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Los Angeles

Investigating the mechanisms and functional consequences of DNA damage response
engagement by the lentiviral accessory gene Vpr

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy in Molecular Biology

by

Karly Ann Nisson

2025

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ABSTRACT OF THE DISSERTATION

Investigating the mechanisms and functional consequences of DNA damage response

engagement by the lentiviral accessory gene Vpr

by

Karly Ann Nisson

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2025

Professor Oliver I. Fregoso, Co-Chair

Professor Kathrin Plath, Co-Chair

Diverse viruses modulate the host DNA damage response (DDR) to enhance viral replication or evade innate immune surveillance. Among primate lentiviruses, the conserved and essential accessory gene Vpr engages the DDR at multiple, potentially unique steps: Vpr induces DNA damage, activates and represses the DDR, and causes cell cycle arrest at the G2/M checkpoint. Of the three major DDR signaling kinases, DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and Rad3 (ATR), Vpr activates ATM and ATR. The precise nature of this activation remains unclear, however, as do the downstream pathways triggered as a consequence. In this dissertation, I investigate Vpr-mediated ATM and ATR signaling and explore how each might remodel the cell in favor of lentiviral replication. In Chapter 2, I determine that Vpr activates canonical ATM signaling and triggers ATM-NEMO dependent NF- κ B/ RelA transcription to remodel the host transcriptome. In Chapter 3, I determine that Vpr-induced ATR activation causes a novel phenotype, nucleolar stress, and characterize the Vpr host target CCDC137 as an important nucleolar stress sensor. Overall, this work encompasses many foundational discoveries that will have important implications for diverse fields, including HIV, nucleolar stress, genome integrity and cancer biology.

The dissertation of Karly Ann Nisson is approved.

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This dissertation is dedicated to all – people and creatures alike – whose paths have crossed
with the little red house with the orange grove on Newport Avenue.

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Chapter 1: Introduction

The lentiviral lifecycle

Lentiviruses are a genus of retroviruses, of which the best-characterized is the human immunodeficiency virus (HIV). The defining features of retroviruses are their abilities to reverse transcribe their RNA genomes into linear double-stranded DNA and integrate into the DNA of the host cell (1). Separating lentiviruses from other retroviruses is their capacity to infect non-dividing cells by passing through the nuclear pore complexes of intact nuclear membranes. This specific ability is central to HIV-1 transmission and AIDS pathogenesis (2).

HIV-1 virions harbor two copies of a ssRNA genome that are enclosed in a conical capsid surrounded by the viral envelope. Encoded in the genome are the polyproteins Gag and Pol, structural precursor proteins and viral enzymes, respectively, Env, the viral envelope glycoprotein, and the single proteins Tat, Rev, Nef, Vif, Vpr and Vpu, all flanked by two long terminal repeats (LTRs). Tat and Rev play essential roles in HIV-1 transcription and unspliced viral RNA export, respectively, and the accessory genes Nef, Vif, Vpr and Vpu each uniquely target host cell restriction factors to counter the innate immune response.

During infection, HIV-1 enters the host cell by binding to its primary receptor, CD4, and a co-receptor, typically CXCR4 or CCR5 (**Figure 1**). This triggers the fusion of the viral envelope and host cell plasma membrane, dispelling the viral capsid core into the cytoplasm. At this time, the viral genome is reverse transcribed by virally encoded reverse transcriptase as the capsid makes its way to the nucleus. Whether the capsid is uncoated in the cytoplasm or nucleus is still being worked out, as multiple models depict both possibilities (3). Following nuclear import, the now dsDNA HIV-1 genome is integrated into the host cell DNA via the viral enzyme integrase and host DNA repair enzymes to form the provirus (4). The HIV-1 provirus is replicated by host cell RNA Polymerase II that binds and initiates transcription at the 5'LTR, and unspliced viral RNA genomes are exported to cytoplasm via Rev. Viral mRNAs are translated by the host translation machinery

to produce viral proteins, and these are assembled into new virions with the viral RNA genomes. These virions then bud out from the host cell, taking with them the host plasma membrane, and mature to form new infectious viral particles (5).

Overall, successful HIV-1 infection relies on the manipulation of many host pathways to enhance replication and evade the innate immune response. One of these pathways is the host DNA damage response, which is involved in multiple steps of the viral lifecycle and manipulated by multiple lentiviral proteins (6–9).

The host DNA damage response

The host DNA damage response (DDR) is a complex network of signaling pathways that senses and responds to diverse genomic insults. These insults may be introduced endogenously, through errors generated during DNA replication, for instance, or exogenously, through exposure to ultraviolet and ionizing radiation. Each insult is recognized by DNA damage sensors and early signal transducers that work in concert to relay the DNA damage signal (10) to effectors that can give rise to different downstream cellular consequences.

The exact nature of the factors involved in these pathways depends on the specific type of damage induced, as both endogenous and exogenous exposures can result in double-stranded DNA breaks (DSBs), single-stranded DNA breaks (SSBs) or a myriad of single-base modifications. Responding to each of these different lesions are the three major DDR signaling kinases that represent the major branches of the DDR: DNA-dependent protein (DNA-PK), ataxia-telangiectasia mutated (ATM), and ataxia telangiectasia and Rad3related protein (ATR). Although DNA-PK and ATM both respond to DSBs, the former primarily promotes non-homologous end-joining (NHEJ) whereas the latter orchestrates a global signaling network. Thus, ATM is considered the master regulator of cellular responses to DSBs (11). Conversely, ATR is generally associated with SSBs and is recruited to ssDNA intermediates generated during replication stress.

Additionally, ATR can respond to cellular insults independent of the DDR, including mechanical stress at the nuclear envelope and prolonged nucleolar stress (12, 13). While each kinase has its own functional specificities, there exists a great deal of crosstalk among the major DDR kinases that allows for diverse downstream cellular consequences (**Figure 1**).

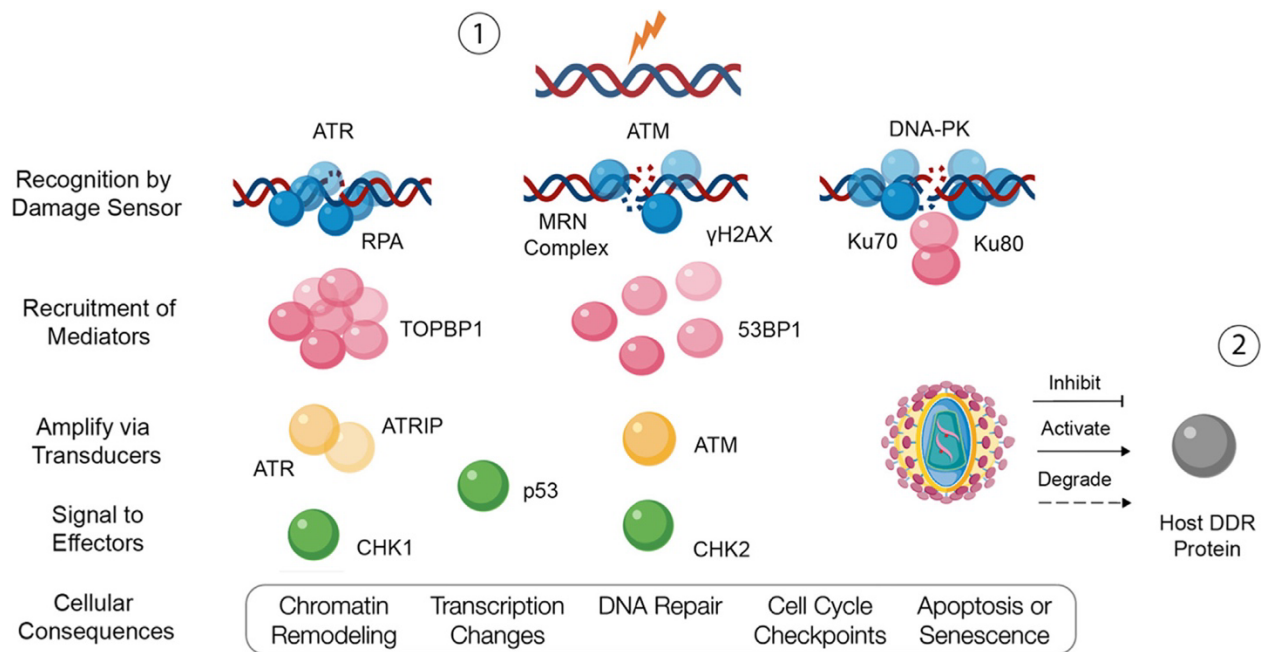


Figure 1: The DDR senses, signals, and responds to aberrant DNA through three primary pathways, ATM, ATR, and DNA-PK, that are modulated by viruses. Figure from Lopez, Nichols Doyle and Sandoval et al., J Mol Biol. (2021) (14)

DDR kinases trigger global cellular responses

Once activated, each DDR kinase defines a downstream response specific to the type and severity of the lesion. Generally, these responses fit into the categories of DNA repair, cell cycle arrest, chromatin and/or transcriptional changes and apoptosis. A crucial function of the ATR kinase, for instance, is to phosphorylate the downstream effector CHK1 to arrest the cell cycle at the G2/M checkpoint following damage in S phase (15). ATM, meanwhile, can induce both global and local transcriptional changes, either altering the chromatin environment near DSBs to initiate *in cis* transcriptional silencing (16) or activating NEMO-dependent NF- κ B signaling to alter global

transcription (17). In addition, cellular responses to severe and/or prolonged DNA damage can include additional stress pathways that modulate translation, including the integrated stress response, which is activated in response to persistent SSBs and DSBs (18).

Activation of different downstream consequences of the DDR may also be influenced by specific nuclear compartments. The nucleolus, for instance, detects and responds to genomic insults that occur *in cis*, in ribosomal DNA (rDNA), as well as *in trans*, in nuclear DNA. When DSBs occur in rDNA, the nucleolar DDR (nDDR) can act locally; ATM signaling can trigger transcriptional silencing that is isolated to the specific nucleolus or nucleolar region containing the break (19). In response to nuclear DSBs, however, ATM and ATR inhibit all cellular RNA-Pol I, resulting in universal, transient rDNA transcriptional repression (20). Prolonged lesions, as may occur upon inhibition of the nDDR, or DNA damage that causes ribosome stress, can result in nucleolar stress that may trigger cell cycle arrest or apoptosis (21).

Viruses activate and manipulate DDR pathways

Because of its role in detecting genomic abnormalities, the DDR can also recognize and respond to viral infections, including foreign viral genetic material and lesions induced by viral proteins. Yet, despite its antiviral capacities, the DDR can also benefit viruses that have evolved to manipulate it. Diverse viruses manipulate the DDR by causing DNA damage, modulating DNA damage sensors, or altering DDR signaling. The retrovirus Human T-lymphotropic virus type 1 (HTLV-1), for instance, encodes Tax, an oncoprotein that induces chromosome abnormalities, inhibits ATM activation, dysregulates cell-cycle checkpoints (22), and induces NF-KB activation through an interaction with NEMO that activates the IKK complex (23). Less complex viruses, such as the autonomous parvoviruses, can also broadly activate the DDR (24). Among these, B19V can activate all three DDR kinases, and its induction of the ATR-Chk1 signaling has been shown to enhance viral replication (25). While individual viruses exploit the DDR via unique mechanisms, most reap the shared benefits of enhanced viral replication and innate immune

evasion. How exactly each virus manipulates the DDR, and how these manipulations give rise to nuclear environments that better favor viral replication remains unclear.

Lentiviral accessory genes modulate host pathways

Primate lentiviruses, including human immunodeficiency virus (HIV), encode accessory genes that antagonize innate immune factors and enhance viral infectivity. In the context of HIV evolution, there are two primary lentiviral lineages defined by different cross-species transmission events: HIV-1, which originated in chimpanzees and is the causative agent of the HIV pandemic, and HIV-2, which originated in sooty mangabeys and is primarily endemic in West Africa (26) (**Figure 2**). Conserved among viruses from both lineages are the lentiviral accessory genes *vif*, *vpr*, and *nef*. In addition, HIV-1 and its predecessors encode *vpu*, and HIV-2 and some SIVs encode *vpx* (27). These accessory genes do not possess inherent enzymatic activity, but instead target specific host restriction factors for proteasomal degradation through the recruitment of host ubiquitin ligase complexes. Each accessory gene has evolved to degrade a specific cellular target(s) via this mechanism, and – in most cases – this degradation is associated with a primary phenotype. The lentiviral accessory gene Vpu, for instance, degrades the host factor Tetherin to prevent the inhibition of viral particle release(27).

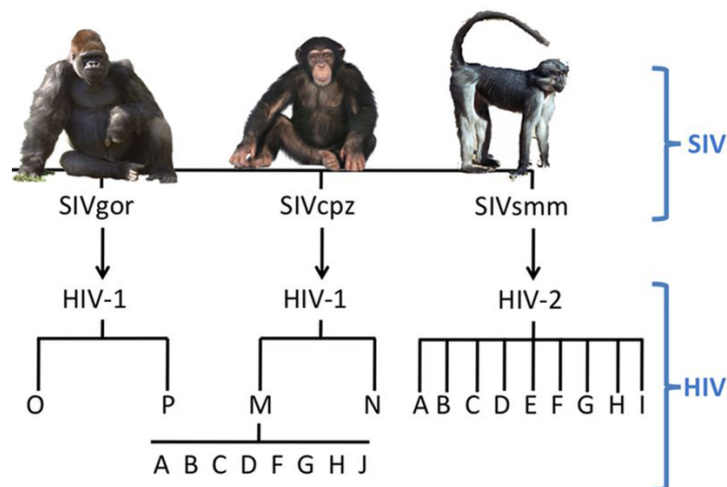


Figure 2: **Origins of the HIV-1 and HIV-2 lineages.** Figure modified from Letko et al., Journal of Virology (2013) (28).

Congruent with the dual roles of many DDR factors in the innate immune response, multiple host proteins targeted by lentiviral accessory genes are involved in DNA repair. Vpx degrades SAMHD1, a host restriction factor involved in the response to DSBs, to prevent the depletion of dNTP pools below those required for reverse transcription (27, 29, 30). Similarly, Vif prevents the inhibition of reverse transcription by depleting APOBEC3G, a cellular cytidine deaminase that both promotes repair of DSBs (31) and inhibits reverse transcription by deaminating cytidine residues in the viral genome (27, 32). While multiple accessory genes modulate the host DDR, either by depleting DDR repair factors or manipulating DDR signaling (7), the accessory gene canonically associated with DDR engagement is Vpr. Yet, precisely how Vpr modulates the DDR is still being investigated.

The enigmatic Vpr

Despite its conservation among primate lentiviruses, Vpr remains the only accessory gene without a known primary host target or function. Although Vpr is known to target a myriad of host factors(33–35), none of these interactions have been able to fully explain its diverse roles, which include: regulation of apoptosis and reverse transcription (36), transactivation of the viral LTR(37), engagement of the DNA damage response (DDR) and arrest of the cell cycle(38, 39). Of these, the latter two are conserved across diverse Vpr orthologs, suggesting that modulation of DDR signaling is crucial to the function of Vpr and represents an important step in the viral lifecycle.

Vpr engages the DDR at multiple steps (6) by directly inducing DNA damage, activating the DDR, repressing DSB repair, and inducing various downstream cellular responses. Although the precise mechanism by which Vpr causes DNA damage is unknown, Vpr-induced DNA damage is associated with stalled replication forks and activates markers of both DSBs and SSBs (6). In line with this, Vpr activates both ATM and ATR kinases (39–41). Yet, Vpr also represses the DDR by

inhibiting the DSB repair pathways homologous recombination (HR) and nonhomologous end joining (NHEJ), and these phenotypes are independent from Vpr-induced damage, and DDR activation. Finally, Vpr also modulates downstream DDR pathways, including ATR-dependent cell cycle arrest at the G2/M checkpoint. Given that HIV Vpr mutants that lack the ability to cause DNA damage can still induce this arrest, there is a specificity to the triggers underlying this phenotype that extend beyond replication stress or total DNA damage. Although the origin of this specificity remains unclear, many have proposed it to be the degradation of a specific, yet unknown host factor. At present, however, how Vpr engages the DDR at multiple, independent stages is unclear, and no phenotype can be explicitly linked to any of the factors Vpr has been found to degrade.

Like other lentiviral accessory genes, Vpr hijacks a host ubiquitin ligase complex to deplete host restriction factors. Vpr specifically co-opts the E3-CRL4^{DCAF1} ubiquitin ligase complex by binding the host protein DCAF1. While the requirement for Vpr-DCAF1 binding in Vpr-mediated DNA damage and DDR activation is unclear, Vpr does bind and degrade various DDR factors via this co-option. These include HTLF (33, 42), UNG2 (35, 43), SLX4 complex proteins MUS81 and EME1 (44, 45), TET2 (46), SAMHD1 (47, 48) and MCM10 (49). In some cases, such as that of HTLF, Vpr is specifically antagonizing a repair factor to prevent it from restricting lentiviral replication, characteristic of a canonical lentiviral accessory gene: host target relationship (50). In other cases, such as that of MCM10, Vpr is binding and enhancing the degradation of a host factor naturally regulated by DCAF1(49). Though different, both scenarios have important implications for Vpr function and viral replication, even if none fully explain how Vpr modulates the DDR.

Vpr degrades the nucleolar protein CCDC137

Recently, Vpr-induced DDR signaling, G2/M arrest and increased viral transcription were associated with the ability of Vpr to degrade the host factor CCDC137 (51, 52). CCDC137 is a nucleolar protein that localizes to the chromosome peripheries during mitosis, though its precise

cellular functions and mechanistic connections to Vpr phenotypes are still relatively unknown. CCDC137 has thus far been associated with a myriad of cellular pathways, including (PI3K)/Akt signaling, RNA binding, E3 ubiquitin ligase co-option, and nuclear hormone receptor (NHR) signaling (53–56). Its involvement with the latter has been implicated in adenoviral infection: the adenoviral E1A protein binds CCDC137 to prevent it from sequestering the estrogen related (ERR) and retinoic acid (RAR) receptors in the nucleolus(53, 54), disrupting their respective signaling pathways. Importantly, CCDC137 has been described as an oncogene (57–59) and CCDC137 overexpression contributes to cancer progression and poor clinical outcomes in multiple cancer types. Determining the exact pathways CCDC137 inhabits, as well as the consequences of their dysregulation, will thus dually contribute to our understanding of tumorigenesis and lentiviral infectivity, specifically with regards to Vpr function.

Significance and Dissertation Overview

Understanding how viruses manipulate the host DDR has the potential to reveal novel insights into virus and host biology, as well as contribute to the development of anti-viral and cancer therapeutics alike. Within the lentiviral field specifically, a clear role for the accessory protein Vpr in both DDR manipulation and infection has remained elusive. Consequently, much of the work in the field has focused on trying to identify 1) a primary Vpr function as well as 2) the primary host target(s) Vpr degrades to cause the numerous phenotypes that have been attributed to it over the years. In my dissertation I explore the mechanisms by which Vpr engages two different branches of the DDR – ATM and ATR signaling – and induces novel downstream cellular stress pathways.

In **Chapter 2**, I describe our group's investigation of the activation of ATM and NFkB/RelA signaling by the HIV-1 accessory protein Vpr. We determine that Vpr activates both signaling cascades in U2OS cells and primary human monocyte-derived macrophages (MDMs). More specifically, we show that HIV-1 Vpr-mediated activation of the ATM-NF-KB essential modulator (NEMO) pathway results in global transcriptional changes. By performing RNA-sequencing of

U2OS cells expressing Vpr mutants that separate different Vpr phenotypes, we show that these transcriptional changes are independent of Vpr-mediated G2/M arrest. We demonstrate that Vpr activates NEMO-dependent NF- κ B transcription: Vpr triggers RelA nuclear localization and enhances expression of NF- κ B target genes, and both phenotypes are abrogated by the loss of NEMO. Finally, we show that these changes are induced early in infection, suggesting that Vpr-induced ATM-NEMO signaling may remodel the cellular environment to create favorable conditions for events that occur later in the lentiviral lifecycle. Overall, this work clarifies how Vpr engages ATM signaling, demonstrates that Vpr alters the transcriptome via the essential transcription factor NF- κ B, and further untangles the relationship between Vpr-mediated DDR activation and cell cycle arrest. This work has been published in *mBio* as:

Sandoval C, **Nisson K**, Fregoso OI. HIV-1 Vpr-induced DNA damage activates NF- κ B through ATM-NEMO independent of cell cycle arrest. *mBio*. 2024 Oct 16;15(10):e0024024. doi: 10.1128/mbio.00240-24. Epub 2024 Sep 13. PMID: 39269169; PMCID: PMC11481869.

In **Chapter 3**, I describe how our investigation into the relationship between Vpr and CCDC137 led us to the discovery of a novel role for Vpr in inducing ATR-dependent nucleolar stress. We separate the ability of Vpr to degrade CCDC137 from its ability to cause G2/M arrest and show that Vpr depletes CCDC137 independent of E3-CRL4^{DCAF1} ubiquitin ligase complex assembly. We further characterize the evolution underlying this degradation and show that robust CCDC137 depletion is a unique feature of Vpr from the pandemic causing HIV-1 lineage. Moreover, we show that CCDC137 shows signatures of longstanding evolutionary antagonism, and its degradation by Vpr is species-specific, together suggesting an important role for CCDC137 depletion in Vpr function and HIV-1 infection. In investigating alternative mechanisms that might underlie Vpr-mediated CCDC137 depletion, we discover that CCDC137 is degraded in response to specific genomic insults that trigger nucleolar stress. We show that Vpr causes nucleolar stress by

depleting the nucleolar proteome, altering the localization of certain nucleolar proteins, augmenting nucleolar size, and repressing ribosome biogenesis. Further, we determine that CCDC137 depletion is one component that comprises an Vpr-induced nucleolar stress response, and these collective nucleolar phenotypes are ATR dependent. In contrast, we show that Vpr orthologs that induce DNA damage but fail to deplete CCDC137 trigger DNA damage that is ATM-dependent. Overall, this work clarifies the association between CCDC137 degradation and a conserved Vpr phenotype, identifies a novel nucleolar ATR-pathway, and determines that Vpr triggers a nucleolar stress response. Moreover, it challenges the field's perception of what "solving Vpr" will look like, suggesting that, instead of degrading a single host factor, Vpr is disrupting global cellular processes to produce the wide array of phenotypes that have been attributed to it over the years.

Finally, in **Chapter 4** I will summarize the findings of both investigations and discuss how they contribute to our understanding of Vpr-mediated DDR engagement and host DDR regulation. Moreover, I will expand this discussion to explore the impacts of these findings on HIV replication, viral pathogenesis, and nucleolar biology.

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**Chapter 2: HIV-1 Vpr-induced DNA damage activates NF- κ B through ATM-NEMO
independent of cell cycle arrest**

**HIV-1 Vpr-induced DNA damage activates NF- κ B through ATM-NEMO independent of cell
cycle arrest**

Short Title: HIV-induced DNA damage alters cellular transcription

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Abstract

Lentiviruses encode a number of multi-functional accessory proteins, however the primary role of the accessory protein Vpr remains unclear. As Vpr engages the host DNA damage response (DDR) at multiple steps, modulation of the DDR is considered central to the function(s) of Vpr. Vpr activates ATR-mediated DDR signaling, resulting in cell cycle arrest. However, the cellular consequences of Vpr-induced DNA damage, and the connection of Vpr-induced DNA damage to other Vpr functions, are unknown. Here, we determined HIV-1 Vpr-induced DNA damage activates the ATM-NEMO pathway and alters cellular transcription via NF- κ B/RelA. Through RNA-sequencing of cells expressing Vpr or mutants that separate the ability of Vpr to induce DNA damage from other DDR phenotypes, we identified that Vpr alters the transcriptome independent of cell cycle arrest. In tissue-cultured U2OS cells and primary human monocyte-derived macrophages (MDMs), we showed Vpr activates both ATM and NF- κ B/RelA signaling cascades. While inhibition of NEMO did not affect Vpr-induced DNA damage, it prevented NF- κ B activation by Vpr, highlighting the importance of NEMO in Vpr-mediated transcriptional reprogramming. Virion delivered Vpr was sufficient to induce DNA damage and activate ATM-NEMO dependent NF- κ B transcription, suggesting that engagement of the DDR and transcriptional changes can occur early during viral replication. Together, our data uncover cellular consequences of Vpr-induced DNA damage and provide a mechanism for how Vpr activates NF- κ B through DNA damage and ATM-NEMO signaling, which occur independent of cell cycle arrest. We propose this is essential to overcoming restrictive environments, such as in macrophages, to enhance viral replication.

importance

The HIV accessory protein Vpr is multi-functional and required for viral replication *in vivo*, yet how Vpr enhances viral replication is unknown. Emerging literature suggests that a conserved function of Vpr is engagement of the host DNA damage response (DDR). For example, Vpr activates DDR signaling, causes DDR-dependent cell cycle arrest, promotes degradation of various DDR proteins, and alters cellular consequences of DDR activation. However, a central understanding of how these phenotypes connect and how they affect HIV-infected cells remains unknown. Here, we found that Vpr-induced DNA damage alters the host transcriptome by activating an essential transcription pathway, NF- κ B. This occurs early during infection of primary human immune cells, suggesting NF- κ B activation and transcriptome remodeling are important for establishing productive HIV-1 infection. Together, our study provides novel insights into how Vpr alters the host environment through the DDR, and what roles Vpr and the DDR play to enhance HIV replication.

