Title
HIV-1 Vpr and HIV-2 Vpr modulate the DNA damage response

Author
Sandoval, Carina

Publication Date
2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

HIV-1 Vpr and HIV-2 Vpr modulate the DNA damage response

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in Molecular Biology

by

Carina Sandoval

2023

ABSTRACT OF THE DISSERTATION

HIV-1 Vpr and HIV-2 Vpr Modulate the DNA Damage Response

by

Carina Sandoval

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2023

Professor Oliver I. Fregoso, Chair

HIV-1 and HIV-2 are two evolutionarily district viruses that encode the accessory protein Vpr. Although many functions have been ascribed to HIV-1 and HIV-2 Vpr, the primary and conserved function of Vpr is unknown. Both HIV-1 and HIV-2 Vpr induce cell cycle arrest, modulate the DNA damage response (DDR), alter transcription, and engage the E3 ubiquitin ligase $CRL4A^{DCAF1}$ complex, causing host protein degradation. Moreover, most of the phenotypes described for Vpr involve the ability of Vpr to modulate the DNA damage response (DDR). Here, we aimed to identify the conserved and divergent functions of HIV-1 and HIV-2 Vpr and determine how Vpr enhances viral replication.

In Chapter 1, we wrote a review on viral modulation of DDR and the similarities between DDR and innate immunity. We report that many diverse viruses modulate the DDR at multiple steps to facilitate viral replication. Furthermore, we compare the DDR and innate immune response, as both pathways are responsible for sensing, signaling, and responding to aberrant nucleic acids.

In Chapter 2, we found that HIV-1 Vpr induces DNA damage and activates ATM-signaling in the absence of cell cycle arrest and engagement with the $CRL4A^{DCAF1}$ complex. These findings were novel as most functions described for accessory genes require host protein degradation. Yet, an

emerging idea in the field is that accessory genes can function independently of co-opting E3 ubiquitin ligase complexes. Furthermore, in primary macrophages, we found that Vpr-induced DNA damage activates NF-κB transcription through the ATM-NEMO pathway. Lastly, we found virion-delivered and *de novo* expressed Vpr induces DNA damage and activates NF-κB. These data propose Vpr enhances viral replication by promoting proviral transcription through NF-κB.

In Chapter 3, we found that HIV-2 Vpr induces DNA damage, activates DDR signaling, and promotes nuclear translocation of RelA, independent of $CRL4A^{DCAF1}$ engagement and cell cycle arrest, as conserved by HIV-1 Vpr. However, HIV-2 Vpr does not upregulate NF-κB target genes that HIV-1 Vpr upregulates. These data suggest that there are both conserved and divergent mechanisms by which HIV-1 and HIV-2 Vpr modulate the DDR.

In Chapter 4, we leveraged our understanding of viral modulation of the DDR to pose the hypothesis that DDR genes are rapidly evolving because they are in conflict with HIV-1. Here, we found that 14.6% of DDR genes show signatures of positive selection, and most of these are involved in homologous recombination. Through a CRISPR screen, we identified DDR genes that, when knocked out, altered HIV-1 infectivity. To investigate further, we focused on the candidate gene MUTYH and found that MUTYH protein levels are refractory to knock-down, overexpression, and interferon treatment.

In Chapter 5, we found that HIV-1 and HIV-2 Vpr induce DNA breaks independent from cell cycle arrest. Furthermore, we found that Vpr inhibits homologous recombination repair and that repression requires the engagement of the $CRL4A^{DCAF1}$ complex. These data propose that Vpr inhibits DNA repair through the degradation of an unknown host factor.

The dissertation of Carina Sandoval is approved.

Melody Li

Alexander Hoffmann

Donald Kohn

Samson Chow

Oliver I. Fregoso, Committee Chair

University of California, Los Angeles

2023

TABLE OF CONTENTS

DEDICATION

For all the women that came before me and for all the women that will come after.
Thanks to you, I am here, and because of you, is why I am here.

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I thank my family for loving and supporting me throughout my Ph.D. To my husband, Kevin Valdez, thank you for all the adventures that were not distractions from science but rather fueled me to dive back into the lab, re-energized and with a new perspective. Thank you for your unwavering support of me and for stress eating with me. To my parents, Jesus and Rosaura Sandoval, thank you for being brave and immigrating to the US. I hope I've made you proud. My mind is now from UCLA, but my heart will always be from Mexico. To my sisters: Mayra, Araceli, and Avery. You three are like a fresh cup of coffee, perfect in the morning and perhaps not a great idea in the evening. Thank you for sharing life's ups and downs with me. To my nieces and nephew: Hailey, Brianna, and Aj. Thank you for teaching me how to be resilient and kind in the face of turmoil.

Next, I thank my advisor Oliver Fregoso for supporting me throughout the most remarkable journey of my life (thus far). Thank you for your patience, encouragement, and confidence in me. You re-wired my brain and now I am an independent scientist. Everyone at UCLA, including me, is lucky to have you. I am also thankful to my committee: Melody Li, Alexander Hoffmann, Donald Kohn, and Samson Chow—special thanks to Melody Li for advocating for me throughout my entire Ph.D. Your happiness and devotion to science are contagious.

Lastly, I want to thank my lab mates. Andrew Lopez, thank you for making your community my community. You gave me a sense of belonging and provided me with the best lunch breaks. Randilea Nichols Doyle, thank you for making me a better person by challenging me to be more accountable. Thank you for all the birthday cakes. Karly Nisson, thank you for making me feel seen scientifically as a fellow Gene Regger. Vivian Yang, thank you for your curiosity-based science approach and endless random facts about nature. Lastly, thank you to my brilliant colleagues for their support, especially Andres Rodriguez and everyone from the Fregoso and Li Labs.

VITA

EDUCATION

University of California, Los Angeles Los Angeles, CA

Ph.D. candidate, Molecular Biology

Advancement to candidacy November 15, 2019

California State University, Fullerton Fullerton, CA

B.S., Biological Science, Molecular Biology & Biotechnology 2017

Minor in Chemistry

cum laude, GPA:3.73

RESEARCH EXPERIENCE

University of California, Los Angeles June 2018 – present

PhD Candidate; Principal Investigator: Dr. Oliver Fregoso

Committee: Dr. Melody Li, Dr. Alexander Hoffmann,

Dr. Donald Kohn, and Dr. Samson Chow

Subject: Lentiviral replication (HIV-1 & HIV-2) and DNA damage

University of California, Los Angeles- Summer NSF Fellowship Summer 2017

Graduate Student Researcher; Principal Investigator: Dr. Jeff Long

Subject: Epigenetic transcriptional repression

Cornell University, Ithaca New York June – August 2016

Undergraduate Researcher; Principal Investigator: Dr. Maureen Hanson

Subject: RNA editing and post transcriptional modifications

California State University, Fullerton August 2014 – July 2017

Undergraduate Researcher; Principal Investigator: Dr. Melanie Sacco

Subject: Host-pathogen interactions of Tomato mosaic virus (ToMV)

California State University, Fullerton

Howard Hughes Medical Institute Research Thesis; Dr. Maria Linder August 2015-June 2017

Subject: Innate immune activation in Arabidopsis

California State University, Fullerton

Ronald E. McNair Post-baccalaureate Achievement Program; Dr. Patricia E. Literte 2015-2017

Subject: Signaling cascades and post translational modifications

California State University, Fullerton

Research Careers Preparatory Program; Director: Dr. Laura Arce August 2014- July 2015

Subject: Molecular biology

FUNDING AWARDS

F31 Ruth L. Kirschstein National Research Service Award, Predoctoral Fellowship February 2022

Percentile 1 and Impact Score 10

T32 Cellular and Molecular Biology NIH Training Grant June 2019 & 2020

Eugene V. Cota-Robles Fellowship Award August 2017

Howard Hughes Medical Institute Research Scholarship June 2015

Ronald E. McNair Research Scholarship June 2014

Research Careers Preparatory Research Scholarship June 2013

PUBLICATIONS

Sandoval C, and Fregoso OI. HIV-1 Vpr-induced DNA damage activates NF-κB independent of cell cycle arrest and $CRL4A^{DCAF1}$ engagement. Manuscript submitted

Lopez A*****, Nichols Doyle R*****, **Sandoval C***, Nisson K, Yang V, Fregoso OI. Viral Modulation of the DNA Damage Response and Innate Immunity: Two Sides of the Same Coin. Journal of Molecular Biology, March 2022 PMID 34695379. ***Contributed equally**

Li D*, Lopez A*, **Sandoval C**, Nichols Doyle R, Fregoso OI. HIV Vpr modulates the host DNA damage response at two independent steps to damage DNA and repress double-strand DNA break repair. mBio, July 2020 PMID 32753492. *Contributed equally

---

HONORS

Sidney C. Rittenberg Award, Microbiology, Immunology and Molecular Genetics, UCLA 2022

Poster Award Recipient, The Twenty-Sixth West Coast Retrovirus Meeting 2022

Teaching Assistant Award, Microbiology, Immunology and Molecular Genetics, UCLA 2020

Diversity, Equity and Inclusion (DEI) Award given to the Association for Multi-ethnic Bioscientist's Advancement with Carina Sandoval as Co-President 2020

Glenn Nagel Undergraduate Research Award Recipient, 2017

29th Annual CSU system-wide Conference

Poster Award Recipient, Society for Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS) 2015

TRAVEL AWARDS

Travel Award, 41th Annual Meeting of the American Society for Virology (ASV) 2023

Travel Award, 40th Annual Meeting of the American Society for Virology (ASV) 2022

Travel Award, 39th Annual Meeting of the American Society for Virology- *Deferred Covid19* 2020

Travel Award, American Society of Plant Biologist (ASPB) 2016

Travel Award, West Coast Biological Sciences Undergraduate Research, Point Loma Nazarene, University 2015

CONFERENCE PRESENTATIONS

Carina Sandoval and Oliver Fregoso. “HIV Accessory Protein Vpr Induces DNA Damage and Alters Cellular Transcription.” Poster presentation, The Twenty-Sixth West Coast Retrovirus Meeting, Palm Springs, CA, October 2022.

Carina Sandoval and Oliver Fregoso. “HIV Accessory Protein Vpr Induces DNA Damage and Alters Cellular Transcription.” Poster presentation, MBI Retreat, University of California Los Angeles, CA, August, 2022.

Carina Sandoval and Oliver Fregoso. “HIV Accessory Protein Vpr Induces DNA Damage and Alters Cellular Transcription.” Poster presentation, American Society for Virology Annual Meeting, Madison, WI, July 2022.

Carina Sandoval and Oliver Fregoso. “HIV Accessory Protein Vpr Induces DNA Damage and Alters Cellular Transcription.” Oral presentation, Virology Seminar, University of California Los Angeles, CA, March 2022.

Carina Sandoval and Oliver Fregoso. “HIV Accessory Protein Vpr Induces DNA Damage and Alters Cellular Transcription.” Poster presentation, Gordon Conference on DNA Damage, Mutation and Cancer, Ventura, CA March 2022.

Carina Sandoval and Oliver Fregoso. “Vpr-induced DNA Damage is Independent of $Cul4A^{DCAF1}$ Recruitment and Alters Cellular Transcription.” Poster presentation, Retroviruses, Cold Spring Harbor Laboratory, virtual, May 2021.

Carina Sandoval and Oliver Fregoso. “Vpr-induced DNA Damage is Independent of $Cul4A^{DCAF1}$ Recruitment and Alters Cellular Transcription.” Poster presentation, MBI Recruitment, University of California Los Angeles, CA January 2021.

Carina Sandoval and Oliver Fregoso. “Engagement of the DNA Damage Response by HIV-1 and HIV-2 Vpr”. Oral Presentation, MBI Graduate Student Seminar, University of California Los Angeles, CA March 2020.

Carina Sandoval, Ricky Padilla Del Valle, Rick McLaughlin and Oliver I. Fregoso. “It Takes Two to Tango: Stories of The Relentless Evolutionary Battle Between Humans and Pathogens.” Oral Presentation, SACNAS, O‘ahu, HI, October 2019.

Carina Sandoval, Alex Salas, Judd Hultquist, Nevan Krogan, and Oliver I. Fregoso. “Identifying Novel HIV-Host Interactions Involved in the DNA Damage Response.” Poster presentation, Symposium on HIV/AIDS, Palm Springs, CA, January 2019.

TEACHING EXPERIENCE

Teaching Assistant

Course: MIMG 102- Introduction to Virology — Winter 2020

University of California, Los Angeles

Teaching Assistant — Winter 2019

Course: MCDB 104AL – Research Immersion Laboratory in Developmental Biology

University of California, Los Angeles

Teaching Assistant Conference — Fall 2019

46th Annual Teaching Assistant Conference, Center for the Advancement of Teaching

University of California, Los Angeles

Teaching Assistant — Spring 2017

Course: Hatha Yoga

California State University, Fullerton

Teaching Assistant Spring 2017

Course: Molecular Biology Techniques Laboratory

California State University, Fullerton

Teaching Assistant

Course: Psychology, Logic and Critical Thinking Spring 2012

California State University, Fullerton

DIVERSITY AND OUTREACH

AMEBA Co-President 2019-2022

University of California, Los Angeles

Led the Association for Multi-ethnic Bioscientist's Advancement (AMEBA), a graduate student group that aims to support underrepresented students in STEM. Successfully co-wrote grants to secure new funds for AMEBA and held five annual events geared towards building community, outreach, wellness and professional development.

Molecular Biology Institute Recruitment Ambassador 2019 – 2021

Graduate Student Resources & Student Life presenter and lead contact for incoming graduate students

University of California, Los Angeles

AMEBA Administrative Chief 2018-2019

University of California, Los Angeles

Organized graduate student events to create an equitable environment to promote the advancement and retention of PhD students in the Life Sciences.

SACNAS General Member 2017-2018

University of California, Los Angeles

Developed science outreach modules for K-12 students. Taught general physics principles to K-12 students.

Research Careers Outreach 2015-2017

Collaborated with Kids to College, Nicholas Academic Center Scholars and Chemical Education to mentor underrepresented students (K-12) to pursuit higher education.

CHAPTER 1: Viral Modulation of the DNA Damage Response and Innate Immunity: Two Sides of the Same Coin

Andrew Lopez[a] † , Randilea Nichols Doyle[b] † , **Carina Sandoval[a] †** , Karly Nisson[a], Vivian Yang[a]

Oliver I. Fregoso[ab]

Molecular Biology Institute[a], Department of Microbiology, Immunology & Molecular Genetics[b] at the University of California, Los Angeles, California, United States of America

† Authors contributed equally

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 to establish a comprehensive understanding of the important of the DDR in the numerous fields of virology.

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.

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.

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. 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.

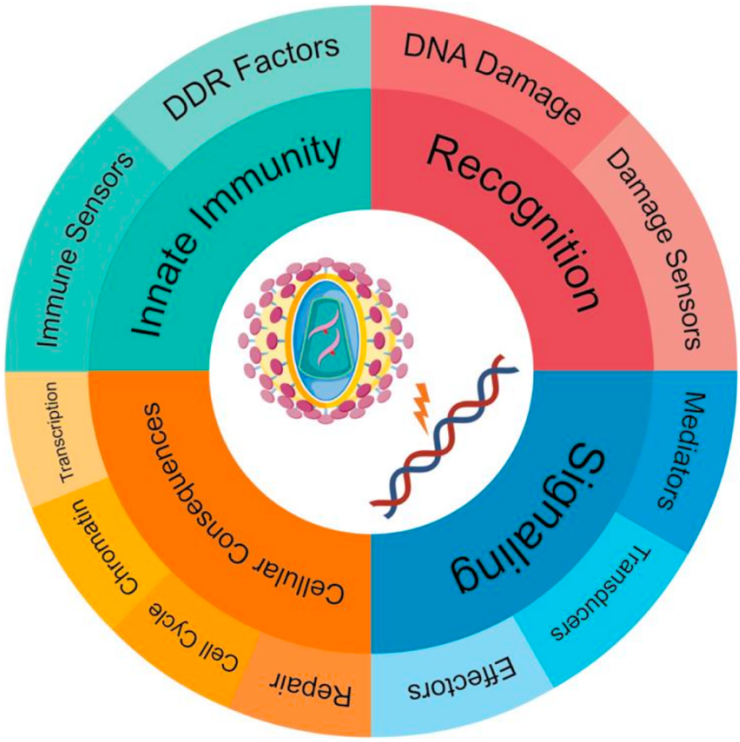
Many questions remain as we are only just beginning to scratch the surface of the role the DDR plays in viral replication and even innate immunity. For example, it is still unclear for many viruses whether steps such as induction of DNA damage or cell cycle arrest are active processes required for viral 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

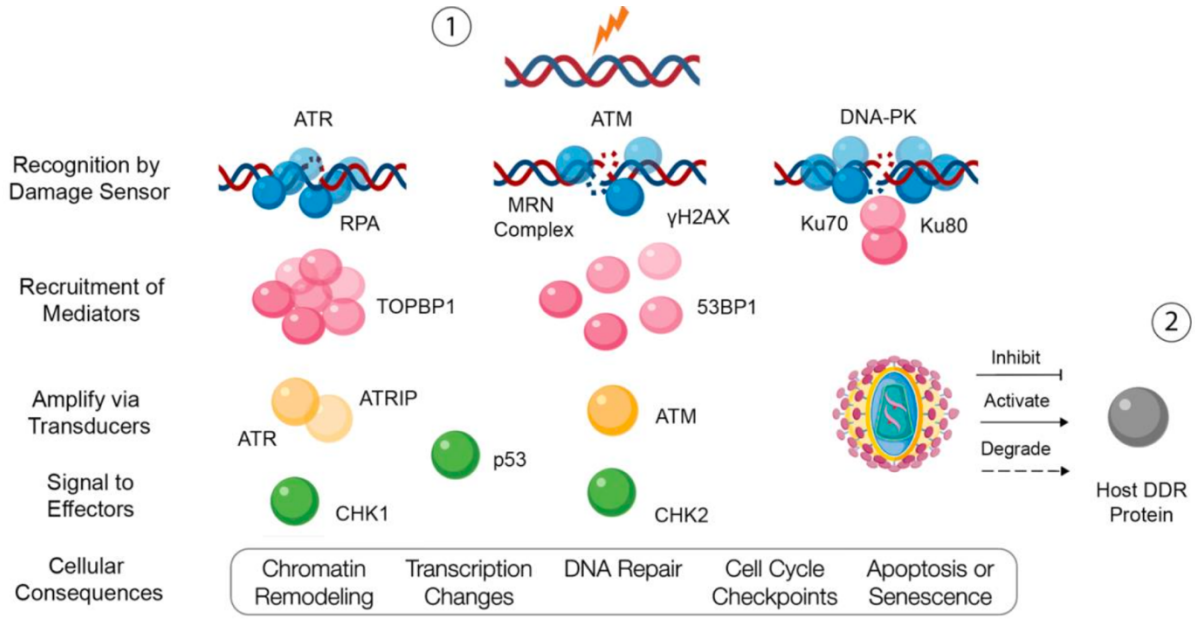
genes that comprise these pathways are marked by signatures of rapid evolution, called positive selection (PS). PS is often the consequence of long-standing evolutionary conflict and can be indicative of an important role in viral replication [179]. These signatures can be found in genes encoding proteins 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, 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.



Chapter 1 Figure 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.



Chapter 1 Figure 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.

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

Table 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.

REFERENCES

[1] Baltimore D. Expression of animal virus genomes. Bacteriol Rev. 1971;35:235-41.

[2] Weitzman MD, Fradet-Turcotte A. Virus DNA Replication and the Host DNA Damage Response. Annu Rev Virol. 2018;5:141-64.

[3] Luftig MA. Viruses and the DNA Damage Response: Activation and Antagonism. Annu Rev Virol. 2014;1:605-25.

[4] Turnell AS, Grand RJ. DNA viruses and the cellular DNA-damage response. J Gen Virol. 2012;93:2076-97.

[5] Blackford AN, Jackson SP. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. Mol Cell. 2017;66:801-17.

[6] Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. Mol Cell. 2010;40:179-204.

[7] Boichuk S, Hu L, Hein J, Gjoerup OV. Multiple DNA damage signaling and repair pathways deregulated by simian virus 40 large T antigen. J Virol. 2010;84:8007-20.

[8] Zur Hausen H. Induction of specific chromosomal aberrations by adenovirus type 12 in human embryonic kidney cells. J Virol. 1967;1:1174-85.

[9] Vijaya Lakshmi AN, Ramana MV, Vijayashree B, Ahuja YR, Sharma G. Detection of influenza virus induced DNA damage by comet assay. Mutat Res. 1999;442:53-8.

[10] Baydoun HH, Bai XT, Shelton S, Nicot C. HTLV-I tax increases genetic instability by inducing DNA double strand breaks during DNA replication and switching repair to NHEJ. PLoS One. 2012;7:e42226.

[11] Skalka AM, Katz RA. Retroviral DNA integration and the DNA damage response. Cell Death Differ. 2005;12 Suppl 1:971-8.

[12] Koyama T, Sun B, Tokunaga K, Tatsumi M, Ishizaka Y. DNA damage enhances integration of HIV-1 into macrophages by overcoming integrase inhibition. Retrovirology. 2013;10:21.

[13] Iijima K, Kobayashi J, Ishizaka Y. Structural alteration of DNA induced by viral protein R of HIV-1 triggers the DNA damage response. Retrovirology. 2018;15:8.
[14] Tachiwana H, Shimura M, Nakai-Murakami C, Tokunaga K, Takizawa Y, Sata T, et al. HIV-1 Vpr induces DNA double-strand breaks. Cancer Res. 2006;66:627-31.

[15] Li D, Lopez A, Sandoval C, Nichols Doyle R, Fregoso OI. HIV Vpr Modulates the Host DNA Damage Response at Two Independent Steps to Damage DNA and Repress Double-Strand DNA Break Repair. mBio. 2020;11.
[16] Zhang S, Pointer D, Singer G, Feng Y, Park K, Zhao LJ. Direct binding to nucleic acids by Vpr of human immunodeficiency virus type 1. Gene. 1998;212:157-66.

[17] Lai M, Zimmerman ES, Planelles V, Chen J. Activation of the ATR pathway by human immunodeficiency virus type 1 Vpr involves its direct binding to chromatin in vivo. J Virol. 2005;79:15443-51.
[18] Murugesapillai D, Bouaziz S, Maher LJ, Israeloff NE, Cameron CE, Williams MC. Accurate nanoscale flexibility measurement of DNA and DNA-protein complexes by atomic force microscopy in liquid. Nanoscale. 2017;9:11327-37.

[19] Zimmerman ES, Sherman MP, Blackett JL, Neidleman JA, Kreis C, Mundt P, et al. Human immunodeficiency virus type 1 Vpr induces DNA replication stress in vitro and in vivo. J Virol. 2006;80:10407-18.
[20] Schrofelbauer B, Yu Q, Zeitlin SG, Landau NR. Human immunodeficiency virus type 1 Vpr induces the degradation of the UNG and SMUG uracil-DNA glycosylases. J Virol. 2005;79:10978-87.

[21] Wu Y, Zhou X, Barnes CO, DeLucia M, Cohen AE, Gronenborn AM, et al. The DDB1-DCAF1-Vpr-UNG2 crystal structure reveals how HIV-1 Vpr steers human UNG2 toward destruction. Nat Struct Mol Biol. 2016;23:933-40.

[22] Hrecka K, Hao C, Shun MC, Kaur S, Swanson SK, Florens L, et al. HIV-1 and HIV-2 exhibit divergent interactions with HLTF and UNG2 DNA repair proteins. Proc Natl Acad Sci U S A. 2016;113:E3921-30.

[23] Lahouassa H, Blondot ML, Chauveau L, Chougui G, Morel M, Leduc M, et al. HIV-1 Vpr degrades the HLTF DNA translocase in T cells and macrophages. Proc Natl Acad Sci U S A. 2016;113:5311-6.

[24] Laguette N, Bregnard C, Hue P, Basbous J, Yatim A, Larroque M, et al. Premature activation of the SLX4 complex by Vpr promotes G2/M arrest and escape from innate immune sensing. Cell. 2014;156:134-45.

[25] Zhou X, DeLucia M, Ahn J. SLX4-SLX1 Protein-independent Down-regulation of MUS81-EME1 Protein by HIV-1 Viral Protein R (Vpr). J Biol Chem. 2016;291:16936-47.

[26] Yan J, Shun MC, Hao C, Zhang Y, Qian J, Hrecka K, et al. HIV-1 Vpr Reprograms CLR4(DCAF1) E3 Ubiquitin Ligase to Antagonize Exonuclease 1-Mediated Restriction of HIV-1 Infection. mBio. 2018;9.

[27] Lv L, Wang Q, Xu Y, Tsao LC, Nakagawa T, Guo H, et al. Vpr Targets TET2 for Degradation by CRL4(VprBP) E3 Ligase to Sustain IL-6 Expression and Enhance HIV-1 Replication. Mol Cell. 2018;70:961-70 e5.

[28] Romani B, Shaykh Baygloo N, Aghasadeghi MR, Allahbakhshi E. HIV-1 Vpr Protein Enhances Proteasomal Degradation of MCM10 DNA Replication Factor through the Cul4-DDB1[VprBP] E3 Ubiquitin Ligase to Induce G2/M Cell Cycle Arrest. J Biol Chem. 2015;290:17380-9.

[29] Withers-Ward ES, Jowett JB, Stewart SA, Xie YM, Garfinkel A, Shibagaki Y, et al. Human

immunodeficiency virus type 1 Vpr interacts with HHR23A, a cellular protein implicated in nucleotide excision DNA repair. J Virol. 1997;71:9732-42.

[30] Lim ES, Fregoso OI, McCoy CO, Matsen FA, Malik HS, Emerman M. The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. Cell Host Microbe. 2012;11:194-204.

[31] Fregoso OI, Ahn J, Wang C, Mehrens J, Skowronski J, Emerman M. Evolutionary toggling of Vpx/Vpr specificity results in divergent recognition of the restriction factor SAMHD1. PLoS Pathog. 2013;9:e1003496.

[32] Fabryova H, Strebel K. Vpr and Its Cellular Interaction Partners: R We There Yet? Cells. 2019;8.

[33] Greenwood EJD, Williamson JC, Sienkiewicz A, Naamati A, Matheson NJ, Lehner PJ. Promiscuous Targeting of Cellular Proteins by Vpr Drives Systems-Level Proteomic Remodeling in HIV-1 Infection. Cell Rep. 2019;27:1579-96 e7.

[34] Lentz TB, Samulski RJ. Insight into the mechanism of inhibition of adeno-associated virus by the Mre11/Rad50/Nbs1 complex. J Virol. 2015;89:181-94.

[35] Lakdawala SS, Schwartz RA, Ferenchak K, Carson CT, McSharry BP, Wilkinson GW, et al. Differential requirements of the C terminus of Nbs1 in suppressing adenovirus DNA replication and promoting concatemer formation. J Virol. 2008;82:8362-72.

[36] Sarkar R, Patra U, Lo M, Mukherjee A, Biswas A, Chawla-Sarkar M. Rotavirus activates a noncanonical ATM-Chk2 branch of DNA damage response during infection to positively regulate viroplasm dynamics. Cell Microbiol. 2020;22:e13149.

[37] Evans JD, Hearing P. Relocalization of the Mre11-Rad50-Nbs1 complex by the adenovirus E4 ORF3 protein is required for viral replication. J Virol. 2005;79:6207-15.

[38] Liu Y, Shevchenko A, Shevchenko A, Berk AJ. Adenovirus exploits the cellular aggresome response to accelerate inactivation of the MRN complex. J Virol. 2005;79:14004-16.

[39] Forrester NA, Sedgwick GG, Thomas A, Blackford AN, Speiseder T, Dobner T, et al. Serotype-specific inactivation of the cellular DNA damage response during adenovirus infection. J Virol. 2011;85:2201-11.

[40] Cheng CY, Gilson T, Dallaire F, Ketner G, Branton PE, Blanchette P. The E4orf6/E1B55K E3 ubiquitin ligase complexes of human adenoviruses exhibit heterogeneity in composition and substrate specificity. J Virol. 2011;85:765-75.

[41] Pancholi NJ, Weitzman MD. Serotype-specific restriction of wild-type adenoviruses by the cellular Mre11-Rad50-Nbs1 complex. Virology. 2018;518:221-31.

[42] Mariggio G, Koch S, Zhang G, Weidner-Glunde M, Ruckert J, Kati S, et al. Kaposi Sarcoma Herpesvirus (KSHV) Latency-Associated Nuclear Antigen (LANA) recruits components of the MRN (Mre11-Rad50-NBS1) repair complex to modulate an innate immune signaling pathway and viral latency. PLoS Pathog. 2017;13:e1006335.

[43] Baydoun HH, Pancewicz J, Nicot C. Human T-lymphotropic type 1 virus p30 inhibits homologous recombination and favors unfaithful DNA repair. Blood. 2011;117:5897-906.

[44] Blackford AN, Bruton RK, Dirlik O, Stewart GS, Taylor AM, Dobner T, et al. A role for E1B-AP5 in ATR signaling pathways during adenovirus infection. J Virol. 2008;82:7640-52.

[45] Ducu RI, Dayaram T, Marriott SJ. The HTLV-1 Tax oncoprotein represses Ku80 gene expression. Virology. 2011;416:1-8.

[46] Wang J, Kang L, Song D, Liu L, Yang S, Ma L, et al. Ku70 Senses HTLV-1 DNA and Modulates HTLV-1 Replication. J Immunol. 2017;199:2475-82.

[47] Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer. 2008;8:671-82.

[48] Joerger AC, Fersht AR. The p53 Pathway: Origins, Inactivation in Cancer, and Emerging Therapeutic Approaches. Annu Rev Biochem. 2016;85:375-404.

[49] Bieging KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. Nat Rev Cancer. 2014;14:359-70.

[50] Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science. 1989;243:934-7.

[51] Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol. 1989;63:4417-21.

[52] Moody CA, Laimins LA. Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. PLoS Pathog. 2009;5:e1000605.

[53] Baker A, Rohleder KJ, Hanakahi LA, Ketner G. Adenovirus E4 34k and E1b 55k oncoproteins target host DNA ligase IV for proteasomal degradation. J Virol. 2007;81:7034-40.

[54] Boyer J, Rohleder K, Ketner G. Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. Virology. 1999;263:307-12.

[55] Gluck S, Buttafuoco A, Meier AF, Arnoldi F, Vogt B, Schraner EM, et al. Rotavirus replication is correlated with S/G2 interphase arrest of the host cell cycle. PLoS One. 2017;12:e0179607.

[56] Jiang W, Wang Q, Chen S, Gao S, Song L, Liu P, et al. Influenza A virus NS1 induces G0/G1 cell cycle arrest by inhibiting the expression and activity of RhoA protein. J Virol. 2013;87:3039-52.

[57] Li N, Parrish M, Chan TK, Yin L, Rai P, Yoshiyuki Y, et al. Influenza infection induces host DNA damage and dynamic DNA damage responses during tissue regeneration. Cell Mol Life Sci. 2015;72:2973-88.

[58] Wallace NA, Khanal S, Robinson KL, Wendel SO, Messer JJ, Galloway DA. High-Risk Alphapapillomavirus Oncogenes Impair the Homologous Recombination Pathway. J Virol. 2017;91.
[59] Mehta K, Laimins L. Human Papillomaviruses Preferentially Recruit DNA Repair Factors to Viral Genomes for Rapid Repair and Amplification. mBio. 2018;9.

[60] Park JW, Nickel KP, Torres AD, Lee D, Lambert PF, Kimple RJ. Human papillomavirus type 16 E7 oncoprotein causes a delay in repair of DNA damage. Radiother Oncol. 2014;113:337-44.
[61] Nakai-Murakami C, Shimura M, Kinomoto M, Takizawa Y, Tokunaga K, Taguchi T, et al. HIV-1 Vpr induces ATM-dependent cellular signal with enhanced homologous recombination. Oncogene. 2007;26:477-86.

[62] Chambers BS, Heaton BE, Rausch K, Dumm RE, Hamilton JR, Cherry S, et al. DNA mismatch repair is required for the host innate response and controls cellular fate after influenza virus infection. Nat Microbiol. 2019;4:1964-77.
[63] Sato Y, Tsurumi T. Noise cancellation: viral fine tuning of the cellular environment for its own genome replication. PLoS Pathog. 2010;6:e1001158.
[64] Bagga S, Bouchard MJ. Cell cycle regulation during viral infection. Methods Mol Biol. 2014;1170:165-227.
[65] Ben-Israel H, Kleinberger T. Adenovirus and cell cycle control. Front Biosci. 2002;7:d1369-95.
[66] McFadden K, Luftig MA. Interplay between DNA tumor viruses and the host DNA damage response. Curr Top Microbiol Immunol. 2013;371:229-57.
[67] Fan Y, Sanyal S, Bruzzone R. Breaking Bad: How Viruses Subvert the Cell Cycle. Front Cell Infect Microbiol. 2018;8:396.
[68] Ahuja D, Saenz-Robles MT, Pipas JM. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. Oncogene. 2005;24:7729-45.

[69] Yuan X, Yao Z, Wu J, Zhou Y, Shan Y, Dong B, et al. G1 phase cell cycle arrest induced by SARS-CoV 3a protein via the cyclin D3/pRb pathway. Am J Respir Cell Mol Biol. 2007;37:9-19.
[70] Yuan X, Wu J, Shan Y, Yao Z, Dong B, Chen B, et al. SARS coronavirus 7a protein blocks cell cycle progression at G0/G1 phase via the cyclin D3/pRb pathway. Virology. 2006;346:74-85.

[71] Surjit M, Liu B, Chow VT, Lal SK. The nucleocapsid protein of severe acute respiratory syndrome-coronavirus inhibits the activity of cyclin-cyclin-dependent kinase complex and blocks S phase progression in mammalian cells. J Biol Chem. 2006;281:10669-81.
[72] Li FQ, Tam JP, Liu DX. Cell cycle arrest and apoptosis induced by the coronavirus infectious bronchitis virus in the absence of p53. Virology. 2007;365:435-45.
[73] Dayaram T, Lemoine FJ, Donehower LA, Marriott SJ. Activation of WIP1 phosphatase by HTLV-1 Tax mitigates the cellular response to DNA damage. PLoS One. 2013;8:e55989.
[74] Haoudi A, Daniels RC, Wong E, Kupfer G, Semmes OJ. Human T-cell leukemia virus-I tax oncoprotein functionally targets a subnuclear complex involved in cellular DNA damage-response. J Biol Chem. 2003;278:37736-44.
[75] Gupta SK, Guo X, Durkin SS, Fryrear KF, Ward MD, Semmes OJ. Human T-cell leukemia virus type 1 Tax oncoprotein prevents DNA damage-induced chromatin egress of hyperphosphorylated Chk2. J Biol Chem. 2007;282:29431-40.
[76] Salamango DJ, Ikeda T, Moghadasi SA, Wang J, McCann JL, Serebrenik AA, et al. HIV-1 Vif Triggers Cell Cycle Arrest by Degrading Cellular PPP2R5 Phospho-regulators. Cell Rep. 2019;29:1057-65 e4.
[77] Stivahtis GL, Soares MA, Vodicka MA, Hahn BH, Emerman M. Conservation and host specificity of Vpr-mediated cell cycle arrest suggest a fundamental role in primate lentivirus evolution and biology. J Virol. 1997;71:4331-8.

[78] Planelles V, Jowett JB, Li QX, Xie Y, Hahn B, Chen IS. Vpr-induced cell cycle arrest is conserved among primate lentiviruses. J Virol. 1996;70:2516-24.
[79] Clark E, Santiago F, Deng L, Chong S, de La Fuente C, Wang L, et al. Loss of G(1)/S checkpoint in human immunodeficiency virus type 1-infected cells is associated with a lack of cyclin-dependent kinase inhibitor p21/Waf1. J Virol. 2000;74:5040-52.

