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# HIV Vpr Engages the Host DNA Damage Response and Chromatin-Associated Proteome

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Biology

by

Andrew Lopez

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ABSTRACT OF THE DISSERTATION

HIV Vpr Engages the Host DDR and Chromatin-Associated Proteome

by

Andrew Lopez

Doctor of Philosophy in Molecular Biology

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Professor Oliver I. Fregoso

Engagement of the DDR by viruses in a broadly conserved mechanism among viruses and understanding how viruses modulate the DDR has paved the way to understanding several basic biological processes in molecular biology. Despite this, the role of engaging and modulating the DDR in the context of HIV remains poorly understood. For many years it has been apparent that the viral accessory gene Vpr plays a role in engaging the DDR during the viral lifecycle, however, the molecular processes and phenotypes associated with modulation remain poorly understood. For my dissertation research, we examine the function of HIV Vpr in the context of the DDR. First, we explore how Vpr engages and modulates the DDR by determining distinct cellular phenotypes specific to Vpr function to generate a model. We next explore the chromatin-associated proteome of Vpr to understand the underlying molecular mechanisms governing modulation of the DDR. Altogether, this will broaden our understanding HIV molecular biology in the context of the DDR and at chromatin.

The dissertation of Andrew Lopez is approved.

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2021

# DEDICATION

This dissertation is dedicated to my grandmother who taught me to never give up and to always keep learning and growing as a person. Although she isn't here to see me achieve this, this dissertation is a testament to everything she taught me.

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This entire dissertation is the combined effort of all the people that have been in my life, and I want to say thank you to all you. I wouldn't have achieved this without you all.

VITA

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## PEER-REVIEWED PUBLICATIONS

**A. Lopez***, R. Nichols Doyle*, C. Sandoval*, K. Nisson, V. Yang, O. I Fregoso. Viral Modulation of the DNA Damage Response and Innate Immunity: Two Sides of the Same Coin, Journal of Molecular Biology (2021). (* indicates equal contribution)

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# Chapter 1: Introduction

Viruses are obligate parasites that can only persist by infecting a cell and usurping cellular machinery to replicate and disseminate. They are both the most simple and complex parts of the tree of life. There is such an immense diversity of viruses, that they can't be classified through the canonical means that everything else in the tree of life is. There are both DNA as well as RNA viruses and are categorized by a combination of what sense the genetic material is and how many strands they possess. They are ubiquitous and infect almost all living organisms. In turn, viruses have driven much of the evolution of living organisms.

Specifically, lentiviruses have been co-evolving alongside living organisms such as felines, cattle, and primates for millions of years. This evolution has been shaping their evolution and can be traced back to ERVs which comprise a significant amount of the human genome and have brought about new genes in humans such as Syncytin-1. Here specifically we focus on primate lentiviruses, which infect a range of organisms including humans. These primate lentiviruses were originally distributed amongst hundreds of Old-World monkeys in the form of various Simian Immunodeficiency Viruses (SIV) across Africa before eventually crossing the species barrier into humans. From this, Human Immunodeficiency Virus (HIV) arose and has been co-evolving among humans for at least the past hundred years. Throughout this time, several molecular aspects of the virus have been characterized and we understand that accessory genes are critical for progression of the viral lifecycle. In addition to this, they are also drivers of evolution as they are in a constant arms race with antiviral restriction factors.

*Transmission & Evolution*

Primate lentiviruses have been evolving alongside their hosts for millions of years. Before arising in primates, lentiviruses can be traced back to infecting several mammals such as cattle,

felines, and horses. This diversity and long history of viruses traces back to millions of years and represents the vast and complicated evolution of lentiviruses. Throughout the evolution of these viruses, there are clear host barriers that the virus has overcome. By overcoming these host antiviral barriers, the lentiviruses have been able to not only evolve, but also jump into other organisms. To overcome these host barriers, viruses have evolved to counteract specific host antiviral genes that would otherwise prevent or seriously hinder their replication and/or transmission.

Before jumping into primates, it is suspected that lentiviruses jumped from lemurs into primates. The breadth of primates that are infected with lentiviruses is expansive and includes several Old-World monkeys that comprise primates such as African green monkeys, chimpanzees, and gorillas. In these primates, the lentivirus Simian Immunodeficiency Virus (SIV) arose and has spread and become endemic in large populations of these primates. This has resulted in a very diverse amount of SIV across the continent and resulted in viruses that represent distinct transmission events but are also very similar in their architecture and biology. This balance between conservation and adaptation has allowed for lentiviruses to evolve and persist. More importantly, it has also allowed for viruses to transmit, adapt, and result in the manifestation of new viruses such as Human Immunodeficiency Virus (HIV).

*Human Immunodeficiency Virus*

The virus that is responsible for acquired immunodeficiency syndrome (AIDS) was officially discovered in 1983 and was originally named HTLV-III/LAV (human T-cell lymphotropic virus-type III/lymphadenopathy-associated virus) and was later renamed as Human Immunodeficiency Virus (HIV). Today HIV remains pandemic and affects millions of people every year. Despite several advances in medicine and science, HIV remains incurable and can only be suppressed by drugs.

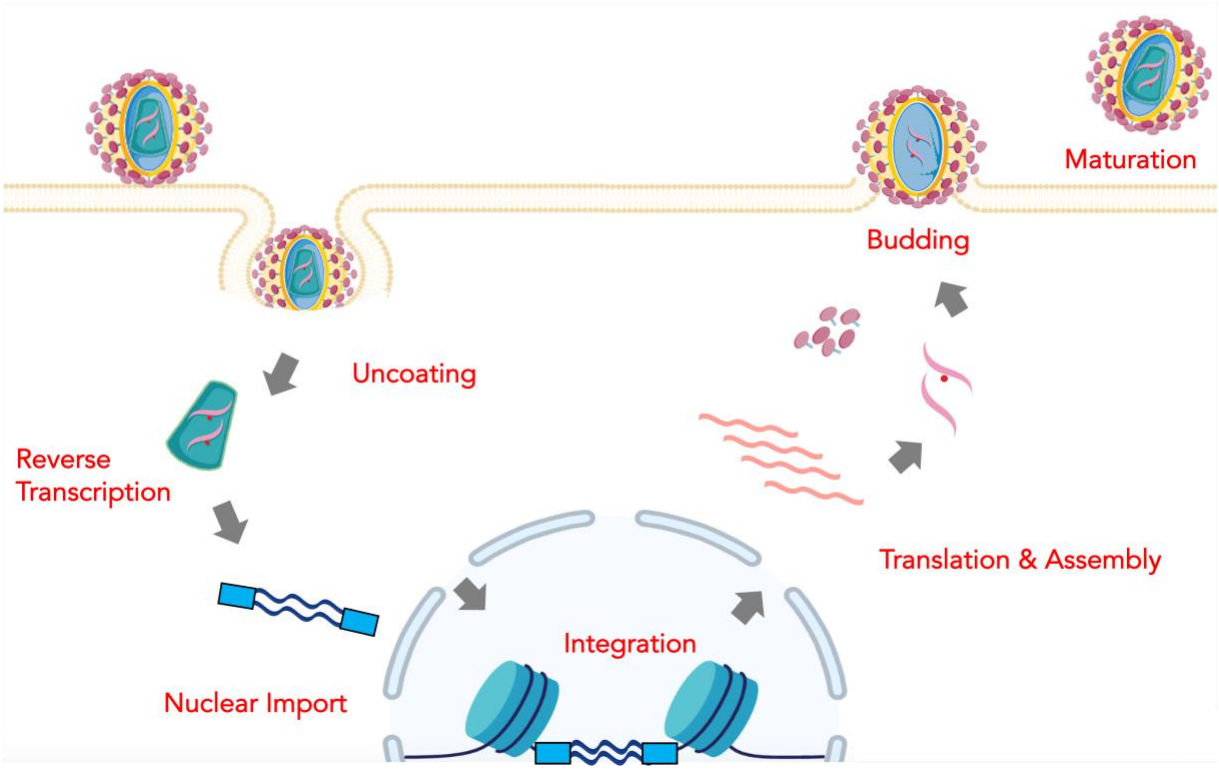
Throughout its complex evolution, SIV has crossed over from primates into humans on multiple occasions and has resulted in two distinct types of HIV: HIV-1 and HIV-2. Specifically, HIV-1 is believed to have emerged from cross-species transmission events from SIVs infecting chimpanzees (SIVcpz), whereas HIV-2 emerged from SIV infecting the sooty mangabey monkey (SIVsmm). Both HIV types have several groups that are considered both geographically and molecularly distinct from one another. Whereas HIV-1 tends to be more pathogenic and is responsible for a pandemic infection of humans, HIV-2 is generally less pathogenic and remains endemic to more restricted geographic regions and populations. In addition, there is a lot of sequence diversity within and between HIV which has resulted in a remarkable degree of host adaptation and immune evasion and has largely contributed to its success.

Viral Lifecycle

The goal of all viruses is to infect a host cell that will allow it to replicate and ultimately infect another host cell. This is known as the viral lifecycle and results in propagation as well evolution of a virus when successful. Most viruses utilize a specific receptor to facilitate cell entry which congruently determines their host range and governs their ability to stay within a host as well as undergo zoonotic transmission. As you can imagine, successfully infecting a cell and getting through the viral lifecycle is critical for a virus that has no other means to replicate and evolve outside of a host. As such, there are many steps that the virus has established to promote the viral lifecycle and specific viral proteins that safeguard these various steps. In general, a virus must undergo the following steps: 1) **entry**, 2) **uncoating**, 3) **replication**, 4) **assembly**, and 4) **release**. By accomplishing these steps, a virus can efficiently replicate and continue to endure.

Although the lentiviral lifecycle is unique in several ways, there it also utilizes steps that are conserved across several viruses. In short, the lentiviral lifecycle begins when the viral receptor contacts the host. From here, the virus binds and fuses with the cell membrane where it

then undergoes receptor mediated endocytosis and enters the cell. This allows the virus to undergo uncoating as well as reverse transcription, which results in a cDNA intermediate that associates with the viral integrase and primes it for the next major step of the viral lifecycle. Once in complex with the integration complex, the virus is then able to integrate into the host genome. After the viral genome is integrated into the host genome, it is then permanently a part of the host and utilizes the host's cellular machinery to replicate via several viral transcripts. These viral mRNAs are then used to produce several viral proteins that are ultimately used to promote the viral lifecycle as well as viral assembly. From here, viral proteins are trafficked to the cellular membrane where new viral particles begin to assemble. After enough proteins accumulate and assemble, viral particles begin to bud from the membrane and mature into infectious HIV particles that then go on to repeat the viral lifecycle.




*Entry*

The process of binding is unique for every virus because it is the result of hundreds if not millions of years of viral evolution. Every virus has evolved to recognize a very specific receptor

that it has evolved to efficiently recognize through numerous rounds of selection. This ultimately results in a relationship between the virus and a very specific cellular target that determines its host range and is a direct result of a virus's specific evolutionary route. Specifically, HIV's host range determinant and primary receptor is the CD4 chemokine receptor. In addition to this, HIV also utilizes the CXCR4 and CCR5 co-receptors to facilitate entry and fusion. HIV is able to interact with and bind to the CD4 receptor through its envelope (Env), which is composed of Gp120 and Gp 41 and manifests itself as a trimer on the viral membrane surface that is decorated by various sites of glycosylation. This trimer is particularly important for the virus because it allows the virus to undergo a specific mechanism of endocytosis mediated cell entry. During this process, HIV Env binds to CD4 along with either CXCR4 or CCR5 and results in several conformational changes that facilitate uptake of the virus by the cell. Once binding of the receptor occurs, the virus is then taken up by the cell for endocytosis. This mechanism is conserved across several viruses and leads to an internalized viral capsid. For the viral capsid to properly enter and infect the cell, the capsid must enter the cytosol environment. To accomplish this, HIV membrane fuses with the cellular host membrane. This process leads to the contents of the virus to be expelled into the cytosol where the next step of the viral lifecycle, uncoating, can occur.

*Uncoating*

A conserved theme across all viruses is that they all function to turn their incoming genetic material into mRNA into order to produce proteins essential for driving the viral lifecycle. This generally involves releasing genetic material into the cytoplasm and then altering their RNA or DNA genome into mRNA. The viral lifecycle of Retroviruses, including HIV, is particularly unique compared to the viral lifecycles of any other type of virus. Whereas other viruses generally only utilize RNA or DNA throughout the viral lifecycle, HIV employs both RNA and DNA intermediates. The HIV genome enters the cell as positive-sense RNA and undergoes reverse transcription (RT),

turning the genome into cDNA which eventually is imported into the nucleus and is integrated into the host genome. This process is unique to retroviruses and allows the virus to permanently become a part of the cell. Despite the linearity of the route, this process is quite dynamic and not completely understood. We know that HIV capsid uncoating and RT are coupled, however, the extent to which these processes are linked to other key processes such as nuclear import and integration remains unclear.

What is known is that upon fusing with the cellular membrane and expelling viral contents into the cytoplasm, HIV begins the process of uncoating and RT and eventually makes its way into the nucleus and integrates into the host genome. For this process to be successful, RT is critical for the viral genome as is one of the major processes that occurs throughout the course of uncoating. The initiation of RT is dependent on the availability of a cellular tRNA primer, Lys 3 and can be summarized in 9 major steps: 1) tRNA priming, 2) Synthesis of 3' LTR complementary DNA, 3) 5' LTR degradation, 4) tRNA-cDNA primer jumping 5) minus-strand cDNA synthesis, 6) RNase H plus-strand RNA degradation, 7) plus-strand DNA synthesis, 8) tRNA jump and cDNA hybridization, and 9) complete synthesis of cDNA genome. The tRNA allows for the RT enzyme to initiate transcription at the at the PBS near the 5' end to create a short minus-strand DNA template that can be used at the 3' RNA end to create a complete minus-strand. The complete minus-strand DNA can then be used to create a plus-strand by degrading the previously utilized RNA genome template through RT's RNase H activity. This ultimately results in a double-stranded DNA genome that can then be utilized by the pre-integration complex (PIC) to be integrated into the host genome.

The process of integration is the final major component of viral uncoating. Once RT has completed, a double-stranded DNA genome has been generated, and the PIC has formed and the viral genome is then primed to integrate into the host genome. Although it remains unclear whether the viral capsid remains intact upon nuclear import or whether the process RT continues

in the nucleus, it is well understood how integration occurs and what factors are involved. The PIC is composed of two integrase (IN) dimers bound to the LTRs of the newly synthesized HIV DNA genome which results in an intasome complex. The intasome complex utilizes IN tetramer to cleave the ends of the bound DNA genome and create a final complex consisting of cleaved LTR ends that are primed for a nucleophilic attack resulting in integration. Once nucleophilic attack occurs through the primed intasome complex, a strand transfer complex forms which then allows for repair at the site of integration and, if successful, results in a proviral genome. Although the exact mechanism behind site of integration remains unclear, it is known that several host factors such as LEDGF are critical for this process and allow for integration to occur at preferred positions which are generally accessible sites that are highly transcribed. Furthermore, integration is not always successful. In such cases, the DNA bound to the intasome reacts with itself and results in 2-LTR circles which, for the most part, are indicative of a dead end for the viral cycle.

The process of viral uncoating is complex and essential for the viral lifecycle of HIV. Although there are several steps at which the viral lifecycle can be impeded or ended, once it integrates into the genome and becomes proviral it is permanently established as a part of the cell. This is significant because this allows for the next part of the viral lifecycle, replication, to continue almost indefinitely.

*Replication*

Like other viruses, HIV utilizes a plethora of host machinery to replicate effectively. Once the virus has integrated into the host genome, it is able to usurp several host transcription factors primarily through its flanking LTR regions and viral proteins Tat and Rev. In order to lead into the other parts of the viral lifecycle, HIV must first transcribe and translate proteins involved in various other processes of the viral lifecycle. To do so, it utilizes a combination of viral proteins as well as RNA secondary structures to kick start these processes. The viral LTR itself has several functions and acts as an enhancer, promoter, transcription initiator and terminator. It’s able to accomplish

this by encoding sites for binding of transcription factors such as NF-$\kappa$B and SP1 which promote basal viral transcription. In addition to this, the viral genome also encodes its own protein, tat, which is expressed early in viral lifecycle to act as a viral transcription factor by binding to the TAR element found within the LTR. It does so by recruiting P-TEFb after binding to the TAR element, which promotes transcriptional elongation via RNAPII. This leads to the production of several different RNA splicing variants that encode a variety of different viral proteins and are critical to the viral lifecycle.

Because these transcripts are initially unprocessed, they must be exported from the nucleus. In general, the virus must find a method to produce essential proteins for the other parts of the viral lifecycle. To accomplish this, the virus evolved the viral protein Rev to help it circumvent the export restriction placed by the cell on unprocessed mRNAs. Rev accomplishes this by usurping the cell's nuclear import and export system via importinB and Crm1/RanGTP respectively. Doing so allows the virus to translate unprocessed viral RNAs and produce proteins that are vital for the later steps of the viral lifecycle. It accomplishes this by utilizing the RRE sequence, which binds Rev to unprocessed viral RNA and allows it to be trafficked in and out of the nucleus. Together, the LTR, Tat, and Rev represents a robust system for the virus to not only ensure that the viral lifecycle proceeds effectually, but also bypass and usurp a preexisting cellular system early in the viral lifecycle. By accomplishing this, HIV is then able to produce other proteins essential for steps further along in the viral lifecycle. This eventually allows the virus to assemble new viral particles to be released to infect another host cell.

*Assembly & Release*

The final major step of viral replication is assembly and release. After transcription and translation have successfully occurred, viral structural proteins are then able to begin assembling into a virion. Gag and GagPol multimerize and bind to the cellular membrane where they can begin the process of RNA packaging. At the same time, Env is trafficked from the rough ER to

the cellular membrane via the Golgi secretory pathway. This allows Env to incorporate into multimerized Gag and assemble into an immature virion on the cellular membrane. Once enough viral Env and Gag have assembled, the ESCRT complex is recruited. This facilitates viral budding and allows the immature virion to release from the cellular membrane. Once this has occurred, viral maturation through proteolytic cleavage occurs via GagPol. This results in fully processed viral proteins and a capsid core safeguarding viral RNA. From here, the virus is matured and is able to infect another cell through the same steps and processes.

*Accessory Genes*

The success of HIV is largely due to the unique accessory genes the virus has evolved over time to counteract host antiviral factors that would otherwise impede viral replication. Four of these proteins are shared across all HIV and one is unique to HIV-2. Together, these genes define HIV from all other retroviruses. More specifically, all HIV can encode for the accessory genes Vif, Vpu, Vpr, and Nef, whereas Vpx, which is a paralog of Vpr, is unique to HIV-2. Collectively, these proteins can bypass several antiviral barriers at various steps of the viral lifecycle (Fig). In doing so, several barriers for replication are alleviated and the virus can effectively replicate. They primarily accomplish this by hijacking a variety of ubiquitin ligase complexes to degrade specific host restriction factors. The host targets and functions for accessory genes have been extensively characterized for Vpu, Vif, and Vpx and effectively degrade Tetherin, ABOBEC3G, and SAMHD1 respectively. Despite this, however, the primary role and target for Vpr remains poorly understood relative to the other accessory genes discussed here.

*Viral Protein R*

Despite many years of research, the primary role and function of Vpr in the viral lifecycle remains elusive. Vpr has been described to play a role in almost every part of the viral lifecycle, from entry to assembly and packaging and has made understanding the primary role particularly difficult (Fig). Despite this, Vpr has a very robust G2/M arrest cell cycle phenotype that is conserved across HIV as well as closely related SIV which suggests that this is an important component of the primary role of Vpr. Like the other accessory genes, this robust phenotype seems to be dependent on hijacking of an E3 ubiquitin ligase complex. More specifically, Vpr recruits the CRL4-DDB1 through engagement with DCAF1. Through this interaction, many host proteins have been characterized as being potential targets for Vpr-mediated degradation. These include, but are not limited to UNG2, HLTF, SLX4, and EXO1. However, although the target of Vpr remains elusive we can appreciate that all these previously characterized potential targets are all associated with cellular roles involved in DNA repair and damage. This leads us to believe that the primary role of Vpr is nuclear and is further evidenced by Vpr localization in the nucleus as well as its capacity to cause replication stress. Altogether, this suggests that the primary role of Vpr is likely tied to nuclear functions involved in DNA damage and repair.

Significance & Dissertation Overview

For many years, the HIV field has utilized many techniques and assays to decipher the primary role of Vpr in viral replication. Although the field has produced many potential targets and roles for Vpr, the field remains muddled with inconsistencies and disagreements. Because the role of Vpr seems to be heavily tied to chromatin-associated functions pertaining to DNA repair and damage, it is imperative that we fully understand the primary function given that the virus integrates into the host genome and relies heavily on host cellular functions tied to DNA repair, damage, and chromatin dynamics. Although we have a thorough understanding of what most

accessory genes do, the primary function and role of Viral Protein Regulatory (Vpr) remains elusive. For my dissertation, I focus on the process of understanding the primary role of Vpr in the viral lifecycle through molecular and evolutionary biology.

In **Chapter 2**, I focus on discussing the importance of viral engagement of the DDR across several diverse viruses. The DDR consists of multiple pathways that sense, signal, and respond to anomalous DNA. To promote efficient replication, viruses have evolved to engage and even modulate the DDR. In this chapter, we will discuss a select set of diverse viruses and the range of mechanisms they evolved to interact with the DDR and some of the subsequent cellular consequences. There is a dichotomy in that the DDR can be both beneficial for viruses yet antiviral. We will also highlight some of the connections between the DDR and innate immunity. Previously believed to be disparate cellular functions, more recent research is emerging that links these processes. By doing so, we aim to establish a convincing foundational understanding of how broadly mechanisms and phenotypes related to engaging the DDR are conserved among all viruses. This will also highlight the crucial importance it plays for human health since understanding how viruses manipulate the DDR presents an important and tractable target for antiviral therapies. A version of this work has been published in *Journal of Molecular Biology* as:

A. Lopez*, R. Nichols Doyle*, C. Sandoval*, K. Nisson, V. Yang, O. I Fregoso. Viral Modulation of the DNA Damage Response and Innate Immunity: Two Sides of the Same Coin, Journal of Molecular Biology (2021). (* indicates equal contribution)

In **Chapter 3**, we establish a two-part model for how HIV Vpr engages the DNA damage response (DDR) which is a signaling cascade that is vital to ensuring the fidelity of the host genome in the presence of genotoxic stress. Growing evidence has emphasized the importance of both activation and repression of the host DDR by diverse DNA and RNA viruses. Previous

work has shown that HIV-1 is also capable of engaging the host DDR, primarily through the accessory protein Vpr. However, the extent of this engagement has remained unclear. Here we show that HIV-1 and HIV-2 Vpr directly induce DNA damage and stall DNA replication, leading to the activation of several markers of double- and single-strand DNA breaks. Despite causing damage and activating the DDR, we found that both HIV-1 and HIV-2 Vpr repress the repair of double-strand breaks (DSB) by inhibiting homologous recombination (HR) repair. Mutational analyses of Vpr revealed that damage of DNA and activation of the DDR are independent from repression of HR, showing that Vpr globally modulates the host DDR at multiple independent steps. Finally, our data indicate that repression of HR repair does not require Vpr-mediated cell-cycle arrest, but instead suggest that repression of HR repair may precede this long-standing enigmatic Vpr phenotype. Together, our data offers novel insight into the function of Vpr and the roles of the DNA damage response in lentiviral replication. A version of this work has been published in *mBio* as:

**Lopez A***, Li D*, Sandoval C, Nichols Doyle R, Fregoso OI. 2020. HIV Vpr modulates the host DNA damage response at two independent steps to damage DNA and repress double-strand DNA break repair. mBio 11:e00940-20. (* indicates equal contribution)

In Chapter 4, we investigate how HIV Vpr engages the DDR to induce cellular consequences such as cell cycle arrest, DNA damage, and repression of DNA repair. Because our current understanding of HIV Vpr interactions is unable to explain our two-part model, we further explored the proteome of Vpr. Previous Vpr proteomic studies have relied heavily on IP-MS and have been unable to comprehensively assess protein interactions that occur at chromatin, which we think are critical for the primary role of Vpr. By utilizing proximity labeling to fully explore the chromatin-associated proteome, we determined that Vpr heavily engages chromatin-

associated proteins involved in chromatin assembly and disassembly such as histones. We also discovered that the nuclear proteasome is highly enriched, suggesting that the previously characterized degradation mechanisms of Vpr could restructure the chromatin-associated proteome. Finally, this provides new insight to Vpr function and highlights the importance of understanding the remaining cellular consequences of engaging the DDR: transcription and chromatin dynamics.