Introduction

The DDR is a signaling cascade activated in response to exogenous and endogenous genotoxic stressors, such as DNA breaks. The DDR consists of sensor proteins that sense the damaged DNA, 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 (60, 61). However, a significant amount of crosstalk exists between these pathways. DDR signaling leads to various cellular responses, such as DNA repair, cell cycle arrest, transcriptional changes, apoptosis, or senescence (62–65).

Many diverse RNA and DNA viruses have evolved to modulate the DDR to enhance viral replication (66, 67). Primate lentiviruses, such as HIV-1, primarily engage the DDR through the accessory protein Vpr (68, 69). Vpr is evolutionarily conserved by extant primate lentiviruses and is both delivered by the incoming virion, allowing it to act early in viral replication, and expressed *de novo* from the integrated provirus, allowing it to act later in viral replication (70). Viruses lacking Vpr have no appreciable replication defects in T cells and most transformed cell lines, however Vpr is required for replication *in vivo* (71, 72) and viruses lacking Vpr have decreased proviral transcription in monocyte derived macrophages (MDMs) (73–75) and dendritic cells (76). Vpr also enhances chromatin accessibility and expression of unintegrated viral DNA (77, 78). This suggests an important role for Vpr in viral transcription. Moreover, studies of Vpr orthologs and mutants in isolation, whether overexpressed or delivered by virus-like particles (VLPs), identified that Vpr engages the host DDR at multiple, potentially unique, steps.

For example, ATR activation by Vpr leads to cell cycle arrest. This requires recruitment of the Cul4A-DDB1 cullin-RING E3 ubiquitin ligase complex, via the DCAF1 adaptor protein (CRL4A^{DCAF1}), which has a primary role in DNA repair (79). CRL4A^{DCAF1} complex recruitment by Vpr also leads to the degradation of many host proteins involved in the DDR (18-27) and is required for Vpr-mediated repression of DSB repair (80). In contrast, we have previously shown that the ability of Vpr to induce DNA damage does not correlate with ATR activation, cell cycle arrest, or repression of DSB repair (80), suggesting that Vpr-induced DNA damage may have unique roles in enhancing lentiviral replication.

Vpr, DNA damage, and proviral transcription are connected through NF- κ B in that both Vpr and DNA damage promote transcriptional changes involving NF- κ B (81, 82), which is an essential transcription factor for HIV-1 proviral transcription (83, 84). In response to DSBs, ATM signaling promotes NF- κ B transcriptional upregulation through the ATM-NEMO pathway (85). ATM and

NEMO interact in the nucleus before translocating to the cytoplasm (86, 87) to subsequently activate RelA nuclear translocation, promoter binding, and NF- κ B transcriptional activation. Previous literature suggests that HIV-1 Vpr activates and represses NF- κ B pathways through phosphorylation and ubiquitylation of TAK1 to enhance NF- κ B signaling (88) and altering the availability of the NF- κ B p50-RelA heterodimer to inhibit NF- κ B signaling (89), respectively. 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- κ B. Here, we aimed to identify the cellular consequences of Vpr-induced DNA damage and the connection between DNA damage and ATM activation, cell cycle arrest, and transcriptional changes.

To determine the consequences of Vpr-induced DNA damage on cellular transcription, we used previously described Vpr mutants that allow us to uncouple DNA damage from the large-scale cellular changes caused by cell cycle arrest. We found that virion-associated or *de novo* expressed Vpr does not require cell cycle arrest 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 still alter NF- κ B associated cellular transcription. In support of this, we showed that Vpr proteins that induce DNA damage activate RelA nuclear translocation and upregulate NF- κ B target genes, such as BIRC3 and CXCL8. We further assessed the requirement for ATM and NEMO signaling in Vpr-mediated NF- κ B activation in U2OS cells and primary human MDMs. We found that Vpr-induced DNA damage activates canonical ATM signaling, and that inhibition of NEMO resulted in loss of NF- κ B transcriptional upregulation. HIV-1 infection and virus-like particle (VLP) delivery of Vpr in MDMs validated the Vpr-dependent upregulation of NF- κ B target genes early during infection. Together, our data support a model where Vpr-induced DNA damage activates NF- κ B through the ATM-NEMO pathway, independent of cell cycle arrest and associated phenotypes. This study further informs how lentiviral accessory proteins engage the DDR at multiple and distinct steps, and

clarifies how this engagement remodels the host environment and immune pathways to promote viral replication.

Results

HIV-1 Vpr alters cellular transcription independent of 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 alters cellular transcription, and whether these transcriptional changes are distinct from those caused by Vpr-mediated cell cycle arrest. To do this, we leveraged a subset of previously established HIV-1 Vpr mutants that inhibit Vpr-mediated cell cycle arrest: Q65R, H71R, and S79A (all generated in the transmitted founder HIV-1 Q23-17 background) (80). These mutants have often been used interchangeably throughout the Vpr literature; however, they have not been fully characterized in the same system. We therefore first evaluated the ability of HIV-1 Q23-17 Vpr wild type (WT) and mutants to induce DNA damage, activate DDR signaling, cause cell cycle arrest, and interact with DCAF1 (through which Vpr binds the CRL4A^{DCAF1} E3 ubiquitin ligase complex (90)), as well as their subcellular localization. U2OS cells were infected with a recombinant adeno-associated virus (rAAV) expressing either HIV-1 Q23-17 Vpr WT or mutants (69, 80) (Fig. S1A), which expressed comparable levels of Vpr to infection with HIV-1 ΔEnv pseudotyped with VSV-G (HIV-1 ΔEnv) (S1B). We confirmed that all mutants lost the ability to cause cell cycle arrest (Fig. S1C), as has been previously described (80, 91–93). DNA damage was assessed by the comet assay while DDR activation was assessed by immunofluorescence 24 hours post infection. Consistent with our previous results (80), HIV-1 Vpr WT and S79A induce DNA breaks (Fig. 1A) and activate the DDR marker γH2A.x (Fig. 1B), while Q65R 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. The Q65R and H71R mutants are proposed to lose interaction with the DCAF1 component of the CRL4A^{DCAF1} E3 ubiquitin ligase complex as they fall within the DCAF1 binding domain (94, 95), and thus further lose the ability to cause cell cycle

arrest and degrade host proteins (80, 96–98). In our hands, HIV-1 Q23-17 Vpr WT and S79A recruit similar amounts of DCAF1, H71R recruits a decreased amount of DCAF1 when compared to Vpr WT and S79A, and Q65R recruits background levels of DCAF1 (Fig. S1D). Finally, we found that all Vpr mutants except for Vpr Q65R show similar subcellular localization as Vpr WT (Fig. S1E). Together, our data indicate that, when compared to Vpr WT, Vpr S79A can be used to assess the aspects of Vpr phenotypes that are specific to cell cycle arrest, Vpr H71R can be used to assess those aspects specific to cell cycle arrest and diminished DCAF1 recruitment, and Vpr Q65R can be used as a functionally dead control as it has lost all Vpr-associated phenotypes and is improperly localized (Table 1).

We next asked if Vpr mutants that induce DNA damage in the absence of cell cycle arrest (H71R and S79A) alter the cellular transcriptome. U2OS cells were infected with rAAV expressing HIV-1 Vpr WT, H71R, S79A, or Q65R, and RNA was collected at 12, 24, and 36 hours post infection for analysis by bulk RNA-sequencing (RNA-seq). Vpr WT, H71R, and S79A significantly altered cellular transcription 36 hours post infection when compared to empty vector or untreated cells, while Q65R was indistinguishable from empty vector or untreated cells (Fig. 1C-E, Fig. S2A-B, and Supplemental Files S1 & S2). The largest effect on cellular transcription was seen with Vpr WT (950 differentially expressed genes, $\text{Log}_2(2)$ fold change p-value < 0.004 and FDR < 0.01), while H71R and S79A showed an intermediate effect (361 differentially expressed genes, $\text{Log}_2(1.35)$ p-value < 0.0007 and FDR < 0.01 and 233 differentially expressed genes, $\text{Log}_2(1.5)$ p-value < 0.0008 and FDR < 0.006), respectively. All cutoffs failed to identify significant differentially expressed genes in cells expressing Vpr Q65R, empty vector or untreated cells. These data suggest that Vpr alters the cellular transcriptome via mechanisms that are dependent and independent of cell cycle arrest.

We focused on genes differentially expressed among Vpr WT, H71R, and S79A, as these genes are indicative of changes in cellular transcription that occur independent of cell cycle arrest and are potentially altered by Vpr-induced DNA damage. Out of the top 200 differentially expressed genes for each condition, 68 genes with $p\text{-value} < 0.01$ and $\text{FDR} < 4.50\text{E-}05$ were shared among Vpr WT, H71R, and S79A, while 100 additional genes were shared by two out of three conditions (Fig. 1D and 1E, Supplemental Files S1 & S2). Gene ontology (Fig. 1F) and Transcriptional Regulatory Relationships Unraveled by Sentence-based Text-mining (TRRUST) (99) (Fig. 1G) analyses of shared upregulated genes identified that Vpr WT, S79A, and H71R mutants were significantly enriched for positive regulation of RelA/NF- κ B signaling and NF- κ B signaling pathways (Fig. 1F-G, Fig. S2 A-B and Supplemental Files S1 & S2). TRRUST analysis further identified upregulation of transcription factors that bind and activate the HIV-1 LTR, such as CEBPB (100), SP1 (101), and JUN (102), as well as HDAC1, which directly modifies the HIV-1 LTR (103) (Fig. 1G). Moreover, Protein-protein Interaction Enrichment Analysis of the shared differentially expressed genes identified that Vpr downregulates expression of histones belonging to the H2A, H2B, H4, and H1 family that are involved in nucleosome assembly and DNA Damage/Telomere Stress Induced Senescence (Fig. S2C); consistent with previous literature that Vpr modulates components of the chromatin environment (104, 105). Collectively, our data show that Vpr alters cellular transcription in the absence of cell cycle arrest and suggest that Vpr-induced DNA damage robustly activates RelA/NF- κ B-mediated signaling.

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

Given NF- κ B signaling was the strongest upregulated pathway by Vpr WT, H71R, and S79A mutants, that NF- κ B signaling is activated by DNA damage, and the precedence for Vpr-mediated modulation of NF- κ B (65, 85, 86, 88), we next directly assayed whether Vpr activates RelA/NF- κ B in the absence of cell cycle arrest. We focused on H71R because it has lost the ability to cause cell cycle arrest (Fig. S1C), has diminished DCAF1 binding (Fig. S1D) and host protein

degradation (96–98), but maintains the ability to induce DNA damage and activate DDR signaling (Fig 1A-B) – thus allowing us to assess the effects of Vpr-induced DNA damage in the absence of cell cycle arrest and decreased protein degradation when compared to Vpr WT. NF- κ B activation was first validated by qRT-PCR for two NF- κ B target genes identified in our RNA-seq, BIRC3 and CXCL8 (Fig. 1E), which play important roles in innate immunity and cell survival (106, 107). 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 at 36hpi (Fig. 2A). To directly assess NF- κ B 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). To directly assess NF- κ B promoter activation, we tested the ability of Vpr to activate transcription from two NF- κ B reporter plasmids with unique NF- κ B binding motifs (108, 109). U2OS cells were co-transfected with a NF- κ B reporter plasmid and either a Vpr WT or mutant expressing plasmid. Consistent with the RNA-seq and immunofluorescence data, Vpr WT and H71R, but not Q65R, upregulate NF- κ B driven expression 48 hours post transfection (Fig. 2C-D). Together, these data demonstrate that Vpr activates RelA and NF- κ B transcription in the absence of cell cycle arrest, suggesting that Vpr-induced DNA damage is sufficient to drive NF- κ B activation.

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

NF- κ B is activated in response to many pathways and signals involved in DNA repair and innate immunity (110). One such pathway is ATM-NEMO, where ATM stimulates NEMO to activate RelA and thus NF- κ B signaling (87). Previous work has shown that Vpr activates markers of both ATR and ATM signaling (80, 111). While ATR is required for Vpr-induced cell cycle arrest, the extent to which Vpr activates ATM and the cellular consequences of ATM activation without cell cycle arrest

are unclear. We hypothesized that Vpr-induced DNA damage activates the ATM-NEMO pathway, which consequently promotes RelA/NF- κ B transcription.

To determine if Vpr-induced DNA damage activates ATM signaling in the absence of cell cycle arrest, we measured activation and colocalization of the DNA damage sensors NBS1 and MRE11 and the downstream signaling transducers, 53BP1 and γ H2A.x. U2OS cells stably expressing NBS1-GFP or 53BP1-GFP (112, 113) were infected with rAAV expressing Vpr WT, H71R, and Q65R and DDR activation was assessed through live-cell imaging over 56 hours. We found that Vpr WT and H71R promote the formation of NBS1 and 53BP1 foci similar to the positive control etoposide, while the non-functional Q65R Vpr mutant was indistinguishable from untreated control cells (Fig. 3A and Fig. S3A-C). Moreover, both damage sensors, MRE11 and NBS1, and transducers, 53BP1 and γ H2A.x, colocalize in Vpr WT and H71R expressing cells, similar to etoposide treated cells (Fig. 3B-C). This suggests that Vpr-induced DNA damage activates, but does not dysregulate, classical ATM signaling.

To determine if NEMO is required for Vpr to upregulate NF- κ B transcription, we asked whether Vpr could upregulate NF- κ B target genes BIRC3 and CXCL8 when NEMO was absent or inhibited. U2OS parental and NEMO knockout cells (114) were infected with rAAV expressing Vpr WT and mutants (Fig. S4A). In the absence of NEMO, Vpr WT and H71R, but not Q65R, still activated the DDR, indicating DNA damage occurs upstream of NEMO signaling and NEMO is not required for Vpr-induced DDR signaling (Fig. 4A and Fig. S4B). As expected, NEMO knockout inhibited TNF α and etoposide-induced TNF α expression, confirming loss of NEMO function (Fig. 4B). In the absence of NEMO, Vpr WT and H71R lost the ability to upregulate BIRC3 and CXCL8 (Fig 4C), suggesting that NEMO is required for Vpr-induced NF- κ B signaling. To further confirm this observation, we inhibited NEMO using a cell-permeable NEMO binding domain (NBD) inhibitor peptide (115). Similar to NEMO knockout cells, NBD peptide, but not a cell permeable

peptide negative control, inhibited $\text{TNF}\alpha$ expression following $\text{TNF}\alpha$ or etoposide treatment (Fig. 4D). To assess whether NBD peptide inhibited Vpr-induced NF- κ B activation, U2OS cells were pretreated with NBD peptide and infected with rAAV expressing Vpr WT and mutants. Consistent with NEMO knockout, the NBD peptide inhibited upregulation of BIRC3 and CXCL8 by Vpr WT and H71R (Fig. 4E), and this inhibition was indistinguishable from loss of NEMO (Fig. S4C). Together, this data suggests that Vpr requires NEMO activation to upregulate transcription of NF- κ B target genes.

Virion delivered HIV-1 Vpr activates ATM-NEMO NF- κ B signaling in primary MDMs

Together our data proposes a model where Vpr-induced DNA damage activates ATM and NEMO signaling, resulting in RelA nuclear translocation and NF- κ B transcriptional upregulation. We next tested this model in primary human MDMs (Fig. 5A), where Vpr enhances viral transcription and replication (116). Primary human MDMs (Fig. S5A) were infected with virus-like particles (VLPs) carrying physiological levels of Vpr WT, H71R, or Q65R (Fig. 5B), and assayed for DNA damage, NF- κ B activation, and NEMO dependence (Fig. 5A). Similar to expression of Vpr in U2OS tissue culture cell lines, VLP-delivered Vpr WT and H71R mutant, but not Q65R or empty VLPs, induced DNA breaks and activated $\gamma\text{H2A.x}$ in primary human MDMs (Fig. 5C-D). Consistent with our data in U2OS cells, VLP-delivered Vpr WT and H71R, but not Q65R or empty VLPs, upregulated BIRC3 and CXCL8 similar to the $\text{TNF}\alpha$ positive control in primary human MDMs (Fig. 5E and Fig. S5C). Finally, NBD peptide delivery prior to VLP infection blocked Vpr WT and H71R-mediated BIRC3 and CXCL8 transcriptional activation, but not Vpr-induced DNA damage in primary human MDMs (Fig. 5D-E and Fig. S5B-C). These data indicate that physiological levels of virion-delivered Vpr in primary human MDMs induce DNA damage that activates NF- κ B transcription in a NEMO dependent manner.

To determine if HIV-1 infection activates NF- κ B target genes, we infected primary human MDMs from four different donors with HIV-1 Δ Env 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 qRT-PCR for unspliced HIV-1 genomic transcripts (Fig. S6A-B), while NF- κ B activation was measured by qRT-PCR for CXCL8 and BIRC3. HIV-1 Δ Env infection resulted in upregulation of CXCL8 and BIRC3 in MDMs as early as 8 hours post infection (hpi) in all donors, despite some donor-to-donor variability in the magnitude of upregulation (Fig. 6A and Fig. S6C), suggesting that HIV-1 infection upregulates NF- κ B target genes early during infection, consistent with VLP delivery of Vpr.

To test whether Vpr was responsible for the upregulation of NF- κ B target genes early during HIV-1 Δ Env infection, we generated two additional viruses: HIV-1 Δ Vpr and HIV-1 Δ Vpr + Vpr packaged in trans (Fig. 6B). We infected primary human MDMs from three additional donors with either HIV-1 Δ Env, HIV-1 Δ Env Δ Vpr, HIV-1 Δ Env Δ Vpr + Vpr, or mock and assayed for infection and NF- κ B activation at 8 hpi. All three viruses infected cells similarly at 8hpi as shown by qRT-PCR for unspliced HIV-1 genomic transcripts (Fig. 6C) and by flow cytometry for HIV-1 core proteins at 48hpi (Fig. S6D). HIV-1 infection upregulated CXCL8 and BIRC3 gene expression, while HIV-1 Δ Vpr did not (Fig. 6C and Fig S6E-F). Moreover, upregulation of CXCL8 and BIRC3 were rescued by infection of HIV-1 Δ Vpr + Vpr (Fig. 6C). These data suggest that Vpr is necessary and sufficient for upregulation of NF- κ B target genes early during HIV-1 infection in primary human MDMs. Jointly, our data support a model where both incoming and *de novo* HIV-1 Vpr induce DNA damage that activates ATM-NEMO signaling to upregulate NF- κ B transcription (Fig. 7).

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 Vpr mutants that separate Vpr-induced DNA damage from cell cycle arrest, we identified a current model where Vpr-induced DNA damage activates ATM-NEMO signaling, which stimulates RelA and upregulates NF- κ B mediated transcription. Using U2OS and primary human MDMs, the latter of which allowed us to investigate how HIV-1 Vpr engages the DDR in this important cell type for the first time, we showed that Vpr induces DNA breaks and activates markers of ATM signaling independent of cell cycle arrest. Moreover, we showed that Vpr-induced DNA damage correlates with RelA/NF- κ B activation, as assayed by bulk RNA-seq, RelA immunofluorescence, and activation of NF- κ B reporter constructs. We validated the upregulation of two NF- κ B target genes, BIRC3 and CXCL8, and showed that inhibition of NEMO ablates the ability of Vpr to upregulate NF- κ B transcription. Finally, VLP delivery of Vpr and HIV-1 infection show that incoming virion-associated Vpr is sufficient to induce DNA damage and activate RelA/NF- κ B transcription. In complement, rAAV expression of Vpr show that *de novo* expressed Vpr can also induce DNA damage and activate RelA/NF- κ B transcription. These data suggest that Vpr-induced DNA damage can play roles in both early and late stages of viral replication to alter cellular transcription. Overall, our data support a model where Vpr-induced DNA damage activates ATM-NEMO signaling and upregulates RelA/NF- κ B 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 (80, 117). As shown here, Vpr-induced DNA damage directly correlates with NF- κ B transcriptional activation that is independent

of cell cycle arrest and dependent on ATM signaling. We have demonstrated that Vpr activates markers of ATM signaling, such as MRE11, NBS1, γ H2A.x, and 53PB1, in a manner that resembles host activation rather than dysregulation. We have also shown that DNA damage and DDR activation occur in primary human MDMs and with virion-delivered, physiologically relevant levels of Vpr protein. Antagonism or dysregulation of DNA damage sensors through relocalization or sequestration is a conserved mechanism among many viruses to evade innate immune detection (118). While we have shown that Vpr-induced DNA damage in MDMs activates markers of ATM signaling in a manner that seems to facilitate classical ATM activation, it remains to be seen whether Vpr-mediated activation of ATR, which correlates with CRL4A^{DCAF1} recruitment, replication stress, and cell cycle arrest in CD4⁺ T cells (119), is classically activated or dysregulated. These observations align with the growing literature suggesting Vpr phenotypes are cell-type dependent and potentially distinct in T cells, monocyte-derived dendritic cells (MDDCs), and MDMs (120). Moreover, how Vpr induces DNA breaks leading to both ATM and ATR activation in cycling and non-cycling cells is not understood.

Complementary studies have shown that Vpr modulates cellular (121) and viral transcription (77, 122, 123). In the context of cellular transcription, Vpr activates genes associated with innate immunity and proliferation in CD4⁺ T cells (124) and promotes the expression of proinflammatory cytokines in MDMs and MDDCs (76, 125). 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.

Our data also indicate that Vpr activates the RelA/NF- κ B immune pathway without induction of cell cycle arrest. This is consistent with previous reports showing that Vpr activates NF- κ B

transcription via phosphorylation of TAK1 (88), an upstream regulator of NF- κ B. We further found that Vpr-induced DNA damage upregulates RelA/NF- κ B transcription via ATM-NEMO signaling, adding to the mechanistic understanding of NF- κ B activation by Vpr. Through engagement with the CRL4A^{DCAF1} complex, Vpr has also been found to repress NF- κ B activation by altering the availability of the NF- κ B p50-p65 heterodimer, thus limiting proinflammatory cytokine expression (89). While it remains unclear what differentiates Vpr-mediated NF- κ B activation from repression, engagement of the CRL4A^{DCAF1} complex may be a distinguishing factor. Furthermore, it is clear that in all cases Vpr carefully modulates NF- κ B activation without globally activating interferon, which would inhibit viral replication. Thus, further studies understanding how Vpr manages to activate only aspects of NF- κ B signaling (126, 127) 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 has been associated with CRL4A^{DCAF1} dependent mechanisms such as cell cycle arrest, as the LTR is most transcriptionally active in G2/M (128, 129), the degradation of host proteins such as CCDC137 (130) and SLF2, the latter of which counteracts SMC5/6 silencing of unintegrated viral DNA (77), as well as CRL4A^{DCAF1} independent mechanisms (15). However, Vpr also promotes LTR transcription in noncycling cells (132, 133) where induction of G2/M 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- κ B signaling via ATM is the potential direct enhancement of LTR transcription, as the HIV-1 LTR contains multiple NF- κ B binding sites essential for viral gene expression. In addition to NF- κ B, we further identified that Vpr activates CEBPB and JUN transcription factors, which have known roles in LTR activation (134). This potential direct effect on the LTR is similar to Vpr-mediated de-repression of the LTR by removal of the transcriptional repressor ZBTB2 following ATR activation (135). Overall, in the absence of cell cycle arrest, Vpr-

induced DNA damage globally alters cellular transcription and upregulates NF- κ B signaling and transcription factors that promote LTR-driven transcription, suggesting that Vpr-induced DNA damage is also important for promoting LTR transcription to enhance viral replication.