[80] Goh WC, Rogel ME, Kinsey CM, Michael SF, Fultz PN, Nowak MA, et al. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nat Med. 1998;4:65-71.
[81] Miller CM, Akiyama H, Agosto LM, Emery A, Ettinger CR, Swanstrom RI, et al. Virion-Associated Vpr Alleviates a Postintegration Block to HIV-1 Infection of Dendritic Cells. J Virol. 2017;91.
[82] Nakada S. Opposing roles of RNF8/RNF168 and deubiquitinating enzymes in ubiquitination-dependent DNA double-strand break response signaling and DNA-repair pathway choice. J Radiat Res. 2016;57 Suppl 1:i33-i40.
[83] Lilley CE, Chaurushiya MS, Boutell C, Landry S, Suh J, Panier S, et al. A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses. EMBO J. 2010;29:943-55.
[84] Sitz J, Blanchet SA, Gameiro SF, Biquand E, Morgan TM, Galloy M, et al. Human papillomavirus E7 oncoprotein targets RNF168 to hijack the host DNA damage response. Proc Natl Acad Sci U S A. 2019;116:19552-62.
[85] Yang J, Deng W, Hau PM, Liu J, Lau VM, Cheung AL, et al. Epstein-Barr virus BZLF1 protein impairs accumulation of host DNA damage proteins at damage sites in response to DNA damage. Lab Invest. 2015;95:937-50.

[86] Ho YK, Zhi H, Bowlin T, Dorjbal B, Philip S, Zahoor MA, et al. HTLV-1 Tax Stimulates Ubiquitin E3 Ligase, Ring Finger Protein 8, to Assemble Lysine 63-Linked Polyubiquitin Chains

for TAK1 and IKK Activation. PLoS Pathog. 2015;11:e1005102.

[87] Squatrito M, Gorrini C, Amati B. Tip60 in DNA damage response and growth control: many tricks in one HAT. Trends Cell Biol. 2006;16:433-42.

[88] Sun Y, Jiang X, Chen S, Fernandes N, Price BD. A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proc Natl Acad Sci U S A. 2005;102:13182-7.

[89] Kamine J, Elangovan B, Subramanian T, Coleman D, Chinnadurai G. Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 Tat transactivator. Virology. 1996;216:357-66.

[90] Creaven M, Hans F, Mutskov V, Col E, Caron C, Dimitrov S, et al. Control of the histone-acetyltransferase activity of Tip60 by the HIV-1 transactivator protein, Tat. Biochemistry. 1999;38:8826-30.

[91] Col E, Caron C, Chable-Bessia C, Legube G, Gazzeri S, Komatsu Y, et al. HIV-1 Tat targets Tip60 to impair the apoptotic cell response to genotoxic stresses. EMBO J. 2005;24:2634-45.

[92] Zhang SM, Song M, Yang TY, Fan R, Liu XD, Zhou PK. HIV-1 Tat impairs cell cycle control by targeting the Tip60, Plk1 and cyclin B1 ternary complex. Cell Cycle. 2012;11:1217-34.

[93] Awasthi S, Sharma A, Wong K, Zhang J, Matlock EF, Rogers L, et al. A human T-cell lymphotropic virus type 1 enhancer of Myc transforming potential stabilizes Myc-TIP60 transcriptional interactions. Mol Cell Biol. 2005;25:6178-98.

[94] Gupta A, Jha S, Engel DA, Ornelles DA, Dutta A. Tip60 degradation by adenovirus relieves transcriptional repression of viral transcriptional activator EIA. Oncogene. 2013;32:5017-25.

[95] Li R, Zhu J, Xie Z, Liao G, Liu J, Chen MR, et al. Conserved herpesvirus kinases target the DNA damage response pathway and TIP60 histone acetyltransferase to promote virus replication. Cell Host Microbe. 2011;10:390-400.

[96] Reitsma JM, Savaryn JP, Faust K, Sato H, Halligan BD, Terhune SS. Antiviral inhibition

targeting the HCMV kinase pUL97 requires pUL27-dependent degradation of Tip60 acetyltransferase and cell-cycle arrest. Cell Host Microbe. 2011;9:103-14.

[97] Shamay M, Liu J, Li R, Liao G, Shen L, Greenway M, et al. A protein array screen for Kaposi's sarcoma-associated herpesvirus LANA interactors links LANA to TIP60, PP2A activity, and telomere shortening. J Virol. 2012;86:5179-91.

[98] Jha S, Vande Pol S, Banerjee NS, Dutta AB, Chow LT, Dutta A. Destabilization of TIP60 by human papillomavirus E6 results in attenuation of TIP60-dependent transcriptional regulation and apoptotic pathway. Mol Cell. 2010;38:700-11.

[99] Subbaiah VK, Zhang Y, Rajagopalan D, Abdullah LN, Yeo-Teh NS, Tomaic V, et al. E3 ligase EDD1/UBR5 is utilized by the HPV E6 oncogene to destabilize tumor suppressor TIP60. Oncogene. 2016;35:2062-74.

[100] Hong S, Dutta A, Laimins LA. The acetyltransferase Tip60 is a critical regulator of the differentiation-dependent amplification of human papillomaviruses. J Virol. 2015;89:4668-75.

[101] Smith JA, Haberstroh FS, White EA, Livingston DM, DeCaprio JA, Howley PM. SMCX and components of the TIP60 complex contribute to E2 regulation of the HPV E6/E7 promoter. Virology. 2014;468-470:311-21.

[102] Nishitsuji H, Ujino S, Harada K, Shimotohno K. TIP60 Complex Inhibits Hepatitis B Virus Transcription. J Virol. 2018;92.

[103] Ma G, Chen L, Luo J, Wang B, Wang C, Li M, et al. Histone acetyl transferase TIP60 inhibits the replication of influenza a virus by activation the TBK1-IRF3 pathway. Virol J. 2018;15:172.

[104] Heine GF, Horwitz AA, Parvin JD. Multiple mechanisms contribute to inhibit transcription in response to DNA damage. J Biol Chem. 2008;283:9555-61.

[105] Huang TT, Wuerzberger-Davis SM, Wu ZH, Miyamoto S. Sequential modification of

NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. Cell. 2003;115:565-76.

[106] Medunjanin S, Putzier M, Nothen T, Weinert S, Kahne T, Luani B, et al. DNA-PK: gatekeeper for IKKgamma/NEMO nucleocytoplasmic shuttling in genotoxic stress-induced NF-kappaB activation. Cell Mol Life Sci. 2020;77:4133-42.

[107] Rashi-Elkeles S, Warnatz HJ, Elkon R, Kupershtein A, Chobod Y, Paz A, et al. Parallel profiling of the transcriptome, cistrome, and epigenome in the cellular response to ionizing radiation. Sci Signal. 2014;7:rs3.

[108] McCool KW, Miyamoto S. DNA damage-dependent NF-kappaB activation: NEMO turns nuclear signaling inside out. Immunol Rev. 2012;246:311-26.

[109] Millen S, Meretuk L, Gottlicher T, Schmitt S, Fleckenstein B, Thoma-Kress AK. A novel positive feedback-loop between the HTLV-1 oncoprotein Tax and NF-kappaB activity in T-cells. Retrovirology. 2020;17:30.

[110] Kinjo T, Ham-Terhune J, Peloponese JM, Jr., Jeang KT. Induction of reactive oxygen species by human T-cell leukemia virus type 1 tax correlates with DNA damage and expression of cellular senescence marker. J Virol. 2010;84:5431-7.

[111] Mitchell S, Vargas J, Hoffmann A. Signaling via the NFkappaB system. Wiley Interdiscip Rev Syst Biol Med. 2016;8:227-41.

[112] Heusinger E, Kirchhoff F. Primate Lentiviruses Modulate NF-kappaB Activity by Multiple Mechanisms to Fine-Tune Viral and Cellular Gene Expression. Front Microbiol. 2017;8:198.

[113] Nakahara T, Tanaka K, Ohno S, Egawa N, Yugawa T, Kiyono T. Activation of NF-kappaB by human papillomavirus 16 E1 limits E1-dependent viral replication through degradation of E1. J Virol. 2015;89:5040-59.

[114] Irwan ID, Cullen BR. Tax Induces the Recruitment of NF-kappaB to Unintegrated HIV-1 DNA To Rescue Viral Gene Expression and Replication. J Virol. 2021;95:e0028521.

[115] Harhaj EW, Giam CZ. NF-kappaB signaling mechanisms in HTLV-1-induced adult T-cell leukemia/lymphoma. FEBS J. 2018;285:3324-36.

[116] Valerie K, Delers A, Bruck C, Thiriart C, Rosenberg H, Debouck C, et al. Activation of human immunodeficiency virus type 1 by DNA damage in human cells. Nature. 1988;333:78-81.

CHAPTER 2: HIV-1 Vpr-induced DNA damage activates NF-κB independent of cell cycle arrest and CRL4A^{DCAF1} engagement

Carina Sandoval[a], Aleksandr Gorin[b], Alexander Hoffmann[ab], and Oliver I. Fregoso[ab]

Molecular Biology Institute[a], Department of Microbiology, Immunology & Molecular Genetics[b] at the University of California, Los Angeles, California, United States of America

ABSTRACT

Lentiviral accessory genes enhance replication through diverse mechanisms. HIV-1 accessory protein Vpr modulates the host DNA damage response (DDR) at multiple steps through the degradation of host proteins, cell cycle arrest, DNA damage, and both activation and repression of DDR signaling. Vpr also alters host and viral transcription; however, the connection between Vpr-mediated DDR modulation and transcriptional activation remains unclear. Here, we determined the cellular consequences of Vpr-induced DNA damage using Vpr mutants that allow us to separate the ability of Vpr to induce DNA damage from $CRL4A^{DCAF1}$ complex dependent phenotypes including cell cycle arrest, host protein degradation, and repression of DDR. In both tissue-cultured U2OS cells and primary human monocyte-derived macrophages (MDMs), we found that Vpr induces DNA breaks and activates DDR signaling in the absence of cell cycle arrest and $CRL4A^{DCAF1}$ complex engagement. Moreover, through RNA-sequencing, we found that Vpr-induced DNA damage alters cellular transcription via activation of NF-κB/RelA signaling. NF-κB/RelA transcriptional activation was dependent on ATM-NEMO, as inhibition of NEMO resulted in loss of NF-κB transcriptional upregulation by Vpr. Furthermore, HIV-1 infection of primary MDMs validated NF-κB transcriptional activation during infection. Both virion delivered and *de novo* expressed Vpr induced DNA damage and activated NF-κB transcription, suggesting that engagement of the DDR can occur during early and late stages of viral replication. Together, our data support a model where Vpr-induced DNA damage activates NF-κB through the ATM-NEMO pathway, independent of cell cycle arrest and $CRL4A^{DCAF1}$ engagement. We propose this is essential to overcoming restrictive environments, such as macrophages, to enhance viral transcription and replication.

INTRODUCTION

The DDR is a signaling cascade activated in response to exogenous and endogenous genotoxic stress, such as DNA breaks. The DDR consists of sensor proteins that sense the damaged DNA, mediator and transducer proteins that transmit the signal of damaged DNA, and effector proteins that elicit a cellular response. Typically, the DDR is divided into three main pathways based on the mediator kinases activated: Ataxia telangiectasia and Rad3 related (ATR), ataxia telangiectasia mutated (ATM), and DNA-dependent protein kinase (DNA-PK). Single-strand DNA breaks (SSBs) are primarily sensed by RPA to activate ATR signaling, while double-strand DNA breaks (DSBs) can be sensed by the MRN (MRE11, RAD51, and NBS1) or Ku70/80 complexes to activate either ATM or DNA-PK signaling, respectively (1,2). However, a significant amount of crosstalk exists between these pathways. DDR signaling leads to various cellular responses, such as DNA repair, cell cycle arrest (3), transcriptional changes (4,5), apoptosis, or senescence (6).

Many diverse RNA and DNA viruses have evolved to modulate the DDR to enhance viral replication (7,8). Primate lentiviruses, such as HIV-1, primarily engage the DDR through the accessory protein Vpr (9,10). Vpr is evolutionarily conserved amongst extant primate lentiviruses and is required for replication *in vivo* (11,12) and in macrophage (13,14) and dendritic cells (15) *in vitro*. Vpr is largely unique among the lentiviral accessory genes in that it is delivered by the incoming virion, allowing it to act early in viral replication, and is also expressed *de novo* from the integrated provirus, allowing it to act later in viral replication (16). Although the primary conserved function of Vpr remains elusive, viruses lacking Vpr have decreased proviral transcription in monocyte derived macrophages (MDMs) and dendritic cells (15), suggesting an important role for Vpr in viral transcription.

Emerging literature suggests that Vpr engages the DDR at multiple, potentially unique, steps. ATR activation by Vpr leads to cell cycle arrest and requires recruitment of the $Cul4A^{DCAF1}$

complex, which has a primary role in DNA repair (17). Cul4A^{DCAF1} complex recruitment by Vpr also leads to the degradation of many host proteins involved in the DDR, including CCDC137 (18), HLTF (19), UNG2 (20,21), SAMHD1 (22), Mus81/EME1 (23), EXO1 (24), TET2 (25), and MCM10 (26), as well as more global proteome remodeling (27). Moreover, Cul4A^{DCAF1} complex recruitment is required for Vpr-mediated repression of DSB repair (28). In contrast, we have previously shown that the ability of Vpr to induce DNA damage does not correlate with cell cycle arrest or repression of DSB repair (28), suggesting that Vpr-induced DNA damage may have unique roles in enhancing lentiviral replication.

Both DNA damage and HIV-1 Vpr promote transcriptional changes involving NF-κB (29,30). In response to DSBs, ATM signaling promotes NF-κB transcriptional upregulation through the ATM-NEMO pathway (31). ATM and NEMO interact in the nucleus before translocating to the cytoplasm (32,33) to subsequently activate RelA nuclear translocation, promoter binding, and NF-κB transcriptional activation. Previous literature suggests that HIV-1 Vpr modulates NF-κB pathways through phosphorylation and ubiquitylation of TAK1 to enhance NFkB signaling (34) and altering the availability of the NF-κB p50-RelA heterodimer to inhibit NF-kB signaling (35). This proposes a testable model where Vpr-induced DNA damage alters the cellular environment to enhance viral replication by altering transcription through ATM-NEMO and NF-κB. Here, we aimed to identify the cellular consequences of Vpr-induced DNA damage and the connection between DNA damage and ATM activation, CRL4A^{DCAF1} engagement, and transcriptional changes.

To determine the consequences of Vpr-induced DNA damage on cellular transcription, we used Vpr mutants that allow us to uncouple DNA damage from cell cycle arrest and Cul4A^{DCAF1} complex host protein degradation. We found that Vpr does not require CRL4A^{DCAF1} engagement to induce DNA breaks and activate DDR signaling in U2OS tissue-culture cells and primary human MDMs.

RNA-sequencing (RNA-seq) identified that wild-type HIV-1 Vpr and Vpr mutants that do not induce cell cycle arrest or engage the $CRL4A^{DCAF1}$ complex alter NF-κB associated cellular transcription. In support of this, we showed that Vpr proteins that only induce DNA damage all activated RelA nuclear translocation and upregulated NF-kB target genes BIRC3 and CXCL8. We further assessed the requirement for ATM and NEMO signaling in Vpr-mediated NF-κB activation. We found that inhibition of NEMO resulted in loss of NF-κB transcriptional upregulation in primary MDMs. HIV-1 infection and virus-like particle (VLP) delivery of Vpr in MDMs validated the upregulation of NF-κB target genes early during infection. Together, our data support a model where Vpr-induced DNA damage activates NF-κB through the ATM-NEMO pathway, independent of cell cycle arrest and $CRL4A^{DCAF1}$ complex host protein degradation. This study further informs how lentiviral accessory proteins engage the DDR at multiple and unique steps, which remodels host environment and immune pathways to promote viral replication.

RESULTS

HIV-1 Vpr induces DNA damage and alters cellular transcription independent of CRL4A^{DCAF1} engagement and cell cycle arrest

Given the connections between the DDR and cellular transcription, we set out to understand whether the ability of Vpr to induce DNA damage gives rise to transcriptional changes, and whether these transcriptional changes are distinct from those caused by Vpr-mediated cell cycle arrest and/or degradation of host proteins. We have previously shown that a Vpr mutant that is unable to cause cell cycle arrest (HIV-1 Q23-17 S79A) causes DNA damage (28), however this mutant still engages the DCAF1 component of the CRL4A^{DCAF1} E3 ubiquitin ligase complex (36). To test the connection between transcriptional changes, cell cycle arrest, and host protein degradation, we used the HIV-1 Q23-17 Vpr mutant H71R, which does not bind the DCAF1 component of the CRL4A^{DCAF1} E3 ubiquitin ligase complex and thus loses the ability to cause cell cycle arrest and degrade host proteins (28) (Fig. S1A). U2OS cells were infected with rAAV expressing either HIV-1 Q23-17 Vpr wild type (WT), Vpr H71R, Vpr Q65R, a mutant of Vpr that is largely functionally dead (37), or etoposide (positive control). DDR activation was assessed by immunofluorescence while DNA damage was assessed by the comet assay 24 hours post infection. Consistent with our previous results, HIV-1 Vpr WT induces DNA breaks (Fig. 1A) and activates the DDR marker γH2A.x (Fig. 1B), while the nonfunctional Q65R mutant does not. In addition, we found that H71R induces DNA breaks (Fig. 1A) and activates γH2A.x (Fig. 1B) at levels similar to Vpr WT. To validate this, we knocked-down DCAF1 in U2OS cells with shRNAs and found that Vpr can still induce DNA breaks and activate DDR signaling when DCAF1 is knocked down (Fig. S1B-D). Together, our data show that Vpr does not require DCAF1 or associated cellular responses to induce DNA damage and activate the DDR, and that the H71R Vpr mutant allows us to investigate the ability of Vpr to induce DNA damage in the absence of host protein degradation and cell cycle arrest.

Next, we asked if Vpr mutants that induce DNA damage in the absence of cell cycle arrest (H71R and S79A) and CRL4A^{DCAF1} recruitment (H71R) alter cellular transcription. U2OS cells were infected with rAAV expressing HIV-1 Vpr WT, H71R, S79A, and Q65R, and RNA was collected at 12, 24, and 36 hours post infection. RNA-seq identified that Vpr WT, H71R and S79A, but not Q65R or empty vector, significantly alter cellular transcription 36 hours post infection when compared to untreated cells (Fig. 1C-E and Fig. S2A-B). Sixty-eight differentially expressed genes with pvalue <0.01 and FDR < 4.50E-05 were shared among Vpr WT, H71R, and S79A, indicative of genes potentially altered by Vpr-induced DNA damage (Fig. 1D and 1E). Gene ontology indicated the shared upregulated genes were enriched for positive regulation of NF-κB signaling and NF-κB signaling pathways, and transcriptional regulatory relationships unravelled by sentence-based text-mining (TRRUST) analysis (38) further identified RelA/NF-κB as a top transcription factor activated by Vpr (Fig. 1F-G, Fig. S2 A-B and Supplemental Files 1&2). Finally, we identified differentially expressed genes exclusively altered in Vpr WT expressing cells, Vpr WT and S79A expressing cells, and unique to both Vpr H71R and S79A mutant expressing cells (Supplemental Files 1 & 2). Collectively, our data show that Vpr alters cellular transcription in the absence of cell cycle arrest and host protein degradation, and further suggest that Vpr-induced DNA damage specifically alters RelA/NF-κB-mediated transcription.

HIV-1 Vpr-induced DNA damage activates RelA and promotes NF-κB transcription

We next directly assayed whether Vpr mutants that can only induce DNA damage activate RelA/NF-κB. NF-κB activation was first validated by qRT-PCR for two NF-κB target genes identified in our RNA-seq, BIRC3 and CXCL8 (Fig. 1E), which are important for innate immunity and cell survival (39,40). U2OS cells were infected with rAAV expressing Vpr WT, H71R and Q65R for 24 or 36 hours. Consistent with the RNA-seq, Vpr WT and H71R upregulate BIRC3 and CXCL8 compared to untreated cells or Q65R mutant (Fig. 2A). qRT-PCR for BIRC3 and CXCL8

in DCAF1 knock-down cells further confirmed that DCAF1 recruitment is not required for HIV-1 Vpr to activate these two NF-κB target genes (Fig. S1E). To directly assess NF-κB activation, we assayed for RelA localization by immunofluorescence. As RelA is cytoplasmic when inactive and nuclear when active, we expect that Vpr WT and H71R will lead to nuclear translocation of RelA. Indeed, we found that Vpr WT and H71R activate nuclear translocation of RelA similar to the positive control, etoposide, while Vpr Q65R and empty vector do not (Fig 2B). Our data suggests that Vpr-induced DNA damage activates RelA and promotes NF-κB transcription.

To directly assess the global binding of RelA at NF-κB promoters we performed Cleavage Under Targets and Tagmentation (CUT&Tag) for RelA. U2OS cells were infected with rAAV expressing Vpr-T2A-mCherry WT, mutants, empty vector, etoposide, or TNFα (positive control). Expression of mCherry served as a proxy for Vpr levels in the cell; we found that Vpr WT and empty vector expressed similarly in U2OS cells (Fig. S3A). As seen previously, Vpr WT upregulated the NF-kB target gene, BIRC3, compared to empty vector (Fig. S3A), indicating that Vpr WT activated cellular transcription as expected. Furthermore, we found that Vpr WT caused global enrichment of RelA binding at NF-kB motifs, as normalized to empty vector (Fig. S3B). Gene browser tracks show that Vpr WT causes RelA binding at the NF-kB motif within the BIRC3 promoter (Fig. S3C), consistent with the expectation that Vpr is activating RelA and driving BRIC3 gene expression. We observed that global activation of RelA by Vpr mostly resembles etoposide-induced activation rather than TNFα-induced activation, as Vpr WT RelA binding sites correlate with etoposide RelA binding sites ($R^2=0.72$) but not TNFa ($R^2=0.47$) (Fig. S3C&D).

Even though equal titers of rAAV expressing Vpr WT and mutants were used, Vpr WT and mutants did not express to similar levels. Vpr S79A, H71R, and Q65R did not express well in this experiment, as assessed by mCherry transcript levels (Fig. S3A). Furthermore, Vpr S79A and H71R did not robustly activate BIRC3 (Fig. S3A). These data suggest that the mutants did not

express well enough to function as expected, and we were therefore unable to measure the effects of Vpr mutants on RelA promoter binding (Fig. S3 B, C & D). Taken together, our immunofluorescence and CUT&Tag data indicate that Vpr activates RelA and NF-κB transcription in the absence of cell cycle arrest, DCAF1 recruitment, and host protein degradation. Our data suggest that Vpr-induced DNA damage is sufficient to drive NF-κB activation.

HIV-1 Vpr-induced DNA damage activates ATM-NEMO signaling

NF-κB is activated in response to many pathways and signals involved in DNA repair and innate immunity (41). One such pathway is ATM-NEMO, where ATM stimulates NEMO to activate RelA and thus NF-κB signaling (33). Previous work has shown that Vpr activates markers of both ATR and ATM signaling (28,42). While ATR is required for Vpr-induced cell cycle arrest, the extent of ATM activation and the cellular consequences of ATM activation without cell cycle arrest are unclear. We hypothesize that Vpr-induced DNA damage activates the ATM-NEMO pathway, which consequently promotes RelA/NF-κB transcription. To determine if Vpr-induced DNA damage activates ATM signaling in the absence of cell cycle arrest and DCAF1 recruitment, we measured activation of multiple DDR proteins including two DNA damage sensors, NBS1 and MRE11, and two downstream signaling transducers, 53BP1 and γH2A.x. U2OS cells stably expressing NBS1-GFP or 53BP1-GFP were infected with rAAV expressing Vpr WT, H71R, and Q65R to assess DDR activation through live-cell imaging throughout infection. We found that Vpr WT and H71R promote the formation of NBS1 and 53PB1 foci at 26, 33, and 48 hours post infection (hpi), similar to the positive control etoposide, while the non-functional Q65R Vpr mutant was indistinguishable from untreated control cells (Fig. 3A and Fig. S4A-C). As many viruses activate or dysregulate DDR signaling through relocalization or sequestration of markers of the DDR, we next assayed for colocalization of the DNA damage sensors MRE11 and NBS1, and transducers 53BP1 and γH2A.x. Both damage sensors MRE11 and NBS1, and transducers 53PB1 and γH2A.x, colocalize in Vpr WT and H71R, similar to etoposide treated cells (Fig. 3B&C),

suggesting that Vpr-induced DNA damage activates, but does not dysregulate, classical ATM signaling.

To determine if NEMO is required for Vpr to upregulate NF-κB transcription, we inhibited NEMO using a cell-permeable NEMO binding domain (NBD) inhibitor peptide (43). U2OS cells were pretreated with NBD peptide and infected with rAAV expressing Vpr WT and mutants. We assessed upregulation of the NF-κB target genes BIRC3 and CXCL8 using qRT-PCR. As before, Vpr WT and H71R upregulated both BIRC3 and CXCL8, while Q65R and empty vector did not (Fig. 4). Furthermore, with the addition of the NBD, Vpr WT and H71R mutant lost the ability to upregulate BIRC3 and CXCL8 (Fig. 4), suggesting that Vpr requires NEMO activation to upregulate transcription of NF-κB target genes.

Virion delivery of Vpr, through VLPs or HIV-1 ΔENV infection, in primary MDMs activates ATM-NEMO signaling and promotes RelA/NF-κB transcription

Together our data proposes a model where Vpr-induced DNA damage activates ATM and NEMO signaling resulting in RelA nuclear translocation and NF-κB transcriptional upregulation. We next tested this model in primary human MDMs, where Vpr enhances viral transcription and replication (14). Primary MDMs (Fig. S5A) were infected with virus-like particles (VLPs) carrying physiological levels of Vpr WT, H71R, or Q65R (Fig. S5B), and assayed for DNA damage, NF-κB activation, and NEMO dependence (Fig 5A). Similar to overexpression of Vpr in U2OS tissue culture cell lines, Vpr WT and H71R mutant induced DNA breaks and activated γH2A.x compared to Vpr Q65R and empty VLPs (Fig. 5B-C). Consistent with our data in U2OS cells, Vpr WT and H71R, but not Q65R or empty VLPs, upregulated BIRC3 and CXCL8 similar to the TNFα positive control when compared to empty cells (Fig. 5D and Fig. S5C). Finally, NBD delivery prior to VLP infection blocked Vpr WT and H71R-mediated BIRC3 and CXCL8 transcriptional activation, but not Vpr-induced DNA damage (Fig 5C-D). These data indicate that physiological levels of virion-

delivered Vpr induces DNA damage that activates NF-κB transcription in a NEMO dependent manner in primary MDMs.

To determine if HIV-1 infection activates NF-κB target genes, we infected primary human MDMs from six different donors with HIV-1 ΔENV and assayed for transcriptional changes at early (8 hours), intermediate (16 hours), and late (24 and 48 hours) timepoints post infection. HIV-1 infection was monitored by flow cytometry for HIV-1 core proteins and qPCR for unspliced transcripts (Fig. S5D-E), while NF-κB activation was measured by qRT-PCR for CXCL8 and BIRC3. TNFα was used as positive control (Fig. S5F). HIV-1 ΔENV infection resulted in upregulation of CXCL8 and BIRC3 in MDMs as early as 8hpi in all donors, despite some donor-to-donor variability in the magnitude of upregulation (Fig. 5E and Fig. S5G-H), suggesting that HIV-1 infection upregulates Vpr-induced NF-κB target genes early during infection, consistent with VLP delivery of Vpr. Jointly, our data support a model where both incoming HIV-1 Vpr and *de novo* produced Vpr induces DNA damage that activates ATM-NEMO signaling to upregulate NF-κB transcription (Fig. 6).

DISCUSSION

In this study, we tested the hypothesis that HIV-1 Vpr alters cellular transcription through induction of DNA damage and activation of DDR signaling. By leveraging well-characterized Vpr mutants that separate Vpr-induced DNA damage from cell cycle arrest and $CRL4A^{DCAF1}$ complex host protein degradation, we identified a current model where Vpr-induced DNA damage activates ATM-NEMO signaling, which stimulates RelA and upregulates NF-κB mediated transcription. Using U2OS and primary human MDMs, which allowed us for the first time to investigate how HIV-1 Vpr engages the DDR in this important cell type, we showed that Vpr induces DNA breaks and activates markers of ATM signaling independent of cell cycle arrest, $CRL4A^{DCAF1}$ complex recruitment, and host protein degradation. Moreover, we showed that Vpr-induced DNA damage correlates with RelA/NF-κB activation, as assayed by bulk RNA-seq, and RelA immunofluorescence. We validated the upregulation of two NF-κB target genes, BIRC3 and CXCL8, and showed that inhibition of NEMO ablates the ability of Vpr to upregulate NF-κB transcription. Finally, VLP delivery of Vpr and HIV-1 infection show that incoming virion-associated Vpr is sufficient to induce DNA damage and activates RelA/NF-κB transcription. In complement, rAAV expression of Vpr shows that *de novo* expressed Vpr can also induce DNA damage and activate RelA/NF-κB transcription. These data suggest that Vpr-induced DNA damage can play roles in both early and late stages of viral replication. Overall, our data support a model where Vpr-induced DNA damage activates ATM-NEMO signaling and upregulates RelA/NF-κB transcription in cell lines and primary human MDMs.

Although Vpr is a multifunctional and enigmatic protein, the DNA damage response is central to many of the phenotypes associated with Vpr. Previous reports from our lab and others have worked to untangle how and why Vpr engages the DDR. A consensus is emerging that Vpr engages the DDR at multiple potentially unique steps. Through recruitment of the $CRL4A^{DCAF1}$ ubiquitin ligase complex, Vpr degrades various DDR proteins, activates ATR signaling, represses

double-strand DNA break repair, and causes cell cycle arrest (28,44). However, as shown here, Vpr induces DNA damage and alters cellular transcription independent of its ability to engage the $CRL4A^{DCAF1}$ ubiquitin ligase complex and associated phenotypes. Functions independent of host protein degradation are unique among most lentiviral accessory genes, as their typical primary functions depend on subverting antiviral mechanisms through host protein degradation (45). These data further define the multifunctional nature of Vpr in enhancing lentiviral infection and support the model where Vpr engages the DDR at multiple independent steps.

Here, we have also shown that Vpr-induced DNA damage directly correlates with NF-κB transcriptional activation that is dependent on ATM signaling. We have demonstrated that Vpr activates markers of ATM signaling, such as MRE11, NBS1, γH2A.x, and 53PB1, in a manner that resembles host activation rather than dysregulation. Antagonism or dysregulation of DNA damage sensors through relocalization or sequestration is a conserved mechanism among many viruses to evade innate immune detection (46). While we have show that Vpr-induced DNA damage activates markers of ATM signaling in a manner that seems to facilitate classical ATM activation, with the end goal of activating transcription, it remains to be seen whether Vpr-mediated activation of ATR, which correlates with $CRL4A^{DCAF1}$ recruitment and cell cycle arrest, is classically activated or dysreglated. Moreover, how Vpr induces DNA breaks leading to both ATM and ATR activation is not understood.

Complementary studies have shown that Vpr modulates cellular (47) and viral transcription (48,49). In the context of cellular transcription, Vpr activates genes associated with innate immunity and proliferation in CD4+ T cells (50) and promotes the expression of proinflammatory cytokines in MDMs and monocyte-derived dendritic cells (MDDCs) (15,25). Together this suggests that Vpr has a vital role in transcriptional reprogramming to potentially create a proinflammatory environment that is conducive for viral replication. Consistent with these studies,

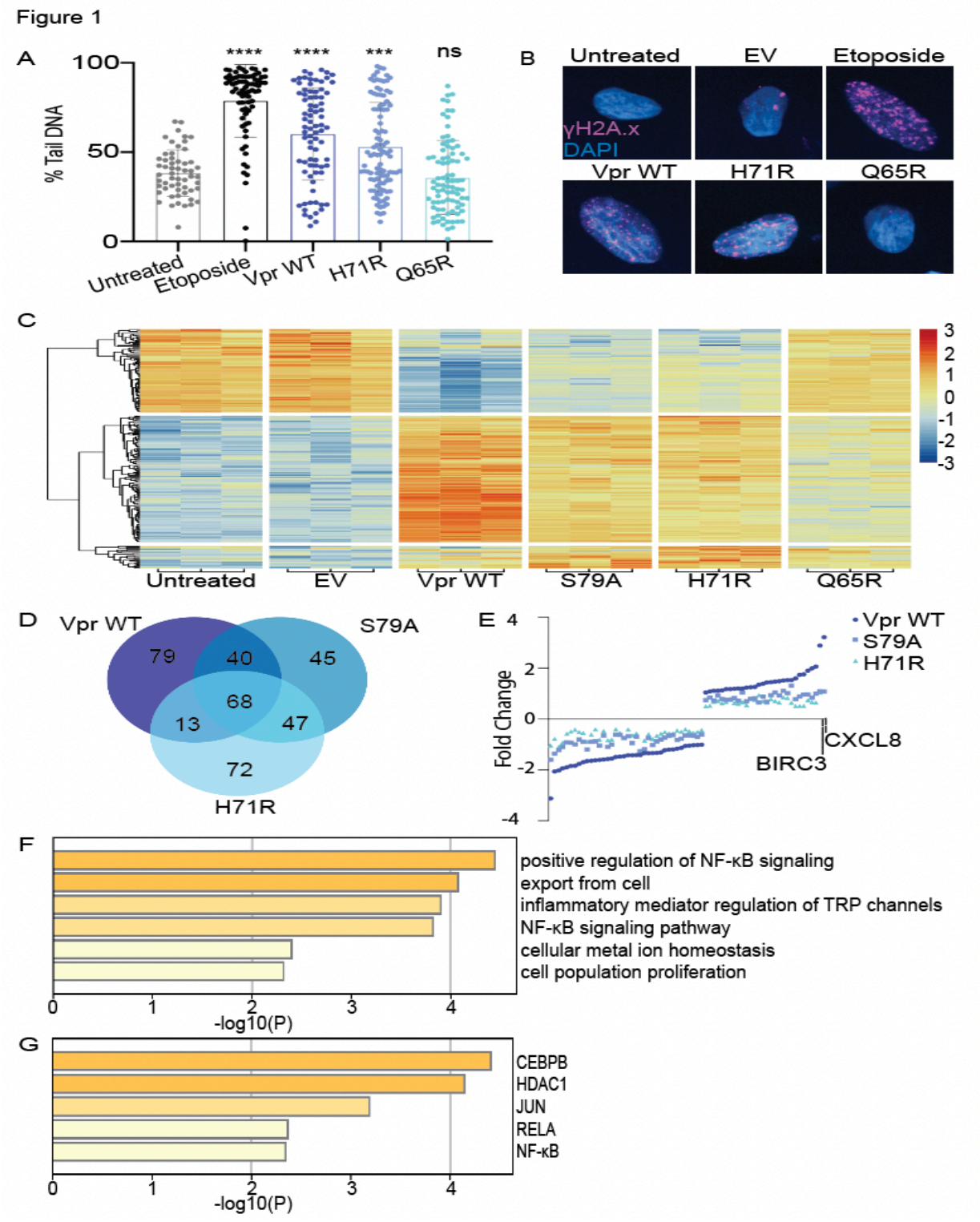
we identified by bulk RNA-seq that Vpr regulates innate immune pathways that are either independent of or dependent on cell cycle arrest and CRL4A^{DCAF1} engagement and host protein degradation. Our data shows that Vpr downregulates TASOR target genes (Fig S2 D-F), which is consistent with the literature suggesting that SIV and HIV-2 Vpr counteract the human silencing hub (HUSH) repressor complex (51,52). Furthermore, we found that Vpr downregulates CXCL14, which aligns with the data suggesting that human papillomaviruses downregulates CXCL14 to enhance replication (53).