Finally, in **Chapter 5** I will summarize and synthesize these chapters to discuss the importance of engagement of the DDR by HIV Vpr and expand upon how this body of work could be extended and potentially lead to novel antiviral therapies and benefit curing HIV infection.

# Chapter 2: Viral Engagement of the DDR is Broadly Conserved Across Viruses

## 2.1 Introduction

To maintain genomic integrity, cells possess various mechanisms to repair, protect, and replicate genetic material. At the heart of this is the DNA damage response (DDR), a signaling cascade that functions to sense, signal, and respond to aberrant nucleic acid. However, in addition to maintaining genomic integrity, the DDR is exquisitely poised to regulate viral infection, since to the cell, viruses are essentially aberrant nucleic acids. In support of this, the connection between viral replication and the DDR has emerged in two primary roles: (1) viruses modulate DDR proteins and pathways required for viral replication; (2) there is significant crosstalk between the DDR and the innate immune response against viruses. These connections have been observed extensively across viral classifications and are relevant to a variety of both DNA and RNA viruses, including single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) viruses, positive (+) and negative (-) stranded RNA viruses, and retroviruses (a (+) RNA virus that relies on a dsDNA intermediate, which we separately classify here according to the Baltimore Classification [1].

Here, we will break down the interaction of viruses with the DDR into four primary sections (**Figure 1**). First, we will discuss how viruses induce DNA damage and antagonize the sensing of this DNA damage. Second, we will discuss how viruses modulate the DDR signaling cascade – from mediators, to transducers, to effectors – with a focus on specific DNA, RNA, and retroviruses. Third, we will highlight the many cellular consequences of viral-induced modulation of the DDR. However, this will not be able to cite all the work that has gone into understanding the connections between viral replication and the DDR (for additional specialized topic reviews, see [2, 3] and others highlighted throughout the text). Additionally, some of the data we cite is limited and has yet to be corroborated. We understand the limitations this brings, yet we have included the work

to demonstrate that examples exist across diverse viruses to substantiate the larger themes and concepts we discuss. We aim for this chapter to establish a comprehensive understanding of the important of the DDR in the numerous fields of virology.

## 2.2 Overview of the DNA Damage Response (DDR)

The first step in activation of the DDR is induction of DNA damage. DNA damage occurs through endogenous pathways, such as DNA replication errors and reactive oxygen species generated during cellular metabolism, as well as exogenous factors, such as ultraviolet and ionizing irradiation, chemical mutagens, and viral replication. These genotoxic stresses can result in double-strand DNA breaks (DSBs), single-strand DNA breaks (SSBs), and single-base modifications such as mismatched bases, DNA adducts, or intra-strand crosslinks. Depending upon the type of damage, specific protein sensors are responsible for recognizing damaged DNA and initiating the DDR signaling. We will focus on sensing and signaling associated with DSBs and SSBs (**Figure 2**). DSBs are primarily recognized by the MRE11, Rad50, NBS1 (MRN) complex, leading to ATM activation and recruitment to sites of genotoxic stress. Active ATM phosphorylates various downstream effector proteins including histone variant γH2AX, CHK2, and 53PB1 [4-6]. Alternatively, DSBs can be recognized by the DNA-PK holoenzyme (Ku70, Ku80, and DNA-PKcs) to be repaired by non-homologous end joining (NHEJ), a more error-prone process than homologous recombination (HR) repair and primarily occurs in the G1 phase of the cell cycle [4-6]. SSBs are sensed by RPA; when bound to ssDNA, RPA activates ATR, which stimulates downstream signaling proteins such as CHK1 [4-6]. Depending on the type and severity of the lesion, activation of the DDR results in various cellular outcomes such as DNA repair, cell cycle arrest, chromatin dynamics, and transcriptional changes.

## 2.3 Viral Manipulation of the DDR

In the following section, we will discuss examples of how diverse viruses modulate all steps of the DDR, including induction of DNA damage, recognition by damage sensors, signaling via mediator, transducer, and effector proteins, and cellular consequences of the DDR (**Figure 2** and **Table 1**). We have focused on specific examples which we hope will convey three main points: (1) modulation of the DDR is conserved by diverse viruses, regardless of viral genome type or location of replication; (2) the DDR both enhances and inhibits viral replication; (3) specific proteins as well as DDR signaling pathways play important roles in viral replication. In addition, while not explicit to this section, we will also highlight some important examples of how the DDR and innate immunity are directly linked.

### I. Induction and Recognition of Host DNA Damage

Viruses induce host DNA damage

Many examples exist that demonstrate induction of DNA damage during viral replication. Simian Virus 40 (SV40), a dsDNA virus, induces DNA damage via the large T antigen [7], and Human adenovirus type 12 (Ad12) induces chromosomal aberrations in human embryonic kidney cells (HEK) [8]. Influenza A (IAV) subtype H3N2, a segmented (-) RNA virus that replicates in the cytoplasm and the nucleus, causes DNA damage in leukocytes early during infection [9]. Human T-lymphotropic virus type 1 (HTLV-1), a retrovirus, induces DSBs during DNA replication through Tax [10]. While retroviruses may induce DNA damage through the process of integration [11, 12], it is becoming more apparent that retroviruses also induce DNA damage independently of integration, which we will describe throughout this review. However, what remains unclear for many of these viruses is how DNA damage occurs, whether viral-induced DNA damage is sensed and signaled by canonical cellular DDR pathways, and what role induction of DNA damage plays in viral replication and disease pathogenesis.

One example of a viral protein that potentially induces DNA damage is the lentiviral protein Vpr. Human Immunodeficiency Virus 1 (HIV-1) Vpr induces both SSBs and DSBs, independent of other lentiviral proteins [13-14]. While Vpr localizes to chromatin and is reported to bind DNA [16-18], it does not display any nuclease activity, suggesting Vpr may induce DNA damage through an indirect mechanism [14]. One possibility is that Vpr induces DNA damage indirectly by binding to chromatin and inhibiting DNA replication [19], leading to DNA damage following replication fork collapse. Another leading hypothesis is that Vpr induces DNA damage as a consequence of degradation of a DNA repair protein. Vpr recruits the host $Cul4A^{DCAF1}$ ubiquitin ligase complex and interacts with many host DDR proteins – including UNG2 [20, 21], HLTF [22, 23], SLX4 complex proteins MUS81 and EME1 [24, 25], EXO1 [26], TET2 [27], MCM10 [28], hHR23A [29], and SAMHD1 [30, 31] – yet degradation of most of these proteins has not been shown to be required for induction of DNA damage likely because the function of Vpr is complex and induces proteomic changes across the entire cellular landscape [ 32, 33].

One of the problems we face in the viral DDR field is that many of the central phenotypes of viral DDR modulation have not been tested directly or with methods that are easily reproducible. For example, induction of DNA damage has often been identified through detection of γH2AX activation rather than probing for DNA damage directly. Utilizing γH2AX in lieu of detecting DSBs or SSBs is problematic because activation of γH2AX is not necessarily a direct indicator of DSBs or SSBs, and γH2AX could potentially be activated by viruses in the absence of DNA damage. To ameliorate this, we recommend that the virologists move toward directly testing for DNA damage through more specific DNA damage assays such as the comet assay.

Viruses modulate DDR sensors

Subsequent to induction of DNA damage, viruses also modulate the primary sensors of this damage, including the MRN complex, RPA, and Ku70/80 (**Figure 2**). The MRN complex,

which is a major sensor of DSBs, has been shown to inhibit replication of many diverse viruses [34-36]. Thus, many viruses inhibit MRN. For DNA viruses such as adenoviruses, the MRN complex inhibits viral replication primarily by impairing viral DNA replication. To overcome this inhibition, Ad5 employs multiple E proteins to both relocalize and degrade components of the MRN complex [35, 37, 38]. Interestingly, not all Ad serotypes can overcome the MRN complex [39-41], indicating differences in the evolution of MRN antagonism. Another dsDNA virus, the herpesvirus Kaposi's Sarcoma-associated Herpesvirus (KSHV), antagonizes MRN through the viral LANA protein to block innate immune inhibition of viral replication and to support lytic reactivation [42]. Similar to adenoviral E proteins, LANA facilitates the relocation of the MRN complex to the cytoplasm. Additionally, RNA viruses such as rotavirus antagonize MRN by relocalizing the complex to the cytoplasm via viral proteins NSP2 and NSP5 [36], and the retrovirus HTLV-1 p30 directly binds to MRN components Rad50 and NBS1 to sequester and inhibit MRN complex formation [43]. MRN antagonism through sequestration and/or relocalization is conserved among diverse viruses, suggesting that evading damage detection by MRN is a strategy beneficial for productive infection.

Viruses also modulate the heterodimeric SSB sensor RPA (composed of RPA70, RPA32, and RPA14). However, unlike MRN antagonism, viruses primarily activate RPA – indicating that RPA enhances viral replication. For example, the Ad5 and Ad12 E1B-55K protein directly interacts with the host E1B-AP5 protein, which binds to the RPA component RPA32 in adenovirus replication centers. This is essential for ATR-dependent phosphorylation of RPA32, suggesting Ad5 and 12 regulate the ATR pathway through direct modulation of RPA phosphorylation [44]. For HIV-1, Zimmerman *et al.* showed that Vpr is responsible for inducing activated RPA foci in primary human CD4+ lymphocytes [19]. However, Vpr does not colocalize with RPA32 foci, suggesting Vpr may indirectly modulate RPA activity [15]. Thus, while less well defined than MRN antagonism, some viruses have evolved to activate RPA through direct and indirect mechanisms.

Finally, viruses antagonize the DSB sensor Ku70/80 complex, which is required for NHEJ-mediated DNA repair. For example, HTLV-1 transcriptionally silences Ku80 expression, inhibiting DNA-PK and innate immune activation [45]. Antagonism of Ku70/80 is important to the viral lifecycle as Ku70 directly recognizes a HTLV-1 reverse transcription intermediate (ssDNA90) and stimulates type 1 IFN and cytokine production, which together limit HTLV-1 infection before retroviral integration [46]. Therefore, Ku70/80 complex antagonism may be necessary to overcome innate immune sensing and to promote viral replication.

**II. DDR Signaling**

Downstream of sensing DNA damage, there is a vast signaling cascade consisting of mediator, transducer, and effector proteins (**Figure 2**). Despite differences in genome type and where they replicate in the cell, DNA, RNA, and retroviruses have evolved several mechanisms to modulate the DDR effectively. And although virus-induced DDR signaling is broadly conserved across viruses, the viral classification does not necessarily correlate with the proteins and pathways modulated. Here we will discuss and exemplify the three primary mechanisms that viruses use to engage DDR signaling: activation, inhibition, and degradation (**Figure 2**).

Human Papilloma Virus (HPV) strains modulate many aspects of the DDR associated with ATR and ATM, predominantly through the early viral proteins E1, E2, E6, and E7. Most notable is the capacity of E6 and E7 to directly interact with p53 and Rb, two important regulator proteins in DDR signaling [47-49], respectively [50, 51]. By inhibiting p53, E6 directly affects the ATR and ATM pathways, which in turn alters cellular processes such as cell cycle, DNA repair, and transcription. In addition to Rb binding, Moody and Laimins showed that high-risk HPV-37 E7 directly interacts with ATM leading to phosphorylation of Ser1981, causing activation and further downstream phosphorylation of CHK2. Moreover, they observed phosphorylation of CHK1 by E7, which is typically associated with ATR signaling, further suggesting a potential role for ATR in

HPV replication [52]. Many of these signaling pathways are activated directly by viruses through alternative mechanisms and are important for viral replication. However, whether ATM and ATR are both activated by diverse HPV subtypes, whether this activation occurs in conjunction with inhibition of either p53 or Rb, and whether this is dependent on antagonism of MRN remains to be studied.

As previously discussed, adenoviruses impair ATM signaling through sequestration of the MRN complex. However, adenoviruses also directly impair DNA-PK signaling, highlighting the necessity to target multiple arms of the DDR. Because all adenoviruses encode a linear double-stranded genome, they are particularly vulnerable to DNA-PK, which functions by re-ligating DSBs with exposed ends. As a response, adenoviruses disable the DNA-PK pathway by proteasome mediated degradation of DNA ligase IV via the interaction of E4 and E1b proteins with the host Cul5 ubiquitin ligase complex [53, 54]. Consequently, this allows the virus to replicate efficiently without being antagonized by host repair machinery, which acts as an antiviral defense mechanism and highlights the capacity of canonical DDR associated proteins to exhibit antiviral innate immune functions.

The viral lifecycle of many RNA viruses is primarily cytosolic. Despite this, RNA viruses still take advantage of the DDR machinery that largely reside and function in the nucleus. Rotavirus, a double-stranded RNA virus, is an interesting example of an RNA virus that modulates the DDR. The viral proteins NSP2 and NSP5 noncanonically activate ATM signaling independent of DNA damage and γH2AX activation and further relocalize ATM, CHK2, and the MRN complex from the nucleus to the cytoplasm [36]. Strikingly, ATM and CHK2 only interact with NSP5 in the presence of replicating viral genomes and inhibition of the ATM pathway reduces viral replication, suggesting that activation of these signaling pathways is important for viral genome replication [55]. Orthomyxoviruses are also of particular interest as they consist of a segmented (-) RNA genome that, unlike many RNA viruses, is shuttled to the nucleus for genome replication and

transcription. As such, these viruses also encode nuclear viral proteins to modulate the host DDR effectively. Specifically, the IAV viral protein NS1 suppresses RhoA and pRb signaling, directly activating the ATM signaling cascade [56]. In addition to these pathways, IAV infection modulates the protein abundance of various fundamental DDR proteins, such as Ku70, Ku80, Rad51, γH2AX, and PCNA, all of which are critical for ATM, ATR, and DNA-PK signaling [57]. Altogether, this exemplifies the evolutionary importance of modulating nuclear DDR factors for all viruses and could suggest that engagement of the DDR drove nuclear replication of some RNA viruses such as orthomyxoviruses and retroviruses. Despite cellular localization of viral replication, viruses require host DDR factors that they do not encode to replicate and thus evolving to activate the DDR through both canonical and noncanonical mechanisms is crucial for viral replication.

## III. Cellular Consequences of DDR

Depending on the type and severity of the genomic lesion, activation of the DDR results in a myriad of cellular consequences, including but not limited to DNA repair, cell cycle arrest, chromatin dynamics, and transcriptional changes (**Figure 2**). In this section, we will highlight how diverse viruses utilize common mechanisms to alter the cellular consequences of the DDR. We will specifically focus on how DNA, RNA, and retroviruses dysregulate DNA repair, promote cell cycle arrest, confer changes in chromatin organization, and induce transcriptional changes.

### Repair

One of the major consequences of modulating the DDR is the disruption of the five primary repair pathways: BER, NER, and MMR, which repair single-strand lesions, and HR and NHEJ, which repair DSBs. Disruption of DNA repair has been observed for many of the viruses that we have discussed thus far. Despite how much is known about viral modulation of DDR signaling, repair as a cellular consequence remains poorly understood. HR is one of the major repair

pathways a cell utilizes extensively during late S and G2 phases of the cell cycle and can be activated in response to DSBs, primarily via ATM signaling. Fittingly, many viruses that regulate ATM signaling also regulate HR. For example, HPV represses HR efficiency by 50-60% through the recruitment of a variety of DNA repair host factors such as Rad51, RPA70, BRCA1, and BRCA2 [58] away from chromatin and relocalization to the viral genome [59]. Consequently, these cells are more sensitive to exogenous genotoxic stress [60]. The role of DNA repair pathways in HIV infection is not well understood. In one system, HT1080 cells were transfected with pBHRF, a plasmid vector used to measure HR, in the presence or absence of transfected Vpr to assess HR repair of truncated GFP. In the presence of Vpr, GFP expression increased, suggesting Vpr enhances HR [61]. However, the effects of Vpr on HR remain unclear and it is crucial that the field uses similar systems and assays, such as the DR-GFP assay, to create reproducible and comparable data. This will also help to determine whether functions such as repression or activation of HR are directly beneficial to viral replication or a consequence of redistributing host DDR factors.

In addition to HR, other repair mechanisms can also play an important role in viral replication. As previously discussed, adenoviruses broadly inhibit the DNA-PK pathway by disrupting DNA ligase IV activity via proteasomal degradation, leading to the downregulation of NHEJ, which affects processes such as V(D)J recombination [54]. Though the role of DNA repair in RNA viruses remains understudied, it has been proposed that DNA repair is exploited during RNA virus infections. For example, IAV modulates and exploits MMR to promote cell survival during infection. An MMR activity assay, which utilizes a mismatch start codon on a luciferase expression plasmid, revealed that maintaining MMR activity is important for the IAV viral life cycle [62]. Strikingly, unlike HR or NHEJ, MMR activity leads to decreased transcription of antiviral innate immune factors, suggesting that affecting this particular DNA repair pathway has the

additional cellular effect of dampening the innate immune response that would otherwise inhibit viral replication.

Cell Cycle

Many viruses utilize the DDR to inhibit or activate cell cycle progression to facilitate an environment conducive to viral replication. Our current understanding is that certain cell cycle phases, such as S-phase, can promote viral replication, whereas the roles of others, such as G1 or G2/M, are still less clear. Here we will highlight different strategies viruses have evolved to both inhibit and promote cell cycle progression to benefit viral replication.

dsDNA viruses are the textbook example of cell cycle control by a virus, as they require a cell to be in S-phase in order to replicate their genomes [63]. This is because dsDNA viruses utilize much of the same machinery as the host to replicate DNA, including cellular DNA replication proteins and dNTPs. To achieve this, almost all dsDNA viruses encode early proteins that directly inhibit the master cell cycle regulators Rb and p53 [47-49] through degradation, relocalization, and/or sequestration [64, 65]. Some examples include HPV E6 and E7 proteins, adenovirus E1A and E1B proteins, and SV40 large T antigen, and has been extensively reviewed elsewhere [3, 66-68]. By studying how dsDNA viruses regulate S-phase, we have not only learned about mechanisms of viral replication but have also uncovered many molecular mechanisms underlying cell cycle regulation and the cellular consequences of dysregulation, such as cancer. Thus, viruses have been an instrumental tool in understanding viral and host biology.

Unlike the aforementioned DNA viruses that primarily push cells into a single cell cycle stage, coronaviruses represent a single family of RNA viruses that have all evolved to differentially regulate the cell cycle and display a range of cell cycle phenotypes. This is accomplished through an assortment of viral proteins, such as CoV-N, nsp13, p28, and ORF3/M, which converge on inhibiting cyclin-CDK complexes or upstream signaling cascades, such as p53, to induce cell

cycle arrest. Consistent with the different viral proteins, the type of arrest induced varies between G0/G1, S, and G2/M [69-72]. Despite these differences, induction of cell cycle arrest is conserved among coronaviruses and allows viruses to exploit host resources, such as translation and replication factors, that are essential for viral replication but are not encoded by the viral genome.

Many retroviruses also alter cell cycle; though the effects on cell cycle progression and viral replication seem to be distinct. For example, HTLV-1 infection allows cells to bypass the G1/S checkpoint despite DNA damage [73]. This is regulated by the interaction of HTLV-1 Tax with the cellular phosphatase Wip1, which dephosphorylates γH2AX and RPA to bypass the DDR-initiated G1/S checkpoint [73]. Interestingly, Tax has more than one function and also mediates G2 accumulation through the direct binding and activation of Chk2 independent of ATM [74, 75]. Primate lentiviruses induce arrest, with at least three HIV-1 proteins implicated in a G1 (Tat) or G2/M (Vif and Vpr) arrest [76-79]. The primary role of cell cycle arrest in HIV-1 replication is unclear, but G2/M arrest has been proposed to promote viral expression [80] and/or prevent nuclear breakdown to exploit nuclear factors in cycling T cells. Lentiviruses also have the ability to infect non-dividing cells, such as macrophages and dendritic cells. While at least one study has shown that prevention of cell cycle progression into mitosis in monocyte-derived dendritic cells is important for LTR-mediated viral transcription [81], it will be important for the field to directly address the role of activating cell cycle-associated pathways in noncycling cells.

Chromatin dynamics

Chromatin bound to damaged DNA must reorganize to allow DDR proteins to access damaged DNA and facilitate repair. Histone proteins bound to damaged DNA undergo post-translational modifications (PTMs), such as methylation, acetylation, phosphorylation, and ubiquitylation. This alters chromatin structure, DNA repair, and the local transcriptional environment. Thus, many viruses directly target histone modifying proteins to influence the

availability and abundance of nuclear factors and ultimately enhance viral replication. Some of the more widely conserved viral targets, which we will specifically discuss here, are the ubiquitin ligase proteins RNF8 and RNF168 and the acetyltransferase Tip60.