Together, our data suggest that during HIV infection incoming Vpr and *de novo* expressed Vpr prime the cellular environment by activating RelA/NF- κ B signaling to promote transcription and enhance viral replication in macrophages. Our data align with the growing body of literature that supports the role of accessory proteins to modulate the host environment and the DDR to promote viral replication.

FIGURE LEGENDS

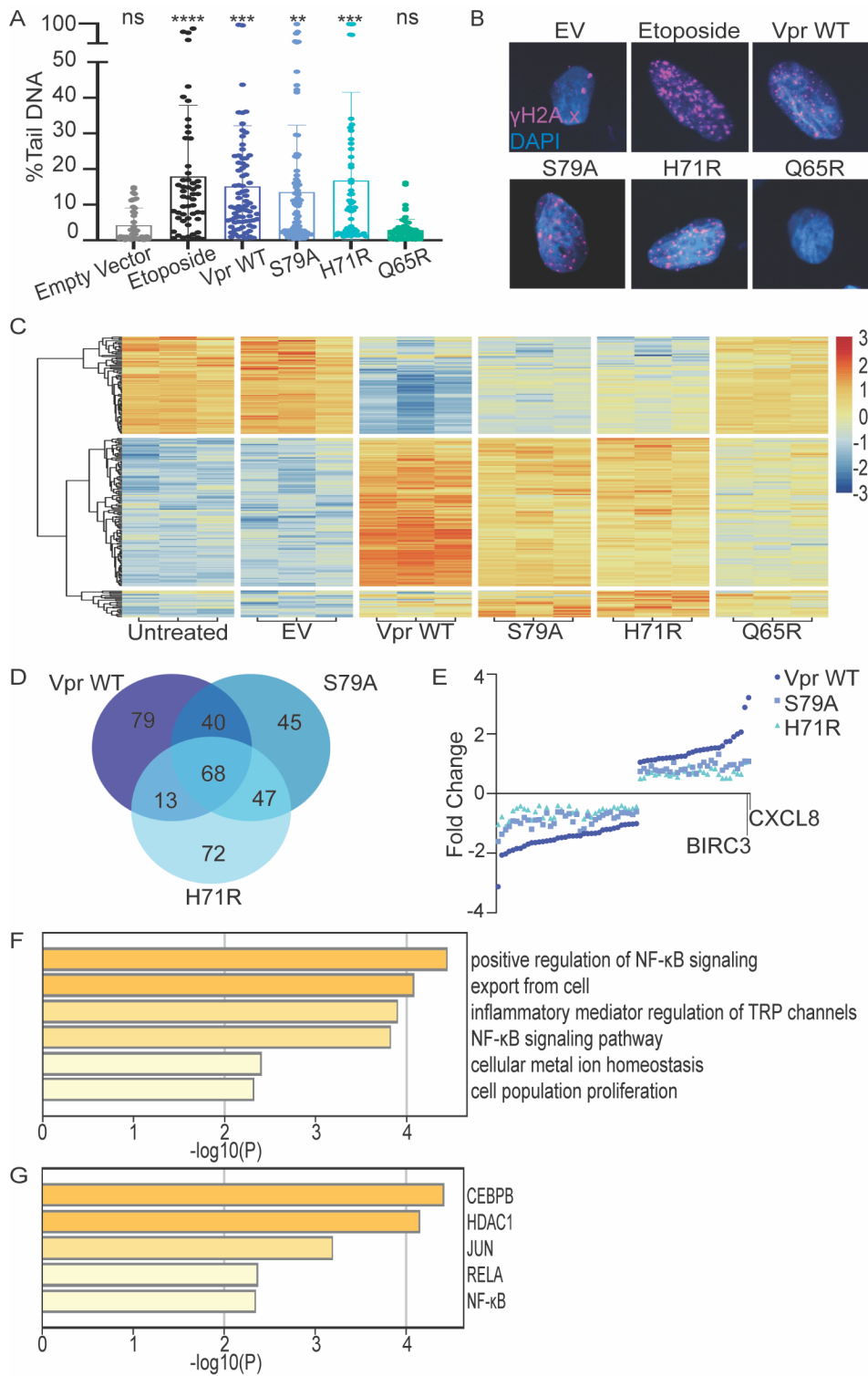


Figure 3: HIV-1 Vpr induces DNA damage and alters cellular transcription independent of cell cycle arrest.

(A) Comet assay of U2OS cells infected with rAAV expressing 3xFLAG-tagged Vpr WT, S79A, H71R, Q65R, empty vector (EV) (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; γ H2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 63x magnification. N=3, one representative experiment shown. (C) RNA-seq of U2OS cells at 36 hpi. Heat map displays Log_2 fold changes of upregulated genes in red and downregulated genes in blue. Each column represents a biological replicate. (D) Venn diagram of the top 100 upregulated and top 100 downregulated genes among Vpr WT, S79A, H71R, and Q65R. (E) 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 EV control, as determined by one-way ANOVA test (NS, nonsignificant; ** $P < 0.001$, *** $P < 0.0004$, **** $P < 0.0001$). Related to Figures S1 and S2.

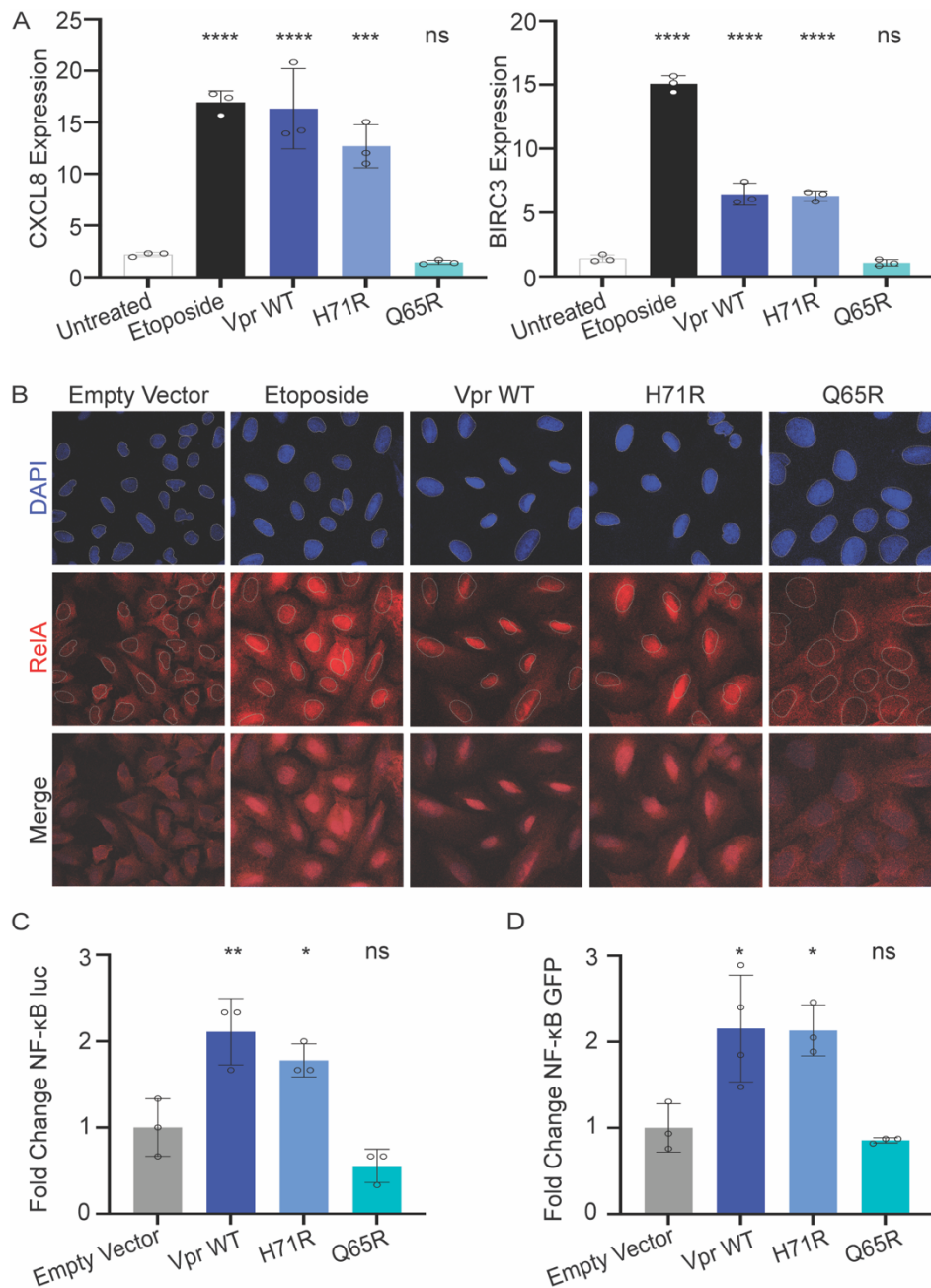


Fig 2: HIV-1 Vpr activates RelA and promotes NF-κB transcription independent of cell cycle arrest. (A) qRT-PCR validation of upregulated NF-κB target genes BIRC3 and CXCL8. Normalized expression (ΔΔCt) was calculated by normalizing to GAPDH followed by calculating fold change to untreated or empty vector treated cells. Cells treated under the same conditions as Fig. 1C. N=3, one representative experiment shown with technical triplicates. (B) Representative immunofluorescence images of U2OS cells infected under the same conditions

as Fig. 1A; RelA (red), nuclei stained with DAPI (blue), and nuclei outlined (grey dashed line). Images were taken at 63x magnification. (C) Representative NF- κ B firefly luciferase reporter assay at 48 hours post transfection. Relative firefly luciferase was normalized to renilla. (D) Representative NF- κ B green fluorescence protein (GFP) reporter assay at 48 hours post transfection. N=3, one representative experiment shown. Asterisk indicate statistical significance compared to negative control, as determined by one-way ANOVA test (NS, nonsignificant; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0003$, **** $P < 0.0001$).

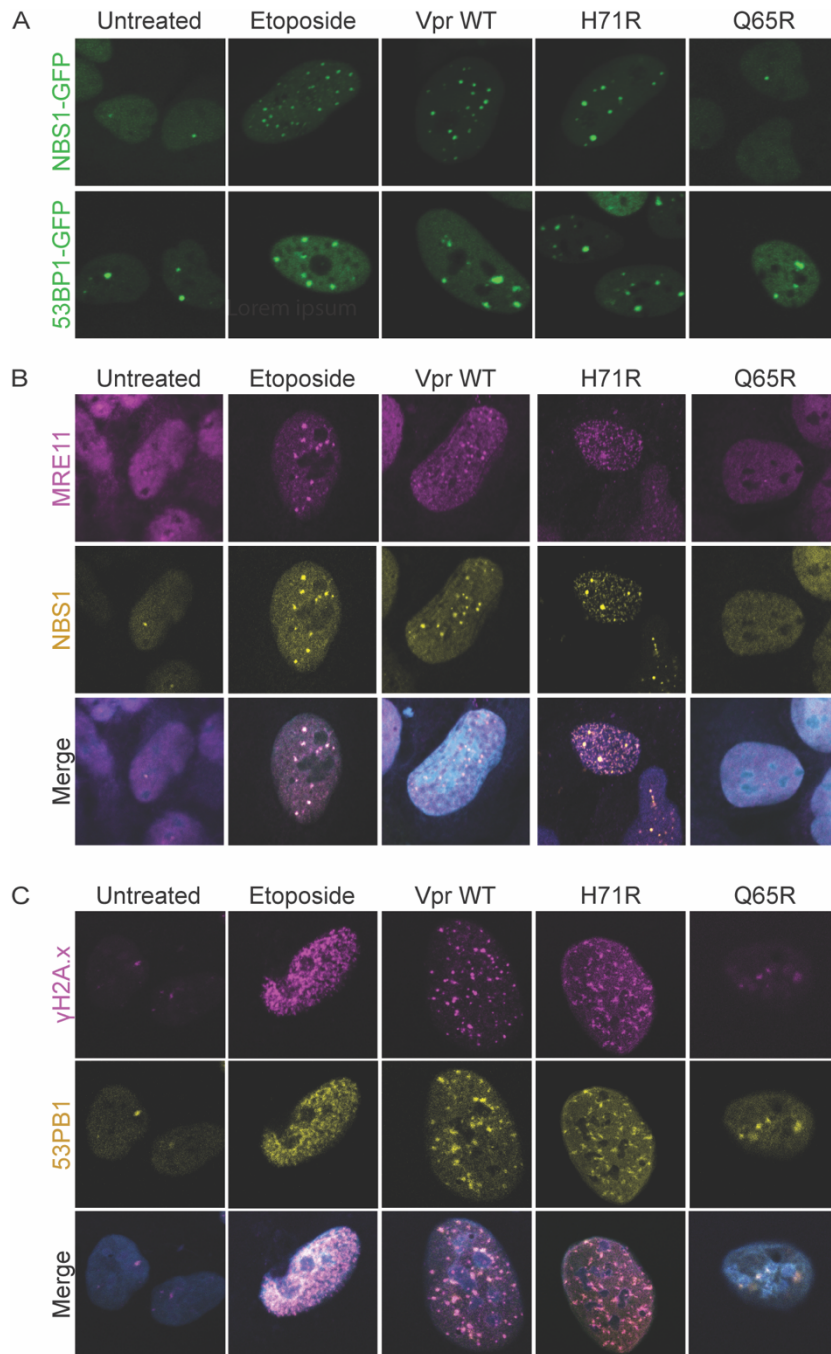


Fig 3: Vpr-induced DNA damage activates ATM signaling independent of cell cycle arrest.

(A) Live-cell imaging of U2OS cells stably expressing NBS1-GFP or 53BP1-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 γ H2A.x (magenta) and 53PB1 (yellow) in U2OS 53PB1-GFP cells infected under the same conditions as Fig. 1A. Images taken at 24 hpi at 63x magnification. N=3, one representative experiment shown. Related to Figure S3.

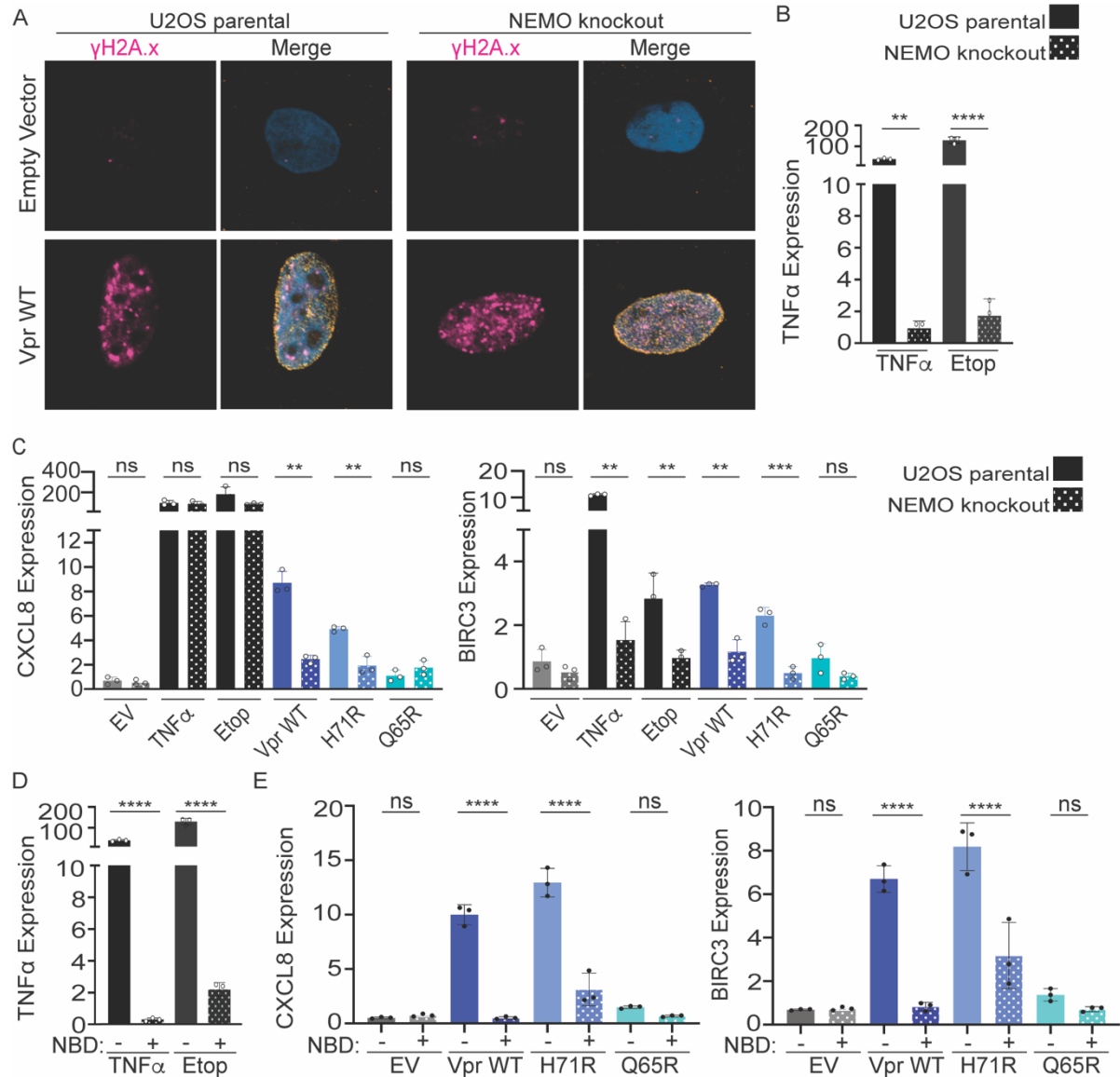


Fig 4: Vpr upregulates NF- κ B transcription dependent on NEMO, yet independent of cell cycle arrest. (A) Representative images of U2OS parental or NEMO knockout cells infected with EV or Vpr WT displaying γ H2A.x (magenta), Vpr FLAG (yellow), and DAPI (blue). Images taken at 24hpi at 63x magnification. N=3, one representative experiment shown. (B) qRT-PCR of TNF α in U2OS parental or U2OS NEMO knockout cells treated with TNF α or Etoposide (positive

controls). RNA was isolated at 36 hpi. Asterisk indicate statistical significance compared to U2OS parental cells. (C) qRT-PCR of BIRC3 and CXCL8 in U2OS parental or NEMO knockout cells. Cells were treated under the same conditions as Fig. 1C. N=3, one representative experiment shown with technical triplicates. Asterisk indicate statistical significance compared to U2OS parental cells. (D) qRT-PCR of $\text{TNF}\alpha$ in the presence of Nemo-Binding inhibitor peptide (NBD, +) or the NBD negative control peptide (-) at 36 hpi. U2OS cells were pretreated with 50 μM of NBD peptide or NBD negative control peptide for 2 hours prior treatment with $\text{TNF}\alpha$ or etoposide. Asterisk indicate statistical significance compared to U2OS parental cells. (E) qRT-PCR of BIRC3 and CXCL8 in the presence of NBD inhibitor peptide (+) or the NBD negative control peptide (-) at 36 hpi. U2OS cells were pretreated as in Fig. 4D and infected with rAAV expressing 3xFLAG-tagged Vpr WT, H71R, Q65R, EV (negative control). Normalized expression to GAPDH. N=3, one representative experiment shown with technical triplicates. Asterisk indicate statistical significance compared to NBD negative control, as determined by one-way ANOVA test (NS, nonsignificant; ** $P < 0.001$, *** $P < 0.0003$, **** $P < 0.0001$). Related to Figure S4.

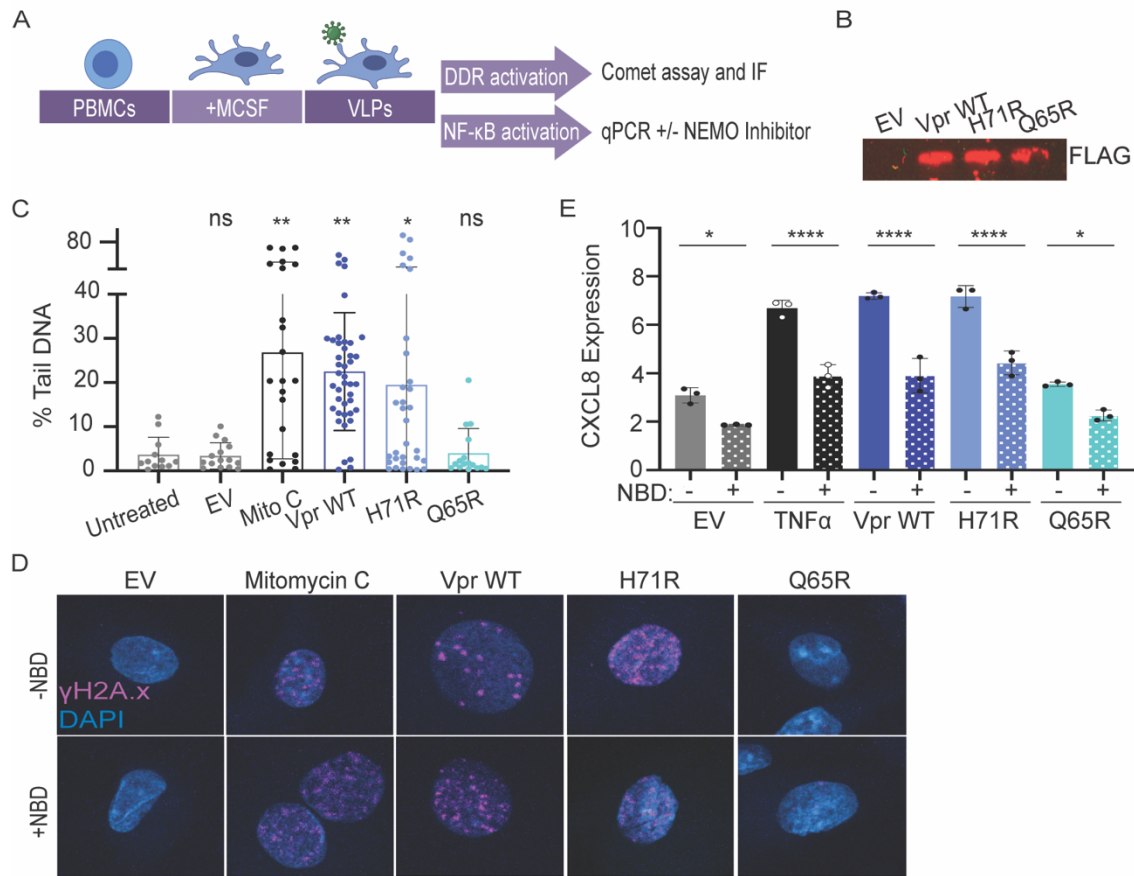


Fig 5: Vpr-induced DNA damage activates ATM-NEMO signaling and NF-κB transcription in primary human MDMs early during infection. (A) Experimental schematic depicting the isolation and differentiation of primary human monocyte-derived macrophages (MDMs) from peripheral blood mononuclear cells (PBMCs) followed by delivery of Vpr via Virus-like Particles (VLPs). 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-Binding inhibitor peptide (NBD) or the NBD negative control peptide. (B) Western blot of 3X FLAG-Vpr WT and mutants packaged in VLPs. (C) Comet assay of MDMs treated with VLPs packaging 3xFLAG-tagged Vpr WT, H71R, Q65R, empty VLPs (negative control), or 25uM Mitomycin C (Mito C, positive control) for 8 hours. Comet assay analysis was performed as in Fig. 1A. N=2, one representative experiment shown. (D) Representative immunofluorescence images of MDMs infected with VLPs as in Fig. 5B and treated with the NBD inhibitor peptide (+) or the NBD negative control peptide

(-). γ H2A.x (magenta) and nuclei stained with DAPI (blue). Images were taken at 100x magnification. N=3, one representative experiment shown. (E) qRT-PCR for CXCL8 of MDMs treated with NBD peptide as in Fig. 5C with TNF α positive control. N=3, one representative experiment shown with technical triplicates. Normalized expression to GAPDH. Asterisk indicate statistical significance compared to NBD negative control, as determined by one-way ANOVA test (NS, nonsignificant; * P<0.03, ** P < 0.005, **** P< 0.0001). Related to Figure S5.

