol*, and *env* (numbered 1, 2, 3, respectively). *Gag* and *pol* are further processed into Matrix (MA), Capsid (CA), Nucleocapsid (NC), Protease (PR), Reverse Transcriptase (RT), and Integrase (IN). Correspondingly, *env* codes for the surface (SU) and transmembrane (TM) proteins, gp41 and gp120. The accessory proteins (light gray) *vif*, *vpr*, *vpu*, and *nef*, function in immunosuppression, cell survival, and viral budding pathways. *Rev* and *tat* (black) code for regulatory proteins that participate in splicing events and viral transcriptional transactivation.

3.2.2 Structure

HIV-1 is a member of the lentiviral *Retroviridae* family [20]. Retroviruses consist of small enveloped viruses that assemble into distinct immature and mature viral particles. The spherical mature virion diameter ranges between 80-100 nm, and contains all the essential components to guarantee early replication upon infection [21]. Stabilized by NC proteins [22], two genomic copies of positive sense single-stranded RNA are encased within a CA core, which is typically cone-shaped with a low percent of conical cylinders infrequently observed [23]. Viral proteins included within this core are NC, RT, IN, PR; and the viral accessory proteins Nef, Vif, and Vpr [18,24]. In addition, host cell $tRNA_3^{Lys}$ is packaged into virions during assembly to serve as a primer for viral cDNA synthesis during reverse transcription [25]. The core structure is encased in a MA protein shell that is associated with the inner viral membrane, and the exterior of the viral particle is covered with envelope (Env) glycoprotein composed of an ectodomain (gp120) and a transmembrane domain (gp41). The structure of a mature HIV-1 viral particle is shown in Figure 2.

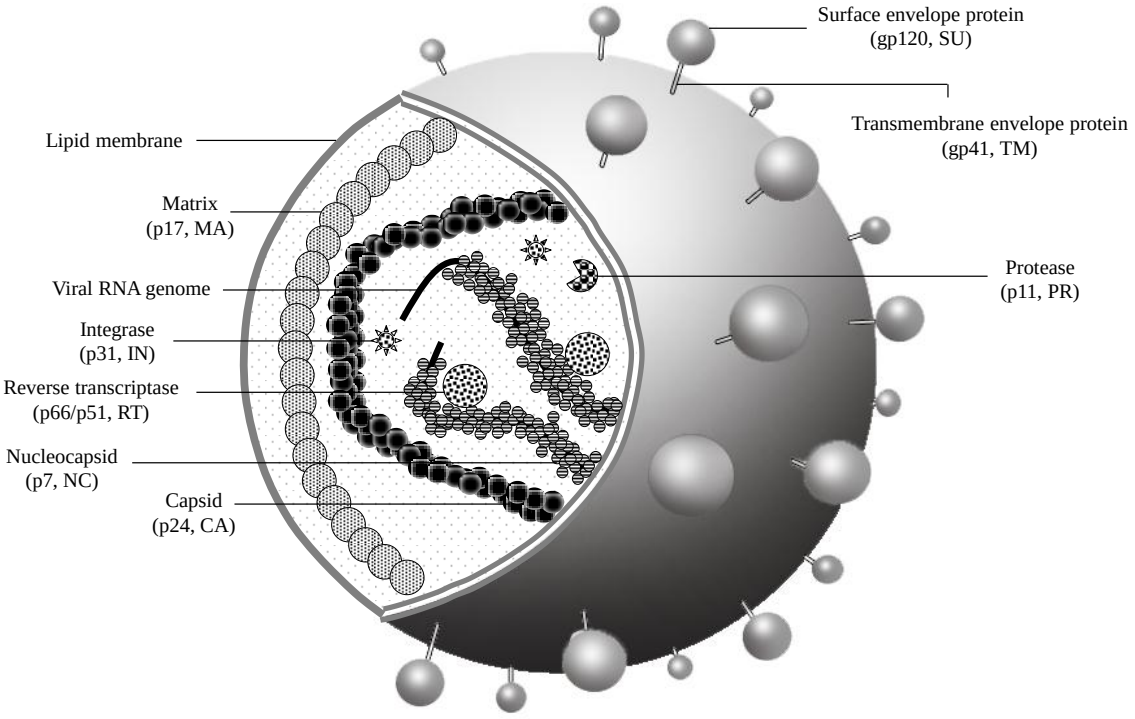

Figure 2 Diagrammatic representation of a mature HIV-1 particle.

The surface and contents of a mature viral particle are depicted. Enveloped and spherical in shape, the 80-100 nm particle is surrounded by a host-derived lipid membrane expressing the surface and transmembrane proteins gp120 and gp41. The viral core consists of a membrane-associated p17 Matrix protein layer enclosing the typical cone-shaped condensed mature p24 capsid. Each viral particle encases two copies of ssRNA, which are associated with two Reverse Transcriptase units and Nucleocapsid proteins. Included are protease and Integrase viral enzymes required for viral replication. Figure concept adapted from Freed, 2001 [26].

3.3 HIV-1 PROTEINS

3.3.1 Gag Polyprotein

The unspliced $Pr55^{Gag}$ polyprotein precursor is cleaved by the viral PR to the mature Gag proteins MA, CA, NC, two smaller spacer peptides (SP1, SP2), and a smaller peptide (p6) [26]. The p17 MA protein lines the inner membrane of the virion and targets both Gag and Gag-Pol polyproteins to the plasma membrane prior to viral assembly, the p24 CA composes the conical-shaped viral core and functions in viral assembly and uncoating, and the p9 NC coats the RNA genome targets it for assembly as well [23,27]. p6 is responsible for binding Vpr and functions in viral release [28]. Aided by its high affinity to viral RNA containing an intact packaging signal, Gag polyprotein expression is sufficient for assembly of immature viral-like particles [22]. Upon budding, the immature virion undergoes major structural changes during the process of maturation. Activated during assembly, viral PR cleaves the polyprotein into its constituent elements which reassemble to form diverse layers of the mature viral particle [23].

3.3.2 Gag-Pol Polyprotein

The unspliced $Pr160^{GagPol}$ polyprotein originates from a rare frameshift event during translation of $Pr55^{Gag}$ [26]. Like the Gag polyporotein, the unspliced $Pr160^{GagPol}$ polyprotein precursor is cleaved by the viral PR to the mature *pol*-econded viral enzymes PR (p10), RT (p66/p51) and IN (p31). These enzymatic proteins are essential for initial infection as required for cleavage of viral polyproteins and viral particle maturation, reverse transcription synthesis of a DNA copy from the viral RNA genome, and integration of proviral DNA into the host genome, respectively [28,29]. Because these *pol*-encoded enzymes are essential for HIV-1 infection but non-essential to cellular

processes, they have been targets for the development of cART. However, the most serious side effects associated with cART is the appearance of multi-drug resistant HIV-1 strains, in particular to protease inhibitors. In combination with an error prone RT enzyme (lacking proofreading capability) and a high turnover viral replication rate, drug selected mutations can only add to the significant viral heterogeneity found within a single patient [30,31].

3.3.3 Env

The p160 Env precursor is processed by a host cellular protease during trafficking to the cell surface, distinctly than the PR-cleaved Gag and Gag-Pol polyproteins [26]. The singly spliced *env* mRNA is translated as an aa845-870 gp160 precursor protein in the rough endoplasmic reticulum and migrates through the Golgi complex, where it undergoes extensive glycosylation that is required for infectivity [27]. After cleavage and oligomerization into trimers, gp41 functions as the transmembrane glycoprotein and gp120 as exterior envelope glycoprotein [27,32,33]. The primary function of gp120 is to recognize the specific target cellular receptors, CD4 glycoprotein and members of the chemokine receptor family, CCR_5 and $CXCR_4$ [34-36]. gp41 on the other hand, serves as an anchor for the gp120/gp41 membrane complex and contains critical domains for membrane fusion catalysis after viral docking [26]. The CCR_5 receptor binds macrophage-tropic, non-syncytium forming viral isolates (R5 virus strains) and $CXCR_4$ binds T cell tropic, syncytium forming isolates (R4 virus strains) [18,37]. Although binding to CD4 alone does not trigger membrane fusion, CD4 binding induces

conformational changes in the gp120 glycoprotein that forms or exposes CCR_5/$CXCR_4$ binding sites [27]. It is this second binding event that leads to membrane fusion [37].

3.3.4 Tat

The HIV-1 *transactivator of transcription*, Tat, is an essential regulatory protein that modulates the viral chromatin landscape and transcriptional activation by association with the *transactivation response RNA* loop region, TAR, present on the proviral 5' region at the transcriptional initiation site (nt +1 to +57) on the HIV-1 long terminal repeat (LTR) [38]. Produced from multiply spliced mRNA, it functions primarily as a nuclear protein. Exon 1 encodes aa1-72 and exon 2 encodes aa73-86 or 73-101 depending on the viral strain [38]. Both Tat isoforms (Tat_{1-86} and Tat_{1-101}) effectively transactivate the LTR and are expressed from early multiply spliced mRNA or late incompletely spliced messages [39]. As previously mentioned, in the case of our study, we utilize viral strains NL4.3 and 89.6. HIV-1 NL4.3 strain contains a truncated Tat_{1-86}, while a majority of clinical isolates, including 89.6, contain Tat_{1-101} [40]. The Tat/TAR complex is able to recruit various critical host cell factors such as the pTEF-b complex (Cdk9/Cyclin T1) to the RNA polymerase II (RNAP II) complex that is occupying the LTR [41-48]. Chiefly, Tat promotes transcriptional elongation for the generation of full length viral mRNAs [49,50].

3.3.5 Rev

The fully spliced *rev* mRNA encodes the *regulator of expression of virion proteins* Rev, an early viral gene product and sequence-specific RNA binding protein that aids in early-to-late phase transition of HIV-1 gene expression [51,52]. Normally, export

of unspliced pre-mRNAs is prevented until intron removal is completed to circumvent the expression of anomalous and potentially harmful proteins [53]. Rev binds a complex secondary RNA structure termed the Rev response element (RRE) located within the second HIV-1 intron, permitting the nuclear export of unspliced and incompletely spliced viral transcripts that code for the late structural and maturation proteins [54]. Export occurs via specific pathway used by small nuclear RNAs (snRNAs) and the ribosomal 5s RNA, thus the Rev activation domain functions as a NES that redirects RRE-containing viral RNAs to a non-mRNA export pathway [55]. Because of this critical function, Rev is unequivocally obligatory for HIV-1 replication as proviruses that lack functional Rev are transcriptionally active but do not express viral late genes resulting in no virion production [56].

3.3.6 Nef

The doubly spliced *nef* mRNA encodes *negative factor* Nef, unique to primate immunodeficiency viruses. Regarded as a multifunctional viral protein involved in downregulation of CD4 cell surface expression, pleiomorphic perturbation T cell activation, and HIV-1 infectivity stimulation [57-60]. Briefly, Nef increases CD4 endocytosis and lysosomal degradation to enhance viral production by increasing Env incorporation and virion budding, actions antagonized by CD4 surface expression [61,62]. In addition, Nef has been shown to decrease MHC Class I resulting in inefficient cytotoxic T cell killing of HIV-1 infected cells [63]. Additionally, Nef has been shown to both obstruct induction of NF-κB and IL-2 expression contrarily to elevating T cell activation, depending on expression context [58,64]. To elaborate, the expression of a

CD8-Nef chimeric molecule had antagonizing or positive effects depending of its cytoplasmic (T cell receptor signaling block) or cell surface localization (spontaneous activation). Lastly, though the mechanism is unclear, Nef deficiency results in viral particles that are 10-30-fold less infectious than those produced in the presence of wild type Nef [65]. Furthermore, it is packaged into the viral particle and has been shown to be processed by PR during virion maturation [65].

3.3.7 Vpu

The singly spliced *vpu* mRNA is expressed from *env* mRNA, yet Vpu (*viral protein U*) is translated in lesser levels than Env due to a non-efficient translation intiation codon [52,66]. Functionally, Vpu supports intracellular CD4 ubiquitin-mediated degradation within the trafficking pathway in the endoplasmic reticulum and enhances Env transport to the cell surface for virion assembly [67]. Also, Vpu has been shown to increase viral release from the infected cells, where a *vpu* deletion mutation resulted in the accumulation of cell-associated viral proteins and impairment of progeny virion release [68]. Vpu has been further implicated as a viral interfering protein of the classic MHC class I pathway which utilizes endoplasmic reticulum trafficking to the cell surface as well [69]. Here, it is thought that Vpu induces degradation of newly synthesized MHC class I via retrograde translocation and the proteasome.

3.3.8 Vpr

The singly spliced *vpr* mRNA encodes a 15 kDa protein that plays several key functions of viral infection. In addition to Vif, Vpr (*viral protein R*) is packaged into the viral particle [70,71]. Vpr plays an important role by facilitating nuclear translocation of

the PIC, providing its ability to infect non-dividing cells such as those of macrophage lineage, for which it has become a druggable target of interest [72,73]. However, Vpr has been found to be dispensable for cycling cells [74]. Of equal importance, Vpr exerts tremendous modulation of the host cell cycle by inducing a G_2/M cell cycle arrest, through which it is thought to prevent the establishment of chronic infection [75,76]. One of the cell cycle arrest mechanisms includes inhibition of p34cdc2/Cyclin B complex activation, an important factor for entry into mitosis [77,78]. Because G_2 cell cycle arrest has been thoroughly studied in the context of DNA damage, the elucidation of DNA damage sensors and cell cycle check points have shed new light on novel pathways by which Vpr acts to induce cell cycle arrest [79]. Key players in early damage response include proteins Ataxia telangiectasia-mutated and Rad3-related (ATR) and Ataxia telangiectasia-mutated (ATM), both of which act as regulators of the G_2/M checkpoint in response to genotoxic stress [79,80]. These interactions will be discussed later in relation to BRCA1 function in viral infection (Chapter 4, section 4.4).

3.3.9 Vif

The singly spliced *vif* mRNA codes for the crucial accessory protein *virion infectivity factor*, involved in infectivity enhancement of viral particles, stability of viral DNA, and possibly virion assembly [81]. Vif exerts its essential function in a cell-specific manner, permitting the production of infectious viral particles in relevant non-permissive cell lines such as $CD4^+$ T cells and macrophages [82,83]. However, its function is dispensable in more permissive cell lines such as adherent cells and some T cell lines, suggesting that these cells may express a protein that can compensate for Vif

function. Of interest, viral particles produced in permissive cells can infect non-permissive cells but these in turn, produce non-infectious particles [84]. Until recently, this function majorly composed Vif participation in viral mechanisms. However, APOBEC3G was found to be incorporated into virions defective for Vif, a finding that has led to a new focused field of study involving HIV-1 restriction factors [85]. APOBEC3G exerts inhibitory effects at the reverse transcription phase of the viral life cycle by cytosine deamination, although studies have shown that APOBEC3G can achieve inhibition in a deamination independent mechanism [85-87]. Therefore, Vif functions to overcome this APOBEC3G-block to viral infection and to prevent APOBEC3G incorporation into viral particles, partially through APOBEC3G proteosomal degradation [88,89]. Finally, Vif was recently described to play a small role in G_2/M cell cycle arrest [90].

3.4 VIRAL LIFE CYCLE

In the absence of cART, HIV infects and progressively depletes $CD4^+$ T lymphocytes, resulting in compromised immunity [91]. Specifically, it can produce more than ~$1x10^{10}$ viral particles per day in a chronically infected individual naïve to ART.

Briefly, initial infection of HIV-1 is disseminated to lymphoid tissue [92-94]. Primary infection is marked by an initial burst in viral replication and acute viremia in peripheral blood that is partially contained by immune response to infection [95], marking a detectable decrease in blood viral load in addition to an initial loss of circulating $CD4^+$ T cells, activation of $CD8^+$ T cells for targeted HIV-1 infected cell killing, and seroconversion [92,96,97]. At this point, the establishment of clinical latency

or chronic stage of infection occurs, where infected cells enter a state of quiescence. After latency establishment, an eventual and progressive loss of $CD4^+$ T cells will continue until the system reaches a cell count of <200 cells/μL of blood, the clinical definition of the onset of AIDS [96]. Despite major research efforts focused on the elucidation of latency establishment and immune evasion, these are mechanisms that remain poorly understood. However, current molecular basic research is interested in further understanding mechanisms by which the integrated provirus can be reversibly repressed [98]. Reversible repression can occur in several ways including proviral repression by neighboring cis-acting sequences such as integration tied to expressed genes (via LEDGF/p75) possibly leading to active expression, or into unfavorable chromatin environments possibly leading to latency. Independently of integration site, proviral transcription is closely linked to the activation status of the cell. The transcription of cellular genes is regulated through a highly organized chromatin structure which will make gene promoters accessible or not to transcriptional machinery [10], and it is a variety of enzymes and protein complexes that will exert differential levels of regulation in order to mediate the opening or closing of chromatin structure.

The primary transcript of 9.3 kb is processed into unspliced message (constituting genomic material for new viral progeny), singly spliced (4-5 kb) and multiply spliced (1.7-2 kb) mRNA [39]. As described earlier, viral infection starts with CD4-CCR_5/$CXCR_4$ binding, docking and consequent Env-mediated viral-host membrane fusion [99]. Upon fusion, the core Capsid is released from the viral particle followed by p24-Capsid uncoating and release of contents into the host cytoplasm, where the viral RT

begins the conversion of the viral RNA genome into cDNA [99]. Approximately 2 hours post-entry [100], the RT synthesizes a negative DNA strand from the genomic template and creates a RNA-DNA intermediate hybrid, from which the positive DNA strand is synthesized [99]. Ultimately, the genomic RNA template is degraded by RNAse H resulting in a double stranded viral DNA copy [101]. It is at this step that the previously discussed APOBEC3G restriction factor exerts its effect (subsection 3.3.9). The viral DNA copy is trafficked to the nucleus in a complex with the viral proteins MA, IN, and Vpr [102]. IN mediates integration into host DNA, and host enzymes complete the integration process by repairing the single strand gaps connecting the unjoined viral DNA 5′ ends, finally resulting in the establishment of a stable provirus [99,102]. After proviral transcription (will be discussed in section 3.5), early multiply spliced mRNAs provide the regulatory proteins that permit the export of incompletely spliced and unspliced viral mRNAs (as discussed in subsection 3.3.5). The expression of Gag, Gag-Pol, Env, and accessory proteins is dependent on the cytoplasmic accumulation of these spliced and non-spliced transcript variants. Accumulation of these proteins within the cytoplasm and on the cell surface, results in favorable conditions for virion assembly and budding [99]. Released viral particles undergo maturation through the proteolytic activity of the viral PR as discussed in subsection 3.3.2 [99]. These processes are depicted in Figure 3.

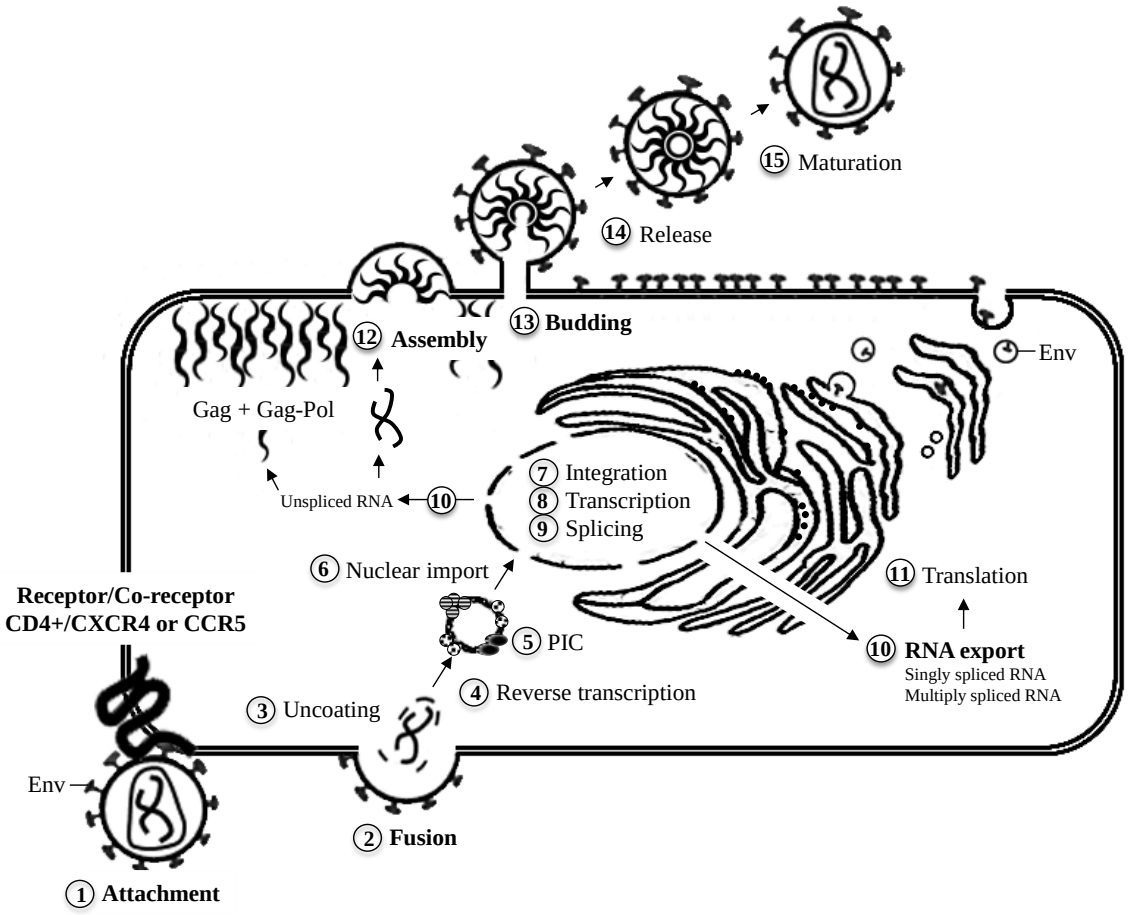

Figure 3 HIV-1 viral life cycle.