RNF8 and RNF168 are ubiquitin-protein ligases that play key roles in DNA damage signaling by catalyzing and amplifying ubiquitylation of histones H2A and H2AX to promote the recruitment of DNA repair proteins at DSBs [82]. Viruses inhibit RNF8 and RNF168 through several diverse mechanisms, including degradation or relocation of RNF8 and limiting the recruitment of DDR proteins to sites of damage. For example, HSV-1 ICP0 degrades RNF8 and RNF168, causing the loss of H2A ubiquitylation and DNA repair factor recruitment to DNA damage sites; thus, inhibiting DDR signaling [83]. Similarly, HPV E7 directly binds to and inhibits RNF8, which again limits the recruitment of 53PB1 to radiation-induced damage sites and increases repair by HR [84]. The EBV immediate-early protein BZLF1/ZEBRA similarly antagonizes RNF8 by relocating RNF8 and 53BP1 away from sites of DNA damage and consequently inhibiting DNA damage repair [85]. HTLV-1 Tax relocalizing RNF8 from the nucleus to the cytoplasm stimulates the DDR and induces assembly of K63-pUb chains that also activate NF-κB [86]. Together, this exemplifies the central role ubiquitylation plays in viral modulation of the host DDR to alter the availability of DDR factors.

Viruses also alter the chromatin environment by modulating the host acetyltransferase Tip60, which is a component of the NuA4 complex that acetylates histones to regulate gene expression and DNA repair [87]. Tip60 also directly regulates DNA repair by acetylation and activation of ATM, independent of NuA4 [88]. Tip60 was first identified through its interaction with the HIV-1 transcriptional activator Tat [89]. While the precise role of the Tat-Tip60 interaction in HIV-1 replication remains unclear [90-92], binding of HTLV-1 $p30^{II}$ to Tip60 promotes acetylation, chromatin remodeling, and transcription of c-Myc target genes [93], suggesting this could be a conserved and important retroviral-host interaction.

In addition to altering the chromatin environment, Tip60 inhibits gene expression of several dsDNA viruses, including adenoviruses [94], herpesviruses [95-97], papillomaviruses [98-101], and the hepadnavirus HBV [102], and thus, DNA viruses have evolved diverse mechanisms to antagonize Tip60. For example, adenovirus antagonizes Tip60 through the viral EIB55K and E4orf6. Both EIB55K and E4orf6 bind to Tip60 during infection and target it for proteasome-mediated degradation, causing cellular chromatin inaccessibility and promoting viral early gene transcription [94]. Recently, Tip60 was indicated to be upregulated in response to IAV infection and to activate type I IFN [103]. It remains to be seen whether Tip60 and other histone modifying proteins may have additional roles in response to viral infection.

Transcriptional Changes

Another major consequence of DNA damage is modulation of the cellular transcriptome. Specifically, DNA damage limits global transcription by inhibiting RNA polymerase II [104] and promoting activation of specific transcriptional pathways, such as NF-κB. Activation of NF-κB by DNA damage is dependent on the ATM-NEMO pathway and upregulates NF-κB-regulated genes important for facilitating cell survival by inhibiting apoptosis and mediating DNA repair [105-107] (reviewed in [108]).

Many viruses induce DNA damage causing transcriptional changes that benefit viral replication and are often linked to NF-κB [42, 109-112]. For example, HPV regulates transcription initiation by recruiting NF-κB through the viral helicase E1. Activation of NF-κB leads to the destabilization of E1, establishing a negative feedback loop to regulate E1-dependent genome amplification and NF-κB transcriptional changes [113]. Similarly, during HTLV-1 and HIV-1 co-infection, HTLV-1 Tax can regulate transcription initiation by facilitating the recruitment of NF-κB to the unintegrated HIV-1 LTR. Mechanistically, HTLV-1 Tax promotes the recruitment of the NF-κB subunits RelA and RelB to the HIV-1 LTR, which induces viral gene expression and facilitates

viral replication [114]. HTLV-1 can also activate NF-κB through Tax-independent mechanisms, which promote survival and proliferation of HTLV-1 infected cells by upregulating genes responsible for proliferation and clonal expansion [115]. Interestingly, transcriptional changes induced by DNA damage enhance viral gene expression and promote viral replication. For example, DNA damage via ultraviolet light or mitomycin C enhances transcription of the HIV-1 LTR [116], further suggesting that DNA damage can also alter viral transcription.

## 2.4 Discussion

Engagement and modulation of the DDR are central to the life cycles of a range of diverse viruses and are a phenotype that is broadly conserved beyond the viruses which we have discussed in this chapter. Despite the diversity in viral genomes, mechanisms of replication, and subcellular localization, the commonality of DDR engagement collectively highlights the importance of engaging ATM, ATR, and DNA-PK signaling pathways as well as the individual proteins in these pathways to promote the viral lifecycle. Converse to the benefits of utilizing the DDR, many of these factors themselves have antiviral activity as well as connections to innate immunity. As such, it is evolutionarily imperative for viruses to engage and modulate the DDR.

Many questions remain as we are only just beginning to scratch the surface of the role the DDR plays in viral replication and even innate immunity. For example, it is still unclear for many viruses whether steps such as induction of DNA damage or cell cycle arrest are active processes required for viral replication or consequences of other steps in viral replication. One way we propose to tackle this is to look for evolutionary conservation within viral genera. While conserved “byproducts” may exist, conservation of function is a strong indicator of significance in viral replication. It will also be important to directly address the causal relationships between the many ways a specific virus engages the DDR. Many steps in DDR signaling are intertwined, and most viruses we discussed activate and/or repress multiple aspects of the DDR. Thus, how one

phenotype may influence another, such as how viruses induce transcriptional changes as a cellular consequence of DNA damage or whether there is a correlation between repression of DNA repair and changes in chromatin dynamics, will be essential to identify important DDR-associated drivers of viral replication. Moreover, determining what viral proteins overcome DDR proteins acting as innate immune proteins, and what additional DDR factors have important roles in innate immunity, is paramount to uncovering the connection between these two interconnected signaling pathways. At the technical level, one aspect that must be addressed to help answer many of these questions is the use of consistent and reproducible assays that have been pioneered by the DDR field but are often overlooked by virologists.

We should consider how we can leverage the interconnection with the DDR to establish new therapeutics to treat viral infection. As the DDR is a major therapeutic target for cancer therapy, many drugs already exist that could be screened for antiviral roles, such as those found to inhibit SARS-CoV-2 replication [157]. Viral dysregulation of the DDR could also be used to selectively deplete infected cells. For example, a direct outcome of DNA damage is that infected cells are hypersensitive to additional DNA damage. In both HTLV-1 and HIV infected cells, induction of DNA damage or repression of DNA repair makes infected cells hypersensitive to additional low levels of exogenous DSBs [10, 15]. Moreover, patients with HPV+ head and neck tumors have increased sensitivity and long-term survival when treated with chemotherapy that induces DNA damage [158]. This concept of “synthetic lethality” has been proposed to treat many types of cancer that are deficient in DNA repair [159]. For example, BRCA1/2-deficient tumors are highly susceptible to inhibition of the DNA repair protein PARP1 due to the inability to repair double-strand breaks by HR or NHEJ [160, 161]. Given the ability of diverse viruses to induce DNA damage and/or antagonize DDR signaling and repair, we propose that a synthetic lethality approach may be feasible to selectively deplete infected cells. Based on the breadth of drugs available to induce low levels of genotoxic stress (including orphaned drugs and others that never

made it to clinical trials), it will be important to thoroughly assess the efficacy of a synthetic lethality approach to killing infected cells *in vitro* and *in vivo*.

Lastly, DNA damage signaling and repair pathways critically maintain genomic integrity and exhibit high evolutionary conservation across eukaryotes. Despite this, the sequences of many genes that comprise these pathways are marked by signatures of rapid evolution, called positive selection (PS). PS is often the consequence of long-standing evolutionary conflict and can be indicative of an important role in viral replication [179]. These signatures can be found in genes encoding proteins involved in specific DDR pathways, including HR and NHEJ, which have been shaped by recurrent PS [180]. Crystal structures of primate NHEJ factors XRCC4, Nbs1, and Polλ reveal PS sites located exclusively on exposed protein surfaces, supporting the idea that virus-host interactions are driving the rapid evolution of residues at these interfaces. Yet, due to the critical nature of NHEJ factors in cell survival, the functions of these genes are evolutionarily constrained, and sites of PS don't appear to alter their primary roles in DNA repair. In the context of viral evolution, however, persistent PS may lead to altered functions of viral genes, even major transformations of the viral lifecycle. For example, in this chapter we briefly exemplified how a subset of RNA viruses like Orthomyxovirus deviate from many other RNA viruses in that they have evolved to replicate in the nucleus and contain viral genes that have evolved to carry out nuclear processes. This is also exemplified further by Retroviruses, which have evolved to reverse transcribe their RNA genome into DNA and effectively become a part of the host through integration of the viral DNA genome into the host genome. Together, this could suggest that evolution could be driving viruses to engage the DDR as we can observe that even RNA viruses such as Rotavirus that are predominantly cytosolic necessitate sequestration of nuclear DDR factors to replicate efficiently. As such, it's imperative that we further investigate and understand how RNA and Retroviruses like HIV engage and modulate the DDR.

## 2.5 Figures

**Figure 2.1:** Four major features of interplay between viruses and the DDR.

Viral engagement of the DDR embodies four major characteristics: Recognition, Signaling, Cellular Consequences, and Innate Immunity. Each of these four pillars can be further broken down into specific components that make up the larger characteristics that viruses have evolved to modulate either through a precise mechanism or as a consequence of infection. (1) Recognition initiates the DDR and is activated by DNA damage. Sensors recognize this DNA damage to activate ATM, ATR, or DNA-PK signaling. Viruses have evolved to modulate this step by inducing DNA damage and antagonizing damage sensors. (2) Signaling then occurs through a variety of mediator, transducer, and effector proteins. Viruses modulate downstream signaling by activating or inhibiting mediators, transducers, and effectors in the DDR. (3) Cellular consequences occur in response to recognition and signaling in the form of DNA repair, cell cycle checkpoints, chromatin remodeling, and transcriptional changes. Viruses can modify repair pathways, elicit specific cell cycle arrest, alter chromatin organization, and induce transcriptional changes (4) Innate immunity is directly tied to these processes as DDR factors can elicit antiviral activity. Together, these four pillars represent the interplay between viruses and the DDR and exemplify how the DDR and innate immunity are directly interconnected and central to viral replication.

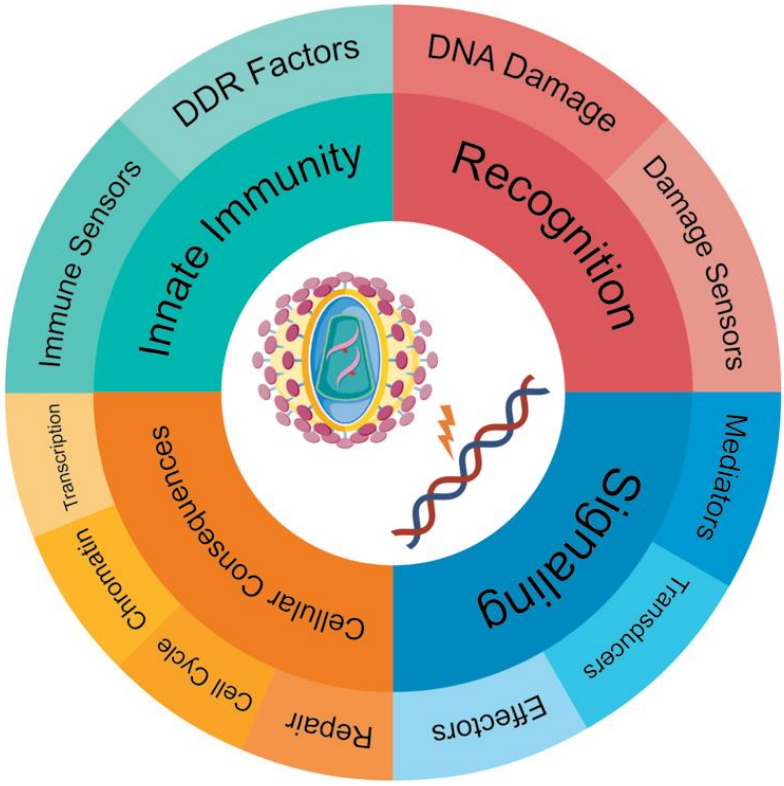
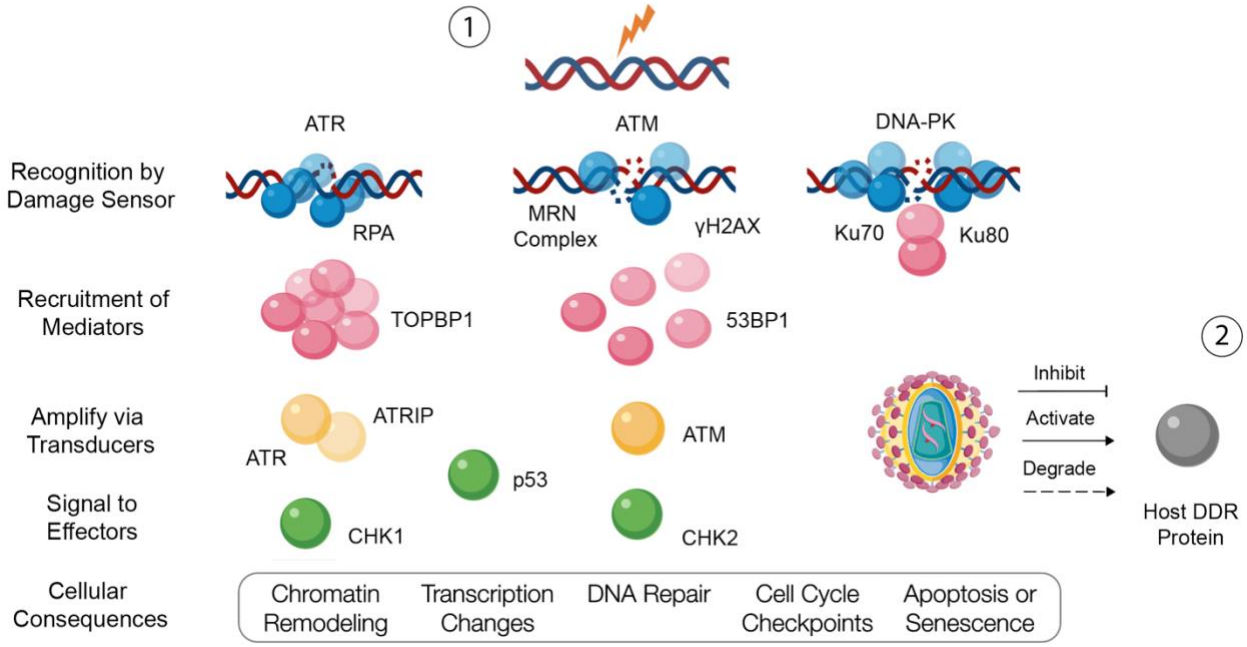


**Figure 2.2:** The DDR senses, signals, and responds to aberrant DNA through three primary pathways, ATM, ATR, and DNA-PK, that are modulated by viruses.

(1) The DDR is a protein signaling cascade that maintains genome integrity. The DDR consists of sensors, which recognize specific DNA lesions, mediators and transducers that transmit this signal of damaged DNA, and effectors, which directly execute a cellular response. While many of these pathways are interconnected, in general, the ATR pathway is activated in response to SSBs, and ATM and DNA-PK pathways are activated in response to DSBs. DDR signaling induces cellular consequences, including DNA repair, cell cycle arrest, chromatin dynamics, and transcriptional changes. Shown are representative DDR proteins, pathways, and cellular consequences that are highlighted here with the exception of apoptosis and senescence. (2) Viruses have developed several mechanisms to activate, inhibit, or degrade various parts of these core signaling pathways. Modulation of these pathways ultimately leads to several cellular consequences which are beneficial to the viral lifecycle.



## 2.6 Tables

**Table *2*.1** A summary of various viruses and the cellular consequences caused by viral modulation of the DDR.

This table summarizes the various viruses and the cellular consequences caused by viral modulation of the DDR. Damage, signaling, cell cycle, and DNA repair are four major phenotypes central to characterizing the ability of viruses to modulate the DDR that we recommend the field focus on moving forward. N/S indicates not shown.

| Genome Type | Virus | Viral Proteins | Damage | Signaling | Repair | Cell Cycle |
|---|---|---|---|---|---|---|
| **(+) RNA** | CoV | CoV-N, nsp13, p125 | Stalled Forks | ATR | N/S | G0/G1, G2/M |
| **(-) RNA** | IAV | NS1 | DSB | ATM, DNA-PK | Modulate MMR | G0/G1 |
| **dsRNA** | Rotavirus | NSP2, NSP5 | N/S | ATM | N/S | G2 |
| **Retro** | HIV | Tat, Vif, Vpr | SSB, DSB | ATR ATM | Repress HR | G1, G2/M |
| **Retro** | HTLV | Tax, p30 | DSB | ATM | Repress HR, Promote NHEJ | G1, G2 |
| **DNA** | HPV | E1, E2, E6, E7 | DSB, Stalled forks | ATM, ATR | Repress HR | G2 |
| **DNA** | EBV | LMP-1, EBNA3C, ZEBRA | DSB | ATM | N/S | G2/M |
| **DNA** | Adenovirus | E4, E1a, E1b, EIB55K, E4orf6 | N/S | ATM, ATR, DNA-PK | Repress NHEJ | Dysregulation |

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# Chapter 3: HIV Vpr Modulates the DDR through Several Mechanisms

## 3.1 Introduction

Primate lentiviruses encode accessory proteins that enhance viral infectivity (1). This is achieved through direct interactions with host proteins to usurp their cellular functions, or to antagonize their antiviral activity. HIV-1 encodes four accessory factors: Vpr, Vif, Vpu, and Nef. In addition, a subset of lentiviruses, including HIV-2, encode a paralog of Vpr called Vpx (2). Of all the lentiviral accessory genes, *vpr* is the only gene with a still unknown primary function.

Despite this, Vpr is critical for the infectivity of HIV and related primate lentiviruses. *In vivo*, viruses lacking Vpr are attenuated compared to wild type viruses, and the dominant viral species to emerge (i.e. most fit) have restored Vpr protein expression (3, 4). Furthermore, *vpr* is evolutionarily conserved by all extant primate lentiviruses (5). Together, this indicates that lentiviruses have maintained *vpr* for a highly important function. Of the many potential roles assigned to Vpr, activation of the host DNA damage response (DDR) and subsequent cell cycle arrest are the only phenotypes conserved by diverse Vpr orthologs (6–8). This conservation of function suggests that engagement of the DDR is central to Vpr function.

The DNA damage response (DDR) is a protein signaling cascade that ensures the fidelity of the genome. It consists of sensors, which recognize specific DNA lesions, mediators and transducers that transmit this signal of damaged DNA, and effectors, which directly execute a cellular response. Ataxia telangiectasia and Rad3 (ATR) (9), Ataxia telangiectasia mutated (ATM) (10), and DNA dependent protein kinase (DNA-PK)(11) are kinases at the head of the complex network that makes up the host DDR. The ATR kinase primarily responds to UV damage and replication stress, while ATM and DNA-PK repair double-strand breaks (DSB) through homologous recombination (HR) and non-homologous end joining (NHEJ), respectively (12). However, due to the essential role of DDR, a tremendous amount of crosstalk and redundancy exists between these kinases (13).

As discussed in **Chapter 2**, there is growing evidence that the DDR is important for viral replication, where it acts to both enhance and inhibit replication (14). For example, the DNA virus herpes simplex virus 1 (HSV-1) induces replication fork collapse at sites of oxidative damage (15). This leads to double-strand breaks (DSB), which initiate activation of the ATM repair pathway. HSV-1 infection also activates ATR, and the inactivation of either pathway severely compromises HSV-1 replication. RNA viruses also engage the DDR; for example, Rift Valley Fever Virus activates markers of DNA damage such as $\gamma$H2AX and upregulates the ATM pathway but represses the ATR pathway (16). Contrary to enhancing viral replication, DDR proteins, such as DNA-PK (17), can activate an antiviral state upon sensing cytoplasmic DNA, while etoposide-induced DNA damage stimulates interferon via STING, ATM, and NF-kB (18–22). Together, this highlights the potential roles for the DDR in innate antiviral immunity and in enhancing viral replication.

Vpr engages the DDR at multiple steps. First, it causes G2 cell cycle arrest both *in vivo* and *in vitro* (7, 23–26). This arrest is dependent on ATR signaling, as it is blocked by chemical inhibition of ATR (27). Moreover, Vpr-mediated cell cycle arrest requires interaction of Vpr with the Cul4A/DCAF1/DDB1 ($CUL4A^{DCAF1}$) E3 ubiquitin ligase complex (28, 29), a cellular complex that is involved in many mechanisms of DNA repair (30, 31). Second, Vpr induces the expression, activation, and recruitment of DDR proteins, as assessed by immunofluorescence and western blot analysis (32–34). Finally, in addition to the $CUL4A^{DCAF1}$ ubiquitin ligase complex, Vpr interacts with and degrades many host DDR proteins, including UNG2 (35, 36), HLTF (37, 38), SLX4 complex proteins MUS81 and EME1 (34, 39), EXO1 (40), TET2 (41), MCM10 (42), and SAMHD1 (5, 43). Yet despite being one of the most highly conserved and robust phenotypes associated with Vpr, how Vpr engages the DDR at so many levels remains unclear.

Using a combination of DNA damage response assays, we monitored the induction of DNA damage, the early signaling events following DDR activation, and the cellular consequences

associated with DNA damage and DDR activation. We found that Vpr engages the DNA damage response at two independent steps: it causes DNA damage and activates DDR signaling, and it represses double-strand DNA break repair. Using a panel of HIV-1 and HIV-2 Vpr mutants, we were able to separate these Vpr functions to show that while Vpr-induced DNA damage is independent of host-factor recruitment, repression of double-strand break repair and cell cycle arrest are not. Our data indicates that lentiviruses both activate and repress the DDR via Vpr, and further characterize a novel phenotype of Vpr that can explain many of the roles that have long been associated with Vpr.

## 3.2 Methods and Materials

*Plasmids*

pscAAV-mCherry-T2A-Vpr plasmids were generated by replacing GFP with mCherry from pscAAV-GFP-T2A-Vpr (6). HIV-2 A.PT (A.PT.x.ALI.AF082339) and HIV-2 G.CI.92 (G.CI.92.Abt96.AF208027) were synthesized as gBlocks (IDT) and subcloned into the pscAAV-mCherry-T2A-Vpr construct using standard cloning techniques. Vpr mutants were generated using site directed mutagenesis (Q5 site directed mutagenesis kit, NEB). pCBASceI was a gift from Maria Jasin (Addgene plasmid # 26477) (84).

*Cell lines & cell culture*

Human embryonic kidney (HEK) 293, HEK 293T, and human bone osteosarcoma epithelial (U2OS) cells were cultured as adherent cells directly on tissue culture plastic (Greiner) in DMEM growth medium (high glucose, L-glutamine, no sodium pyruvate; Gibco) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% $CO_2$. All cells were harvested using 0.05% Trypsin-EDTA (Gibco). The panel of U2OS cells containing an integrated

reporter (DR-GFP, SA-GFP, EJ2-GFP, EJ5-GFP) used in the I-SceI repair assays were kindly provided by Jeremy M. Stark (Beckman Research Institute of the City of Hope) (59).