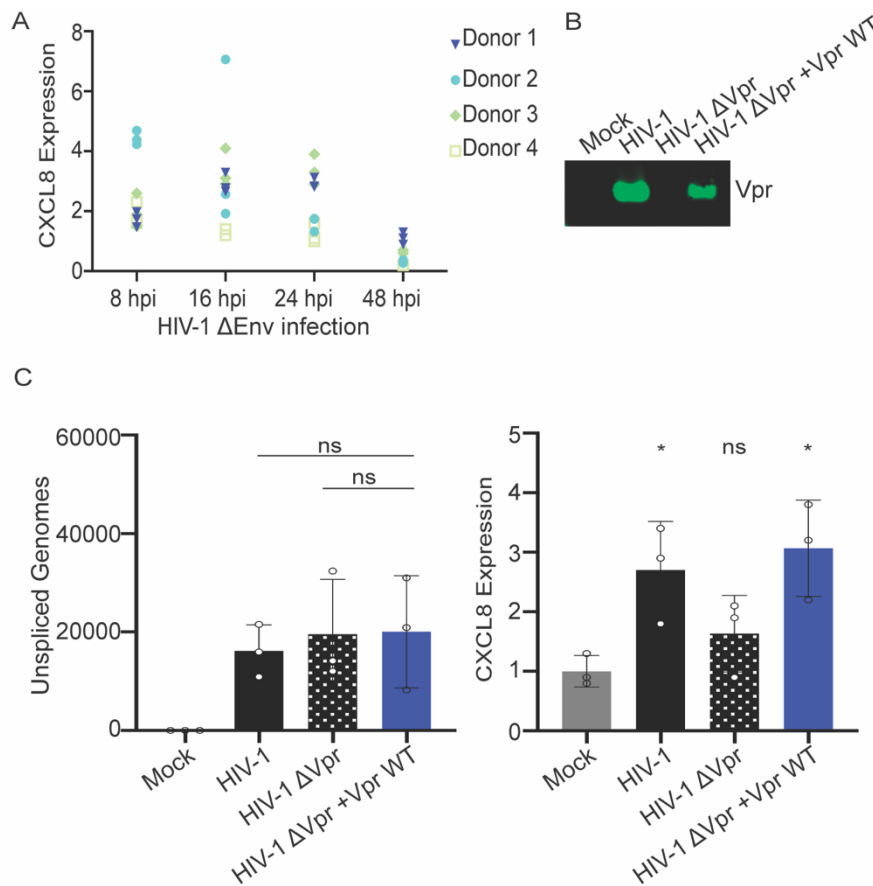


Fig 6: Vpr is necessary and sufficient for activation of NF- κ B target genes by HIV-1 in primary human MDMs. (A) qRT-PCR for CXCL8 of MDMs infected with HIV-1 Δ Env for 8, 16, 24, and 48 hpi from four separate donors. Normalized expression to GAPDH. (B) Western blot of Vpr packaged in mock, HIV-1, HIV-1 Δ Vpr, HIV-1 Δ Vpr + Vpr WT viruses. (C) qRT-PCR for unspliced HIV-1 genomic transcripts and CXCL8 from MDMs infected with 5,000 U/mL RT activity of HIV-1, HIV-1 Δ Vpr, HIV-1 Δ Vpr + Vpr WT viruses or mock (negative control). The mean (N=3)

of three separate donors is shown. Normalized expression to GAPDH. Asterisk indicate statistical significance compared to mock negative control, as determined by one-way ANOVA (NS, nonsignificant; * $P < 0.005$). Related to Figure S6.

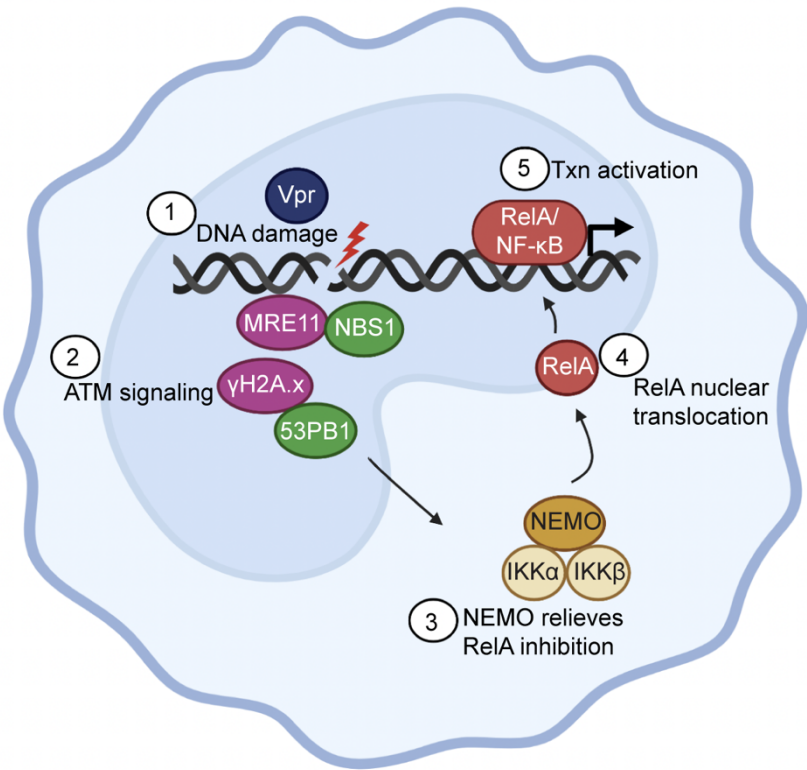


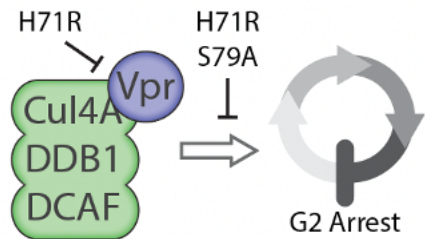
Fig 7: Model showing Vpr-induced DNA damage activates ATM and NEMO signaling resulting in RelA nuclear translocation and NF-κB transcriptional upregulation. 1. Vpr induces double-strand DNA breaks independent of cell cycle arrest. 2. Vpr-induced DNA damage activates markers of ATM signaling including 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).

Table 1: HIV-1 Vpr WT and mutant phenotypes. (+) indicates a phenotype conferred by Vpr, (-) indicates a phenotype not conferred by Vpr, and (-/+) indicates an intermediate Vpr phenotype.

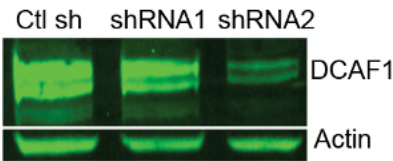
	Localization as WT	Cell Cycle Arrest	Engages DCAF1	DNA Damage	NF- κ B activation
HIV-1 Vpr WT	+	+	+	+	+
S79A	+	-	+	+	+
H71R	+	-	- / +	+	+
Q65R	-	-	-	-	-

Figure Supplement 1

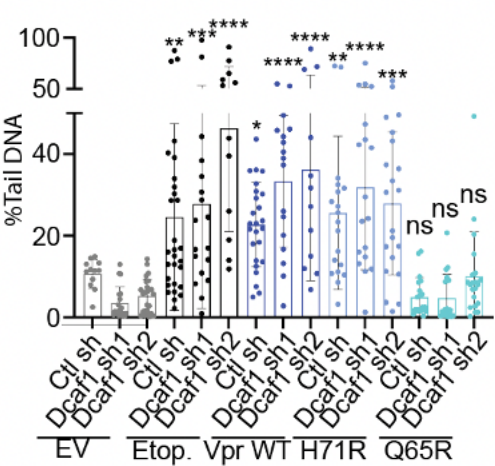
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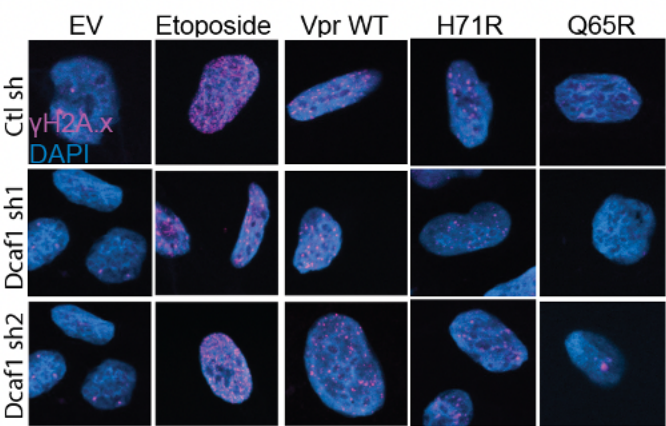
B



C



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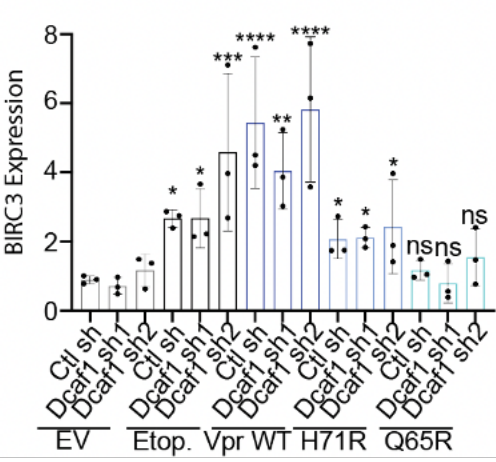
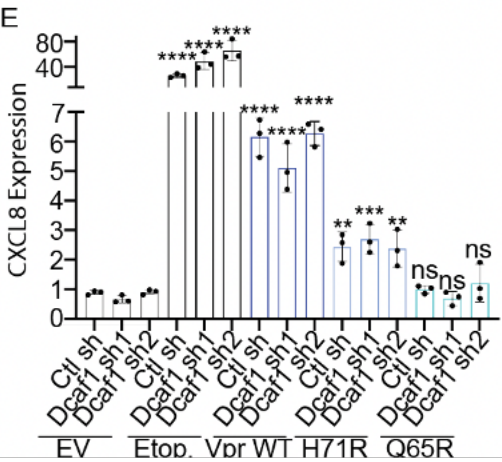


Figure Supplement 1. Knockdown of DCAF1 does not abrogate the ability of Vpr to induce DNA damage, activate DDR signaling, or upregulate NF- κ B target genes.

(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.; γ H2A.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

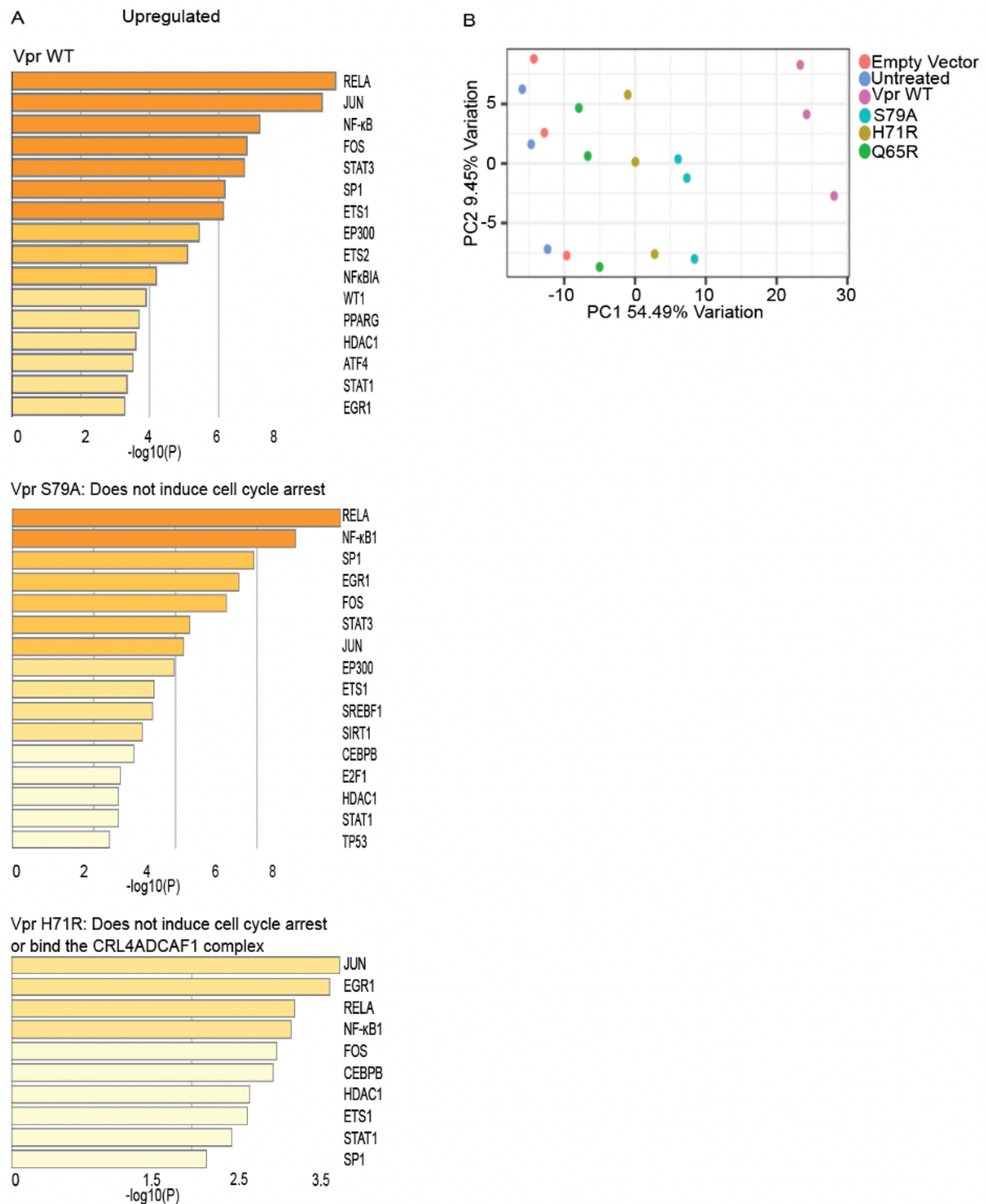


Figure Supplement 2. Vpr alters cellular transcription through diverse mechanisms that are either dependent or independent of cell cycle arrest and CRL4^{DCAF1} engagement.

(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

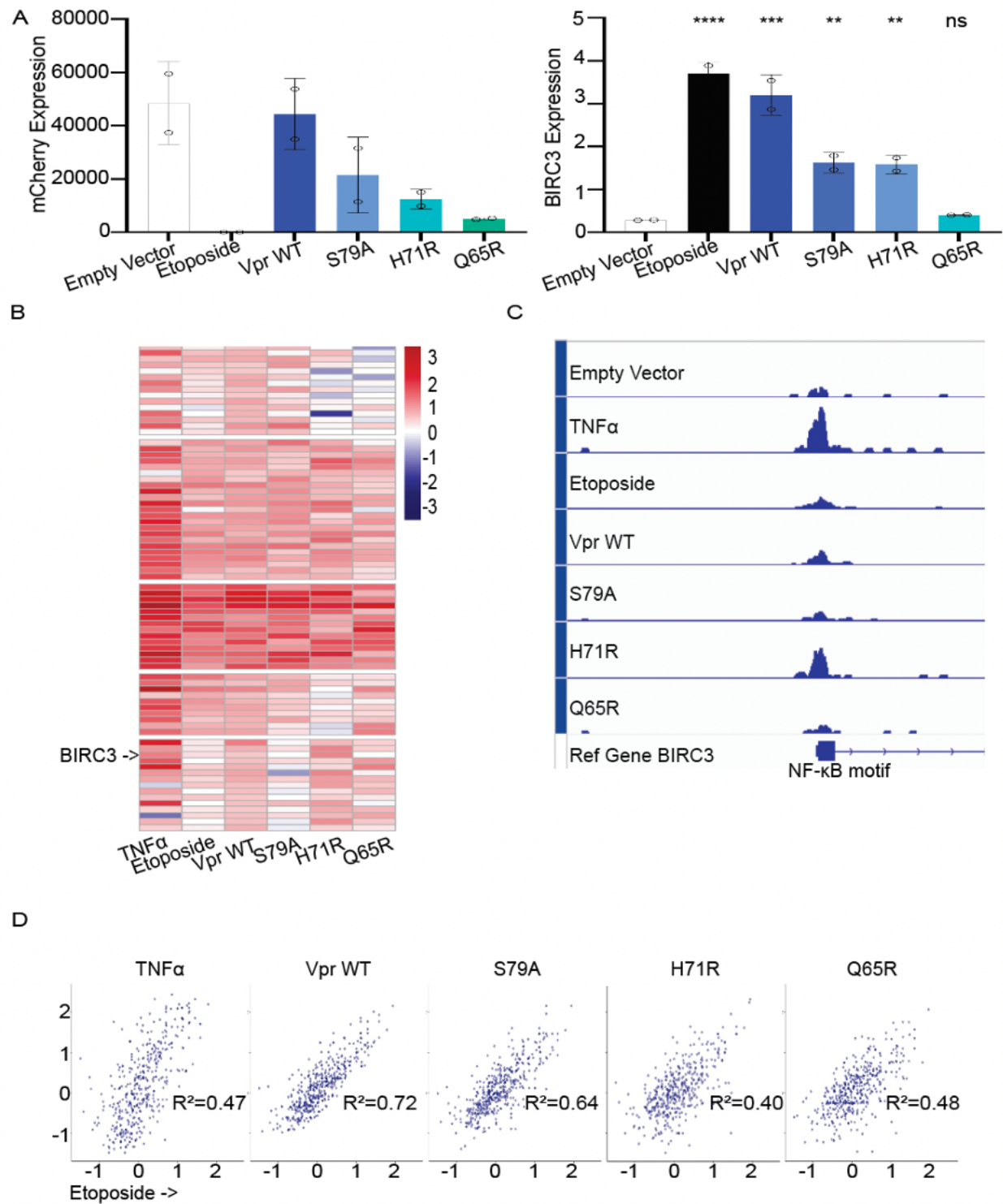


Figure Supplement 3. Vpr WT globally activates RelA to bind NF- κ B promoters.

(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 (1.4×10^8 copies/well) expressing HIV-1 Vpr WT, Vpr mutants, empty vector negative control, 50 μ M etoposide (Sigma) for 36 hours, or stimulated with TNF α (Thermo) for 16 hours. Normalized expression to GAPDH. (B) Heatmap displays Log₂ fold changes depicting RelA enrichment at NF- κ B motifs in red and depletion in blue. Each row represents a single NF- κ B motif. The BIRC3 promoter contains an NF- κ B motif and is found in the 5th cluster. (C) Genome Browser Tracks for the BIRC3 promoter. Reads mapped to the NF- κ B motif in blue. (D) Sample correlations to etoposide. R² displays fittest regression line.

Figure Supplement 4

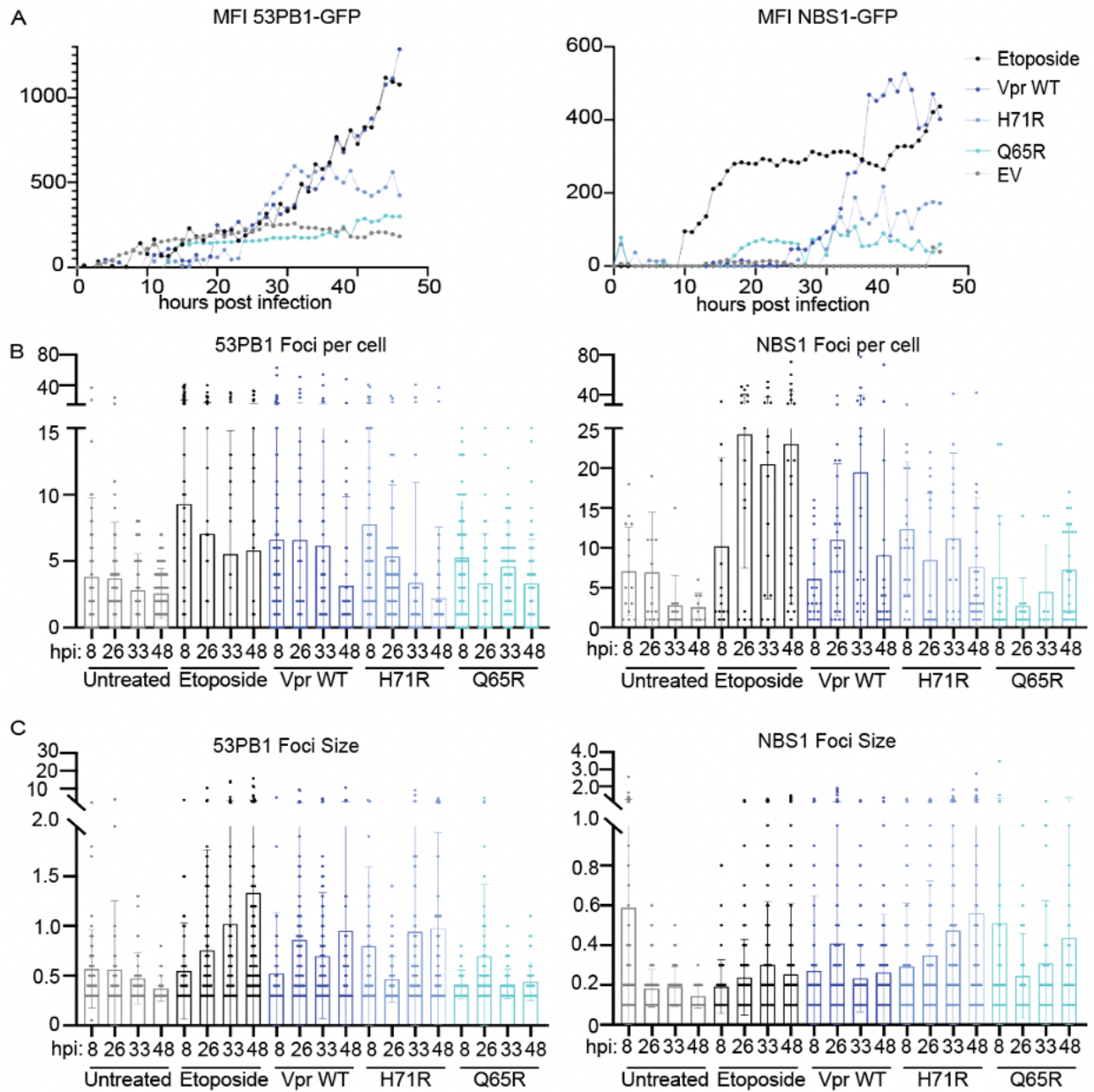


Figure Supplement 4. Vpr-induced DNA damage activates markers of ATM signaling.