A cartoon depicting HIV-1 viral life cycle is shown. Infection starts upon engagement of the CD4 receptor envelope and chemokine co-receptor CCR_5 or $CXCR_4$ by the viral envelope (Env) glycoprotein spikes (step 1). Viral docking leads to membrane fusion and viral entry (step 2). Partial core shell uncoating (step 3) permits reverse transcription (step 4) and generation of the PIC pre-integration complex (step 5). Nuclear import (step 6) is sequentially followed by proviral DNA integration into the host genome (step 7) resulting in the establishment of latency, while proviral transcription (step 8), and viral transcript splicing (step 9) from proviral DNA occur after viral reactivation. Energy-dependent nuclear export (step 10) allows for viral messages to be translated (step 11). Transcription yields different sized viral mRNAs (e.g. depicted are unspliced message → Gag + Gag-Pol or genomic material, singly spliced → Env). Specific protein components and genomic RNA is preferentially packaged by the assembling virion (step 12) and followed by endosomal-mediated immature viral particle budding (step 13) and release (step 14). Protease-mediated virion maturation (step 15) results in the formation of infectious viral particles. Figure concept adapted from Mayo Clinic "HIV-1 REPLICATION, LENTIVIRAL VECTORS" online resource (Dr. Eric M. Poeschla laboratory, original diagram by Dyana Terri Saenz) and Engelman and Cherepanov, 2012 [99].

3.5 HIV-1 TRANSCRIPTION OF THE INTEGRATED PROVIRUS

3.5.1 Chromatin landscape of integrated HIV-1 LTR

The HIV-1 long terminal repeat (LTR) is located at both ends of the integrated provirus. The 5' LTR functions, as with all retroviruses, as the viral promoter and contains several regulatory elements that are required for transcriptional initiation and elongation. Early research during the mid-1990's established the presence of 4 distinct DNase I hypersensitive sites (DHS) at the 5' LTR [103-105], suggesting that these sites in native chromatin are free of histones and readily accessible regions to DNA binding proteins [106]. Post-integration, the 5' LTR is further characterized by a highly organized chromatin structure consisting of 5 nucleosomes (nuc-0 to nuc-4) [10]. These nucleosomes delineate 2 DHS regions localized at nucleotides -255 to -3 and from +141 to +265, which contain nuc-1 (and the transcriptional start site) in between. Region -255 to -3 contains several promoter and enhancer elements that are later occupied by transcription factors [107]. Upon stimulation by using inducers such as TNF-α or histone deacetylase (HDAC) inhibitors (TSA and sodium butyrate are commonly used), DSH3 and DHS4 extend resulting in remodeling of nuc-1 [103,107,108]. Logically, the integrated viral genome is subject to the same epigenetic regulation of the host. Thus, posttranslational modifications become an important key player that has the ability to fine tune the regulation of this organized chromatin structure. Various enzymatic complexes exert DNA methylation, modification of histone tails, or mechanical chromatin remodeling via ATP-dependent enzyme complexes in order to alter DNA-histone contacts [109]. Trono *et al*. [110] successfully depicted how the chromatinized integrated viral genome is subject to host cell chromatin regulatory elements that dictate the state of

latent or activated provirus. In a resting T cell, chromatin regulatory elements at the viral promoter such as nuc-1 carry heterochromatic markers such as lysine 9 trimethylated histone 3 (H3K9me3), heterochromatin protein 1 (HP1), and low levels of histone acetylation [103,110]. The presence of specific sequences at the 5' LTR can bind negative regulators such as NF-κB sites that in a resting cell bind p50 homodimers, C-promoter binding factor-1 (CBF-1), and Sp1. In turn, these proteins are able to recruit HDACs and histone methyl transferases (HMTs) and a variety of other repressive complexes such as C-TIP2 [111,112]. Inversely, upon T cell activation, a cascade of signals revert the repressive state. Events such IκB degradation permits nuclear migration of p50-p65 heterodimer (active NF-κB) and consequent binding to the respective NF-κB site, stimulating viral expression and recruitment of histone acetyltransferases (HATs) that result in nuc-1 remodeling. Additional changes such as calcium flux triggers a signaling cascade by activation of calcineurin which in turn causes NFAT nuclear migration, a positive regulator of HIV-1 transcription. HIV-1 gene expression involves a complex interplay between chromatin-associated proviral DNA, cellular transcription factors, and the viral encoded Tat.

3.5.2 Transcriptional initiation and elongation

Viral transcription is initiated by intracellular cascades such as cytokine signaling that results in transcription factor activation [113]. The HIV-1 5' LTR promoter is considered a faulty promoter given that it lacks regulatory elements and strong enhancer elements required for efficient RNAP II processivity, thus transcription of the HIV-1 LTR initiates with deficient RNAP II activity [114,115]. Tat is an important regulatory

protein that unlike typical activators binding to promoter DNA, binds to nascent viral RNA through an RNA-binding site termed the TAR element at the transcriptional initiation site (nt +1 to +57) on the 5' LTR, resulting in transcriptional elongation [116]. The Tat/TAR complex is able to recruit various critical host cell factors such as the pTEF-b complex (Cdk9/Cyclin T1) to the RNAP II holoenzyme that is occupying the LTR [41-45,47]. Figure 4 shows basic events taking place in Tat-dependent transcription. pTEF-b is an ubiquitous positive acting elongation factor that actively phosphorylates the carboxyl-terminal domain (CTD) of RNAP II, and has been shown to be a critical cofactor for Tat activation of elongation [48,117,118]. Upon Tat pTEF-b binding, Cdk9 undergoes conformational changes that result in enzyme activation yielding the modification of both negative and positive cellular elongation factors [48,119-121]. RNAP II recruitment to the LTR occurs via the 5' LTR U3 region. As depicted in Figure 1, the viral promoter harbors a TATA box element that binds TFIID that consequently binds TFIIB and recruits RNAP II [116]. A model of cellular transcription reasons that RNAP II CTD is phosphorylated at serine 5 (S5) at the initiation complex and serine 2 (S2) on the elongating complex, however, the RNAP II associated with the HIV-1 5' LTR promoter is phosphorylated at S5 and S2 in the presence of Tat, both at the promoter and in downstream coding sequences. These findings suggest that Tat/TAR-dependent kinase activity of pTEF-b plays an important role in diverse steps in transcription elongation and chromatin modification. In the current model [116,122], Tat stimulates elongation rather than initiation since in its absence, there is initiation by RNAP II from the LTR but it stalls ~20-40 nt downstream of the transcriptional start site. In the

presence of Tat, recruitment of pTEF-b results in phosphorylation of negative elongation factors NELF and DSIF [123,124], where NELF dissociates and DSIF becomes a positive elongation factor. Together with further RNAP II S2 and S5 CTD hyperphosphorylation, these events ensue transcriptional processivity. It is important to note that once elongation is triggered, pTEF-b travels with the elongation complex but its CTD kinase activity is not required [125]. In addition to pTEF-b and as part of the super elongation complex (SEC), Tat recruits ELL2 which directly stimulates RNAP II processivity by suppressing its transient stalling [126].

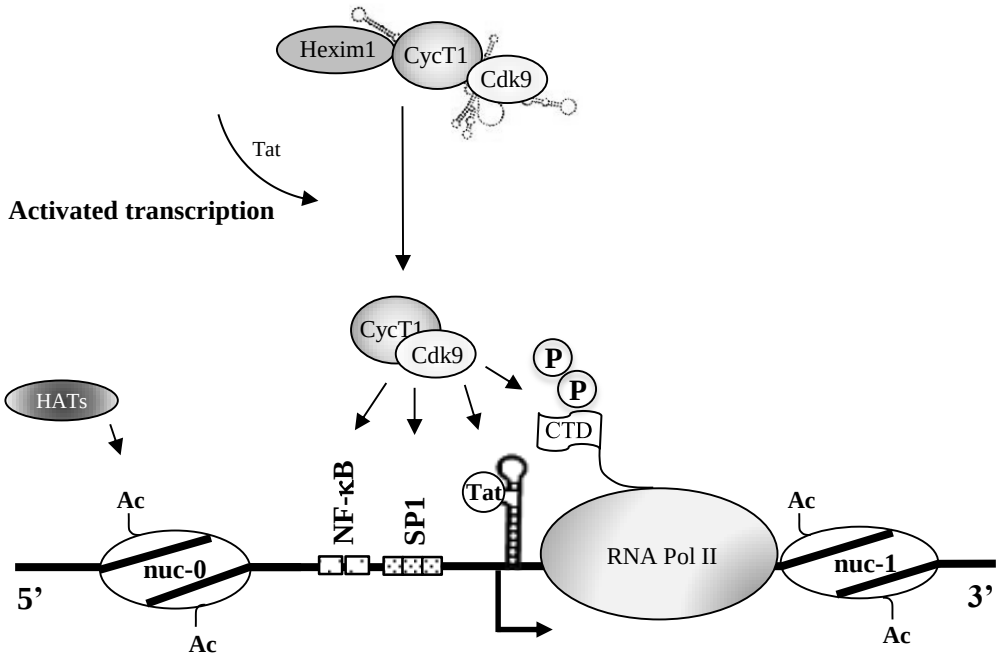

Figure 4 HIV-1 Tat-dependent transcription.

A basic diagrammatic representation of activated HIV-1 proviral transcription is shown. The 7SK snRNA-sequestered Cdk9/Cyclin T1 provides a reservoir of nuclear pTEF-b that can be tapped into upon stress or growth stimulating signals (will be discussed in subsection 3.5.3). In this inhibitory complex, Hexim1-mediated inhibition pTEF-b kinase activity is 7SK snRNA-dependent. Upon proviral transcriptional activation, histone acetyltransferases (HATs) and pTEF-b are recruited to the 5' long terminal repeat harboring the viral promoter through promoter-binding of NF-κB (p50-p65), Sp1, and Tat (through TAR sequence of nascent RNA transcripts). Histone acetylation results in a more permissive chromatin conformation for transcriptional elongation upon RNAP II C-terminal domain (CTD) phosphorylation by Cdk9. Figure concept adapted from He and Zhou, 2011, and Carpio *et al*. 2010 [14,127].

3.5.3 p-TEFb complexes in HIV-1

pTEF-b is a heterodimer composed from Cdk9 and a C-type Cyclin (T1, T2a, T2b, and K), with Cyclin T1 being the most common and abundant partner [128]. Cdk9 exists in two functional isoforms, 42- and 55 kDa, which are differentially expressed within tissues in the body. Both isoforms associate with the respective Cyclin component and exhibit kinase activity for the RNAP II CTD [128]. In quiescent cells, pTEF-b is sequestered by Hexim1/7SK snRNP complex that includes Hexim1 or Hexim2, LARP7 protein, and MePCE [31,47,129-133]. pTEF-b sequestration in this complex prevents basal transcriptional activation of proviral DNA by pTEF-b recruited in a Tat-independent manner [134]. In the presence of stress, pTEF-b dissociates from 7SK snRNA/Hexim1 and associates with Brd4, forming a small transcriptionally active complex that is then recruited to cellular promoters [135,136]. Despite its central role in the transcription of cellular genes, this complex has recently been shown to inhibit Tat transactivation as it can actively compete with Tat for binding of pTEF-b [126]. In infection, pTEF-b is recruited to the viral promoter, initially via p50-p65 and later via Tat-TAR interaction [38,137]. Additional complex regulation has been suggested by Zhou *et al*. [138] where TAR RNA actively displaces 7SK snRNA in an effort to transition inactive pTEF-b into the activated Tat/TAR-bound form for HIV-1 transcription stimulation.

Originally, pTEF-b is known to exist in 3 distinct complexes: the *core complex* composed of Cdk9/Cyclin T1 or Cyclin T2/Brd4, the *7SK snRNP complex* composed of Cdk9/Cyclin T1 or Cyclin T2/7SK snRNA/Hexim1 or Hexim2/LARP7/MepCE, and the *super elongation complex* composed of Cdk9/Cyclin T1 or Cyclin T2/ELL2/Aff4/

Enl/AF9 [139,140]. Collective work by Narayanan *et al*. [141] and Van Duyne *et al*. [10,142] has resulted in the characterization of pTEF-b-containing complexes in HIV-1 infection. A composite of these findings is summarized in Figure 5. Through size exclusion chromatography, we have demonstrated that pTEF-b exists as part of 4 differently sized complexes. Complex I (C-I) has an estimated multimeric high molecular weight of ~2.2-1.2 MDa, complex II (C-II) is situated at ~670-600 kDa, complex III (C-III) ranges from ~600-300 kDa, and complex IV (C-IV) is composed of elements <300 kDa. In infection, C-I has been shown to contain subunits of SWI/SNF chromatin remodeling complexes, Cyclin T1, Cdk9, and importantly, Tat. C-II contains Cyclin T1, Cdk9, Hexim1, and the largest amount of 7SK snRNA, suggesting that it could represent the inactive fraction of pTEF-b. C-III contains Cyclin T1, Cdk9, ELL2, and Tat, suggesting that it could represent the SEC fraction of pTEF-b. Lastly, the smaller C-IV contains re-distributed Cyclin T1 and Cdk9. Interestingly, both ELL2 and especially Brd4 did not elute from the whole cell protein lysate preparations, possibly indicating that these proteins are mostly nuclear and DNA-bound. Additionally, we have demonstrated the effect of diverse small molecule inhibitors of HIV-1 Tat-dependent transcription exclusively on components of C-IV of infected but not uninfected cells, including Cdk inhibitors Flavopiridol and CR8#13, as well as GSK-3β 18BIOder [141,143].

Similarly, the Rice lab recently published convincing data on the existence of diverse pTEF-b-containing complexes [140]. Here, the authors have mined human endogenous complexome data described by Malovannaya *et al*. [144] and identified 12 distinct multiprotein complexes containing Cdk9 and Cyclin T1 (Cdk9/Cyclin T1-

associated protein complexes, CCAPs). Of these, 5 novel pTEF-b complexes negatively regulated HIV-1 Tat function and viral gene expression. The authors argue that since siRNA depletions of CCAPs enhanced Tat function, it is possible that these complexes diminish Cdk9 and Cyclin T1 levels available for Tat, in a way comparable to the negative regulation exerted by the 7SK snRNP. Of our interest, is the finding of a prominent pTEF-b-containing CCAP in a very large multiprotein complex containing CBP/p300, 20 subunit SWI/SNF complex, and Mediator complex, which regulates transcription initiation and chromatin remodeling. However, this complex was not further discussed within this study.

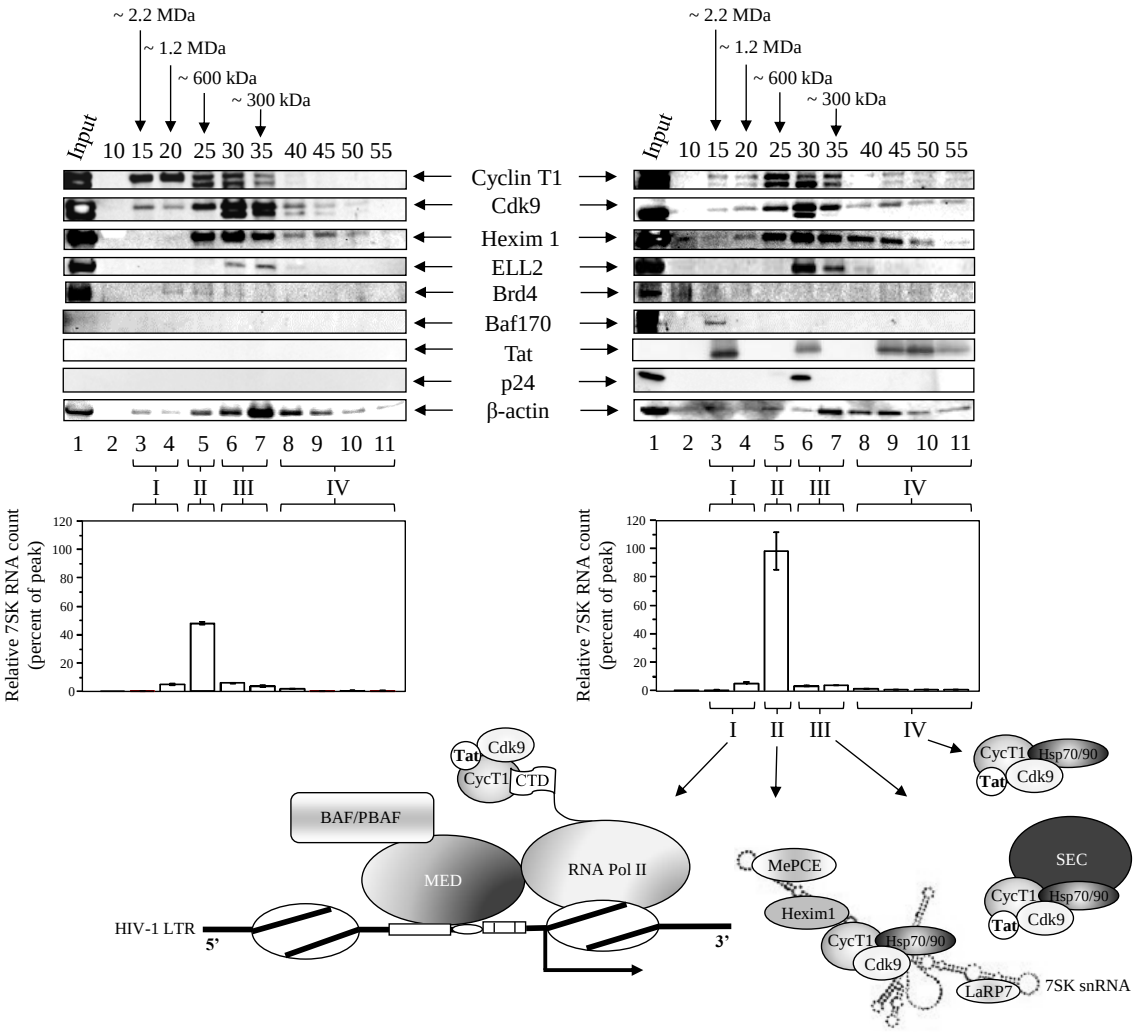

Figure 5 P-TEFb complexes in HIV-1 infection.

Raw data graphical summary of recently reported findings showing a panel of western blots from uninfected and infected T cell samples (Jurkat and J1.1, respectively) that were processed over a size-exclusion chromatography column in presence of 500 mM salt. Fraction numbers correspond to elution of proteins from the column, from highest molecular weight complexes to lowest. Every fifth fraction was used to precipitate proteins and western blot for presence of Cyclin T1, Cdk9, Hexim1, ELL2, Brd4, Baf170, Tat, p24 and β-actin. Total RNA isolated from equal aliquots of size exclusion chromatography fractions 10–55 of the lysates for both cell lines were quantitated by qRT-PCR with 7SK cellular snRNA sequence-specific primers. A total of 4 pTEF-b-containing complexes are designated as complexes I, II, III, and IV. Possible configurations of pTEF-b-containing complexes I-IV are illustrated based on data mining annotations. Complex I schematic more closely depicts hypothetical complex assembly at the HIV-1 long terminal repeat (LTR). Figure adapted from Van Duyne *et al*. 2013, Narayanan *et al*. 2012, and Van Duyne *et al*. 2011 [10,141,142].

3.6 TAT INVOLVEMENT IN VIRAL AND CELLULAR TRANSCRIPTION

3.6.1 Role of Tat in HIV-1 gene expression

In viral gene expression, Tat plays an important role in assembling required transcription factors that function in transcriptional initiation and elongation. As discussed previously in subsection 3.5, Tat recruits pTEF-b and induces CTD phosphorylation of RNAP II that permits transcriptional elongation and the synthesis of full-length viral transcripts and downstream activation of cotranscriptional mRNA capping [117]. Also, Tat induces phosphorylation of other transcription factors including Sp1, CREB, eIF2a, and NF-κB [145-148]. Additionally, the host cell presents natural repressive barriers at the integrated proviral promoter [149]. Given that the integrated 5' LTR exhibits a high order chromatin structure consisting of 5 nucleosomes (nuc-0 to nuc-4) that regulates viral gene expression by access restriction, several other possible interactors have been elucidated as measured by assessing Tat-dependent transcription or direct ternary complex formation. It is known that multiple factors that are associated with chromatin remodeling are important to Tat transactivation. Including but not limited are HATs (e.g. CBP/p300 and p/CAF), SWI/SNF components (e.g. BRG1, Baf53, and Baf170), and microRNA machinery [14,112,150-153]. Lastly, in terms of viral protein-protein interactions, Tat has been found to associate with Vpr resulting in a synergistic upregulation of viral gene expression [154].

3.6.2 Role of Tat in cellular gene expression

Apart from its primary 5' LTR transactivating function, Tat is known to reprogram cellular gene expression of infected and uninfected cells [155]. A number of studies have indicated that Tat can considerably affect transcription when bound to DNA

and that it possesses functional similarities with other known transcription factors [156-158]. For example, Tat exists as a Zn^{2+}- or Cd^{2+}-linked dimer bridging cysteine-rich regions of each monomer, which is characteristic of DNA binding proteins [159]. Furthermore, Tat has been found to directly bind to NF-κB enhancer sequences [155]. In infected cells, Tat increases the expression of several cytokines, CCR_5, and CD25, and decreases MHC class I expression to exert a suppressive immune effect [160-162]. Uninfected bystander cells are affected by extracellular Tat released from infected cells, when upon uptake, it exerts its effects on responsive genes [143,163]. Such is the case of the extracellular Tat-induced upregulation of matrix metalloproteinases that are linked to the destruction of extracellular matrix proteins and the brain-blood barrier, a fact that entails the progression of HIV-1 associated neurocognitive disorders (HAND) [143,164]. A recent genome-wide binding map of Tat to the human genome showed, through chromatin immunoprecipitation and deep sequencing, that ~53% of the Tat target regions are within DNA repeat elements (>50% Alu sequences) and in introns and distal intergenic regions [165]. Surprinsingly, only ~7% of Tat-bound regions are near gene promoter transcription start sites. In addition, Tat-bound promoters in uninfected Jurkat T cells included those bound by ETS1 transcription factor, CBP histone acetyltransferase and those enriched for histone H3K4me3 and H3K27me3. Interestingly, although Tat has not been implicated, exosomal TAR RNA has been shown to downregulate apoptosis by decreasing expression of cellular genes including Bim and Cdk9 [166].

3.6.3 Other roles

Additional functions linked to HIV-1 Tat involve various cellular responses. Firstly, Tat may induce or suppress apoptosis that is dependent upon concentration, cell type, and the nature of Tat localization (intracellular *versus* extracellular) [167,168]. Secondly, Tat may incur induction of DNA repair deficiencies which are thought to play a role in AIDS associated cancer pathologies [169]. In addition, current popular researched topics relate to Tat oxidative stress and neurotoxic effects and contribution to HAND [143,163,170]. Moreover, recent studies have reported some distinct Tat immunoregulatory activities including its use as an immunogen adjuvant for vaccine design or triggering a Th1-type dominant adaptive immune response [171-174]. Additional reported functions include inducing maturation of monocyte-derived dendritic cells, varying the subunit composition of the proteasome, and modulating the humoral responses against unrelated antigens [171,174-176]. Furthermore, Tat has been linked to the DNA repair pathway by its repression of DNA-PKcs and interaction with Tip60, pointing to a mechanism of double strand break (DSB) repair impairment [169]. Finally, additional regulatory functions include the cooperation between Tat and Vpr with pTEF-b for the superactivation of viral transcription, and participation in the reverse transcription step of the viral life cycle [154,177].