*Generation of Viruses*

AAV vectors were generated by transient transfection of HEK 293 cells using polyethyleneimine (PEI) as previously described (85). Levels of DNase-resistant vector genomes were quantified by inverted terminal repeat (ITR)-specific quantitative PCR (qPCR) using a linearized plasmid standard according to the method of Aurnhammer et al. (86).

*Western Blots*

Cells we lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, Benzonase, Protease inhibitor). Lysates were clarified by centrifugation at 14,500g for 10 min and boiled in 4X sample buffer (4 ml glycerol, 2.4 ml 1M Tris pH 6.8, 0.8 g SDS, 0.5 ml β-mercaptoethanol, 3.1 ml ddH2O, bromophenol blue) in preparation for SDS-PAGE using 4-12% Bis-Tris polyacrylamide gels and subsequently transferred onto a PVDF membrane. Immunoblotting was performed using: mouse anti-FLAG (1:2000; Sigma), mouse anti-Actin (1:50,000), rabbit-anti-VPRBP (Cell Signaling), goat anti-mouse HRP (1:5000; Invitrogen), goat anti-rabbit HRP (1:5000; Invitrogen).

*DNA Combing Assay*

DNA combing assay was adapted from (52). Cells were plated in 6-well tissue culture treated plates (Greiner) at 1.75 x $10^6$ cell/ well and allowed to rest overnight. Cells were then infected with rAAV 2.5 at equal titers (1.4 x $10^8$ copies/ well) or 500uM hydroxyurea (Sigma) for 20 hrs. Following infection, cells were incubated with 10uM EdU (Invitrogen) for 20 min., then harvested, spun down, and resuspended in 1X PBS (Gibco). The cell suspension was added and lysed with

lysis buffer (50mM EDTA, 0.5% SDS, 200mM Tris-HCl pH 7.5) directly on a silane-coated slide (Electron Microscopy), then incubated for 5 to 8 min. After incubation, the slide was tilted at a 45° angle to allow the droplet to roll down then fixed with 3:1 methanol acetic acid for 15 min. after the slide was completely dry. Then the slide was washed with 1X PBS, blocked with 3% BSA for 30 min., and stained with secondary EdU mixture (Click-IT EdU imaging kit; Invitrogen) and DNA (Yoyo-1; Life Technologies). Microscopy was performed using the Zeiss Axioimager Z1 and images were analyzed using ImageJ.

*Alkaline Comet Assay*

Alkaline Comet Assay was performed as previously described (50) with some minor changes. Cells were plated in 6-well tissue culture treated plates (Greiner) at 1.75 x $10^6$ cell/ well and allowed to rest overnight. Cells were then infected with rAAV 2.5 at equal titers (1.4 x $10^8$ copies/ well) or 50uM etoposide (Sigma) for 20 hrs. Following infection, cells were then harvested, spun down, and resuspended in 0.5% low melting point agarose at 37˚C. Samples were then spread onto agarose-coated slides (Cell Biolabs) and allowed to solidify for 20 min at 4˚C. After agarose solidification, samples were incubated in lysis buffer (10 mM Tris-HCl pH 10, 2.5M NaCl, 0.1M EDTA, 1% Triton X-100) for 1 hr., then in the alkaline running buffer (0.3M NaOH, 1 mM EDTA) for 30 min., and finally electrophoresed at 300mA for 30min – all done at 4˚C. Samples were then washed in $ddH_2O$ and fixed in 70% ethanol at 4˚C. Cells were stained with Yoyo-1 (Life Technologies) for 15 min at room temperature, then washed with $ddH_2O$ and dried overnight. Images were acquired on the Zeiss Axioimager Z1. Images were analyzed using the OpenComet plug in for ImageJ.

*Cell Cycle Analysis*

U2OS cells were plated and synchronized using serum starvation with 0.05% FBS DMEM (Gibco) for at least 12 hours. Cells were simultaneously released from synchronization and infected with AAV2.5 (600 copies/cell) for 38 hours. For univariate labeling, cells were fixed with ice-cold ethanol and DNA was stained with 0.01 g/ml propidium iodide (Sigma-Aldrich) and RNase A in PBS. For bivariate labeling, cells were additionally pulse labeled with 10 μM EdU (Invitrogen) for at least 30 minutes. Pulse labeled cells were then permeabilized with 0.01% Triton X-100 for 3.5 minutes and fixed with 4% PFA for 20 min. EdU was detected using Click-iT EdU Alexa Fluor 647 Imaging Kit (Invitrogen) and stained with propidium iodide for at least 2 hours and assessed by flow cytometry on a FACSVERSE (BD). At least 10,000 cells were collected each run and data was analyzed using FlowJo software.

*Immunofluorescence*

Cells were plated in 6-well tissue culture treated plates (Greiner) at 1.75 x $10^6$ cell/ well and allowed to rest overnight. Cells were then infected with rAAV 2.5 at equal titers (1.4 x $10^8$ copies/ well) or 50uM etoposide (Sigma) for 20 hrs. For the EdU-IF experiments, EdU was added to the cells for 20 minutes. Cells were then permeabilized with 0.5% Triton X-100 in PBS at 4˚C for 5 min, fixed in 4% PFA for 20 min. Samples were then washed in 1X PBS and incubated with blocking buffer (3% BSA, 0.05% Tween-20, and 0.04 NaN3 in PBS) for 30 minutes. Cells were probed with appropriate primary antibodies (anti-FLAG M2 [Sigma-Aldrich], anti-γH2AX, anti-RPA32, or anti-53BP1 [Cell Signaling]), then washed in PBST (0.05% Tween-20 in PBS), and probed with Alexa- Fluor conjugated secondary antibodies (Life Technologies). Nuclei were stained with diamidino-2-phenylindole (DAPI; Life Technologies). Secondary staining for EdU was added as the last step and stained twice to ensure signal. Images were acquired on the Zeiss Axioimager Z1 and analyzed using ImageJ.

*Sensitivity Assays*

Sensitivity assays were performed as previous described in (87) with minor changes. Cells were plated in 24 well plates at 3 x $10^3$ cells/well and allowed to settle overnight. Done in triplicate per sample, the corresponding amounts of drugs were added and infected with rAAV 2.5 in equal titers (9.9 x $10^6$ copies/well), then incubated for 7 days. On the $7^{th}$ day, cells were washed with 1X PBS, fixed with 10% methanol and 10% acetic acid in water for 10-15 min, and stained with 0.1% crystal violet in methanol for 5 min. Plates were then washed with water, allowed to dry overnight, and the crystal violet was resolubilized with 300uL 0.1% SDS in methanol for 2 hrs. 100uL of the resolubilized dye was added to a 96 well, round bottom plate (Griener) and the absorbance was measured using the Gen5 (Biotek) plate reader at 595nm wavelength.

*I-Sce1 Repair Assays*

I-Sce1 repair assays were performed as previously described (88) with some minor changes. Cells were plated in 6 well plates 1.75 x 10^6 cells/ well and allowed to settle overnight. Cells were transfected with 1.5ug pBASce-1 and 0.5ug of corresponding pscAAV using Lipofectamine 3000 (Invitrogen) in antibiotic and serum free media. Prior to transfection, cell media was changed to DMEM high glucose (Gibco) and L-glutamine (Gibco) and 5% fetal bovine serum (Gibco) without antibiotics. Cells were allowed to incubate with transfection reaction for 30-48 hrs, then harvested, fixed with 4% PFA, and resuspended in FACS buffer (3% BSA in PBS). At least 20,000 cells/ condition were measured through flow cytometry (Attune NxT) and data was analyzed using FlowJo.

## 3.3 Results

*HIV-1 and HIV-2 Vpr activate multiple DNA damage markers*

The extent to which HIV Vpr engages the host DNA damage response (DDR) has not been critically examined. Therefore, we first asked if both HIV-1 and HIV-2 Vpr similarly activate the DDR. HIV-1 and HIV-2 are evolutionarily divergent primate lentiviruses that entered the human population through different non-human primate hosts (44). The Vpr proteins of these two viruses share only about 30-40% similarity, yet both cause cell cycle arrest (5, 7). Thus, if engagement of the DDR was central to the function of Vpr, we would expect that Vpr proteins from these two diverse human lentiviruses would also similarly activate the DDR. To test this, we delivered HIV-1 Q23-17 Vpr and HIV-2 Rod9 Vpr to U2OS cells via a recombinant adeno-associated virus (rAAV) vector system expressing 3X FLAG-tagged Vpr (6) and assayed for DDR markers twenty hours post infection by immunofluorescence (IF) for $\gamma$H2AX, a marker for DNA double- and single-strand breaks (DSB and SSB, respectively) (45), RPA32, a marker of SSB (46), and 53BP1, a late marker of DSB that is recruited to sites of damage by $\gamma$H2AX (47). In the presence of HIV-1 and HIV-2 Vpr, there were increased amounts of $\gamma$H2AX foci compared to the uninfected and empty vector controls (Fig. 1), which correlated with G2 arrest (Fig. S1). Similar to $\gamma$H2AX, HIV-1 and HIV-2 Vpr expression also lead to increased levels of RPA32 and 53BP1 foci compared to uninfected and empty vector control cells, and produced fewer yet larger foci compared to the etoposide positive control, a topoisomerase II inhibitor (Fig. 1). We also observed a distinct lack of colocalization between Vpr and markers of DNA damage (Fig. 1A), indicating that Vpr is not present at the potential sites of damage at this time point. Additionally, cells that expressed higher levels of Vpr did not have appreciably more DNA damage foci or fluorescence intensity of damage marker per cell when compared to cells with lower Vpr expression (Fig. 1A asterisks and Fig. Sn, respectively), suggesting that activation of these markers is saturated with low levels of Vpr. And while HIV-1 had significantly higher ($P<0.03$) levels of 53BP1 compared to

HIV-2 Vpr, the levels of γH2AX and RPA32 activation were the same for HIV-1 and HIV-2 as measured by mean fluorescence intensity (MFI) of individual cells (Fig. 1B).

We also tested several HIV-1 and HIV-2 Vpr isolates to determine if activation of the DDR by HIV-1 and HIV-2 Vpr were isolate specific or conserved by the greater diversity of HIV Vpr proteins. These include representative Vpr isolates from HIV-1 groups M (subtype G), N, O, and P consensus sequences, as well as HIV-2 Vpr isolates from groups A, B, and divergent. We found that all HIV-1 and HIV-2 Vpr proteins tested caused cell cycle arrest and increased the number of γH2AX foci, indicative of DDR activation (Fig. S2). In total, our data highlights that a conserved function of HIV-1 and HIV-2 Vpr is the activation of the same markers of DNA damage.

*HIV-1 and HIV-2 Vpr expression damages DNA and induces replication stress*

The formation of γH2AX, RPA32, and 53BP1 foci in cells expressing HIV-1 and HIV-2 Vpr suggests the presence of both SSB and DSB. However, it is also possible that Vpr leads to activation of these markers without causing actual DNA damage. Previous studies to identify Vpr-induced DNA damage using pulse field gel electrophoresis, which only reveals DSB, have been contradictory (48, 49). Here we used the alkaline comet assay, which uses a high pH (>10) buffer to denature supercoiled DNA and single-cell gel electrophoresis to reveal damaged DNA fragments, including both SSB and DSB (50). U2OS cells were infected with rAAV-Vpr for twenty hours and the extent of DNA damage within individual cells was measured by calculating the percent tail DNA, which is proportionate to the amount of damaged DNA within a cell (Fig. 2A). While uninfected and empty vector control cells had little appreciable damage, both HIV-1 and HIV-2 Vpr expression significantly increased levels of percent tail DNA, indicative of an increase in damaged DNA (Fig. 2B). These results also correlate well with the IF data for γH2AX, RPA32, and 53BP1 that show lower MFI for Vpr-induced DNA damage markers when compared to etoposide treatment (Fig. 1B). We segregated the samples into two populations, below 20% and

above 20% tail DNA, to highlight the population of cells within each sample with a greater extent of damage (Fig. 2A and 2C). Whereas approximately 1% of uninfected and empty vector control cells had tail DNA above 20%, HIV-1 and HIV-2 Vpr expression resulted in 5% and 8% of cells above 20% tail DNA, respectively, indicating that expression of Vpr leads to significant DNA damage.

As replication stress has been proposed to be a driver of this Vpr-induced DDR (51), and the activation of the DNA damage markers and cell cycle arrest (Fig. 1A and Fig. S1) are hallmarks of stalled DNA replication forks, we next determined whether Vpr expression leads to replication fork stalling via the DNA combing assay (52). This assay quantities the length of replication tracks by incorporation of EdU into nascent DNA. U2OS cells were infected with rAAV-Vpr for 20 hours, at which point EdU was added to the cells for 20 minutes. We found that HIV-1 and HIV-2 Vpr significantly decreased EdU track lengths compared to the uninfected and empty vector controls (Fig. 2D). There was no direct correlation between levels of Vpr expression and DNA replication during this 20-minute window. However, cells expressing the highest levels of Vpr were not in S-phase during this window (Fig. Sn), consistent with Vpr-mediated G2 arrest and our bivariate cell cycle assay (Fig. S1). Like the comet and IF assays, the greatest amount of damage was exhibited by the positive control, hydroxyurea (HU), which stalls DNA replication by depleting dNTP pools (53), suggesting that while the impairment of normal DNA replication by HIV-1 and HIV-2 Vpr is significant, it is not as detrimental to the cell as HU. Overall, our alkaline comet and DNA combing data show that Vpr directly engages the DDR by inducing DNA breaks and stalling DNA replication.

*ATR senses stalled replication forks downstream of Vpr-induced DNA damage*

Our results indicate that Vpr directly damages DNA and stalls DNA replication (Fig. 2). However, whether DNA damage occurs prior to replication fork stalling or as a consequence of

stalled replication forks is unclear. To differentiate between these two possibilities, we inhibited the fundamental DNA damage repair kinase ATR via the selective ATR inhibitor (ATRi) VE-821 (54). ATR acts as the primary signaling axis for replication stress and cell cycle checkpoints, where it is recruited during S phase through RPA to stalled replication forks (9, 54). Here, it stabilizes replication forks from collapse, initiates the recruitment of repair proteins, and activates critical cell cycle checkpoints (9, 54). If Vpr-mediated DNA damage is due to stalled replication, we would expect ATR inhibition to increase DNA damage as the cells would not be able to guard against replication fork collapse or initiate repair. However, if damage occurs before replication stress, we would expect the inhibition of ATR to alter fork progression, but not DNA damage.

We first confirmed ATR inhibition mitigated Vpr-mediated cell cycle arrest for both HIV-1 and HIV-2 Vpr isolates tested (Fig. S3). We also assayed for an effect of ATM inhibition (ATMi), as we found activation of repair markers associated with ATM activation (such as γH2AX and 53BP1 in Fig. 1) but found no effect of ATMi on Vpr-mediated cell cycle arrest, consistent with previously published results (REFS). Next, to determine the effect of ATR inhibition on DNA damage by Vpr, we again used the alkaline comet assay. While all samples had proportionately increased levels of damage when ATR was inhibited, there was no significant difference for either HIV-1 or HIV-2 Vpr with or without ATRi (Fig. 3A and 3B). This suggests that ATR inhibition does not affect the ability of Vpr to generate DNA lesions.

In contrast, the DNA combing assay, which we used to determine the effect of ATR inhibition on stalled replication fork progression by Vpr, showed that replication track lengths were significantly shorter for HIV-1 and HIV-2 Vpr expressing cells when ATR was inhibited (Fig. 3C), presumably due to fork collapse. Though the overall effects of ATRi are modest, which is likely due to the intertwined nature of DNA damage, sensing, and repair, our data from the comet and DNA combing assays shows that while Vpr mediated DNA damage is independent of ATR signaling, the ability to stall DNA replication is not. Moreover, it indicates that Vpr first induces

DNA damage, leading to the activation of ATR and subsequent stalled replication forks, presumably to mitigate replication stress.

*Vpr sensitizes cells to additional double-strand breaks*

As we established with the immunofluorescence and alkaline comet assay, HIV-1 and HIV-2 Vpr induce DNA damage activating markers related to a wide variety of DNA lesions, such as SSB and DSB. While our data suggest that Vpr directly damages DNA, it is also possible that damage results from the inability of cells to repair pre-existing damage, such as damage due to replication stress. To address this question, we tested the sensitivity of cells expressing Vpr against various chemotherapeutics that directly damage DNA or inhibit a repair mechanism to cause damage.

We began by testing the sensitivity of Vpr treated cells to etoposide, which generates DSB by preventing the enzyme topoisomerase II from properly removing knots formed from DNA over-winding (55). Cells expressing Vpr were highly sensitized to etoposide treatment, where survival at even the lowest concentration (0.01uM) decreased to 60-70% compared to uninfected and empty vector control cells (Fig. 4). This indicates that Vpr expressing cells are unable to repair etoposide-induced DSB. We next tested sensitivity to hydroxyurea (HU). Prolonged exposure of HU to cells at high concentrations results in replication fork collapse and extensive DSB (56). Although Vpr expressing cells were not sensitized to HU treatment at low concentrations, at higher concentrations of HU (>3.90uM) where DSB are presumably present, survival of cells expressing HIV-1 and HIV-2 was significantly decrease compared to control cells (Fig. 4). Similar results were seen for the PARP1/2 inhibitor, Olaparib, which also leads to DSB due to the inability to repair DNA lesions (57) (Fig. 4). In contrast to the other chemotherapeutics, HIV-1 and HIV-2 Vpr expression did not dramatically hyper-sensitize cells to the interstrand-crosslinking agent cisplatin (58) (Fig. 4) despite activating markers associated with interstrand-crosslink (ICL) repair (6, 34).

Altogether, the sensitivity assays indicate that Vpr expressing cells specifically show increased sensitivity to multiple chemotherapeutics that are capable of generating DSBs by inhibiting crucial host repair mechanisms, suggesting that Vpr may also inhibit the ability of cells to repair this damage.

*Vpr inhibits double-strand break repair*

Because we observed that Vpr expressing cells display hypersensitivity to the induction of exogenous DSB, we hypothesized that Vpr itself inhibits DNA break repair. To test this hypothesis, we used multiple GFP-based U2OS reporter cell lines that monitor repair of an I-SceI induced DSB by either homologous recombination (HR), non-homologous end joining (NHEJ), alternative NHEJ (alt-NHEJ), or single-strand annealing (SSA) (59, 60). Each cell line contains a GFP gene that is uniquely disrupted by an I-SceI restriction site and does not express GFP, as well as a truncated GFP donor sequence. Upon transfection and expression of I-SceI, this site is cut and only proper repair by the indicated pathway results in GFP expression. In addition to transfecting I-SceI alone, we also used combinations that included empty vector, HIV-1, or HIV-2 Vpr that express mCherry via a T2A ribosomal skipping sequence. Thirty hours later, we measured repair on a per-cell basis using flow cytometry for successful repair (GFP) and transfection efficiency (mCherry) (Fig. 5A and Fig. S4A).

We first tested the I-SceI reporter cell line for HR. While transfection of I-SceI alone or with empty vector control resulted in similar amounts of HR, we found that cells transfected with HIV-1 and HIV-2 Vpr decreased HR efficiency by 66% and 49%, respectively, when normalized to wild type cells at 100% (Fig. 5A and 5B). This indicates that HIV-1 and HIV-2 Vpr repress HR. Based on these results, we next tested the I-SceI reporter cell line for NHEJ, which is often utilized by cells to repair DSBs when HR is repressed (61). Similar to HR, HIV-1 Vpr expression also decreased NHEJ efficiency by 51% compared to wild type cells. In contrast to HIV-1, HIV-2 Vpr

did not significantly decrease NHEJ, as these cells were able to repair via NHEJ at 90% of wild type levels (Fig. 5C and Fig. S4A), highlighting potential mechanistic differences between HIV-1 and HIV-2 Vpr. Finally, we tested the I-SceI reporter cell lines for alt-NHEJ and SSA repair mechanisms but found no significant change in repair when compared to control cells (Fig. S4B and S4C). Thus, based on the data from the four different I-SceI reporter cell lines, we have identified that both HIV-1 and HIV-2 Vpr repress double-strand break repair, in addition to inducing DNA damage.

*Disconnect between induction of DNA damage and downregulation of repair machinery*

Our findings demonstrate that both HIV-1 and HIV-2 Vpr are capable of inducing DNA damage, stalling DNA replication, downregulating homologous recombination repair, and causing cell cycle arrest. However, it is unclear how these phenotypes are linked and what role(s) host protein interactions play. To address these questions, we further tested a subset of well characterized HIV-1 and HIV-2 Vpr mutants for their ability to induce, signal, and respond to DNA damage via the alkaline comet assay, EdU immunofluorescence, HR I-SceI repair assays, and bivariate cell cycle analysis, respectively. We tested four mutants for each HIV-1 and HIV-2 Vpr. These include: HIV-1 W54R/HIV-2 L59A Vpr mutants, which block the ability of HIV-1 Vpr to recruit and degrade the DNA glycosylase UNG2 (62); HIV-1 Q65R/HIV-2 Q70R Vpr, which renders Vpr unable to properly localize, multimerize, or recruit known host proteins such as the $Cul4^{DCAF1}$ complex or UNG2 and is therefore largely functionally dead (33, 63); HIV-1 S79A/ HIV-2 S84A mutants, which renders Vpr unable to cause cell cycle arrest or interact with TAK1 to activate canonical NF-kB (64, 65); HIV-1 R80A/ HIV-2 R85A Vpr mutants, which can still interact with $Cul4^{DCAF1}$ and degrade TET2 (41) but does not cause cell cycle arrest, presumably due to the requirement of an additional unknown host protein(s) (66). Moreover, HIV-1 Vpr, but not HIV-2 Vpr, interacts with UNG2, HLTF, and the SLX4 complex proteins MUS81 and EME1, allowing

us to further dissect the requirement(s) for previously reported Vpr-interacting proteins in inducing DNA damage, stalling DNA replication, downregulating HR repair, and causing cell cycle arrest.

Consistent with previously published results, all mutants except HIV-1 W54R/HIV-2 L59A Vpr failed to induce cell cycle arrest (Fig. 6A). In contrast to cell cycle arrest, only HIV-1 Q65R/HIV-2 Q70R Vpr lost the ability to damage DNA (Fig. 6B), indicating that damage of DNA occurs independent of the Vpr-host protein-protein interactions tested here and that this phenotype is independent of cell cycle arrest. When testing for the effects of Vpr on DNA replication, we found that, in addition to HIV-1 Q65R/HIV-2 Q70R Vpr, HIV-1 S79A/ HIV-2 S84A Vpr mutants were unable to stall DNA replication (Fig. 6C), suggesting that activation of TAK1 is integral in the ability of Vpr to stall DNA replication. Finally, in concert with cell cycle arrest, all mutants except the HIV-1 W54R/HIV-2 L59A Vpr mutants failed to repress homologous recombination (Fig. 6D), suggesting that both of these phenotypes require $Cul4^{DCAF1}$ ubiquitin ligase complex recruitment in addition to potentially unidentified host protein(s). A summary of these results can be found in Table 1.