(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

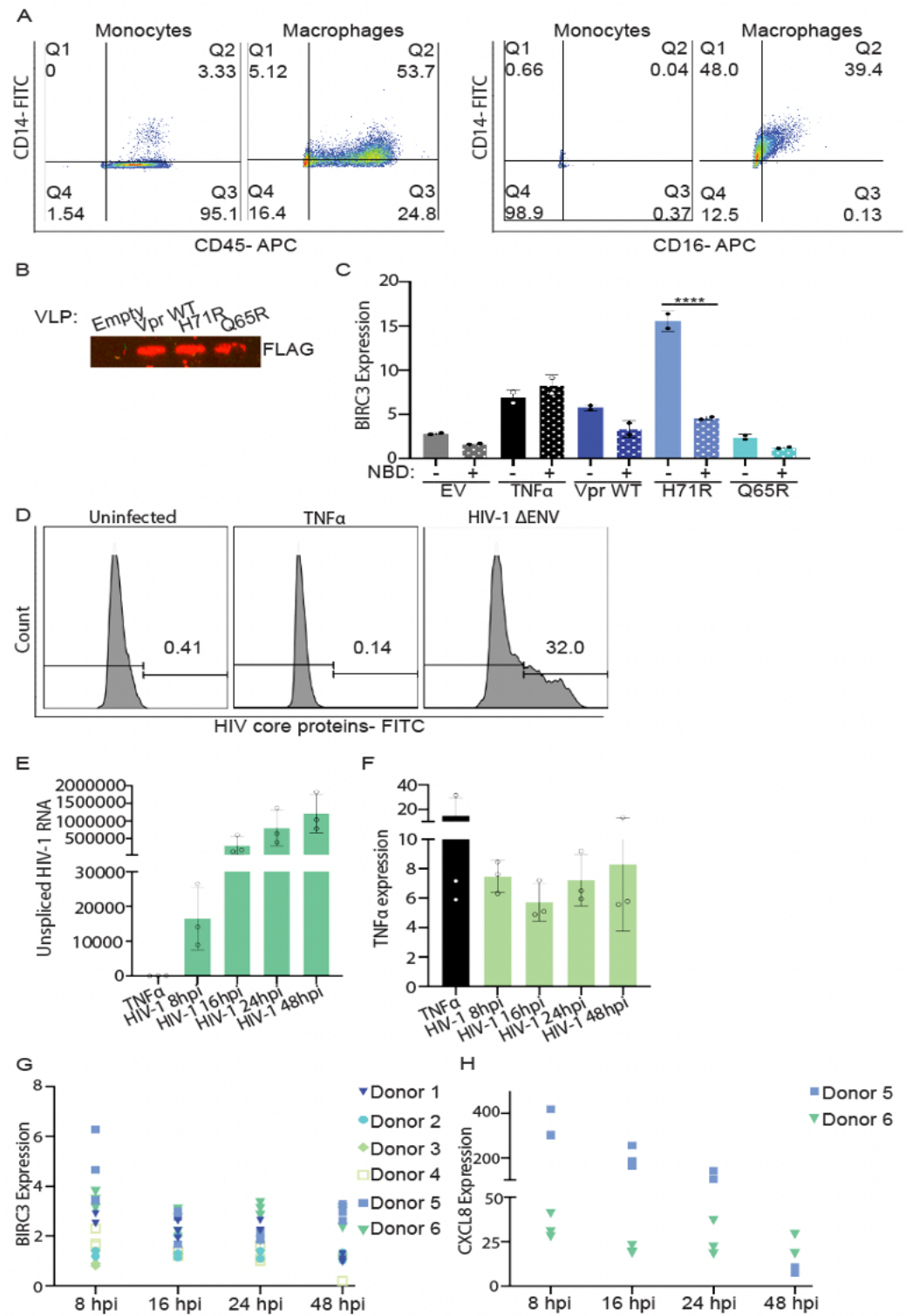


Figure Supplement 5. Infection of primary MDMs with VLPs packaging Vpr protein or HIV-1 infection upregulate NF- κ B transcription dependent on NEMO.

(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 TNF α as positive control. n=3, one representative experiment shown. (D) Histogram quantifying percent of infected MDMs with HIV-1 Δ ENV 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 TNF α of MDMs infected with HIV-1 Δ ENV 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 Δ ENV 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

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 53BP1-GFP (113) and NBS1-GFP (112) were kindly provided by Claudia Lukas (University of Copenhagen, Denmark). The U2OS parental and NEMO knockout cells were kindly provided by Zhijian J. Chen (University of Texas Southwestern Medical Center). U2OS cells were pretreated with 50 μ M of Nemo-Binding inhibitor (NBD) peptide or NBD negative control peptide (Thermo) for 2 hours prior to infection with rAAV or VLPs.

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™ Monocyte Isolation Kit (STEM CELL) and were differentiated into monocyte-derived macrophages (MDMs) by stimulation with 20 ng/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% CO₂ for 7 days while replenishing media every 3 days.

Plasmids

pcDNA-3xFLAG-Vpr and pscAAV-mCherry-T2A-Vpr WT and mutant plasmids were generated as previously described (80). For rAAV production, pHelper and pAAV-2.5 capsid plasmids were used (Addgene) (80). For VLP production, psPAX2 and pmD2.G were used (Addgene). For HIV-1 Δ Env pseudotyped with VSV-G production, HIV-1 Bru-GFP Δ Env was used (136) and pseudotyped with pmD2.G VSV-G (Addgene). For NF- κ B reporter assays, the NF- κ B RedFirefly luciferase (Addgene, plasmid #124530) and pSIRV-NF κ B-eGFP (Addgene, plasmid #118093) plasmids were used.

Generation of Viruses

rAAV virus packaging the pscAAV-mCherry-T2A-Vpr WT or mutant vectors were generated by transient transfection of HEK 293 cells using polyethyleneimine (PEI) as previously described (137). Virus-like particles (VLPs) packaging Vpr WT or mutant proteins were generated by transient transfection of HEK 293T cells using TransIT-LT1 (Mirus). VLPs were harvested 48 hrs 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 Δ Env

pseudotyped with VSV-G were generated by transient transfection of HEK 293T cells using TransIT-LT1 (Mirus). For HIV-1 Δ Env Δ Vpr with Vpr WT packaged in trans (HIV-1 Δ Vpr +Vpr WT), 40 μ g of pcDNA-3xFLAG-Vpr WT was also co-transfected in producer 293T cells. Virus was collected 48 hrs post media change and filtered through a 0.45 μ m PES filter. Viral titer was identified by measuring activity of reverse-transcription through qPCR (138).

HIV-1 Δ Env Infection

MDMs were plated in 96-well tissue culture plates (10,000 cells per well) (Greiner) and allowed to adhere overnight. MDMs were infected and centrifuged ("spininfected") with 5,000 U/mL RT activity of HIV-1 Δ Env pseudotyped with VSV-G (HIV-1), HIV-1 Δ Env Δ Vpr pseudotyped with VSV-G (HIV-1 Δ Vpr) , HIV-1 Δ Env pseudotyped with VSV-G packaging Vpr WT (HIV-1 Δ Vpr +Vpr WT) or mock at 1200 $\times$ g for 90 min at 37°C. Infection was assessed 48 hours after infection via qRT-PCR and Flow Cytometry.

Alkaline Comet Assay

The alkaline comet assay and data analysis was performed as previously described (80), with minor changes. MDMs were infected with VLPs delivering Vpr WT and mutants at equal protein levels or 25 μ M 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 (rpkm) were calculated for samples compared to untreated cells. Heat map was generated using pheatmap hierarchical clustering on z-score $\log_2$ rpkm. Gene ontology, Protein-protein Interaction Enrichment Analysis, and TRRUST analysis were done on Metascape.

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was isolated using the PureLink™ RNA Mini Kit (Invitrogen). RNA was reverse transcribed using SuperScript IV First-Strand Synthesis System (Invitrogen) with Oligo(dT) primers. qRT-PCR 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 AACCTCTGCACCCAGTTTTC, TNF α CTCTTCTGCCTGCTGCACTTTG and ATGGGCTACAGGCTTGTCCTC, HIV-1 unspliced RNA Genome GCGACGAAGACCTCCTCAG and GAGGTGGGTTGCTTTGATAGAGA, GAPDH CAAGATCATCAGCAATGCCT and AGGGATGATGTTCTGGAGAG, and Vpr AGGCCATGGCTTCATGGATTA and GATCTACCGGGTCCATTCCTG. mRNA levels were quantified by calculating $\Delta\Delta C_t$. Target transcript C_t values were normalized to the C_t 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. 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 NaN₃ 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 and Co-immunoprecipitations

Protein was collected from cells as previously described (80). For co-immunoprecipitations, cells were washed with cold phosphate-buffered saline (PBS) and lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, Benzodase, protease inhibitor) and clarified by centrifugation 15,500 × g for 15 min. Immunoprecipitations were performed overnight at 4°C with Dynabeads Protein G beads (Invitrogen) conjugated to mouse anti-FLAG M2 (Sigma-Aldrich). For western blotting, 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 12% Bis-Tris polyacrylamide gels and subsequently transferred onto a polyvinylidene difluoride membrane. Membranes were blocked in intercept blocking buffer (Li-COR Biosciences). Immunoblotting was performed using rabbit anti-Vpr (Proteintech), mouse anti-FLAG M2 (Sigma-Aldrich), rabbit anti-Actin (Bethyl), mouse alpha-Tubulin (Sigma), or rabbit anti-DCAF1 (Proteintech) for 1 hr or overnight at 4°C. 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).

NF-κB reporter assays

For luciferase-based readout, U2OS cells were plated in a 24-well plate, allowed to adhere overnight, then co-transfected (Mirus) with the NF-κB Firefly luciferase plasmid and pcDNA-3xFLAG-Vpr WT, mutant, or empty vector control plasmids. Protein was collected in the passive lysis buffer (Promega) 48 hours post transfection. Relative firefly luciferase and renilla luminescence were measured using manufacturer's instruction of the Dual-Luciferase Reporter Assay System (Promega) on the Synergy BioTek plate reader. For GFP-based readout, U2OS cells were co-transfected (Mirus) with pSIRV-NF-κB-eGFP and pscAAV-mCherry-T2A-Vpr WT and mutant plasmids. 48 hours post transfection, 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 GFP conjugated to 647 (Biolegend) and mCherry conjugated to 488 (R&D) for 1 hr 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. Double population of GFP and mCherry positive events were identified and quantified using FlowJo software.

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 with the HIV-1 core antigen-FITC KC57 antibody (Beckman Coulter), which recognizes HIV-1 p24 as well as the precursor p55 and intermediate p39 and p41 products, for 1 hr 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.

Data access

The bulk RNA-sequencing data in this publication was deposited in NCBI's Gene Expression Omnibus and can be accessed through GEO series accession number GSE253779 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE253779>).

AUTHOR CONTRIBUTIONS

Conceptualization C.S. and O.I.F.; methodology C.S. and K.N.; writing, reviewing, and editing C.S., K.N. 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.

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COMPETING INTERESTS

The author(s) declare(s) they have no competing interests.

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Chapter 3: HIV Vpr activates a nucleolar-specific ATR pathway to degrade the nucleolar stress sensor CCDC137

HIV Vpr activates a nucleolar-specific ATR pathway to degrade the nucleolar stress sensor CCDC137

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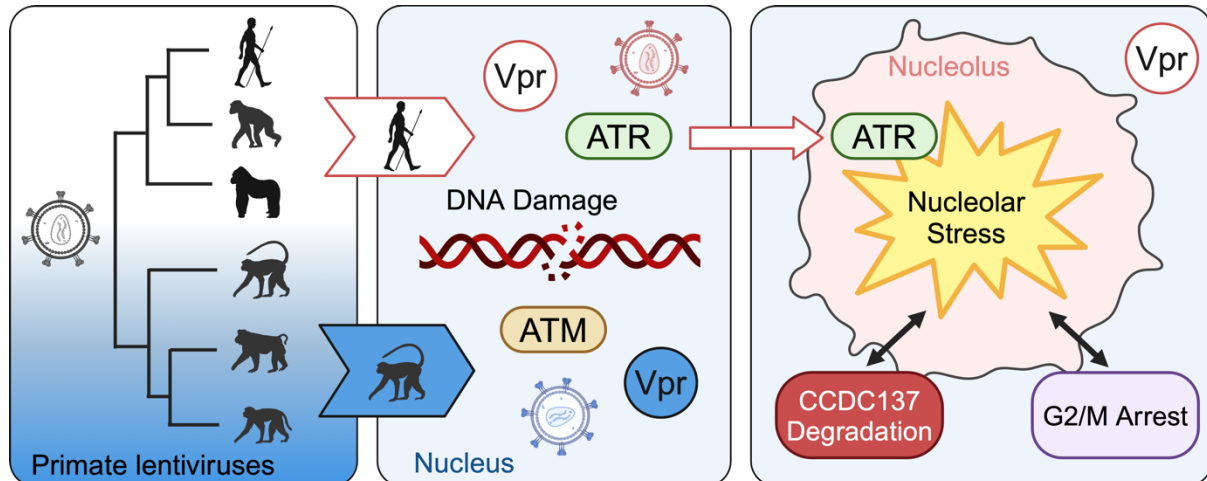
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GRAPHICAL ABSTRACT



ABSTRACT

The lentiviral accessory protein Vpr engages an extensive network of cellular pathways to drive diverse host consequences. Of its many phenotypes, CRL4A-E3 ubiquitin ligase complex co-option, DNA damage response (DDR) engagement, and G2/M arrest are conserved and thus proposed to be functionally important. How Vpr effects these functions and whether they explain

how Vpr dysregulates additional cellular pathways remain unclear. Here we leverage the ability of Vpr to deplete the nucleolar protein CCDC137 to understand how Vpr-induced DDR activation impacts nucleolar processes. We characterize CCDC137 as an indirect Vpr target whose degradation does not correlate with Vpr-induced G2/M arrest. Yet, degradation is conserved among Vpr from the pandemic HIV-1 and related SIVcpz/SIVgor, and it is triggered by genomic insults that activate a nucleolar ATR pathway in a manner similar to camptothecin. We determine that Vpr causes ATR-dependent features of nucleolar stress that correlate with CCDC137 degradation, including redistribution of nucleolar proteins, altered nucleolar morphology, and repressed ribosome biogenesis. Together, this data distinguishes CCDC137 as a non-canonical Vpr target that may serve as a sensor of nucleolar disruption, and in doing so, identifies a novel role for Vpr in nucleolar stress.

INTRODUCTION

Lentiviral accessory proteins inhibit cellular factors and pathways that interfere with viral replication to counteract host innate immunity or augment pro-viral signaling (1). Of the four HIV-1 accessory proteins – Vpr, Vif, Vpu and Nef – Vpr is the only factor that has not yet been associated with a primary target(s) or function(s). Despite this, Vpr is evolutionarily conserved among all extant primate lentiviruses (2, 3) and essential for *in vivo* infection by HIV and SIV (4–7).

Although lentiviral Vpr has not yet been assigned a primary phenotype, the many targets and functions ascribed to it have cemented Vpr as a highly versatile protein with extensive cellular consequences. Its diverse roles include binding the host factor DCAF1 to co-opt the CRL4A^{DCAF1} E3 ubiquitin ligase complex and subsequently tag host proteins for degradation, arresting the cell cycle, promoting apoptosis, augmenting viral transcription, and engaging the DNA damage response (DDR) (8). Central to these diverse functions is the host DDR, which Vpr engages through multiple, distinct mechanisms. Of the three primary DDR signaling cascades – DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), and Ataxia telangiectasia and Rad3 (ATR) kinases – Vpr activates both ATM and ATR (9–13), triggering downstream ATM-mediated NF- κ B activation (14) and ATR-dependent G2/M cell cycle arrest (11), respectively. Yet, despite requiring ATR activation (11, 15), Vpr-induced cell cycle arrest cannot be explained by damage

alone, as HIV-1 mutants lacking the ability to cause G2/M arrest are able to activate DDR signaling (13). In addition to activating these kinases, Vpr further engages the host DDR by causing DNA damage, inhibiting DNA repair, and binding and degrading many proteins involved in the DDR (reviewed in (16)).

CCDC137 is a recently identified Vpr target whose degradation has been connected to Vpr-induced DNA damage, G2/M arrest, and increased viral transcription (17). The precise cellular pathways that connect CCDC137 to these phenotypes, however, are unclear. Although CCDC137 functions are still emerging, current literature depicts CCDC137 as a multi-faceted nucleolar protein associated with diverse functions and processes. Multiple studies implicate CCDC137 overexpression in cancer progression and poor clinical outcomes (18–22), suggesting it plays an important role in regulating cellular homeostasis. In hepatocellular carcinoma (HCC), for instance, RNA binding (20) and E3 ubiquitin ligase co-option (19) by CCDC137 alter phosphoinositide 3-kinase (PI3K)/Akt signaling to promote cancer progression. In addition to its roles in tumorigenesis, CCDC137 modulates nuclear receptor signaling by sequestering the estrogen related (ERR) and retinoic acid (RAR) receptors in the nucleolus (23, 24). The adenoviral E1A protein binds CCDC137 to disrupt its association with RAR, thus releasing RAR from the nucleolus to engage in downstream signaling (23). Together these findings characterize CCDC137 as a versatile, though enigmatic, protein with ties to cellular proliferation. None, however, fully explain how Vpr-mediated CCDC137 degradation might contribute to Vpr-associated phenotypes, nor do they describe how CCDC137 might operate in the nucleolar compartment it inhabits.

The nucleolus is a dynamic subnuclear structure that serves as the site for the initial stages of ribosome biogenesis. Its organization is intrinsically linked to this process, encompassing three different layers representing successive steps of ribosome biogenesis, including RNA Pol I-driven ribosomal RNA (rRNA) transcription, rRNA processing, and ribosome assembly (25). Because of the cell's reliance on this process, the nucleolus is an essential central hub for coordinating the stress response to a variety of cellular insults (26). Nucleolar perturbation by insults such as DNA damage, nucleotide depletion, oncogenic abnormalities, or viral infections (26, 27) are sufficient

to rapidly trigger nucleolar stress that can lead to cell cycle arrest or apoptosis (28). In addition, more recent findings demonstrate a role for the nucleolus in orchestrating cellular stress pathways that go beyond a response related to ribosome biogenesis, including DDR regulation and maintenance of genome stability (29). In line with this, a tremendous amount of crosstalk exists between the nucleolus and DDR, as many DNA repair proteins localize to the nucleolus and, conversely, many nucleolar proteins function in the DDR (29). Both ATM and ATR have been implicated in the nucleolar response to DDR activation depending on the cellular insult (30).

ATM and ATR are activated in the nucleolus by insults that occur both *in trans*, such as those in nuclear chromatin, as well as those that develop *in cis*, in ribosomal DNA (rDNA) (31–34). Although the mechanisms that lead to nucleolar DDR (nDDR) activation overlap with those that trigger the global DDR, there are several distinguishing factors that render nucleolar ATR and ATM pathways unique. In response to nuclear double-stranded breaks (DSB), for instance, the MRE11-RAD50-NBS1 complex recruits and activates ATM (35, 36); in nucleoli, NBS1 and subsequent ATM recruitment require the nucleolar protein Treacle (33). Nucleolar ATR activation in response to rDNA damage also involves Treacle but is unique in its dependence on the ATR activator TOPBP1 (33, 34). Upon global ATR activation, TOPBP1 and other ATR activating factors are recruited to scaffolds of single-stranded DNA (ssDNA) coated with the ssDNA binding complex RPA. In nucleoli, TOPBP1-Treacle foci act as these scaffolds, and recruit stress response factors to rDNA lesions.

Although Vpr activates and dysregulates many of the pathways implicated in nucleolar stress, a role for Vpr in nucleolar processes has yet to be defined. Here we leverage the connections between CCDC137, the nucleolus, and the DDR to test the hypothesis that Vpr modulates nucleolar pathways. We find that CCDC137 is an indirect Vpr target whose degradation is associated with, but not a direct cause or consequence of, Vpr-induced G2/M arrest. Additionally, we characterize CCDC137 as an evolutionarily important Vpr target that bridges multiple Vpr phenotypes, including a newly identified Vpr function, nucleolar stress. We further define CCDC137 as a specific component of nucleolar stress sensing that is degraded when Vpr-induced DNA damage triggers the activation of a nucleolar-specific ATR pathway. Together, these results

characterize the evolutionary importance of CCDC137 depletion to Vpr function, define DDR activation as required for this depletion, and identify a role for Vpr in nucleolar processes.

MATERIAL AND METHODS

Plasmids and generation of viruses

pcDNA3.1-3XFLAG-Vpr, pcDNA3.1-3XFLAG-Vpx, pscAAV-GFP-T2A-Vpr, pscAAV-mCherry-T2A-Vpr, and LPCX-HA-SAMHD1 plasmids were described previously (2, 37). The pBABE-HA-ER-IPpol plasmid was a gift from Michael Kastan (Addgene #32565) (38). Vpr and Vpx sequences represent the following isolates: HIV-1 Bru (H1B.FR.83.HXB2_LAI_IIIB_BRU; GenBank K03455.1), HIV-1 Q23-17 (H1A.Q2317; GenBank AF004885.1), HIV-1 ETH220 (H1C.ET.86.ETH2220; GenBank U46016.1), HIV-1 SE6165 (H1G.SE.93.SE6165 GenBank: AF061642.1), HIV-1 SE9280 (H1J.SE.93.SE9280; GenBank AF082394.1), HIV-1 P Fr (P RBF168; GenBank GU111555.1), HIV-2 Rod9 (H2A.Rod9; GenBank M15390.1), HIV-2 7312a (H2B.7312a; GenBank L36874.1), HIV-2 A.PT.x.ALI (H2A.PT.x.ALI; GenBank AF082339.1); HIV-2 AB.JP.04 (H2_01_AB.JP.04.NMC307_20; GenBank AB731738.1), HIV-2 B.GH.86 (H2B.GH.86.D205; GenBank X61240.1), HIV-2 ABT96 (H2G.CI.92.ABT96; GenBank AF208027.1), SIVcpz MB897 (CPZ.CM.x.SIVcpzMB897; GenBank EF535994.1), SIVcpz EK505 (CPZ.CM.05.SIVcpzEK505; GenBank DQ373065.1), SIVcpz CAM13 (CPZ.CM.01.SIVcpzCAM13; GenBank AY169968.1), SIVgor CP684 (GOR.CM.04.SIVgorCP684com.; GenBank FJ424871), SIVsmm SL9 (SMM.SL.92.SL92B; GenBank AF334679.1), SIVsmm CFU212 (SMM.US.86.CFU212; GenBank JX860407.1), and SIVmac 239 (MAC.US.x.239; GenBank M33262). In addition, the following consensus Vpr sequences were used: HIV-1 N con (39), HIV-1 O con (39), and HIV-1 P con (MEQAPEDQGPPREPFNEWMLQTLEELKAEAVRHFPMPWLHSLGQYIYNTYGDTWEGVTAIRILQQLIFIH YRIGCQHRSRIGILTLRRGGRHGPRSRS).

LPCX-HA-CCDC137 plasmids were generated by synthesizing CCDC137 as gBlocks (IDT) that were subcloned into LPCX-SAMHD1-HA (2) using standard cloning techniques. CCDC137 protein sequences are available at Figshare: <https://doi.org/10.6084/m9.figshare.26841640> and <https://doi.org/10.6084/m9.figshare.26841634>.

pscAAV-mCherry-T2A Vpr and pscAAV-GFP-T2A Vpr plasmids were packaged into rAAV vectors through transient transfections of Human embryonic kidney (HEK) 293 cells using polyethyleneimine (PEI) as previously described (40). Inverted terminal repeat (ITR)-specific quantitative PCR (qPCR) was used to quantify levels of DNase-resistant vector genomes as previously described (41).

Cell lines and cell culture

HEK 293T and human bone osteosarcoma epithelial (U2OS) cells were cultured as adherent cells in Dulbecco's modified Eagle's medium (DMEM) growth medium (high glucose, L-glutamine, no sodium pyruvate) with 10% fetal bovine serum (Peak Serum) 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), and rAAV infections were performed by adding rAAV directly to the growth medium. For inhibitor assays, cells were incubated with 5nM Actinomycin D (Gibco) for 5 hours (hr), 5µM or 10µM MLN4924 (Cell Signalling) for 8hr, 10µM MG132 (Sigma) for 5hr, or 10µM ATMi (KU-55933) (Cayman Chemicals) or ATRi (VE-821) (Cayman Chemicals) for 24hr. For genotoxic agent assays, cells were incubated with titrating amounts of camptothecin (Cayman Chemicals), cisplatin (Tokyo Chemical Industry), etoposide (Cayman Chemicals), hydroxyurea (Sigma) or Olaparib (MedChemExpress) for 24hr.

For CCDC137 knockdown experiments, gRNAs targeting unique sites within CCDC137 (gRNA 1: GCGACTAGATAAAGTCCGA; gRNA 2: TCAGCAAGAACCAGGCCATC) or a non-targeting gRNA control (CAGCTCATCGGTGTCCTACT) were subcloned into lentiCRISPRv2 puro (pLCV2), a gift from Brett Stringer (Addgene plasmid # 98290; <http://n2t.net/addgene:98290>; RRID:Addgene_98290) (42), using NEBridge Golden Gate Assembly (New England Biolabs). Lentivirus was produced as previously described (39). Cells stably expressing each gRNA were selected for via 2µg/mL puromycin (Gibco).