Our data also indicate that Vpr activates the RelA/NF-κB immune pathway without induction of cell cycle arrest and CRL4A^{DCAF1} recruitment and host protein degradation. This is consistent with previous reports showing that Vpr activates NF-κB transcription via phosphorylation of TAK1 (34), an upstream regulator of NF-κB. We further found that Vpr-induced DNA damage activates ATM-NEMO signaling to upregulate RelA/NF-κB transcription, adding to the mechanistic understanding of NF-κB activation by Vpr. Through engagement with the CRL4A^{DCAF1} complex, Vpr has also been found to repress NF-κB activation by altering the availability of the NF-κB p50-p65 heterodimer, thus limiting proinflammatory cytokine expression (35). While it remains to be understood what differentiates Vpr-mediated NF-κB activation from repression, whether Vpr engages CRL4A^{DCAF1} complex seems to be a distinguishing factor. Furthermore, it is clear that in all cases Vpr carefully modulates NF-κB activation without globally activating interferon, which would inhibit viral replication. Thus, further studies understanding how Vpr manages to activate only aspects of NF-κB signaling (54,55) will help to define how Vpr may contribute to the ability of HIV to subvert the innate immune response.

Although various studies have shown that Vpr also alters viral transcription the mechanisms are unclear and disparate. For example, Vpr-mediated LTR activation is associated with cell cycle arrest where the LTR is most transcriptionally active (56,57), degradation of host proteins such

as CCDC137 (18), and CRL4A^{DCAF1} independent mechanisms (58). However, studies have shown that Vpr also promotes LTR transcription in noncycling cells (59,60) where induction of cell cycle arrest is absent and the necessity for degradation of specific host proteins in LTR activation has not been extensively examined. One benefit of Vpr activating NF-κB signaling via ATM is the potential direct enhancement of LTR transcription, as the HIV-1 LTR contains multiple NF-κB binding sites essential for viral gene expression. In addition to NF-κB, we further identified that Vpr activates CEBPB and JUN transcription factors involved in LTR activation. This is similar to Vpr-mediated de-repression of the LTR by removal of the transcriptional repressor ZBTB2 following ATR activation (61). Overall, Vpr WT and mutants that only induce DNA damage globally alter cellular transcription, upregulate NF-κB signaling and transcription factors that promote LTR-driven transcription, suggesting that Vpr-induced DNA damage is important for promoting LTR transcription to enhance viral replication.

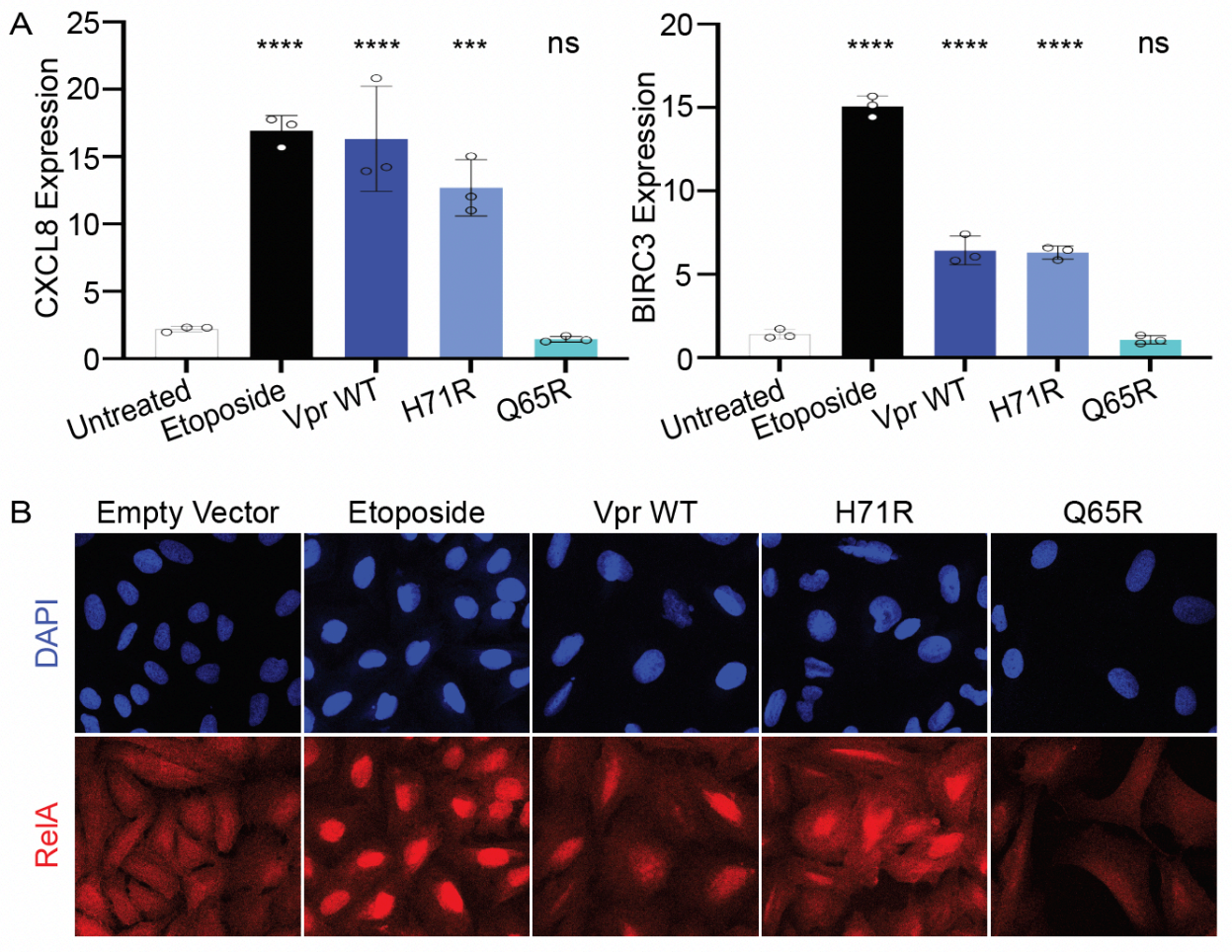
Overall, our data suggest that during HIV infection, incoming Vpr and *de novo* expressed Vpr primes the cellular environment by activating RelA/NF-κB signaling to promote LTR transcription and enhance viral replication in macrophages. Our data aligns with the growing body of literature that supports the role of accessory proteins modulating the host environment and the DDR to promote viral replication through mechanisms independent of host protein degradation.



Chapter 2 Figure 1. HIV-1 Vpr induces DNA damage and alters cellular transcription independent of cell cycle arrest and CRL4A^{DCAF1} engagement.

(A) Comet assay of U2OS cells infected with rAAV expressing 3xFLAG-tagged Vpr WT, H71R, Q65R, untreated (negative control), or 50uM etoposide (positive control). Percent tail DNA was quantified at 24 hours post infection (hpi) using the OpenComet plug-in for the ImageJ software. Each circle represents one cell. N=3, one representative experiment shown. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; γH2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. N=3, one representative experiment shown. EV, empty vector. (C) RNA-seq of U2OS cells at 36 hpi. Heatmap displays Log_2 fold changes of upregulated genes in red and downregulated genes in blue. Each column represents a biological replicate, n=3 (D) Venn diagram of the top 100 upregulated and top 100 downregulated genes among Vpr WT, S79A, H71R, and Q65R. € Dot plot of the 68 conserved differentially expressed genes among Vpr WT, S79A, and H71R. BIRC3 and CXCL8 are highlighted as the two most upregulated genes (Log_2 fold change) under all conditions. (F) Gene ontology and (G) TRRUST analysis of the 30 upregulated genes performed with Metascape software. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; *** P< 0.0004, **** P< 0.0001). Related to Figures S1 and S2.

Figure 2

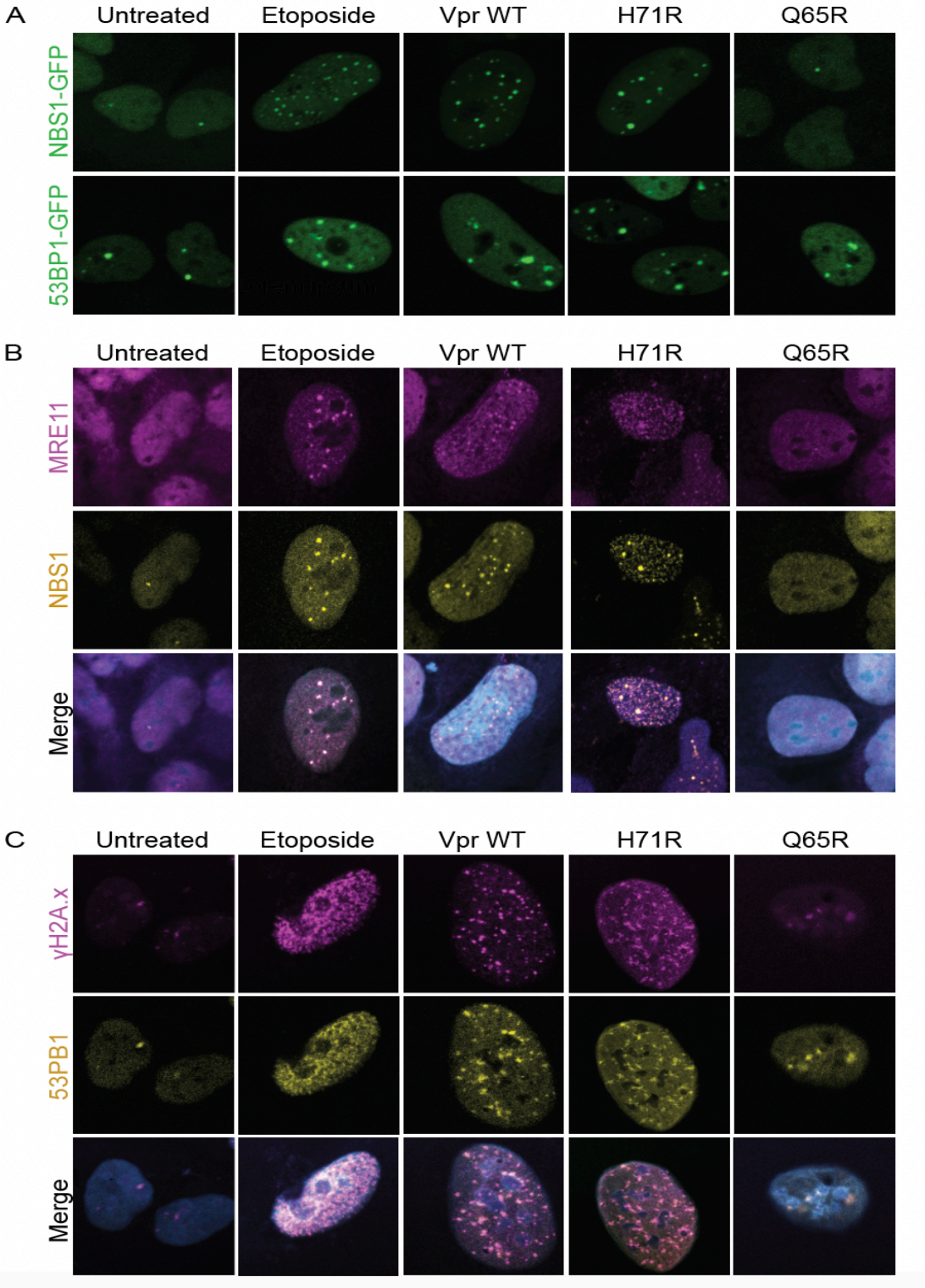


Chapter 2 Figure 2. HIV-1 Vpr activates RelA and promotes NF-κB transcription independent of cell cycle arrest and CRL4A^{DCAF1} engagement.

(A) qRT-PCR validation of upregulated NF-κB target genes BIRC3 and CXCL8. Normalized expression (ΔΔCt) was calculated by normalizing to GAPDH followed by calculating fold change to untreated or empty vector treated cells. n=3, one representative experiment shown. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; RelA (red) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. n=3, one representative experiment shown. Asterisk indicate statistical significance

compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; *** P< 0.0003, **** P< 0.0001). Related to Figure S3.

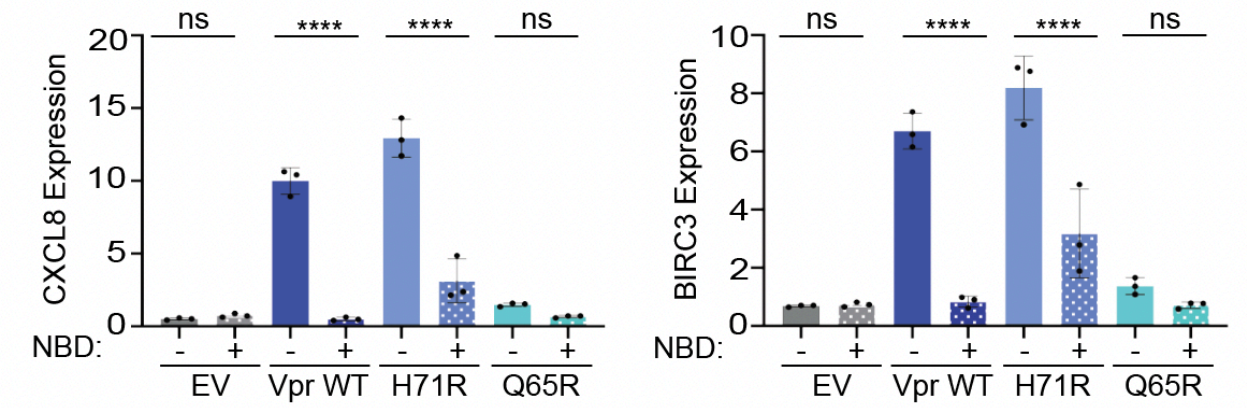
Figure 3



Chapter 2 Figure 3. Vpr-induced DNA damage activates ATM signaling independent of cell cycle arrest and CRL4A^{DCAF1} engagement.

(A) Live-cell imaging of U2OS cells stably expressing NBS1-GFP or 53PB1-GFP. Cells were infected under the same conditions as Fig. 1A. and representative images were taken at 33 hpi at 63x magnification. n=3, one representative experiment shown. (B) Representative images of co-localization of DNA damage sensors MRE11 (magenta) and NBS1 (yellow) in U2OS NBS1-GFP or (C) DNA damage transducers yH2A.x (magenta) and 53PB1 (yellow) in U2OS 53PB1-GFP cells infected under the same conditions as Fig. 1A. Representative images taken at 24 hpi at 63x magnification. n=3, one representative experiment shown. Related to Figure S4.

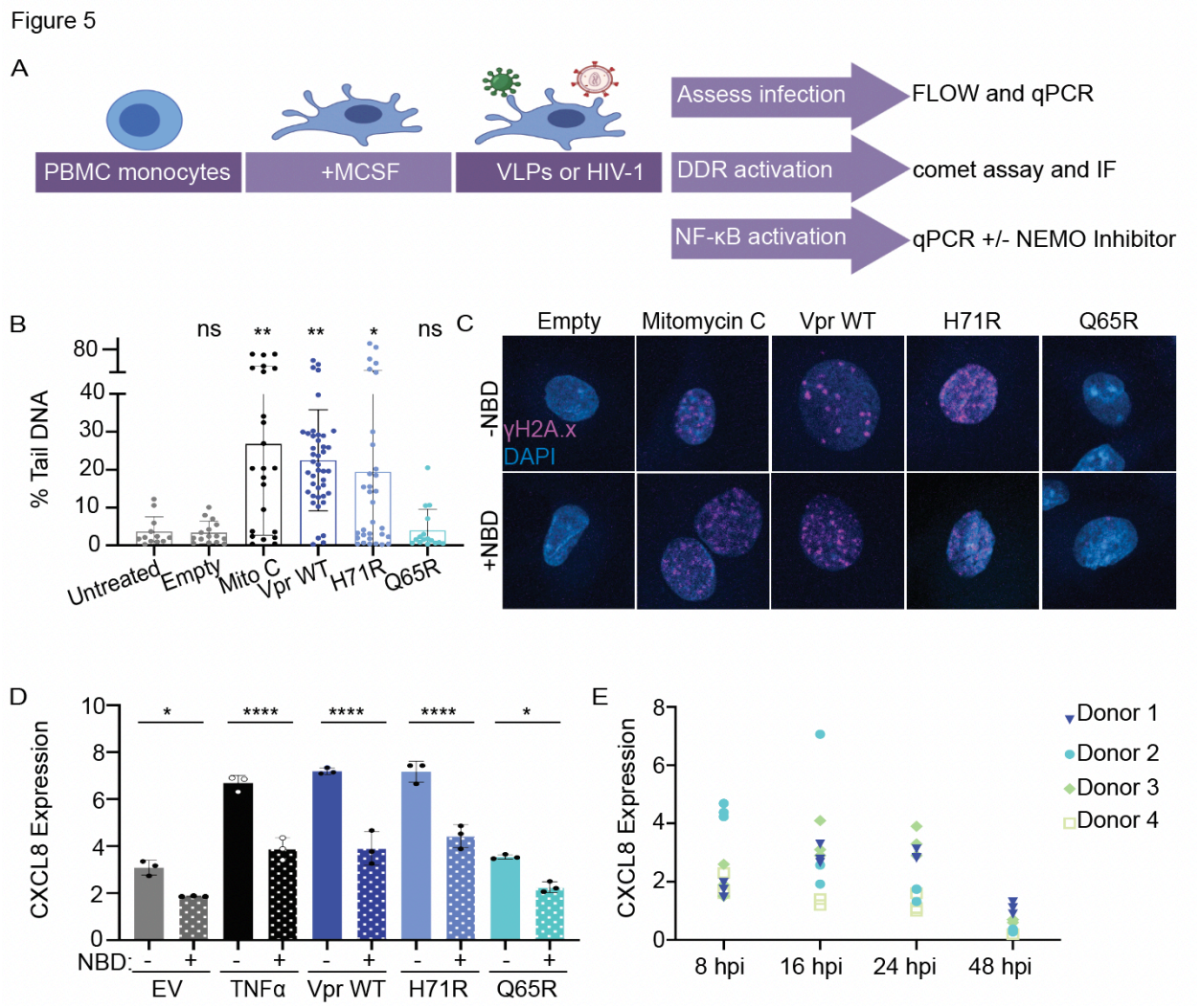
Figure 4



Chapter 2 Figure 4. Vpr upregulates NF-κB transcription dependent on NEMO, yet independent of cell cycle arrest and CRL4A^{DCAF1} engagement.

qRT-PCR of BIRC3 and CXCL8 in the presence or absence of Nemo-Binding Inhibitor peptide (NBD) at 36 hpi. U2OS cells were pretreated with 100uM of NBD for 2 hours prior to infection with rAAV expressing 3xFLAG-tagged Vpr WT, H71R, Q65R, empty vector (negative control). Normalized expression to GAPDH. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; **** P< 0.0001).

Figure 5

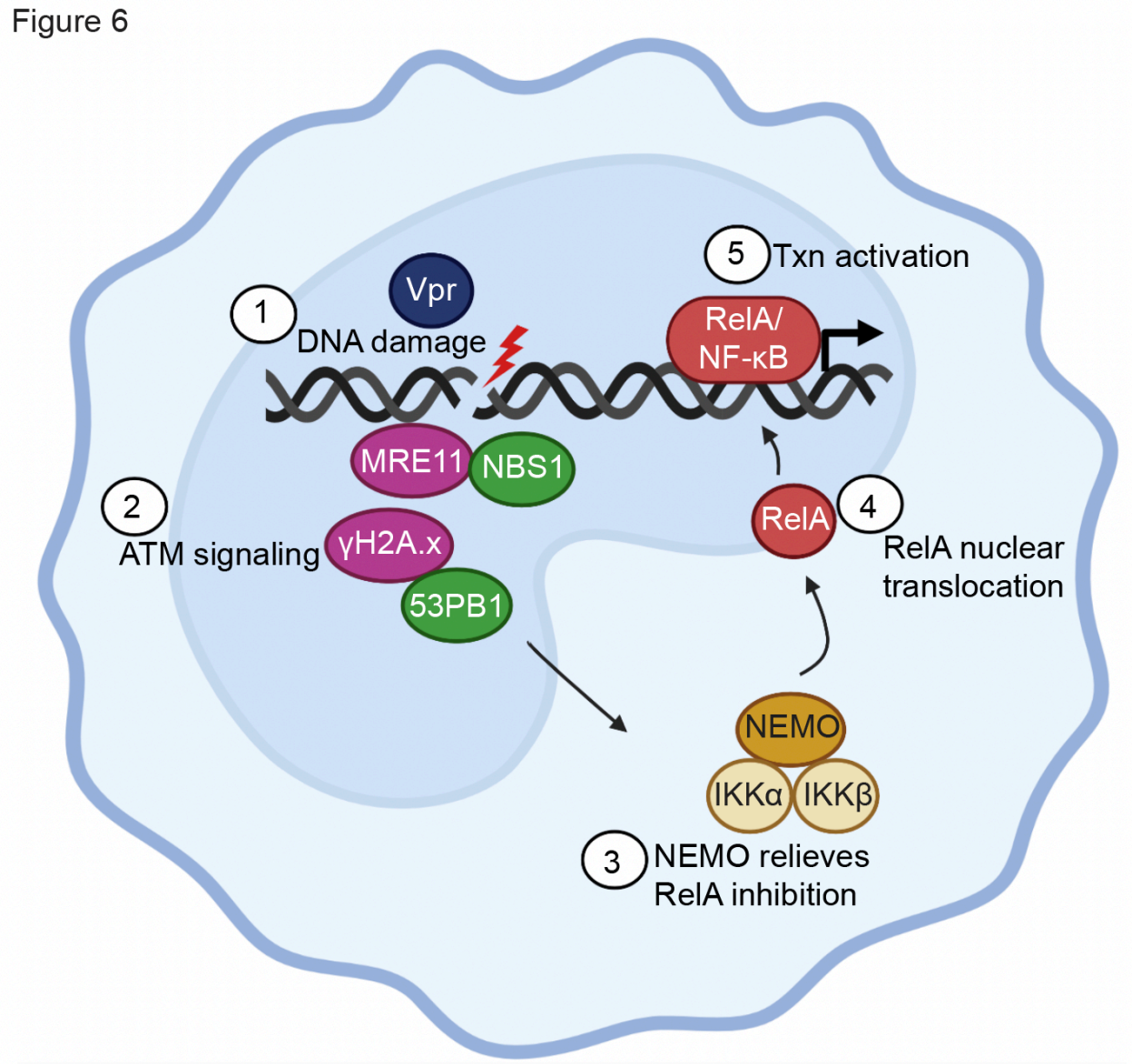


Chapter 2 Figure 5. Vpr-induced DNA damage activates ATM-NEMO signaling and NF-κB transcription in primary MDMs early during infection.

(A) Experimental schematic depicting the isolation and differentiation of primary human monocyte-derived macrophages (MDMs) from peripheral blood mononuclear cells (PBMCs) followed by delivery of Vpr via Virus-like Particles (VLPs) or HIV-1 infection. Infected MDMs were assayed for induction of DNA damage, activation of DDR signaling, and the upregulation of NF-κB target genes in the context of NEMO inhibitor. (B) Comet assay of MDMs treated with VLPs packaging 3xFLAG-tagged Vpr WT, H71R, Q65R, empty VLPs (negative control), or 25uM

Mitomycin C (positive control) for 8 hours. Comet assay analysis was performed as in Fig. 1A. n=2, one representative experiment shown. (C) Representative immunofluorescence images of MDMs infected with VLPs and treated or not with Nemo-Binding Inhibitor peptide (NBD) as in Fig. 5B; γH2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 100x magnification. n=3, one representative experiment shown. (D) qRT-PCR for CXCL8 of MDMs treated with NBD as in Fig. 5C with TNFa as positive control. n=3, one representative experiment shown. (E) qRT-PCR for CXCL8 of MDMs infected with HIV-1 ΔENV for 8, 16, 24, and 48 hpi from four separate donors. Normalized expression to GAPDH. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; * P<0.03, ** P < 0.005, **** P< 0.0001). Related to Figure S5.

Figure 6



Chapter 2 Figure 6. Model showing Vpr-induced DNA damage activates ATM and NEMO signaling resulting in RelA nuclear translocation and NF-κB transcriptional upregulation.

1. Vpr induces double-strand DNA breaks independent of cell cycle arrest and $CRL4A^{DCAF1}$ complex engagement. 2. Vpr-induced DNA damage activates markers of ATM signaling including MRE11, NBS1, γH2A.x, and 53PB1. 3. ATM-DDR activation results in NEMO relieving RelA inhibition from inhibitor of NF-κB (IκBα and IκBβ). 4. RelA translocates into the nucleus. 5. RelA

binds to NF-κB promoters and activates transcription (txn) of NF-κB target genes such as BIRC3 and CXCL8. (Schematic made on Biorender).

Figure Supplement 1

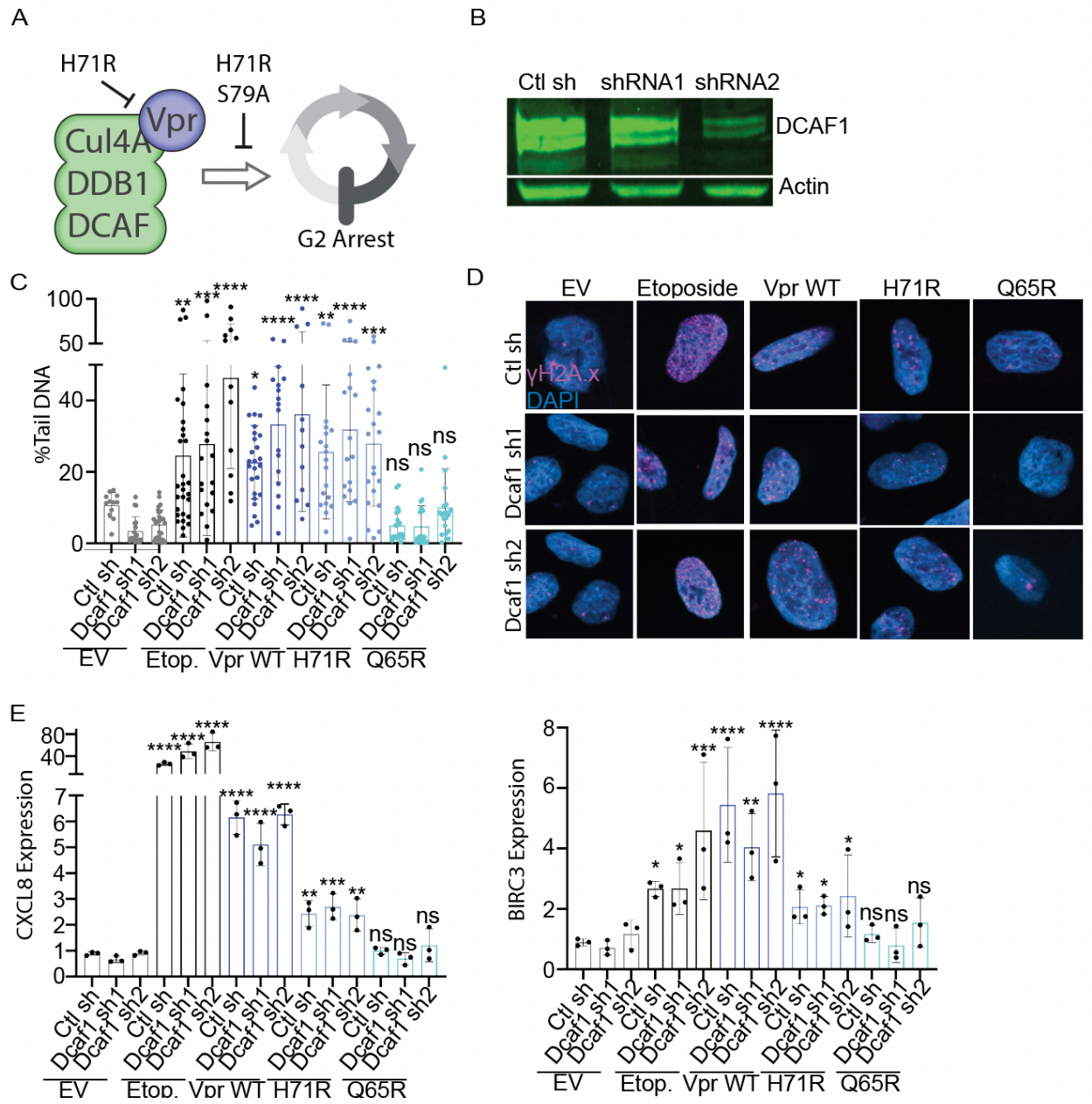


Figure Supplement 1. Knockdown of DCAF1 does not abrogate the ability of Vpr to induce DNA damage, activate DDR signaling, or upregulate NF-κB target genes.

(A) Schematic representation of Vpr mutants used in these studies to decouple some Vpr functions. Vpr S79A and Vpr H71R do not induce cell cycle arrest and H71R also does not bind DCAF1 of the CRL4A^{DCAF1} complex to degrade host proteins or remodel the proteome. Vpr Q65R

(not shown) is a largely non-functional negative control. (B) Western blot of endogenous DCAF1 U2OS cells to test stable knockdown with shRNA. Control (ctl) shRNA and two DCAF1-specific shRNAs (shRNA 1 and shRNA2) were used. Actin is used as a loading control. (C) Comet assay of DCAF1 knock-down cells infected with rAAV and analyzed as in Fig 1A. n=1. (D) Representative immunofluorescence images of DCAF1 knock-down U2OS cells infected under the same conditions as Fig. 1A.; γH2A.x (magenta) and nuclei with DAPI (blue). Images were taken at 63x magnification. n=1, one representative image shown. (E) qRT-PCR for CXCL8 and BIRC3 in DCAF1 knock-down U2OS cells infected under the same conditions as Fig. 1A. n=1. Asterisk indicate statistical significance compared to untreated control, as determined by t-test (NS, nonsignificant; * P< 0.02, ** P<0.004, *** P<0.0003, **** P<0.0001). EV, empty vector negative control; Etop., etoposide positive control.

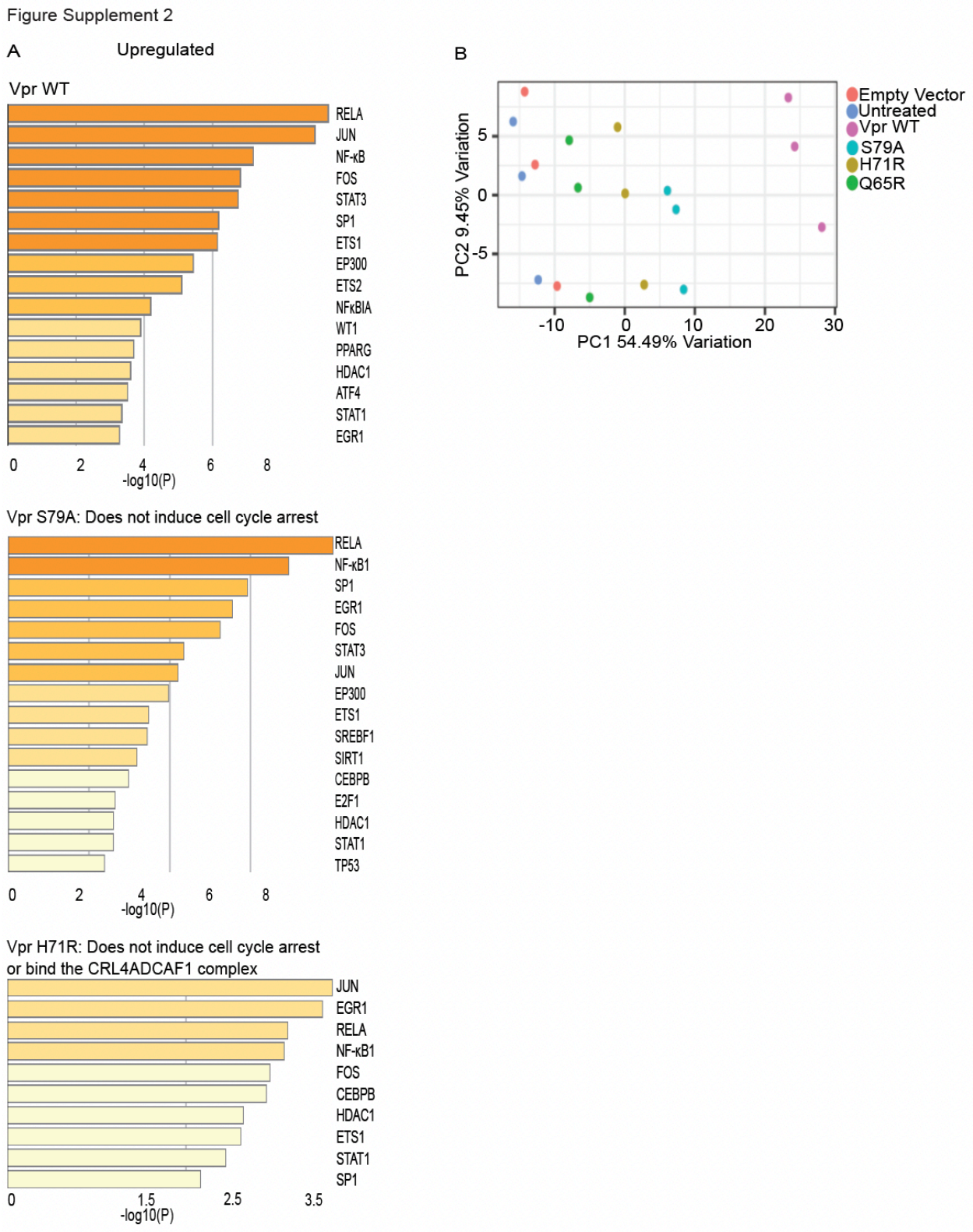


Figure Supplement 2. Vpr alters cellular transcription through diverse mechanisms that are either dependent or independent of cell cycle arrest and CRL4A^{DCAF1} engagement.

(A) Gene ontology using Metascape displaying TRRUST analysis for upregulated genes. (B) Principal component analysis (PCA) of the RNA-seq data showing the relatedness of the three biological replicates compared to empty vector or untreated cells.

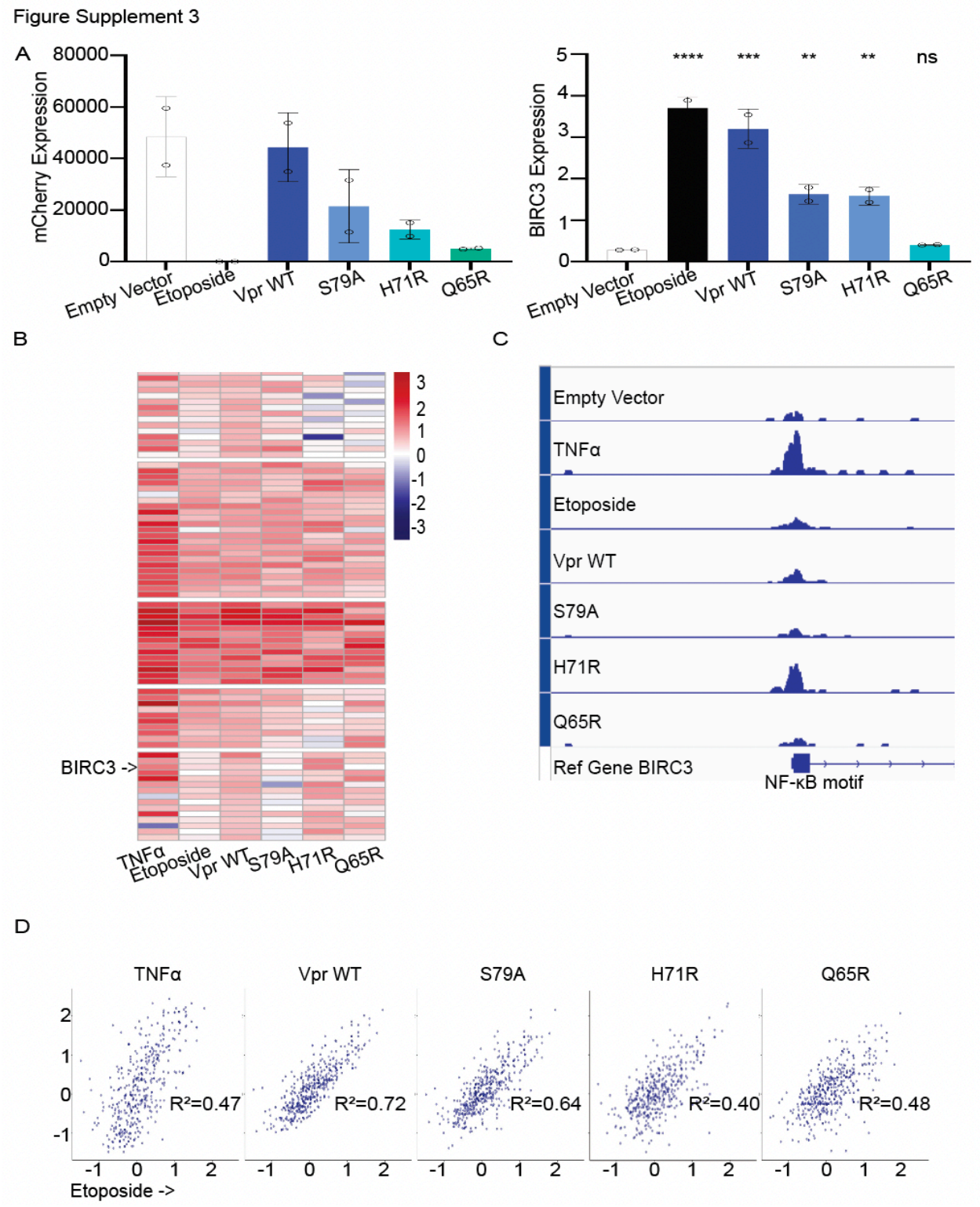


Figure Supplement 3. Vpr WT globally activates RelA to bind NF-κB promoters.