Overall, our mutational analyses of HIV-1 and HIV-2 Vpr indicate that repression of HR and cell cycle arrest are correlated, and these phenotypes are independent of Vpr-induced DNA damage. Moreover, by testing multiple mutants deficient for host factor recruitment, as well as comparing HIV-1 and HIV-2 Vpr orthologs which differentially recruit host proteins, our results rule out most known Vpr-interacting host proteins for a role in induction of DNA damage and repression of HR.

*Repression of HR is not a consequence of Vpr-mediated cell cycle arrest*

The predominant phenotype of Vpr expression *in vivo* and *in vitro* is G2 cell cycle arrest, which depends on recruitment of the $Cul4A^{DCAF1}$ ubiquitin ligase complex through a direct interaction of Vpr with DCAF1. Here, we have identified a new phenotype of Vpr, repression of

HR, that tracks with G2 cell cycle arrest based on our Vpr mutant data (Fig X and Table 1). However, whether repression of HR by Vpr is a consequence or potential driver of Vpr-mediated arrest remains unclear.

To address this, we first asked if $Cul4A^{DCAF1}$ complex recruitment is also required for repression of HR by Vpr. We selected two mutants that have been previously shown to alter HIV-1 Vpr binding to DCAF1, L64A and H71R, and further generated those mutants in HIV-2 Vpr (L69A and H76R). Whereas both HIV-1 H71R/ HIV-2 H76R blocked interaction with endogenous DCAF1, HIV-1 L64A/ HIV-2 L69A was still able to recruit the DCAF1 adaptor protein, though at a slightly lower level than wild type Vpr (Fig. 7A). Consistent with recruitment of DCAF1, HIV-1 H71R/ HIV-2 H76R, but not HIV-1 L64A/ HIV-2 L69A, lost their ability arrest cells (Fig. XB). We next tested these mutants for their ability to repress HR. As we saw with DCAF1 binding and cell cycle arrest, HIV-1 H71R/ HIV-2 H76R failed to repress HR, whereas HIV-1 L64A/ HIV-2 L69A repressed HR to near WT Vpr levels. These data suggest that, similar to cell cycle arrest, repression of HR by Vpr requires DCAF1 binding.

To determine if repression of HR requires G2 arrest, we analyzed DNA content of DR-GFP HR cells with Hoechst dye and determined which population of Vpr-expressing cells showed a repression of HR: G1 or G2. We would expect that if Vpr-mediated G2 arrest is required to repress HR repair, then only Vpr-expressing cells in G2, and not G1, would show repressed HR repair. However, if G2 arrest is not required for Vpr to repress HR repair, then either G1 or G1 and G2 cells would show a repression of HR repair in the presence of Vpr.

As seen previously, both HIV-1 and HIV-2 Vpr repressed total cellular HR when compared to empty vector control. Vpr-expressing cells also showed strong repression of HR repair in G1. However, Vpr-expressing cells in G2 were statistically indistinguishable from control. Together, this data indicates that Vpr-mediated repression of HR does not require G2 arrest, but instead occurs primarily in the G1 phase of the cell cycle. Moreover, as G1 precedes G2, this may suggest

that repression of HR precedes cell cycle arrest, and therefore may be more central to the primary function of Vpr.

## 3.3 Discussion

Here we show that HIV-1 and HIV-2 Vpr induce both double- and single-strand DNA breaks, leading to the recruitment of repair factors, including $\gamma$H2AX, RPA32, and 53BP1. These Vpr-induced DNA lesions are sensed by ATR and require NF-kB signaling to stall DNA replication. However, contrary to the induction of DNA damage and the activation of the DNA damage response, Vpr represses essential mechanisms of double-strand break repair, including homologous recombination repair. Moreover, mutational analysis of Vpr has identified that there is a disconnect between mutants that can damage DNA and those that can repress DNA repair and activate cell cycle arrest. Finally, repression of HR requires DCAF1 and is not a consequence of G2 cell cycle arrest but is potentially a driver of this arrest. Overall, our data support a model where Vpr has two unique and independent mechanisms to modulate the host DDR: first, Vpr has the inherent ability to induce DNA damage, which is independent of known Vpr-binding host factors. This Vpr-induced damage is sensed by ATR, and signals through NF-kB to block DNA replication fork progression. Second, through recruitment of the $Cul4^{DCAF1}$ complex and an unknown host protein(s), Vpr represses DNA repair machinery, leading to a prolonged cell cycle to deal with the inability to repair DNA lesions.

While it may seem counterintuitive to both activate and repress the DDR through unique mechanisms, Vpr is not the only viral protein, and lentiviruses are not the only viruses, to both activate and repress the DDR at different steps in viral replication (14). Moreover, as Vpr has two unique phases in an infected cell – it is delivered early via the incoming virion and expressed *de novo* following integration and gene expression – it is possible that these two distinct DDR-associated functions of Vpr are separated in the viral lifecycle of an infected cell.

But why would Vpr engage the DDR at two unique steps, and how would this help lentiviral replication? Many of these roles in viral replication attributed to Vpr (67–73) may be directly explained by either activation or repression of the DDR. For example, DNA damage promotes nucleotide biosynthesis (74) and thus may enhance reverse transcription. This is analogous to the degradation of SAMHD1 by lentiviral Vpx/Vpr (5, 75, 76) and could help to explain why Vpr from HIV-2, which encodes both Vpr and the paralogous Vpx protein, does not attenuate host repair machinery, or recruit host DDR proteins (6, 36, 37, 40, 41, 77), as efficiently as HIV-1 Vpr. The stalling of replication forks could enhance integration by remodeling histones and prolonging S-phase. Integration could also be enhanced by attenuating double-strand break repair, similar to the repression of HR and base excision repair by Human T-lymphotropic Virus 1 (HTLV-1) to facilitate viral integration (78–80). Moreover, the induction of DNA breaks could enhance LTR-driven transcription by activating important DDR-responsive transcription factors, such as NF-kB and AP-1 (65, 81).

However, as the primary role of lentiviral accessory genes is to overcome antiviral restriction factors, our data also support a model where DDR proteins and/or pathways restrict HIV replication and are overcome by Vpr. Whether modulation of the DDR is a direct effect of Vpr or a consequence of degradation of an antiviral host protein that is also integral to the DDR is unclear from our data. We have shown that, like Vpr-mediated G2 arrest, DCAF1 interaction and potentially the $Cul4^{DCAF1}$ ubiquitin ligase complex is required for repression of HR repair, which supports the model in which a host factor is degraded by Vpr. However, through the combination of our mutant data and use of HIV-1 and HIV-2 Vpr, we are able to rule out most known DDR-associated Vpr-interacting proteins for roles in modulating the DDR as described herein. Whether some of the remaining proteins we were unable to characterize are required for Vpr-mediated engagement of the DDR, such as the endonuclease Exo1, or whether novel undiscovered host proteins are required remains unclear. However, this overall hypothesis is consistent with the

growing appreciation of the DDR in the innate immune response to pathogens (17) (18–22), and is consistent with the role of lentiviral accessory genes in viral replication, such as Vpx-mediated degradation of the antiviral DDR protein SAMHD1(82, 83).

Thus, while it is clear that the DDR is a central hub that is essential for replication of many viruses in different phases of their lifecycle, the precise roles of Vpr-mediated activation and repression of the DDR in HIV replication remain unclear and warrant further investigation. In establishing that Vpr activates and represses the DDR, we have clarified the multiple ways that Vpr modulates the host DDR and uncovered a new phenotype for Vpr that may precede cell cycle arrest, suppression of double-strand break repair, which will allow us to better define the primary role of Vpr. Finally, our data indicate that Vpr expression could have important implications for the development and treatment of cancer in HIV-positive individuals, where induction of DNA damage and deregulation of repair could serve to complicate tumorigenesis but also sensitize cells to chemotherapeutics; further highlighting the importance of Vpr in HIV replication and associated diseases.

Lastly, despite showing here that HIV Vpr induces cell cycle arrest and represses repair independent of DNA damage, it remains unclear how these processes occur. Our mutational analyses were unable to explain not only how these processes might occur, but also whether they are linked. As such, the underlying molecular mechanisms governing these phenotypes remain to be explained. It is possible that there remains an unknown protein or group of proteins that are central to these processes and could potentially be the missing link that could bridge and fully explain these seemingly independent phenotypes.

## 3.5 Figures

**Figure 3.1** Activation of the DNA damage response is conserved between HIV-1 and HIV-2 Vpr.

(A) Representative immunofluorescence images of U2OS cells infected with rAAV expressing 3× FLAG-tagged HIV-1 and HIV-2 Vpr, control empty vector (no Vpr), or uninfected control for 20 h. Blue (DAPI) shows the nuclei, 3×-FLAG Vpr is shown in green, and the phosphorylated DNA damage markers (γH2AX, RPA32, and 53BP1) are shown in red. Asterisks indicate cells with either high or low Vpr expression. The single-cell images show only 3×-FLAG Vpr and corresponding DNA damage marker. Images were taken at ×63 magnification. (B) Mean fluorescence intensity (MFI) of 100 cells per condition was quantified for all markers. Asterisks indicate statistical significance compared to empty vector control, as determined by Kruskal-Wallis tests (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 2$, one representative experiment shown).

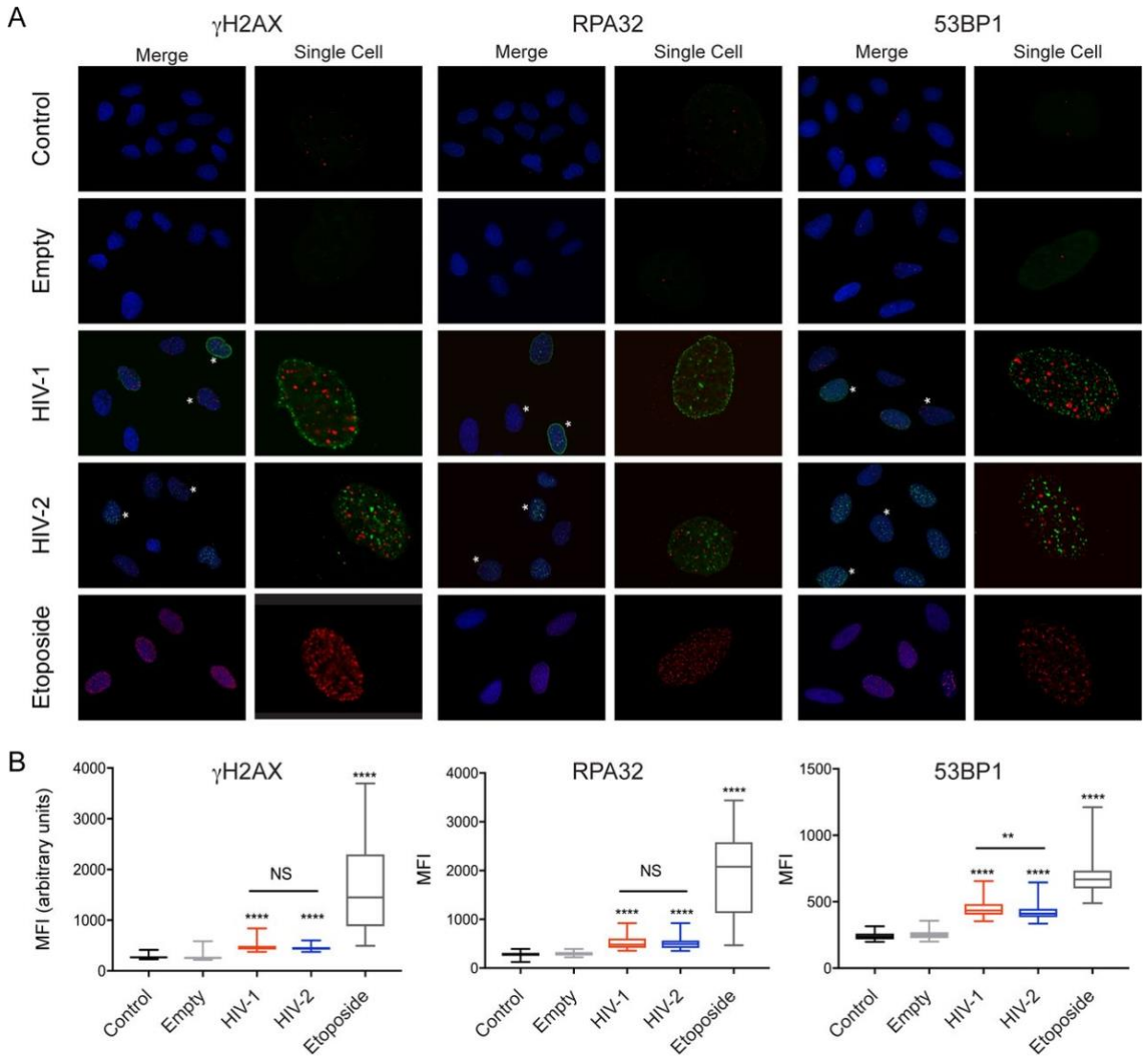


**Figure 3.2** HIV-1 and HIV-2 Vpr damage DNA and stall DNA replication.

(A) Visual representation taken from the alkaline comet assay of the four degrees of damage measured by percent tail DNA. Intensity profiles, lines, and numbers on the images were automatically generated by the *OpenComet* plug-in for the ImageJ software. (B) Distribution of the percent tail DNA measured for 100 cells per condition from one independent experiment using the *OpenComet* plug-in. U2OS cells were treated under the same conditions as those for Fig. 1A prior to being harvested for the comet assay. The bars represent the median with interquartile range; $n = 3$, one representative experiment shown. (C) A bar graph representation of the cells in panel B separated into two populations, below 20% tail DNA (unshaded) and above 20% tail DNA (shaded). Asterisks indicate statistical significance compared to empty vector control or HIV-1 compared to HIV-2 Vpr, as determined by chi-square test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$, one representative experiment shown). (D) A box and whiskers representation of the distribution of EdU track lengths (μm). U2OS cells were treated under the same conditions as those shown in Fig. 1A. Asterisks indicate statistical significance for HIV-1, HIV-2, and hydroxyurea (HU) compared to the empty vector control, as determined by the Kruskall-Wallis test, while statistical difference between HIV-1 and HIV-2 was determined by the Mann-Whitney test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$, one representative experiment shown).

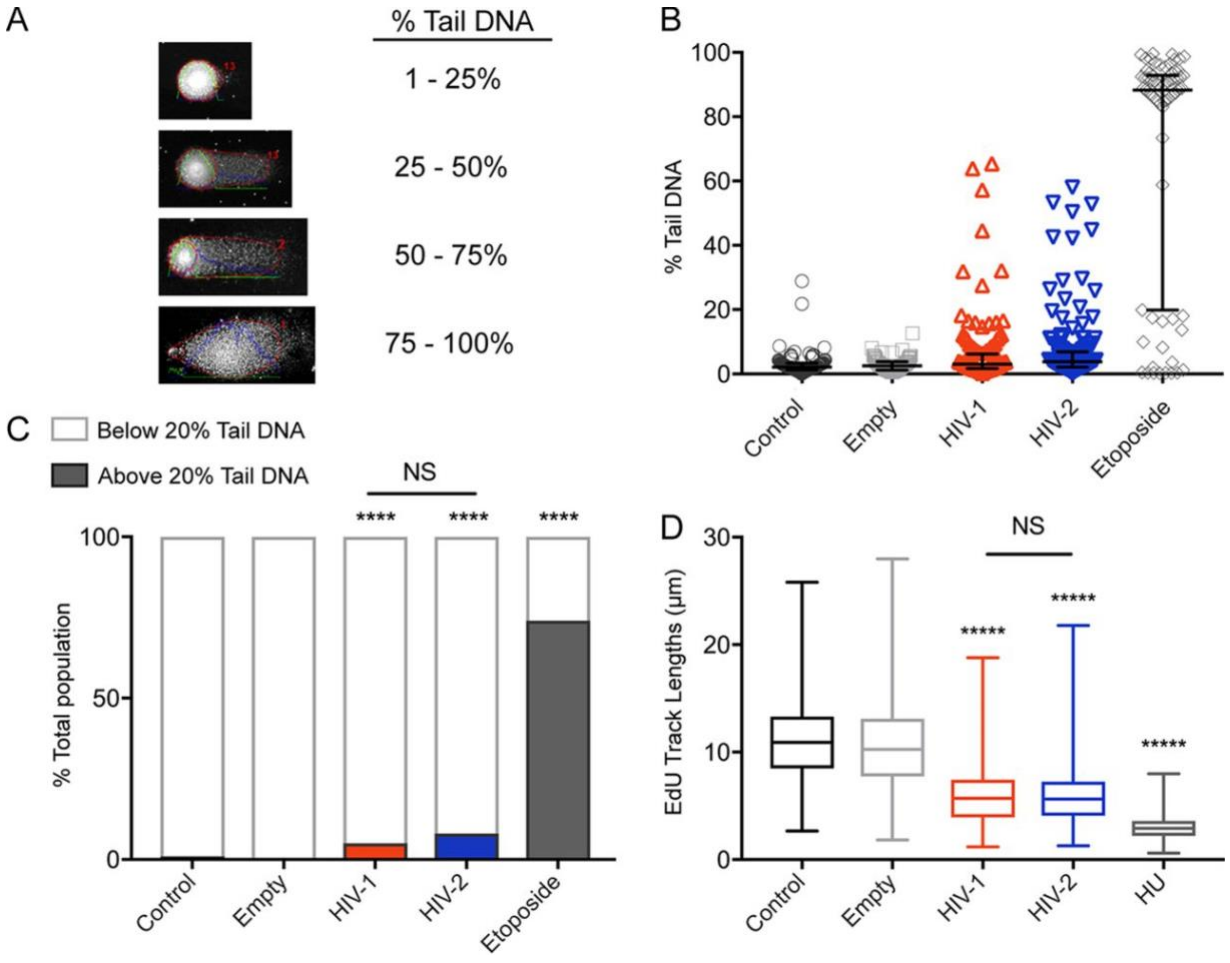


**Figure 3.3** Vpr-induced DNA damage occurs prior to replication fork stalling and is independent of ATR.

(A) U2OS cells treated under the same conditions as those for Fig. 1A were incubated with or without 10 μM VE-821 ATR inhibitor (ATRi) for 20 h and then subjected to the alkaline comet assay as described for Fig. 2B. Graph shows quantification of percent tail DNA of 100 cells measured per condition, with the bars representing the medians and interquartile ranges. ATRi-treated conditions are shown in filled shapes ($n = 3$, one representative experiment shown). (B) A bar graph representation of the data from panel A, with the population separated as shown in Fig. 2C. Cells treated with ATRi above 20% tail DNA are represented as the shaded regions with dots. Asterisks indicate statistical significance as determined by chi-square test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$, one representative experiment shown). (C) Distribution of EdU track lengths (μm) from cells treated under the same conditions as those for panel A. Cells treated with ATRi are represented as box plots with dots. Asterisks indicate statistical significance of empty vector with ATRi, HIV-1 with or without ATRi, HIV-2 with or without ATRi, and etoposide compared to empty vector without ATRi, as determined by the Kruskall-Wallis test, while statistical difference between empty vector, HIV-1, and HIV-2 with or without ATRi was determined by the Mann-Whitney test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$, one representative experiment shown).

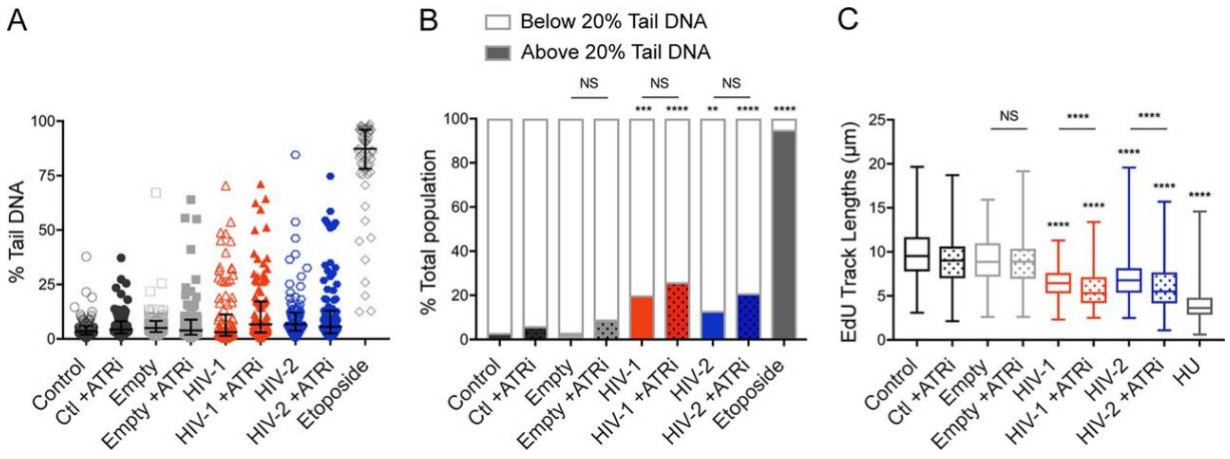


**Figure 3.4** Cells expressing HIV-1 or HIV-2 Vpr are hypersensitive to exogenous double-strand DNA breaks.

Sensitivities of the untreated control, empty vector control, HIV-1, and HIV-2 Vpr-expressing U2OS cells to etoposide, hydroxyurea, olaparib, and cisplatin were tested by incubating cells for 7 days in the corresponding drug at the indicated concentrations. Survival was analyzed by crystal violet staining for live cells compared to the no drug treatment. Sensitivity results are the means from three independent experiments ($n = 3$), and error bars represent ± standard deviations. Asterisks indicate statistical significance compared to empty vector control, as determined by 2-way analysis of variance (*, $P < 0.05$; **, $P < 0.03$;***, $P < 0.002$; ****, $P < 0.0001$).