Flow Cytometry

Approximately 6.0×10^6 cells per condition were harvested at 24 hours post infection (hpi) and stained with Hoescht 33342 Ready Flow™ Reagent (Invitrogen) according to the manufacturer's instructions for cell cycle analysis. Event counts were measured on the AttuneNXT (Thermo Fisher Scientific), and data were analyzed using FlowJo software. G1 and G2 values were obtained using the Dean-Jett-Fox Univariate model (43, 44), and G2/G1 ratios were calculated from these percentages.

Western blots and co-immunoprecipitations

Cells were lysed for western blot in radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl [pH 8.0], 150mM NaCl, 1mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, Universal Nuclease (Pierce), and EDTA-free protease inhibitor (Pierce) and clarified by centrifugation at $15,000 \times g$ for 15 min. Immunoprecipitations were performed as previously described (39) using anti-HA affinity gel (Millipore Sigma) in NP-40 lysis buffer (200mM NaCl, 50mM Tris [pH 7.4], 0.5% NP-40, 1mM DTT, Universal nuclease, protease inhibitor). All samples were boiled in 4X sample buffer (40% glycerol, 240mM Tris, pH 6.8, 8% SDS, 0.5% β -mercaptoethanol, and bromophenol blue), run on 4 to 12% Bis-Tris polyacrylamide gels, and subsequently transferred onto polyvinylidene difluoride membranes (Sigma). Membranes were blocked in intercept blocking buffer (Li-COR Biosciences) and probed with the indicated primary antibodies: mouse anti-FLAG M2 1:3000 (Sigma), mouse anti-HA 1:10000 (Invitrogen), rabbit anti-CC137 1:2000 (Atlas and Abcam), rabbit anti-VprBP 1:2000 (Proteintech), mouse anti- β -tubulin 1:5000 (Cell Signaling), rabbit anti- γ H2AX 1:5000 (Cell Signaling), rabbit anti-p53 1:5000 (Proteintech), rabbit anti-UTP11 1:3000 (Antibodies Online), rabbit anti-NOP14 1:1000 (Proteintech), rabbit anti-RRP36 1:1000 (Proteintech), mouse anti-TOPBP1 1:500 (Santa Cruz Biotechnology), and rabbit anti-Ape1 1:1000 (Cell Signaling). Membranes were incubated with secondary antibodies IRDye 800CW anti-Rabbit and IRDye 680RD anti-mouse 1:20000 (Li-COR Biosciences) and visualized using the Li-COR Odyssey M (Li-COR Biosciences).

Quantification of CCDC137 protein levels. Degradation experiments were performed as biological triplicates ($n=3$), and protein levels were quantified using ImageStudio (Li-COR Biosciences). Each condition was normalized to a corresponding β -tubulin loading control. CCDC137 degradation

values for a given Vpr are reported as the percentage of CCDC137 protein remaining relative to an empty vector control, averaged across three separate experiments. From these percentages, each degradation phenotype is then classified as robust (0-35% CCDC137 remaining), intermediate (36-80% CCDC137 remaining) or absent (81-100% CCDC137 remaining); these descriptions are used throughout the text to represent calculated values that fall within these ranges. Degradation heatmaps were generated based on the averaged percentages from three biological replicates ($n=3$).

Quantitative Reverse Transcription PCR (qRT-PCR)

Cells were harvested at 24hpi and RNA was isolated using the PureLink™ RNA Mini Kit (Invitrogen). cDNA was generated using the SuperScript IV First-Strand Synthesis System (Invitrogen) with Oligo(dT) primers. qPCR reactions were prepared using PowerTrack SYBR Green Master Mix (Thermo Fisher Scientific) and the following primers (5' to 3'): CCDC137 TATGAGGAGCCGCCAAGAGATG and CCTCTCCCTTTGCTTCCTTCTC, and GAPDH CAAGATCATCAGCAATGCCT and AGGGATGATGTTCTGGAGAG (IDT). All reactions were carried out on the LightCycler 480 System (Roche). To quantify mRNA levels, $\Delta\Delta C_t$ values were calculated for each sample, and CCDC137 values were normalized to $\Delta\Delta C_t$ values for the housekeeping gene GAPDH. Statistical data were based the averaged normalized $\Delta\Delta C_t$ values from triplicate reactions performed for three different biological replicates.

Immunofluorescence

Cells were plated onto glass slides in 24-well plates (Greiner Bio-One) at 1.5×10^5 cells per well and allowed to expand overnight. For localization and nucleolar size assays, cells were harvested at 24hr post-treatment. Cells were permeabilized (0.5% Triton-X 100 in PBS) for 5min on ice prior to fixation (4% paraformaldehyde in PBS) for 20min at 20°C and then incubated overnight in PBS at 4°C. Cells infected with the rAAV HIV-1 Q65R mutant were fixed prior to permeabilization. Coverslips were then washed three times in PBS before blocking (30% BSA, 0.1% Tween 20 in PBS) for 30min at 20°C. Cells were incubated with the following primary antibodies diluted in blocking buffer overnight at 4°C: rabbit anti-CC137 1:200 (Abcam), mouse anti-Nucleolin 1:100 (Cell Signaling), mouse anti-UBTF 1:100 (Santa Cruz Biotechnology), mouse anti-TOPBP1 1:200 (Santa

Cruz Biotechnology), mouse anti-RPA32 1:5000 (GeneTex), rabbit anti-NPM1 1:500 (Atlas), rabbit anti-Nucleolin 1:1000 (Cell Signaling) and mouse anti-FLAG M2 1:5000 (Sigma). Coverslips were then washed three times in 0.1% Tween 20 in PBS for 5min each at 20°C and incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies) for 1hr at 20°C. Nuclei were stained with Hoescht 33342 1:2000 (Invitrogen) diluted in PBS. Coverslips were mounted onto glass slides using antifade mounting media (Invitrogen).

For 5-ethynyl uridine (EU) experiments, cells were harvested at 28 hpi or 24hr post drug treatment following a 1hr incubation with 0.8mM EU (Invitrogen). Coverslips were prepared according to manufacturer's instructions for the Click-iT™ RNA Alexa Fluor™ 488 Imaging Kit (Invitrogen).

Representative images for all IF experiments were acquired on the LSM 980 using Airyscan. For quantification of Nucleophosmin 1 (NPM1) and Nucleolin (NCL) nucleoplasmic translocation, Fiji was used to quantify nucleolar or total nuclear signal measured as mean fluorescence intensity (MFI). Nucleoplasmic signal was calculated by subtracting nucleolar MFI from total nuclear MFI. Statistical analyses were based on at least 50 cells per condition. For 5-EU and nucleolar size assays, images for quantification were obtained using the Zeiss Axio Imager Z1. Fiji was used to quantify nucleolar area and EU signal, measured as MFI of total nuclei as previously described (45). Statistical analyses were based on approximately 100-200 cells per condition. For nucleolar DDR assays, Fiji was used to quantify total vs. nucleolar number of TOPBP1 or RPA32 foci with at least 25 cells per condition. Nucleolar foci were defined by the overlap of these markers with the stable nucleolar marker NCL.

Phylogenetics and positive selection (PS) analyses

Phylogenetics, positive selection analysis, and detection of other potential genetic innovations (such as recombination, insertions/deletions and duplications) were performed using the Detections of Genetic Innovations (DGINN) pipeline, as previously described (46). Briefly, homologous codon sequences to human CCDC137 in primates were automatically retrieved using NCBI blastn implemented in DGINN. Sequences were aligned using Prank (47) and recombination events were detected using Genetic Algorithm for Recombination Detection (GARD) (48). Amino

acid alignment at the primate level was also generated using Muscle (49). Positive selection marks were detected in a simian codon Prank alignment of CCDC137 orthologs from 23 species using the following tests of selection in DGINN: HYPHY BUSTED and MEME, PAML codeml (M0, M1, M2, M7, M8, M8a), and Bio++ (M0, M1^{NS}, M2^{NS}, M7^{NS}, M8^{NS}, M8a^{NS})(50–53). Sites under positive selection were identified using HYPHY MEME (P value < 0.05), from the codeml M2 and M8 model BEB statistics (BEB > 0.95), and Bio++ Bayesian posterior probabilities (PP) from the M2^{NS} and M8^{NS} models (PP > 0.95). In addition, Fast, Unconstrained Bayesian AppRoximation for Inferring Selection (FUBAR) web-based method was also used to detect site-specific positive selection (PP > 0.90) (54). Alignments are openly available at FigShare: <https://doi.org/10.6084/m9.figshare.26841640> and <https://doi.org/10.6084/m9.figshare.26841634>.

Protein structure prediction with AlphaFold

The AlphaFold predicted structure of the human CCDC137 protein (accession Q6PK04) was obtained from the AlphaFold database (<https://alphafold.ebi.ac.uk/entry/Q6PK04>) as no experimentally determined structures were available. ChimeraX was used to annotate the default CCDC137 predicted structure.

Proteomic Analyses

For gene set over-representation analysis (GSOA), genes from the Greenwood *et al.* dataset (55) or the Johnson *et al.* dataset (56) were selected if they possessed a log2 fold change of at least 0.5 in either direction. GSOA was performed using the enricher function of clusterProfiler package (version 3.12.0) in R with default parameters. Significant gene ontology (GO) terms (adjusted p value < 0.05) were identified and further refined to select non-redundant terms. To select non-redundant gene sets, a GO term tree was first constructed based on distances (1-Jaccard Similarity Coefficients of shared genes in MSigDB) between the significant terms. The GO term tree was cut at $h = 0.2$ to identify clusters of non-redundant gene sets. For results with multiple significant terms belonging to the same cluster, the most significant term was selected (i.e., lowest adjusted p value) with the largest number of genes (i.e., to select the broadest categories). For the gene set enrichment analysis (GSEA), non-thresholded log2 fold changes from the Johnson *et al.* dataset

were used as inputs. GO terms were not further refined. GO terms were obtained from the c5 category of Molecular Signature Database (Human MSigDB v2024.1.Hs) (57). Cytoscape (58) was used to visualize CCDC137 interactions determined using STRING (59).

RESULTS

CCDC137 degradation is neither a direct cause nor consequence of G2/M arrest.

To investigate the connection between CCDC137 degradation and Vpr-induced G2/M arrest, we first tested whether previously established (14) HIV-1 Vpr mutants that lack the ability to cause cell cycle arrest could still degrade CCDC137. These mutants are made in the backbone of the primary HIV-1 isolate Q23-17 (WT) and crucially separate conserved Vpr phenotypes. Consequently, they allow us to correlate CCDC137 degradation with individual Vpr functions, including G2/M arrest (H71R, S79A, R80A) and decreased DCAF1 binding (H71R) (60–62). As a negative Vpr control, we also assayed the degradation capacity of the functionally dead Q65R mutant, which does not cause arrest, bind DCAF1, or properly localize within the nucleus (14). To test this, we delivered either HIV-1 WT Vpr or mutants to U2OS cells via a recombinant adeno-associated virus (rAAV) vector system (13) and assayed Vpr-induced cell cycle arrest via flow cytometry (**Figure 1A and S1A**) and endogenous CCDC137 levels via quantitative western blot (**Figure 1B-C**).

In the presence of HIV-1 WT Vpr, we observed a correlation between G2/M arrest and CCDC137 degradation as previously reported (17, 63). HIV-1 WT Vpr degraded endogenous CCDC137 (50%) and resulted in potent cell cycle arrest ($G2/G1 = 1.36$) when compared to an empty vector control ($G2/G1 = 0.46$) (**Figure 1A-C**). The functionally dead HIV-1 Q65R Vpr mutant failed to either cause arrest ($G2/G1 = 0.42$) or degrade CCDC137 (96%), as expected. When we introduced other HIV-1 Vpr mutants, however, we saw that all significantly degraded CCDC137 despite their inability to arrest cells in G2/M. The cell cycle arrest-deficient mutants HIV-1 R80A, S79A, and H71R resulted in arrest profiles similar to the empty vector control ($G2/G1 = 0.41, 0.59, \text{ and } 0.33$, respectively) but degraded CCDC137 to levels induced by HIV-1 WT Vpr (38%, 51%, and 66%, respectively).

Comparing the degradation and G2/G1 values side-by-side in a heatmap further highlighted a lack of correlation among the two phenotypes (**Figure 1D**).

To further investigate a connection between CCDC137 depletion and G2/M arrest, we next generated stable CCDC137 knockdown (KD) cell lines and assayed their arrest profiles. Consistent with CCDC137 being essential for proliferating cell viability (17), we were able to knockdown CCDC137 to levels below those induced by HIV-1 WT Vpr using two unique gRNAs (23% for KD 1 and 21% for KD 2) (**Figure 1E-F**) but could not generate full CCDC137 knockouts in the context of either gRNA pools or single-cell clones. Although expression of HIV-1 Vpr resulted in potent G2/M arrest in cells expressing a non-targeting control (NTC) gRNA ($G2/G1 = 2.5$) or two separate CCDC137-targeting gRNAs ($G2/G1 = 2.7$ for KD 1 and 2.0 for KD 2), knocking down CCDC137 in the absence of Vpr was not sufficient to trigger arrest ($G2/G1 = 0.53$ for NTC, 0.72 for KD 1, and 0.38 for KD 2) (**Figure 1G and S1B**).

Overall, the lack of correlation among G2/M arrest profiles and CCDC137 levels induced by either HIV-1 Vpr mutants or CCDC137-targeting gRNAs (**Figure 1H**) shows that Vpr-mediated CCDC137 degradation is neither a direct cause nor consequence of cell cycle arrest.

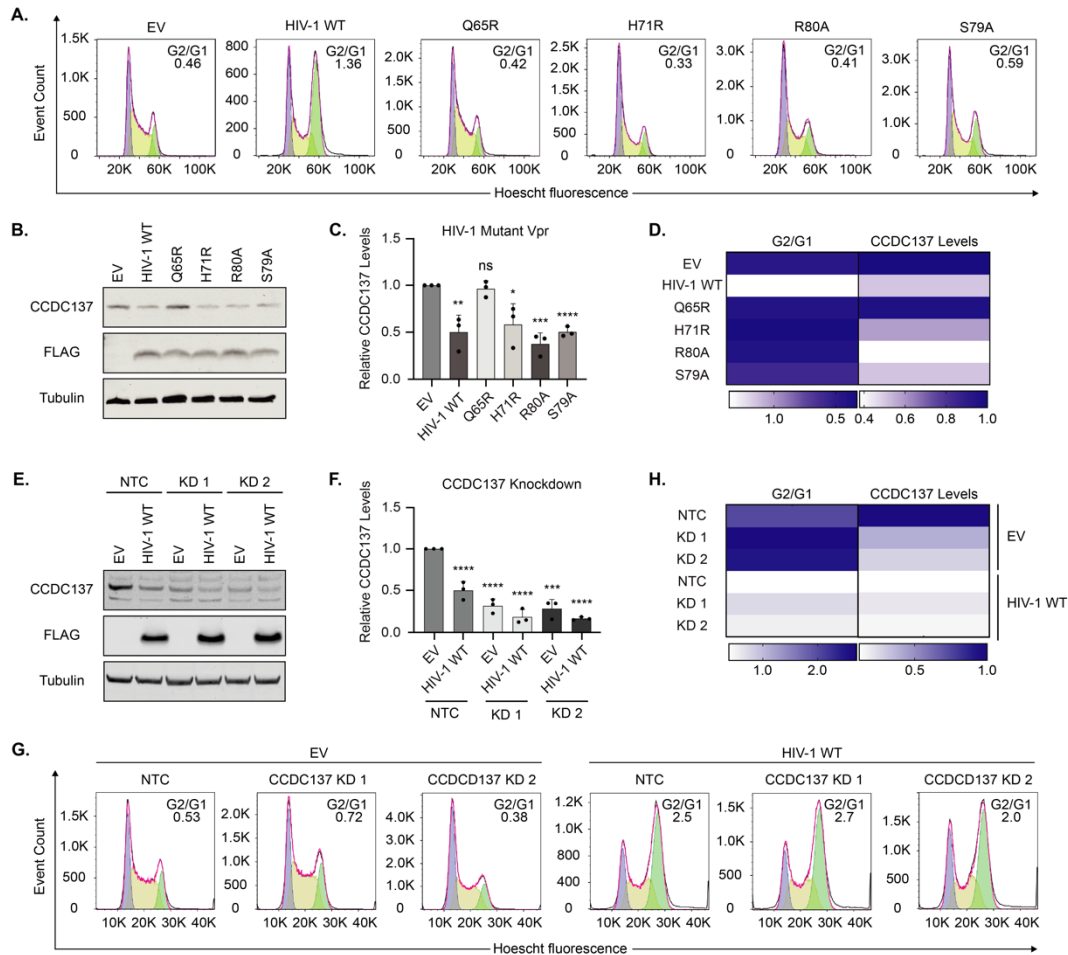


Figure 1. CCDC137 degradation does not correlate with Vpr-mediated G2/M arrest. (A) Representative flow cytometry cell cycle plots of U2OS cells infected with the indicated WT or mutant Vpr and gated for Vpr (mCherry) expression at 28 hours post infection (hpi). G2/G1 values were calculated from the percentages of cells in G2 and G1 in each condition based on DNA content (Hoechst fluorescence) ($n=3$). (B) Representative western blot of cells harvested from the same experiment as in (A) probed for endogenous CCDC137 and 3XFLAG Vpr. (C) Quantification of CCDC137 protein levels from (B) normalized to Tubulin, and relative to the empty vector control. Data from 3 biological replicates are shown. (D) Heatmap of G2/G1 values from (A) and average CCDC137 levels from (B) in the presence of the indicated Vpr. Color gradients were set to empty vector values (dark blue) and either the lowest CCDC137 level or highest G2/M value (white). (E) Representative western blot of CRISPR-Cas9 edited U2OS cells stably expressing gRNAs targeting CCDC137 (KD 1 or 2) or an NTC and probed and quantified (F) as in (B-C). (G) Representative flow cytometry cell cycle plots of control or knockdown U2OS cells infected with EV or WT Vpr as in (A). (H) Heatmap of G2/G1 values from (G) and average CCDC137 levels from (E) treated with EV or WT Vpr in the indicated U2OS lines. Color gradients set as in (D). All results were analyzed by unpaired t-tests. Asterisks indicate statistical significance from empty vector control; error bars represent $\pm$ standard deviation (ns = not significant, $*P \leq 0.0332$, $**P \leq 0.0021$, $***P \leq 0.0002$, $****P < 0.0001$). EV=empty vector; NTC= non-targeting control; KD = knockdown.

CCDC137 degradation is not mediated by canonical Vpr engagement of the CRL4-E3 ubiquitin ligase complex.

We next asked whether recruitment of the CRL4A^{DCAF1} E3 ubiquitin ligase complex was required for CCDC137 degradation, consistent with other canonical Vpr targets (64). First, we assayed whether this degradation was cullin-ring ligase (CRL) dependent by co-expressing hemagglutinin (HA)-tagged human CCDC137 and 3XFLAG-tagged Vpr in the presence or absence of increasing concentrations of the neddylation inhibitor MLN4924. As a positive control, we assayed MLN4924 rescue of Vpx-mediated SAMHD1 degradation, which is also CRL4A^{DCAF1}-dependent. MLN4924 increased CCDC137 levels in the presence of Vpr by 3.75-fold (12% to 45% increase), similar to the positive control SAMHD1 (4.5-fold, from 15% to 66%) (**Figure 2A-B**). However, unlike SAMHD1, MLN4924 increased CCDC137 levels in cells lacking Vpr (1.45-fold change, from 100% to 145%), suggesting that basal CCDC137 levels may be regulated by a cullin-dependent pathway independent of Vpr. We observed similar trends when we assayed MLN4924 rescue of endogenous CCDC137 depleted by HIV-1 Vpr (**Figure S1C-D**). Moreover, Vpr-mediated depletion of both overexpressed and endogenous CCDC137 levels was also rescued by the proteasome inhibitor MG132 (**Figure S1E-H**), and CCDC137 RNA levels were not decreased in Vpr-treated samples (**Figure 2C**). Together, this suggests that CCDC137 levels are regulated post-transcriptionally in a cullin-dependent manner, both dependent and independent of Vpr.

To further clarify a role for CRL4A^{DCAF1} ubiquitin ligase complex recruitment in Vpr-mediated CCDC137 degradation, we next performed co-immunoprecipitations (co-IPs) to probe for the recruitment of DCAF1, the adaptor protein between Vpr and CRL4A-DDB1 (65), to a Vpr-CCDC137 complex. In the presence of MLN4924, we transiently co-expressed HA-tagged human CCDC137 and 3X-FLAG tagged Vpr in U2OS cells, performed IPs against HA-CCDC137, and probed for interactions with 3X-FLAG Vpr and endogenous DCAF1 (**Figure 2D**). As has been previously reported, HIV-2 Rod9 Vpx robustly interacted with human SAMHD1, and DCAF1 was only recruited in the presence of HIV-2 Rod9 Vpx (66, 67). In contrast, we only detected minimal interaction between CCDC137 and HIV-1 WT Vpr. The intensity of this interaction was no greater than that observed between CCDC137 and HIV-2 Rod9 Vpx, which does not degrade CCDC137. Moreover, low levels of DCAF1 were already bound to CCDC137 in the absence of Vpr when compared to an

empty vector control, and these levels did not increase in the presence of HIV-1 WT Vpr. HIV-1 Vpr mutants that could or could not degrade CCDC137, H71R and Q65R, respectively, showed similar weak band intensities for 3XFLAG-Vpr and DCAF1 that we observed with WT Vpr. Performing the reverse-IP, we confirmed that neither HIV-1 WT nor HIV-1 Q65R Vpr interacted with CCDC137, despite the former robustly interacting with DCAF1 (**Figure S11**). Consistent with this minimal interaction, we also did not observe co-localization among Vpr and CCDC137 via immunofluorescence (IF), despite dimmer nucleolar CCDC137 signal in cells expressing Vpr orthologs that were capable of degradation (**Figure S2A**).

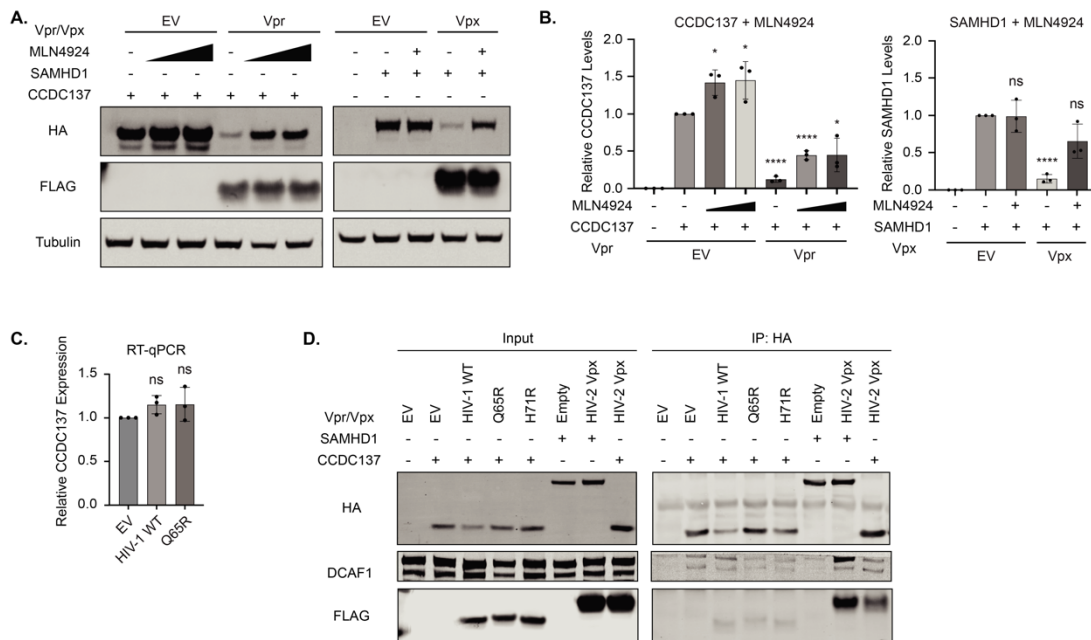


Figure 2. CCDC137 degradation does not correlate with Vpr-mediated CRL4^{DCAF1} E3 ubiquitin ligase complex recruitment. (A) MLN4924 rescue of Vpr-mediated CCDC137 degradation or Vpx-mediated SAMHD1 degradation in U2OS cells treated with MLN4924 (10 μM for SAMHD1 and 5 μM or 10 μM for CCDC137, as indicated). Western blot is representative of three separate experiments ($n=3$) and probed for HA-CCDC137 or HA-SAMHD1, 3XFLAG Vpr or Vpx, and Tubulin. (B) Quantification of (A) as described for (Figure 1C). (C) qRT-PCR of endogenous CCDC137 mRNA levels in the presence of HIV-1 WT or Q65R Vpr. (D) Co-immunoprecipitation assays against HA-SAMHD1 or HA-CCDC137 in U2OS cells treated with 10 μM MLN4924, probed for HA, 3X FLAG Vpr or Vpx, and DCAF1. All results were analyzed by unpaired t-tests. Asterisks indicate statistical significance from empty vector control; error bars represent $\pm$ standard deviation (ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$). EV=empty vector.