(A) qRT-PCR for mCherry (pscAAV-mCherry-T2A-Vpr) as proxy for Vpr expression in U2OS cells and for BIRC3. U2OS cells were infected with rAAV-2.5 (1.4 × 108 copies/well) expressing HIV-1 Vpr WT, Vpr mutants, empty vector negative control, 50 μM etoposide (Sigma) for 36 hours, or stimulated with TNFα (Thermo) for 16 hours. Normalized expression to GAPDH. (B) Heatmap displays Log_2 fold changes depicting RelA enrichment at NF-κB motifs in red and depletion in blue. Each row represents a single NF-κB motif. The BIRC3 promoter contains an NF-κB motif and is found in the 5th cluster. (C) Genome Browser Tracks for the BIRC3 promoter. Reads mapped to the NF-κB motif in blue. (D) Sample correlations to etoposide. R2 displays fittest regression line.

Figure Supplement 4

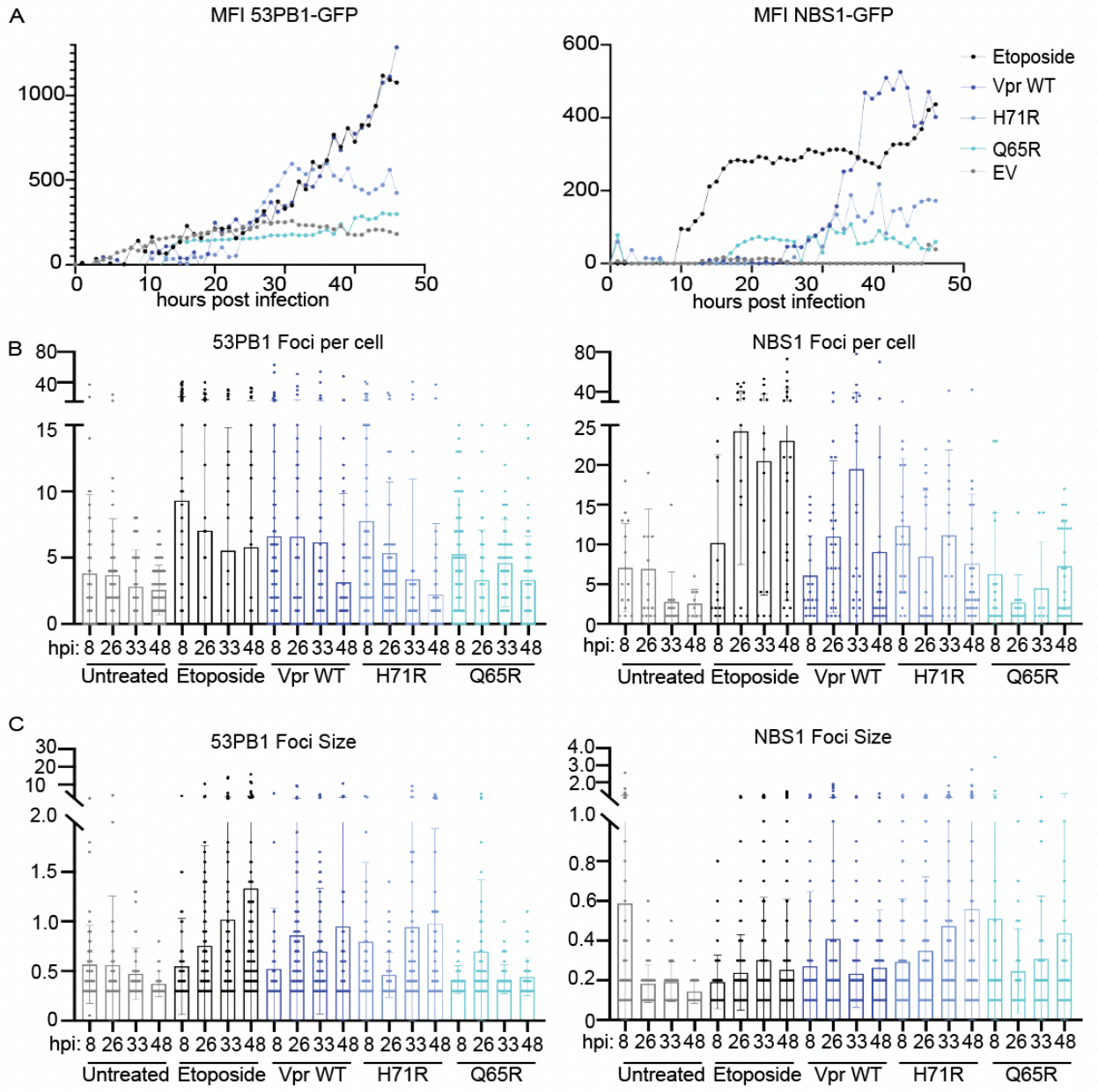


Figure Supplement 4. Vpr-induced DNA damage activates markers of ATM signaling.

(A) Live-cell imaging taken on the IncuCyte of U2OS NBS1-GFP or 53PB1-GFP cells infected as in Fig. 1A for 48 hrs. n=2, one representative experiment shown. (B & C) Quantification of DNA damage induced in the live-cell imaging experiment in Fig. 3A at 8, 26, 33, and 48hpi. Foci per

cell and foci size were quantified using ImageJ software. Images were taken at 63x magnification using the LSM 900. n=3, one representative experiment shown. EV, empty vector

Figure Supplement 5

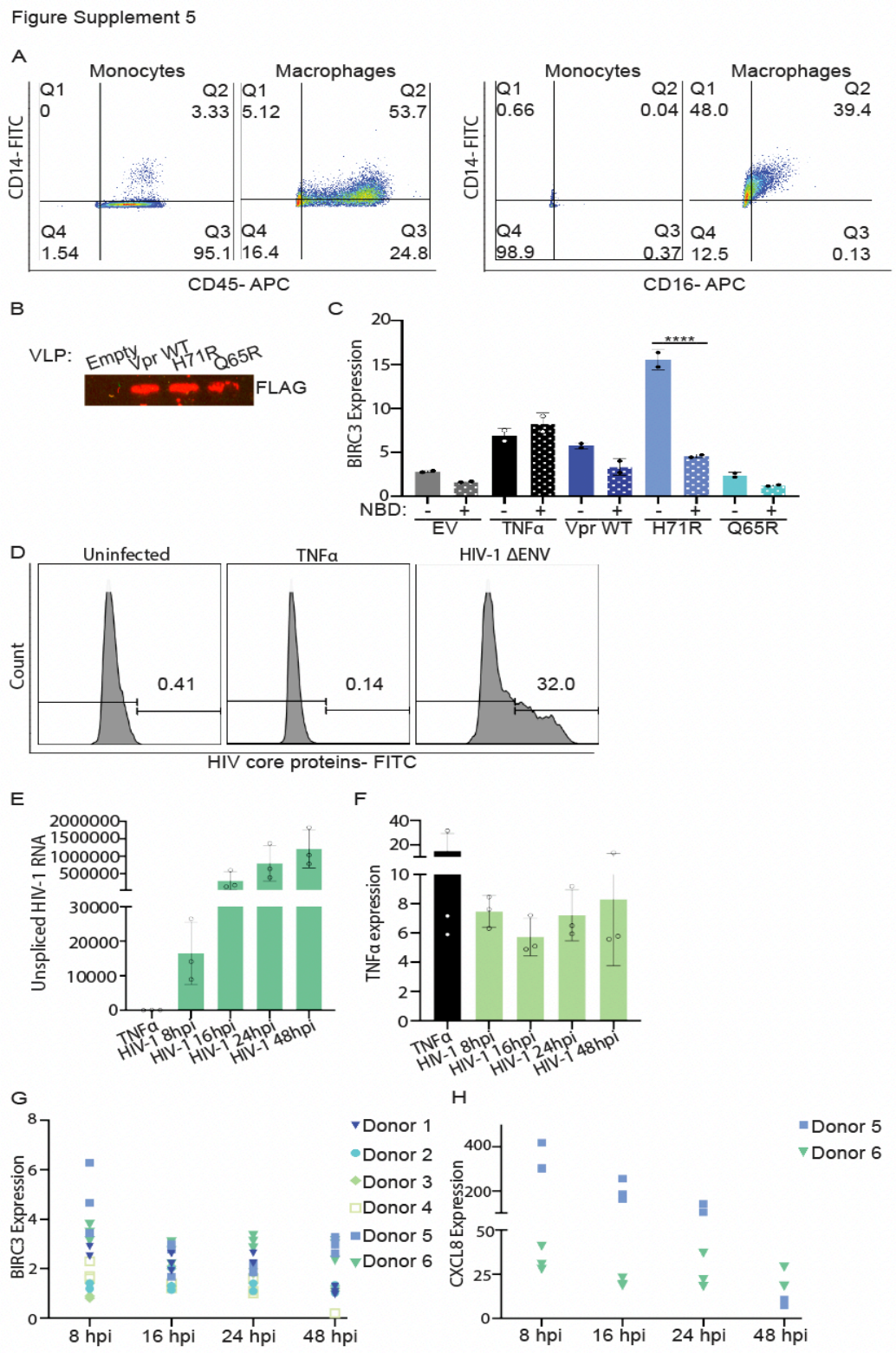


Figure Supplement 5. Infection of primary MDMs with VLPs packaging Vpr protein or HIV-1 infection upregulate NF-κB transcription dependent on NEMO.

(A) Flow cytometry plots displaying differentiation of PBMC-derived monocytes to macrophages. Primary MDMs are CD14+, CD45+ and CD16+. n=6, one representative experiment shown. (B) Western blot of 3X FLAG-Vpr WT and mutants packaged in Virus-like particles (VLPs). (C) qRT-PCR for BIRC3 of MDMs treated with NBD as in Fig. 5C with TNFa as positive control. n=3, one representative experiment shown. (D) Histogram quantifying percent of infected MDMs with HIV-1 ΔENV at 48hpi. n=6, one representative experiment shown. (E) qRT-PCR for unspliced HIV-1 RNA at 8, 16, 24 and 48hpi. n=6, one representative experiment shown. (F) qRT-PCR for TNFa of MDMs infected with HIV-1 ΔENV at 8, 16, 24 and 48hpi. n=6, one representative experiment shown. (G & H) qRT-PCR for BIRC3 or CXCL8 of MDMs infected with HIV-1 dENV at 8, 16, 24 and 48hpi in separate donors. n=6, (G) is all 6 donors, (H) is donors 5 and 6 in addition to those shown in Fig. 5E. Asterisk indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; **** P<0.0001).

MATERIALS AND METHODS

Plasmids.

pcDNA-3xFLAG-Vpr and pscAAV-mCherry-T2A-Vpr WT and mutant plasmids were generated as previously described (28). For rAAV production, pHelper and pAAV-2.5 capsid plasmids were used (Addgene and (28). For VLP production, psPAX2 and pmD2.G were used (Addgene). For HIV-1 ΔENV production, Bru-GFP ΔENV was generated as previously described (62) and pmD2.G (Addgene). For lentivirus stable knock-down of DCAF1, pLKO.1 (Addgene), psPAX2(Addgene) and pmD2.G (Addgene) were used.

Generation of Viruses.

rAAV vectors packaging the pscAAV-mCherry-T2A-Vpr WT or mutant plasmids were generated by transient transfection of HEK 293 cells using polyethyleneimine (PEI) as previously described (63). Virus-like particles (VLPs) packaging Vpr WT or mutant proteins were generated by transient transfection of HEK 293T using TransIT-LT1 (Mirus). VLPs were harvested 48hrs post transfection, concentrated through a 25% sucrose cushion at 24,000 rpm for 3 hrs at 4˚C, and resuspended in PBS. Protein packaging was validated through western blot. HIV-1 ΔENV pseudotyped with VSV-G were generated by transient transfection of HEK 293T using TransIT-LT1 (Mirus). Virus was collected 48hrs post transfection. Lentivirus expressing DCAF1 shRNAs were generated by transfection of HEK 293T using TransIT-LT1 (Mirus). To target the DCAF1 sequence (5' to 3') (AGCACTTCAGATTATCATCAA) we used shRNA 1: AATTCAAAAAGCTGAGAATACTCTTCAAGAACTCGAGTTCTTGAAGAGTATTCTCAGC. To target the DCAF1 sequence (GCTGAGAATACTCTTCAAGAA) we used the shRNA 2: AATTCAAAAAAGCACTTCAGATTATCATCAACTCGAGTTGATGATAATCTGAAGTGCT. Media was changed 18 hours post transfection. Lentiviruses were harvested 48 hours post media change and filtered through a 0.45 μm PES filter.

Cell Lines and Cell Culture.

Human bone osteosarcoma epithelial (U2OS), Human embryonic kidney (HEK) 293, and HEK 293T cells were cultured as adherent cells directly on tissue culture plastic (Greiner) in Dulbecco's modified Eagle's medium (DMEM) growth medium (high glucose, L-glutamine, no sodium pyruvate; Gibco) with 10% fetal bovine serum (FBS) (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 stably expressing 53PB1-GFP (64) and NBS1-GFP (65) were kindly provided by Claudia Lukas (University of Copenhagen, Denmark). Stable DCAF1 knock-down cells were generated by delivering fresh lentiviruses expressing DCAF1 shRNAs or empty vector to U2OS cells. U2OS with integrated lentiviral construct were selected for with 1ug/ml of Puromycin. Knock-down of DCAF1 was assessed through western blots.

Monocyte-Derived Macrophages (MDMs).

Human peripheral blood mononuclear cells (PBMCs) were obtained from human donors at the UCLA/CFAR Virology Core Laboratory. Primary monocytes were isolated from PBMCs by negative selection using the EasySep™ Human T Cell Isolation Kit (STEM CELL) and were differentiated into monocyte-derived macrophages (MDMs) by stimulation with 20ng/mL macrophage colony-stimulating factor (M-CSF) (R&D Systems). MDMs were cultured in Roswell Park Memorial Institute (RPMI) 1640 growth medium (L-Glutamine) with 10% fetal bovine serum (Gibco) at 37°C and 5% CO_2 for 7 days while replenishing media every 3 days.

Alkaline Comet Assay.

The alkaline comet assay and data analysis was performed as previously described (28), with minor changes. MDMs were infected with VLPs delivering Vpr WT and mutants at equal protein levels or 25uM Mitomycin C (Cayman). Cells were harvested with Accutase (STEM CELL) at 10

hrs post infection and resuspended in 0.5% low-melting-point agarose at 37°C. Images were acquired on the Zeiss Axio Imager Z1. Images were analyzed using the OpenComet plug-in for ImageJ.

RNA-sequencing.

Total RNA from U2OS cells was isolated using TRIzol® reagent (Invitrogen). RNA integrity was assessed using the Bioanalyzer TapeStation 4200 RNA High Sensitivity (Agilent). Library Preparation was completed using the KAPA mRNA HyperPrep Kit (Illumina) enriching for Poly(A) RNA with magnetic oligo-dT beads and attaching unique dual indexed adapters. Quality control of the library was done with the Bioanalyzer TapeStation 4200 D1000 High Sensitivity (Agilent). RNA was sequenced using the Hiseq3000 1x50 at the UCLA Technology Center for Genomics & Bioinformatics core. Reads were aligned to the Human h38 STAR genome and gene counts were determined using edgeR. $\log_2$ reads per kilobase of transcript per million reads mapped (rpkms) were calculated for samples compared to untreated cells. Heatmap was generated using pheatmap hierarchical clustering on z-score log2 rpkms. Gene ontology and TRRUST analysis were done on Metascape.

Quantitative Reverse Transcription PCR (RT-qPCR).

Total RNA was isolated using the PureLink™ RNA Mini Kit (Invitrogen). RNA was reverse transcribed using SuperScript IV First-Stand Synthesis System (Invitrogen) with Oligo(dT) primers. qPCR was performed with PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific) on the LightCycler 480 System (Roche) with the following primers (5' to 3') BIRC3 AAGCTACCTCTCAGCCTACTTT and CCACTGTTTTCTGTACCCGGA, CXCL8 TTTTGCCAAGGAGTGCTAAAGA and AACCCTCTGCACCCAGTTTTC, TNFa CTCTTCTGCCTGCTGCACTTTG and ATGGGCTACAGGCTTGTCACTC, HIV-1 unspliced RNA GCGACGAAGACCTCCTCAG and GAGGTGGGTTGCTTTGATAGAGA, and GAPDH

CAAGATCATCAGCAATGCCT and AGGGATGATGTTCTGGAGAG. mRNA levels were quantified by calculating ΔΔCt. Target transcript Ct values were normalized to the Ct value of the housekeeping gene GAPDH followed by calculating fold change to untreated or empty vector treated cells.

CUT&Tag.

U2OS cells were infected with rAAV-2.5 (1.4 × 108 copies/well) expressing HIV-1 Vpr WT, Vpr mutants or empty vector for 36 hours, or stimulated with TNFα (Thermo) for 16 hours. Cells were trypsinized and collected in PBS. CUT&Tag for human RelA was performed per epicypher CUTANA CUT&Tag protocol with 100,000 starting cells. SC-109 anti-human RelA antibody (Santa Cruz Biotechnology) was used at a dilution of 1:1 as the primary antibody per manufacturer protocols. The resulting library was sequenced on the HiSeq 3000 platform (Illumina) with a read length of 50bp. Low quality ends of reads were trimmed (cutoff q=30), remaining adapter sequences were removed with cutadapt. Reads were aligned to the human genome (hg38) with bowtie with the following options: --non-deterministic --mm --phred33 --very-sensitive -x hg38. Aligned reads were deduplicated using picard to keep only unique reads with quality ≥30 for subsequent analysis. CUT&Tag peaks were called using MACS3 with the following settings: -f BAM -g hs -q 0.01 --keep-dup all --call-summits –nomodel. Read counts were normalized to library size by conversion to CPM with edgeR. To ensure that only high-quality peaks were analyzed, only genomic regions with reads <50th percentile in the TNFα and HIV1 Vpr WT were analysis. CPM fold changes were calculated relative to empty vector control and log2 transformed.

Live-Cell Imaging.

U2OS-53PB1-GFP and U2OS-NBS1-GFP cells were imaged using the IncuCyte S3 Live-Cell Analysis Instrument (Sartorius). Mean Fluorescence Intensity (MFI) was calculated using the

Sartorius software. For higher resolution, U2OS-53PB1-GFP and U2OS-NBS1-GFP cells were imaged using the LSM 900. Foci per cell and foci size were analyzed using ImageJ.

Immunofluorescence.

Cells were plated in 6- or 24-well tissue culture plates (Greiner) and allowed to adhere overnight, then infected with VLP (equal protein expression), rAAV-2.5 (1.4×10^8 copies/well), or etoposide (Sigma). U2OS cells were permeabilized with 0.5% Triton X-100 in PBS at 4°C for 5 min and fixed in 4% PFA for 20 min, MDM cells were permeabilized with 0.1% Saponin (Fisher) in PBS at 4°C for 15 min and fixed in 4% PFA for 15 min. Cells were washed, incubated with blocking buffer (3% BSA, 0.05% Tween 20, and 0.04 NaN_3 in PBS for U2OS cells or 3% FBS in 0.1% Saponin for MDM cells) for 30 min. Cells were probed with appropriate primary antibodies (anti-γH2A.x Ser139, anti-53BP1, or anti-RelA(p65) [Cell Signaling], anti-GFP [Takara], and anti-MRE11 [Novous]) and then washed and probed with Alexa Fluor-conjugated secondary antibodies (Life Technologies). Nuclei were stained with diamidino-2-phenylindole (DAPI; Life Technologies). Images were acquired on the LSM 980.

Western Blots.

Protein was collected from cells as previously described (28). Membranes were blocked in intercept blocking buffer (Li-COR Biosciences). Immunoblotting was performed using mouse anti-FLAG M2 (Sigma-Aldrich), rabbit anti-Actin (Bethyl), or mouse anti-DCAF1 (Proteintech) for 1hr. Blots were incubated with secondary antibodies IRDye 800CW anti-Rabbit and IRDye 680RD anti-Mouse (Li-COR Biosciences) for 1 hr and then visualized using the Li-COR Odyssey M (Li-COR Biosciences).

HIV-1 ΔENV Infection.

MDMs were plated in 96-well tissue culture plates (Greiner) and allowed to adhere overnight. MDMs underwent spinfection with HIV-1 ΔENV pseudotyped with VSV-G at 1200× *g* for 90 min at 37°C. Infection was assessed 48 hrs after infection via RT-qPCR and Flow Cytometry.

Flow Cytometry.

Isolated monocytes and infected MDMs were lifted from tissue culture plates using accutase (STEM CELL). Cells were stained for CD14-FITC, CD45-APC, or CD16-APC (STEM CELL) for 30 min at 4°C, washed with PBS, fixed in 4% PFA for 15 min, permeabilized with 0.1% Triton X-100 in PBS at 4°C for 15 min, then washed with PBS. Cells were probed for HIV-1 core antigen-FITC KC57 (Beckman Coulter) for 1hr at 4°C then washed with PBS and resuspended in FACS buffer (5% FBS in PBS). Events were assessed by flow cytometry on the AttuneNxT (Thermo Fisher Scientific). At least 10,000 events were collected per run. Data was analyzed using FlowJo software.

Statistical Analyses.

All statistical analyses were performed using GraphPad Prism 9.

Author Contributions.

Conceptualization, C.S. and O.I.F; methodology, C.S.; Cut and Tag, A.G,; writing original draft C.S and O.I.F; writing, review and editing C.S. and O.I.F; visualization C.S. and O.I.F.; supervision and funding acquisition O.I.F. All authors have read and agreed to the published version of the manuscript.

Funding.

This work was supported by NIH NIAID grant R01AI147837 to O.I.F. C.S was supported by NIH NIAID F31 Ruth L. Kirschstein National Research Service Award Predoctoral Fellowship F31-

AI165286, and the NIH NIGMS Grant GM007185 T32 Ruth L. Kirschstein National Research Service Award Cell and Molecular Biology Training Grant. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Acknowledgements.

We like to thank Dr. Julia Mack and Julianne Ashby for their assistance and expertise with Live-cell imaging using the LSM900. We thank Dr. Alexander Hoffmann and Dr. Diane Lefaudeux for her instruction on RNA-seq analysis. We thank Dr. Claudia Lukas for providing U2OS cells that stably express 53PB1-GFP and NBS1-GFP. We thank Dr. Steven Bensinger for the LightCycler 480 System, Dr. Jesse Zamudio for the Qubit fluorometer, Dr. Peter Bradley for the Zeiss Axio Imager Z1, Dr. Matteo Pellegrini for the TapeStation, Dr. Steve Jacobsen and Dr. Jefferey Long for the LSM980. We thank Dr. Randilea Nichols Doyle, Karly Nisson, and Vivian Yang for providing comments on the manuscript.

REFERENCES

1. Blackford AN, Jackson SP. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. Mol Cell. 2017 Jun 15;66(6):801–17.

2. Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. Nat Rev Mol Cell Biol. 2013 Apr;14(4):197–210.

3. Matthews HK, Bertoli C, de Bruin RAM. Cell cycle control in cancer. Nat Rev Mol Cell Biol. 2022 Jan;23(1):74–88.

4. Valerie K, Delers A, Bruck C, Thiriart C, Rosenberg H, Debouck C, et al. Activation of human immunodeficiency virus type 1 by DNA damage in human cells. Nature. 1988 May;333(6168):78–81.

5. Dunphy G, Flannery SM, Almine JF, Connolly DJ, Paulus C, Jønsson KL, et al. Non-canonical Activation of the DNA Sensing Adaptor STING by ATM and IFI16 Mediates NF-κB Signaling after Nuclear DNA Damage. Mol Cell. 2018 Sep 6;71(5):745-760.e5.

6. Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, et al. Cellular Senescence: Defining a Path Forward. Cell. 2019 Oct 31;179(4):813–27.

7. Ryan EL, Hollingworth R, Grand RJ. Activation of the DNA Damage Response by RNA Viruses. Biomolecules. 2016 Jan 6;6(1):2.

8. Weitzman MD, Fradet-Turcotte A. Virus DNA Replication and the Host DNA Damage Response. Annu Rev Virol. 2018 Sep 29;5(1):141–64.

9. Tachiwana H, Shimura M, Nakai-Murakami C, Tokunaga K, Takizawa Y, Sata T, et al. HIV-1 Vpr Induces DNA Double-Strand Breaks. Cancer Res. 2006 Jan 15;66(2):627–31.

10. Fregoso OI, Emerman M. Activation of the DNA Damage Response Is a Conserved Function of HIV-1 and HIV-2 Vpr That Is Independent of SLX4 Recruitment. Prasad VR, editor. mBio. 2016 Nov 2;7(5):e01433-16.

11. Lang SM, Weeger M, Stahl-Hennig C, Coulibaly C, Hunsmann G, Müller J, et al. Importance of vpr for infection of rhesus monkeys with simian immunodeficiency virus. J Virol. 1993 Feb;67(2):902–12.

12. Beaumont T, Van Nuenen A, Broersen S, Blattner WA, Lukashov VV, Schuitemaker H. Reversal of Human Immunodeficiency Virus Type 1 IIIB to a Neutralization-Resistant Phenotype in an Accidentally Infected Laboratory Worker with a Progressive Clinical Course. J Virol. 2001 Mar;75(5):2246–52.

13. Balliet JW, Kolson DL, Eiger G, Kim FM, McGann KA, Srinivasan A, et al. Distinct Effects in Primary Macrophages and Lymphocytes of the Human Immunodeficiency Virus Type 1 Accessory Genes vpr, vpu, and nef: Mutational Analysis of a Primary HIV-1 Isolate. Virology. 1994 May 1;200(2):623–31.

14. Mashiba M, Collins DR, Terry VH, Collins KL. Vpr Overcomes Macrophage-Specific Restriction of HIV-1 Env Expression and Virion Production. Cell Host Microbe. 2014 Dec 10;16(6):722–35.

15. Miller CM, Akiyama H, Agosto LM, Emery A, Ettinger CR, Swanstrom RI, et al. Virion-Associated Vpr Alleviates a Postintegration Block to HIV-1 Infection of Dendritic Cells. Sundquist WI, editor. J Virol. 2017 Jul;91(13):e00051-17.

16. Paxton W, Connor RI, Landau NR. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. J Virol. 1993 Dec;67(12):7229–37.

17. Roshal M, Kim B, Zhu Y, Nghiem P, Planelles V. Activation of the ATR-mediated DNA Damage Response by the HIV-1 Viral Protein R. J Biol Chem. 2003 Jul;278(28):25879–86.

18. Zhang F, Bieniasz PD. HIV-1 Vpr induces cell cycle arrest and enhances viral gene expression by depleting CCDC137. eLife. 9:e55806.

19. Lahouassa H, Blondot ML, Chauveau L, Chougui G, Morel M, Leduc M, et al. HIV-1 Vpr degrades the HLTF DNA translocase in T cells and macrophages. Proc Natl Acad Sci. 2016 May 10;113(19):5311–6.

20. Schröfelbauer B, Yu Q, Zeitlin SG, Landau NR. Human Immunodeficiency Virus Type 1 Vpr Induces the Degradation of the UNG and SMUG Uracil-DNA Glycosylases. J Virol. 2005 Sep;79(17):10978–87.

21. Ahn J, Vu T, Novince Z, Guerrero-Santoro J, Rapic-Otrin V, Gronenborn AM. HIV-1 Vpr Loads Uracil DNA Glycosylase-2 onto DCAF1, a Substrate Recognition Subunit of a Cullin 4A-RING E3 Ubiquitin Ligase for Proteasome-dependent Degradation *. J Biol Chem. 2010 Nov 26;285(48):37333–41.

22. Lim ES, Fregoso OI, McCoy CO, Matsen FA, Malik HS, Emerman M. The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. Cell Host Microbe. 2012 Feb 16;11(2):194–204.

23. Zhou X, DeLucia M, Ahn J. SLX4-SLX1 Protein-independent Down-regulation of MUS81-EME1 Protein by HIV-1 Viral Protein R (Vpr) *♦. J Biol Chem. 2016 Aug 12;291(33):16936–47.

24. Yan J, Shun MC, Hao C, Zhang Y, Qian J, Hrecka K, et al. HIV-1 Vpr Reprograms CLR4^{DCAF1} E3 Ubiquitin Ligase to Antagonize Exonuclease 1-Mediated Restriction of HIV-1 Infection. Smithgall TE, editor. mBio. 2018 Nov 7;9(5):e01732-18.

25. Lv L, Wang Q, Xu Y, Tsao LC, Nakagawa T, Guo H, et al. Vpr Targets TET2 for Degradation by CRL4VprBP E3 Ligase to Sustain IL-6 Expression and Enhance HIV-1 Replication. Mol Cell. 2018 Jun 7;70(5):961-970.e5.

26. Romani B, Shaykh Baygloo N, Aghasadeghi MR, Allahbakhshi E. HIV-1 Vpr Protein Enhances Proteasomal Degradation of MCM10 DNA Replication Factor through the Cul4-

DDB1[VprBP] E3 Ubiquitin Ligase to Induce G2/M Cell Cycle Arrest. J Biol Chem. 2015 Jul 10;290(28):17380–9.

27. Greenwood EJD, Williamson JC, Sienkiewicz A, Naamati A, Matheson NJ, Lehner PJ. Promiscuous Targeting of Cellular Proteins by Vpr Drives Systems-Level Proteomic Remodeling in HIV-1 Infection. Cell Rep. 2019 Apr 30;27(5):1579-1596.e7.

28. Li D, Lopez A, Sandoval C, Nichols Doyle R, Fregoso OI. HIV Vpr Modulates the Host DNA Damage Response at Two Independent Steps to Damage DNA and Repress Double-Strand DNA Break Repair. mBio. 2020 Aug 4;11(4):e00940-20.

29. Brzostek-Racine S, Gordon C, Van Scoy S, Reich NC. The DNA Damage Response Induces IFN. J Immunol. 2011 Nov 15;187(10):5336–45.

30. Roux P, Alfieri C, Hrimech M, Cohen EA, Tanner JE. Activation of Transcription Factors NF-κB and NF-IL-6 by Human Immunodeficiency Virus Type 1 Protein R (Vpr) Induces Interleukin-8 Expression. J Virol. 2000 May 15;74(10):4658–65.

31. Wu ZH, Shi Y, Tibbetts RS, Miyamoto S. Molecular Linkage Between the Kinase ATM and NF-κB Signaling in Response to Genotoxic Stimuli. Science. 2006 Feb 24;311(5764):1141–6.

32. Huang TT, Wuerzberger-Davis SM, Wu ZH, Miyamoto S. Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. Cell. 2003 Nov 26;115(5):565–76.

33. Miyamoto S. Nuclear initiated NF-κB signaling: NEMO and ATM take center stage. Cell Res. 2011 Jan;21(1):116–30.

34. Liu R, Lin Y, Jia R, Geng Y, Liang C, Tan J, et al. HIV-1 Vpr stimulates NF-κB and AP-1 signaling by activating TAK1. Retrovirology. 2014 Jun 9;11(1):45.

35. Kogan M, Deshmane S, Sawaya BE, Gracely EJ, Khalili K, Rappaport J. Inhibition of NF-κB activity by HIV-1 Vpr is dependent on Vpr binding protein. J Cell Physiol. 2013 Apr;228(4):781–90.

36. Belzile JP, Duisit G, Rougeau N, Mercier J, Finzi A, Cohen ÉA. HIV-1 Vpr-Mediated G2 Arrest Involves the DDB1-CUL4AVPRBP E3 Ubiquitin Ligase. PLOS Pathog. 2007 Jul 13;3(7):e85.

37. Le Rouzic E, Belaïdouni N, Estrabaud E, Morel M, Rain JC, Transy C, et al. HIV1 Vpr arrests the cell cycle by recruiting DCAF1/VprBP, a receptor of the Cul4-DDB1 ubiquitin ligase. Cell Cycle Georget Tex. 2007 Jan 15;6(2):182–8.

38. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun. 2019 Apr 3;10(1):1523.

39. Russo RC, Garcia CC, Teixeira MM, Amaral FA. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. Expert Rev Clin Immunol. 2014 May 1;10(5):593–619.

40. Frazzi R. BIRC3 and BIRC5: multi-faceted inhibitors in cancer. Cell Biosci. 2021 Jan 7;11(1):8.

41. Mitchell S, Vargas J, Hoffmann A. Signaling via the NFκB system. WIREs Syst Biol Med. 2016 May;8(3):227–41.

42. Nakai-Murakami C, Shimura M, Kinomoto M, Takizawa Y, Tokunaga K, Taguchi T, et al. HIV-1 Vpr induces ATM-dependent cellular signal with enhanced homologous recombination. Oncogene. 2007 Jan;26(4):477–86.

43. Strickland I, Ghosh S. Use of cell permeable NBD peptides for suppression of inflammation. Ann Rheum Dis. 2006 Nov;65(Suppl 3):iii75–82.

44. Liang Z, Liu R, Lin Y, Liang C, Tan J, Qiao W. HIV-1 Vpr protein activates the NF-κB pathway to promote G2/M cell cycle arrest. Virol Sin. 2015 Dec 1;30(6):441–8.

45. Strebel K. HIV Accessory Proteins versus Host Restriction Factors. Curr Opin Virol. 2013 Dec;3(6):10.1016/j.coviro.2013.08.004.

46. Lopez A, Nichols Doyle R, Sandoval C, Nisson K, Yang V, Fregoso OI. Viral Modulation of the DNA Damage Response and Innate Immunity: Two Sides of the Same Coin. J Mol Biol. 2022 Mar 30;434(6):167327.

47. Virgilio MC, Miguel Disbennett W, Chen T, Lubow J, Welch JD, Collins KL. HIV-1 Vpr combats the PU.1-driven antiviral response in primary human macrophages. BioRxiv Prepr Serv Biol. 2023 Mar 21;2023.03.21.533528.

48. Joseph AM, Ladha JS, Mojamdar M, Mitra D. Human immunodeficiency virus-1 Nef protein interacts with Tat and enhances HIV-1 gene expression. FEBS Lett. 2003 Jul 31;548(1–3):37–42.

49. Sawaya BE, Khalili K, Gordon J, Taube R, Amini S. Cooperative Interaction between HIV-1 Regulatory Proteins Tat and Vpr Modulates Transcription of the Viral Genome *. J Biol Chem. 2000 Nov 10;275(45):35209–14.