3.6.4 Tat-binding proteins

Multiple Tat-binding proteins have been described for their role in Tat-dependent transcription. The most studied factor binding Tat consists of pTEF-b, which was initially characterized as a Tat-associated kinase or TAK [38,134]. Specifically, Tat presents a strong interaction with Cyclin T1 but not Cdk9 [38]. Additional proteins that have been

studied include basal transcription factors TBP, TFIIB, TFIID, TFIIH, Sp1, and TAF55 [38]. Recently, Tat has been shown to specifically bind TLR4-MD2, promoting proinflammatory TNF-α expression and the immunosuppressive cytokine IL-10, both of which play a role in immune dysregulation during early HIV-1 infection and AIDS progression [178].

CHAPTER FOUR
BREAST CANCER PROTEIN 1: A BRIEF BACKGROUND

4.1 GENERAL BIOLOGY AND FUNCTION OF BRCA1

Largely characterized in cancer, the breast cancer susceptibility gene, BRCA1, is a multi-domain protein that acts as a specific tumor suppressor for breast and ovarian cancer [179]. The inheritance of one defective allele predisposes an individual to the development of breast, ovarian, or T cell cancers [180]. Hereditary breast cancer represents ~5-10% of all cases, however from these ~80% display mutations in BRCA1 [181]. Although inactivation by mutation is rarely observed in sporadic cancers (the remaining 90-95%), it has been shown that ~10-15% display BRCA1 promoter hypermethylation and ~30-40% exhibit decreased BRCA1 mRNA or protein expression [182].

4.1.1 Structure

The BRCA1 gene is composed of 24 exons encoding a large 1,863 amino acid protein [182]. The protein structure consists of an N-terminal RING finger domain and two BRCT domain tandem repeats situated at the C-terminal. Of interest, *brca1* mutations often occur at these domains in addition to the exon 11-13 region [183]. The RING domain encompasses aa1-109 (exons 2-7), is responsible for the E3 ubiquitin ligase activity exerted by BRCA1 and facilitates interactions with other proteins Additionally, it harbors a nuclear export signal (NES) at aa88-99 at the N-terminal

region. The NES constitutes an active Rev-type signal mediating CRM-1 dependent nuclear export of BRCA1 [184].

The C-terminal region contains an acidic transcriptional activation domain (TAD) that is transcriptionally active when fused to a Gal4 DNA binding domain [185]. The TAD domain contains the BRCT tandem repeats that span aa1650-1863 and mediate interaction between BRCA1 and proteins phosphorylated by ATR/ATM kinases [183]. Additional structural elements include 2 nuclear localization signals (NLS) spanning aa501-507 and 607-614 (exon 11), a DNA binding domain spanning aa452-1079 (exon 11), a serine cluster domain encompassing aa1,280-1,524 (exons 11-13), and a highly conserved coiled-coil motif that has been shown to be critical for the functional synergy between the 2 BRCT transcriptional activation domains [186,187]. The NLS signals are recognized by importin-α, though NLS1 has been shown to be critical for mediating BRCA1-importin-α interaction [183,188]. Figure 6 summarizes the structural organization of these functional domains as well as interacting proteins.

It is important to note that a relative high number of BRCA1 splice variants have been identified, however, many of these may not be functional since they are sourced from tumor samples [189]. It is established that translation of the complete *brca1* ORF, or canonical sequence, results in the primary p220 BRCA1 protein [190]. Issues with antibody specificity for these various isoforms have complicated their focused study, thus, for this project we have focused on the full-length 220 kDa BRCA1 isoform.

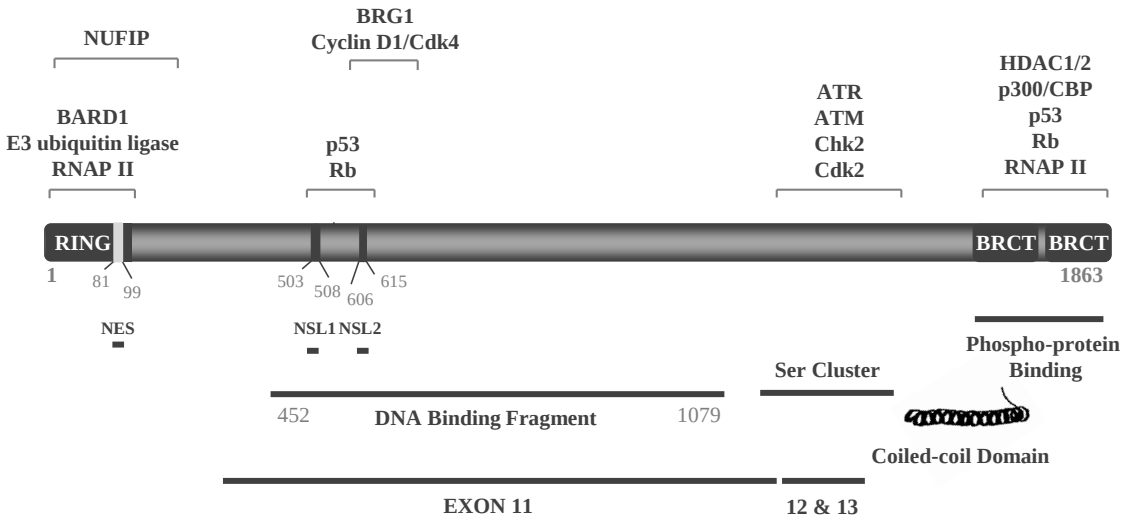

Figure 6 Diagrammatic representation of BRCA1 protein structure, functional domains, and selected binding partners.

BRCA1 consists of a 1,863 amino acids and contains various functional domains that are functionally important. The RING domain harbors the nuclear export signal (NES). BARD1, E3 ubiquitin ligase, and RNAP II bind the RING domain. The region 503-615 containing the two nuclear localization signals (NLS) binds p53 and Rb. The DNA binding domain spans aa452-1079 and interacts with chromatin remodeling and cell cycle factors BRG1 and Cyclin D1/Cdk4. The serine cluster domain at aa1,280-1,524 interacts with multiple kinases including ATR, ATM, Chk2, and Cdk2. The BRCT domains span aa1,64-1,859 are contained within the transcriptional activation domain and interact with various proteins including HDAC1 and HDAC2, p300/CBP, p53, Rb, and RNA polymerase II holoenzyme. Figure adapted from Rosen *et al*. 2003 [182].

4.1.2 Regulation and function

BRCA1 is classified as a multifaceted tumor suppressor [191]. Primarily, it functions in genome maintenance and cell division in healthy cells. In breast cancer, truncated or mutated versions abolish functional normalcy and contribute to the development of tumorigenesis [179,182]. Its main tumor suppressor function is achieved by the upkeep of genome integrity mediated by DNA replication, repair, transcription, and cell cycle checkpoint regulation [192]. All these functions are accomplished by BRCA1 interactions with cellular factors in addition to a tight regulatory mechanism that includes autoregulation and subcellular localization (see Figure 6 for a short illustration of important interacting partners) [190,193]. As part of its transcriptional coregulator function, BRCA1 has been shown to assemble in complexes containing transcription factor E2F-1 and Rb, forming a multicomponent transcriptional complex that binds the BRCA1 promoter itself resulting in decreased expression [193]. Of interest, this repressive complex is disrupted by genotoxic stress, which in turn results in BRCA1 displacement from its promoter and consequent upregulation. In conjunction with ubiquitination and proteasome-dependent degradation as the cell enters G_1, this mechanism may explain BRCA1 expression throughout the cell cycle, given that its expression peaks at S phase, with decreased G_2 and mitosis levels, and markedly reduced quantities in G_0/G_1 [191,194-196]. Recently however, Dimitrov *et al*. [190] have shown that microRNA miR-545 directly downmodulates endogenous BRCA1 mRNA and protein levels in both G_0/G_1 and S/G_2. Moreover, upon miR-545 inhibition, resulting BRCA1 upregulation resulted in aberrant BRCA1-associated DNA damage. These data indicate that when the physiological BRCA1 level control is lost, BRCA1 incurs *de novo*

DNA damage in partnership with Rad51, further highlighting the complexity and importance of this genome surveillance protein in cellular health. Taken together, these regulatory processes may help to explain the difficulties sometimes encountered when working with this protein.

Its interaction with BARD1 is perhaps one of the best described [191,197,198]. Upon heterodimer formation, BARD1 promotes nuclear localization and stimulates SUMO-dependent ubiquitin E3 ligase activity. This complex has been shown to function in the ubiquitinylation process, and recent studies indicate that BRCA1-mediated ubiquitination occurs in response to replication or genotoxic stress, thus linking this function to DNA damage response (DDR) [198-200]. Likewise, its function in DDR complexes has been well documented. Implicated in homologous recombination, BRCA1 associates with Rad51 at the damaged region [201]. In addition, both BRCA1 and Rad51 share cell cycle-dependent expression, suggesting that they function during S and maybe G_2/M phases [202]. Furthermore, in response to ionizing radiation, BRCA1 initiates homologous recombination and double-strand break repair [186]. Several studies have suggested its implication in nucleotide-excision repair, which involves transcription-coupled repair or global genome repair [203,204].

Its involvement in chromatin remodeling may arise due to the fact that heterochromatin modification occurs around DSBs. BRCA1 has been shown to interact with SWI/SNF complex catalytic core unit, BRG1 [205], suggesting that these proteins function in chromatin remodeling at DNA damage sites. Additional links include the ability of BRCA1 to function as a histone deacetylase and interact with other chromatin

remodeling units like BRCA1-associated genome surveillance (BASC) complex and the BRCA1-associated protein BACH1 DNA helicase [186,206].

In addition to the complex list of functions and regulatory mechanisms, BRCA1 has been recently reported to modulate gene expression through interaction with the Drosha microprocessor complex and Smad3/p53/DHX9 [207]. Drosha is a type III ribonuclease that functions by processing pri-miRNA into pre-miRNA that is later cleaved into mature miRNA in the cytoplasm by Dicer. Importantly, microRNAs from cellular or viral origin are key post-transcriptional regulators of gene expression.

BRCA1 is primarily characterized as a nuclear phospho-protein, and is actively transported to the nucleus to regulate DNA replication and gene transactivation, relocates in the nucleus to sites of DNA damage for repair, and is translocated to the cytoplasm to participate in centrosome integrity maintenance to ensure optimal mitotic cell division and cytoskeletal shape [191]. Furthermore, BRCA1 is found at the mitochondria, where it is thought to participate in mitochondrial genome repair and apoptosis.

Its transcriptional functions and further regulatory mechanisms by post-translational modification and are addressed in sections 4.2 and 4.3, respectively.

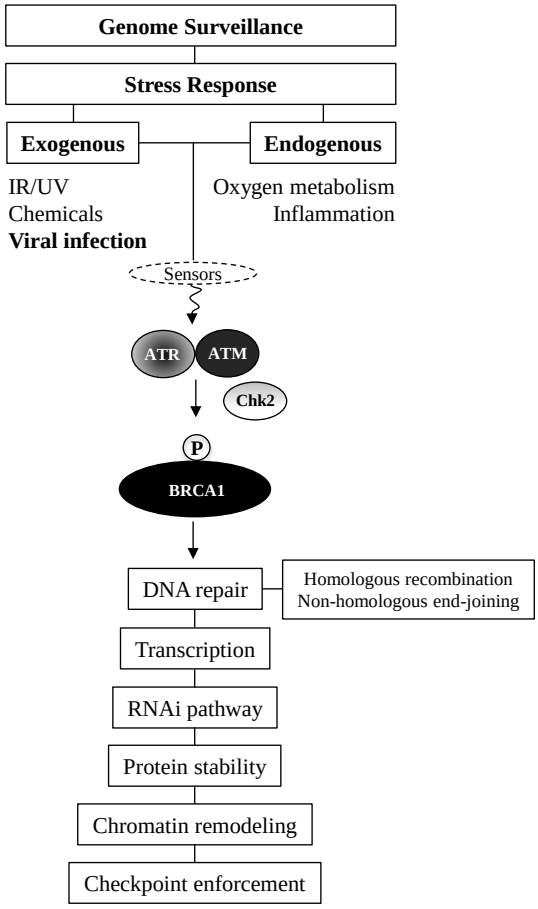

Figure 7 BRCA1 function schematic.

BRCA1 is a multifaceted tumor suppressor protein whose main function is surveillance for genome stability that is achieved by its participation in DNA repair, transcription, RNAi pathway regulation, maintenance of protein stability, chromatin remodeling, and cell cycle regulation. Exogenous agents such as ionizing and ultraviolet radiation (IR/UV), chemical insults (e.g. carcinogens, teratogens), or in the case of this study, viral infection (e.g. HIV-1); initiate BRCA1-mediated stress response. Likewise, endogenous events such as inflammation or oxygen metabolism can generate conditions that trigger DNA damage. Moreover, endogenous events can be a response to external events. DNA damage activates upstream effector kinases that result in the activation of BRCA1 by phosphorylation and the consequent regulation of afore mentioned cell signaling pathways. Figure concept adapted from Rosen *et al*. 2003 and Narod and Foulkes 2004 [182,186].

4.2 TRANSCRIPTIONAL ROLE OF BRCA1

BRCA1 was first implicated in transcription in 1996, when the C-terminus (aa1560-1863) fused to reporter gene Gal4 was able to activate transcription [185], with aa1760-1863 being the minimal transactivation domain (TAD). Within this TAD are two BRCA1 C-terminus (BRCT) motifs that are found in a large family of proteins important for DDR, such as DNA ligase IV, p53BP1, and base excision response scaffold protein XRCC1 [208]. Since then, numerous other findings have served to strengthen the connection between transcription and BRCA1. For example, BRCA1 is part of the RNAP II holoenzyme complex and has been shown to modulate its phosphorylation status [209-212]. Association with RNAP II occurs through subregions of the BRCT domain mediated by RNA Helicase A (RHA), an enzyme involved in the unwinding of RNA and DNA [211]. As part of the RNAP II holoenzyme, BRCA1 is involved through the recruitment of various cofactors and transcription factors, in transcriptional activation or repression. For instance, the transcriptional coactivators and acetyltransferases CBP/p300 have been shown to interact with BRCA1 in a cell cycle-dependent manner, resulting in the activation of the Rous sarcoma virus long terminal repeat promoter [213]. On the other hand, BRCA1 has been shown to downregulate p300 expression and inhibit ER-mediated transcription, an action reversed by ectopic expression of either p300 or CBP [214]. An additional factor associated with the RNAP II holoenzyme includes the chromatin modifying BRG1 ATPase, who along with BRM, form the catalytic subunits of SWI/SNF chromatin remodeling complexes [215]. This is of interest because as mentioned in the previous section, BRCA1 can directly interact with BRG1 and associates itself within a multiprotein complex displaying chromatin remodeling activity,

indicating a direct function of BRCA1 in transcriptional control through chromatin structure modulation [205]. An additional layer of regulation is achieved by BRCA1/BARD1, which ubiquitinates the hyperphosphorylated form of RNAP II [197], resulting in the targeted proteasomal degradation of RNAP II following DNA damage [216]. It has been demonstrated that in order for BRCA1 to directly stimulate transcription, both the N-and C-termini of BRCA1, but not BARD1, are required [191]. Similarly, BRCA1 acts as a p53 transcriptional coactivator in transiently transfected mouse fibroblasts [217]. BRCA1 has also been shown to interact with RNAP II holoenzyme components RPB2 and RPB10α [218]. Other important interacting proteins include transcription factors Sp1 and STAT1, and histone deacetylases HDAC1 and HDAC2 [182,206,212,213]. BRCA1 stimulates STAT1/2 and type I IFNs in response to IFN-γ [219], NF-κB in response to TNF-α or IL-1β [220], and p53 responsive genes [221]. Additional genes found to be transactivated by BRCA1 include p53-response pro-apoptotic mediators Mdm2 and Bax, and independently of p53, critical DNA-damage responsive genes p21/Waf1, p27/Kip1, and GADD45α [217,222-226]. BRCA1 can also act as a CTD kinase inhibitor contributing to the activation of p21/WAF1 gene expression [222]. BRCA1 is able to inhibit transcription of estrogen responsive genes as well as telomerase reverse transcriptase gene expression, where BRCA1 has been found to bind pS2 (estrogen responsive gene) and hTERT promoters *in vivo* [227-229]. Therefore, the current literature suggests that based on the promoter of interest, BRCA1 can have either activating or inhibitory effects through multiple mechanisms. Of

importance, most of these interacting factors have been implicated in HIV-1 transcription and replication [149,151,152,230-237].

Importantly, BRCA1 has been shown to be in complex with pTEF-b to activate RNAP II-dependent transcription [218]. Precisely, BRCA1 interacts with NUFIP, a nuclear human zinc-finger-containing protein. In turn, NUFIP associates with pTEF-b through interaction with the regulatory Cyclin T1 subunit, strengthening the role BRCA1 plays in cellular transcription. More importantly, Cyclin T1 was shown to be an essential factor for BRCA1- and NUFIP-dependent activation of RNAP II transcription. The fact that NUFIP has been associated with preinitiation, open, and elongation transcriptional complexes, allows for a more complex BRCA1 function.

The significance of some of these interactions is not fully understood, but they highlight context-dependent BRCA1 functionality in transcription. Many fo these interactions are depicted in Figure 6.

4.3 POST-TRANSLATIONAL MODIFICATIONS OF BRCA1

Covalent protein modifications create regulatory and functional mechanisms that waive the requirement for new protein synthesis. Proteins involved in transcription are often regulated through post-translational modifications (PTMs). Although epigenetic changes to the BRCA1 gene have been extensively researched at the DNA-level, proteome research has focused on phosphorylation of BRCA1 by DNA damage protein kinases, while little information is known about the effects of other PTM events. To date, the known PTMs of BRCA1 include phosphorylation, ubiquitinylation, and methylation [182]. In a healthy cell, phospho-BRCA1 predominates in S phase and subsequently becomes dephosphorylated after mitosis (Rosen et al 2003). Total known phosphorylation events are effected by multiple protein kinases including Aurora A kinase (Aur A), Cdk4/Cyclin D1, Chk2, ATR, Cdk2/Cyclin A or E, and ATM [182]. Both Aur A and BRCA1 localize to the centrosome, and its kinase activity is known to regulate G_2/M cell cycle transition. Phosphorylation by Aur A at serine 308 is thought to play a role in this transition [238], while Cdk4/Cyclin D1 phosphorylation of serine 632 was shown to change BRCA1 target gene specificity [239]. Phosphorylation of serine 988 by Chk2 is known to regulate DSB repair, where inhibition of this PTM resulted in disruption of BRCA1-dependent homologous and non-homologous recombination [240]. Cdk2/Cyclin A/E has been shown to be phosphorylate BRCA1 at serine 1497, an event in accordance with the G_1/S-specific increase in BRCA1 phosphorylation and the activation of CDK2-cyclin complexes, and at threonine 967 with no reported functional relevance [182,241]. Additional less studied phosphorylation events occur at threonine 508 by AKT and at serine 1572 by CK2. Phospho-T508 affects NLS1 and results in BRCA1

cytoplasmic accumulation, while S1572 implicates CK2 as a mediator of BRCA1 activity [182].

ATR and ATM both phosphorylate serine 1423 in response to DNA damage, whereas ATM phosphorylates serine cluster residues S1387, S1457, and S1524 [182]. Characterization of these phosphorylation events has shown that ATR phosphorylates BRCA1 in response to UV radiation-induced adducts or crosslinks while ATM does so in response to ionizing radiation-induced DSBs. Moreover, in a healthy cell, these kinases phosphorylate BRCA1 as part of DNA repair mechanisms during cell replication [242]. Importantly, the G_2/M phase checkpoint requires S1423 phosphorylation, whereas the intra-S phase checkpoint requires S1387 phosphorylation [243].

Recently, we have described a novel BRCA1 PTM by PRMT1, the most abundant mammalian arginine methyltransferase [244]. Briefly, we have shown that BRCA1 associates with PRMT1 at the region spanned by aa504-802, and *in silico* sequence analysis prediction indicates that the methylation event is occurring at R610. Functional consequences of this PTM are similar to those exerted by Cdk4/Cyclin D1 phosphorylation of S632, where BRCA1 occupancy at the promoter of BRCA1 responsive genes is altered according to its methylation status.

Lastly, the E3 ubiquitin ligase activity acquired by binding to BARD1 results in non-lysine 48-linked chains as auto-ubiquitination chain polymerization at lysines 6 and 29, implying that these chains may serve an alternative role such as in DNA repair signaling other than as a marker for degradation [245].

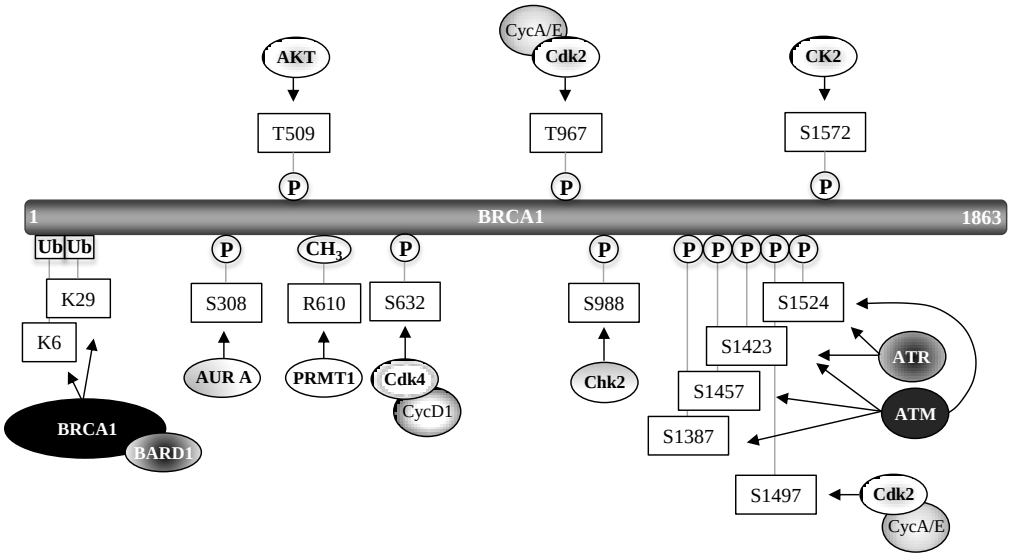

Figure 8 Post-translational modifications of BRCA1.