To elucidate whether Vpr-mediated CCDC137 depletion might derive from altered CCDC137 dynamics within the nucleolus, we further visualized CCDC137 localization in the context of two other nucleolar proteins, nucleolin (NCL) and upstream binding transcription factor (UBF1), via IF. NCL localizes to dense fibrillar component (DFC) and granular component (GC) layers of the nucleolus, reflecting its roles in rRNA processing (68), whereas UBF1 is an essential component of the RNA Polymerase I pre-initiation complex found in the fibrillar center (FC) (69). Consistent with its localization to the FC, CCDC137 signal overlaps with that of NCL (**Figure S2B**) and borders that of UBTF (**Figure S2C**). Relative to these markers, CCDC137 appeared on the exterior of nucleoli, where it may have the potential to interact with nucleoplasmic factors. HIV-1 Vpr did not alter CCDC137 localization in the context of either protein, despite dampening CCDC137 signal. While the addition of MLN4924 rescued this loss of signal, it did not reveal CCDC137 localization in any other nuclear compartments, countering the possibility that Vpr might be mis-localizing CCDC137 prior to degradation. UBF1 signal was enhanced in the context of MLN4924, however, suggesting an additional example of a nucleolar protein whose levels may be cullin-regulated.

Overall, Vpr depleted CCDC137 in the absence of a robust direct interaction, additional DCAF1 recruitment, or altered CCDC137 localization. While cellular CCDC137 levels may be regulated via a DCAF1-dependent mechanism, Vpr is not enhancing this interaction. Together, this suggests that Vpr does not degrade CCDC137 via canonical co-option of the CRL4A^{DCAF1} ubiquitin ligase complex.

The ability to deplete CCDC137 is evolutionarily conserved among Vpr orthologs from the HIV-1 lineage.

Although we did not find evidence for a direct correlation between Vpr-mediated CCDC137 degradation and G2/M arrest, we reasoned that this degradation may still be an evolutionarily conserved, and thus important, capacity of Vpr. We first asked whether CCDC137 degradation was conserved within the broader Vpr diversity from HIV-1 and HIV-2, which both infect humans but have distinct evolutionary origins: HIV-1 originating from spillovers of SIVcpz and SIVgor, and HIV-2 from SIVsmm (70). Using transient co-transfections of HA-CCDC137 and 3X-FLAG Vpr, we determined that four Vpr isolates from the pandemic HIV-1 group M viruses robustly degraded human CCDC137 (all < 20%), as did a consensus Vpr from group O (3.5%). A consensus Vpr from

group N and two Vpr isolates from group P (one a primary sequence and one a consensus Vpr) did not significantly degrade CCDC137 (68%, and 96 and >100%, respectively) (**Figure 3A-B**). In contrast to HIV-1 Vpr, HIV-2 Vpr isolates showed intermediate or no degradation of human CCDC137, while Vpx showed no degradation (>35% CCDC137 remaining) (**Figure S3A-B**), suggesting this function is not present among HIV-2 viruses.

We next explored whether the ability of Vpr to degrade CCDC137 was conserved among the ancestral lineages of HIV-1 and HIV-2 viruses. Within the HIV-1 lineage, we found that all Vpr isolates tested from SIV infecting chimpanzees (SIVcpz) and gorilla (SIVgor) robustly degraded chimpanzee (*Pan troglodytes troglodytes*) CCDC137 at each concentration tested (**Figure 3C-D**). Among the HIV-2 lineage, SIV sooty mangabeys (SIVsmm) Vpr isolate SL9, which is phylogenetically distant from the other HIV-2 and SIVsmm isolates, robustly degraded CCDC137 from sooty mangabeys (*Cercocebus atys*). However, SIVsmm CFU212 and SIVmac239 (from macaque) Vpr had intermediate degradation phenotypes, in line with other HIV-2 isolates (**Figure S3C-D**). These results suggest that the ability to degrade CCDC137 is conserved among Vpr from HIV-1/SIVcpz/gor but not the HIV-2 lineage and may thus be a specific and important function of HIV-1 and SIVcpz/gor (**Figure 3E**).

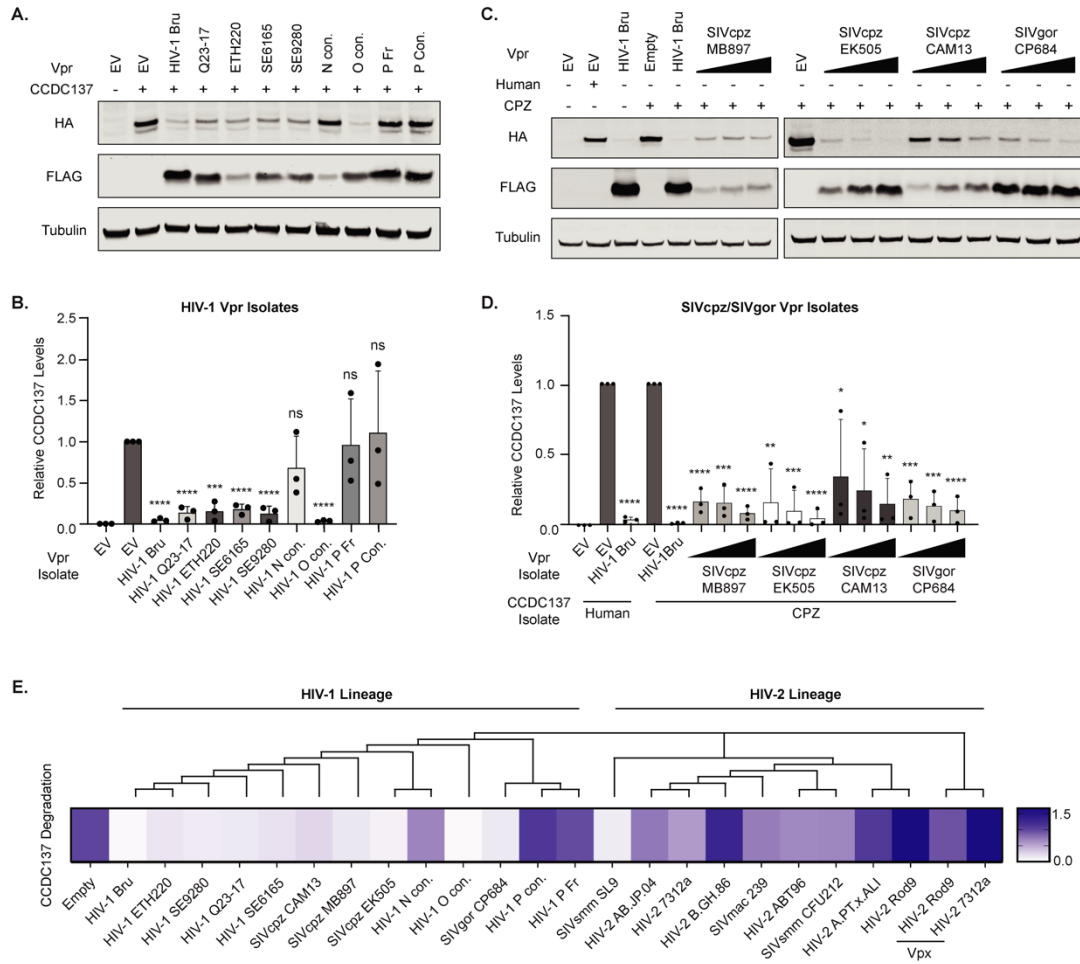


Figure 3. Degradation of CCDC137 is conserved among the HIV-1/SIVcpz/SIVgor Vpr lineage. (A) Representative western blots of U2OS cells transiently transfected with human HA-CCDC137 and 3XFLAG HIV-1 Vpr isolates. Cells were harvested at 28 hours post-transfection, and western blots were probed and (B) quantified as in Figure 1C ($n=3$). (C) Representative western blots and (D) quantification of chimpanzee (CPZ) HA-CCDC137 degradation by 3XFLAG SIVcpz or SIVgor Vpr isolates as described in (A) and (B) ($n=3$). (E) Heatmap of HA-CCDC137 levels in the presence of Vpr/Vpx from the HIV-1 or HIV-2 lineage. The color gradient was set to the highest (dark blue) and lowest (white) CCDC137 levels measured. The corresponding cladogram represents the aligned sequences of the Vpr/Vpx orthologs used in (A-D) and SF3 (A-D) as indicated. Results were analyzed by unpaired t-tests. Asterisks indicate statistical significance from empty vector control; error bars represent $\pm$ standard deviation (ns = not significant, $*P \leq 0.0332$, $**P \leq 0.0021$, $***P \leq 0.0002$, $****P < 0.0001$). EV = empty vector; con. = consensus.

Specificity of the Vpr-mediated CCDC137 degradation is conferred by both the Vpr and CCDC137 sequence.

To determine whether the degradation capacity of a given Vpr was inherent to the Vpr or dependent on the CCDC137, we challenged a panel of Vpr orthologs and isolates that could or could not degrade CCDC137 against the natural diversity of CCDC137 present in primates. We selected four Vpr orthologs based on their diverse abilities to degrade their autologous CCDC137 (**Figure 3**): HIV-1 Bru (robust), HIV-2 7312a (intermediate), SIVcpz MB897 (robust), and SIVsmm CFU212 (intermediate). We assayed degradation against the breadth of CCDC137 sequence variability from humans, chimpanzees, sooty mangabeys, African Green Monkeys (AGMs) (*Chlorocebus sabaeus*), a prosimian (PRO, *Propithecus coquereli*), and a New World monkey (NWM, *Callithrix jacchus*). We found that HIV-1 and SIVcpz Vpr robustly degrade nearly all CCDC137 orthologs tested, including those from prosimians and NWM. However, degradation capacities of HIV-2 7312a and SIVsmm CFU212 Vpr varied, depending on the CCDC137 ortholog tested (**Figure 4A-D and S3E-F**). Together, these data suggest that degradation is an innate capacity of certain Vpr orthologs that is also influenced by unique properties of certain CCDC137 proteins (**Figure 4E**). Although these phenotypes are not reminiscent of typical virus-host molecular arms-races, in which we might expect robust degradation phenotypes in the context of autologous hosts, there is some specificity conferred by both the CCDC137 (host) and Vpr (viral) sequence.

We therefore characterized the evolutionary history of CCDC137 in primates and tested whether it has experienced episodes of rapid evolution and positive selection (PS). We performed PS analyses using the Detection of Genetic INNovations (DGINN) pipeline, which performs phylogenetic and positive selection analyses from five complementary methods to increase the sensitivity and specificity in the detection of genetic innovation. Among simians, we did not find evidence of strong PS in CCDC137 (**File S1**). However, performing detailed site-specific analyses, we identified five regions undergoing rapid evolution (**Figure 4F-G**). Most are in predicted disordered regions, except a 269-272 region in the C-terminal helix of CCDC137 (**Figure S3G**). Furthermore, site 194 overlapped with a region linked to adenoviral E1A interaction (23), which could be reminiscent of a past evolutionary conflict. However, no signatures of positive selection or rapid evolution overlapped with nuclear localization sequences (NLS), a nuclear-hormone receptor binding motif (LXXLL), or a sequence associated with Vpr-mediated CCDC137

degradation (17). Remarkably, the latter was entirely conserved amongst primate sequences (**Figure 4F-G**). Given the functional host-specificity observed, it is thus likely that other CCDC137 determinants beyond sequence identity are also involved in CCDC137-induced degradation by Vpr.

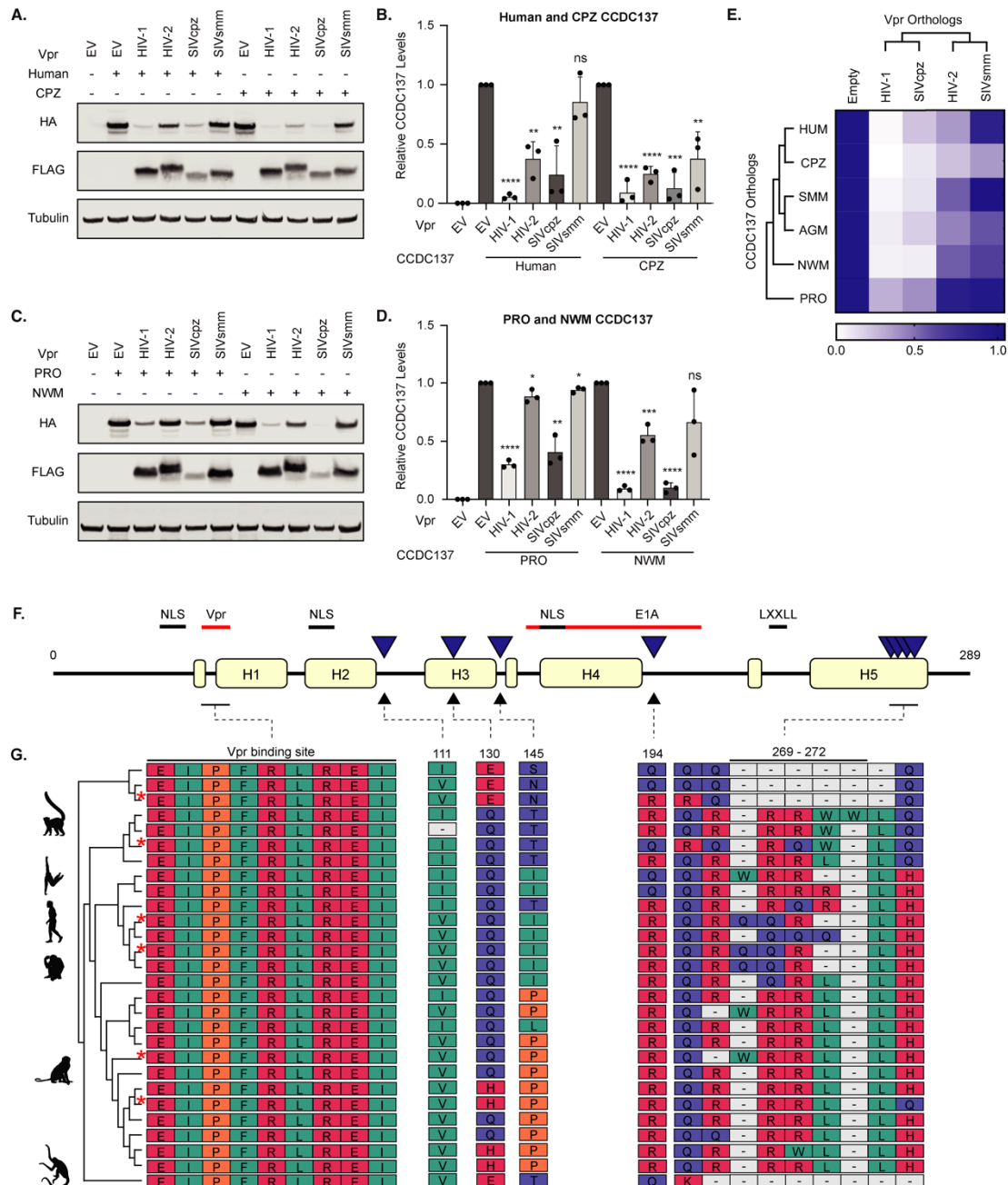


Figure 4. Host species- and viral lineage-specific determinants influence CCDC137 degradation. (A) Representative western blot and (B) quantification of human and chimpanzee (CPZ) HA-CCDC137 degradation by a panel of 3XFLAG HIV-1 Bru, HIV-2 7312a, SIVcpz MB897 and

SIVsmm CFU212 Vpr orthologs. Assays were performed as described in Figure 3 (A-D) ($n=3$). **(C)** Representative western blot and quantification of **(D)** prosimian (PRO) and New World monkey (NWM) HA-CCDC137 degradation by the indicated Vpr ($n=3$). **(E)** Heatmap depicting degradation of the different CCDC137 orthologs (rows) by the indicated Vpr ortholog (columns). Results were analyzed by unpaired t-tests. Asterisks indicate statistical significance from empty vector control; error bars represent $\pm$ standard deviation (ns = not significant, $*P \leq 0.0332$, $**P \leq 0.0021$, $***P \leq 0.0002$, $****P < 0.0001$). **(F)** Schematic representation of the CCDC137 protein. Helices are noted as H1-5 and sites of PS (File S1 for details and methods) are represented as solid blue triangles. CCDC137 sequence motifs and viral protein binding sites are represented as labeled black and red lines, respectively. **(G)** Residues corresponding to the proposed Vpr binding site and marks of PS. Amino acids are labeled by property (pink = charged, teal = hydrophobic uncharged, blue = polar uncharged, and orange = special case). Grey boxes containing (-) indicate a lack of corresponding amino acid in the alignment. Sequences are organized based on an evolutionary tree (left) constructed from 28 primate CCDC137 sequences. Red asterisks indicate sequences used in (A-E). Primate icons are from Adobe Stock Images. EV = empty vector. NLS = nuclear localization sequence.

CCDC137 depletion is a consequence of specific genomic insults.

Despite our findings that CCDC137 is not a canonical target of Vpr, it is clear that Vpr orthologs from the HIV-1 lineage potentially deplete this host factor. Given the absence of a direct CCDC137-Vpr interaction, we reasoned that CCDC137 depletion might result from another important HIV-1 Vpr function. Considering that CCDC137 degradation has been linked to activation of the DDR (17) and Vpr both induces DNA damage and modulates DDR signaling, we asked whether CCDC137 degradation is a consequence of DNA damage.

To test this, we investigated whether a panel of different genotoxic agents were sufficient to deplete CCDC137. Within this panel, we assayed CCDC137 levels in the context of γ H2AX levels, which we used to measure the amount of global DDR activation induced by each agent. We found that titrating amounts of camptothecin (**Figure 5A-B**) and etoposide (**Figure S4A-B**), which cause DNA damage by inhibiting Topoisomerase I and II, respectively, robustly depleted CCDC137. In contrast, cisplatin (**Figure 5C-D**), an agent that forms intra-strand DNA crosslinks that inhibit DNA synthesis, and hydroxyurea (**Figure S4C-D**), a ribonucleotide reductase inhibitor, did not deplete CCDC137, even at the highest concentrations tested. Olaparib, a PARP1/2 inhibitor that prevents DNA damage repair and leads to an accumulation of DSBs, degraded CCDC137 only at high concentrations (**Figure S4E-F**) that may induce off-target effects. Among conditions that induced

CCDC137 degradation, degradation inversely correlated with amount of DNA damage. However, CCDC137 degradation did not correlate with the absolute amount of damage present (**Figure 5E**). For example, in instances where camptothecin and cisplatin both induced higher γ H2AX levels than Vpr, only camptothecin resulted in CCDC137 degradation. Degradation was also independent of the ability to cause G2/M arrest (**Figure 5F**) – consistent with the degradation of CCDC137 by Vpr mutants lacking this ability (**Figure 1**) – or activate p53 (**Figure 5G-H**). Moreover, CCDC137 depletion itself did not cause DDR activation, nor was CCDC137 required for Vpr-induced DDR activation (**Figure S4G**). Together, these results suggest that CCDC137 depletion is a consequence of specific genomic insults.

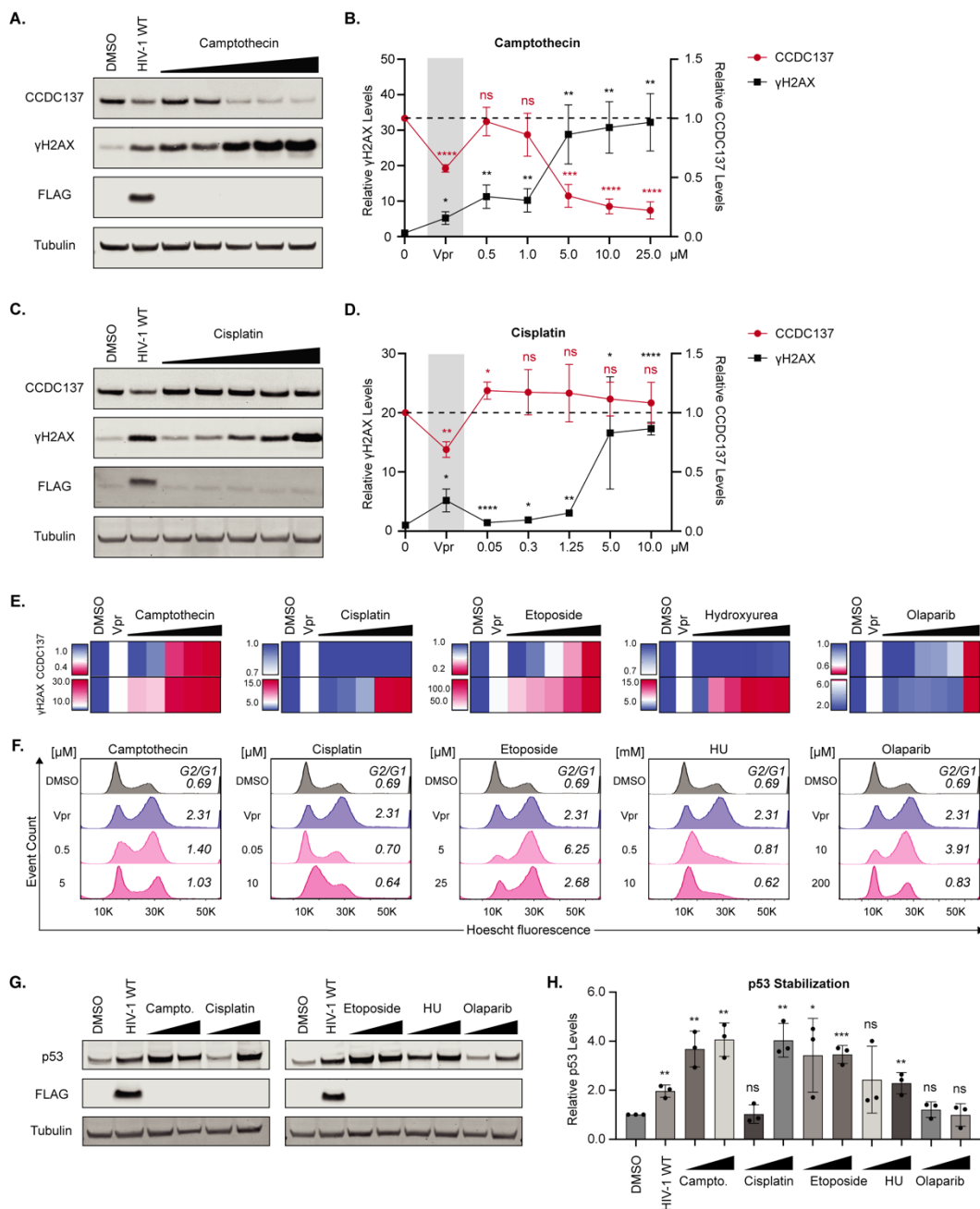


Figure 5. CCDC137 depletion is a consequence of specific types of DNA damage. (A) Representative western blot of CCDC137 depletion following 24hr of treatment with DMSO, HIV-1 WT, or titrating amounts camptothecin performed and (B) quantified as described in Figure 1C. ($n=3$) (C) Representative western blot and (D) quantification of CCDC137 and γ H2AX levels in the presence of titrating amounts of cisplatin ($n=3$). (E) Heatmaps indicating CCDC137 and γ H2AX levels in U2OS cells treated with DMSO, HIV-1 WT Vpr, or titrating amounts of the indicated genotoxic agents. Values for CCDC137 or γ H2AX in a given condition are averaged from three separate experiments ($n=3$). Top row: color gradients depict the percentage of CCDC137 remaining in the empty vector control (dark blue), the lowest percent of CCDC137 measured

among the represented conditions (dark pink), and CCDC137 levels in Vpr-treated cells as a midpoint (white). Bottom row: color gradients depict γ H2AX induced by empty vector (dark blue), the highest γ H2AX value measured among the given conditions (dark pink) and γ H2AX levels induced by Vpr as a midpoint (white). **(F)** Representative flow cytometry plots of cell cycle profiles under the indicated conditions as described in Figure 1A. **(G)** Representative western blots and **(H)** quantification of p53 levels following 24hr of treatment with DMSO, HIV-1 WT, camptothecin (0.5 μ M or 5 μ M), cisplatin (0.05 μ M or 10 μ M), etoposide (5 μ M or 25 μ M), hydroxyurea (0.5 μ M or 10mM), or olaparib (10 μ M or 200 μ M) ($n=3$). Results were analyzed by unpaired t-tests. Asterisks indicate statistical significance from empty vector control; error bars represent $\pm$ standard deviation (ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$). Campto. = camptothecin; HU = hydroxyurea.