50. Bauby H, Ward CC, Hugh-White R, Swanson CM, Schulz R, Goujon C, et al. HIV-1 Vpr Induces Widespread Transcriptomic Changes in CD4 $^{+}$ T Cells Early Postinfection. Goff SP, editor. mBio. 2021 Jun 29;12(3):e01369-21.

51. Chougui G, Munir-Matloob S, Matkovic R, Martin MM, Morel M, Lahouassa H, et al. HIV-2/SIV viral protein X counteracts HUSH repressor complex. Nat Microbiol. 2018 Aug;3(8):891–7.

52. Yurkovetskiy L, Guney MH, Kim K, Goh SL, McCauley S, Dauphin A, et al. Primate immunodeficiency virus proteins Vpx and Vpr counteract transcriptional repression of proviruses by the HUSH complex. Nat Microbiol. 2018 Dec;3(12):1354–61.

53. Cicchini L, Westrich JA, Xu T, Vermeer DW, Berger JN, Clambey ET, et al. Suppression of Antitumor Immune Responses by Human Papillomavirus through Epigenetic Downregulation of CXCL14. Imperiale MJ, editor. mBio. 2016 Jul 6;7(3):e00270-16.

54. Romero N, Favoreel HW. Pseudorabies virus infection triggers NF-κB activation via the DNA damage response, but actively inhibits NFkB-dependent gene expression. J Virol. 2021 Oct 6;JVI.01666-21.

55. Harman AN, Nasr N, Feetham A, Galoyan A, Alshehri AA, Rambukwelle D, et al. HIV Blocks Interferon Induction in Human Dendritic Cells and Macrophages by Dysregulation of TBK1. Silvestri G, editor. J Virol. 2015 Jul;89(13):6575–84.

56. Gummuluru S, Emerman M. Cell Cycle- and Vpr-Mediated Regulation of Human Immunodeficiency Virus Type 1 Expression in Primary and Transformed T-Cell Lines. J Virol. 1999 Jul;73(7):5422–30.

57. Goh WC, Rogel ME, Kinsey CM, Michael SF, Fultz PN, Nowak MA, et al. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: A mechanism for selection of Vpr in vivo. Nat Med. 1998 Jan;4(1):65–71.

58. Nodder SB, Gummuluru S. Illuminating the Role of Vpr in HIV Infection of Myeloid Cells. Front Immunol [Internet]. 2019 [cited 2023 Apr 3];10. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01606

59. Varin A, Decrion AZ, Sabbah E, Quivy V, Sire J, Van Lint C, et al. Synthetic Vpr Protein Activates Activator Protein-1, c-Jun N-terminal Kinase, and NF-κB and Stimulates HIV-1 Transcription in Promonocytic Cells and Primary Macrophages*. J Biol Chem. 2005 Dec 30;280(52):42557–67.

60. Fabryova H, Kao S, Sukegawa S, Miyagi E, Taylor L, Ferhadian D, et al. HIV-1 Vpr Induces Degradation of Gelsolin, a Myeloid Cell-Specific Host Factor That Reduces Viral Infectivity by Inhibiting the Expression and Packaging of the HIV-1 Env Glycoprotein. Goff SP, editor. mBio. 2023 Feb 28;14(1):e02973-22.

61. Bruce JW, Bracken M, Evans E, Sherer N, Ahlquist P. ZBTB2 represses HIV-1 transcription and is regulated by HIV-1 Vpr and cellular DNA damage responses. PLOS Pathog. 2021 Feb 26;17(2):e1009364.

62. Yamashita M, Emerman M. Capsid Is a Dominant Determinant of Retrovirus Infectivity in Nondividing Cells. J Virol. 2004 Jun;78(11):5670–8.

63. Choi VW, Asokan A, Haberman RA, Samulski RJ. Production of Recombinant Adeno-Associated Viral Vectors for In Vitro and In Vivo Use. Curr Protoc Mol Biol [Internet]. 2007 Apr [cited 2023 May 3];78(1). Available from: https://onlinelibrary.wiley.com/doi/10.1002/0471142727.mb1625s78

64. Bekker-Jensen S, Lukas C, Melander F, Bartek J, Lukas J. Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1. J Cell Biol. 2005 Jul 18;170(2):201–11.

65. Lukas C, Melander F, Stucki M, Falck J, Bekker-Jensen S, Goldberg M, et al. Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. EMBO J. 2004 Jul 7;23(13):2674–83.

CHAPTER 3: Conserved and Divergent Engagement of the DDR by HIV-1 and HIV-2 Vpr

Carina Sandoval[a] and Oliver I. Fregoso[ab]

Molecular Biology Institute[a], Department of Microbiology, Immunology & Molecular Genetics[b] at the University of California, Los Angeles, California, United States of America

INTRODUCTION

HIV-1 and HIV-2 encode accessory proteins that enhance viral replication through various mechanisms(1). Accessory proteins can directly interact with host proteins to usurp their cellular function or antagonize their antiviral activity. HIV-1 and HIV-2 are two evolutionarily district viruses that resulted from specific cross-species events. HIV-1 group M originated from cross-species transmission from chimpanzees infected with Simian immunodeficiency virus (SIV) into humans. In contrast, HIV-2 originated from cross-species transmission from sooty mangabeys infected with SIV into humans(2). Although HIV-1 and HIV-2 have two distinct lineages, they encode many similar accessory proteins. For example, HIV-1 encodes the accessory proteins Vpr, Vif, Vpu, and Nef, while HIV-2 encodes Vpr, Vif, Nef, and Vpx (3). Previous research has led to the understanding of most lentiviral accessory proteins. However, Vpr is the last accessory protein encoded by HIV-1 and HIV-2 without a known primary conserved function. Here, we aim to understand whether HIV-1 and HIV-2 Vpr are functionally similar.

Most phenotypes described for Vpr involve the ability of Vpr to modulate the DNA damage response (DDR)(4,5). First, HIV-1 and HIV-2 Vpr were found to induce G2/M cell cycle arrest, which requires engagement with the E3 ubiquitin ligase $CRL4A^{DCAF1}$ complex (6). Later, HIV-1 Vpr was described to cause degradation of many host proteins involved in the DDR, including CCDC137 (7), HLTF (8), UNG2 (9), SAMHD1 (10), Mus81/EME1 (11), EXO1 (12), TET2 (13), and MCM10 (14), as well as more global proteome remodeling (15). However, HIV-2 Vpr engages host DDR proteins differentially than HIV-1. HIV-2 Vpr does not degrade HLTF or UNG2 (16), two components of the DNA repair machinery. Furthermore, unlike HIV-1 Vpr, HIV-2 Vpr does not bind the host endonuclease SLX4 (4). Most recently, our lab identified that HIV-1 and HIV-2 Vpr inhibits DNA repair, which requires engagement with the E3 ubiquitin ligase $CRL4A^{DCAF1}$ complex (5). It is becoming clear that although there are many similarities in how HIV-1 and HIV-2 Vpr

engage the DDR, there are also differences that may explain the distinct cellular consequences caused by HIV-1 and HIV-2 Vpr. Overall, Vpr modulates the DDR at multiple and potentially unique steps. However, it is unknown if modulation of the DDR is conserved among HIV-1 and HIV-2 Vpr.

Recently, we have shown that HIV-1 and HIV-2 Vpr activates the DNA damage response (DDR). Both HIV-1 and HIV-2 Vpr induce double and single-strand DNA breaks, which activate markers of ATM signaling, such as γH2A.X, MRE11, NBS2, and 53BP1 (see Chapter 1). Employing Vpr mutants that decouple Vpr functions (Table 2), we have shown that HIV-1 Vpr induces DNA breaks and activates classical ATM signaling without cell cycle arrest and engagement with the $CRL4A^{DCAF1}$ complex. Furthermore, we identified that Vpr-induced DNA damage activates RelA/NF-κB transcription, causing upregulation of two NF-κB target genes (BIRC3 and CXCL8) (see Chapter 1). However, it is unknown whether the ability to activate ATM signaling and cause upregulation of NF-κB target genes BIRC3 and CXCL8 are conserved by HIV-2 Vpr. Lastly, because Vpr is packaged in the virion, we asked if physiological levels of virion-delivered Vpr can activate the DDR and ATM signaling.

Here, we identified that HIV-2 Vpr induces DNA breaks and activates markers of ATM-signaling, suggesting that activation of the DDR is conserved among HIV-1 and HIV-2 Vpr. Furthermore, we found that HIV-2 Vpr WT and H76R mutant, which does not cause cell cycle arrest or engage the $CRL4A^{DCAF1}$ complex, promote translocation of RelA into the nucleus, suggesting activation of NF-kB. However, HIV-2 Vpr and mutants did not upregulate NF-κB target genes, BIRC3 and CXCL8, distinct from HIV-1 Vpr. These data suggest there are divergent cellular consequences among HIV-1 and HIV-2 Vpr engagement with the DDR. Lastly, we found that virion delivered HIV-1 and HIV-2 Vpr-activated markers of ATM signaling that resembled classical DDR activation rather than dysregulation. Our data suggest that HIV-1 and HIV-2 Vpr engage the DDR and

activate ATM signaling, yet the downstream consequences are divergent. Our work offers novel insight into the primary conserved function of Vpr and the ability to engage the DDR to enhance viral replication (as shown in Chapter 1).

RESULTS

HIV-2 Vpr induces DNA damage and activates DDR signaling independent of CRL4A^{DCAF1} engagement and cell cycle arrest, as conserved by HIV-1 Vpr

We set out to understand whether the ability of HIV-1 Vpr to induce DNA damage, activate DDR signaling, and promote RelA/NF-κB transcription was conserved among HIV-2 Vpr. We have previously shown that the HIV-2 Rod9 Vpr mutant S84A causes DNA damage without cell cycle arrest (Li and Lopez). We have also shown that HIV-2 Rod9 Vpr H76R does not bind the DCAF1 component of the CRL4A^{DCAF1} E3 ubiquitin ligase complex and does not induce cell cycle arrest (5), while the HIV-2 Rod9 Vpr Q70R is non-functional. These HIV-2 Vpr mutants allowed us to determine if HIV-2 Vpr engages the DDR without cell cycle arrest and CRL4A^{DCAF1} engagement as seen with HIV-1 Vpr. U2OS cells were infected with rAAV expressing either HIV-2 Rod9 Vpr wild type (WT), Vpr H76R, Vpr Q70R, or etoposide (positive control). Induction of DNA damage was assessed by comet assay, and activation of DDR signaling was assessed by immunofluorescence 24 hours post-infection. We found that HIV-2 Vpr WT induces DNA breaks (Fig. 1A) and activates the DDR marker γH2A.x (Fig. 1B), while the nonfunctional Q70R mutant does not. In addition, we found that H76R induces DNA breaks (Fig. 1A) and activates γH2A.x (Fig. 1B) at levels similar to Vpr WT. Our data indicate that HIV-2 Vpr induces DNA breaks and activates DDR signaling without cell cycle arrest and CRL4A^{DCAF1} engagement, as seen with HIV-1 Vpr (see Chapter 1). These data suggest that the mechanism of engagement of the DDR is conserved by HIV-1 and HIV-2 Vpr.

HIV-2 Vpr activates RelA as HIV-1Vpr

To determine if HIV-2 Vpr activates RelA, we employed immunofluorescence for RelA in U2OS cells. As RelA is cytoplasmic when inactive and nuclear when active, therefore we expected HIV-2 Vpr to promote RelA translocation similar to HIV-1 Vpr. We found that HIV-2 Vpr WT and H76R mutant promote RelA nuclear translocation similar to etoposide (positive control), while the Vpr Q70R and empty vector do not (Fig 1 C). These data suggest that the ability of Vpr to activate the DDR and activate RelA is conserved among HIV-1 and HIV-2 Vpr.

HIV-2 Vpr does not upregulate NF-κB target genes that HIV-1 Vpr upregulates

Next, we tested the ability of HIV-2 Vpr to upregulate NF-κB transcription using qRT-PCR for NF-κB target genes BIRC3 and CXCL8. Although HIV-2 Vpr WT and mutants were expressed similarly to HIV-1 Vpr (Fig 1E), HIV-2 Vpr WT and mutants did not upregulate CXCL8 and BIRC3 (Fig 1D). These data were unexpected for two reasons, HIV-2 Vpr WT and H76R activate RelA, and HIV-1 Vpr WT promotes the expression of BIRC3 and CXCL8. These data suggest that the cellular consequences of Vpr activating the DDR are distinct among HIV-1 and HIV-2 Vpr.

Virion-delivered HIV-2 Vpr activates DDR signaling

We next tested our hypothesis that virion-delivered (incoming) HIV-2 Vpr can activate the DDR. We generated VLPs that package HIV-1 Vpr WT or HIV-2 Vpr WT, although at different levels, and empty VLPs (Fig. 2D). Using live-cell imaging via IncuCyte, we tracked DDR activation throughout infection. U2OS cells stably expressing 53PB1-GFP or NBS1-GFP were infected with VLPs that packaged HIV-1 Vpr WT, HIV-2 Vpr WT, or empty particles, and cells were images up to 48hpi. Cells were treated with etoposide (positive control). We found that VLPs delivering HIV-1 and HIV-2 Vpr WT activated NBS1 and 53PB1, damage sensor and transducer, respectively (Fig 2A). Furthermore, since many viruses dysregulate DDR signaling through relocalization of

DDR markers, we assayed for co-localization of damage sensors and transducers in the context of HIV-1 and HIV-2 Vpr-induced DNA damage. We found both damage sensors, MRE11 and NBS1, and transducers 53PB1 and γH2A.x, colocalize in cells treated with HIV-1 Vpr WT and HIV-2 Vpr WT VLPs, similar to etoposide-treated cells (Fig. 2B&C). These data suggest that incoming Vpr from HIV-1 and HIV-2 Vpr activates ATM signaling and do not dysregulate the DDR.

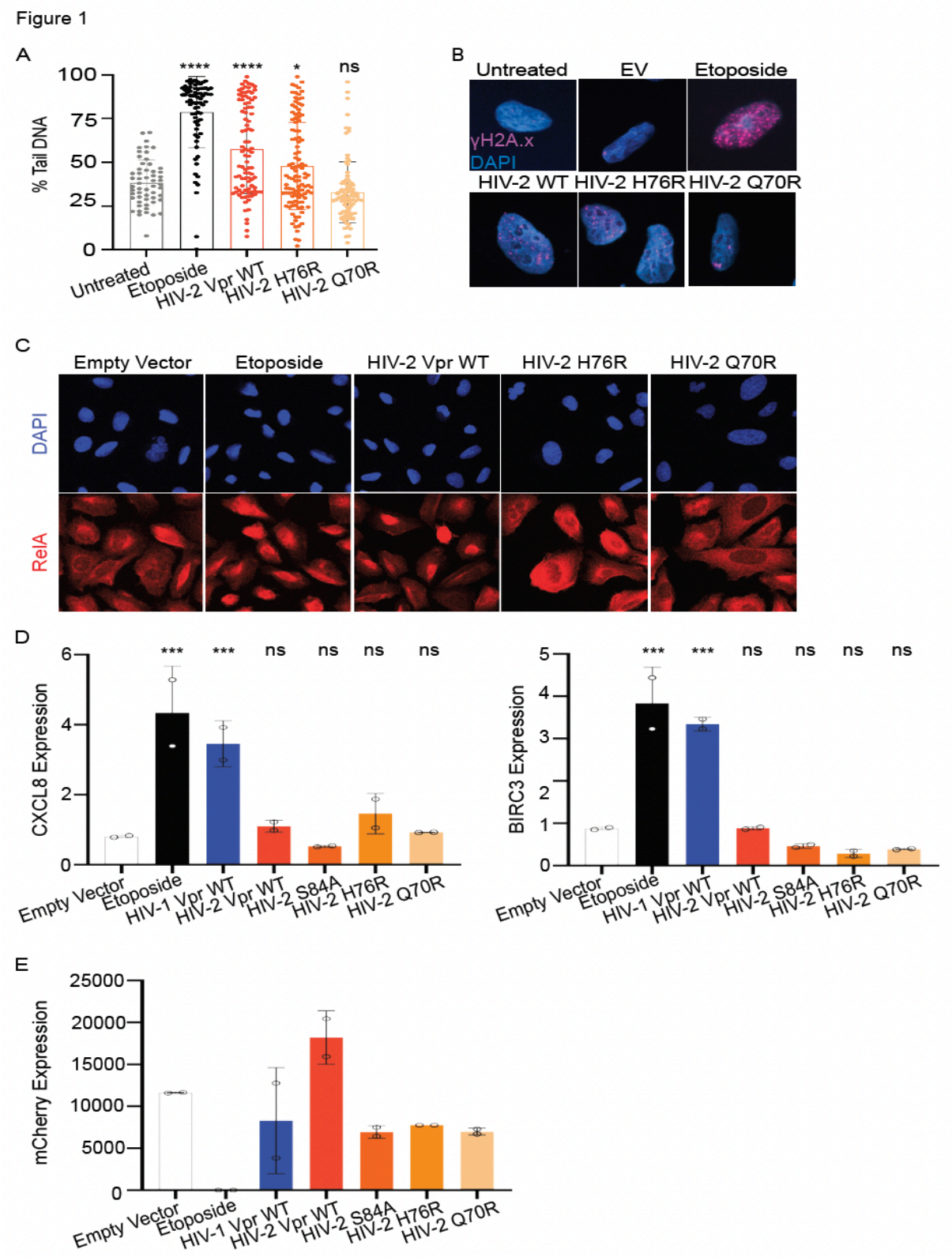
DISCUSSION

We tested the hypothesis that the ability of Vpr to activate the DDR and promote RelA/NF-κB transcription is conserved among HIV-1 and HIV-2 Vpr. Here we found that HIV-2 Vpr induces DNA damage and activates DDR signaling independent of $CRL4A^{DCAF1}$ engagement and cell cycle arrest similar to HIV-1 Vpr. Furthermore, we showed that HIV-2 Vpr WT and Vpr mutants that only induce DNA damage promote RelA translocation yet do not upregulate known NF-κB target genes that HIV-1 Vpr upregulates. These data suggest that although HIV-1 and HIV-2 Vpr similarly activate the DDR, the cellular consequence of HIV-1 and HIV-2 Vpr-induced DNA damage is distinct. Therefore, it will be essential to perform RNA-sequencing of HIV-2 Vpr WT and Vpr mutant infected cells to determine the effect of HIV-2 Vpr activating RelA on cellular transcription.

Vpr is delivered in the incoming virion and is thought to play an important role early during infection to enhance viral replication. We investigated whether virion-delivered HIV-1 and HIV-2 Vpr activate or regulate the DDR. We showed that VLPs delivering either HIV-1 or HIV-2 Vpr promote classical activation of ATM signaling. These data suggest that the virion-delivered HIV-1 and HIV-2 Vpr evolved a common mechanism to promote ATM activation, yet how activation of ATM signaling promotes viral replication is unknown. Furthermore, whether a common ancestor of HIV-1 and HIV-2 Vpr can also activate ATM signaling is unknown. Understanding if related SIV Vpr's engagement the ATM signaling will help determine if this is a novel or conserved function of Vpr. Our data suggest that incoming HIV-1 and HIV-2 Vpr activate DDR signaling. Furthermore, we found that HIV-2 Vpr induces DNA damage and activates DDR signaling independent of $CRL4A^{DCAF1}$ engagement and cell cycle arrest, similar to HIV-1 Vpr. Although HIV-2 WT and H76R mutants promote RelA nuclear translocation, HIV-2 Vpr does not upregulate known NF-κB target genes that HIV-1 Vpr upregulates. These data suggest that HIV-1 and HIV-2 Vpr have evolved to

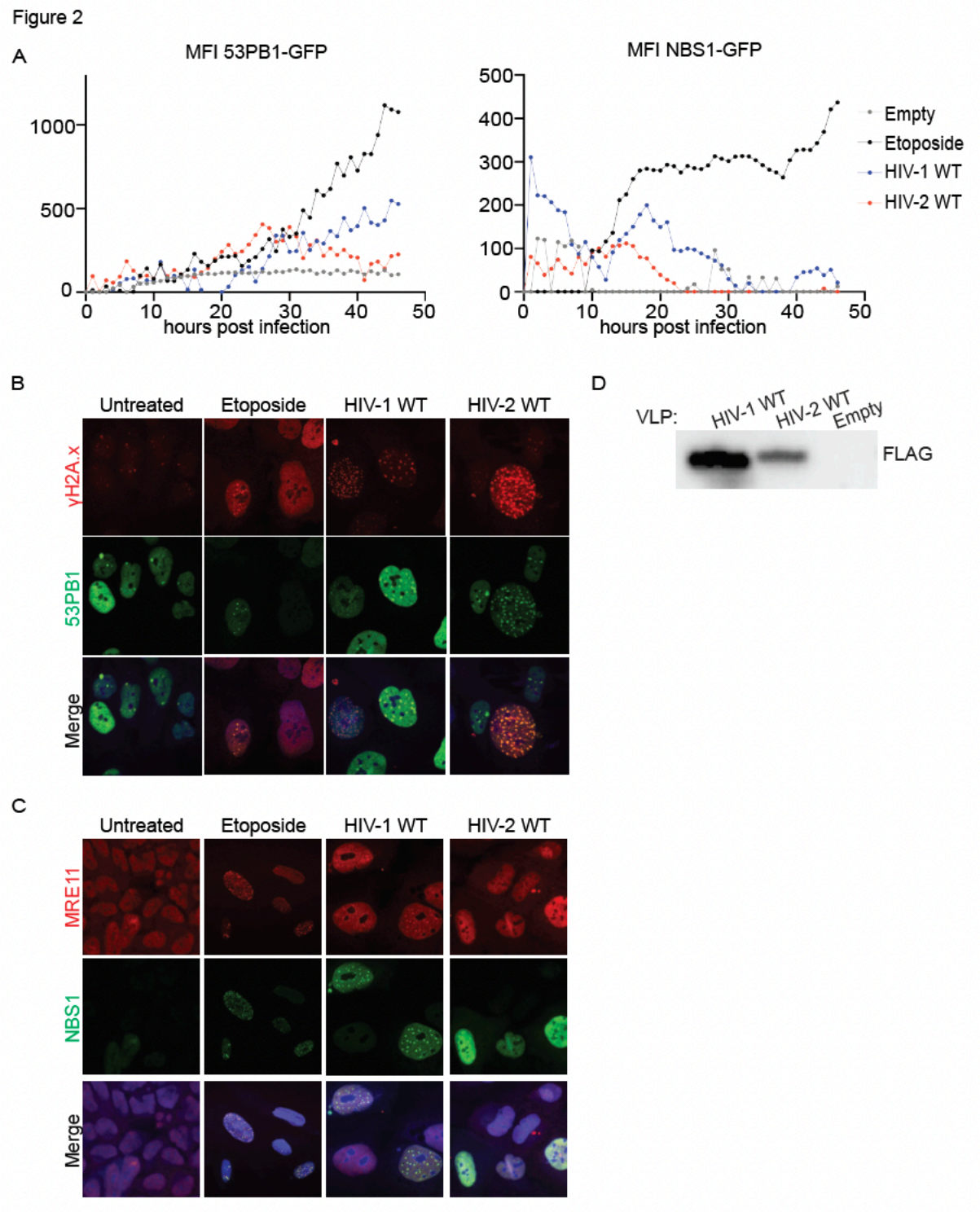
activate ATM signaling and modulate RelA, yet the effects on NF-κB by HIV-2 Vpr are unclear and distinct from HIV-1 Vpr.

HIV-1 and HIV-2 activate the DDR through similar mechanisms, yet the effect on cellular transcription is distinct. HIV-1 and HIV-2 Vpr activate the DDR and promote RelA activation without engagement of the $CRL4A^{DCAF1}$ complex and associated phenotypes. These data suggest that HIV-1 and HIV-2 Vpr are not degrading an unknown host factor to activate the DDR. Alternatively, HIV-1 and HIV-2 Vpr may co-opt a different E3 ubiquitin ligase to target host proteins for degradation independent of DCAF1 binding. Nonetheless, it will be essential to determine how HIV-1 and HIV-2 Vpr activate RelA yet have distinct effects on cellular transcription. One reason why HIV-1 and HIV-2 Vpr have distinct cellular functions could be because HIV-2 encodes Vpx, a paralog of Vpr, that is described to function in similar ways as HIV-1. However, HIV-2 Vpx does not affect the cell cycle and has evolved to enhance viral replication in non-cycling cells. Therefore, it is necessary to determine which functions of HIV-1 Vpr are shared among HIV-2 Vpr versus HIV-2 Vpx. Overall, our work identified conserved and divergent functions among HIV-1 and HIV-2 Vpr that start to unravel the evolutionarily conserved function of Vpr.



Chapter 3 Figure 1. HIV-2 Vpr induces DNA damage and promotes RelA translocation yet does not upregulate NF-κB target genes (BIRC3 and CXCL8) as HIV-1 Vpr.

(A) Comet assay of U2OS cells infected with rAAV expressing 3xFLAG-tagged HIV-2 Vpr WT, H76R, Q70R, untreated (negative control), or 50uM etoposide (positive control) at 24 hours. Percent tail DNA was quantified using the OpenComet plug-in for the ImageJ software. Each circle represents one cell. n=3, one representative experiment is shown. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; γH2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. n=3, one representative experiment shown. EV, empty vector. (C) Representative immunofluorescence images of U2OS cells infected under the same conditions as Fig. 1A; RelA (red) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. n=3, one representative experiment shown. (D) qRT-PCR of upregulated NF-κB target genes BIRC3 and CXCL8 represented as log2 normalized expression to GAPDH. 50uM etoposide and HIV-1 Vpr WT (positive controls) and untreated cells (negative control). n=3, one representative experiment shown. Asterisks indicate statistical significance compared to untreated control, as determined by one-way ANOVA test (NS, nonsignificant; *P<0.01, *** P< 0.0003, **** P< 0.0001).



Chapter 3 Figure 2. Virus-like particles delivering HIV-1 and HIV-2 Vpr WT activate markers of ATM signaling.

(A) Live-cell imaging taken on the IncuCyte of U2OS NBS1-GFP or 53PB1-GFP cells infected with virus-like particles delivering HIV-1 and HIV-2 Vpr WT or empty virus-like particles (negative control) and 50uM etoposide (positive control). Cells were imaged for 48 hp. n=2, one representative experiment is shown. (B) Cells were treated as in Figure 2B. Representative images of co-localization of DNA damage transducers yH2A.x (red) and 53PB1 (green) in U2OS 53PB1-GFP cells. Representative images were taken at 24 hp at 63x magnification. n=3, one representative experiment shown. (C) Cells were treated as in Figure 2B. Representative images of co-localization of DNA damage sensors MRE11 (red) and NBS1 (green) in U2OS NBS1-GFP cells. Representative images were taken at 24 hp at 63x magnification. n=3, one representative experiment shown. (D) Western blot of 3X FLAG-HIV-1 Vpr WT and 3X FLAG-HIV-2 Vpr WT protein packaged in VLPs.

	DNA Damage	Engages the CRL4A^{DCAF1}	Cell Cycle Arrest	NF-κB transcription
HIV-1/HIV-2 Vpr WT	+/+	+/+	+/+	+/-
S79A/S84A	+/+	+/+	-/-	+/-
H71R/H76R	+/+	-/-	-/-	+/-
Q65R/Q70R	-/-	-/-	-/-	-/-

Table 2. HIV-1 and HIV-2 Vpr mutants that decouple cellular effects caused by Vpr.

HIV-1 and HIV-2 Vpr mutatns that decouple cellular efffects caused by Vpr. Highlighted in yellow are discoveries that were made through the work in Chapters 2 & 3 driven by Carina Sandoval. DNA damage was assessed via the comet assay (see Chapter 2). Engagement of CRL4A^{DCAF1} was assessed via co-immunoprecipitation (see Chapter 5). Cell cycle arrest was asssessed via propidium iodide stain flow cytometry (see Chapter 5). NF-kB transcriptional activation was assessed via RNA-seq for HIV-1 Vpr WT and mutants, while NF-kB transcriptional activation for HIV-2 Vpr WT and mutants was assessed by qRT-PCR for known HIV-1 target genes (see Chapter 2 &3).

MATERIALS AND METHODS

Plasmids.

pcDNA-3xFLAG-Vpr and pscAAV-mCherry-T2A-Vpr WT and mutant plasmids were generated as previously described (22). For rAAV production, pHelper and pAAV-2.5 capsid plasmids were used (Addgene and 22). For VLP production, psPAX2 and pmD2.G were used (Addgene).

Generation of Viruses.

rAAV vectors packaging the pscAAV-mCherry-T2A-Vpr WT or mutant plasmids were generated by transient transfection of HEK 293 cells using polyethyleneimine (PEI) as previously described (91). Virus-like particles (VLPs) packaging Vpr WT or mutant proteins were generated by transient transfection of HEK 293T using TransIT-LT1 (Mirus). VLPs were harvested 48hrs post-transfection, concentrated through a 25% sucrose cushion at 24,000 rpm for 3 hrs at 4˚C, and resuspended in PBS. Protein packaging was validated through Western blot.

Cell Lines and Cell Culture.

Human bone osteosarcoma epithelial (U2OS), Human embryonic kidney (HEK) 293, and HEK 293T cells were cultured as adherent cells directly on tissue culture plastic (Greiner) in Dulbecco's modified Eagle's medium (DMEM) growth medium (high glucose, l-glutamine, no sodium pyruvate; Gibco) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO2. All cells were harvested using 0.05% trypsin-EDTA (Gibco). The panel of U2OS cells stably expressing 53PB1-GFP and NBS1-GFP were kindly provided by Claudia Lukas (University of Copenhagen, Denmark).

Alkaline Comet Assay.

The alkaline comet assay and data analysis were performed as previously described (22), with minor changes. U2OS cells were infected with VLPs delivering Vpr WT and mutants at equal protein levels. Images were acquired on the Zeiss Axio Imager Z1. Images were analyzed using the OpenComet plug-in for ImageJ.

Quantitative Reverse Transcription PCR (RT-qPCR)

Total RNA was isolated using the PureLinkTM RNA Mini Kit (Invitrogen). RNA was reverse transcribed using SuperScript IV First-Stand Synthesis System (Invitrogen) with Oligo(dT) primers. qPCR was performed with PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific) on the LightCycler 480 System (Roche) with the following primers (5' to 3') BIRC3 AAGCTACCTCTCAGCCTACTTT and CCACTGTTTTCTGTACCCGGA, CXCL8 TTTTGCCAAGGAGTGCTAAAGA and AACCCTCTGCACCCAGTTTTC, and GAPDH CAAGATCATCAGCAATGCCT and AGGGATGATGTTCTGGAGAG. mRNA levels were quantified by calculating $\Delta\Delta$Ct. Target transcript Ct values were normalized to the Ct value of the housekeeping gene GAPDH followed by calculating fold change to untreated or empty vector-treated cells.

Live-Cell Imaging.

U2OS-53PB1-GFP and U2OS-NBS1-GFP cells were imaged using the IncuCyte S3 Live-Cell Analysis Instrument (Sartorius). Mean Fluorescence Intensity (MFI) was calculated using the Sartorius software.

Immunofluorescence.

Cells were plated in 6- or 24-well tissue culture plates (Greiner) and allowed to adhere overnight, then infected with VLP (equal protein expression), rAAV-2.5 (1.4 × 108 copies/well), or etoposide (Sigma). U2OS cells were permeabilized with 0.5% Triton X-100 in PBS at 4°C for 5 min and fixed

in 4% PFA for 20 min. Cells were washed, incubated with blocking buffer (3% BSA, 0.05% Tween 20, and 0.04 NaN3 in PBS for U2OS cells. Cells were probed with appropriate primary antibodies (anti-γH2A.x Ser139, anti-53BP1, or anti-RelA(p65) [Cell Signaling], anti-GFP [Takara], and anti-MRE11 [Novous]) and then washed and probed with Alexa Fluor-conjugated secondary antibodies (Life Technologies). Nuclei were stained with diamidino-2-phenylindole (DAPI; Life Technologies). Images were acquired on the LSM 980.

Western Blots.

Protein was collected from cells as previously described (5).

Statistical Analyses.

All statistical analyses were performed using GraphPad Prism 9.

REFERENCES

1. HIV-1 Accessory Proteins—Ensuring Viral Survival in a Hostile Environment | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/EEKW67V3/items/6AA2DAE3/item-details

2. Origins of HIV and the AIDS Pandemic | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/EEKW67V3/items/EFFIWVDB/item-details

3. Strebel K. HIV Accessory Proteins versus Host Restriction Factors. Curr Opin Virol. 2013 Dec;3(6):10.1016/j.coviro.2013.08.004.

4. Fregoso OI, Emerman M. Activation of the DNA Damage Response Is a Conserved Function of HIV-1 and HIV-2 Vpr That Is Independent of SLX4 Recruitment. Prasad VR, editor. mBio. 2016 Nov 2;7(5):e01433-16.

5. Li D, Lopez A, Sandoval C, Nichols Doyle R, Fregoso OI. HIV Vpr Modulates the Host DNA Damage Response at Two Independent Steps to Damage DNA and Repress Double-Strand DNA Break Repair. mBio. 2020 Aug 4;11(4):e00940-20.

6. Roshal M, Kim B, Zhu Y, Nghiem P, Planelles V. Activation of the ATR-mediated DNA Damage Response by the HIV-1 Viral Protein R. J Biol Chem. 2003 Jul;278(28):25879–86.

7. Zhang F, Bieniasz PD. HIV-1 Vpr induces cell cycle arrest and enhances viral gene expression by depleting CCDC137. eLife. 9:e55806.

8. Lahouassa H, Blondot ML, Chauveau L, Chougui G, Morel M, Leduc M, et al. HIV-1 Vpr degrades the HLTF DNA translocase in T cells and macrophages. Proc Natl Acad Sci. 2016 May 10;113(19):5311–6.

9. Schröfelbauer B, Yu Q, Zeitlin SG, Landau NR. Human Immunodeficiency Virus Type 1 Vpr Induces the Degradation of the UNG and SMUG Uracil-DNA Glycosylases. J Virol. 2005 Sep;79(17):10978–87.

10. Lim ES, Fregoso OI, McCoy CO, Matsen FA, Malik HS, Emerman M. The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. Cell Host Microbe. 2012 Feb 16;11(2):194–204.

11. Zhou X, DeLucia M, Ahn J. SLX4-SLX1 Protein-independent Down-regulation of MUS81-EME1 Protein by HIV-1 Viral Protein R (Vpr) *♦. J Biol Chem. 2016 Aug 12;291(33):16936–47.

12. Yan J, Shun MC, Hao C, Zhang Y, Qian J, Hrecka K, et al. HIV-1 Vpr Reprograms CLR4 ^{DCAF1} E3 Ubiquitin Ligase to Antagonize Exonuclease 1-Mediated Restriction of HIV-1 Infection. Smithgall TE, editor. mBio. 2018 Nov 7;9(5):e01732-18.

13. Lv L, Wang Q, Xu Y, Tsao LC, Nakagawa T, Guo H, et al. Vpr Targets TET2 for Degradation by CRL4VprBP E3 Ligase to Sustain IL-6 Expression and Enhance HIV-1 Replication. Mol Cell. 2018 Jun 7;70(5):961-970.e5.

14. Romani B, Shaykh Baygloo N, Aghasadeghi MR, Allahbakhshi E. HIV-1 Vpr Protein Enhances Proteasomal Degradation of MCM10 DNA Replication Factor through the Cul4-DDB1[VprBP] E3 Ubiquitin Ligase to Induce G2/M Cell Cycle Arrest. J Biol Chem. 2015 Jul 10;290(28):17380–9.

15. Greenwood EJD, Williamson JC, Sienkiewicz A, Naamati A, Matheson NJ, Lehner PJ. Promiscuous Targeting of Cellular Proteins by Vpr Drives Systems-Level Proteomic Remodeling in HIV-1 Infection. Cell Rep. 2019 Apr 30;27(5):1579-1596.e7.