The upstream effector kinases ATM, ATR and Chk2 are activated upon DNA damage and subsequently activate BRCA1 by phosphorylation. ATM kinase phosphorylates BRCA1 at 4 different serine residues S1387, S1457, S1423, and S1524 in response to double strand breaks (DSBs). Similarly, Chk2 phosphorylates serine residue S988 in response to DSBs. ATR kinase also phosphorylates S1423 and S1524 in response to DNA adducts or crosslinks. Cdk2/Cyclin A/E phosphorylates S1497 possibly as part of G_1/S-specific phosphorylation. Cdk4/Cyclin D1 phosphorylates S632 as transcriptional regulation of BRCA1 activity. The arginine methyltransferase PRMT1 has been linked to methylation of the BRCA1 region spanning aa504-802, with a putative methylation site at R610 in breast cancer. Aurora A kinase phosphorylates S308 as a regulatory mechanism in the G_2/M cell cycle transition. BRCA1/BARD1 in complex with E3 ubiquitin ligase activity forms K6 and K29 ubiquitin chains and has been shown to assume auto-ubiquitination. Additional phosphorylation events occur at T509 by AKT, T697 by Cdk2/Cyclin A/E, and S1572 by casein kinase CK2. These post-translational modifications have several implications in the function of BRCA1. Figure adapted from Rosen *et al*. 2003 [182].

4.4 BRCA1 IN VIRAL INFECTION

BRCA1 participation in viral infection has been scarcely documented and most studies involve its function as part of the DDR. Many viruses with distinct replication approaches activate DDR pathways in a bid to manage DNA repair, recombination, and cell survival; and include HIV-1, EBV, HSV-1, adenovirus and SV40 [246].

An initial study linking BRCA1 to adeno-associated virus type 2 Rep proteins reported *brca1* as a novel Rep recognition sequence (RSS). Rep proteins exercise multiple functions including DNA binding, site-specific endonuclease activity, DNA-DNA and DNA-RNA helicase activity, and ATPase activities. Thus, this putative BRCA1 RSS may function as an alternate viral integration site or as a mean of Rep-mediated effects in host cells [247]. Another early study researching BRCA1 subcellular distribution reported differential BRCA1 redistribution after infection with herpes simplex virus type 1 and adenovirus 5. In the presence of infection, BRCA1-containing domains as observed by confocal microscopy, were dispersed (HSV-1) or recruited to regions of viral transcription and replication (AV5) [248]. More recently, HTLV-1 Tax oncoprotein was shown to form damage-independent nuclear foci that contain DDR factors DNA-PK, BRCA1, and MDC1, suggesting a model for virus-induced genomic instability wherein viral oncogene-induced foci compete with normal cellular DDR [249]. Interestingly, infection with La Crosse encephalitis virus (LACV) did not trigger DDR markers including p53 or BRCA1 phosphorylation [250]. Further reports are qualitative and mention BRCA1 loss, along with other tumor suppressor proteins, in human papilloma virus (HPV)-linked cervical cancers [251,252]. BRCA1 and other DDR proteins have been shown to be significantly upregulated in high- and low-risk HPV and

to interact with the HPV E6 and E7 oncoproteins [253,254]. Likewise, HPV infection has been shown to activate the ATM damage pathway as measured by phosphorylation of Chk2, BRCA1, and Nsb1 [255]. Activation was shown to be required for viral genome amplification and formation of viral replication foci.

Though limited, various studies have linked BRCA1 to HIV-1 infection. Primarily, they assess BRCA1 involvement from the perspective of the DDR. In summary, Vpr-induced BRCA1-H2AX foci formation have been shown to be required for G_2 cell cycle arrest [256]. Later, BRCA1 was shown to undergo phosphorylation at S1423 in response to genotoxic stress and to increase transcription of its target gene, GADD45α [257]. Furthermore, alterations of various genes contributing to cell cycle transition at the G_2/M checkpoint were identified in HIV-1 infection, which included PP2A, BRCA1, and GADD45α [237].

Of these, the principal response studied involved BRCA1 activation by ATR. In conjunction with ATR, several studies have implicated ATM to the HIV-1 life cycle. For example, ATR has been shown to be dispensable for retroviral integration but is necessary for checkpoint activation in Vpr-induced G_2/M arrest [258,259]. However, these findings are controversial given that a recent study has demonstrated that ATR and probably other PIKK family members including ATM and DNA-PKcs are required for retroviral replication in a cell-specific way [260]. Similarly, ATM has been linked to Vpr where Vpr causes dysregulation of ATM-dependent signaling and consequent homologous recombination regulation loss, serving as a possible explanation to the high incidence of malignant tumor development in HIV-1 positive patients [261]. Moreover,

ATM has been shown to enhance Rev function resulting in enhanced HIV-1 replication [262]. Importantly, it is this emerging role of DNA repair in HIV-1 infection that advocates for the search of therapeutic intervention [263].

In addition to the Vpr link to BRCA1 phosphorylation at S1423 and foci formation in infection [256,257], recent findings further implicate BRCA1 as an important modualtor of ATR/ATM activation in response to DNA damage [264]. Here, the authors found that BRG1 depletion impairs BRCA1 recruitment to damage sites and weakens DNA damage-induced BRCA1 phosphorylation. Moreso, ATR/ATM activation was also observed to be attenuated, suggesting that BRG1 is upstream of both BRCA1 recruitment to damage sites and ATR/ATM activation. Further links that may involve BRCA1 include DDR roles of PIKK family members in the cellular response to oxidative stress given that Tat increases lung oxidative burden in transgenic mice and HAND patients [265,266].

Taken together, this information depicts that BRCA1 may be a target differentially modulated between viral infections. Interestingly, while all DNA viruses discussed display a BRCA1 phenotype in response to infection, it is worthy of noting that the RNA viruses have contrasting responses. Replicating in the cytoplasm, LACV does not elicit activation of DDR markers, whereas the retroviruses HIV-1 and HTLV-1, both involved BRCA1 and DDR.

CHAPTER FIVE
CHARACTERIZATION OF BRCA1 EXPRESSION IN HIV-1 INFECTION

5.1 INTRODUCTION

It is common knowledge that an infected host cell undergoes changes in protein network dynamics. Consequently, the discovery of novel protein interactions in infected but not in uninfected cells, is necessary to further understand viral mechanisms and host cell machinery manipulation. We have previously characterized various protein expression profiles and complex alterations in the presence of infection including chromatin remodeling complex subunits and components of pTEF-b [10,141-143]. In detail, we have defined four different complexes I-IV that contain Cdk9, Cyclin T1, and Tat in infected cells [141,142]. Of particular interest is complex I (molecular weight range ~1-2 MDa), which has been shown to contain Baf170, Baf53, pTEF-b, Tat, and Actin by western blot (refer to Figure 5). Based on these and previously published data characterizing BRCA1 presence in a predominant high molecular weight multiprotein complex in conjunction with BRG1 [10,205,267], we first wanted to examine the status of BRCA1 expression and association in higher order multimeric complexes in HIV-1 uninfected and infected cells. To this end, size-exclusion chromatography serves as a tool to efficiently fractionate functional protein complexes of interest for further downstream analysis [10,143,163,268]. In fact, several recent studies have utilized this technique to successfully discern novel complex changes in SWI/SNF chromatin remodeling PBAF

subunits (Baf170 and Baf53) and pTEF-b in HIV-1 infection, GSK-3β redistribution in HIV-1 and Venezuelan Equine Encephalitis Virus (VEEV) infection, and IKK complex variations in Rift Valley Fever Virus (RVFV) infection [10,141-143,269,270].

5.2 RESULTS

5.2.1 BRCA1 is differentially expressed in HIV-1 infected T cells

Initially, we asked whether BRCA1 expression is altered in HIV-1 infection. To address this question we first performed size-exclusion chromatography separation of HIV-1 infected cell extracts and analyzed eluted fractions for BRCA1 distribution by western blot. We prepared total protein extracts from matched parental uninfected Jurkat and chronically infected J1.1 cell lines and loaded the samples on a Superose 6 size-exclusion column in the presence of a stringent 500 mM salt concentration. The high salt concentration provides specificity for stable protein complexes. Samples were fractionated in water soluble buffer followed by precipitation to concentrate protein fractions and remove excess salt. The Superose 6 sizing column used in this study is designed to separate higher order molecular complexes based on aggregate molecular weight, as indicated in the sample A_{280} chromatogram trace of the processed whole cell extracts in Figure 9A. At first, every 5^{th} fraction was assayed by western blot for BRCA1 and β-actin (data not shown). As expected, based on previous findings [205], the resulting immunoblots revealed that BRCA1 was present in a high molecular weight fraction associated with the left-hand side of the eluted fractions. As the inset depicts, the total protein lysate separates in the shape of a bell curve, with the majority of proteins eluting around fraction 30 and reduced amount of proteins eluting in the high and low molecular

weight regions (data not shown). In order to further examine BRCA1-containing neighboring fractions, high molecular weight fractions #17, #21, and #22 from both uninfected and infected cells were assayed by western blot for the presence of BRCA1, Baf170, Cdk9, Cyclin T1, and β-actin. The results in Figure 9B show differential BRCA1 expression in fraction #17 between uninfected and infected cells (compare lanes 3 and 6). Although BRCA1 is present to a lesser extent in uninfected cells, infected cells showed a stronger predominant band in addition to an upper band that is suggestive of possible protein modification in the presence of infection. While β-actin analysis was used as a loading control between matched uninfected and infected fractions (e.g. Jurkat #17 *versus* J1.1 #17), we wanted to verify the loading of similar protein amounts. To this end, we concentrated a small sample of fraction #17 for both Jurkat and J1.1 used in panel B, resolved them by SDS-PAGE and silver stained the gel. Results in Figure 9C showed that fractions #17 of uninfected and infected cells contained relatively similar amounts of total protein. To finalize our conclusions, we performed microBCA analysis of fractions #17 of uninfected and infected cells, and quantified them to be ~0.25 µg/µl and ~0.15 µg/µl, respectively. Taken together, these results imply differential BRCA1 modulation in chronic HIV-1 infection.

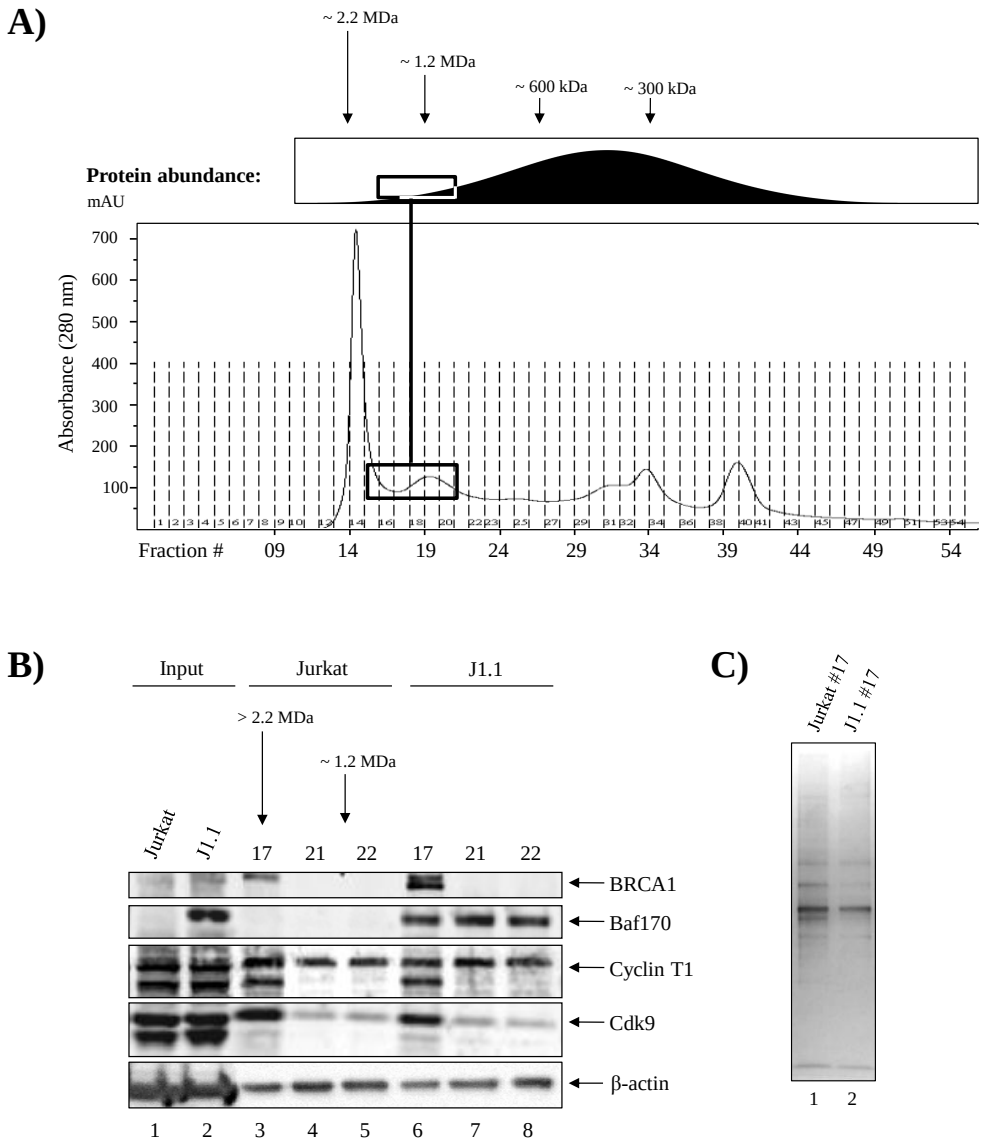

Figure 9 BRCA1 expression is upregulated in HIV-1 infection.

A) Two and half milligrams of whole cell extract from uninfected Jurkat and infected J1.1 T cells were loaded in a size-exclusion chromatography column and separated in the presence of 500 mM salt buffer. No detergents were used during fractionation. Fraction numbers correspond to elution of proteins from size-exclusion chromatography column, from highest molecular weight complexes to lowest. The chromatogram shows a sample A_{280} trace of the processed whole cell extract sample. The inset depicts the overall bell-shaped elution profile of the sample as confirmed by western blot. **B)** A sampling (250 µl) of high molecular weight fractions (#17, 21, 22) was acetone precipitated, resuspended in 30 µl of lysis buffer, and boiled for 10 min. Fifteen microliters were run on a gel for western blotting for the presence of BRCA1, Baf170, Cyclin T1, Cdk9, and β-actin. Western blots were performed in duplicate. **C)** Fifty microliters of Jurkat and J1.1 fractions #17 were acetone precipitated and processed as in panel A. Fifteen microliters were run on a gel and silver stained to visualize total protein content in these relevant fractions as a measure of loading control. Additional quantification by microBCA protein assay revealed Jurkat and J1.1 sample concentrations to be ~0.25 µg/µl and ~0.15 µg/µl, respectively.

5.2.2 BRCA1 is present in a high molecular weight complex in HIV-1 infected PBMCs

To further investigate previous observations in a more relevant system, we next examined the BRCA1 expression profile in uninfected and infected primary blood mononuclear cells (PBMCs). PBMCs were spinoculated with mock inoculum or the dual-tropic HIV-1 89.6 strain, and cells were harvested at day 3 post-infection. The results in Figure 10A indicate that in the input PBMC whole cell lysates used for size-exclusion chromatography separation of the uninfected and infected samples, BRCA1 expression levels were higher (~2.3 fold) in HIV-1 infected cells compared to uninfected cells. The membrane was probed for p24 as a marker of successful infection and β-actin as a loading control. Roughly every 5^{th} fraction was assayed by western blot for BRCA1 and β-actin. The results in Figure 10B show a dramatic differential BRCA1 signature in the high molecular weight fraction #19 between uninfected (upper panel) and infected (lower panel) cells. Similarly, p24 and β-actin analysis were used as infection and loading controls. To confirm equal loading of samples, we concentrated a small sample of fraction #19 for both uninfected and infected PBMCs used in panel B, resolved them by SDS-PAGE and silver stained the gel. Results in Figure 10C showed that both uninfected and infected fractions #19 contained relatively similar amounts of total protein. To finalize our conclusions, we again performed microBCA analysis of fractions #19 of uninfected and infected cells, and quantified them to be ~0.18 µg/µl and ~0.16 µg/µl, respectively. The overlaid A_{280} chromatogram in Figure 10D shows similar traces between uninfected (black line) and infected (gray overlay) PBMCs. Collectively, these

results support the differential modulation of BRCA1 observed in established cell lines and confirm events in primary cells.

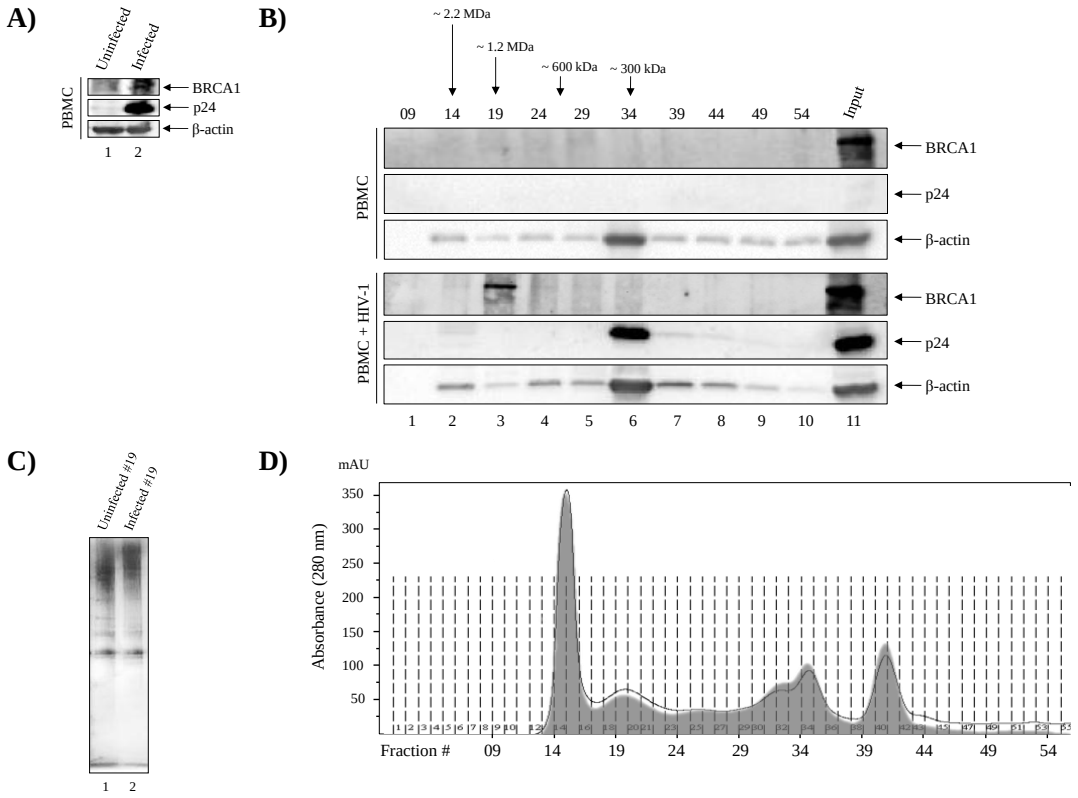

Figure 10 BRCA1 is present in a high molecular weight complex in HIV-1 infection.

A) Primary mononuclear cells (PBMCs) were infected with HIV-1 89.6 for 3 days, and assayed by western blot for the presence of BRCA1, p24, and β-actin. **B)** Samples from uninfected and infected PBMCs were loaded in a size-exclusion chromatography column and separated in the presence of 500 mM salt buffer. No detergents were used during fractionation. Fraction numbers correspond to elution of proteins from size-exclusion chromatography column, from highest molecular weight complexes to lowest. A sampling (250 µl) of every 5th fraction from #9 to 54 was acetone precipitated, resuspended in 30 µl of lysis buffer, and boiled for 10 min. Fifteen microliters were run on a gel for western blotting for the presence of BRCA1, p24, and β-actin. Western blots were performed in duplicate. **C)** Fifty microliters of uninfected and infected PBMC fractions #19 were acetone precipitated and processed as in panel B. Fifteen microliters were run on a gel and silver stained to visualize total protein content in these relevant fractions as a measure of loading control. Additional quantification by microBCA protein assay revealed uninfected and infected PBMC sample concentrations to be ~0.18 µg/µl and ~0.16 µg/µl, respectively. **D)** The chromatogram shows A_{280} traces of the processed whole cell extract samples. The uninfected PBMC trace appears as a black line and the gray overlay corresponds to infected cells.

5.3 MATERIALS AND METHODS

5.3.1 Ethics statement

All protocols involving human subjects were reviewed and approved by the George Mason University (GMU) Institutional Review Board. Informed written consents from the human subjects were obtained in this study.

5.3.2 Cell culture

Suspension infected J1.1 and uninfected Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 1% streptomycin/penicillin. The J1.1 cell line is a Jurkat E6.1 derivative chronically infected with HIV-1 (LAI strain), and contains a single integrated copy of HIV-1 genome [271,272]. PBMCs were obtained from healthy donors at the Student Health Center (GMU), Fairfax, VA. PBMCs were isolated from the blood of healthy donors by Ficoll-Paque density gradient centrifugation (Amersham 6BIOsciences, Uppsala, Sweden) and were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

5.3.5 Infections

PBMCs were PHA/interleukin-2 stimulated for 24 hours and infected with HIV-1 89.6 by spinoculation for 2 hours at 3,000 rpm. Cells were washed twice with medium to remove unbound virus. Cells were collected and processed for downstream analysis 3 days post-infection. HIV-1 89.6 strain inoculum was a kind gift of Dr. Fatah Kashanchi, and originally obtained from the NIH AIDS Research and Reference Reagent Program (AIDS RRRP, Germantown, MD, USA). PHA was obtained from Sigma Aldrich (St.

Louis, MO, USA), and IL-2 was purchased from Roche-Applied-Science (Indianapolis, IN, USA).