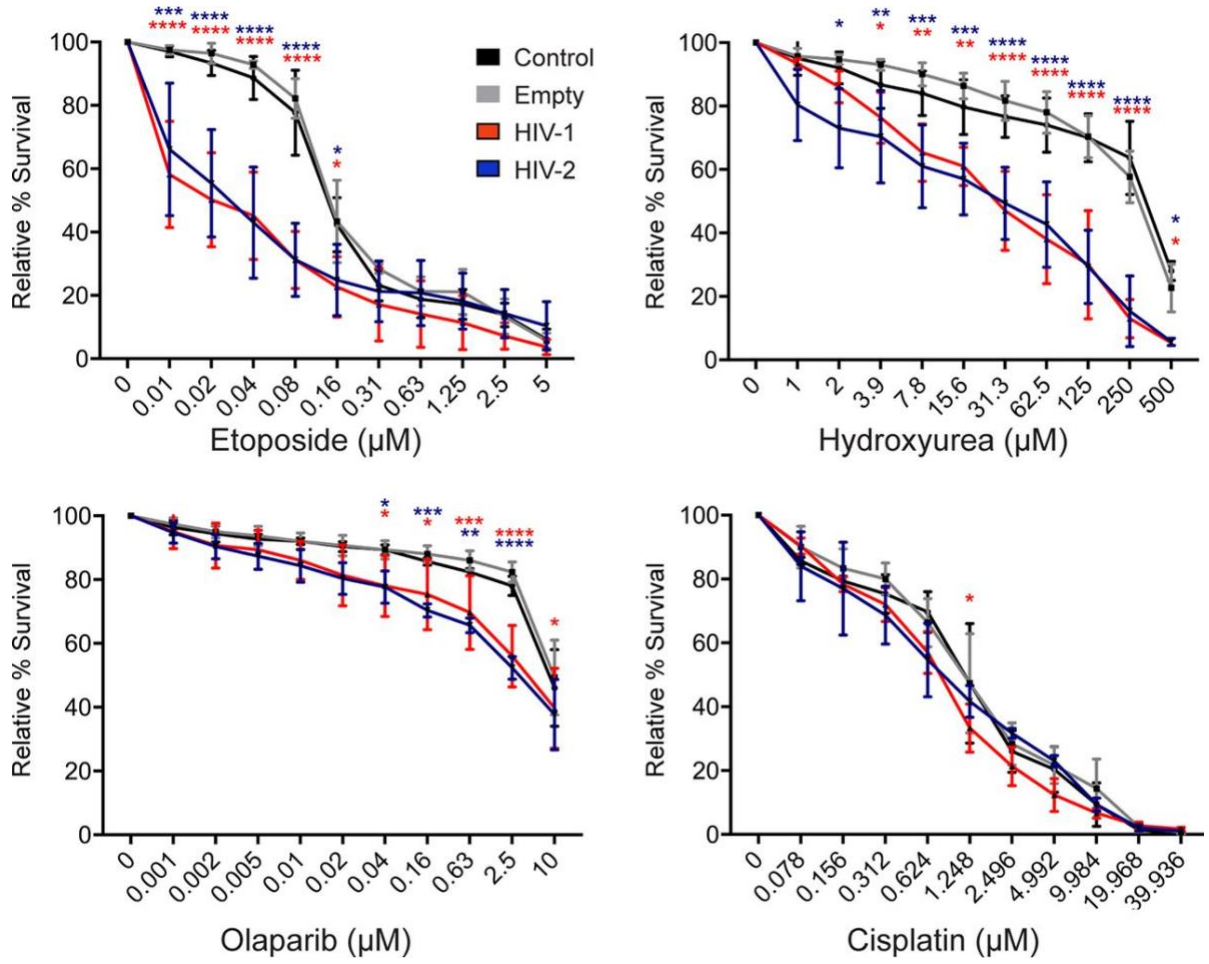


**Figure 3.5** HIV-1 and HIV-2 Vpr repress double-strand break repair.

(A, left) Schematic of I-SceI-based homologous recombination (HR) U2OS reporter cell line (DR-GFP assay). (Right) Representative flow cytometry plots of one I-SceI repair assay experiment for HR repair. Cells were transfected for 30 h with the I-SceI plasmid alone or with either empty vector, HIV-1, or HIV-2 Vpr that expresses mCherry via a T2A ribosomal skipping sequence. Twenty thousand cells were measured per condition and gated for homologous recombination-mediated DSB repair (GFP). (B, left) Schematic of I-SceI-based classical NHEJ U2OS reporter cell line (EJ5-GFP assay). (Right) Representative flow cytometry plots of one I-SceI repair assay experiment for NHEJ repair. Cells were treated and measured under the same conditions as those described for panel A. (C) I-SceI HR repair assay representing the average percent repair by homologous recombination from four experiments ($n = 4$), normalized to the I-SceI-only condition. Cells were treated and measured using the same conditions as those described for panel A. Asterisks indicate statistical significance compared to empty vector control, as determined by a one-sample *t* test (theoretical mean set to the average value of the empty vector control), while statistical difference between HIV-1 and HIV-2 was determined by the Mann-Whitney test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Error bars represent ± standard deviations. (D) I-SceI NHEJ repair assay representing average percent repair by classical nonhomologous end joining from four experiments ($n = 4$), normalized to the I-SceI-only condition. Cells were treated and measured under the same conditions as those described for panel A. Statistical analysis was determined with the same methods as those shown for panel C. Error bars represent ± standard deviations.

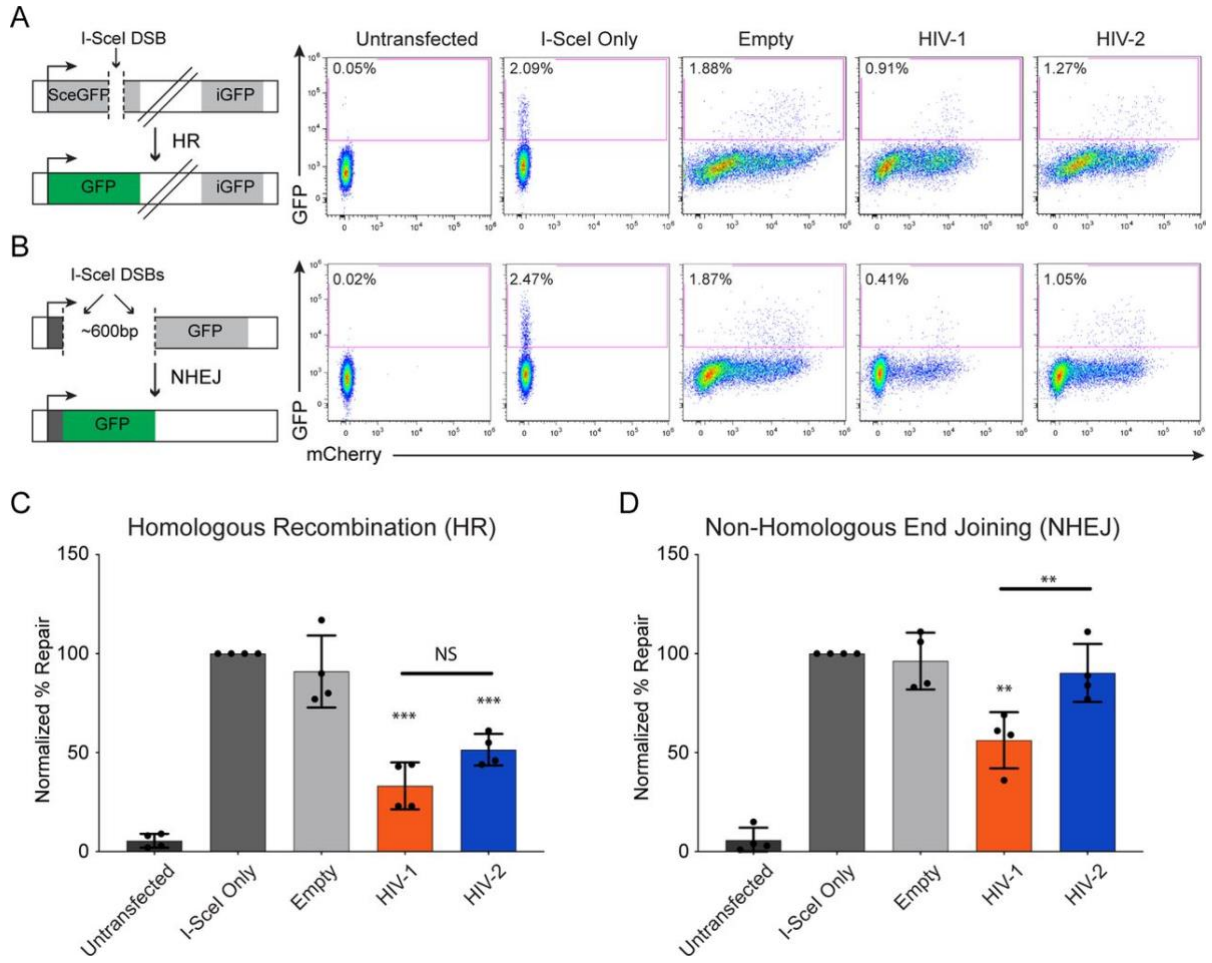


**Figure 3.6** Vpr-induced DNA damage is independent of repression of homologous recombination and cell cycle arrest.

(A) Bivariate cell cycle analysis of synchronized U2OS cells infected with rAAV expressing 3× FLAG-tagged HIV-1 and HIV-2 Vpr, empty vector, or control uninfected cells for 38 h. The graph shows the percentage of the population of 10,000 cells per condition in $G_1$, S, and $G_2$, measured using flow cytometry of cells stained for propidium iodide (PI; total DNA content) and EdU (DNA synthesis). Asterisks indicate statistical significance compared to empty vector control, as determined by Tukey's multiple-comparison test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$). Error bars represent ± standard deviations. (B) The alkaline comet assay for HIV-1 and HIV-2 Vpr mutants as represented in Fig. 2C with 100 cells measured per condition. U2OS cells were treated under conditions similar to those for Fig. 1A. Asterisks indicate statistical significance to empty vector control, as described for Fig. 2C ($n = 3$, one representative image shown). (C) Box and whisker plot representation of the distribution of EdU mean fluorescence intensity (MFI) for HIV-1 and HIV-2 Vpr mutants with cells treated under the same conditions as those for panel B. Asterisks indicate statistical significance to empty vector control, as determined by the Dunn's multiple-comparison test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$, one representative experiment shown). (D) Experimental results from the I-SceI DR-GFP assay representing average percent repair by homologous recombination for HIV-1 and HIV-2 mutants, as described for Fig. 5C. Asterisks indicate statistical significance from empty vector control, as described for Fig. 5C ($n = 3$).

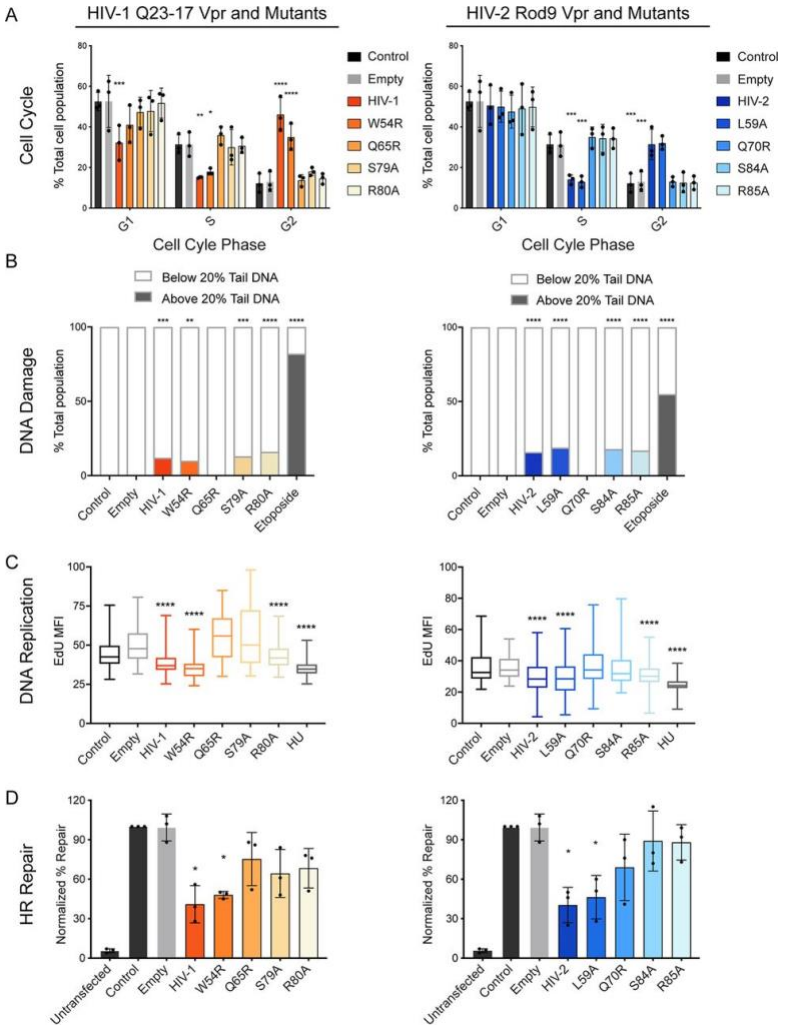
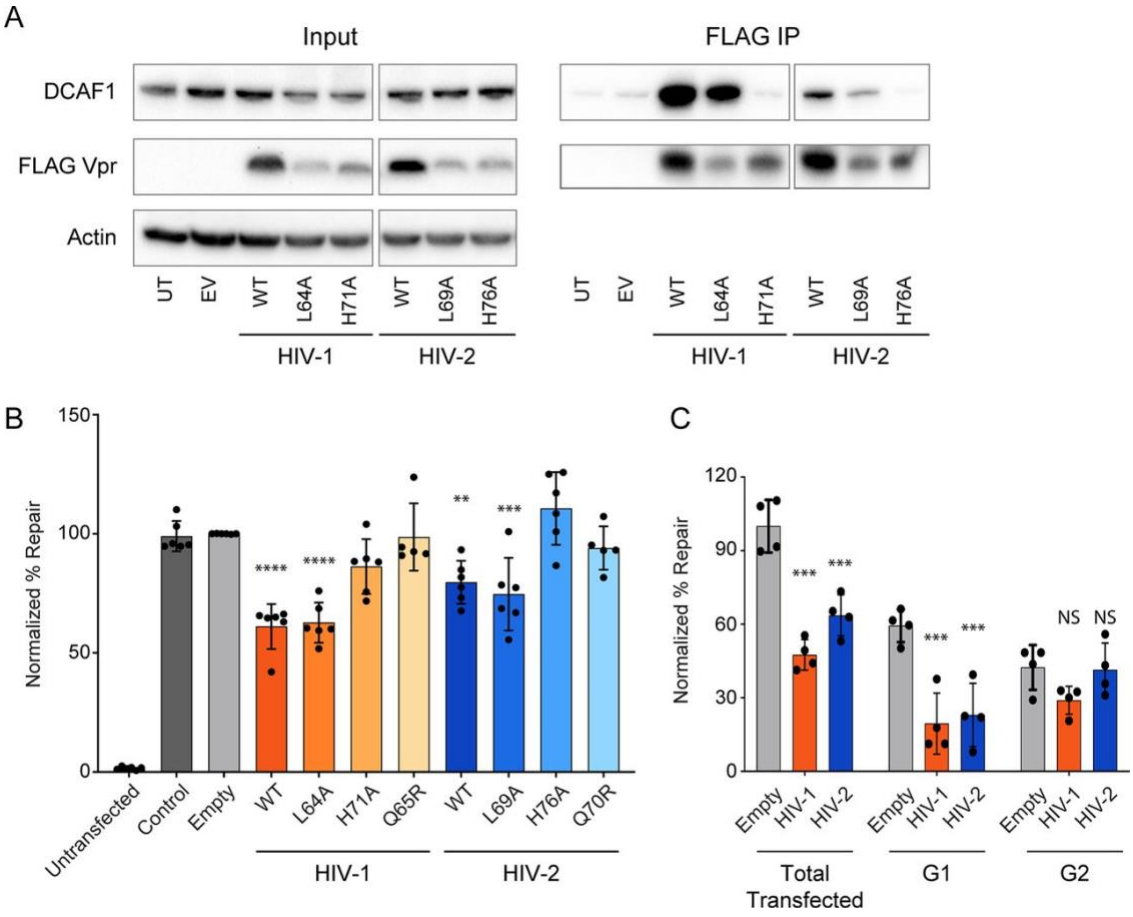


**Figure 3.7** Repression of homologous recombination by Vpr requires DCAF1 but does not require cell cycle arrest.

(A, left) Representative Western blots of U2OS cells for endogenous DCAF1, transiently transfected 3×-FLAG Vpr, and endogenous actin as a loading control. (Right) Immunoprecipitations against 3×-FLAG, probed for endogenous DCAF1 and transiently transfected 3×-FLAG Vpr. (B) Experimental results from the I-SceI DR-GFP assay representing average percent repair by homologous recombination for HIV-1 and HIV-2 mutants, as described for Fig. 5C. Asterisks indicate statistical significance from empty vector control, as described for Fig. 5C ($n = 6$). (C) Experimental results from bivariate I-SceI DR-GFP-cell cycle assay. Cells were transfected for 30 h with the I-SceI plasmid alone or with either empty vector, HIV-1, or HIV-2 Vpr that expresses mCherry via a T2A ribosomal skipping sequence and then labeled with Hoechst dye to label total DNA content. Twenty thousand cells were measured per condition. Total, $G_1$, and $G_2$ mCherry-expressing cell populations were gated for homologous recombination-mediated DSB repair (GFP). Asterisks indicate statistical significance from empty vector control, as described for Fig. 5C ($n = 4$).



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# Chapter 4: HIV Vpr Engages Several Chromatin-Associated Proteins

## 4.1 Introduction

Our current understanding of HIV Vpr, as illustrated by the previous chapter, is that the viral accessory protein activates and modulates the host DDR. This can be summarized by two independent major phenotypes: DNA damage and repression of DNA repair. We have established that Vpr phenotypes that are conserved between both HIV-1 and HIV-2, such as degradation of host proteins and activation of the DDR have robust cellular consequences associated with them. However, the model we generated in **chapter 3** is not fully explained by these phenotypes and remains incomplete. Despite our thorough understanding of the cellular consequences induced by Vpr and the many proteins potentially involved in Vpr function, none of these currently completely explain the model we have generated. Although our current model remains incomplete, it clearly defines the major phenotypes for Vpr function that are largely driven by nuclear repair factors and the DDR and begets the hypothesis that the primary role and function of Vpr is largely at chromatin. As such, it is likely that we are still missing key protein-protein interactions (PPIs) central to Vpr function. Recent literature has suggested that Vpr causes large-scale cellular proteomic changes that are conserved across both HIV-1 and HIV-2 (1). Despite these studies, our understanding of Vpr function remains unclear largely because they have primarily utilized IP MS approaches that have heavily relied on direct PPIs (2–4). Given that we now understand that Vpr functions in the nucleus and at or near chromatin, this could explain why IP MS approaches have not been able to identify that there is a single and primary target for Vpr due to the limitations of such approaches. Therefore, we hypothesize that Vpr primarily functions and alters the proteome at chromatin and that the function of Vpr is likely more complex than previously described altering several proteins and pathways rather than just a single protein target. This would also explain why previous literature have identified an array of targets for Vpr that are not conserved across Vpr orthologs, but are all involved in DNA repair and chromatin

functions (2–5). Despite the numerous previous MS studies and approaches, none to date have specifically interrogated proteomic changes at the chromatin level. As such, to get around these limitations of previous IP-MS approaches we required an approach that will allow us to bypass these limitations and properly interrogate the proteome of Vpr at chromatin.

Here, we utilized an APEX proximity labeling approach to determine what changes were occurring at chromatin in the presence of Vpr. This system was especially well suited for testing our hypothesis because it allowed us to circumvent many of the issues that have previously plagued understanding Vpr protein interactions at chromatin. Historically, there have been 4 major problems that have hindered our understanding of Vpr. 1) To sufficiently solubilize chromatin-bound and nuclear proteins necessitates harsh conditions that lose PPI required for IP-MS, it is extremely difficult to maintain direct PPIs that are critical for IP-MS approaches while sufficiently solubilizing the sample to free these proteins. 2) Previously utilized IP-MS approaches only capture direct protein-protein interactions and are unable to detect proteins that only transiently interact. 3) Little is currently known about Vpr localization and trafficking in the context of host proteins in or at chromatin, including proteins that are critical for Vpr function such as DCAF1. 4) The primary role of Vpr induces large-scale proteomic changes that completely change the cellular landscape and makes it critical that we understand what is occurring at a variety of different timepoints to fully understand how Vpr functions. Together, these obstacles have severely hindered the field and prevented us from understanding what the protein interactors of Vpr are at chromatin.

To get around all these obstacles, we developed a system using a APEX2 proximity labeling. This is because APEX proximity labeling covalently attaches a biotin molecule to proteins that are in direct contact with or near the bait protein. In doing so, these proteins are labeled with a robust molecular tag that is resistant to harsh solubilization techniques. This system also allowed us to characterize and pursue very specific timepoints. Because the biotinylating reaction

itself takes only approximately 30 seconds, this results in a very precise snapshot at a very specific timepoint. As such, this can be varied to look at very early and late stages of the lifecycle. By utilizing this system, we are not only able to avoid the problems that have plagued the Vpr field, but we can also by-pass restrictions and ask questions that could not previously be asked using conventional IP-MS approaches. This strategy not only allowed us to completely solubilize the chromatin fraction using techniques that otherwise would not be possible using conventional IP-MS techniques, but also allowed us to detect proteins that are transiently interacting with our bait protein. By extension, this also allowed us to ask and test for many other questions beyond just quantitative MS. Proximity labeling allowed us to observe where our specific bait protein is labeling and localizing by detection of biotinylation via immunofluorescence and CryoEM (7). This is particularly important for Vpr because we still do not have a complete understanding of its localization and trafficking especially in the context of chromatin. Most importantly, using an APEX proximity labeling-based system allowed us to completely disregard the limitations on solubility brought upon by classical IP-MS (6). Unlike a conventional IP, APEX labeling allowed us to ignore any limitations caused by solubility. In addition to all this, the system is easily amenable to other Vpr orthologs and mutants that can be utilized as baits.

Here, we generate HIV-1 Q23-17 and HIV-2 Rod9 APEX-Vpr constructs to further investigate the proteome of HIV Vpr at the chromatin. In addition to this, we generated a HIV-2 Rod9 Vpx construct to act as a spatial control. Because Vpx engages similar proteins to Vpr and localizes to the nucleus but has distinct mechanisms from Vpr we use this to subtract noise that would arise from nuclear proximity labeling and engaging DCAF1 (8–11). This way, we not only improve our confidence in which proteins are distinctly important for Vpr, but also learn about Vpx function which has primarily been studied in the context of the cytoplasm. We utilized proximity labeling with immunofluorescence to examine where in the nuclear compartment Vpr labels along with APEX-MS (AP-MS) to establish a chromatin-associated proteome. Importantly, these

techniques can be repurposed for the various Vpr mutants that we used to characterize Vpr function and build a model with as discussed in **chapter 3**. By using these mutants, we can obtain a much more precise representation of what PPIs are occurring at chromatin to better understand the differences that lead to the phenotypes we have extensively characterized. Importantly, the scope of these experiments can be further expanded in the future by utilizing the other aspects of proximity labeling such as CryoEM and exploiting the temporal flexibility of the system by examining other timepoints of the viral lifecycle.