16. HIV-1 and HIV-2 exhibit divergent interactions with HLTF and UNG2 DNA repair proteins | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/U4EDQXEH/items/RQQRGW5G/item-details

CHAPTER 4: Identifying Novel HIV-1 Restriction and Dependency Factors Involved in the DNA Damage Response

Carina Sandoval[a], Judd F. Hultquist[c], Janet Young[d], Ian Perrone[b], Alex Salas[b], Nevan J. Krogan[c], and Oliver I. Fregoso[b]

[a]Molecular Biology Institute Interdepartmental Doctoral Program, [b]Department of Microbiology, Immunology & Molecular Genetics at the University of California, Los Angeles, California, United States of America

[c]Department of Cellular and Molecular Pharmacology at the University of California, San Francisco, California, United States of America

[d]Division of Basic Sciences, Fred Hutch Cancer Center, Seattle, Washington, United States of America

INTRODUCTION

Various host proteins modulate HIV replication. Antiviral restriction factors are host proteins that limit replication, while dependency factors are host proteins that facilitate replication. For example, LEDGF is a dependency factor because it facilitates integration, an essential step of viral replication (1). In addition, the host DNA damage response (DDR) is a signaling cascade often modulated by diverse viruses to enhance viral replication(2,3). However, it is unknown whether proteins of the DDR can act as antiviral restriction factors or dependency factors during HIV-1 replication. Therefore, we aimed to identify novel HIV-1 antiviral restriction and dependency factors involved in the DDR to characterize new targets for HIV/AIDS therapeutics.

The DNA damage response (DDR) is a highly conserved and essential pathway responsible for safeguarding the genome from genotoxic assault (4). As the DDR must repair DNA with high fidelity to ensure cell survival, DDR pathways, and proteins are evolutionarily conserved. DDR genes are thought to be under strong purifying selection because mutations that change amino acids (non-synonymous) are selected against, as they may be detrimental to the organism. In contrast, genes under strong selective pressures to rapidly evolve will accumulate non-synonymous changes at a rate faster than expected, called positive selection (5). While most rapidly evolving genes are in conflict with pathogens such as viruses, some genes involved in the DDR show signatures of positive selection. Therefore, we wanted to explore this apparent paradox and test our hypothesis that DDR genes are rapidly evolving because they are in conflict with HIV-1.

We aim to identify the DDR proteins that regulate HIV-1 replication and understand mechanistically how these proteins regulate viral replication. To identify candidate DDR genes, we first hypothesized they would show signatures of rapid evolution, indicative of long-standing

evolutionary conflict with pathogens. Therefore, we performed a high-throughput assay to assess the selective pressure on every codon of 321 DDR genes, specifically looking for signatures of rapid evolution called positive selection. We identified that 14.6% (47/321) of these genes show signatures of positive selection on at least two or more amino acids. These data suggest that the DDR genes are being driven to evolve rapidly. We next hypothesized that the positive selection observed on DDR genes resulted from their essential roles in viral replication. To test this hypothesis, we knocked out DDR genes with signatures of positive selection using CRISPR/Cas9 in primary CD4+T cells from two independent donors and assayed for changes in HIV-1 infectivity. We identified candidate genes that increase viral infectivity compared to the non-targeting control, suggesting these are potential antiviral restriction factors. We also identified candidate genes that decreased viral infectivity compared to the non-targeting control, suggesting these are potential viral dependency factors.

To further investigate, we focused on two candidate genes, MUTYH and POLG. Both MUTYH and POLG increased viral replication when knocked-out in CD4+T cells, suggesting they may act as antiviral restriction factors during HIV-1 infection. We validated our two candidate genes, MUTYH and POLG, in Jurkat and THP-1 cells using a three-prong approach; knock-down, overexpression, and asked if they were induced by interferon as part of the antiviral interferon response. We found that knock-down of POLG was lethal and thus could not assess changes in infectivity. Furthermore, we found that shRNAs targeting both isoforms of MUTYH did not knock down protein levels despite the cells being puromycin resistant. Next, overexpression of MUTYH using the Dox inducible system resulted in a transient upregulation of MUTYH protein levels visualized through western blot. Lastly, we found that up to 100ng/ml of either interferon (IFN) IFNα or IFNβ did not modulate MUTYH protein levels over time. Together, these data suggest that MUTYH cellular levels seem tightly regulated in the cell and are unaffected by knock-down, overexpression, or interferon. It will be important to determine how MUTYH protein levels are

regulated and why MUTYH has six sites of positive selection. Following up on this study will identify DDR host factors that regulate HIV-1 replication, which will help further our understanding of DDR proteins and can inform the development of novel antiviral factors that may benefit human health.

RESULTS

Identifying candidate HIV-1 host factors within the DDR that contain signature(s) of positive selection and are induced by IFN

To systematically test the hypothesis that DDR genes are rapidly evolving due to conflict with HIV-1, our lab curated a list of 321 DDR genes and assessed the selective pressure of every codon. To do this, we first identified candidate genes and processed open reading frames (ORFs), obtained primate ortholog sequences, generated sequence alignments, and tested for codon evolution using PAML (6) (Fig. 1A). Strikingly, we identified that 14.6% (47/321) of these genes show signatures of positive selection on at least two or more amino acids, and most of these genes are involved in Homologous Recombination (HR) repair (Fig. 1B, (7)). Furthermore, we identified which of the 321 DDR genes are also induced by interferon (IFN) since many viruses activate IFN. In total, we found that 22 DDR genes have signatures of positive selection, 16 DDR genes are induced by IFN, and 6 DDR genes have both signatures of positive selection and are induced by IFN (Fig. 1C). Together, these data suggest that many DDR genes rapidly evolve even though they have a conserved role in safeguarding the genome and DNA repair.

To determine which of the 44 DDR genes identified are potential novel HIV-1 host factors, we performed a CRISPR screen and knocked-out each DDR gene using four gRNAs in primary CD4+T cells. Four non-targeting gRNAs (NTC) were used as negative controls. The gRNAs were complexed with Cas9 protein and electroporated into CD4+T cells, followed by infection with HIV-1 GFP reporter virus. Infectivity was assessed by GFP readout using flow cytometry. We expected that knockout of a restriction factor will enhance viral replication, while knockout of a dependency factor will decrease viral replication (Fig. 1D). Our approach was validated, as we were able to identify the previously known dependency factors LEDGF (Plum circles) and CXCR4 (Blue

circles) (1,8). As expected, our NTC (Brown circles) did not significantly change infectivity. In total, we identified many DDR genes that when knocked-out altered HIV-1 infectivity (Fig. 1E). We found that MUTYH (Green circles) knockout enhanced viral replication in three out of four gRNAs (Fig. 1F). We also identified POLG as a potential antiviral restriction factor. However, we could not validate these findings as knock-down of POLG was lethal in Jurkat and THP-1 cells (data not shown). Here, we tested the hypothesis that DDR genes show signatures of rapid evolution as a consequence of their conserved role in viral replication. We decided to focus on one candidate gene, MUTYH, to validate our findings and determine the mechanism by which MUTYH restricts viral replication.

The candidate gene, MUTYH, is rapidly evolving and is a potential HIV-1 antiviral restriction factor

To validate our screen, we focused on the DDR gene called MUTYH. MUTYH is a base excision repair (BER) protein that repairs damaged DNA throughout the cell cycle (9). DNA lesion 8-oxo guanine (G^0) is a product of oxidative stress that is repaired by BER. The Mono-functional DNA glycosylase (MUTYH) excises adenines that are misincorporated opposite to G^0 to restore the original sequence (Fig. 2A). Positive selection analysis of MUTYH reveals 6 sites under rapid evolution. MUTYH contains two domains, HhH-GPD and NUDIX_4, and displays positive selection at 35R, 51S, 89V, 320Q, 313R, and 425R (Fig. 2B). We decided to focus on MUTYH because, in our screen, the editing efficiency of MUTYH was calculated to be 42.1% by Tracking of Indels by Decomposition (Tide) analysis (Fig. 2C) and 3 of our 4 MUTYH gRNAs showed enhancement of viral replication when MUTYH was knocked-out (Fig. 2D). Therefore, we hypothesize that MUTYH is an antiviral restriction factor of HIV-1. We expect that knock-down of MUTYH will enhance viral replication, overexpression of MUTYH to decrease viral replication, and expect MUTYH to be IFN inducible.

MUTYH protein levels are refractory to change via knock-down, overexpression or treatment with IFN

To determine if MUTYH is an antiviral restriction factor, we first knocked-down MUTYH using lentiviral shRNAs in HIV-1 relevant cell types, Jurkat and THP-1 cells. Using three different shRNAs targeting MUTYH or three PLKO non-targeting shRNAs, we did not detect knock-down of MUTYH in both Jurkat or THP-1 cells. To test the possibility that only a few cells successfully knocked-down MUTYH, we generated single cell clones that harbored the integrated shRNA targeting MUTYH. Although all cells collected were puromycin resistant, suggesting that they harbor the integrated shRNA, we did not detect consistent knock-down of MUTYH in Jurkat or THP-1 cells (Fig. 3 A &B). Only group 5 of the single cell clones targeted with shRNA3 (3-5) displayed a significant knock-down of MUTYH in Jurkat cells (Fig. 3A). The 3-5 Jurkat cells grew slowly and have not been tested for their effect on HIV-1 replication yet.

Furthermore, MUTYH-FLAG tagged was cloned into a lentiviral doxycycline (Dox) inducible plasmid (pMD145). Lentiviruses expressing the MUTYH or empty Dox inducible plasmids were generated and delivered to Jurkat or THP-1 cells and were selected for using puromycin. Jurkat or THP-1 cells stably expressing either MUTYH or empty vector (negative control) were treated with 1μg/ml of Dox, and endogenous MUTYH and MUTYH-FLAG protein was detected via western blot, and actin was used as loading control. Dox inducible overexpression of MUTYH was detected at 12 hrs post-Dox delivery. However, overexpression of MUTYH was lost at 24 and 36 hrs post-Dox delivery (Fig. 3C). These data suggest that MUTYH is only transiently overexpressed and that MUTYH levels are tightly regulated by the cell. Jurkat or THP-1 cells stably expressing either MUTYH or empty vector were induced with Dox and infected with HIV-1 replicating virus. Infectivity was assessed 2, 4, and 6 days post infection. Overexpression of MUTYH with Dox did not alter infectivity at day 2 (Fig. 3D), day 4, or day 6 post infection (data

not shown). Therefore, it will be important to test if adding Dox every twelve hours during infection, to keep MUTYH levels high, has an effect on viral replication.

Lastly, we wanted to understand if MUTYH was IFN inducible. We treated THP-1 and Jurkat cells with 1ng/ml, 10ng/ml, 20ng/ml, 50ng/ml, and 100ng/ml with human IFNα and IFNβ and assessed MUTYH protein levels via western blot 8 hrs post-delivery. We assessed protein levels for MxB (positive control) since IFNα and IFNβ stimulate MxB. As expected, we found MxB to be induced by IFNα and IFNβ in a titratable fashion. We found that MUTYH was not induced by either IFNα or IFNβ (Fig. 3E). These data suggest that MUTYH protein levels are not affected by IFNα and IFNβ at these concentrations within an 8 hour timeframe. Overall, we found that knock-down of MUTYH was not detected consistently in THP-1 and Jurkat cells. Inducible overexpression of MUTYH was transient and reached maximum protein levels at 12 hours post-Dox treatment. Lastly, MUTYH is not induced by either IFNα or IFNβ (Fig. 4). Together, MUTYH protein levels were difficult to modulate, which suggests that there are cellular mechanisms that tightly regulate MUTYH protein levels.

DISCUSSION

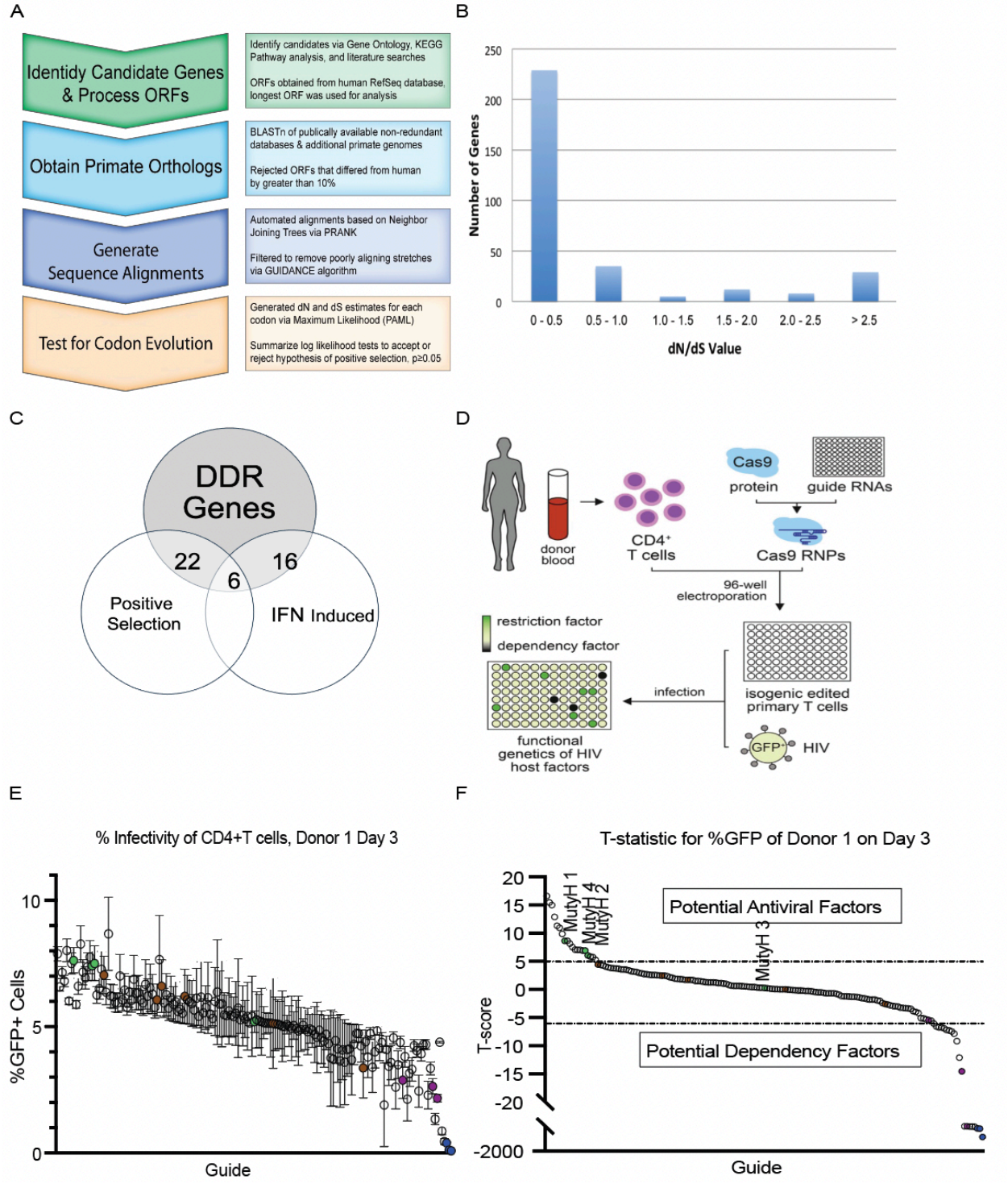
In this study, we found that the DDR genes possess signatures of positive selection and are induced by IFN. We tested the hypothesis that rapidly evolving and/or IFN induced DDR genes are in evolutionary conflict with HIV-1. Through a screen in primary CD4+T cells, we found many DDR genes modulate HIV-1 replication and are potential restriction or dependency factors. We focused on one candidate gene, MUTYH, yet did not conclusively determine if MUTYH is or is not an HIV-1 restriction factor. We did find that MUTYH protein levels are refractory to change via knock-down, overexpression or treatment with interferon.

Future work will look at other candidate genes to determine which DDR genes are host factors involved in HIV-1 replication. Moreover, future work will determine the functional consequences of rapid evolution on MUTYH cellular function. To address the hypothesis that the rapid evolution we have observed on MUTYH is necessary to maintain the important cellular functions in BER, we will generate MUTYH with ancestral codons at sites of positive selection and test these "ancestral MUTYH" constructs for their ability to interact with other DNA repair machinery and to facilitate BER. We expect that if positive selection on MUTYH is essential for its role in BER, then reverting the sites to ancestral sequences will cause MUTYH to lose the ability to facilitate BER in the context of the modern human DDR. To identify ancestral MUTYH codons, we will use maximum likelihood-based ancestral reconstruction. We will clone human MUTYH from cDNA, and ancestral mutants will be generated by site-directed mutagenesis. In all experiments, modern human MUTYH will be used as a positive control, and a mutant MUTYH will be used as a negative control.

Together, our approach suggests that the DDR genes show signatures of rapid evolution and stimulation by the antiviral interferon response. These data propose that the DDR is in an evolutionary conflict with pathogen(s). Most DDR genes that show signatures of positive selection play roles in DNA repair and homologous recombination. Since many diverse RNA and DNA

viruses modulate the DDR, it is possible that viruses drive rapid evolution of DDR genes. For example, Adenovirus inhibits DNA repair by degradation of DNA ligase IV, thus preventing genome concatenation and facilitating viral replication (10). Additionally, HIV-1 Vpr inhibits DNA repair through an unknown mechanism that is likely dependent on host protein degradation. Yet, it is unknown how the DDR maintains its essential role of safeguarding the genome while rapidly evolving. Overall, future work will unravel the marvels of the DDR and shed light on the multifaceted job of the DDR with respect to genome maintenance and innate immune response.

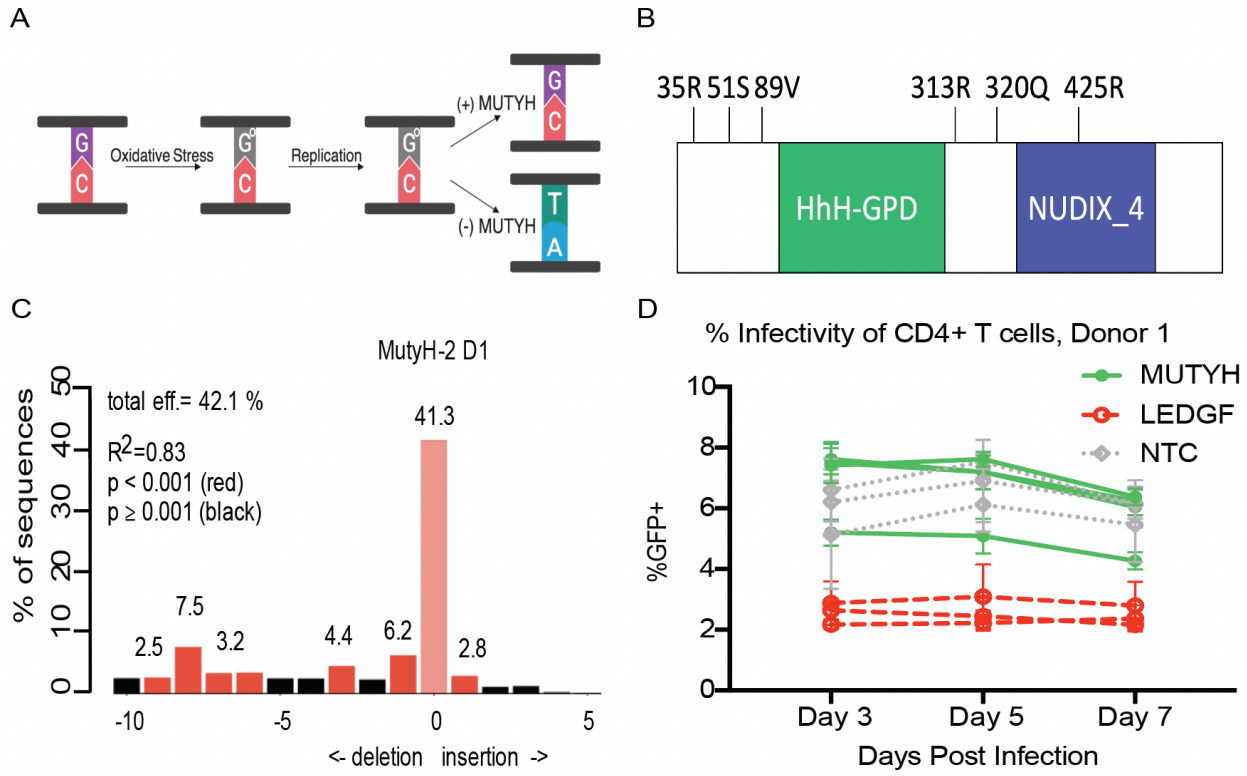
Figure 1



Chapter 4 Figure 1. Identifying candidate HIV-1 host factors that are DDR genes that contain signature(s) of positive selection and are induced by IFN.

(A) Overview of pipeline used to assess signatures of positive selection. This pipeline assessed positive selection of 321 DDR genes across an average of 15 primate species. (B) DDR genes show signatures of positive selection. Positive selection was measured via dN/dS values (rates of non-synonymous to synonymous codon substitutions). dN/dS values above 1.0 are indicative of positive selection. (C) Venn diagram depicting that there are 6 DDR proteins that are under positive selection and interferon induced. 22 DDR proteins that are only under positive selection. 16 DDR proteins that are only interferon induced. (D) CRISPR Cas9 approach to knockout DDR genes and assess HIV infectivity. Cas9 and gRNAs were delivered into primary CD4+T cells and subsequently infected with NL4-3 with IRES:GFP. Infectivity was assessed via Flow cytometry. (E) CRISPR/Cas9 knockout screen identified genes that alter HIV-1 infectivity. Data points represent average infected CD4+T cells, shown by GFP. MutyH (Green), LEDGF (Plum), and CXCR4 (Blue), Non-targeting controls (TC) (Brown). Error bars represent standard deviation from three replicates. (F) T-statistic analysis revealed both antiviral factors that increased viral infectivity and dependency factors that decreased viral infectivity. Data points represent T-score for %GFP+ cells. MutyH (Green), LEDGF (Plum), and CXCR4 (Blue).

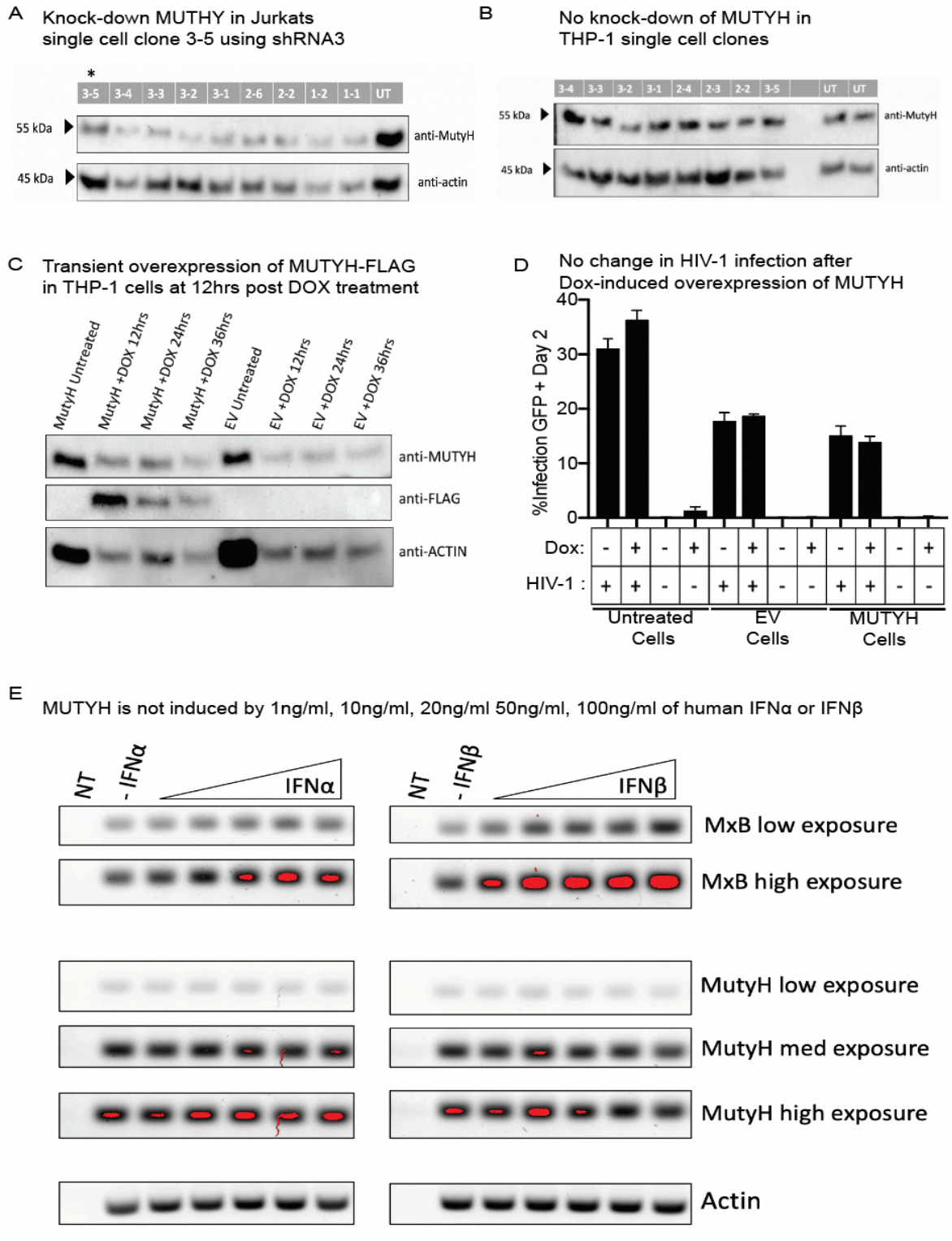
Figure 2



Chapter 4 Figure 2. The candidate gene, MUTYH, rapidly evolves and is a potential HIV-1 antiviral restriction factor.

(A) MutyH is a base excision repair (BER) protein that repairs damaged DNA throughout the cell cycle. DNA lesion 8-oxo guanine (G^0) is a product of oxidative stress that is repaired by BER. The Mono-functional DNA glycosylase (MUTYH) excises adenines that are misincorporated opposite to G^0 to restore the original sequence. (B) Positive selection analysis of MUTYH reveals 6 sites under rapid evolution. MUTYH contains two domains, HhH-GPD and NUDIX_4, and displays positive selection at 35R, 51S, 89V, 320Q, 313R, and 425R. Reference sequence *Angolan colobus.* (C) Tracking of Indels by Decomposition (Tide) analysis. Tide estimates the frequency of insertions and deletions (indels) in a pool of cells delivered with Cas9 and gRNAs. (D) CRISPR/Cas9 knockout of MUTYH caused an increase in infectivity at Day 3 compared to most non-target controls (NTC). Data points represent avg. infected CD4+T cells.

Figure 3

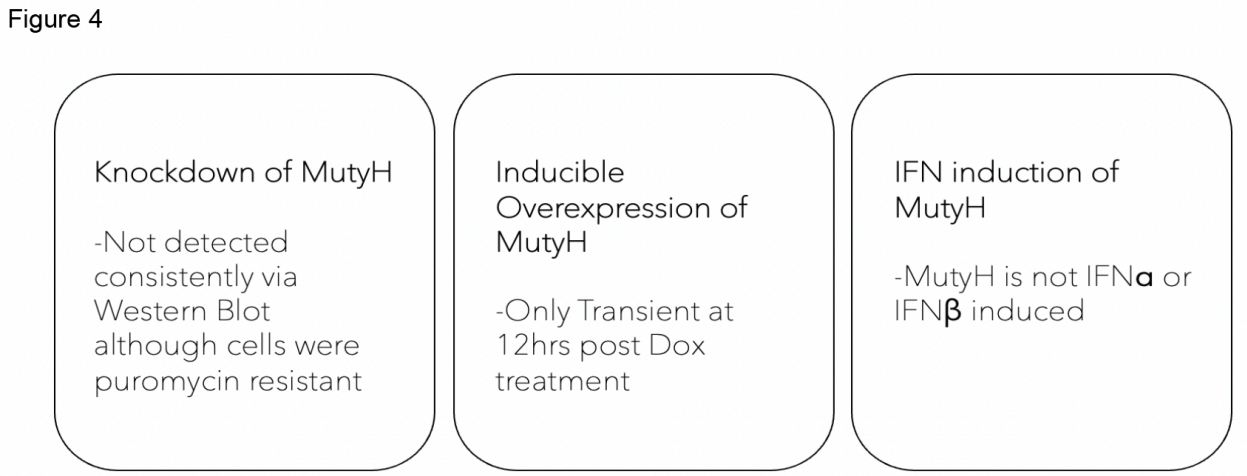


Chapter 4 Figure 3. MUTYH protein levels are refractory to change via knock-down, overexpression or treatment with IFN.

(A) Knock-down of MUTYH using three different shRNAs in Jurkat single cell clones. For example, a sample called 3-5 comes from cells integrated with shRNA3 from the 5th well. Untreated cells (UT) (B) Knock-down of MUTYH in THP-1 single cell clones.

(C) Dox inducible overexpression of MUTYH. Lentiviral delivery of MUTYH: FLAG or Empty vector: FLAG into THP-1 cells, followed by induction with 1μg/ml of doxycycline. (D) HIV-1 infected THP-1 cells stably expressing MUTYH or empty vector treated with or without 1μg/ml Dox. (E) THP-1 cells treated with IFNα or IFNβ did not stimulate MUTYH. THP-1 cells were treated with 1ng/ml, 10ng/ml, 20ng/ml, 50ng/ml, and 100ng/ml of human IFNα or IFNβ for 8 hours.

Figure 4



Chapter 4 Figure 4. Conclusion for MUTYH in THP-1 and Jurkat cells

MUTYH protein levels are refractory to change via knock-down, overexpression or treatment with IFN.

MATERIALS AND METHODS

Plasmids.

For lentivirus stable knock-down of MUTYH, pLKO.1 (Addgene), psPAX2(Addgene) and pmD2.G (Addgene) were used. For Dox-inducible overexpression of MUTYH, pCW57, pMD2.G, and JK3 (Addgene) were used**.** For HIV-1 Bru-GFP production, Bru-GFP was generated as previously described (11) and pMD2.G (Addgene).

Generation of Viruses.

HIV-1 Bru-GFP virus was generated by transient transfection of HEK 293T using TransIT-LT1 (Mirus). Virus was collected 48hrs post transfection and filtered through a 0.45 μm PVDF filter. Lentivirus expressing MUTYH shRNAs were generated by transfection of HEK 293T using TransIT-LT1 (Mirus). MUTYH shRNAs were provided by Molecular Screening Shared Resource (MSSR) at UCLA. Media was changed 18 hours post transfection. Lentiviruses were harvested 48 hours post media change and filtered through a 0.45 μm PES filter. Lentivirus expressing the Dox inducible MUTYH plasmid were generated by transfection of HEK 293T using TransIT-LT1 (Mirus). Media was changed 18 hours post transfection. Lentiviruses were harvested 48 hours post media change and filtered through a 0.45 μm PES filter.

Cell Lines and Cell Culture.

Jurkat T lymphocytes and THP-1 monocytes were cultured as suspension cells in Roswell Park Memorial Institute (RPMI) 1640 growth medium (L-Glutamine) with 10% fetal bovine serum (Gibco) at 37°C and 5% CO_2. Stable MUTYH knock-down cells were generated by delivering fresh lentiviruses expressing MUTYH shRNAs or empty vector to Jurkat or THP-1 cells. Stable inducible overexpression MUTYH cells were generated by delivering fresh lentiviruses expressing MUTYH inducible construct or empty vector to Jurkat or THP-1 cells. Cells with integrated lentiviral

construct were selected for with 2ug/ml of Puromycin for THP-1, 0.6ug/ml Puromycin for Jurkats. Knock-down of MUTYH or overexpression of MUTYH was assessed through western blot. HEK 293T cells were cultured as adherent cells directly on tissue culture plastic (Greiner) in Dulbecco's modified Eagle's medium (DMEM) growth medium (high glucose, L-glutamine, no sodium pyruvate; Gibco) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO_2. Cells were harvested using 0.05% trypsin-EDTA (Gibco).

Western Blots.

Protein was collected from cells as previously described (12). Immunoblotting was performed using mouse anti-FLAG M2 (Sigma-Aldrich) and rabbit anti-Actin (Bethyl).

HIV-1 Bru-GFP Infection.

Jurkat or THP-1 cells were plated in 96-well tissue culture plates (Greiner). Cells underwent spinfection with HIV-1 Bru_GFP at 1200× *g* for 90 min at 37°C. Infection was assessed 48 hrs after infection via Flow Cytometry for HIV-1 core proteins.

Flow Cytometry.

Jurkat or THP-1 cells were washed with PBS, fixed in 4% PFA for 15 min, permeabilized with 0.1% Triton X-100 in PBS at 4°C for 15 min then washed with PBS. Cells were probed for HIV-1 core antigen-FITC KC57 (Beckman Coulter) for 1hr at 4°C then washed with PBS and resuspended in FACS buffer (5% FBS in PBS). Events were assessed by flow cytometry on the AttuneNxT (Thermo Fisher Scientific). At least 10,000 events were collected per run. Data was analyzed using FlowJo software.

REFERENCES

1. Maertens G, Cherepanov P, Pluymers W, Busschots K, De Clercq E, Debyser Z, et al. LEDGF/p75 Is Essential for Nuclear and Chromosomal Targeting of HIV-1 Integrase in Human Cells*. J Biol Chem. 2003 Aug 29;278(35):33528–39.

2. Lopez A, Nichols Doyle R, Sandoval C, Nisson K, Yang V, Fregoso OI. Viral Modulation of the DNA Damage Response and Innate Immunity: Two Sides of the Same Coin. J Mol Biol. 2022 Mar 30;434(6):167327.

3. Ryan EL, Hollingworth R, Grand RJ. Activation of the DNA Damage Response by RNA Viruses. Biomolecules. 2016 Jan 6;6(1):2.

4. Blackford AN, Jackson SP. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. Mol Cell. 2017 Jun 15;66(6):801–17.

5. Rules of engagement: molecular insights from host-virus arms races | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/U4EDQXEH/items/QWZ4KEF9/item-details

6. Human PAML browser: a database of positive selection on human genes using phylogenetic methods | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/U4EDQXEH/items/F7ZU9KTB/item-details

7. A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/U4EDQXEH/items/FUMEG3AH/item-details

8. HIV coreceptors: from discovery and designation to new paradigms and promise | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from:

https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/U4EDQXEH/items/N92T9SFZ/item-details

9. Base excision repair and the role of MUTYH | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/U4EDQXEH/items/N89UGTIP/item-details

10. Adenovirus E4 34k and E1b 55k oncoproteins target host DNA ligase IV for proteasomal degradation | CS Vpr paper - 2023 | Zotero [Internet]. [cited 2023 May 11]. Available from: https://www.zotero.org/groups/5026855/cs_vpr_paper_-_2023/collections/U4EDQXEH/items/2INT43BC/item-details

11. Yamashita M, Emerman M. Capsid Is a Dominant Determinant of Retrovirus Infectivity in Nondividing Cells. J Virol. 2004 Jun;78(11):5670–8.

12. Li D, Lopez A, Sandoval C, Nichols Doyle R, Fregoso OI. HIV Vpr Modulates the Host DNA Damage Response at Two Independent Steps to Damage DNA and Repress Double-Strand DNA Break Repair. mBio. 2020 Aug 4;11(4):e00940-20.

Chapter 5: HIV Vpr Modulates the Host DNA Damage Response at Two Independent Steps to Damage DNA and Repress Double-Strand DNA Break Repair

Donnal Li[b] †, Andrew Lopez[a] † , **Carina Sandoval[a]** , Randilea Nichols Doyle[b], and Oliver I. Fregoso[ab]

Molecular Biology Institute[a], Department of Microbiology, Immunology & Molecular Genetics[b] at the University of California, Los Angeles, California, United States of America

† Authors contributed equally

ABSTRACT

The DNA damage response (DDR) 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 conserved 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 Vpr represses the repair of double-strand breaks (DSB) by inhibiting homologous recombination (HR) and nonhomologous end joining (NHEJ). Mutational analyses of Vpr revealed that DNA damage and DDR activation are independent from repression of HR and Vpr-mediated cell cycle arrest. Moreover, we show that repression of HR does not require cell cycle arrest but instead may precede this long-standing enigmatic Vpr phenotype. Together, our data uncover that Vpr globally modulates the host DDR at at least two independent steps, offering novel insight into the primary functions of lentiviral Vpr and the roles of the DNA damage response in lentiviral replication.