5.3.6 Protein extracts and immunoblotting

Cells were collected, washed once with PBS and pelleted. For size exclusion chromatography and immunoprecipitation, cells were lysed in a buffer containing Tris-HCl pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM DTT and one tablet complete protease inhibitor cocktail per 50 ml. Lysis was performed under ice-cold conditions, incubated on ice for 30 min and spun at 4°C for 5 min at 14,000 rpm. The protein concentration for each preparation was determined with by Bradford assay (Sigma Aldrich Aldrich). For immunoblotting, lysis buffer consisted of a 1:1 mixture of T-PER reagent (Pierce, Rockford, IL, USA) and 2X Tris-glycine SDS sample buffer (Novex, Life Technologies, Carlsbad, CA, USA), 33 mM DTT, and protease and phosphatase inhibitor mixture (1X Halt mixture, Pierce). Cells were collected directly in lysis buffer and boiled for 10 min. Cell extracts were resolved by SDS-PAGE on a 4-20% tris-glycine gel (Invitrogen, Life Technologies). Proteins were transferred to PVDF microporous membranes by overnight transfer as described by the manufacturer (Invitrogen, Life Technologies). Membranes were blocked with PBS 0.1% Tween-20 + 3% BSA. Primary antibodies against specified proteins were incubated with the membrane in blocking solution overnight at 4°C. Antibodies against Cdk9 (sc-484), BRCA1 (sc-642), Baf170 (sc-10757), and Cyclin T1 (sc-10750) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The β-actin antibody (ab49900) was purchased from Abcam (Boston, MA, USA). Anti-p24 (4121) was obtained from the

AIDS RRRP. Membranes were washed twice with PBS + 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody for 1 hour in blocking solution. Presence of secondary antibody (#32430 and #32460, Pierce) was detected by SuperSignal West Dura Extended Duration Substrate (Pierce). Luminescence was visualized on a Molecular Imager ChemiDoc XRS system Bio-Rad station (Bio-Rad, Hercules, CA, USA).

5.3.7 Size exclusion chromatography

Jurkat and J1.1 cell lines ($5x10^8$) were cultured to early-mid log phase of growth and pelleted at 4°C for 5 min at 1800 rpm. PBMCs were obtained from healthy donors and infected with dual tropic 89.6 HIV-1 as previously described [270]. The cell pellets were washed twice with PBS without Ca^{2+} and Mg^{2+}. Cell pellets were resuspended in lysis buffer and incubated on ice for 20 min, with gentle vortexing every 5 min. Cell lysates were centrifuged at 4°C at 10,000 rpm for 10 min. Supernatants were transferred to a fresh tube, where protein concentrations were determined using the Bradford assay (Sigma Aldrich). Five milligrams of protein from each cell line was acetone precipitated using 4 volumes of ice-cold 100% acetone, incubating for 15 min on ice. Lysates were centrifuged at 4°C for 10 min at 12,000 rpm, supernatants were removed, and the pellet was allowed to dry. Pellets were resuspended, equilibrated, and degassed in chromatography running buffer (0.2 M Tris-HCl pH 7.5, 0.5 M NaCl, and 5% glycerol). Five milligrams of lysates were run on a Superose 6 HR 10/30 size-exclusion chromatography column using the ÄKTA purifier system (GE Healthcare, Piscataway, NJ, USA). Flow-through was collected at 4°C at a flow rate of 0.3 ml/min at 0.5 ml for approximately 90 fractions. High molecular weight fractions were analyzed by

immunoblotting for BRCA1, Cyclin T1, Cdk9, Baf170, and Actin in order to determine the point of protein complex elution.

5.4 SUMMARY

In this chapter we focused on defining BRCA1 expression in the context of HIV-1 infection. Here we have described the general expression signature of BRCA1 in HIV-1 infection (Figures 9B and 10A-B). We found that endogenous BRCA1 level is upregulated in chronically infected T cells and importantly, in PBMC cells. Furthermore, though there is no detectable redistribution amongst fractions obtained by size-exclusion chromatography of BRCA1 between uninfected and infected cells in established cell lines, there is a dramatic change in infected PBMCs (Figure 10B). In addition, results imply possible post-translational BRCA1 modification during infection (Figures 9B, 10A-B).

CHAPTER SIX
CHARACTERIZATION OF BRCA1 IN TAT-DEPENDENT TRANSCRIPTION

6.1 INTRODUCTION

BRCA1 was first implicated in transcription when the C-terminus (aa1560–1863) fused to Gal4 was able to activate transcription [185], with aa1760–1863 being the minimal transactivation domain (TAD). Within this TAD are two BRCA1 C-terminus (BRCT) motifs that are found in a large family of proteins important for DDR, such as DNA ligase IV, p53BP1, and base excision response scaffold protein XRCC1 [208]. Since then, numerous other findings have served to strengthen the connection between transcription and BRCA1. For instance, BRCA1 is part of the RNAP II holoenzyme complex and has been shown to modulate its phosphorylation status [209-212]. BRCA1 also interacts with multiple cofactors and transcription factors, including CBP/p300, Sp1, STAT1, ER and BRG1 [182,212,213]. Among the genes found to be transactivated by BRCA1 are Mdm2, Bax, p21/Waf1, p27/Kip1, and GADD45α (with p21/Waf1 and GADD45α transactivation being independent of p53) [217,222-226]. Of importance, most of these interacting factors have been implicated in HIV-1 transcription and replication [149,151,152,230-237]. Moreover, BRCA1 may function as a transcriptional coactivator or corepressor, a function that varies depending on its ability to recruit both the basal transcription machinery and proteins implicated in chromatin remodeling [205,206].

6.2 RESULTS

6.2.1 HIV-1 Tat-dependent transcription is more efficient in cells containing BRCA1

Consequently, we were interested in characterizing BRCA1 function in HIV-1 transcription. First, we started our functional transcription studies in a cell system null for BRCA1 expression. UWB1.289 cells are derived from ovarian cancer in a germ line BRCA1 mutation carrier and lack expression of BRCA1 [273]. UWB1.289+BRCA1 cells are a stable UWB1.289 derivative cell line carrying a pcDNA3 plasmid coding for wild-type BRCA1 [274]. Here we transfected both set of cells with HIV-1 LTR-Luc, Tat, and Renilla reporter plasmid as a control for transfection efficiency between cell lines. Based on scored luciferase activity, results in Figure 11A indicate that there is ~11 fold difference in Tat transactivation of the HIV-1 LTR in cells expressing BRCA1 (lane 2, black bar) when compared to the BRCA1 null cells (lane 2, white bar). To elaborate these findings in our transcriptional assay cell system (TZM-bl cells), we obtained plasmid constructs coding for wild type BRCA1 or a mutant form (BRCA1 4P) with alanine amino acid substitutions for the ATR and ATM target serine residues (S1387A/S1423A/S1457A/S1524A). First, we verified efficient expression of the plasmids by transfection into UWB1.289 cells followed by immunoblotting. Western blot results in Figure 11B demonstrate that both BRCA1 plasmids express effectively in these cells. The membrane was probed for β-actin as a loading control. Next, we were interested in assessing the effect of BRCA1 overexpression in the context of integrated proviral DNA. To this end, we co-transfected a combination of Tat, pcDNA or wild type BRCA1 into TZM-bl cells for 48 hours for transcriptional luciferase assays. The TZM-bl

cell line is a widely used reporter system for the study of Tat-dependent transcription as it harbors the luciferase gene under the control of the HIV-1 LTR. Additionally, it has been engineered to express CD4, CCR_5, and $CXCR_4$ to more closely resemble primary target cells [275]. Results in Figure 11C show a significant transcriptional enhancement (~89% increase) in the presence of wild type BRCA1 overexpression when compared to pcDNA-transfected cells (compare lanes 2 and 3). Taken together, these results suggest that BRCA1 functions as an enhancer in Tat-dependent transcription of both an unintegrated (UWB1.289/ UWB1.289+BRCA1 cells) and integrated proviral LTR (TZM-bl cells).

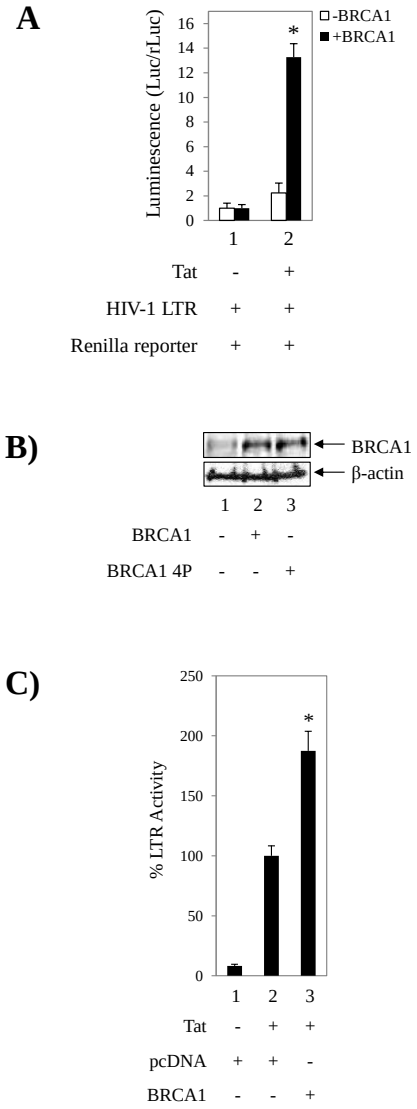

Figure 11 HIV-1 Tat-dependent transcription is more efficient in cells containing BRCA1.

To assess HIV-1 Tat-dependent transcription in the absence or presence of BRCA1, a series of overexpression experiments were performed. **A)** UWB1.289 BRCA1 null and UWB1.289+BRCA1 cells and were co-transfected with a combination of pcDNA, pcTat, LTR-Luc, and CMV-Luc (Renilla) plasmid DNA. Dual-Glo luciferase assay was performed 48 hours later as described by the manufacturer, to determine the effect of BRCA1 expression status on HIV-1 LTR activity. Transfections were performed in triplicate and raw data was normalized to Renilla luciferase expression in both cell lines. **B)** UWB1.289 BRCA1 null cells were co-transfected with a combination of pcDNA, BRCA1 wild type (BRCA1), and BRCA1 mutant (BRCA1 4P) for S1387/1423/1457/1524A serine residues. Plasmid expression was verified by western blot of whole cell extract. Samples were run on 4-20% SDS-PAGE and immunoblotted against BRCA1 and β-actin as a loading control. Western blots were performed in duplicate. **C)** TZM-bl cells were co-transfected with a combination of pcTat, pcDNA, and BRCA1 (wt) plasmid DNA. Bright-Glo luciferase assay was performed 48 hours as described by the manufacturer, to determine the effect of BRCA1 overexpression on HIV-1 LTR activity. The assays were performed in triplicate. (*) indicates statistically significant difference (unpaired t-test of triplicates) $p<0.05$. Error bars indicate standard deviation.

6.2.2 BRCA1 selective depletion impairs HIV-1 Tat-dependent transcription

Based on these findings, we next wanted to examine the functional consequences of BRCA1 selective depletion in Tat-dependent transcription. To assess the effect of BRCA1 knockdown in HIV-1 transcription, we first screened various siRNAs against BRCA1 and selected for the one producing more effective depletion results (data not shown). TZM-bl cells were co-transfected with Tat and siRNA against BRCA1 or GFP as a non-specific control, and luciferase assays were performed 48 hours post-transfection. Results in Figure 12 show that luciferase activity was significantly reduced by 40% in cells transfected with siBRCA1 when compared to cells transfected with siGFP (compare lanes 1 and 2). As additional controls, BRCA1 levels in these cells were assayed by qRT-PCR and western blot. BRCA1 was successfully repressed (~90%) at the transcriptional level (compare lanes 3 and 4) and protein levels were decreased by ~68%. To discard the possibility that the decrease in Tat-dependent transcription was due to cytotoxic effects upon BRCA1 knockdown, we performed viability assays on these samples. No changes in cell viability were observed between samples transfected with control siRNA or BRCA1 siRNA (compare lanes 5 and 6), indicating that the transcriptional efficiency loss is specific to BRCA1 selective depletion. Collectively, these data support the permissive role of BRCA1 for Tat-dependent HIV-1 transcription.

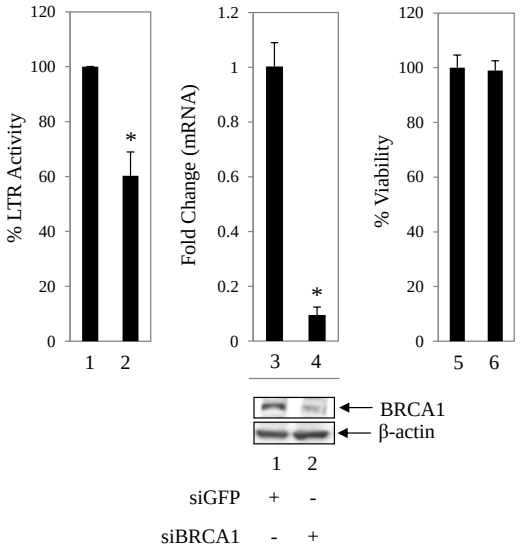

Figure 12 BRCA1 is required for efficient Tat-dependent transcription.

A) TZM-bl cells were co-transfected with pcTat or siRNA against GFP (control) and BRCA1. Bright-Glo luciferase assay was performed 48 hours as described by the manufacturer, to determine the effect of BRCA1 selective depletion on HIV-1 LTR activity (left panel). BRCA1 depletion was confirmed by qRT-PCR and western blot (middle panel). Total RNA was isolated from cells using RNeasy Mini Kit extraction. Three hundred nanograms were used to generate cDNA with the High-Capacity RNA-to-cDNA Kit following manufacturer's recommendations. Fold changes were calculated relative to Actin using the ΔΔCt method. The assays were performed in triplicate. For western blot, samples were run on 4-20% SDS-PAGE and immunoblotted against BRCA1 and β-actin as a loading control. CellTiter-Glo cell viability (right panel) was performed as described by the manufacturer, to verify that LTR transcriptional activity was not a result of cytotoxicity. Western blots were performed in duplicate. Transcriptional and viability assays were performed in triplicate. (*) indicates statistically significant difference (unpaired t-test of triplicates) $p<0.05$. Error bars indicate standard deviation.

6.2.3 BRCA1 is in complex with Tat

Accumulating studies have revealed nearly 1,500 molecular interactions between HIV-1 and human proteins [162]. As discussed in subsection 3.6.4, the most studied Tat binding protein is its interaction with the pTEF-b Cyclin T1 subunit [38]. Because of the fact that Tat is known to interact with various transcription factors and is present within the high molecular weight BRCA1-containing fraction, we were interested in determining whether these proteins were in complex. To this end, GST-BRCA1 constructs spanning the full length of the protein (Figure 13A) and GST-Tat were utilized in a pull-down assay from whole TZM-bl cell lysates that had been transfected with Flag-Tat. TZM-bl cells were chosen as they express robust levels of BRCA1 (Figure 12), and are our base cell line for our transcriptional assays. Western blot results in Figure 13B indicate that the only detectable association between BRCA1 and Tat occurs at the aa504-802 region (lane 4, upper panel), while GST-Tat inversely confirmed the BRCA1-Flag Tat observed association with the BRCA1 fragment (lane 8, middle panel). Cdk9 seemed to overlap at regions aa1-1276 and aa1501-1861 (lanes 3-5, and 7, bottom panel). Additionally, we saw GST-Tat binding to the Flag-Tat present in the cell lysate, serving as a positive binding control given that Tat exists as a Zn^{2+}- or Cd^{2+}-linked dimer [159]. GST protein beads (lane 2, all panels) were used as a background control. Membranes were probed with antibodies against Cdk9, BRCA1, and Flag. To further corroborate this interaction, these whole cell extracts were immunoprecipitated with BRCA1 or IgG and western blotted with anti-BRCA1. Results in Figure 13C show that BRCA1-Tat interaction was observed specifically with the BRCA1 immunoprecipitation and not with the IgG background control. Probing against BRG1 was used as an immunoprecipitation control

since it has been shown to be a BRCA1-binding partner [205]. Collectively, these results indicate that physical interaction of BRCA1-Tat is detectable at the aa504-802 region of BRCA1, suggesting that these proteins including Cdk9, are associated and supporting BRCA1 participation in Tat-dependent transcription.

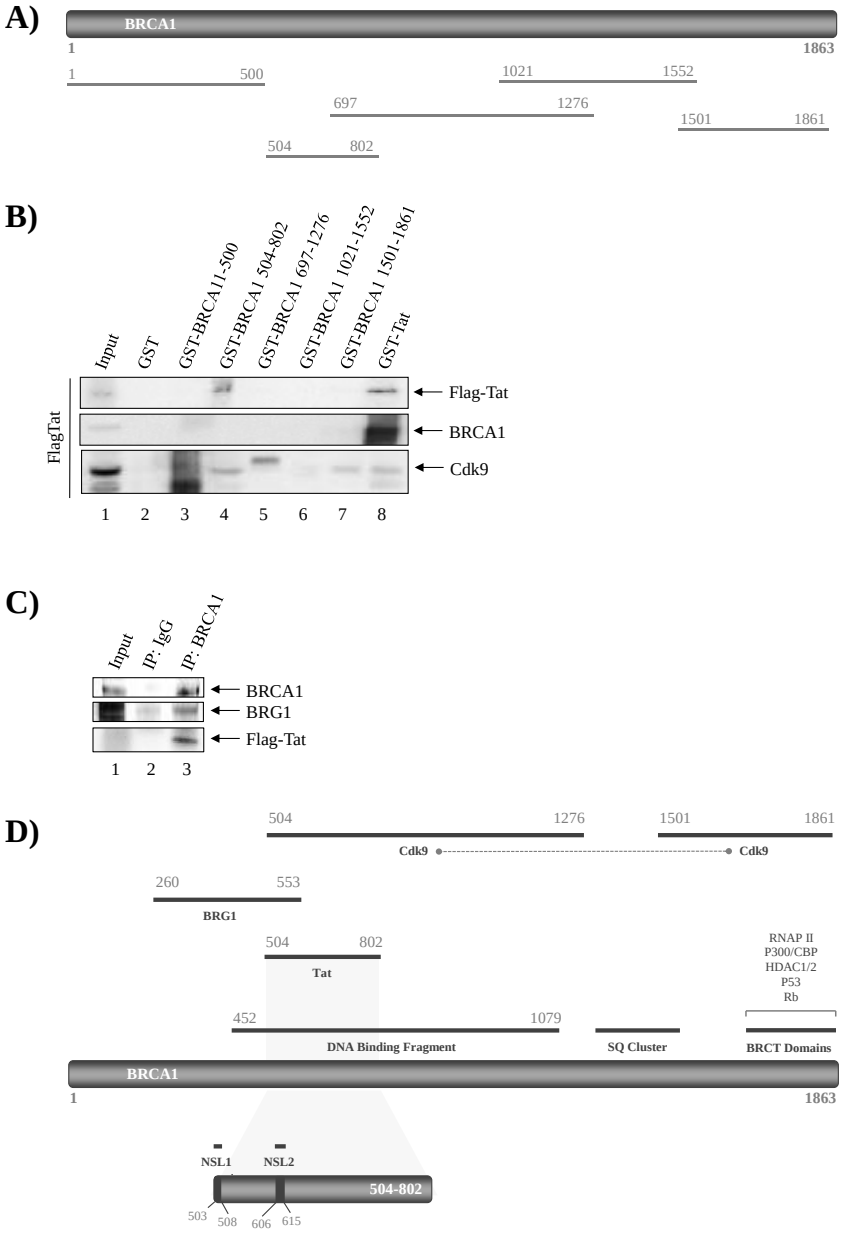

Figure 13 BRCA1 associates with Tat.

A) Schematic of five GST-BRCA1 fragments spanning the whole length protein. **B)** TZM-bl cells were transfected with Flag-Tat plasmid DNA for 48 hours. One milligram of whole cell protein extract was incubated with 1 µg GST-BRCA1 and GST-Tat constructs. For Cdk9 binding, beads were washed once with TNE_{300} + 0.1% NP-40, once with TNE_{150} + 0.1% NP-40, and once with TNE_{50} + 0.1% NP-40. For Tat binding, beads were washed once with TNE_{150} + 0.1% NP-40, and once with TNE_{50} + 0.1% NP-40. Beads were resuspended in blue lysis buffer and boiled for 10 min. Samples were separated on a 4-20% gel by SDS-PAGE, and probed with antibodies against Cdk9, BRCA1, and Flag. **C)** TZM-bl cells were transfected with Flag-Tat plasmid DNA for 48 hours. One milligram of TZM-bl whole cell protein extract were immunoprecipitated with anti-BRCA1 and anti-IgG antibodies, beads were washed once with TNE_{150} + 0.1% NP-40 and twice with TNE_{50} + 0.1% NP-40, separated on a 4-20% by SDS-PAGE, and probed against BRCA1, BRG1, and Flag. Western blots were performed in duplicate. **D)** Schematic of $BRCA1_{aa1\text{-}1,863}$ indicative of significant domains and protein-protein interactions.

6.2.4 Small molecule inhibition of BRCA1 gene expression affects HIV-1 Tat-dependent transcription

Currently, there are no therapeutic agents specifically targeting BRCA1. However recently, it has been documented that curcumin reduces expression of the BRCA1 gene by histone acetylation impairment at the BRCA1 promoter [276]. Generally, curcumin is categorized as a multifactorial compound characterized by tolerable doses linked to antioxidant therapy, anti-tumor effects, and anti-viral effects [269,276-279]. Moreover, curcumin has been shown to suppress pathways concomitant to HIV-1 replication and HIV-1 associated neurocognitive disorders (HAND) [280-282]. In addition, a recent study demonstrated multi-pathway involvement in curcumin-induced inhibition of Tat-dependent transcription [283]. Here, the authors showed modulation of the HDAC1/NF-κB pathway used by HIV-1 to exert chromatin remodeling at the viral promoter and further promoter activation by NF-κB. Despite that curcumin is not BRCA1-specific, we were interested in using the compound as a model for small molecule inhibition of BRCA1. To confirm previous findings in our cell-based system, TZM-bl cells were treated for 24 hours with vehicle (DMSO) or a titration of curcumin designed from concentrations used in the literature (0.5, 1, 10, and 20 μM). Western blot was then performed using anti-BRCA1 and anti- β-actin antibodies to determine the expression levels of BRCA1. Results in Figure 14A show a dose-dependent loss of BRCA1 expression with curcumin treatment when compared to DMSO (compare lanes 1 to 2-5). Next, we confirmed Tat-dependent transcription inhibition by treating Tat-transfected TZM-bl cells with 20 μM of curcumin. Results in Figure 14B show a significant decrease in transcription (~45%) with curcumin when compared to DMSO (compare lanes 1 and

2) that is specific to treatment and not due to compound cytotoxicity (compare lanes 3 and 4) and likens previously published data [283]. To examine the effects of curcumin on BRCA1 occupancy of the HIV-1 promoter, we next performed ChIP analysis. Preliminary ChIP studies with total and p-BRCA1 S1423 in unstimulated and TNF-α stimulated cells (basal transcription) indicated that BRCA1 could be detected primarily at the LTR (nt -116 to +4) and nuc-1 regions (nt +30 to +134), and in lesser amounts at the nuc-2 (nt +283 to +390) and *env* regions following TNF-α stimulation (data not shown). Moreover, phospho-BRCA1 S1423 was predominantly found at the nuc-1 region after cytokine induction. ChIP assays from Tat-transfected DMSO- and curcumin-treated (20 μM) TZM-bl cells were performed using antibodies against RNA RNAP II (positive control), IgG (negative control) and BRCA1. Interestingly in Figure 14C results, we observed BRCA1 occupancy loss from the activated HIV-1 LTR with curcumin treatment when compared to the DMSO control (compare lanes 7 and 8). Collectively, these results are suggestive of BRCA1 playing a role in transcriptional initiation or initiation of elongation, and implies the use of chemotherapeutic agents treating BRCA1 as a druggable target.