## 4.2 Results

*Generation and validation of APEX constructs*

Previous literature indicated that Vpr is sensitive to fusions with large proteins, especially at the C- terminus. However, it has previously been shown to tolerate proteins such as GFP fused to the N-terminus, which are relatively small, and still allow Vpr to localize and function properly. Because of this, it was imperative determine whether Vpr could tolerate a fusion with APEX at either terminus. Therefore, we generated HIV-1 Vpr, HIV-2 Vpr, and HIV-2 Vpx constructs with APEX at either the N- or C-terminus. To validate these constructs and to determine whether N- or C-terminal APEX tagging would be tolerated by Vpr/Vpx, we verified that four primary characteristics were preserved: localization, interactions, labeling, and function.

Both C- and N-terminally fused Vpr-APEX constructs were generated to assay for viability of the constructs. We transiently transfected U2OS cells with Vpr-APEX constructs and assayed for localization by immunofluorescence. We observed that both HIV-1 and HIV-2 Vpr localized properly when N-terminally fused, however, were unable to localize properly to the nucleus or at chromatin when fused at the C-terminus. Additionally, HIV-1 and HIV-2 Vpr localized in a perinuclear and pan-nuclear pattern as expected, respectively. Engagement of DCAF1 was assayed by performing a co-IP for DCAF1 with the APEX-Vpr constructs. We determined that all

the APEX-Vpr constructs were able to pull down DCAF1 (Figure). Proximity labeling was assayed by performing western blot and immunofluorescence to detect hyperbiotinylation which would indicate that proximity labeling had occurred. Through western blot, we detected hyperbiotinaylation for all APEX-Vpr constructs. Using immunofluorescence we also observed hyperbiotylation, however, only the N-terminally fused constructs labeled at the appropriate nuclear and chromatin cellular compartments whereas the C-terminally fused constructs labeled and localized cytosolically. In summary, our findings corroborated previous Vpr studies showing that Vpr is only able to tolerate fusions at the N-terminus otherwise it becomes experimentally nonfunctional (Table). The same assays were used for validating our APEX-Vpx constructs and revealed that, like APEX-Vpr, the N-terminally fused construct met our criteria. We thus proceeded with N-terminally tagged APEX-constructs for downstream MS analysis.

To determine whether we could quantify and identify biotinylated proteins via MS, we performed a proximity labeling reaction followed by an IP with streptavidin beads. Here we detected several proteins previously characterized to interact with Vpr such as SLX4, HLTF, CCDC137, and EXO1. In addition, we detected some insoluble chromatin-associated proteins such as various DNA binding proteins like histones. This validated that our system could detect previously characterized Vpr interactors as well as a diversity of chromatin-associated proteins.

We next developed a method that could effectively solubilize the chromatin fraction to improve chromatin-associated protein enrichment. To achieve this, we utilized a combination of sonication and harsh chemical denaturation using chaotropic agents such as SDS to solubilize proteins in the insoluble chromatin fraction. This approach resulted in increased solubilization of chromatin-associated proteins but hindered downstream MS detection due to utilizing detergents that were incompatible with MS (data not shown). To circumvent this, we utilized salt-based fractionation to first isolate nuclei and then solubilize the chromatin fraction via MNase treatment and a series of washes containing increasing amounts of NaCl. Salt-based fractionation highly

enriched for chromatin-associated proteins without introducing MS-incompatible detergents into the system. This resulted in much higher detection of insoluble chromatin-associated proteins such as histones via MS, however, this approach also revealed that we were detecting too much background noise in our controls samples (data not shown). To ameliorate this, we optimized the IP conditions by varying the number of beads and the stringency of the washes by utilizing harsher salt, pH, and chaotropic agent conditions. By performing an IP followed by a silver stain and MS, we determined that increasing the wash stringency and reducing the amount of streptavidin beads during the IP reduced background noise while preserving enrichment of our proteins of interest (figure). This resulted in an approach that entailed isolating nuclei and solubilizing chromatin-associated proteins via MNase and increasing salt washes, followed by an IP with stringent washes.

*HIV Vpr Engage Proteins involved in Chromatin Remodeling and Proteasomal Degradation*

Proximity labeling by APEX-Vpr followed by AP-MS of the chromatin fraction revealed that both HIV-1 and HIV-2 Vpr engage several components of the chromatin-associated proteome. As expected, majority of these proteins have not been previously characterized due to their highly insoluble chromatin environment. Despite this, MSstats of our proximity labeling approach identified over 240 chromatin-associated proteins that were significantly enriched or depleted. To further analyze these hits, we used Metascape network analyses to identify several significant PPIs and networks belonging to chromatin assembly/disassembly, DNA damage response, transcription, and proteasomal degradation. A caveat of this study, however, is that due to the nature of APEX proximity labeling we cannot distinguish between cellular and localized depletion/enrichment or proteins. As such, when we refer to enrichment or depletion, we refer to the data analyses that show statistically significant enrichment or depletion.

Of the groups listed, one of the most significant groups was related to chromatin/nucleosome dynamics. In the context of chromatin/nucleosome assembly and disassembly, we identified numerous histones including H1, H2, H3, and H4. Importantly, we identified various histones belonging to the H2A and H2B family, suggesting that these play an important role in Vpr function. Furthermore, we also identified H2AX as a statistically significant hit for Vpr which is significant in that we have previously characterized the importance of engaging the DDR and activating phosphorylation of H2AX. In addition to this, we also identified H1.2 as a significant interactor for HIV Vpr, which is significant because one previous study has suggested that H1.2 plays an important role for HIV-1 Vpr function. Despite this, however, we observed opposite phenotypes for HIV-1 and HIV-2 in our system. Whereas nucleosome and chromatin remodeling factors seemed to be enriched for HIV-2, only a few of these were enriched for in HIV-1 and most were depleted.

Another prominent group that emerged from Metascape analyses were proteins associated with nuclear proteasomal degradation. In particular, the PA28y-20S proteasome and its corresponding subunits were highly enriched for both HIV-1 and HIV-2 Vpr. More specifically we detected several PSMA, PSMB, and PSME subunits of the nuclear proteasome involved in in regulation and assembly of PA28$\gamma$-20S. This is especially significant due to the ability of Vpr to engage the CRL4A$^{DCAF1}$ E3 ubiquitin ligase complex. Despite this, however, we did not detect any components of the E3 ligase complex or DCAF1 itself for either HIV-1 or HIV-2 Vpr. Despite this, we detected significant enrichment and depletion of factors related to transcription such as TAF1 and TAF8 which are involved in initiating transcription. In addition to this, we observed that several of our top hits are regulated by transcriptional activators such as SP1 and NF-$\kappa$B.

Here, we revealed that APEX proximity labeling is an effective tool to identify the nuclear proteome of HIV Vpr. Importantly, developing this technique for a viral chromatin associated protein has allowed us to study the proteome of Vpr in a way that has not been previously

achievable. Previous methods have been restricted by the limitations of conventional IP-MS, which rely heavily on direct PPIs and have limited the resolution of the chromatin-associated proteome for Vpr. However, here we have circumvented this problem and removed the limitations of necessitating preserving direct PPIs. By doing so, we have been able to effectively interrogate the chromatin-associated proteome of HIV Vpr and identified several factors that Vpr engages. We have identified four major groups of proteins and networks that are significant and enriched by HIV Vpr in the chromatin: chromatin assembly/disassembly, proteasomal degradation, and transcription. Together, these not only support the hypothesis that engagement of the DDR is central to the role of HIV Vpr, but also suggest that the role of Vpr is likely more complex than simply degrading a target protein as previously thought.

Like another previous Vpr proteome study, our findings suggest that Vpr engages numerous proteins and likely causes considerable proteomic changes. When we interrogate the deeper insoluble chromatin fraction, we detect several histone proteins and subunits of the nuclear proteasome complex. This could suggest that through engagement of the E3 ubiquitin ligase complex, Vpr is causing substantial changes in the proteome via degradation of chromatin-associated factors. This could explain why so many diverse DDR associated proteins such as UNG2, HLTF, and SLX4 have previously been associated with Vpr function, but have not been conserved across Vpr orthologs. Vpr could potentially be utilizing DDR associated factors to localize to regions of the chromatin in a non-specific manner to induce greater proteome-wide changes that could aid in processes such as transcription. Indeed, here we observed that both HIV-1 and HIV-2 induced changes in transcriptional machinery and signaling pathways. We also observed that many of the proteins enriched by HIV Vpr are associated with SP1 and NF-KB signaling, which have previously been extensively characterized to be important for viral transcription. Despite this, however, we don't observe a seamless parallel between HIV-1 and HIV-2 protein enrichment.

Like most phenotypes that we have characterized thus far, HIV-1 and HIV-2 Vpr deviate in their protein enrichment profiles. Here we observed that although we see a significant increase in enrichment for histones for HIV-2 Vpr, this is not paralleled by HIV-1 Vpr. Although we identify enrichment of some histones, we also identify a depletion of many histones for HIV-1 Vpr. Although unclear from the current data, this could be due to differences in localization of HIV-1 and HIV-2 Vpr or potentially deviations in the rate of proteasomal degradation by HIV-1 Vpr. This would require sampling different timepoints to determine whether there are changes at earlier or later timepoints and utilizing proteasome inhibitors to assess whether there are changes in enrichment or depletion. This could also help better explain why we don't observe factors that have previously been characterized to be degraded by Vpr. During the initial optimization of proximity labeling solubilization and MS where we weren't fully solubilizing chromatin or isolating nuclei, we detected several Vpr-associated components of the $CRL4A^{DCAF1}$ E3 ligase complex as well as targets of degradation such as HLTF, EXO1, and CCDC137. However, as we optimized and improved our solubilization of chromatin, we observed less of these until we could eventually not detect them. This could be because these factors are degraded early or that their degradation is a result of more complex chromatin dynamic changes that occur due to the presence and localization of Vpr at chromatin.

Overall, we have determined that engagement of the chromatin-associated proteome is extensive for HIV Vpr. While previously characterized processes such as engagement of the DDR, repression of HR, and cell cycle arrest are further supported be these observations, we also observe novel insight into the function of Vpr. We observe that engagement of histones and the nuclear proteasome is likely a fundamental component of Vpr function. This could suggest that Vpr could be localizing the nuclear proteasome to specific sites at chromatin and inducing nuclear-wide changes to chromatin dynamics via degradation. This could help explain why we observe changes in repair and induction of markers of damage and potentially bridge the gap that exists

in our model that separates DNA damage from the other cellular consequences we have observed. It is possible that drastic and dysregulated chromatin dynamics induced by Vpr could lead to DNA damage. The differences we observe here between HIV-1 and HIV-2 could also potentially relate to the ability of HIV-2 to infect non-cycling cells more efficiently, where the chromatin environment is likely less dynamic. It is likely the function of Vpr is far more complex than has previously been assumed, and here we have begun to observe the extent of that. From the data, we also observe that the other pillars of viral engagement of the DDR such as chromatin dynamics and transcription are likely also important for Vpr function and the greater HIV viral lifecycle and merit further investigation.

## 4.3 Methods and Materials

*Plasmids*

pcDNA APEX-Vpr plasmids were generated by inserting synthesized gBlocks (IDT) with APEX2 and either HIV-1 Q23-17 or HIV-2 Rod9 Vpr into pcDNA 3.1 using standard cloning techniques. pcDNA APEX-Vpx construct was generated by replacing Vpr with HIV-2 Rod9 Vpx that was amplified using PCR and subcloned using standard cloning techniques. Vpr mutants were generated using site directed mutagenesis (Q5 site directed mutagenesis kit, NEB).

*Cell lines & Cell Culture*

Human embryonic kidney (HEK 293T) and human bone osteosarcoma epithelial (U2OS) cells were cultured as adherent cells directly on tissue culture plastic (Greiner) in DMEM growth medium (high glucose, L-glutamine, no sodium pyruvate; Gibco) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% $CO_2$. All cells were harvested using 0.05% Trypsin-EDTA (Gibco).

*Western Blots*

Cells we lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, Benzonase, Protease inhibitor). Lysates were clarified by centrifugation at 14,500g for 10 min and boiled in 4X sample buffer (4 ml glycerol, 2.4 ml 1M Tris pH 6.8, 0.8 g SDS, 0.5 ml β-mercaptoethanol, 3.1 ml ddH2O, bromophenol blue) in preparation for SDS-PAGE using 4-12% Bis-Tris polyacrylamide gels and subsequently transferred onto a PVDF membrane. Immunoblotting was performed using: mouse anti-FLAG (1:2000; Sigma), mouse anti-Actin (1:5,000), rabbit-anti-VPRBP (1:2000; Cell Signaling), anti-Biotin conjugated (1:2000; Jackson), anti-Biotin HRP (1:10000; Thermo) goat anti-mouse HRP (1:5000; Invitrogen), goat anti-rabbit HRP (1:5000; Invitrogen).

*APEX Proximity Labeling*

U2OS cells were APEX proximity labeled with biotin as previously described (ref). Briefly, U2OS cells were preincubated with 500 uM biotin-phenol (Adipogene) for 30 min at 37°C. After incubation, the proximity labeling reaction was induced by adding 10uM $H_2O_2$ DMEM at a 1:1 ratio. After 30 seconds, media was removed, and the reaction was quenched using ice cold quenching buffer. Cells were washed 3 times with ice cold quenching buffer and incubated on ice for 20 minutes. The cells were then harvested for nuclear fractionation.

*Salt Fractionation*

U2OS cells were harvested and lysed with a dounce homogenizer in a hypotonic solution. Nuclei were then collected using centrifugation and digested using micrococcal nuclease for 30 min at 37°C. Nuclei were then dissolved and solubilized using salt fractionation. Using increasing NaCl concentrations from 80 mM to 600 mM, chromatin was fractionated, collected, and pooled. Samples were then diluted using a 2X RIPA solution at a 1:1 ratio and immunoprecipitated

overnight at 4°C using magnetic Streptavidin beads. Beads were then washed 3X with RIPA, 1X with 1M KCl, 1X with NaCO3, 1X with 1M Urea, and 2X with Tris-HCl pH 8.0. Beads were collected on a magnetic rack and resuspended in Tris-HCl pH 8.0 in preparation for MS processing.

*Proteomics*

Samples were transferred to a new tube and suspended in 2M urea, 50 mM Tris-HCl. They were then alkylated using 5 mM iodoacetamide and incubated in a shaker at 37°C for 30 min. Samples were then quenched using 5 mM DTT and digested using 1.5 ug Trypsin overnight at 37°C. The following morning the supernatant was collected and digested with additional 1 ug of Trypsin at 37°C for 2 hours. The samples were then collected in a fresh tube and acidified using TFA. They were then desalted using C18 Microspin columns and dried in a speedvac for 1.5 hours.

*Bioinformatics*

Shotgun MS data analyses were performed using statistical models in the MSstats package to calculate fold changes (FC) and *p-values* (Choi et al., 2014). Proteins with a *p-value* ≤ 0.05 and a log2 FC ≥ 1 were considered significant. Four biological replicates were performed, and each biological replicate was grown and processed at the same time. Untreated U2OS cells were used as controls and APEX2-Vpx was used as a spatial reference.

## 4.4 Discussion

Here, we revealed that APEX proximity labeling is an effective tool to identify the nuclear proteome of HIV Vpr. Importantly, developing this technique for a viral chromatin associated protein has allowed us to study the proteome of Vpr in a way that has not been previously achievable. Previous methods have been restricted by the limitations of conventional IP-MS,

which rely heavily on direct PPIs and have limited the resolution of the chromatin-associated proteome for Vpr. However, here we have circumvented this problem and removed the limitations of necessitating preserving direct PPIs. By doing so, we have been able to effectively interrogate the chromatin-associated proteome of HIV Vpr and identified several factors that Vpr engages. We have identified four major groups of proteins and networks that are significant and enriched by HIV Vpr in the chromatin: chromatin assembly/disassembly, proteasomal degradation, transcription, and the DDR. Together, these not only support the hypothesis that engagement of the DDR is central to the role of HIV Vpr, but also suggest that the role of Vpr is likely more complex than simply degrading a target protein as previously thought.

*Vpr is in proximity of several chromatin-associated proteins*

Like another previous Vpr proteome study (1), our findings suggest that Vpr engages numerous proteins and likely causes considerable proteomic changes. When we interrogate the deeper insoluble chromatin fraction, we detect several histone proteins and subunits of the nuclear proteasome complex. This could suggest that through engagement of the E3 ubiquitin ligase complex, Vpr is causing substantial changes in the proteome via degradation of chromatin-associated factors. This could explain why so many diverse DDR associated proteins such as UNG2, HLTF, and SLX4 have previously been associated with Vpr function, but have not been conserved across Vpr orthologs (12). Vpr could potentially be utilizing DDR associated factors to localize to regions of the chromatin in a non-specific manner to induce greater proteome-wide changes that could aid in processes such as transcription. Indeed, here we observed that both HIV-1 and HIV-2 induced changes in transcriptional machinery and signaling pathways. We also observed that many of the proteins enriched by HIV Vpr are associated with SP1 and NF-KB signaling, which have previously been extensively characterized to be important for viral transcription(13–15). Furthermore, we observed that engagement of PTEN is conserved between

both HIV-1 and HIV-2. This is important because, nuclear PTEN is involved in several processes that we have previously determined to be important for Vpr function. In the nucleus, PTEN regulates chromatin remodeling, cell cycle arrest, and double-strand break repair. Strikingly, these are all processes and cellular consequences that we have shown to be central to Vpr function and could be a potential missing link that joins the seemingly disparate model that we currently have for Vpr.

*Previously characterized Vpr targets are absent in our system*

Like most phenotypes that we have characterized thus far, HIV-1 and HIV-2 Vpr deviate in their protein enrichment profiles. Here we observed that although we see a significant increase in enrichment for histones for HIV-2 Vpr, this is not paralleled by HIV-1 Vpr. Although we identify enrichment of some histones, we also identify a depletion of many histones for HIV-1 Vpr relative to HIV-2 Vpr. Although unclear from the current data, this could be due to differences in localization of HIV-1 and HIV-2 Vpr or potentially deviations in the rate of proteasomal degradation by HIV-1 Vpr. This would require sampling different timepoints to determine whether there are changes at earlier or later timepoints and utilizing proteasome inhibitors to assess whether there are changes in enrichment or depletion. This could also help better explain why we don't observe factors that have previously been characterized to be degraded by Vpr. During the initial optimization of proximity labeling solubilization and MS where we weren't fully solubilizing chromatin or isolating nuclei, we detected several Vpr-associated components of the $CRL4A^{DCAF1}$ E3 ligase complex as well as targets of degradation such as HLTF, EXO1, and CCDC137 (16). However, as we optimized and improved our solubilization of chromatin, we observed less of these until we could eventually not detect them. This could be because these factors are degraded early or that their degradation is a result of more complex chromatin dynamic changes that occur due to the presence and localization of Vpr at chromatin.

*The function of Vpr is likely more complex than degrading a singlular target*

Overall, we have determined that engagement of the chromatin-associated proteome is extensive for HIV Vpr. Unlike previously believed, Vpr likely engages a variety of different proteins and does not have only one target protein. This theory is further supported by studies on other accessory genes, that are now showing that they too are also multifunctional. Additionally, while previously characterized core Vpr processes such as engagement of the DDR, repression of HR, and cell cycle arrest are further supported be these observations, we also observe novel insight into the function of Vpr. We observe that engagement of histones and the nuclear proteasome is likely a fundamental component of Vpr function. This could suggest that Vpr could be localizing the nuclear proteasome to specific sites at chromatin and inducing nuclear-wide changes to chromatin dynamics via degradation. Importantly, this parallels work showing that engagement of H1.2 is important for Vpr function (17). Strikingly, we also show that engagement of PTEN is likely important for the role of Vpr. This could help explain why we observe changes in repair and induction of markers of damage and potentially bridge the gap that exists in our model that separates DNA damage from the other cellular consequences we have observed. It is possible that drastic and dysregulated chromatin dynamics induced by Vpr could lead to DNA damage. The differences we observe here between HIV-1 and HIV-2 could also potentially relate to the ability of HIV-2 to infect non-cycling cells more efficiently, where the chromatin environment is likely less dynamic (11, 18, 19). Taken together, the data suggest that it is very likely that the function of Vpr is far more complex than has previously been assumed, and here we have begun to observe the extent of that. From the data, we also observe that the other pillars of viral engagement of the DDR such as chromatin dynamics and transcription are likely also important for Vpr function and possibly the greater scope of the HIV viral lifecycle and merit further investigation. We suggest that the field begins to better understand the role Vpr plays in cellular transcription as well as chromatin dynamics. It will also be crucial to understand the cellular

crosstalk between innate immunity and the DDR, as we observe several chromatin-associated factors that are also involved in immunity and closely entwined with DDR signaling.

## 4.5 Figures

**Figure 4.1** Validation of APEX2 constructs.

(A) Western blots HIV Vpr and Vpx APEX constructs. HIV-1 (H1) Vpr, HIV-2 (H2) Vpr, and HIV-2 Vpx expression in the chromatin fraction were normalized. Biotinylation across all the construts was also assessed in the context of normalized expression. (B) Immunofluorescence of APEX constructs Vpr (myc, green) and biotinylation (red).

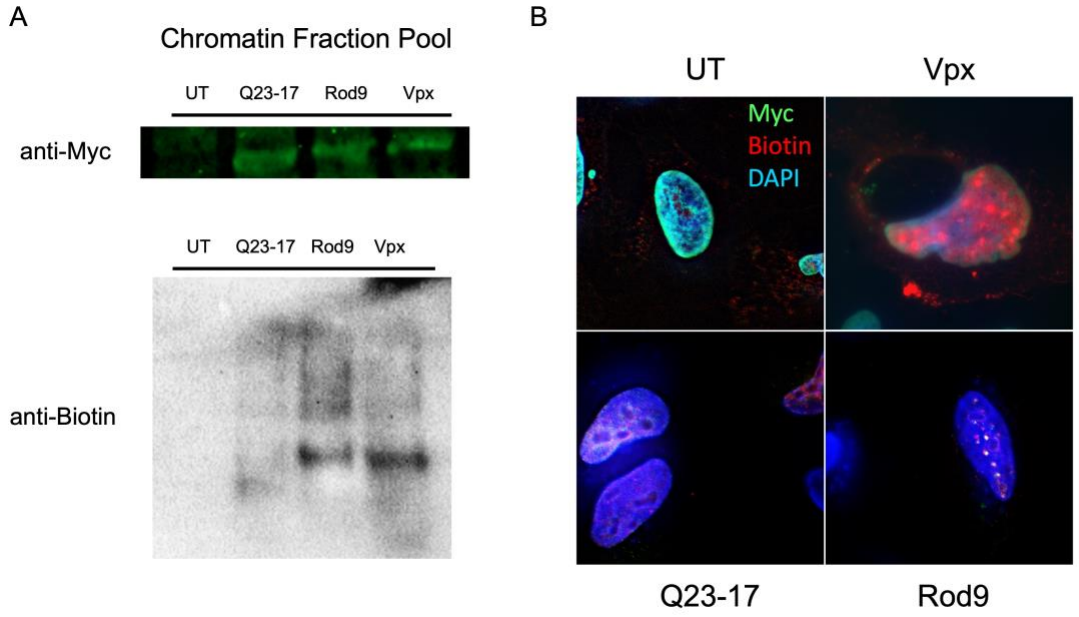


**Figure 4.2** Correlation analyses across APEX2 replicates indicates there are unique differences between samples.

(A) A heatmap of the APEX replicates analyzed using MSstats for Q23-17, Rod9, Vpx, and untreated samples. Sample relatedness is indicted by phylogeny and color. (B) PCA analysis of relatedness across and between APEX2 replicates. Circled clusters indicate samples belonging to the same replicate group.