INTRODUCTION

Primate lentiviruses encode accessory proteins that enhance viral replication (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 (WT) 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 the engagement of the DDR is central to Vpr function.

The DDR is a protein signaling cascade that ensures the fidelity of the genome. It consists of sensors that recognize specific DNA lesions, mediators, and transducers, which 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 participate in the repair of double-strand breaks (DSB) through homologous recombination (HR) and nonhomologous end joining (NHEJ), respectively (12). However, due to the essential role of the DDR, a tremendous amount of cross talk and redundancy exists between these kinases (13).

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 γH2AX 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-κB (18–22). Together, these findings highlight 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 the chemical inhibition of ATR (27). Moreover, Vpr-mediated cell cycle arrest requires interaction of Vpr with the Cul4A/DCAF1/DDB1 (CUL4ADCAF1) 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 CUL4ADCAF1 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). 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 most known Vpr-host protein interactions, repression of double-strand break repair is dependent on DCAF1 recruitment. Finally, we showed that repression of HR repair is not a consequence of Vpr-mediated G2 cell cycle arrest, as it occurs prior to G2 arrest. Our data indicate that lentiviruses both activate and repress the DDR via Vpr and further characterize a novel phenotype of Vpr that can help explain many of the roles that have long been associated with Vpr.

RESULTS

Primate lentiviruses encode accessory proteins that enhance viral replication (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 (WT) 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 the engagement of the DDR is central to Vpr function.

The DDR is a protein signaling cascade that ensures the fidelity of the genome. It consists of sensors that recognize specific DNA lesions, mediators, and transducers, which 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 participate in the repair of double-strand breaks (DSB) through homologous recombination (HR) and nonhomologous end joining (NHEJ), respectively (12). However, due to the essential role of the DDR, a tremendous amount of cross talk and redundancy exists between these kinases (13).

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 γH2AX 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-κB (18–22). Together, these findings highlight 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 the chemical inhibition of ATR (27). Moreover, Vpr-mediated cell cycle arrest requires interaction of Vpr with the Cul4A/DCAF1/DDB1 (CUL4ADCAF1) 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 CUL4ADCAF1 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). 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 most known Vpr-host protein interactions, repression of double-strand break repair is dependent on DCAF1 recruitment. Finally, we showed that repression of HR repair is not a consequence of Vpr-mediated G2 cell cycle arrest, as it occurs prior to G2 arrest. Our data indicate that lentiviruses both activate and repress the DDR via Vpr and further characterize a novel phenotype of Vpr that can help explain many of the roles that have long been associated with Vpr.

We also tested a number of HIV-1 and HIV-2 Vpr isolates to determine if activation of the DDR by HIV-1 and HIV-2 Vpr was isolate specific or conserved by the greater diversity of HIV Vpr proteins. These include representative Vpr isolates from HIV-1 group M (subtype G), N, O, and P consensus sequences, as well as HIV-2 Vpr isolates from groups A and B and divergent groups. 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 highlight that a conserved function of HIV-1 and HIV-2 Vpr is the activation of the same markers of single- and double-strand 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 pulsed-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 20 h, 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, which show lower MFI for Vpr-induced DNA damage markers than etoposide treatment (Fig. 1B). We segregated the samples into two populations, below and above 20% tail DNA, to highlight the population of cells within each sample with a greater extent of damage (Fig. 2A and C). 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 the 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 quantitates the length of replication tracks by incorporation of EdU (5-ethynyl-2′-deoxyuridine) into nascent DNA. U2OS cells were infected with rAAV-Vpr for 20 h, at which point EdU was added to the cells for 20 min. Hydroxyurea (HU), which stalls DNA replication by depleting deoxynucleoside triphosphate pools (53), was used as a positive control. We found that HIV-1 and HIV-2 Vpr significantly decreased EdU track lengths compared to those of the uninfected and empty vector controls (Fig. 2D). Consistent with DNA damage markers, there was no direct correlation between levels of Vpr expression and DNA replication during this 20-min window. However, cells expressing the highest levels of Vpr were largely not in S-phase during this window (Fig. S3), suggesting there is a threshold where Vpr expression robustly excludes cells from S phase. Like the comet and IF assays, the greatest amount of replication fork stalling was exhibited by the positive control, HU, 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 replication protein A (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. S4A). We also assayed for an effect of ATM inhibition (ATMi; KU-55933), 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 (Fig. S4B), consistent with previously published results (32, 49, 55). 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 B). 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. Although 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 show 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, which leads 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 preexisting 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 overwinding (56). Cells expressing Vpr were highly sensitized to etoposide treatment, where survival at even the lowest concentration (0.01 μM) decreased to 60 to 70% compared to that of 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 HU. Prolonged exposure of cells to HU at high concentrations results in replication fork collapse and extensive DSB (57). Although Vpr-expressing cells were not sensitized to HU treatment at low concentrations, at higher concentrations of HU (>3.90 μM), where DSB are presumably present, survival of cells expressing HIV-1 and HIV-2 was significantly decreased compared to that of 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 (58) (Fig. 4). In contrast to the other chemotherapeutics, HIV-1 and HIV-2 Vpr expression did not dramatically hypersensitize cells to the interstrand cross-linking agent cisplatin (59) (Fig. 4), despite activating markers associated with interstrand cross-link (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 DSB by inhibiting crucial host repair mechanisms, suggesting that Vpr also inhibits 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 independent green fluorescent protein (GFP)-based U2OS reporter cell lines that specifically monitor the repair of an I-SceI-induced DSB by either homologous recombination (HR), nonhomologous end joining (NHEJ), alternative NHEJ (alt-NHEJ), or single-strand annealing (SSA) (60, 61). 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 (Fig. 5A and B depict a schematic of HR and NHEJ cell lines, respectively). 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 B).

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 control cells at 100% (Fig. 5A and C). This indicates that HIV-1 and HIV-2 Vpr repress HR. Based on these results, we next tested the I-SceI reporter cell line that measures NHEJ, which is often utilized by cells to repair DSBs when HR is repressed (62). Similar to HR, HIV-1 Vpr expression also decreased NHEJ efficiency by 51% compared to that of 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. 5B and D), highlighting potential mechanistic differences between

HIV-1 and HIV-2 Vpr. Consistent with DNA damage and DNA replication, there was no correlation between Vpr expression (mCherry) and repair (GFP) based on flow plots (Fig. 5A and B). Finally, we tested the I-SceI reporter cell lines for alt-NHEJ and SSA repair mechanisms but found no significant change in repair compared to control cells (Fig. S5). 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 double-strand DNA break 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 assay, 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 (63); HIV-1 Q65R/HIV-2 Q70R Vpr, which renders Vpr unable to properly localize, multimerize, or recruit known host proteins, such as the Cul4ADCAF1 complex or UNG2 and, therefore, is largely functionally dead (33, 64, 65); HIV-1 S79A/HIV-2 S84A mutants, which render Vpr unable to cause cell cycle arrest or interact with TAK1 to activate canonical NF-κB (66, 67); and HIV-1 R80A/HIV-2 R85A Vpr mutants, which can still interact with Cul4ADCAF1 and degrade TET2 (41) but do not cause cell cycle arrest, presumably due to the requirement of an additional unknown host protein(s) (68). Moreover, as HIV-1 and HIV-2 Vpr differentially interact with and/or downregulate UNG2, HLTF, and the SLX4 complex (6, 37), by testing diverse Vpr orthologs we were further able to 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 and Fig. S6B). In contrast to cell cycle arrest, only HIV-1 Q65R/HIV-2 Q70R Vpr lost the ability to damage DNA (Fig. 6B and Fig. S6C), indicating that damage of DNA occurs independently of cell cycle arrest and of the Vpr-host protein-protein interactions assayed here. 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 repair (Fig. 6D). Overall, our mutational analyses of HIV-1 and HIV-2 Vpr indicate that repression of HR and cell cycle arrest are correlated, and that these two phenotypes are independent of Vpr-induced DNA damage and downstream signaling. 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 previously observed 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. While it is unclear what leads to Vpr-mediated cell cycle arrest, G2 arrest depends on recruitment of the Cul4ADCAF1 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. 6). 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 Cul4ADCAF1 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 (28) and H71R (35), and further generated those mutants in HIV-2 Vpr (L69A and H76R, respectively). To validate if these mutants lost the ability to recruit DCAF1, we

immunoprecipitated FLAG-Vpr and probed for endogenous human DCAF1. In our hands, HIV-1 H71R/HIV-2 H76R no longer recruited DCAF1. However, 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, fully lost the ability to arrest cells (Fig. S7). We next tested these mutants for their ability to repress HR using the HR I-SceI repair assay. Again, consistent 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 nearly WT Vpr levels (Fig. 7B). These data suggest that, similar to cell cycle arrest, repression of HR repair by Vpr requires DCAF1 binding.

To determine if repression of HR by Vpr requires G2 arrest or occurs independently of this arrest, we defined the cell cycle status (G1 or G2 phase) of DR-GFP cells that exhibited repair using Hoechst dye. Here, we first performed the DR-GFP assay as before. Cells were then stained with Hoechst dye to label DNA content, gated for Vpr expression (mCherry), and repair was measured for either total Vpr-expressing cells, Vpr-expressing cells in G1, or Vpr-expressing cells in G2. We would expect that if Vpr-mediated G2 arrest is required to repress HR, then Vpr-expressing cells in G2 would primarily show repressed HR when normalized to empty vector control cells. However, if G2 arrest is not required for Vpr to repress HR, then cells in G1 would also show a repression of HR in the presence of Vpr.

As seen previously, both HIV-1 and HIV-2 Vpr repressed total cellular HR, unlike the empty vector control. Vpr-expressing cells also showed strong repression of HR repair in G1 compared to that of empty vector control cells. However, Vpr-expressing cells did not repress HR in G2, as they were statistically indistinguishable from control cells (Fig. 7C). Together, these data indicate that Vpr-mediated repression of HR does not require G2 arrest but instead occurs primarily in the G1 phase of the cell cycle.

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 γH2AX, RPA32, and 53BP1. These Vpr-induced DNA lesions are sensed by ATR and require NF-κB 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 (HR) and nonhomologous end joining (NHEJ). 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, we show that repression of HR is not a consequence of G2 cell cycle 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 largely independent of known Vpr-binding host factors. This Vpr-induced damage is sensed by ATR and signals through NF-κB to block DNA replication fork progression. Second, through recruitment of the Cul4ADCAF1 complex, Vpr represses DNA double-strand break repair machinery, leading to a prolonged cell cycle to deal with the inability to repair DNA lesions.

Why would Vpr engage the DDR at two unique steps, and how would this help lentiviral replication? 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). For example, human papillomaviruses (HPV) upregulate ATM to push cells away from NHEJ and toward HR, which is thought to enhance viral persistence and integration (69, 70). Interestingly, this also sensitizes HPV+ cells to exogenous genotoxic agents due to their inability to repair additional damage (71), as we have shown here for HIV Vpr (Fig. 4). Moreover, as Vpr has two unique phases in an infected cell, i.e., 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 life cycle of an infected cell.

While it is possible that some of these DDR-associated phenotypes are indirect consequences of other effects of Vpr on the cell, such as induction of proinflammatory cytokines (72), this dual function of Vpr in engaging the DDR at multiple independent steps could help clarify some of the discrepancies in the Vpr literature and may directly explain many of the roles in viral replication attributed to Vpr (73–79). For example, DNA damage promotes nucleotide biosynthesis (80) and, thus, may enhance early events in HIV replication, such as reverse transcription. This is analogous to the degradation of SAMHD1 by lentiviral Vpx/Vpr (5, 81, 82) 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, 83), as efficiently as HIV-1 Vpr. The stalling of replication forks (Fig. 2D) could enhance integration by remodeling histones and prolonging the S phase. Integration could also be enhanced by attenuating double-strand break repair (Fig. 5), similar to the repression of HR and base excision repair by human T-lymphotropic virus 1 (HTLV-1), to facilitate viral integration (84–86). Moreover, the induction of DNA breaks (Fig. 2A to C) could enhance long terminal repeat-driven transcription by activating important DDR-responsive transcription factors, such as NF-κB and AP-1 (67, 87).

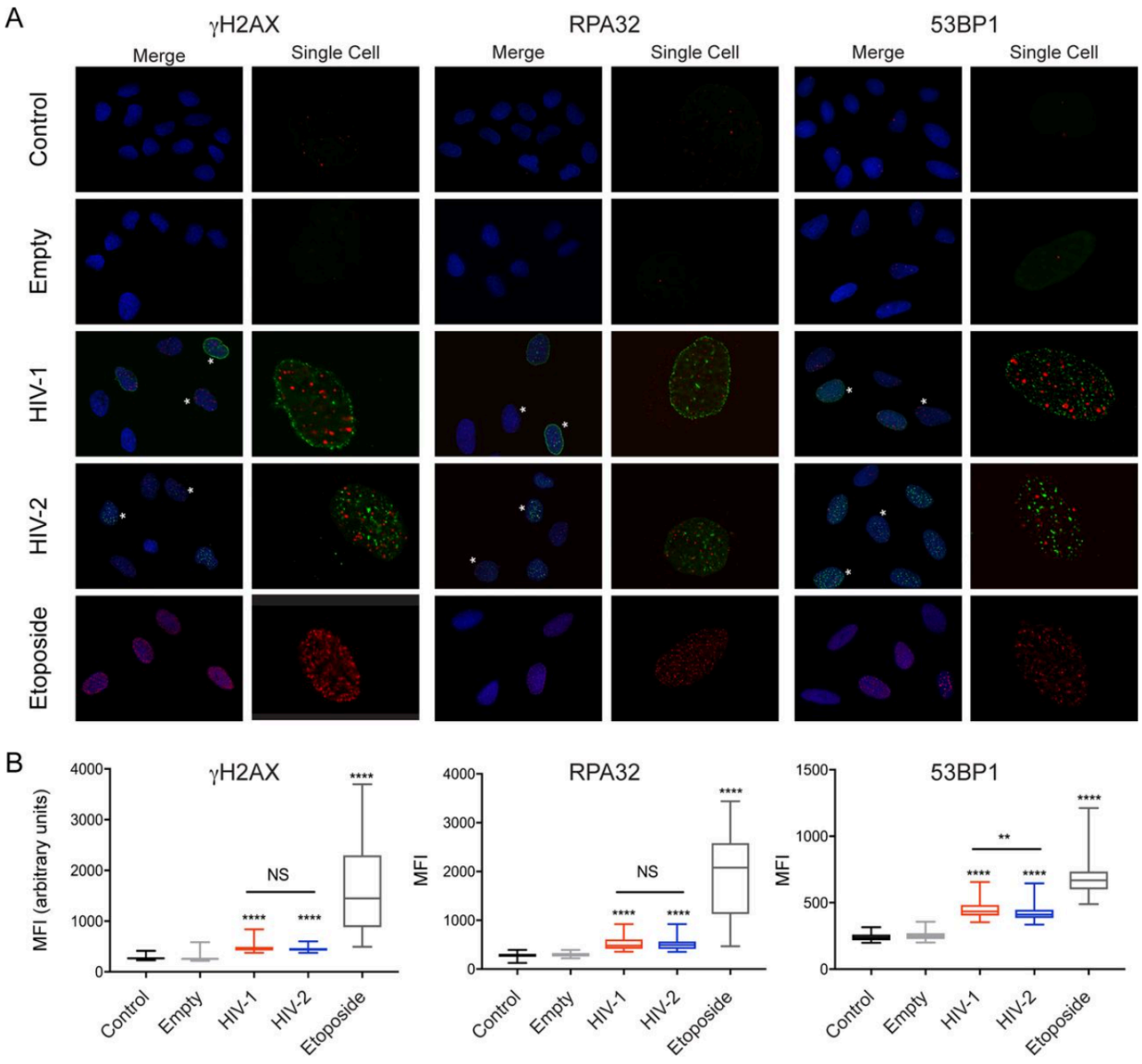
As the primary role of lentiviral accessory genes is to overcome antiviral restriction factors, our data also support a model where DDR proteins and/or pathways restrict HIV replication and are overcome by Vpr. This is consistent with the growing evidence that DDR proteins and pathways contribute to the innate immune response to pathogens (17–22). We have shown that, like Vpr-mediated cell cycle arrest, recruitment of the Cul4A ubiquitin ligase complex adaptor protein DCAF1 is required for repression of HR repair (Fig. 7). Vpr could be recruiting this complex away from a natural target or usurping it to degrade a host protein, which is consistent with the primary role of lentiviral accessory genes in viral replication, such as Vpx-mediated degradation of the antiviral DDR protein SAMHD1 (88, 89). While Vpr has been shown to recruit and degrade many

host proteins, through the combination of our mutant data and use of HIV-1 and HIV-2 Vpr orthologs (Fig. 6 and 7), we are able to rule out most known DDR-associated Vpr-interacting proteins (and potential cellular effects of Vpr) for roles in modulating the DDR as described here. Whether some of the remaining Vpr-interacting proteins we were unable to characterize, such as the endonuclease Exo1, are required for Vpr-mediated engagement of the DDR or whether novel undiscovered host proteins are required remains unclear. Moreover, whether modulation of DDR pathways is a direct primary effect of Vpr or a consequence of degradation of an antiviral host protein that is also integral to the DDR is also unclear. However, our data pinpoint double-strand DNA break repairs as important cellular pathways that warrant further investigation into both innate immunity and Vpr.

Our mutant data also show that the long-standing enigmatic cell cycle arrest caused by Vpr correlates with repression of HR, suggesting these two phenotypes are linked. As HR is upregulated in G2, one might expect Vpr to enhance this repair mechanism instead of inhibit it. Intriguingly, we find the majority of Vpr-mediated repression of HR occurs in cells that are currently in G1, not G2, suggesting that repression of HR precedes, and may initiate, G2 arrest. Based on this, we hypothesize that repression of HR, not cell cycle arrest, is the crucial phenotype associated with Vpr, and that understanding this process will give clearer insight into the primary function of Vpr in viral replication.

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 life cycle, the precise roles of Vpr-mediated activation and repression of the DDR in HIV replication remain obscure. 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. This will allow us to better define the primary evolutionarily conserved role of Vpr. Finally, our data indicate that Vpr expression has important implications for the development and treatment of HIV-associated diseases such as cancer, where induction of DNA damage and

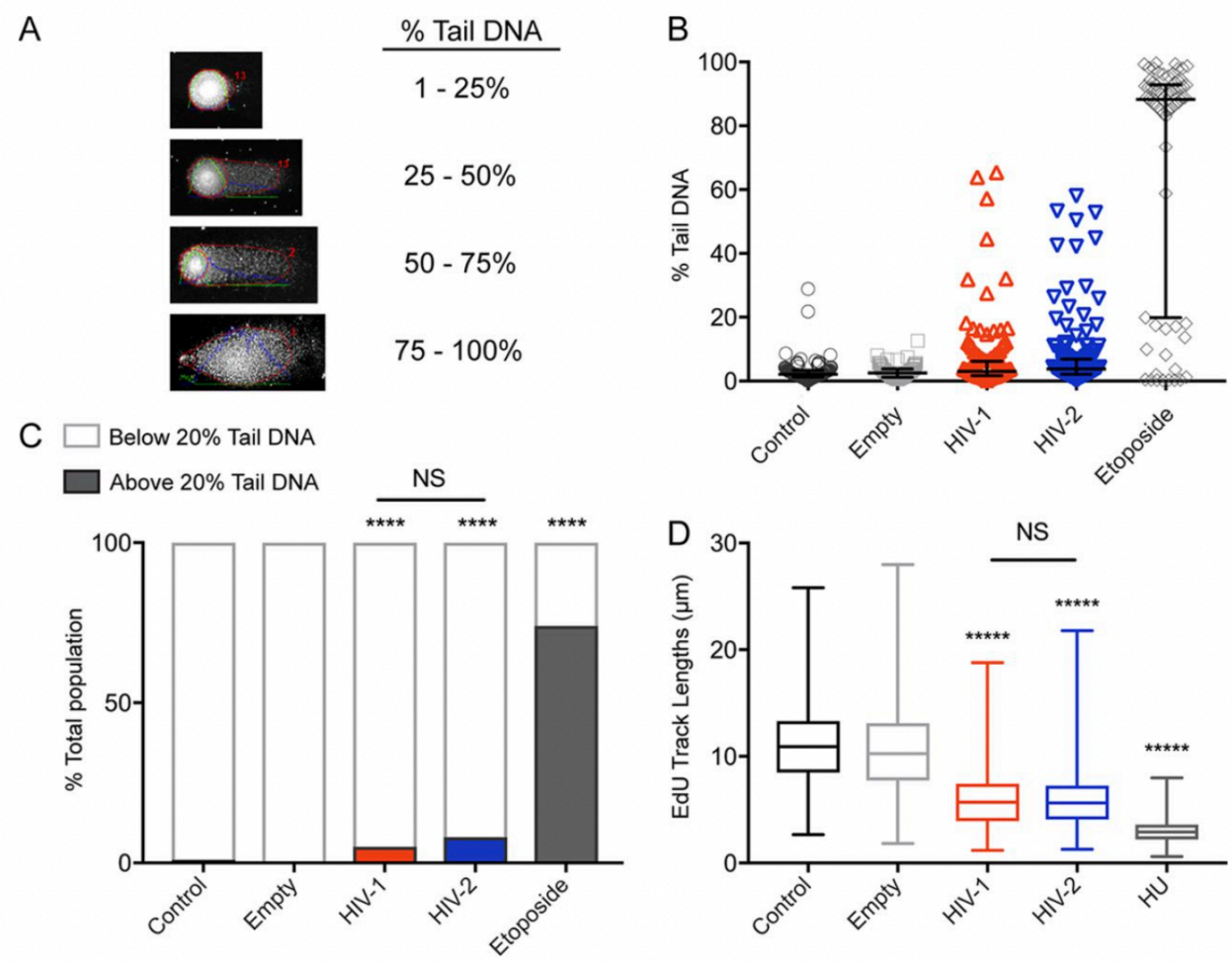
deregulation of repair could serve to complicate tumorigenesis but also sensitize cells to chemotherapeutics, further highlighting the importance of Vpr in HIV replication and associated diseases.



Chapter 5 Figure 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

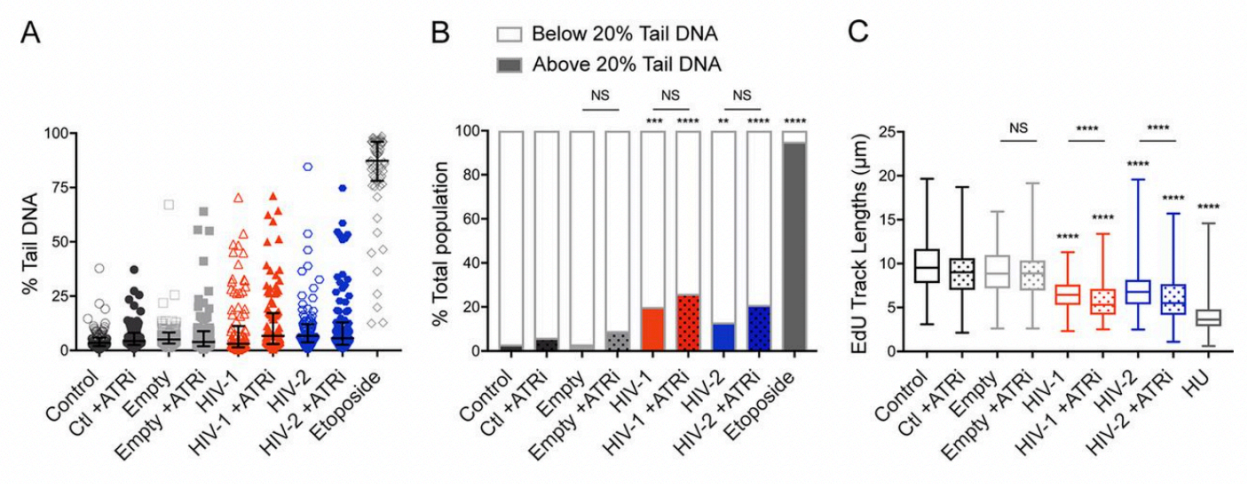
fluorescence intensity (MFI) of 100 cells per condition was quantified for all markers. Asterisks indicate statistical significance compared to empty vector control, as determined by Kruskal-Wallis tests (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 2$, one representative experiment shown).



Chapter 5 Figure 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

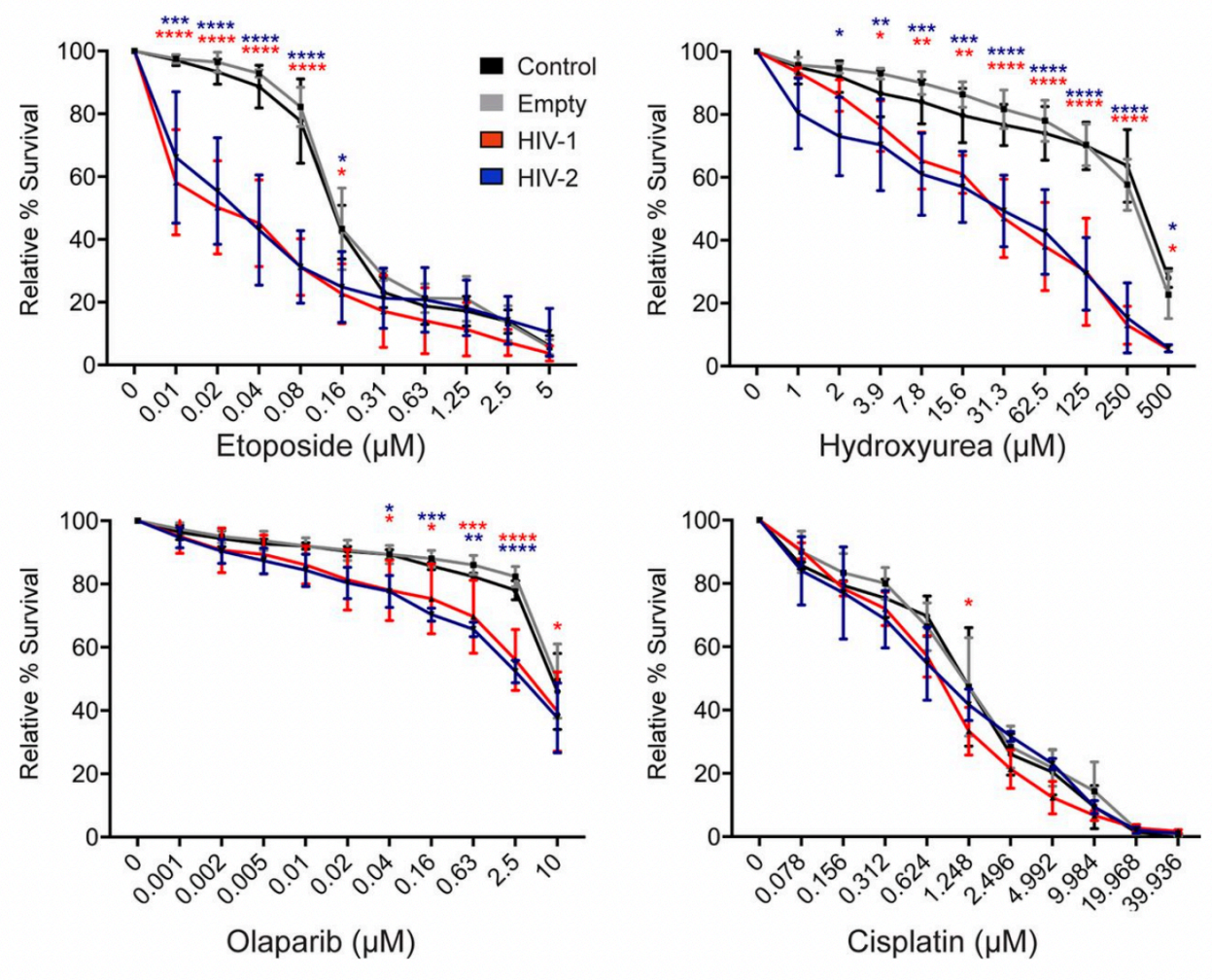
(shaded). Asterisks indicate statistical significance compared to empty vector control or HIV-1 compared to HIV-2 Vpr, as determined by chi-square test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$, one representative experiment shown). (D) A box and 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).



Chapter 5 Figure 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

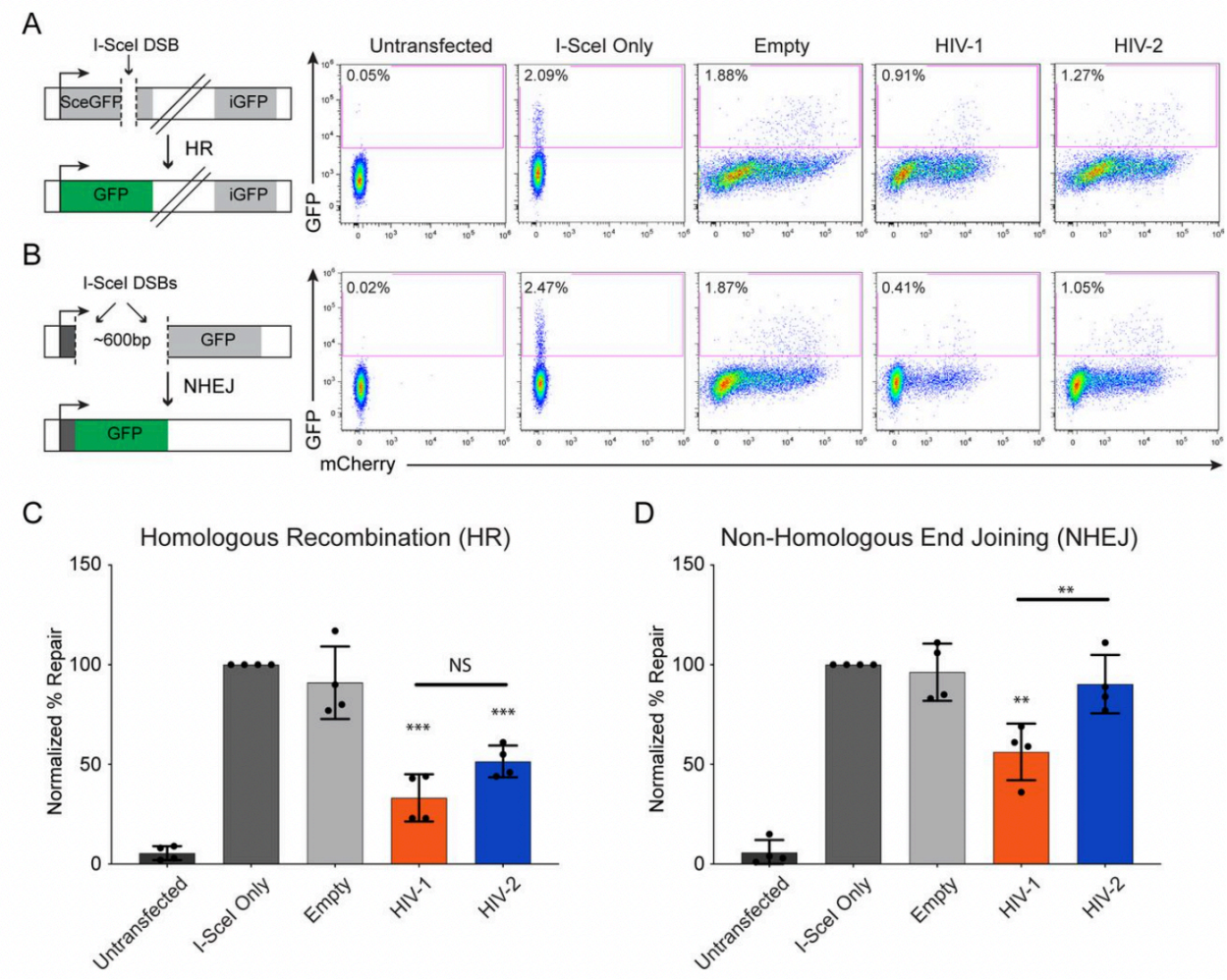
without ATRi was determined by the Mann-Whitney test (NS, nonsignificant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 3$, one representative experiment shown).



Chapter 5 Figure 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.

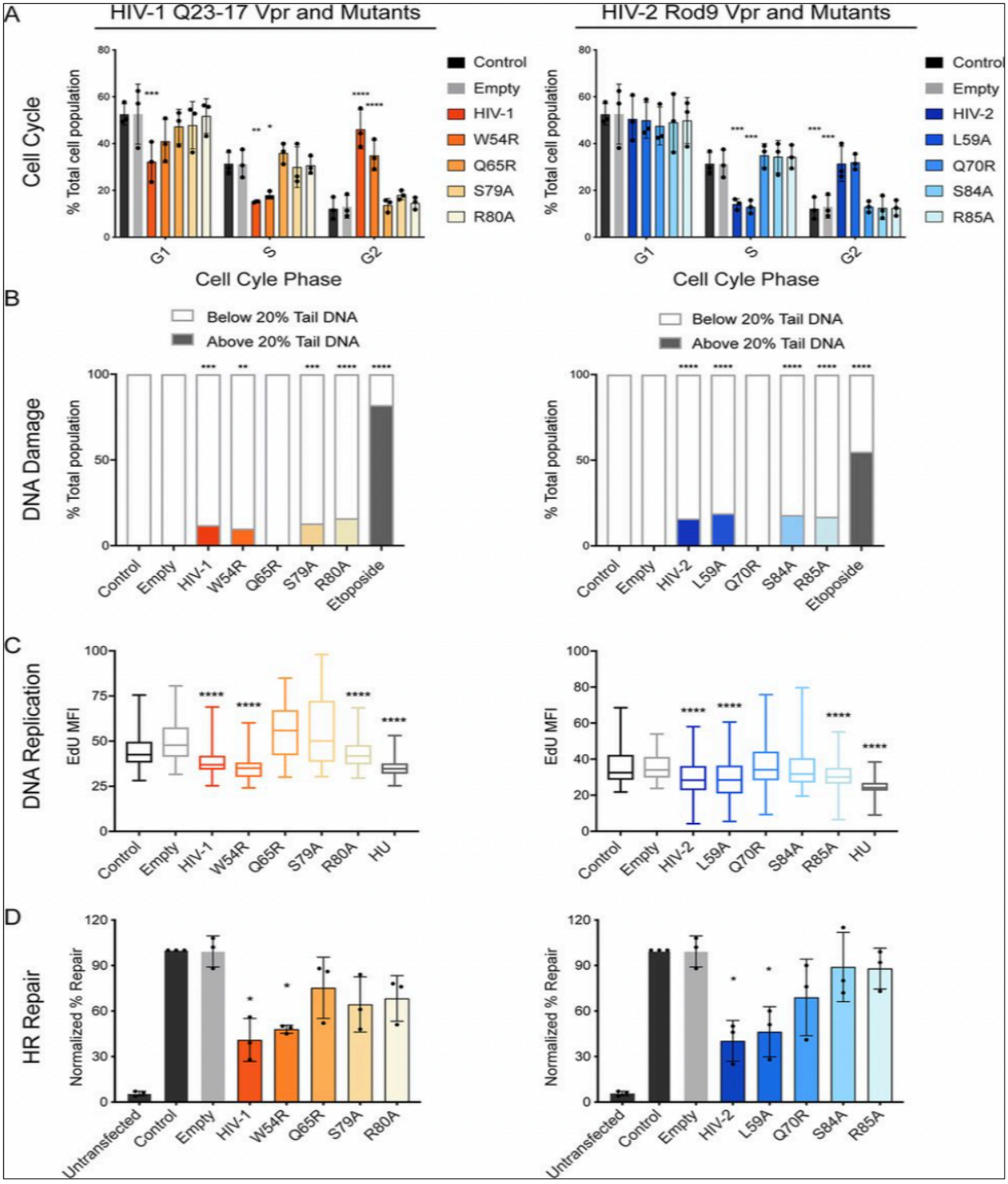
Asterisks indicate statistical significance compared to empty vector control, as determined by 2-way analysis of variance (*, P < 0.05; **, P < 0.03;***, P < 0.002; ****, P < 0.0001).