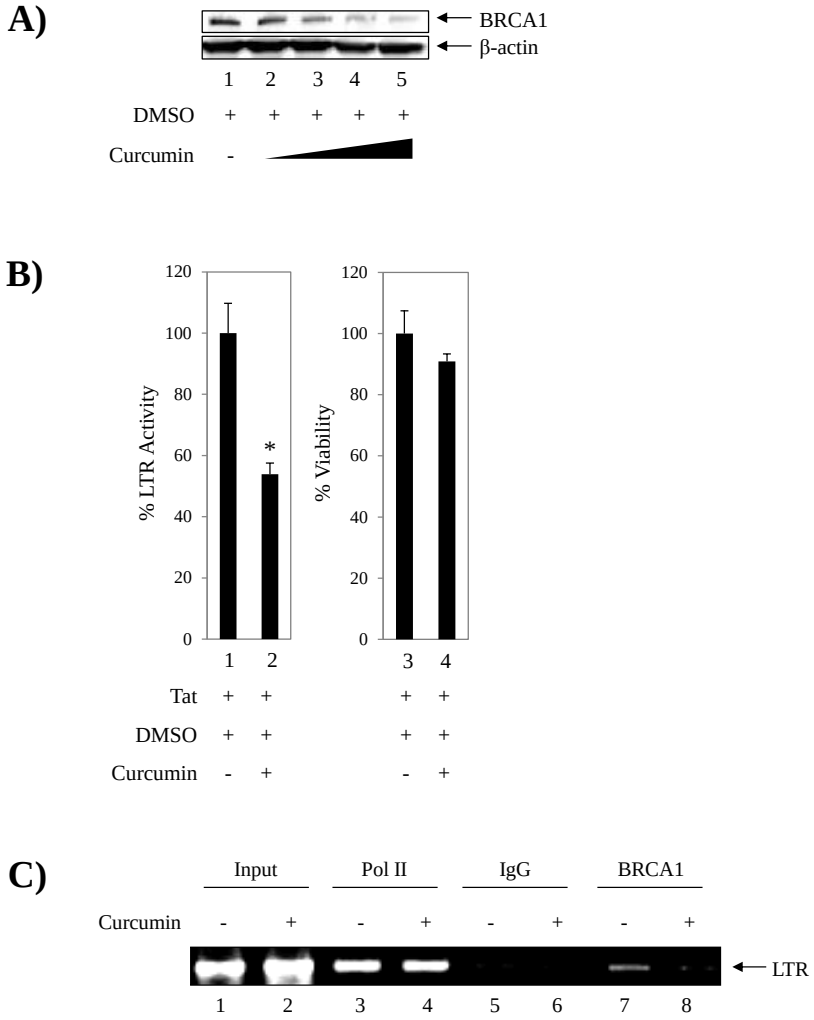

Figure 14 Curcumin decreases BRCA1 occupancy at the HIV-1 LTR.

A) TZM-bl cells were transfected with pcTat and treated the next day with vehicle (DMSO) or a titration of curcumin (0.5, 1, 10, and 20 μM). Samples were run on 4-20% SDS-PAGE and immunoblotted against BRCA1 and β-actin as a loading control. **B)** TZM-bl cells were transfected with pcTat and treated the next day with DMSO or 20 μM curcumin. Bright-Glo luciferase assays and CellTiter-Glo cell viability assays were performed 24 hours post-treatment as described by the manufacturer, to determine the level of curcumin-driven BRCA1 expression downregulation and treatment cytotoxic effects. Assays were performed in triplicate. (*) indicates statistically significant difference (unpaired t-test of triplicates) $p<0.05$. Error bars indicate standard deviation. **C)** TZM-bl cells were transfected with pcTat and treated the next day with DMSO or curcumin (20 μM) for 24 hours prior to being collected for ChIP analysis. Samples were processed as in (C). Results are representative of two independent experiments.

6.3 MATERIALS AND METHODS

6.3.1 Cell culture, transfection, and inhibitor treatment

TZM-bl cells are engineered HeLa cells that express CD4, CCR_5, and $CXCR_4$ and contain integrated reporter genes for Firefly luciferase and β-galactosidase under the control of an HIV-1 long terminal repeat [275,284]. These cells were cultured to confluency in DMEM supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 1% streptomycin/penicillin (Gibco/BRL, Gaithersburg, MD, USA). UWB1.289 cells are derived from ovarian cancer in a germ line BRCA1 mutation carrier and lack expression of BRCA1 [273]. UWB1.289+BRCA1 cells are a stable UWB1.289 derivative cell line carrying a pcDNA3 plasmid coding for wild-type hemagglutinin-tagged BRCA1 [274]. Cells were purchased from the American Type Culture Collection (Manassass, VA, USA) and were maintained in 1:1 RPMI 1640/MEGM (Lonza, Walkersville, MD, USA) supplemented with 3% fetal bovine serum. UWB1.289 and UWB1.289+BRCA1 cells were seeded in a 96-well plate and co-transfected with a 0.6 μg combination of pRL-CMV-luciferase reporter (Renilla), HIV-1 LTR-luciferase reporter (Firefly), and pcTat. The Renilla reporter plasmid was co-transfected to allow correction for differences in transfection efficiency. Cells were collected for luminescence analysis 48 hours post-transfection. UWB1.289 cells were seeded in a 12-well plate and co-transfected using with a 3 μg combination of pNL4.3 and pcDNA, BRCA1 (wt) or the BRCA1 mutant BRCA1 4P [constructed by swapping a cDNA fragment harboring the S1387A and S1423A mutations into the wild type plasmid followed by adding additional mutations with sequential rounds of QuikChange site-directed mutagenesis (Stratagene, Santa Clara, CA, USA) using primer sets: 5'-GCAGTATTAACTG CACAGAAAAGTAGTG-3' and 5'-CACTACT

TTTCTGTGCAGTTAATACTGC-3’ (S1457A); 5’-GAATAGAAACTACCCAGCTCAAGAGG A-3’ and 5’-GAGCTCCTCTTGAGCTGGGTA GTTTCTATTC-3’(S1524A)]. The cells were harvested 24 hours post-transfection and processed for RNA extraction. For siRNA transfection, TZM-bl cells were co-transfected in a 96-well plate for 48 hours with pcTat (0.5 μg) and siRNA against GFP (#P-002048-01-20, Dharmacon, Lafayette, CO, USA) or BRCA1 (Hs_BRCA1_15, #SI02664368, Qiagen, Valencia, CA, USA) using DharmaFECT Duo (#T-2010-01, Dharmacon). For small molecule inhibitor treatment, TZM-bl cells were transfected with pcTat (0.5 μg) in a 96-well plate. Cells were treated the next day with DMSO or the indicated compound at various concentrations. Curcumin was purchased from Santa Cruz Biotechnology (sc-200509, Santa Cruz, CA, USA). Curcumin was prepared in 10 mM stock solution dissolved in DMSO. All transfections unless noted otherwise were performed with Attractene reagent (Qiagen) according to the manufacturer's instructions.

6.3.2 Transcriptional and viability assays

Forty-eight hours post transfection or drug treatment, luciferase activity of the Firefly luciferase was measured with Dual-Glo or Bright-Glo Luciferase Assay (Promega, Madison, WI, USA). Alternatively, CellTiter-Glo (Promega) was used to measure viability following the manufacturer’s recommendations. CellTiter-Glo is a luminescent assay used to measure cell viability by ATP level. The reagent was added to the wells (1:1 reagent:media) and incubated at room temperature for 10 min protected from light. Luminescence was read from a 96-well plate on an EG&G Berthold luminometer (Berthold Technologies, Oak Ridge, TN, USA).

6.3.3 Immunoblotting

Cell extracts were resolved by SDS PAGE on a 4-20% tris-glycine gel (Invitrogen, Life Technologies, Carlsbad, CA, USA). Proteins were transferred to PVDF microporous membranes by overnight transfer as described by the manufacturer (Invitrogen, Life Technologies). Membranes were blocked with PBS 0.1% Tween-20 + 3% BSA. Primary antibody against specified proteins was incubated with the membrane in blocking solution overnight at 4°C. Antibodies against BRCA1 (sc-642), Cdk9 (sc-484), and BRG1 (sc-10768) were purchased from Santa Cruz. Anti-Flag (F3165) and β-actin antibody (ab49900) were obtained from was obtained from Sigma Aldrich (St. Louis, MO, USA) and Abcam (Boston, MA, USA), respectively. Membranes were washed twice with PBS + 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody for 1 hour in blocking solution. Presence of secondary antibody (#32430 and #32460, Pierce, Rockford, IL, USA) was detected by SuperSignal West Dura Extended Duration Substrate (Pierce). Luminescence was visualized on a Molecular Imager ChemiDoc XRS system Bio-Rad station (Bio-Rad, Hercules, CA, USA).

6.3.4 RNA extraction and qRT-PCR

Total RNA was isolated from cell pellets using the RNEasy Kit (Qiagen) according to the manufacturer's protocol. A total of 300 ng of RNA was used to generate cDNA using the High-Capacity RNA-to-cDNA Kit (#4387406, Invitrogen, Life Technologies) following manufacturer’s recommendations. Quantitative PCR was performed with SYBR Green PCR Master Mix (#4309155, Applied Biosystems, Foster City, CA). Fold changes were calculated relative to Actin using the ΔΔCt method.

Primers used are described: BRCA1 ►*Forward* 5'-GGCTATCCTCTCAGAGTG ACATTT3' and *Reverse* 5'GCTTTATC AGGTTATGTT GCATGGT3' [285].

6.3.5 Chromatin immunoprecipitation

Cells were crosslinked with 1% formaldehyde for 10 min and crosslinking was stopped by the addition of 125 mM glycine. Chromatin fragments were prepared from $5x10^6$ cells per sample. Cells were lysed using SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, one tablet complete protease inhibitor cocktail per 50 ml) on ice for 10 min. Cells were sonicated on ice for 6 bursts of 10 seconds to obtain an average DNA length of 500 to 1000 bp (Misonix XL 2000, Misonix, NY, USA). Lysate was clarified by centrifugation at 4°C for 10 min at 14,000 rpm. Supernatant was then diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 167 mM NaCl) and pre-cleared with a mixture of protein A/G agarose (blocked previously with 1 mg/ml salmon sperm DNA and 1 mg/ml BSA, Stratagene) at 4°C for 1 hour. Pre-cleared chromatin was incubated with 10 µg of antibody at 4°C overnight. Next day, 60 µl of 30% slurry of blocked protein A/G agarose was added and complexes incubated for 2 hours. Immune complexes were recovered by centrifugation and washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), twice with high salt buffer (0.1% SDS, 1% Triton X-100 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0), and once with TE buffer. Immune complexes were eluted twice with elution buffer (1% SDS, 0.1 M NaHCO3) and incubating at room temperature for 15 min on a rotating

wheel. Cross-links were reversed by adding 20 µl of 5M NaCl and incubating elutes at 65°C overnight. The next day, proteinase K (100 µg/ml final concentration) was added and samples incubated at 55°C for 1 hour. Samples were extracted with phenol:chloroform twice and ethanol precipitated overnight. Pellets were then washed with 70% ethanol, dried, resuspended in 50 µl of TE and assayed by PCR. Thirty-five cycles of PCR were performed in 50 µl with 10 µl of immunoprecipitated material, 0.2 µM of primers, using the Platinum PCR SuperMix (#11306-016, Invitrogen, Life Technologies). Finally, PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. The antibodies used for immunoprecipitation were as follows: total RNAP II CTD (ab817, Abcam), BRCA1 (sc-642, Santa Cruz), p-BRCA1 S1423 (sc-101647, Santa Cruz), and IgG (sc-2027, Santa Cruz). Primers used are described: HIV-1 LTR (nt -69-+175) *Forward* ► 5'-CTGGG CGGGACTGGGGAG-3' and *Reverse* 5'-TCACACAACAGACGGGCACAC-3'.

6.3.6 GST pull-down and immunoprecipitation

GST tagged proteins were purified as described previously [286]. Constructs were washed three times with PBS + 1% Triton X-100, pelleted and resuspended in PBS + 1% Triton X-100. Bead volume was normalized between samples by the addition of extra bead slurry prepared in the same manner for each condition. Whole cell protein extract from TZM-bl cells that were transfected with Flag-Tat_{101} for 48 hours was brought up to a final volume of 500 µl with lysis buffer and 1 µg of GST-BRCA1 constructs (1–500, 504–802, 697–1276, 1021–1552, 1501–1861) were rotated at 4°C overnight. GST-Tat beads were washed once with TNE_{150} + 0.1% NP-40 and twice with TNE_{50} + 0.1% NP-

40. GST-BRCA1 beads were washed once with TNE_{300} + 0.1% NP-40, once with TNE_{150} + 0.1% NP-40, and once with TNE_{50} + 0.1% NP-40. For IP, 1 mg of whole cell protein was brought up to a final volume of 500 µl with TNE_{50} + 0.1% NP-40 and precleared for 15 min with 50 µl of 30% A/G agarose bead slurry (CalBioChem, La Jolla, CA). Supernatants were transferred to a new tube with 10 µg of BRCA1 or normal rabbit IgG antibodies (Santa Cruz), and the solution was rotated overnight at 4°C. The next day complexes were precipitated with A/G beads for 90 min. Beads were washed once with TNE_{150} + 0.1% NP-40 and twice with TNE_{50} + 0.1% NP-40. Cells were collected directly in lysis buffer and boiled for 10 min. The GST-construct plasmids pDC78 GST-BRCA1 (1-500), pDC80 GST-BRCA1 (1021-1552), pDC81 GST-BRCA1 (1501-1861), pDC99 GST-BRCA1 (504-802), pDC208 GST-BRCA1 (697-1276) were originally a kind gift from Dr. Tanya Paull at the University of Texas/ICMB [287].

6.4 SUMMARY

In this chapter we focused on defining whether BRCA1 functions as a coactivator or corepressor of HIV-1 transcription in established cell line models. Here we have shown that non-integrated Tat-dependent HIV-1 LTR transcription is more effective in cells containing BRCA1 (Figure 11A). Additionally, we have shown that overexpression of wild type BRCA1 in a system containing an integrated HIV-1 LTR enhances the level of Tat transcriptional transactivation (Figure 11C). Expression of plasmid was confirmed in Figure 11B. In support of these data, we found that BRCA1 selective depletion decreases Tat-dependent transcription efficiency from an integrated HIV-1 LTR (Figure 12), further supporting its enhancer role. Further contributing with suggestive evidence

that BRCA1 may be in a transcriptionally active complex with pTEF-b and Tat (see Figures 5 and 9B), we have shown that it is associated with Tat and Cdk9 (Figure 13). Together, these data illustrate the possibility that BRCA1 is enhancing Tat-dependent transcription through a pTEF-b/Tat complex.

These set of experiments also describe the use of curcumin as a therapeutic model for inhibition of BRCA1 expression. Curcumin is a naturally-derived low molecular weight compound widely used in animal and human cancer treatment studies in tolerable doses [276,277,288]. Furthermore, as a multifactorial compound, it is considered to possess the advantages of reduced molecularity, no drug-drug interactions and improved pharmacokinetics and pharmacodynamics [278]. Here, we have confirmed that curcumin decreases BRCA1 gene expression (Figure 14A) as previously reported [276]. More so, we have verified curcumin-mediated HIV-1 transcriptional inhibition (Figure 14B) and have shown that curcumin abrogates BRCA1 occupancy at the HIV-1 LTR (Figure 14C). Collectively, these data directly implicate BRCA1 in Tat-depedent transcription and propose BRCA1 as a possible therapeutic target through the use of curcumin.

CHAPTER SEVEN
BRCA1 PHOSPHORYLATION IN TAT-DEPENDENT TRANSCRIPTION

7.1 INTRODUCTION

In the presence of genotoxic stress, ATR and ATM collaborate to mediate cellular responses to DNA damage [289]. BRCA1 contains a serine cluster domain (SCD) spanning aa1280-1524, a common motif present in ATR/ATM protein targets [290]. ATR phosphorylates BRCA1 on serine 1423 in response to UV damage or HU-induced replication arrest [242], while ATM-mediated phosphorylation events at serines 1387, 1423, 1457 and 1524, have been characterized in response to ionizing radiation-induced damage [286,287]. While the Chk2 phospho-site is not located within the SCD region, phosphorylation of serine 988 occurs in response to the same stressors as with ATM [291]. Like curcumin, pharmacological inhibition of the components of the ATM and ATR regulated signaling pathways are attractive due to their chemosensitizing effects in cancer cells [289,292,293]. Importantly, HIV-1 replication inhibition by caffeine, an ATR and ATM general inhibitor, has been shown to occur at the integration step of the HIV-1 life cycle [294]. However, antagonizing findings show that both ATR and ATM are non-essential for integration and that caffeine did not affect HIV-1 infection [295]. Nevertheless, a more recent study sheds light on this controversy by showing that ATR and possibly other PIKK family members are involved in retroviral replication in a cell context-dependent manner [260]. Likewise, ATM DDR stimulation has been linked to

viral Integrase activity and ATM inhibition has been shown to suppress replication of both wild type and drug-resistant HIV-1 [296,297].

7.2 RESULTS

7.2.1 BRCA1 cell cycle expression is altered in infected cells

As a multifunctional protein, BRCA1 plays a role in cell cycle regulation through diverse protein-protein interactions [298]. Moreover, BRCA1 is expressed in a cell cycle-dependent manner [194,299,300]. Expression increases during late G_1, peaks and becomes phosphorylated during S phase, and progressively decreases and is dephosphorylated upon exit from mitosis. To further characterize BRCA1 expression in infection, TZM-bl cells were synchronized by nocodazole treatment and cell populations collected at various stages of the cell cycle. Due to the nature of this experiment, VSV-G pseudotyped HIV-1 particles (VSV-G-NL4.3) were generated in order to achieve efficient infection and synchronization release. Western blot results in Figure 15A indicate that BRCA1 expression in uninfected cells can be detected following expected expression patterns throughout G_1, S and G_2/M phases (top left panel). However, expression in infected cells shows ~32% less BRCA1 in G_1, similar S phase levels, and markedly increased ~5-fold expression at G_2/M (top right panel), when compared to uninfected cells. Interestingly, BRCA1 expression during infection is again suggestive of possible modification with visible doublet bands. BRCA1 S-phase phosphorylation, in the absence of exogenous stress, occurs in response to DNA DSBs in replication forks and is required as part of the S-phase cell cycle checkpoint [298]. This led us to examine the phospho-status of BRCA1 by probing for S1423 serine phosphorylation, since this

residue is phosphorylated by checkpoint signaling ATR and ATM kinases in response to damaged DNA or stalled DNA replication [195,242,287,301]. Our results indicate that in uninfected cells, p-BRCA1 S1423 phosphorylation occurs predominantly during the S phase, thus following expected patterns (second left panel). In contrast, excepting G_1, p-BRCA1 S1423 phosphorylation is generally increased during infection, particularly at the G_2/M and M phases (second right panel). A slight delay in phosphorylation during infection is noted at G_1 (~46% less p-BRCA1 S1423), mimicking the observed total BRCA1 signature (compare lanes 6-7 to 2-3 for both total and p-BRCA1 S1423 panels). Synchronization was verified by immunoblot for Cyclin A2, while p24 and β-actin were used as infection and loading controls, respectively. Figure 15B summarizes BRCA1 expression in uninfected (dotted curve) and infected cells (red curve). Because HIV-1 Tat-dependent transcription occurs in a cell cycle-dependent manner [231], the diagram was annotated with Tat-dependent transcription dynamics (black curve). Taken together, these results suggest altered BRCA1 expression and post-translational modification throughout the cell cycle in HIV-1 infected cells.

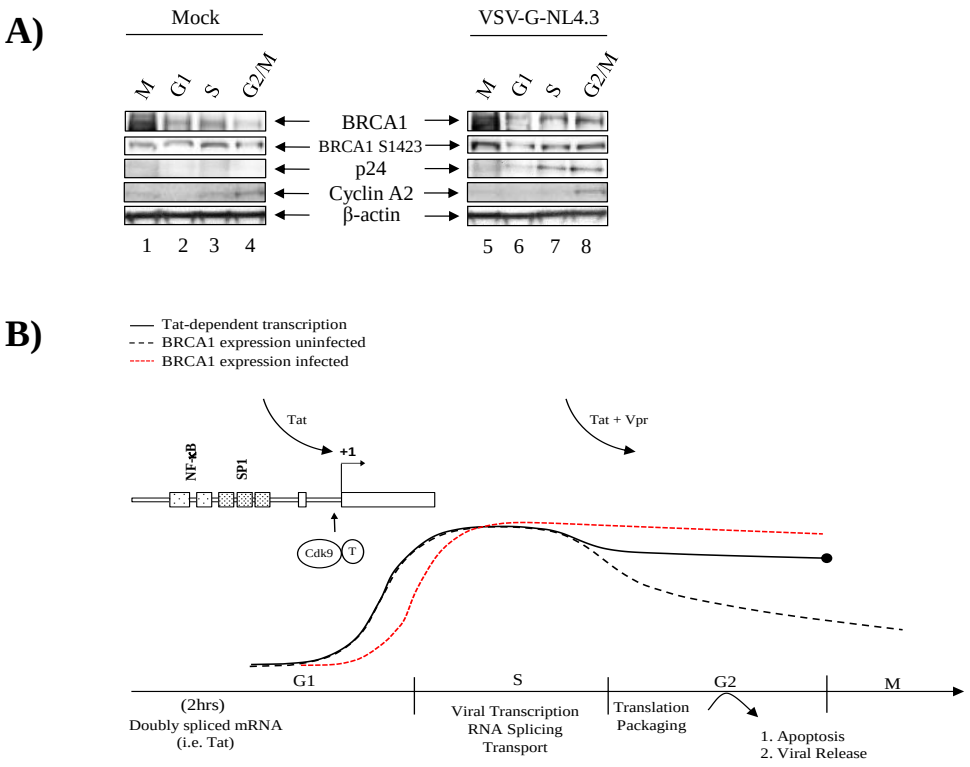

Figure 15 BRCA1 cell cycle expression dynamics in HIV-1 infection.