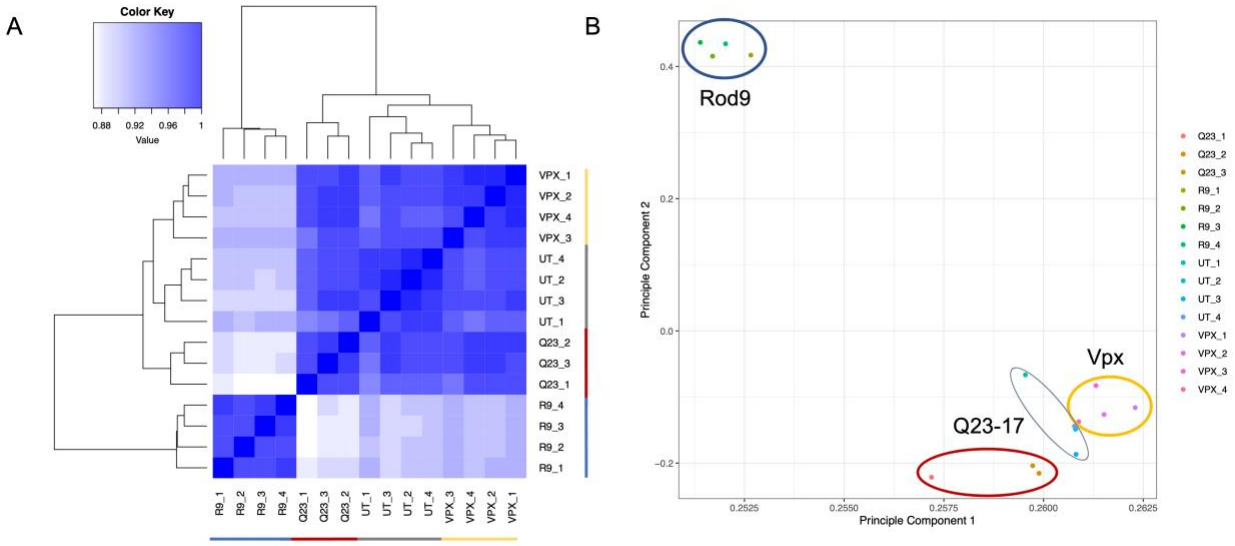


**Figure 4.3** Statistical analyses of proteomics reveals several proteins are enriched and depleted across all APEX2 constructs.

(A) Volcano plots generated by MSstats analyses of proteomics data. Cutoffs for indication of increase or decrease were set to a $p$ *value* ≤ 0.05 and a log2 FC ≥ 1. Samples were compared to untransfected control, APEX2-Vpx spatial reference, and between HIV-1 and HIV-2 Vpr. (B) Bar graphs visualizing the number of differentially abundant proteins for each sample comparison.

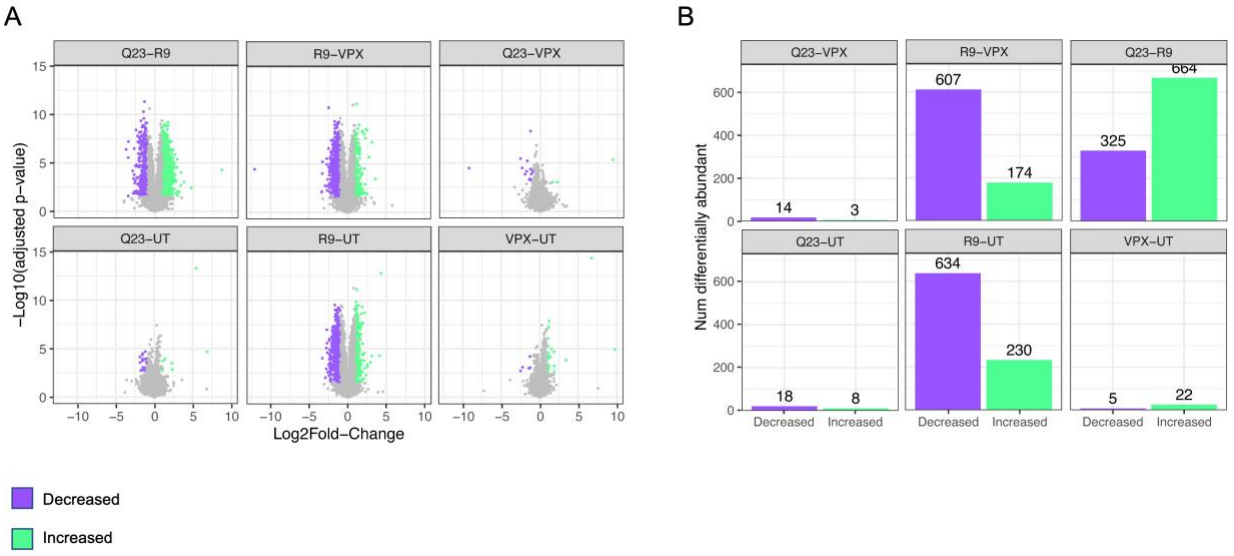


**Figure 4.4** Network analyses reveals protein-protein interactions and networks shared between HIV-1 and HIV-2.

(A) Metascape generated network analyses of enriched proteins determined by MSstats analyses with a *p value* ≤ 0.05 and a log2 FC ≥ 1. Metascape networks reveal that several enriched top hits are shared between HIV-1 and HIV-2. (B) Gene Ontology shows that these networks belong to several categories. Most prominent are DDR, nucleosome assembly, and protesome pathways.

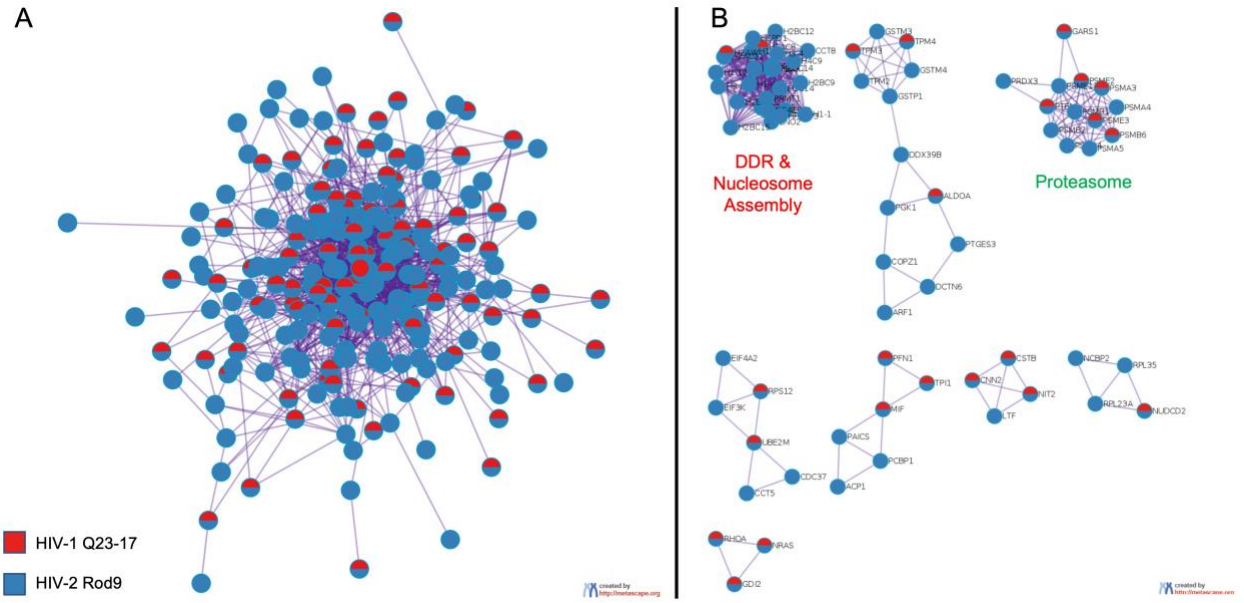


**Figure 4.5** Both HIV-1 and HIV-2 Vpr are in proximity of two major pathways: Nuclear Proteasome and Nucleosome Assembly.

(A) Metascape generated network analyses of enriched proteins determined by MSstats analyses with a *p value* ≤ 0.05 and a log2 FC ≥ 1. Metascape networks reveal that several enriched top hits are shared between HIV-1 and HIV-2. (B) Gene Ontology shows that these networks belong to several categories. Analyses indicated that both HIV-1 and HIV-2 are in close proximity to networks involved in the nuclear proteosome PA28y-20S and several histones involved in nucleosome assembly.

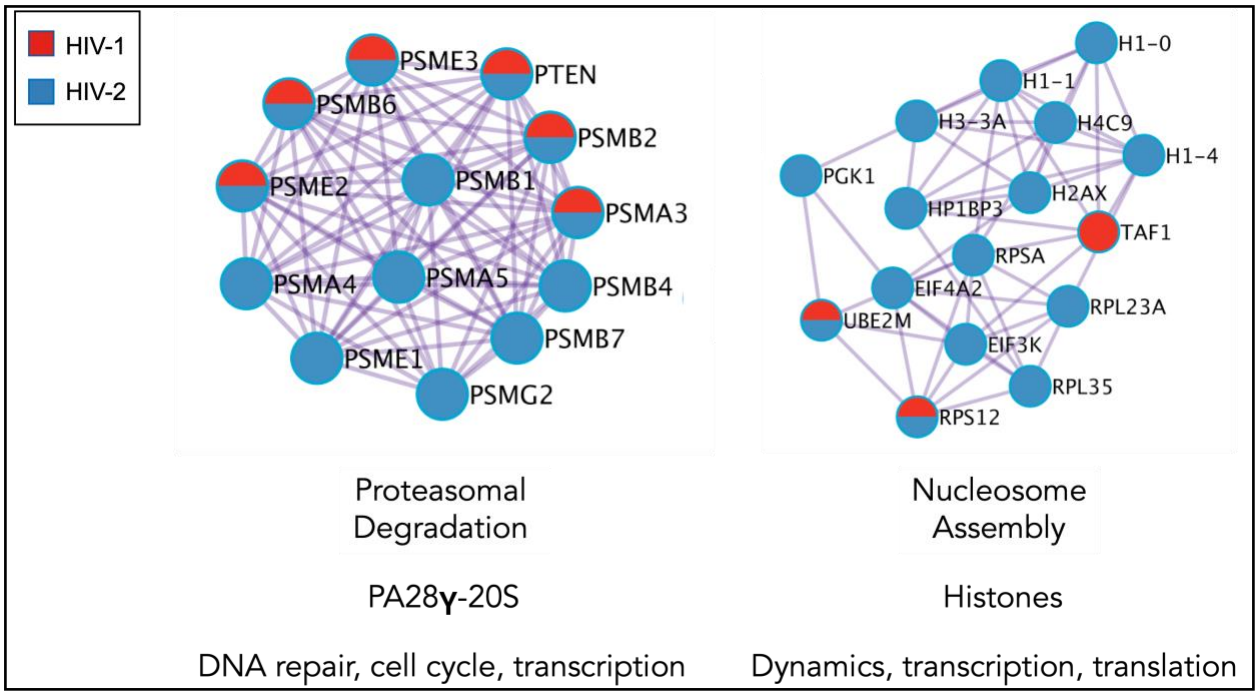


**Figure 4.6** APEX2-Rod9 highly enriches for proteins involved in DDR, nucleosome assembly, and proteasomal pathways.

(A) Metascape generated network analyses of enriched proteins determined by MSstats analyses with a *p value* ≤ 0.05 and a log2 FC ≥ 1. Metascape networks reveal that several enriched top hits for HIV-2 are highly interconnected. (B) Gene Ontology shows that these networks belong to several categories. Most prominent are DDR, nucleosome assembly, and proteasome pathways. Other networks include transcriptional and translational regulation.

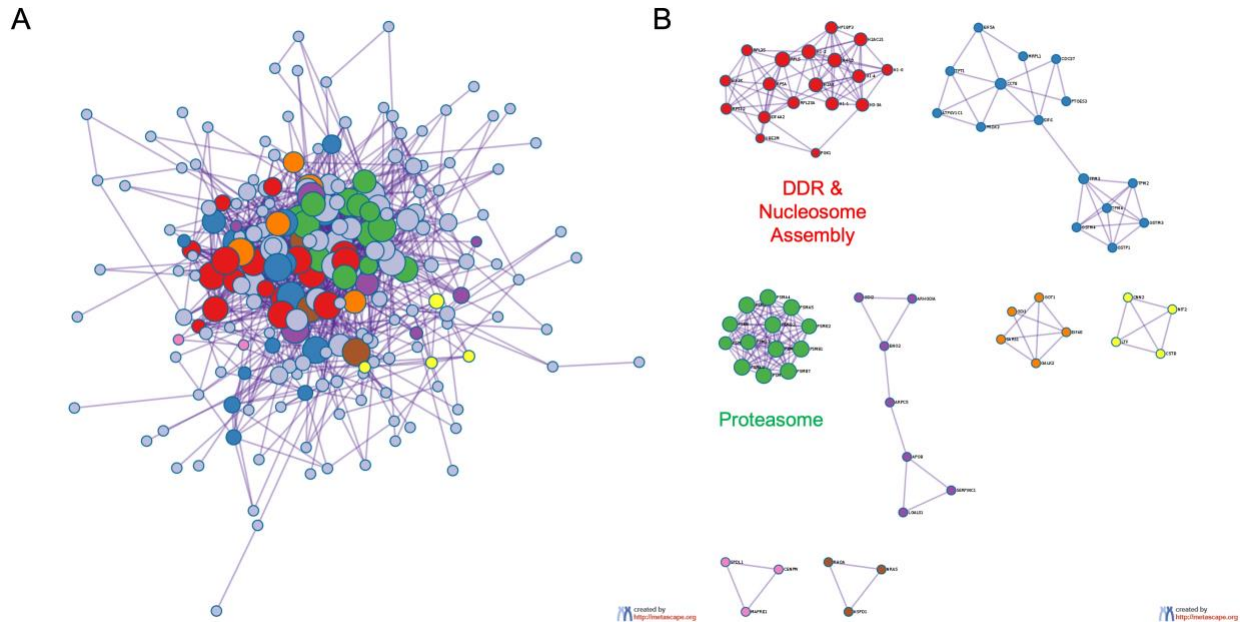
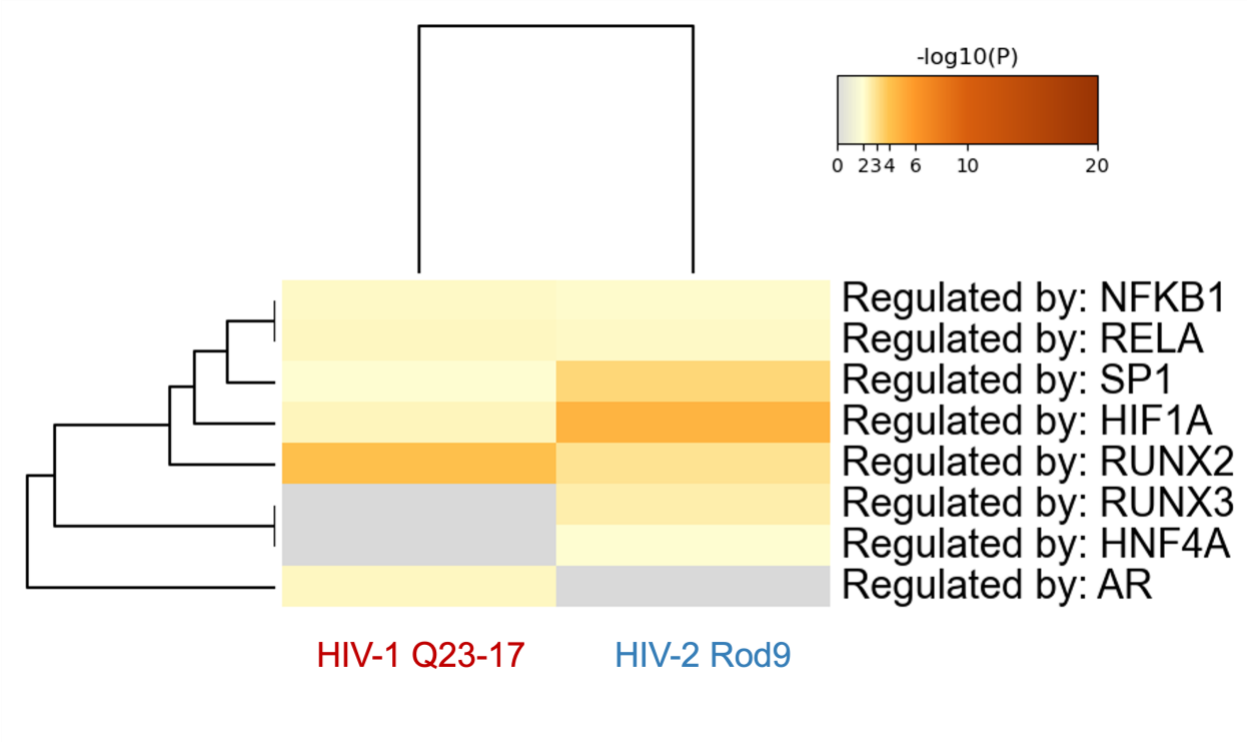


**Figure 4.7** Pathway analyses reveals that several proteins are regulated by activators of transcription.

(A) Further pathway analyses via Metascape revealed that both HIV-1 and HIV-2 Vpr enriched proteins are significantly regulated by regulatory pathways involved in DDR, transcription, cell cycle, and proteasomal degradation. Notably, these include the transcriptional activation pathways NF-KB1 and SP1.



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# Chapter 5: Conclusion

## 5.1 Discussion

The importance that the role the DDR plays in the viral lifecycle has long been recognized and considerably characterized for a broad range of diverse viruses. Despite this, our understanding of whether HIV engages the DDR has been limited. Since the discovery of AIDS, HIV research has heavily focused on preventing progression to AIDS and finding a cure. As such, several highly effective antiviral drugs and potential cures have been realized. However, we still lack a complete understanding of several basic viral molecular processes that would expediate discovering a cure. Because of this, we sought to better understand the basic viral molecular processes that occur in the nucleus and determine whether HIV engages the DDR and the extent of which it does so through the viral accessory gene Vpr. In addition to this, we aimed to describe a model for engagement of the DDR as well as define the mechanisms by which this is accomplished.

In **Chapter 2**, we discussed the importance of engaging the DDR for several well-studied viruses and described the many methods utilized by disparate viruses to accomplish the same objective. Understanding the DDR in this context has not only paved the way to several antiviral therapies but has also been imperative to our understanding of numerous fundamental biological processes such as cell cycle progression and transcription. Furthermore, these mechanisms are conserved across most well-studied viruses including those which replicate in the cytoplasm and suggests a principal evolutionary role. Taken together, this highlights the significance of studying the DDR in the context of viral replication to further our understanding of processes that are critical for viral replication and develop novel antiviral treatments and cures that otherwise would not be possible through our current perspectives.

In **Chapter 3**, we aimed to determine whether HIV Vpr could engage the DDR and define the phenotypes associated with engagement of the DDR to describe a model for HIV. Given that

engagement of the DDR is so well conserved across several viruses and that HIV integrates into the host genome which necessitates usurpation of several nuclear host factors involved in processes such as repair and damage. Furthermore, the viral accessory gene Vpr had been previously characterized to induce cell cycle arrest and activate markers of DNA damage. Altogether, this made HIV an exceptional candidate for these studies. Because HIV Vpr had been shown to activate several markers of DNA damage, we first aimed to further characterize the extent of which this occurred as well as if we could detect actual DNA damage. We found that both HIV-1 and HIV-2 not only activate markers of DNA damage through both the ATR and ATM signaling cascades, but also cause DNA damage in the form of single- and double-stranded breaks. We next further characterized the ability of HIV Vpr to induce cell cycle arrest and found that both HIV-1 and HIV-2 Vpr lead to an accumulation of cells in the late S-phase and G2, which is concurrent with the ability to stall replication forks. In addition to this, we found that both HIV-1 and HIV-2 repress HR-mediated DNA repair. Strikingly, this was linked to its ability to induce cell cycle arrest, but not cause DNA damage. Altogether, this generated a model for engagement of the DDR by HIV Vpr where Vpr causes DNA damage intendent of its ability to induce cell cycle arrest and repress HR. This allowed us to then interrogate the specific mechanisms by which Vpr accomplishes this.

Finally, in **Chapter 4**, we aimed to establish a chromatin-associated proteome for HIV Vpr and discovered that a variety of proteins are involved in Vpr function at chromatin. Because the model we generated in the previous chapter remained incomplete and could not be fully explained by known interactors, we sought to fill these gaps by interrogating the of proteome Vpr at chromatin. We found that Vpr seems to be deeply involved in processes related to nucleosome assembly and proteasomal degradation. We also observed an enrichment of several DDR-related factors and transcriptional machinery. Taken together, this reveals aspects of Vpr function that have not previously been thoroughly explored and begins to potentially explain some of the

missing components of our model. Here, we see that not only is the function of Vpr more complex than previously considered, but also that it is critical that we understand chromatin dynamics and transcriptional changes associated with Vpr.

In summary, this research demonstrates the importance of engaging the DDR by HIV Vpr. We establish that both HIV-1 and HIV-2 Vpr induce an assortment of phenotypes in cells expressing Vpr. We found that although most of these phenotypes are linked and dependent on interacting with host DCAF1, DNA damage is independent of this interaction and demonstrates that Vpr has many potential functions outside of this. Our proteomic data strongly suggest that the function of Vpr is complex and likely induces proteome-wide changes at chromatin that could affect other major process such as transcription and chromatin dynamics.

In the future, my work could be extended to broadening our understanding the lifecycle of HIV at the molecular level in the context of the chromatin environment. Here, we show that engagement of several DDR and chromatin factors are central to the role of HIV Vpr and is conserved across HIV-1 and HIV-2 orthologs. This suggests that engaging and modulating the chromatin environment is likely an important process that must occur during viral replication. This is significant because although we understand how to suppress viral replication using a variety of drugs, we lack an understanding of many viral processes that occur in the chromatin environment such as integration hotspots and latency that are essential to finding a cure. Future work could assist in clarifying these processes, with the understanding that HIV induces changes to the chromatin-associate proteome that are likely essential for viral modulation of the chromatin environment.