Chapter 5 Figure 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-

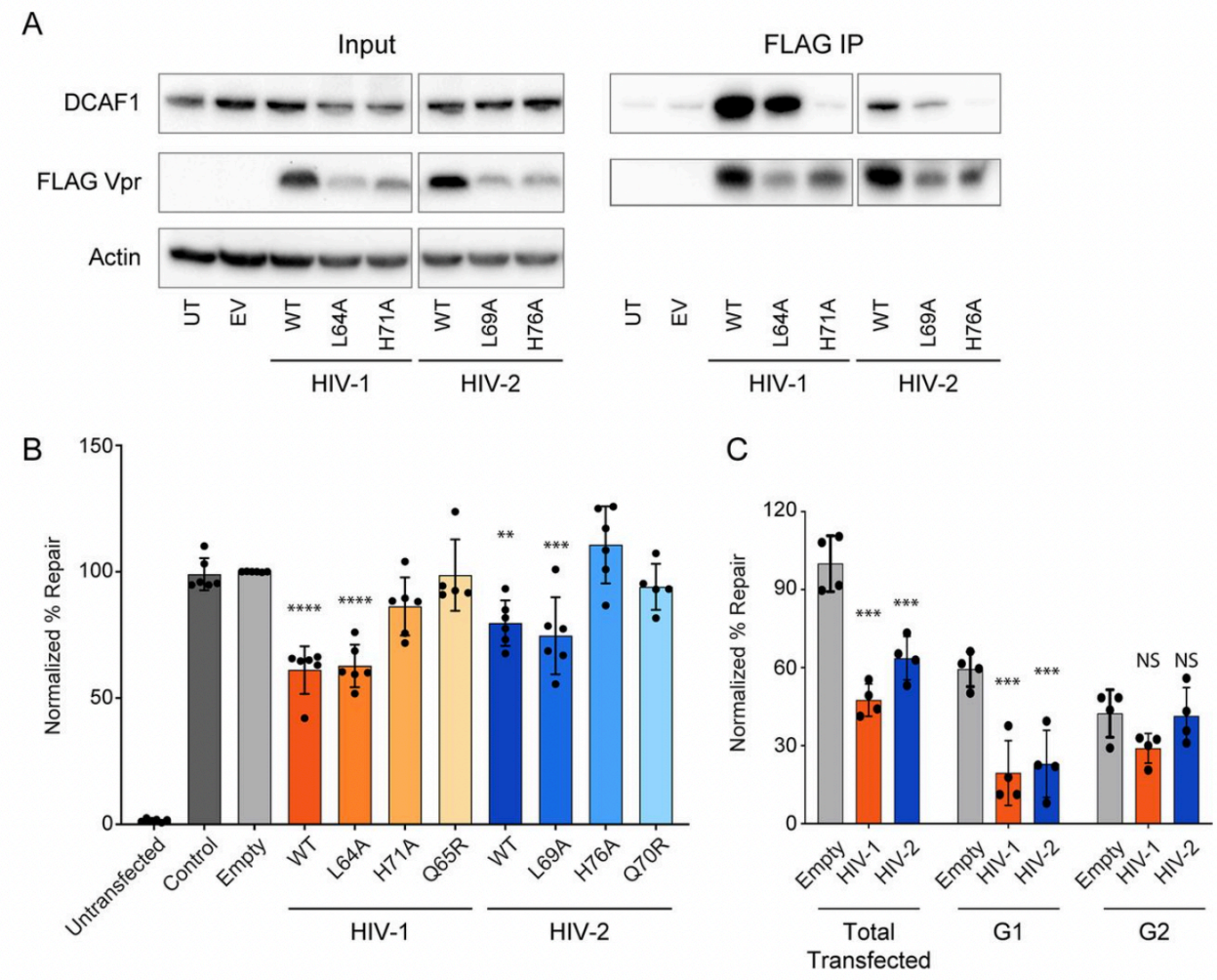
mediated DSB repair (GFP). (B, left) Schematic of I-SceI-based classical NHEJ U2OS reporter cell line (EJ5-GFP assay). (Right) Representative flow cytometry plots of one I-SceI repair assay experiment for NHEJ repair. Cells were treated and measured under the same conditions as those described for panel 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.



Chapter 5 Figure 6. Vpr-induced DNA damage is independent of repression of homologous

(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 G1, S, and G2, 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 ($n = 3$).



Chapter 5 Figure 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, G1, and G2 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).

MATERIALS AND METHODS

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 number 26477) (90).

Cell lines and 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 Dulbecco's modified Eagle's medium (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% CO2. All cells were harvested using 0.05% trypsin-EDTA (Gibco). Transfections were performed with TransIT-LT1 (Mirus). The panel of U2OS cells containing an integrated reporter (DR-GFP, SA-GFP, EJ2-GFP, and EJ5-GFP) used in the I-SceI repair assays were kindly provided by Jeremy M. Stark (Beckman Research Institute of the City of Hope) (60).

Generation of viruses.

AAV vectors were generated by transient transfection of HEK 293 cells using polyethyleneimine (PEI) as previously described (91). 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. (92).

Western blots and coimmunoprecipitations.

Cells were 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) and clarified by centrifugation at 14,500 × g for 10 min. Immunoprecipitations

were performed as previously described (6) using anti-FLAG affinity beads (Sigma). All samples were boiled in 4× sample buffer (40% glycerol, 240 mM Tris, pH 6.8, 8% SDS, 0.5% β-mercaptoethanol, and bromophenol blue) in preparation for SDS-PAGE using 4 to 12% Bis-Tris polyacrylamide gels and subsequently transferred onto a polyvinylidene difluoride membrane. Immunoblotting was performed using mouse anti-FLAG M2 (Sigma), mouse anti-actin (Thermo-Fisher), rabbit anti-DCAF1 (Cell Signaling), goat anti-mouse horseradish peroxidase (HRP; Invitrogen), and goat anti-rabbit HRP (Invitrogen).

DNA combing assay.

The DNA combing assay was adapted from reference 52. Cells were plated in 6-well tissue culture-treated plates (Greiner) at 1.75 × 106 cells/well and allowed to rest overnight. Cells were then infected with rAAV 2.5 at equal titers (1.4 × 108 copies/well) or 500 μM hydroxyurea (Sigma) for 20 h. Following infection, cells were incubated with 10 μM EdU (Invitrogen) for 20 min, harvested, spun down, and resuspended in 1× phosphate-buffered saline (PBS; Gibco). The cell suspension was added and lysed with lysis buffer (50 mM EDTA, 0.5% SDS, 200 mM Tris-HCl, pH 7.5) directly on a silane-coated slide (Electron Microscopy) and then incubated for 5 to 8 min. After incubation, the slide was tilted at a 45° angle to allow the droplet to roll down and then fixed with 3:1 methanol acetic acid for 15 min after the slide was completely dry. The slide then was washed with 1× PBS, blocked with 3% bovine serum albumin (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 Axio Imager Z1, and images were analyzed using ImageJ.

Alkaline comet assay.

The 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 × 106 cells/well and allowed to rest overnight. Cells were then infected with rAAV 2.5 at equal titers (1.4 × 108 copies/well) or 50 μM etoposide (Sigma) for 20 h. Following infection, cells were then harvested,

spun down, and resuspended in 0.5% low-melting-point agarose at 37°C. Samples then were 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, 2.5 M NaCl, 0.1 M EDTA, 1% Triton X-100) for 1 h and then in the alkaline running buffer (0.3 M NaOH, 1 mM EDTA) for 30 min and finally electrophoresed at 300 mA for 30 min, all done at 4°C. Samples then were washed in double-distilled water (ddH2O) and fixed in 70% ethanol at 4°C. Cells were stained with Yoyo-1 (Life Technologies) for 15 min at room temperature and then washed with ddH2O and dried overnight. Images were acquired on the Zeiss Axio Imager Z1. Images were analyzed using the OpenComet plug-in for ImageJ.

Cell cycle analysis.

U2OS cells were plated and either left unsynchronized or synchronized using serum starvation with 0.05% fetal bovine serum (FBS)-DMEM (Gibco) for at least 12 h. Cells were infected with rAAV 2.5 (600 copies/cell) for 38 h. For labeling with Hoechst, cells were incubated with Hoechst ready flow reagent (Invitrogen) as recommended. For labeling with propidium iodide, 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 min. Pulse-labeled cells were then permeabilized with 0.01% Triton X-100 for 3.5 min and fixed with 4% paraformaldehyde (PFA) for 20 min. EdU was detected using Click-iT EdU Alexa Fluor 647 imaging kit (Invitrogen) followed by Hoechst or PI staining. Cells were assessed by flow cytometry on a FACSVERSE (BD). At least 10,000 cells were collected each run, and data were analyzed using FlowJo software.

Immunofluorescence.

Cells were plated in 6-well tissue culture-treated plates (Greiner) at 1.75 × 106 cells/well and allowed to rest overnight. Cells were then infected with rAAV 2.5 at equal titers (1.4 × 108 copies/well) or 50 μM etoposide (Sigma) for 20 h. For the EdU-IF experiments, EdU was added to the cells for 20 min. Cells were then permeabilized with 0.5% Triton X-100 in PBS at 4°C for

5 min and fixed in 4% PFA for 20 min. Samples were then washed in 1× PBS and incubated with blocking buffer (3% BSA, 0.05% Tween 20, and 0.04 NaN3 in PBS) for 30 min. Cells were probed with appropriate primary antibodies (anti-FLAG M2 [Sigma-Aldrich], anti-γH2AX, anti-RPA32 [GeneTex], or anti-53BP1 [Cell Signaling]) and 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 Axio Imager Z1, and mean fluorescence intensity (MFI) was analyzed using ImageJ.

Sensitivity assays.

Sensitivity assays were performed as previously described (93), with minor changes. Cells were plated in 24-well plates at 3 × 103 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 × 106 copies/well) and then incubated for 7 days. On the 7th day, cells were washed with 1× PBS, fixed with 10% methanol and 10% acetic acid in water for 10 to 15 min, and stained with 0.1% crystal violet in methanol for 5 min. Plates were then washed with water and allowed to dry overnight, and the crystal violet was resolubilized with 300 µl 0.1% SDS in methanol for 2 h. A volume of 100 µl of the resolubilized dye was added to a 96-well, round-bottom plate (Greiner), and the absorbance was measured using a Gen5 (Biotek) plate reader at 595-nm wavelength.

I-SceI repair assays.

I-SceI repair assays were performed as previously described (94), with some minor changes. Cells were plated in 6-well plates at 1.75 × 106 cells/well and allowed to settle overnight. Cells were transfected with 1.5 µg pBASce-1 and 0.5 µg of corresponding pscAAV using Lipofectamine 3000 (Invitrogen) in antibiotic- and serum-free medium. Prior to transfection, cell medium 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 to 48 h, harvested, fixed with 4% PFA, and resuspended in fluorescence-activated cell sorting buffer (3%

BSA in PBS). At least 20,000 cells/condition were measured through flow cytometry (Attune NxT), and data were analyzed using FlowJo.

REFERENCES

1. Malim MH, Emerman M. 2008. HIV-1 accessory proteins—ensuring viral survival in a hostile environment. Cell Host Microbe 3:388–398. Crossref. PubMed. ISI.

2. Tristem M, Purvis A, Quicke D. 1998. Complex evolutionary history of primate lentiviral vpr genes. Virology 240:232–237. Crossref. PubMed. ISI.

3. Campbell BJ, Hirsch VM. 1997. Vpr of simian immunodeficiency virus of African green monkeys is required for replication in macaque macrophages and lymphocytes. J Virol 71:5593–5602. Crossref. PubMed. ISI.

4. Somasundaran M, Sharkey M, Brichacek B, Luzuriaga K, Emerman M, Sullivan JL, Stevenson M. 2002. Evidence for a cytopathogenicity determinant in HIV-1 Vpr. Proc Natl Acad Sci U S A 99:9503–9508. Crossref. PubMed. ISI.

5. Lim ES, Fregoso OI, McCoy CO, Matsen FA, Malik HS, Emerman M. 2012. The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. Cell Host Microbe 11:194–204. Crossref. PubMed. ISI.

6. Fregoso OI, Emerman M. 2016. Activation of the DNA damage response is a conserved function of HIV-1 and HIV-2 Vpr that is independent of SLX4 recruitment. mBio 7:e01433-16. Crossref. PubMed.

7. Planelles V, Jowett JBM, Li Q-X, Xie Y, Hahn B, Chen I. 1996. Vpr-induced cell cycle arrest is conserved among primate lentiviruses. J Virol 70:9. Crossref. ISI.

8. Stivahtis GL, Soares MA, Vodicka MA, Hahn BH, Emerman M. 1997. Conservation and host specificity of Vpr-mediated cell cycle arrest suggest a fundamental role in primate lentivirus evolution and biology. J Virol 71:4331–4338. Crossref. PubMed. ISI.

9. Cimprich KA, Cortez D. 2008. ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol 9:616–627. Crossref. PubMed. ISI.

10. Shiloh Y, Ziv Y. 2013. The ATM protein kinase: regulating the cellular response to genotoxic stress and more. Nat Rev Mol Cell Biol 14:197–210. Crossref. ISI.

11. Davis AJ, Chen BPC, Chen DJ. 2014. DNA-PK: a dynamic enzyme in a versatile DSB repair pathway. DNA Repair 17:21–29. Crossref. PubMed. ISI.

12. Blackford AN, Jackson SP. 2017. ATM, ATR, and DNA-PK: the trinity at the heart of the DNA damage response. Mol Cell 66:801–817. Crossref. PubMed. ISI.

13. Awasthi P, Foiani M, Kumar A. 2015. ATM and ATR signaling at a glance. J Cell Sci 128:4255–4262. Crossref. PubMed. ISI.

14. Weitzman MD, Fradet-Turcotte A. 2018. Virus DNA replication and the host DNA damage response. Annu Rev Virol 5:141–164. Crossref. PubMed. ISI.

15. Edwards TG, Bloom DC, Fisher C. 2017. The ATM and Rad3-related (ATR) protein kinase pathway is activated by herpes simplex virus 1 and required for efficient viral replication. J Virol 92:e01884-17. Crossref. ISI.

16. Baer A, Austin D, Narayanan A, Popova T, Kainulainen M, Bailey C, Kashanchi F, Weber F, Kehn-Hall K. 2012. Induction of DNA damage signaling upon Rift Valley Fever Virus infection results in cell cycle arrest and increased viral replication. J Biol Chem 287:7399–7410. Crossref. PubMed. ISI.

17. Ferguson BJ, Mansur DS, Peters NE, Ren H, Smith GL. 2012. DNA-PK is a DNA sensor for IRF-3-dependent innate immunity. Elife 1:e00047. Crossref. PubMed. ISI.

18. Härtlova A, Erttmann SF, Raffi FA, Schmalz AM, Resch U, Anugula S, Lienenklaus S, Nilsson LM, Kröger A, Nilsson JA, Ek T, Weiss S, Gekara NO. 2015. DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity. Immunity 42:332–343. Crossref. PubMed. ISI.

19. Korwek Z, Sewastianik T, Bielak-Zmijewska A, Mosieniak G, Alster O, Moreno-Villanueva M, Moreno-Villaneuva M, Burkle A, Sikora E. 2012. Inhibition of ATM blocks the etoposide-induced DNA damage response and apoptosis of resting human T cells. DNA Repair 11:864–873. Crossref. PubMed. ISI.

20. Dunphy G, Flannery SM, Almine JF, Connolly DJ, Paulus C, Jønsson KL, Jakobsen MR, Nevels MM, Bowie AG, Unterholzner L. 2018. Non-canonical activation of the DNA sensing adaptor STING by ATM and IFI16 mediates NF-κB signaling after nuclear DNA damage. Mol Cell 71:745–760. Crossref. PubMed. ISI.

21. Ahn J, Xia T, Konno H, Konno K, Ruiz P, Barber GN. 2014. Inflammation-driven carcinogenesis is mediated through STING. Nat Commun 5:5166. Crossref. PubMed.

22. Morotti A, Cilloni D, Pautasso M, Messa F, Arruga F, Defilippi I, Carturan S, Catalano R, Rosso V, Chiarenza A, Taulli R, Bracco E, Rege-Cambrin G, Gottardi E, Saglio G. 2006. NF-kB inhibition as a strategy to enhance etoposide-induced apoptosis in K562 cell line. Am J Hematol 81:938–945. Crossref. PubMed. ISI.

23. Jowett JB, Planelles V, Poon B, Shah NP, Chen ML, Chen IS. 1995. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. J Virol 69:6304–6313. Crossref. PubMed. ISI.

24. Re F, Braaten D, Franke EK, Luban J. 1995. Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. J Virol 69:6859–6864. Crossref. PubMed. ISI.

25. Rogel ME, Wu LI, Emerman M. 1995. The human immunodeficiency virus type 1 vpr gene prevents cell proliferation during chronic infection. J Virol 69:882–888. Crossref. PubMed. ISI.

26. He J, Choe S, Walker R, Di Marzio P, Morgan DO, Landau NR. 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J Virol 69:6705–6711. Crossref. PubMed. ISI.

27. Roshal M, Kim B, Zhu Y, Nghiem P, Planelles V. 2003. Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R. J Biol Chem 278:25879–25886. Crossref. PubMed. ISI.

28. Belzile J-P, Duisit G, Rougeau N, Mercier J, Finzi A, Cohen ÉA. 2007. HIV-1 Vpr-mediated G2 arrest involves the DDB1-CUL4AVPRBP E3 ubiquitin ligase. PLoS Pathog 3:e85. Crossref. PubMed. ISI.

29. Hrecka K, Gierszewska M, Srivastava S, Kozaczkiewicz L, Swanson SK, Florens L, Washburn MP, Skowronski J. 2007. Lentiviral Vpr usurps Cul4–DDB1[VprBP] E3 ubiquitin ligase to modulate cell cycle. Proc Natl Acad Sci U S A 104:11778–11783. Crossref. PubMed. ISI.

30. Hannah J, Zhou P. 2009. Regulation of DNA damage response pathways by the Cullin-RING ubiquitin ligases. DNA Repair 8:536–543. Crossref. PubMed. ISI.

31. Liu L, Lee S, Zhang J, Peters SB, Hannah J, Zhang Y, Yin Y, Koff A, Ma L, Zhou P. 2009. CUL4A abrogation augments DNA damage response and protection against skin carcinogenesis. Mol Cell 34:451–460. Crossref. PubMed. ISI.

32. Zimmerman ES, Chen J, Andersen JL, Ardon O, DeHart JL, Blackett J, Choudhary SK, Camerini D, Nghiem P, Planelles V. 2004. Human immunodeficiency virus type 1 Vpr-mediated G2 arrest requires Rad17 and Hus1 and induces nuclear BRCA1 and -H2AX focus formation. Mol Cell Biol 24:9286–9294. Crossref. PubMed. ISI.

33. Belzile J-P, Abrahamyan LG, Gérard FCA, Rougeau N, Cohen ÉA. 2010. Formation of mobile chromatin-associated nuclear foci containing HIV-1 Vpr and VPRBP is critical for the induction of G2 cell cycle arrest. PLoS Pathog 6:e1001080. Crossref. PubMed. ISI.

34. Laguette N, Brégnard C, Hue P, Basbous J, Yatim A, Larroque M, Kirchhoff F, Constantinou A, Sobhian B, Benkirane M. 2014. Premature activation of the SLX4 complex by Vpr promotes G2/M arrest and escape from innate immune sensing. Cell 156:134–145. Crossref. PubMed. ISI.

35. Schröfelbauer B, Yu Q, Zeitlin SG, Landau NR. 2005. Human immunodeficiency virus type 1 Vpr induces the degradation of the UNG and SMUG uracil-DNA glycosylases. J Virol 79:10978–10987. Crossref. PubMed. ISI.

36. Wu Y, Zhou X, Barnes CO, DeLucia M, Cohen AE, Gronenborn AM, Ahn J, Calero G. 2016. The DDB1–DCAF1–Vpr–UNG2 crystal structure reveals how HIV-1 Vpr steers human UNG2 toward destruction. Nat Struct Mol Biol 23:933–940. Crossref. PubMed. ISI.

37. Hrecka K, Hao C, Shun M-C, Kaur S, Swanson SK, Florens L, Washburn MP, Skowronski J. 2016. HIV-1 and HIV-2 exhibit divergent interactions with HLTF and UNG2 DNA repair proteins. Proc Natl Acad Sci U S A 113:E3921–E3930. Crossref. PubMed. ISI.

38. Lahouassa H, Blondot M-L, Chauveau L, Chougui G, Morel M, Leduc M, Guillonneau F, Ramirez BC, Schwartz O, Margottin-Goguet F. 2016. HIV-1 Vpr degrades the HLTF DNA translocase in T cells and macrophages. Proc Natl Acad Sci U S A 113:5311–5316. Crossref. PubMed. ISI.

39. Zhou X, DeLucia M, Ahn J. 2016. SLX4/SLX1 independent downregulation of MUS81/EME1 by HIV-1 Vpr. J Biol Chem 291:16936–16947. Crossref. PubMed. ISI.

40. Yan J, Shun M-C, Hao C, Zhang Y, Qian J, Hrecka K, DeLucia M, Monnie C, Ahn J, Skowronski J. 2018. HIV-1 Vpr reprograms CLR4DCAF1 E3 ubiquitin ligase to antagonize exonuclease 1-mediated restriction of HIV-1 infection. mBio 9:e01732-18. Crossref. PubMed.

41. Lv L, Wang Q, Xu Y, Tsao L-C, Nakagawa T, Guo H, Su L, Xiong Y. 2018. Vpr targets TET2 for degradation by CRL4VprBP E3 ligase to sustain IL-6 expression and enhance HIV-1 replication. Mol Cell 70:961–970. Crossref. PubMed. ISI.

42. Romani B, Shaykh Baygloo N, Aghasadeghi MR, Allahbakhshi E. 2015. HIV-1 Vpr protein enhances proteasomal degradation of MCM10 DNA replication factor through the Cul4-DDB1[VprBP] E3 ubiquitin ligase to induce G2/M cell cycle arrest. J Biol Chem 290:17380–17389. Crossref. PubMed. ISI.

43. Fregoso OI, Ahn J, Wang C, Mehrens J, Skowronski J, Emerman M. 2013. Evolutionary toggling of Vpx/Vpr specificity results in divergent recognition of the restriction factor SAMHD1. PLoS Pathog 9:e1003496. Crossref. PubMed. ISI.

44. Sharp PM, Hahn BH. 2011. Origins of HIV and the AIDS pandemic. Cold Spring Harb Perspect Med 1:a006841. Crossref. PubMed. ISI.

45. Kuo LJ, Yang L-X. 2008. Gamma-H2AX–a novel biomarker for DNA double-strand breaks. In Vivo 22:305–309. PubMed. ISI.

46. Ruff P, Donnianni RA, Glancy E, Oh J, Symington LS. 2016. RPA stabilization of single-stranded DNA is critical for break-induced replication. Cell Rep 17:3359–3368. Crossref. PubMed. ISI.

47. Panier S, Boulton SJ. 2014. Double-strand break repair: 53BP1 comes into focus. Nat Rev Mol Cell Biol 15:7–18. Crossref. PubMed. ISI.

48. Tachiwana H, Shimura M, Nakai-Murakami C, Tokunaga K, Takizawa Y, Sata T, Kurumizaka H, Ishizaka Y. 2006. HIV-1 Vpr induces DNA double-strand breaks. Cancer Res 66:627–631. Crossref. PubMed. ISI.

49. Lai M, Zimmerman ES, Planelles V, Chen J. 2005. Activation of the ATR pathway by human immunodeficiency virus type 1 Vpr involves its direct binding to chromatin in vivo. J Virol 79:15443–15451. Crossref. PubMed. ISI.

50. Maiuri T, Mocle AJ, Hung CL, Xia J, van Roon-Mom WMC, Truant R. 2017. Huntingtin is a scaffolding protein in the ATM oxidative DNA damage response complex. Hum Mol Genet 26:395–406. Crossref. PubMed. ISI.

51. Zimmerman ES, Sherman MP, Blackett JL, Neidleman JA, Kreis C, Mundt P, Williams SA, Warmerdam M, Kahn J, Hecht FM, Grant RM, de Noronha CMC, Weyrich AS, Greene WC, Planelles V. 2006. Human immunodeficiency virus type 1 Vpr induces DNA replication stress in vitro and in vivo. J Virol 80:10407–10418. Crossref. PubMed. ISI.

52. Nieminuszczy J, Schwab RA, Niedzwiedz W. 2016. The DNA fibre technique–tracking helicases at work. Methods 108:92–98. Crossref. PubMed. ISI.

53. Yarbro JW. 1992. Mechanism of action of hydroxyurea. Semin Oncol 19:1–10. PubMed. ISI.

54. Prevo R, Fokas E, Reaper PM, Charlton PA, Pollard JR, McKenna WG, Muschel RJ, Brunner TB. 2012. The novel ATR inhibitor VE-821 increases sensitivity of pancreatic cancer cells to radiation and chemotherapy. Cancer Biol Ther 13:1072–1081. Crossref. PubMed. ISI.

55. Andersen JL, Le Rouzic E, Planelles V. 2008. HIV-1 Vpr: mechanisms of G2 arrest and apoptosis. Exp Mol Pathol 85:2–10. Crossref. PubMed. ISI.

56. Montecucco A, Zanetta F, Biamonti G. 2015. Molecular mechanisms of etoposide. EXCLI J 14:95–108. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4652635. PubMed. ISI.

57. Petermann E, Orta ML, Issaeva N, Schultz N, Helleday T. 2010. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. Mol Cell 37:492–502. Crossref. PubMed. ISI.

58. Sunada S, Nakanishi A, Miki Y. 2018. Crosstalk of DNA double-strand break repair pathways in poly(ADP-ribose) polymerase inhibitor treatment of breast cancer susceptibility gene 1/2-mutated cancer. Cancer Sci 109:893–899. Crossref. PubMed. ISI.

59. Dasari S, Tchounwou PB. 2014. Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol 740:364–378. Crossref. PubMed. ISI.

60. Gunn A, Stark JM. 2012. I-SceI-based assays to examine distinct repair outcomes of mammalian chromosomal double strand breaks, p 379–391. In Bjergbæk L (ed), DNA repair protocols. Humana Press, Totowa, NJ. Crossref.

61. Gunn A, Bennardo N, Cheng A, Stark JM. 2011. Correct end use during end joining of multiple chromosomal double strand breaks is influenced by repair protein RAD50, DNA-dependent protein kinase DNA-PKcs, and transcription context. J Biol Chem 286:42470–42482. Crossref. PubMed. ISI.

62. Brandsma I, Gent DC. 2012. Pathway choice in DNA double strand break repair: observations of a balancing act. Genome Integr 3:9. Crossref. PubMed.

63. Mansky LM, Preveral S, Selig L, Benarous R, Benichou S. 2000. The interaction of Vpr with uracil DNA glycosylase modulates the human immunodeficiency virus type 1 in vivo mutation rate. J Virol 74:7039–7047. Crossref. PubMed. ISI.

64. Jacquot G, Rouzic EL, Maidou-Peindara P, Maizy M, Lefrère J-J, Daneluzzi V, Monteiro-Filho CMR, Hong D, Planelles V, Morand-Joubert L, Benichou S. 2009. Characterization of the molecular determinants of primary HIV-1 Vpr proteins: impact of the Q65R and R77Q substitutions on Vpr functions. PLoS One 4:e7514. Crossref. PubMed. ISI.

65. Le Rouzic E, Belaïdouni N, Estrabaud E, Morel M, Rain J-C, Transy C, Margottin-Goguet F. 2007. HIV1 Vpr arrests the cell cycle by recruiting DCAF1/VprBP, a receptor of the Cul4-DDB1 ubiquitin ligase. Cell Cycle 6:182–188. Crossref. PubMed. ISI.

66. Barnitz RA, Wan F, Tripuraneni V, Bolton DL, Lenardo MJ. 2010. Protein kinase A phosphorylation activates Vpr-induced cell cycle arrest during human immunodeficiency virus type 1 infection. J Virol 84:6410–6424. Crossref. PubMed. ISI.

67. Liu R, Lin Y, Jia R, Geng Y, Liang C, Tan J, Qiao W. 2014. HIV-1 Vpr stimulates NF-κB and AP-1 signaling by activating TAK1. Retrovirology 11:45. Crossref. PubMed. ISI.

68. DeHart JL, Zimmerman ES, Ardon O, Monteiro-Filho CM, Argañaraz ER, Planelles V. 2007. HIV-1 Vpr activates the G2 checkpoint through manipulation of the ubiquitin proteasome system. Virol J 4:57. Crossref. PubMed. ISI.

69. Wallace NA, Khanal S, Robinson KL, Wendel SO, Messer JJ, Galloway DA. 2017. High-risk Alphapapillomavirus oncogenes impair the homologous recombination pathway. J Virol 91:e01084-17. Crossref. PubMed. ISI.

70. Anacker DC, Moody CA. 2017. Modulation of the DNA damage response during the life cycle of human papillomaviruses. Virus Res 231:41–49. Crossref. PubMed. ISI.

71. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A, Gillison ML. 2008. Improved survival of patients with human papillomavirus–positive head and neck

squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 100:261–269. Crossref. PubMed. ISI.

72. Roesch F, Richard L, Rua R, Porrot F, Casartelli N, Schwartz O. 2015. Vpr enhances tumor necrosis factor production by HIV-1-infected T cells. J Virol 89:12118–12130. Crossref. PubMed. ISI.

73. Guenzel CA, Herate C, Benichou S. 2014. HIV-1 Vpr—a still “enigmatic multitasker.” Front Microbiol 5:127. Crossref. PubMed. ISI.

74. de Silva S, Planelles V, Wu L. 2012. Differential effects of Vpr on single-cycle and spreading HIV-1 infections in CD4+ T-cells and dendritic cells. PLoS One 7:e35385. Crossref. PubMed. ISI.

75. Vodicka MA, Koepp DM, Silver PA, Emerman M. 1998. HIV-1 Vpr interacts with the nuclear transport pathway to promote macrophage infection. Genes Dev 12:175–185. Crossref. PubMed. ISI.

76. Cohen EA, Terwilliger EF, Jalinoos Y, Proulx J, Sodroski JG, Haseltine WA. 1990. Identification of HIV-1 Vpr product and function. J Acquir Immune Defic Syndr 3:11–18. PubMed. ISI.

77. Connor RI, Chen BK, Choe S, Landau NR. 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206:935–944. Crossref. PubMed. ISI.

78. Felzien LK, Woffendin C, Hottiger MO, Subbramanian RA, Cohen EA, Nabel GJ. 1998. HIV transcriptional activation by the accessory protein, VPR, is mediated by the p300 co-activator. Proc Natl Acad Sci U S A 95:5281–5286. Crossref. PubMed. ISI.

79. Mashiba M, Collins DR, Terry VH, Collins KL. 2014. Vpr overcomes macrophage-specific restriction of HIV-1 Env expression and virion production. Cell Host Microbe 16:722–735. Crossref. PubMed. ISI.

80. Franklin DA, He Y, Leslie PL, Tikunov AP, Fenger N, Macdonald JM, Zhang Y. 2016. p53 coordinates DNA repair with nucleotide synthesis by suppressing PFKFB3 expression and promoting the pentose phosphate pathway. Sci Rep 6:38067. Crossref. PubMed.

81. Laguette N, Sobhian B, Casartelli N, Ringeard M, Chable-Bessia C, Ségéral E, Yatim A, Emiliani S, Schwartz O, Benkirane M. 2011. SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. Nature 474:654–657. Crossref. PubMed. ISI.

82. Hrecka K, Hao C, Gierszewska M, Swanson SK, Kesik-Brodacka M, Srivastava S, Florens L, Washburn MP, Skowronski J. 2011. Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. Nature 474:658–661. Crossref. PubMed. ISI.

83. Zhou X, DeLucia M, Hao C, Hrecka K, Monnie C, Skowronski J, Ahn J. 2017. HIV-1 Vpr protein directly loads helicase-like transcription factor (HLTF) onto the CRL4-DCAF1 E3 ubiquitin ligase. J Biol Chem 292:21117–21127. Crossref. PubMed. ISI.

84. Durkin SS, Guo X, Fryrear KA, Mihaylova VT, Gupta SK, Belgnaoui SM, Haoudi A, Kupfer GM, Semmes OJ. 2008. HTLV-1 tax oncoprotein subverts the cellular DNA damage response via binding to DNA-dependent protein kinase. J Biol Chem 283:36311–36320. Crossref. PubMed. ISI.

85. Baydoun HH, Bai XT, Shelton S, Nicot C. 2012. HTLV-I tax increases genetic instability by inducing DNA double strand breaks during DNA replication and switching repair to NHEJ. PLoS One 7:e42226. Crossref. PubMed. ISI.

86. Rushing AW, Hoang K, Polakowski N, Lemasson I. 2018. The human T-cell leukemia virus type 1 basic leucine zipper factor attenuates repair of double-stranded DNA breaks via nonhomologous end joining. J Virol 92:e00672-18. Crossref. PubMed. ISI.

87. Christmann M, Kaina B. 2013. Transcriptional regulation of human DNA repair genes following genotoxic stress: trigger mechanisms, inducible responses and genotoxic adaptation. Nucleic Acids Res 41:8403–8420. Crossref. PubMed. ISI.

88. Daddacha W, Koyen AE, Bastien AJ, Head PE, Dhere VR, Nabeta GN, Connolly EC, Werner E, Madden MZ, Daly MB, Minten EV, Whelan DR, Schlafstein AJ, Zhang H, Anand R, Doronio C, Withers AE, Shepard C, Sundaram RK, Deng X, Dynan WS, Wang Y, Bindra RS, Cejka P, Rothenberg E, Doetsch PW, Kim B, Yu DS. 2017. SAMHD1 promotes DNA end resection to facilitate DNA repair by homologous recombination. Cell Rep 20:1921–1935. Crossref. PubMed. ISI.

89. Mlcochova P, Caswell SJ, Taylor IA, Towers GJ, Gupta RK. 2018. DNA damage induced by topoisomerase inhibitors activates SAMHD1 and blocks HIV-1 infection of macrophages. EMBO J 37:50–62. Crossref. PubMed. ISI.

90. Richardson C, Moynahan ME, Jasin M. 1998. Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. Genes Dev 12:3831–3842. Crossref. PubMed. ISI.

91. Choi VW, Asokan A, Haberman RA, Samulski RJ. 2007. Production of recombinant adeno-associated viral vectors for in vitro and in vivo use. Curr Protoc Mol Biol 78:16.25.1–16.25.24. Crossref.

92. Aurnhammer C, Haase M, Muether N, Hausl M, Rauschhuber C, Huber I, Nitschko H, Busch U, Sing A, Ehrhardt A, Baiker A. 2012. Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. Hum Gene Ther Methods 23:18–28. Crossref. PubMed. ISI.

93. Wang Y, Huang J-W, Li M, Cavenee WK, Mitchell PS, Zhou X, Tewari M, Furnari FB, Taniguchi T. 2011. MicroRNA-138 modulates DNA damage response by repressing histone H2AX expression. Mol Cancer Res 9:1100–1111. Crossref. PubMed. ISI.

94. Schumacher AJ, Mohni KN, Kan Y, Hendrickson EA, Stark JM, Weller SK. 2012. The HSV-1 exonuclease, UL12, stimulates recombination by a single strand annealing mechanism. PLoS Pathog 8:e1002862. Crossref. PubMed. ISI.