A) TZM-bl cells were synchronized with 200 ng/ml nocodazole for 24 hours. Cells were infected with pseudotyped VSV-G-NL4.3 HIV-1 and released in complete growth medium. Cells were collected at different cell cycle stages (M, G1, S, G_2/M). Whole cell protein extracts from synchronized cells were separated on a 4-20% gel by SDS-PAGE, and western blotted with antibodies against BRCA1, p-BRCA1 S1423, p24, Cyclin A2 as synchronization control, and β-actin as a loading control. B) Schematic summarizing Tat-dependent transcription dynamics [231] and BRCA1 expression throughout the cell cycle.

7.2.2 Inhibition of upstream BRCA1 phosphorylation effectors, ATR/ATM, decreases Tat-dependent transcription

Because of the BRCA1 activation changes observed from the cell cycle data, and the involvement of ATR and ATM kinases in BRCA1 phosphorylation, we next looked at the effect of ATR/ATM inhibition on Tat-dependent HIV-1 transcription. ATR and ATM inhibition have been widely studied in the cancer field because of their role in facilitating resistance of cancer cells to genotoxic treatment [289,302]. To this end, we initially used caffeine, a methylxanthine that has been used extensively to study ATR/ATM signaling as a natural inhibitor of these kinases [303]. TZM-bl cells were transfected with Tat and treated the next day with vehicle (water) and a titration of caffeine (500 μM, 2mM and 5 mM). Forty-eight hours post-treatment the cells were subjected to luciferase assays. As can be observed in Figure 16A, there is a dose-dependent decrease in HIV-1 transcription with increasing concentrations of caffeine of up to ~50% inhibition when compared to vehicle (compare lanes 2 and 4). To confirm compound inhibition specificity, we performed cell viability assays of treated cells to assay for inhibitor-induced cell death. Results in Figure 16B show that caffeine was not toxic at 500 μM or 2 mM. Working concentrations in published literature use caffeine up to 10 mM, however we observed that in our system, 5mM caffeine decreased cell viability by ~60% (lane 5). Taken together, these results suggest that BRCA1 phosphorylation may play a role in Tat-dependent transcription as observed indirectly by the inhibition of upstream BRCA1 activators.

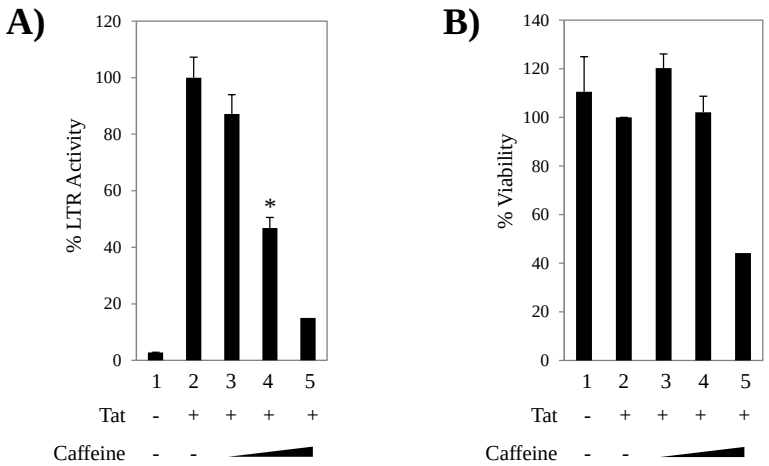

Figure 16 Caffeine decreases Tat-dependent HIV-1 transcription.

A) TZM-bl cells were transfected pcTat and treated the next day with vehicle (water) and caffeine at 500 μM and 2 mM. Bright-Glo luciferase assays were performed 48 hours post-treatment as described by the manufacturer, to determine the level of caffeine-driven inhibition of Tat-dependent transcription. **B)** CellTiter-Glo cell viability assays were performed 48 hours post-treatment as described by the manufacturer, to verify that LTR transcriptional activity decrease was not a result of cytotoxicity. Assays were performed in triplicate. (*) indicates statistically significant difference (unpaired t-test of triplicates) $p<0.05$. Error bars indicate standard deviation.

7.2.3 Inhibition of the upstream BRCA1 phosphorylation effector ATM kinase, but not Chk2, decreases Tat-dependent transcription

Caffeine relative non-specificity for ATR/ATM has led to the elucidation of more specific inhibitors of these kinases. The small molecule inhibitor *2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one* or KU55933 (referred to henceforth as ATM*in*) has been shown to specifically inhibit ATM in the low IC_{50} of 12.9 nM without inhibiting ATR at doses of up to 100 μM [292]. To further explore BRCA1 phosphorylation effectors, we next looked at the effect of specific ATM kinase inhibition on Tat-dependent transcription in parallel with a specific Chk2 kinase inhibitor, *2-(4-(4-chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide* or Chk2*in*. TZM-bl cells were transfected with Tat and treated the next day with vehicle (DMSO) and a titration of ATM*in* or Chk2*in* (0.1, 1 and 10 μM). Forty-eight hours post-treatment the cells were subjected to both luciferase (Figure 17A) and viability assays (Figure 17B). The results show a dose-dependent Tat-dependent LTR transcriptional inhibition of up to 76% in the presence of ATM*in* but not Chk2*in* (compare lanes 6 and 9), demonstrating ATM kinase specificity in this inhibitory process given that Chk2 also phosphorylates BRCA1 in response to DNA damage [240]. Both inhibitors showed no cytotoxicity. To further confirm the specific participation of ATM in Tat-dependent transcription, we performed ATM selective depletion in Tat-transfected TZM-bl cells. Results in Figure 17C indicate that upon ATM knockdown, Tat transactivation of the viral promoter is decreased by ~45%. Selective depletion decreased ATM expression by ~54% as confirmed by western blot against ATM and β-actin as a loading control. Further viability assays revealed no cytotoxic effects upon ATM siRNA-mediated downregulation (data not shown). Taken

together, these results show that LTR transcription is more potently and specifically inhibited by ATM*in* at lower concentrations than those used with caffeine.

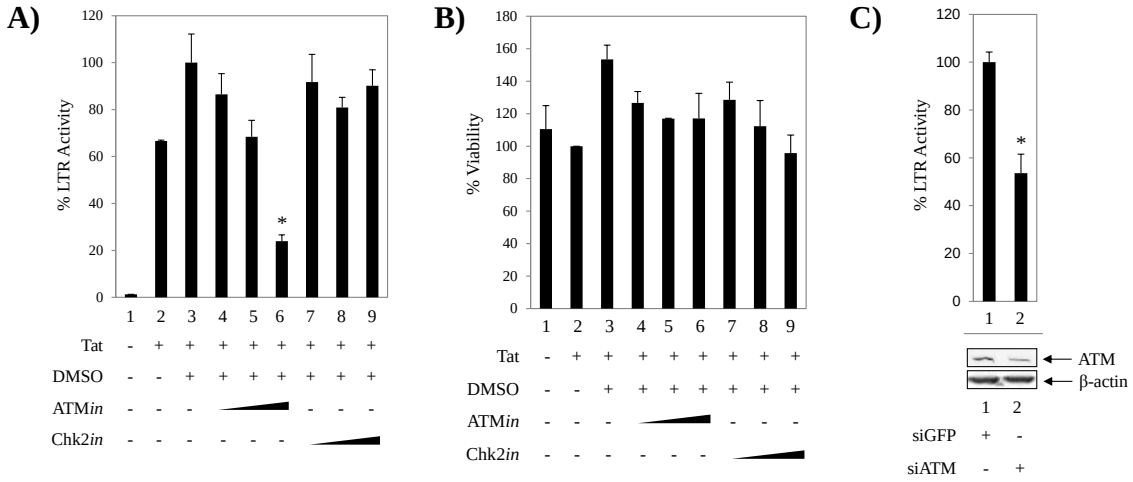

Figure 17 ATM, but not Chk2 inhibition, decreases Tat-dependent HIV-1 transcription.

A) TZM-bl cells were transfected with pcTat and treated the next day with vehicle (DMSO) and a titration of ATM or Chk2 inhibitors (ATM*in* and Chk2*in*) at 0.1 μM, 1 μM and 10 μM. Bright-Glo luciferase assays were performed 48 hours post-treatment as described by the manufacturer, to determine the level of caffeine-driven inhibition of Tat-dependent transcription. **B)** CellTiter-Glo cell viability assays were performed 48 hours post-treatment as described by the manufacturer, to verify that LTR transcriptional activity decrease was not a result of cytotoxicity. **C)** TZM-bl cells were co-transfected with pcTat or siRNA against GFP (control) and ATM. Bright-Glo luciferase assays were performed 48 hours post-treatment as described by the manufacturer, to determine knockdown effects of Tat-dependent transcription. Whole cell extracts from these samples were run on 4-20% SDS-PAGE and immunoblotted against ATM and β-actin as a loading control. Western blots were performed in duplicate. Assays were performed in triplicate. (*) indicates statistically significant difference (unpaired t-test of triplicates) $p<0.05$. Error bars indicate standard deviation.

7.2.4 Inhibition of BRCA1 phosphorylation by ATM kinase decreases BRCA1 LTR occupancy

BRCA1 is a nucleocytoplasmic shuttling protein [304]. In Figure 18A we have shown that the cytoplasmic and nuclear BRCA1 pool in infected J1.1 T cells showed decreased cytoplasmic (~24%) and increased nuclear levels (~76%) when compared to the uninfected Jurkat T cell fractions (~35% and 65%, respectively). Localization of GAPDH (cytoplasmic) serves as positive control of fraction enrichment and doubles as a loading control between fractions. We therefore asked whether Tat alone could induce any changes in BRCA1 cytoplasmic or nuclear levels and phosphorylation. For this, we utilized a fractionation of cytoplasmic *versus* nuclear extracts in the same manner we did for Figure 18A. The results in Figure 18B indicate that there is no major change in BRCA1 cytoplasmic levels between pcDNA- or Tat-transfected TZM-bl cells (upper panel, compare lanes 1 and 3). A modest increase in nuclear BRCA1 in cells containing Tat is observed when compared to cells containing control DNA (compare lanes 2 and 4). Increased p-BRCA1 S1423 levels were observed at ~1.2-fold and ~6.2-fold in the cytoplasmic and nuclear fractions of Tat-containing cells (second panel), further supporting the involvement of phospho-BRCA1 in Tat-dependent transcription. Localization of lamin A (nuclear) and GAPDH (cytoplasmic) serve as positive controls of fraction enrichment, and double as loading control between fractions.

In order to functionally link inhibitor study findings to BRCA1, ChIP assays from Tat-transfected DMSO- and ATM*in*-treated TZM-bl cells (10 μM) were performed using antibodies against RNA RNAP II (positive control), IgG (negative control), BRCA1, and p-BRCA1 S1423. Results in Figure 18C reveal total BRCA1 (compare lanes 7 and 8) and

S1423 phosphorylated BRCA1 (compare lanes 9 and 10) occupancy loss from the activated HIV-1 LTR with ATM*in* treatment when compared to the DMSO control. Taken together, these data confirm the BRCA1-specific effects of ATM*in* in Tat-dependent transcription inhibition and suggests that BRCA1 phosphorylation by ATM is required for its recruitment to the HIV-1 LTR in the presence of Tat.

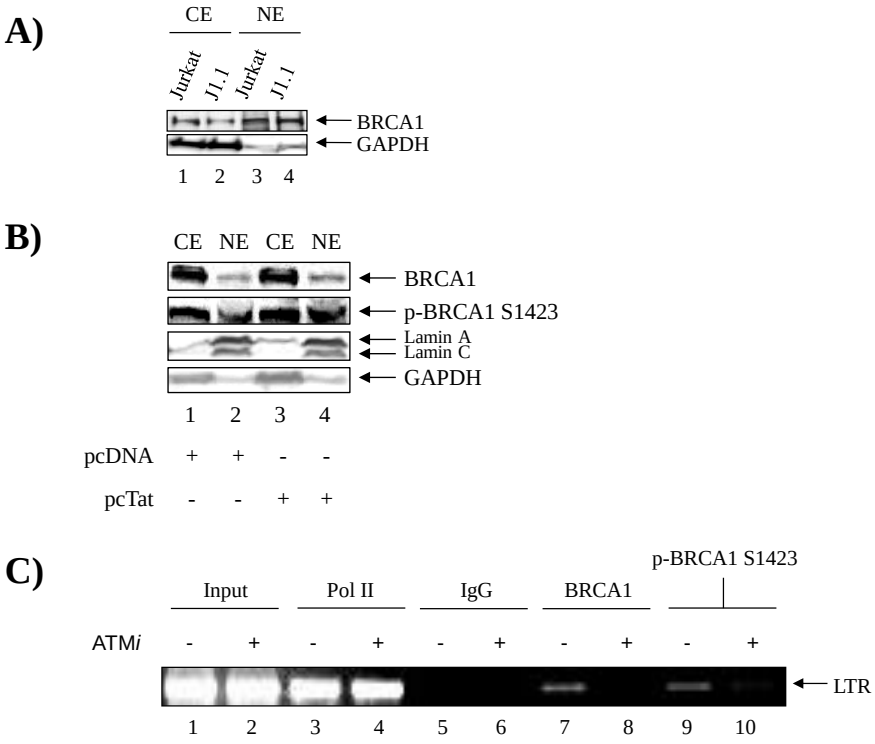

Figure 18 Inhibition of BRCA1 phosphorylation by ATM decreases BRCA1 occupancy at the HIV-1 LTR.

A) Cytosolic and nuclear extracts were prepared from TZM-bl cells. The extracts were analyzed by western blotting using antibodies against BRCA1 and GAPDH. GAPDH was used as control for cytoplasmic fraction enrichment and doubles as a loading control between fractions. Western blots were performed in duplicate. **B)** Cytosolic and nuclear extracts were prepared from TZM-bl cells. The extracts were analyzed by western blotting using antibodies against BRCA1 and p-BRCA1 S1423. Lamin A and GAPDH were used as nuclear and cytoplasmic controls, respectively, and double as loading controls between fractions. Western blots were performed in duplicate. **C)** TZM-bl cells were transfected with pcTat and treated the next day with DMSO or ATM inhibitor (10 μM) for 48 hours prior to being collected for ChIP analysis. Antibodies used for ChIP were anti-BRCA1 (10 μg), anti-IgG (10 μg), and anti-RNAP II (10 μg). PCR products were run on a 2% agarose gel and visualized with ethidium bromide staining. Results are representative of two independent experiments.

7.2.5 Mutation of ATM kinase serine substrates impairs BRCA1 enhancement of Tat-dependent transcription

Based on the observed BRCA1 altered phosphorylation cellular distribution in Tat-containing cells and HIV-1 LTR phosphorylation-dependent BRCA1 occupancy, we next asked whether mutation of ATM kinase serine substrates on BRCA1 would have an effect on HIV-1 transcription in the presence of Tat. To this end, we utilized the BRCA1 mutant construct, BRCA1 4P, which harbors ATM target serine residues ΔS1387A, S1423A, S1457A, and S1524A substitutions (efficient expression confirmed by western blot in Figure 11B). We assessed the effect of BRCA1 4P overexpression in the context of integrated proviral DNA. To this end, we co-transfected a combination of Tat, pcDNA or BRCA1 4P into TZM-bl cells for 48 hours and quantified Tat-dependent HIV-1 LTR-driven luciferase expression. Results in Figure 19 show no transcriptional enhancement between cells containing control DNA or BRCA1 4P. These results are contrasting to the ~89% increase in transcription observed in Figure 11C upon overexpression of wild type BRCA1. Taken together, these results are suggestive of specific ATM-dependent post-translational modification of BRCA1 that interferes with its ability to enhance Tat-dependent transcription.

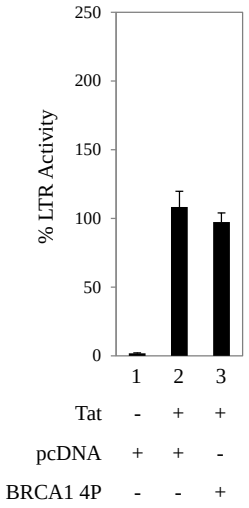

Figure 19 BRCA1 phosphorylation by ATM plays a role in Tat-dependent HIV-1 transcription.

TZM-bl cells were co-transfected with a combination of pcTat, pcDNA, and BRCA1 4P plasmid DNA (mutant for S1387A, S1423A, S1457A, and S1524A). Bright-Glo luciferase assay was performed 48 hours as described by the manufacturer, to determine the effect of BRCA1 overexpression on HIV-1 LTR activity. The assays were performed in triplicate. Error bars indicate standard deviation.

7.3 MATERIALS AND METHODS

7.3.1 Cell culture, transfection, and inhibitor treatment

TZM-bl cells were cultured to confluency in DMEM supplemented with 10% heat-inactivated FBS, 1% L-glutamine, and 1% streptomycin/penicillin (Gibco/BRL, Gaithersburg, MD, USA). TZM-bl cells were transfected as indicated in subsection 6.3.1. siRNA against ATM (Hs_ATM_5, #SI00299299) was obtained from Qiagen (Valencia, CA, USA). TZM-bl cells were treated the next day with DMSO or the indicated compound at various concentrations. The ATM kinase inhibitor (ATM*in*) *2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one* and Chk2 kinase inhibitor (Chk2*in*) *2-(4-(4-chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide*, were purchased from EMD4Biosciences (Gibbstown, NJ, USA). Caffeine was purchased from Sigma Aldrich (St. Louis, MO, USA). All inhibitors were prepared in 10 mM stock solution dissolved in DMSO.

7.3.2 Pseudotyped virus preparation

The Δ*env* mutant, pNL4-3(KFS), was kindly provided by Dr. Yunato Wu and originally obtained from Dr. Eric Freed [305]. pHCMV-G that expresses the vesicular stomatitis virus glycoprotein has been described previously [306]. Virus stocks of HIV-1 VSV-G-NL4.3 were produced by co-transfection of 293T cells ($5x10^6$) with 10 µg of pHCMV-G and 10 µg of plasmid pNL4-3(KFS). Supernatants were collected 24 hours post-transfection, centrifuged for 10 min at 3,000 rpm to remove cellular debris, filtered through a 0.45 µm filter, and concentrated with a size exclusion Vivaspin 20 concentrator (Sartorius-Stedim Biotech, Goettingen, Germany). Levels of p24 in viral supernatant were measured using the Perkin Elmer Alliance p24 antigen ELISA Kit (Perkin Elmer,

Norwalk, CT, USA). Plates were kinetically read using an ELx808 automatic microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 630 nm.

7.3.3 Cell synchronization

TZM-bl cells were synchronized by treatment with nocodazole (200 ng/ml) for 24 hours (Sigma Aldrich). Following treatment, arrested cells were collected by the mitotic shake method. Adherent cells were not collected. Cells were collected (M phase population) and the remaining cells were mock infected or infected with VSV-G-NL4.3 pseudotyped virus for 2 hours. Cells were washed once with DMEM and released in DMEM medium supplemented with 20% FBS, 1% L-glutamine, and 1% streptomycin/penicillin. Cells were collected at 0 hours (M phase), 4 hours (G_1 phase), 12 hours (S phase), and 20 hours (G_2/M phase).

7.3.4 Transcriptional and viability assay

Forty-eight hours post transfection or drug treatment, luciferase activity of the Firefly luciferase was measured with the BrightGlo Luciferase Assay (Promega, Madison, WI, USA). Alternatively, CellTiter-Glo (Promega) was used to measure viability following the manufacturer's recommendations. CellTiter-Glo has been described in subsection 6.3.2. Luminescence was read from a 96-well plate on an EG&G Berthold luminometer (Berthold Technologies, Oak Ridge, TN, USA).

7.3.5 Immunoblotting

Cell extracts were resolved by SDS PAGE on a 4-20% tris-glycine gel (Invitrogen, Life Technologies, Carlsbad, CA, USA). Proteins were transferred to PVDF microporous membranes by overnight transfer as described by the manufacturer (Invitrogen, Life Technologies). Membranes were blocked with PBS 0.1% Tween-20 +

3% BSA. Primary antibody against specified proteins was incubated with the membrane in blocking solution overnight at 4°C. Anti-ATM (2873), anti-Lamin A/C (4777), anti-Cyclin A2 (4656), and GAPDH (2118) was purchased from Cell Signaling Technology (Beverly, MA, USA). BRCA1 (sc-642), and p-BRCA1 S1423 (sc-101647) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were washed twice with PBS + 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody for 1 hour in blocking solution. Presence of secondary antibody (#32430 and #32460, Pierce, Rockford, IL, USA) was detected by SuperSignal West Dura Extended Duration Substrate (Pierce). Luminescence was visualized on a Molecular Imager ChemiDoc XRS system Bio-Rad station (Bio-Rad, Hercules, CA, USA).

7.3.6 Chromatin immunoprecipitation

Cells were processed as previously described in subsection 6.3.5 of Chapter 6. The antibodies used for immunoprecipitation were as follows: total RNAP II CTD (ab817, Abcam, Boston, MA, USA), BRCA1 (sc-642, Santa Cruz), p-BRCA1 S1423 (sc-101647, Santa Cruz), and IgG (sc-2027, Santa Cruz).

7.4 SUMMARY

In this chapter we focused on describing BRCA1 phosphorylation characteristic of DDR executed by ATR and ATM DNA damage sensor kinases. These set of experiments describe altered BRCA1 levels in infection through the stages of the cell cycle that are in similar accordance with previous reports by Coberley *et al*. [237] and Andersen *et al*. [257] (Figure 15A). In addition and supporting the notion that BRCA1 is a phosphoprotein that changes in a qualitative and quantitative manner during cell cycle

progression [194], we have further corroborated modifications in the BRCA1 phosphorylation pattern in infected cells when compared to uninfected cells. Based on the existing evidence linking BRCA1 activation to transcription and chromatin remodeling [185,205,208-211,307,308], one can speculate that the observed changes could be at the viral integration and transcriptional levels since our cell cycle studies were performed in the presence of a new full-length HIV-1 viral infection. For example, it has been shown that the viral life cycle is integrally related to the cell cycle [231]. Here, Kashanchi *et al*. demonstrated viral transcription activation at two distinct stages of the cell cycle by the viral transactivator protein Tat (Figure 15B). A Tat-dependent LTR activation (requiring TAR and functional promoter Sp1 binding sites) occurs at late G_1 and a second phase of transactivation (TAR independent) occurs at G_2, a process aided by the Vpr-induced G_2/M arrest. Our data shows that BRCA1 expression in infection matches this profile.

To further characterize the role of BRCA1 phosphorylation in Tat transcriptional transactivation, we have shown that inhibition of upstream BRCA1 phosphorylation effectors, chiefly ATR and ATM, results in decreased Tat-dependent transcription of integrated HIV-1 LTR (Figures 16 and 17). Importantly, we have shown that Chk2 inhibition of serine residue S988 did not result in LTR transcriptional downregulation, displaying specificity for ATR and ATM serine substrates S1387, S1423, S1457, and S1524, despite the fact that both ATM and Chk2 phosphorylate BRCA1 in response to DSBs [182]. This observation is in correlation with previous studies showing implications of these kinases in HIV-1 viral replication [260,294-297]; however, none specifically looked at the effects on Tat-dependent LTR transcription.

In order to assess any phosphorylation changes of BRCA1 in subcellular fractions, we have further characterized BRCA1 nucleo-cytoplasmic shuttling in HIV-1 uninfected and chronically infected T cells (Figure 18A), as well as in TZM-bls in the absence or presence of Tat (Figures 18B). We have shown that in the presence of Tat, there is a detectable increase in nuclear levels of p-BRCA1 S1423 (Figure 18B, compare lanes 2 and 4). Given that we observed presence of increased nuclear phospho-BRCA1, we linked ATM phosphorylation of BRCA1 to the HIV-1 promoter. Here, we have shown that upon treatment with the specific ATM*in*, there is a decrease of total and phosphorylated BRCA1 occupancy from the HIV-1 LTR region spanning the -69 to +175 sequence (Figure 18C). Lastly, we have further characterized the relevance of ATM serine target residues and have shown that HIV-1 Tat-dependent transcription is no longer enhanced in cells expressing a BRCA1 construct mutant for S1387A, S1423A, S1457A, and S1524A (Figure 19) when compared to results from cells expressing wild type BRCA1 (Figure 11C). These data reveal the effects of ATM-mediated phosphorylation of BRCA1 in Tat-dependent viral transcription. Additionally, they highlight the viability of therapeutic approaches targeting host cell proteins.

CHAPTER EIGHT
DISCUSSION

The molecular function of BRCA1 has been subject of intensive studies since it was cloned in 1994 [179]. Primarily in cancer, it has been characterized as a multifaceted tumor suppressor protein due to its role in cell cycle progression, DNA repair and DDR processes, transcriptional and RNAi pathway regulation, and apoptosis [182,207]. Limited studies have linked BRCA1 to HIV-1. Initially, Zimmerman *et al*. [256] showed BRCA1-H2AX foci formation was required for Vpr-induced G_2 arrest. Later, this same group further elaborated on the role of BRCA1 in infection by showing Vpr-induced ATR-dependent activation of BRCA1 at serine substrate S1423 as a response to genotoxic stress, suggesting a model for Vpr-induced apoptosis via transcriptional regulation of the BRCA1 target gene, GADD45α [257]. Around the same time, Coberley *et al*. [237] demonstrated alterations of various genes contributing to cell cycle transition at the G_2/M checkpoint through a temporal genetic network study in mock or R5-tropic HIV-1 infected primary macrophages. Specifically, infection activated mediators of cell cycling at different times during infection including PP2A, BRCA1 (intermediate), and GADD45α (late). In the current study, we focused on defining unique mechanisms altered by HIV-1 that may be used for its inhibition by expanding the limited characterization of BRCA1's role in HIV-1 infection.

Here we describe the general expression signature of BRCA1 in HIV-1 infection (Figures 9, 10, and 15). We found that endogenous BRCA1 is overexpressed during infection in a variety of cells including T cells and primary PBMC cells (Figures 9B and 10A-B). We found that though there is no detectable BRCA1 complex redistribution between uninfected and infected cells, BRCA1 presence dramatically increases in a high molecular weight complex exclusive to infected PBMCs (Figures 10B). In previous studies, we have characterized four different pTEF-b containing complexes in HIV-1 infection [141,142]. Interestingly, BRCA1 seems to be present in complex I which has also been shown to contain Cdk9, Cyclin T1, Tat, and Baf170 [10,141,142], suggesting participation in transcriptional and chromatin remodeling pathways. Complementing this hypothesis, a recent study characterizing Cdk9 and Cyclin T1-associated protein complexes (CCAPs) has identified a prominent CCAP containing Cdk9/Cyclin T1 in a very large multiprotein complex regulating transcription initiation and chromatin remodeling [140]. Although the authors did not elaborate on the characterization of this particular CCAP, it is mentioned to include CBP/p300, and the INO80, SWI/SNF and Mediator complexes.

In pursue of this notion, we have demonstrated that BRCA1 functions as an enhancer of HIV-1 Tat-dependent transcription in established cell line models (Figure 11). Our studies indicate that non-integrated HIV-1 LTR transcribes better in cells containing BRCA1 than in BRCA1-null cells (Figure 11A). Similarly, overexpression of wild type BRCA1 in a system containing an integrated HIV-1 LTR enhances the level of Tat transcriptional transactivation (Figure 11C). In support of these data, we found that

BRCA1 selective depletion concordantly decreases Tat-dependent transcription efficiency (Figure 12), further signifying its enhancer role. It is known that the majority of proteins responsible for production of viral RNA exist in multiprotein complexes [140]. Importantly, we found that BRCA1 associates with Tat and Cdk9 (Figure 13), promoting the idea of its participation in a multiprotein complex regulating transcription initiation and possibly, chromatin remodeling. BRCA1 has been shown to synergistically participate with SWI/SNF components in heterochromatin and to co-purify with the ATPase catalytic core unit, BRG1 [205,309,310]. We have shown that Baf170 is present in the BRCA1-containing multimeric high molecular weight complex in infection (Figure 9B), implicating the existence of SWI/SNF complexes within this fraction. For example, previously published data revealed that when selectively depleted, loss of Baf170 significantly inhibited HIV-1 replication in a similar way as depletion of other SWI/SNF subunits did [10,112]. Also, we have observed Baf170 in complex with p-BRCA1 S1423 by immunoprecipitation (data not shown), further supporting a transcription-coupled chromatin remodeling complex model. Moreover, SWI/SNF elements have been previously shown to modulate HIV-1 transcription and replication [10,112,150,311]. Additional mass spectrometry preliminary data suggests that BRCA1 is in association with mRNA splicing and processing machinery (data not shown). Interestingly, recent characterization of the RNAP II holoenzyme has shown that it functions as an assembly platform for transcriptional and pre-mRNA processing machineries [141]. Likewise, Tat has been described as a selective regulator of viral splicing and is linked to splicing factors such as SF-1, which implicate its ability to couple both transcriptional elongation

and RNA splicing [312-314]. The idea of a scaffold complex formation of this magnitude may explain the generous size of the high molecular weight complex observed both by the Rice group [140] and our fractionations (Figures 9B and 10B).

To further investigate the effects of BRCA1 loss on viral transcription, we used curcumin as a therapeutic model for inhibition of BRCA1 expression. Curcumin is a low molecular weight polyphenol component of turmeric (*Curcuma longa*). It is considered a multifunctional compound (MFC) due to its ability to elicit multiple pharmacological actions in treatment of multifactorial diseases [278]. Curcumin is considered generally safe and studies in animals and humans have shown it has antineoplastic and chemosensitizing activity in a tolerable dose [276,277], making it an attractive chemotherapeutic agent for cancer treatment despite conflicting findings in certain cancer subtypes [288]. Of particular interest, when administered as adjuvant therapy to existing cART, curcumin has been shown to enhance the protease inhibitor indinavir antiretroviral activity in persistently infected cells [315]. Here, we have confirmed that curcumin decreases BRCA1 gene expression (Figure 14A) and Tat-dependent transcription (figure 14B) as previously reported [276,283]. To further complement these data, we demonstrate the ability of curcumin to abrogate BRCA1 occupancy at the HIV-1 LTR (Figure 14C). Interestingly, a recent study reported modulation of BRCA1 promoter occupancy by SUMO1 [316] through an interplay between the release of BRCA1 and recruitment of HDAC1 to the promoter. Conversely, SUMO1 knockdown led to recruitment of BRCA1 and release of HDAC1 at the BRCA1 target promoters resulting in transcriptional activation of the BRCA1 target genes. Remarkably, a recent study

shows that curcumin treatment decreases SUMO1 conjugated proteins in astrocytes [317]. Thus, curcumin-induced BRCA1 occupancy loss from the LTR could lead to increased HDAC1 occupancy at the LTR in a similar manner.

The viral life cycle is integrally related to the cell cycle. Previously it has been shown that viral transcriptional activation happens at two distinct stages of the cell cycle [231]. A Tat-dependent LTR activation (requiring TAR and functional promoter Sp1 binding sites) occurs at G_1 and a second phase of transactivation (TAR independent) occurs at G_2, as a process aided by the Vpr-induced G_2/M arrest. BRCA1 is regulated through phosphorylation in a cell cycle-dependent manner by multiple kinases including Cdk2, Cdk4, and Aurora-A [182]. DNA damage phosphorylation events by hCds1/Chk2, ATR, and ATM occur following damage produced by ionizing radiation, UV, replication-induced DNA damage or DNA damaging inducing chemicals [182,242,286,287,318]. As suggested by Andersen *et al*. [257], this residue is phosphorylated by ATR and may be a mechanism that contributes to Vpr-induced apoptosis. To fully revise BRCA1 phosphorylation dynamics, we performed a series of studies assessing S1423 phosphorylation throughout the cell cycle in the presence of infection and small molecule-driven inhibitory studies of upstream phosphorylation effectors. In accordance, we found that p-BRCA1 S1423 is markedly increased at the S and G_2/M phases of the cell cycle in response to a single cycle infection when compared to mock-infected cells (Figure 15), an event possibly in synergy with Vpr G_2/M cell cycle arrest [256,257]. BRCA1 contains a serine cluster domain (SCD) spanning aa1280-1524, a common motif present in ATR/ATM protein targets [290]. ATR phosphorylates BRCA1 on serine 1423

in response to UV damage or HU-induced replication arrest [242], whereas ATM-mediated phosphorylation events at serines 1387, 1423, 1457 and 1524, have been characterized in response to ionizing radiation-induced damage [286,287]. While the Chk2 phospho-site is not located within the SCD region, phosphorylation of serine 988 occurs in response to the same stressors as with ATM [291]. We have shown for the first time that inhibition of upstream BRCA1 phosphorylation effectors ATR/ATM by caffeine and ATM*in*, results in decreased Tat-dependent transcription of integrated HIV-1 LTR (Figures 16 and 17). Significantly, we have shown that Chk2 inhibition of serine residue S988 did not result in LTR transcriptional downregulation, exhibiting specificity for ATR and ATM serine substrates S1387, S1423, S1457, and S1524, even though both ATM and Chk2 phosphorylate BRCA1 in response to DSBs [182]. This observation is in correlation with various studies implicating these kinases in HIV-1 viral replication [260,294-297]. For example, caffeine and caffeine-related methylxanthines, including FDA-approved theophylline, have been used to inhibit HIV-1 integration in primary cells [294]. Similarly, HIV-1 IN has been shown to stimulate an ATM-dependent DDR and that in the absence of this enzyme, cells are sensitized to retroviral-induced death [296]. Thus, treatment with ATM*in* KU55933 suppressed viral replication, not only of wild type, but drug-resistant HIV-1. Of interest for this study, caffeine has been found to prevent pTEF-b dissociation from its 7SK snRNP inactive complex, illustrating a possible mode of action in its inhibition of Tat-dependent transcription by enabling sequestration of active nuclear pTEF-b [319]. Collectively, these findings strengthen the

case of using small molecule inhibitors against non-essential host cell proteins that are required for HIV-1 infection.

In order to assess any phosphorylation changes of BRCA1 in subcellular fractions, we have further characterized BRCA1 nucleo-cytoplasmic shuttling in HIV-1 uninfected and chronically infected T cells, as well as in TZM-bls in the absence or presence of Tat (Figure 18A-B). We have shown that in the presence of Tat, there is a detectable increase in nuclear levels of p-BRCA1 S1423 (Figure 18B). Given the presence of increased nuclear phospho-BRCA1, we have linked ATM phosphorylation of BRCA1 to the HIV-1 promoter. We have shown that upon treatment with the specific ATM*in*, there is a decrease of total and phosphorylated BRCA1 occupancy from the HIV-1 LTR region spanning the -69 to +175 sequence (Figure 18C). These results are interesting because in this cell system, there is no viral integration events occurring or Vpr expression, thus ATM phosphorylation of BRCA1 occurs in response to other stimuli. Possible explanations include the generation of reactive oxygen species (ROS) by Tat that could trigger DSBs (DSBs). Tat has been shown to induce ROS production in human neuroblastoma and astroglioma cells as a model to explain brain tissue chronic oxidative stress present in HIV-1 infected patients suffering from HAND [320,321]. Moreover, it has been demonstrated that oxidative stress-induced DSBs arise in hetero- and euchromatic DNA and that ATM is required for their repair [322].

Lastly, we have further characterized the relevance of ATM serine target residues and have shown that overexpression of mutant BRCA1 in a system containing an

integrated HIV-1 LTR does not enhance the level of Tat transcriptional transactivation like overexpression of wild type BRCA1 does (compare Figures 19 and 11C).

BRCA1 phosphorylation in HIV-1 has primarily been described in function of ATR as the effector kinase and as DDR event [257]. These data reveal the effects of ATR/ATM-mediated phosphorylation of BRCA1 in Tat-dependent HIV-1 viral transcription. Additionally, they highlight the viability of therapeutic approaches targeting host cell proteins.

CHAPTER NINE
CONCLUSIONS, SIGNIFICANCE, AND FUTURE RECOMMENDATIONS

Work in this dissertation focuses on understanding the involvement of BRCA1 in HIV-1 Tat-dependent transcription. Collectively, conclusions from the current study are as follows:

AIM I

1. With the use of cell line models for chronic infection and infection of primary cells, BRCA1 was shown to be upregulated.

2. BRCA1 is modified in infection through phosphorylation by ATR/ATM.

3. BRCA1 size exclusion chromatography revealed that it exists as part of a high macromolecular complex in infected cells.

3. BRCA1 is differentially expressed and phosphorylated at S and G_2/M of the cell cycle between uninfected and infected cells.

AIM II

1. Amongst other unknown components, the high molecular weight fraction contains BRCA1, Cyclin T1, Cdk9, Baf170, and Tat.

2. BRCA1 was found to be in complex with Tat and Cdk9.

3. Preliminary data suggests that BRCA1 interacts with Baf170 and proteins involved in mRNA splicing and processing.

AIM III

1. HIV-1 transcription is more effective in cells containing BRCA1.
2. BRCA1 acts as an enhancer of Tat-dependent transcription.
3. BRCA1 inhibition by curcumin results in decreased BRCA1 occupancy at the HIV-1 LTR.
4. Inhibition of phosphorylation effectors decrease Tat-dependent transcription and LTR occupancy by BRCA1.

As thoroughly discussed in this manuscript, BRCA1 dysregulation results in loss of genome stability that may result in cellular apoptosis or tumorigenesis. Because of its differential expression across tissues and in disease, understanding its modulation by HIV-1 infection may shed light on HIV-1 associated patient pathologies such as T cell and other cancers, cellular oxidative stress, and HAND. A more direct way to achieve a global understanding of its transcriptional cofactor functions would be to perform ChIP-chip analysis to fully assess changes in its activation of BRCA1-responsive genes, and explore other BRCA1-regulated pathways that could serve as novel chemotherapy targets. Additionally and as an example of its expression modulation, the use of curcumin as adjuvant for chemotherapy in oncology, and potentially cART, further demands the question of its impact on BRCA1 expression in the patient and what impacts or to what extent will BRCA1 expression be decreased *in vivo* as a side-effect of curcumin treatment [323]. Moreover, it has been suggested that Tat-induced DNA repair deficiencies may play a significant role in the development of AIDS-associated cancer, thus, dysregulation of BRCA1 and the DDR pathway can be conducive to carcinogenesis [169].

This high molecular weight BRCA1-containing complex seems to comprise multiple factors that are not only involved with transcription, but with chromatin remodeling, and possibly RNA splicing and processing. Given that BRCA1 is known to participate in chromatin remodeling, transcriptional regulation, microRNA processing, and others, the amplitude for future studies is vast. As an added example, the regulatory interaction with Drosha could have various implications in the generation of viral miRNAs such as TAR [14,207,324]. One can wonder whether increased activation of BRCA1 in infection would affect miRNA processing in favor of infection, given that changes in miRNA signatures have been reported in primary human cells from seropositive individuals [325].

Most important is the possibility that BRCA1 is functioning as a protein scaffold or mediating factor recruitment to the viral promoter through protein-protein interactions. As mentioned in subsection 4.2, BRCA1-interacting proteins include transcription factors Sp1, HDACs1/2, and HATs CBP/p300 [182,206,212,213]. Given that we have observed transcriptional enhancement of Tat-dependent transcription by BRCA1, it would be of great interest to further examine if this enhancement is occurring by recruitment of Sp1 to its respective binding sites in the U3 region of the 5' LTR. Similarly, it would be of interest to study whether there are any specific dynamics happening at the chromatin level through the recruitment of HATs, specifically CBP/p300 that would exert histone acetylation that results in a more permissive chromatin environment for transcription to occur as discussed in subsection 3.5.1. Such questions can be initially addressed by performing ChIP assays to determine enrichment

of Sp1 at the viral promoter or CBP/p300 at nucleosome-containing regions of the LTR in the absence or presence of BRCA1. Correspondingly, generation of LTR mutant reporter constructs for Sp1 binding sites could also reveal whether part of the observed BRCA1 enhancement is due to transcription factor recruitment. It is important to note that the Sp1 association region spans the area where Tat-BRCA1 association was detected, at aa260-802, including as well part of the region where BRG1-BRCA1 association was detected at aa260-553 [244]. Alternative routes of examining BRCA1 as a novel Tat-binding protein could include functional studies such as the generation of Δaa504-802 mutant BRCA1 constructs to further inspect the relevance of the detected association in this region and how it would interfere with the other known interactions of with BRG1 and Sp1 for example.

Though currently unlinked, we have recently reported that GSK-3β is upregulated in HIV-1 infection and that its inhibition by specific small molecule compounds provides cytoprotection from Tat-induced cell death [143,163]. Members of the lymphoid enhancer factor-1/T-cell factor (LEF-1/TCF) family of transcription factors interact with β-catenin and play a role as nuclear effectors of Wnt signaling, which includes gene targets that impact cell differentiation, communication, apoptosis and survival, and proliferation [326,327]. A possibility of increased cytoplasmic GSK-3β in the presence of activated HIV-1 involves the β-catenin signaling pathway, which has been shown to function as an intrinsic molecular pathway restricting HIV-1 replication in PBMCs [326]. GSK-3β forms part of a repressive multiprotein cytoplasmic complex, in addition to Adenomatous polyposis coli (APC) and Axin

proteins, which target β-catenin for degradation. The TCF-4/β-catenin interaction is linked to HIV-1 replication inhibition in multiple cell types, including astrocytes and lymphocytes. Recently, TCF-4 and β-catenin have been shown to have multiple binding sites relative to the transcriptional initiation site at the 5′ HIV-1 LTR [328]. In particular, TCF-4 and β-catenin at position −143 associate with the nuclear matrix binding protein SMAR1, which may tether the HIV-1 DNA segment into the nuclear matrix and away from transcriptional machinery, resulting in repression of basal HIV-1 LTR transcription. Thus, increased cytoplasmic GSK-3β may be acting as a transcriptional activating complex by repressing the β-catenin HIV-1 restrictive signaling pathway. Interestingly, these findings can be linked back to BRCA1 in two ways. First, cell treatment with the same parental compound used to inhibit GSK-3β in our studies, *6-Bromoindirubin-3′-oxime*, has been shown to repress BRCA1 expression more potently than curcumin and to markedly increase β-catenin expression [329]. Moreover, BRCA1 has been recently shown to regulate the expression of the nuclear form of β-catenin [330]. Thus, the regulation of BRCA1 expression by the Wnt/GSK-3β pathway in HIV-1 infection could be studied to further refine the role of GSK-3β upregulation in infection. Secondly, GSK-3β is known to regulate Drosha nuclear localization through S300 and S302 phosphorylation [331], hence it could be possible that its inhibition could lead to both cytoplasmic retention of Drosha and BRCA1 repression, resulting in miRNA pathway impairment that could upset infection regulatory mechanisms. GSK-3β inhibition and its conferring of cytoprotection has big implications as a possible cART adjuvant therapy for HIV-1 infected patients that suffer from HAND, since other FDA-

approved GSK-3β inhibitors have been used extensively in the treatment of neurocognitive dysfunctions [332]. Few studies have provided any associations between BRCA1 and neurocognitive deficiencies and so, BRCA1 has yet to be linked to cognitive impairments.

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BIOGRAPHY

Irene Güendel Sánchez, raised in San José, Costa Rica, graduated in 2000 from Colegio de La Salle. Irene moved to New Zealand in 2001 as an exchange student through the American Field Service Intercultural Program, and attended Saint Oran's College in Lower Hutt. She seized this great opportunity and remained in New Zealand, where she majored in Cell and Molecular Bioscience at Victoria University of Wellington, culminating her undergraduate studies in 2006. In 2007 she interned at the International Atomic Energy Agency in Austria, where she participated in various projects pertaining genomics for disease resistance in ruminants. As an Organization of American States scholarship grantee, she completed a Master of Science in Genomics and Bioinformatics at The George Washington University, Washington D.C., in 2009. Irene assisted in teaching the undergraduate General Genetics laboratory component for the majority of her Ph.D., and finalized her doctoral degree in Biosciences, with an emphasis in Functional Genomics and Biotechnology, at George Mason University in 2014.

As a J-1 scholar, she will be providing educational services to Costa Rica for an initial period of two years. She is currently collaborating with the Costa Rican National Academy of Sciences on a pilot project that will deliver online science seminars to rural technical high schools to promote interest in scientific vocations in the new generations. Additionally, she will be delivering online courses in virology, epigenetics, and biosciences to Costa Rican state universities.