Vpr orthologs that degrade CCDC137 disrupt the nucleolar proteome.

Considering that CCDC137 is a nucleolar protein, and there is extensive crosstalk between the DDR and nucleolus, we hypothesized that CCDC137 degradation may derive from DNA damage that specifically affects nucleolar pathways. In support of nucleolar involvement, of the genotoxic agents we assayed, only those that degrade CCDC137 – camptothecin, etoposide, and olaparib – perturb the nucleolus (45, 71–75). Cisplatin, however, acts via nucleolar-independent DDR activation (76), in line with its inability to degrade CCDC137. To test this hypothesis, we first asked whether Vpr causes nucleolar stress.

Nucleolar stress is diagnosed based on alterations to the nucleolar proteome and changes in nucleolar morphology and function (27). To assess the effects of Vpr on the nucleolar proteome, we leveraged two pre-existing studies that specifically assayed proteomic changes induced by Vpr (55, 56). Johnson *et al.* infected Jurkat E6.1 T cells with HIV-1 Δ Env NL4-3 (WT), HIV-1 lacking one of each accessory gene Vpr, Vpu or Vif (Δ Vpr, Δ Vpu or Δ Vif, respectively), or uninfected control cells and quantified changes in the total proteome. Gene set enrichment analyses (GSEA) of protein groups differentially increased or decreased among the different infection conditions revealed that the nucleolus and nucleolar processes harbored some of the most significantly decreased protein changes (**Figure 6A and Figure S5A**). When compared to mock infection, these changes were only lost with Δ Vpr infection. When compared to WT infection, only Δ Vpr, but not Δ Vif or Δ Vpu, were statistically significant for the decrease in proteins associated with nucleolus

and nucleolar processes, together showing Vpr is necessary and sufficient for alterations to the nucleolar proteome.

To understand whether Vpr-driven changes in nucleolar pathways correlated with CCDC137 degradation, we next analyzed proteomic data from Greenwood *et al.*, in which CEM-T4 T cells were transduced with Vpr proteins that could or could not degrade CCDC137. We performed gene set overrepresentation analysis (GSOR) of differential protein abundance in each condition. In the presence of Vpr proteins that degrade CCDC137, including HIV-1 Group O, HIV-1 NL4-3, the cell cycle deficient mutant HIV-1 S79A, and SIVcpz Vpr, the most significantly downregulated cellular pathways were nucleolar processes (**Figure 6B**), particularly ribosome biogenesis. Similarly, cellular components associated with nucleolar functions harbored the most significantly depleted genes (**Figure S5B**). In contrast, among changes induced by Vpr that did not degrade CCDC137 – SIVsmm Vpr, the functionally dead mutant HIV-1 Q65R Vpr, and HIV-2 Vpx – neither nucleolar pathways nor cellular compartments harboring nucleolar processes were significantly downregulated. Further, nucleolar proteins make up the majority of proteins significantly altered by CCDC137-degrading Vpr in both the Johnson *et al.* and Greenwood *et al.* datasets (**Figure S5C**). To determine whether CCDC137 interacted with nucleolar genes identified in the Greenwood *et al.* abundance dataset, we performed a functional protein association network analysis using STRING (59) (**Figure 6C**). We discovered that CCDC137 interacted with multiple nucleolar proteins, including those with known roles in rRNA processing and HIV infection (78–81). One of these proteins, nucleophosmin (NPM1), is a prominent nucleolar stress sensor (77). Together, these analyses suggest that nucleolar disruption is a specific function of Vpr, and one unique to Vpr proteins capable of CCDC137 degradation.

Given our findings that Vpr orthologs capable of depleting CCDC137 also decreased levels of other nucleolar proteins, we next sought to validate the results of our proteomic analyses and, using the CCDC137 KD cell lines (**Figure 1F**), distinguish whether CCDC137 degradation was a driver of, or a response to, Vpr-mediated nucleolar disruption. We selected three nucleolar proteins that were represented in multiple nucleoli-associated GO terms in both Johnson *et al.*

and Greenwood *et al.* datasets for further validation: NOP14, a stress-responsive gene involved in 40S ribosome biogenesis (82), RRP36, a component of the 90S preribosome involved in pre-rRNA processing(83), and UTP11, a subunit of the U3-containing small subunit (SSU) processome complex also involved in pre-rRNA processing(84). We assayed degradation of each protein by HIV-1 or SIVsmm Vpr orthologs (Q23-17 and CFU212, respectively), which we focused on because of their contrasting effects on nucleolar pathways (**Figure 6B**) and human CCDC137 degradation phenotypes (**Figure 4A-B**). In line with the proteomic data, HIV-1 WT Vpr depleted RRP36 to levels comparable to those observed with CCDC137 in the NTC cells (46% vs. 50%, respectively) (**Figure 6F-G**) whereas SIVsmm Vpr only induced intermediate degradation phenotypes (74% for RRP36 vs. 73% for CCDC137). Vpr-depleted RRP36 levels were also rescued by MLN4924 and MG132 (**Figure S5D**), and both etoposide and camptothecin were sufficient to induce RRP36 degradation (**Figure S5E**), further mirroring the degradation profiles of CCDC137. Although neither Vpr ortholog depleted NOP14 (**Figure 6H**), both robustly degraded UTP11 (7.7% for HIV-1 WT and 5.2% for SIVsmm Vpr) (**Figure 6I**), suggesting a diversity in the degradation profiles of different nucleolar proteins. CCDC137 knockdown was not sufficient to induce depletion of any of the three nucleolar proteins nor did it inhibit the ability of Vpr to induce this depletion (**Figure 6F-I**), suggesting that CCDC137 is a component, but not a driver, of a larger Vpr-induced nucleolar stress response.

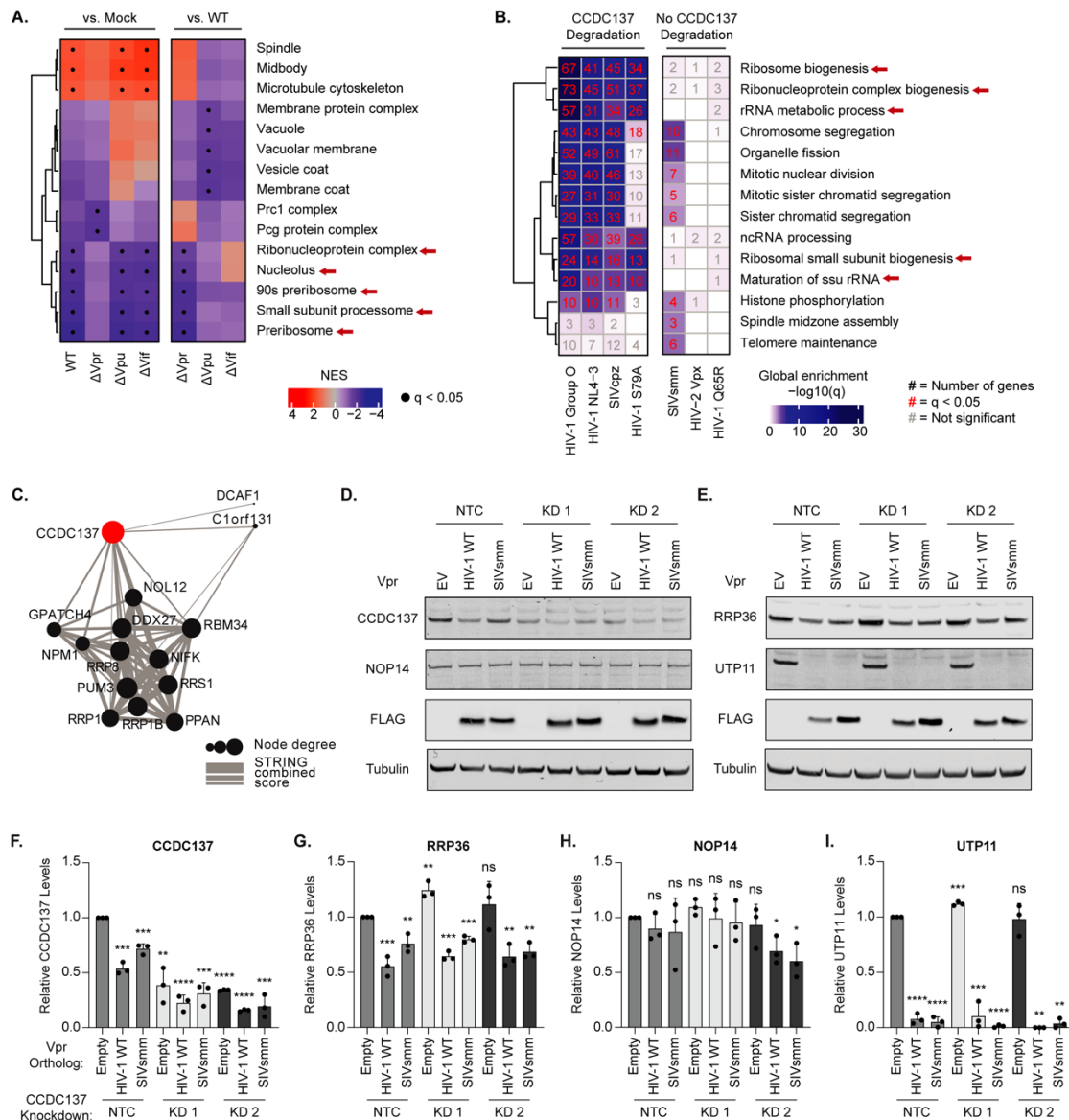


Figure 6. Vpr induced nucleolar stress correlates with CCDC137 degradation. (A) Gene set enrichment analysis (GSEA) of all expressed genes in (56) abundance proteomics dataset, using gene sets from the GO Cellular Component ontology. Graph legend shows normalized enrichment score (NES) computed using the enrichment and adjusted p-value values. Red arrows highlight nucleolar-associated gene sets. (B) GO terms enriched by gene set overrepresentation analysis (GSOA) (adjusted p-value < 0.05) of differentially expressed genes from (55) ($\log_2\text{FC} > 0.5$, Vpr expression versus empty). Top terms extracted from the GO Biological Process ontology. (C) A subnetwork extracted from STRING of CCDC137 interactions possessing a combined score greater than 0.4. (D-E) Representative western blots of nucleolar proteins found to be depleted in both abundance proteomics datasets represented in (A) and (B) in the indicated U2OS control and CCDC137 knockdown cell lines. Cells were harvested at 24hpi following treatment with EV, HIV-1 WT or SIVsmm Vpr and the indicated protein levels were quantified in (F-I) as in Figure 1C ($n=3$). Results were analyzed by unpaired t-tests. Asterisks indicate statistical significance from the empty

vector control in NTC cells; error bars represent $\pm$ standard deviation (ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$).

Vpr alters nucleolar morphology and disrupts nucleolar processes.

We next determined whether Vpr triggered functional changes in nucleolar morphology and processes that are canonically associated with nucleolar stress. We first assayed redistribution of NPM1 and nucleolin (NCL) from the nucleolus to the nucleoplasm by HIV-1 WT or SIVsmm Vpr (**Figure 7A-D**). As positive controls for nucleolar disruption, cells were treated with 5nM Actinomycin D (Act. D), which selectively inhibits rRNA transcription (85), and etoposide or camptothecin (**Figure S6A-F**). Consistent with their abilities to degrade CCDC137, HIV-1 and SIVsmm Vpr led to robust and intermediate NPM1 translocation phenotypes, respectively, (**Figure 7A-B**) despite inducing similar γ H2AX levels (**Figure S6G-H**), suggesting NPM1 dispersal correlates with CCDC137 degradation. Moreover, this dispersal resembled the morphological changes induced by etoposide and camptothecin rather than Act. D, suggesting Vpr-induced nucleolar stress is likely not a direct derivative of RNA Pol I inhibition. While Act. D and etoposide also disrupted NCL distribution, neither the Vpr orthologs nor camptothecin altered NCL localization, further implying specificity to the nucleolar changes observed. Act. D was sufficient to induce CCDC137 depletion in a cullin-dependent manner, however, indicating that direct nucleolar insults result in CCDC137 degradation (**Figure S6I**).

In addition to nucleolar protein redistribution, we also looked at nucleolar size, which is indicative of nucleolar disruptions (27), including those caused by viral infections (26). We measured nucleolar area among the same conditions as in Figure 7A-D in cells targeted with NTC or CCDC137 gRNAs (**Figure 7E-F**). As expected, Act. D reduced nucleolar size, consistent with inhibited RNA Pol I activity. While HIV-1 Vpr significantly increased nucleolar size, we also observed this phenotype for SIVsmm Vpr, suggesting that augmented nucleolar area does not correlate with CCDC137 depletion. In line with this, CCDC137 knockdown was not sufficient to increase nucleolar size.

Finally, we assayed whether Vpr disrupted ribosome biogenesis and whether this correlated with CCDC137 degradation. To determine this, we assayed changes in rRNA transcription, the rate limiting step of ribosome biogenesis (88), by 5-ethynyl uridine (EU) pulse-labeling. As this step

can represent 80% of cellular transcription, general changes in EU signal are indicative of changes in nascent rRNA transcription (45). Reflecting the findings from our nucleolar size assays, we found that HIV-1 and SIVsmm Vpr both significantly decreased EU signal, suggestive of decreased nascent rRNA transcription and thus inhibition of ribosome biogenesis (**Figure 7G-H**). Depletion of CCDC137 had no effect on nascent rRNA transcription, however, further suggesting that CCDC137 degradation does not directly trigger a nucleolar stress response but may be an important component of one.

Overall, these data suggest that Vpr triggers multiple nucleolar stress phenotypes, including NPM1 translocation – the most prominent hallmark of nucleolar stress (77) – increased nucleolar area, and repression of nascent rRNA transcription.

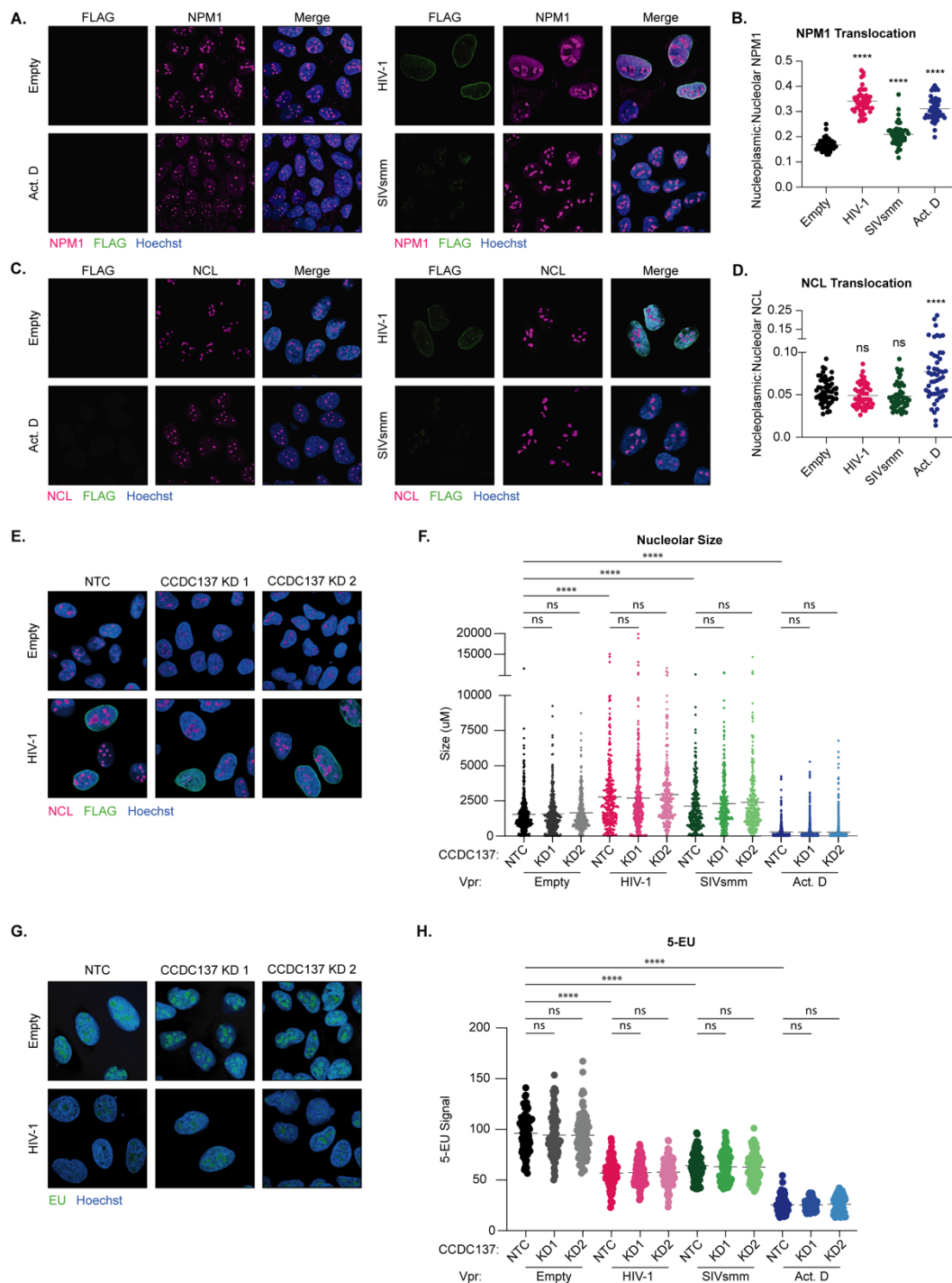


Figure 7. Vpr induces multiple signatures of nucleolar stress. (A) Representative immunofluorescence images of U2OS cells treated with the indicated conditions probed for NPM1 (magenta), 3XFLAG (Vpr) (green), and Hoechst (blue). Images were taken at 63X magnification. (B) Quantification of NPM1 translocation in (A). Nucleolar or total nuclear NPM1 MFI were measured for at least 50 cells per condition. Each dot represents one cell. (C) Representative immunofluorescence images as in (A) probed for NCL (magenta), 3XFLAG (Vpr) (green), and

Hoechst (blue). **(D)** NCL nucleoplasmic:nucleolar translocation quantified as in (B). Results in (B) and (D) were analyzed with a one-way ANOVA with Dunnett's multiple comparisons test. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from the empty vector control. **(E)** Representative IF images of nucleolar size experiments performed in control or CCDC137 knockdown U2OS cell lines probed for nucleolin (NCL) as described in (C). Images were taken at 63X. **(F)** Quantification of nucleolar size experiments performed as in (E) in the indicated cell lines. Nucleolar area was measured for approximately 100 cells per condition. Each dot represents one cell. Representative quantification of two independent experiments ($n=2$). **(G)** Representative IF images of control or CCDC137 knockdown U2OS cells expressing HIV-1 WT Vpr or empty vector and stained for EU. Images were taken at 63X. **(H)** Quantification of total MFI in EU pulsed cells of the indicated conditions. MFI of EU signal was measured for approximately 100 cells per condition. Representative quantification of two independent experiments ($n=2$). Results in (F) and (H) were analyzed with a one-way ANOVA with Šidák's multiple comparison test. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from empty vector control or NTC cells subject to the same treatment condition; ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$.

Vpr induces CCDC137 depletion through ATR-dependent nucleolar stress.

Thus far, our results suggest that specific types of DNA damage are sufficient to disrupt the nucleolus and deplete CCDC137. Consequently, we sought to clarify the pathways connecting these processes. Central to the nucleolar-DDR crosstalk are the DDR signaling kinases ATM and ATR, which can be activated in response to both direct and indirect nucleolar insults (35, 89). To determine whether activation of either kinase was required for CCDC137 depletion, we asked whether Vpr-mediated CCDC137 degradation was rescued by inhibition of ATM via KU-55933 (ATMi) or ATR via VE-821 (ATRi). While ATMi had no effect on CCDC137 degradation, ATRi fully rescued CCDC137 degradation induced by both HIV-1 and HIV-2 Vpr (**Figure 8A-B**). This rescue was only observed for Vpr, given that neither inhibitor rescued CCDC137 degradation induced by camptothecin or etoposide (**Figure S7A**). Overall, these results demonstrate that CCDC137 degradation requires Vpr-induced ATR activation.

To specifically determine if HIV-1 Vpr induces a nucleolar ATR response that correlates with CCDC137 depletion, we assayed the number of RPA32 and TOPBP1 foci found in the nucleoli of cells treated with EV, HIV-1 Vpr or SIVsmm Vpr. As a positive control for rDNA damage, we also measured foci induced by overexpression of I-Ppo1, an endonuclease that cleaves a recognition site within 28S rDNA and a few other locations in the genome (33, 38, 90). We found that both

HIV-1 Vpr and I-Ppo1 significantly induced nuclear and nucleolar foci of both RPA32 and TOPBP1 (**Figure S7B-E**), suggesting the activation of ATR pathways *in trans* and *in cis*. We further assayed whether Vpr activated a nucleolar ATR response by augmenting total protein levels of APE1 – a multifunctional protein whose overexpression induces nucleolar ATR activation and represses rRNA transcription in unperturbed conditions (91) – but found that Vpr did not alter total APE1 protein levels (**Figure S7F-G**). Taken together, these results suggest that HIV-1 Vpr activates multiple nucleolar markers of ATR activation.

Given that ATR activation is necessary but not sufficient for CCDC137 degradation, we hypothesized that this degradation is due to the activation of an ATR-dependent nucleolar response to DNA damage. To test this hypothesis, we asked if Vpr-induced nucleolar stress was also dependent on ATR. We first assayed nucleolar size using the same Vpr and genotoxic agent panel as in **Figure 6A-B** and **S6A-B**. As expected, we detected larger nucleoli among all experimental conditions apart from Act. D (**Figure 8C-E** and **S8A-B**). Consistent with CCDC137 degradation, ATRi rescued the altered nucleolar sizes induced by both HIV-1 and HIV-2 Vpr, but not SIVsmm Vpr. Instead, the increased nucleolar size induced by SIVsmm CFU212 was fully rescued by ATMi, suggesting that Vpr orthologs that degrade CCDC137 do so by triggering a nucleolar ATR pathway. We also found that camptothecin augmented nucleolar size in an ATR dependent manner, whereas etoposide induced changes were ATM dependent (**Figure S8A-B**). This increased nucleolar size was not solely the result of enlarged nuclei, as nucleolar number also decreased, indicative of nucleolar fusion (**Figure S8D**). Moreover, it was not a consequence of G2/M arrest, as it was observed for SIVsmm CFU212 Vpr (**Figure 8D-E**), which does not induce arrest (**Figure S8D**).

We next asked whether the Vpr-mediated repression of rRNA transcription could be similarly rescued by ATR and ATM inhibition. Reflecting the nucleolar size assays, all Vpr orthologs decreased rRNA transcription; repression caused by HIV-1 and HIV-2 Vpr was ATR dependent whereas repression caused by SIVsmm CFU212 was ATM dependent (**Figure 8F-H**). Similarly,

camptothecin and etoposide decreased EU signal, and these changes were ATR and ATM dependent, respectively (**Figure S8E-F**).

Together, these data show that Vpr orthologs that degrade CCDC137 cause nucleolar stress by modulating the nucleolar proteome, altering NPM1 localization and nucleolar size, and decreasing ribosome biogenesis. Moreover, they suggest that HIV Vpr degrades CCDC137 through the activation of a nucleolar-specific ATR pathway, suggesting CCDC137 plays a central role in nucleolar homeostasis.

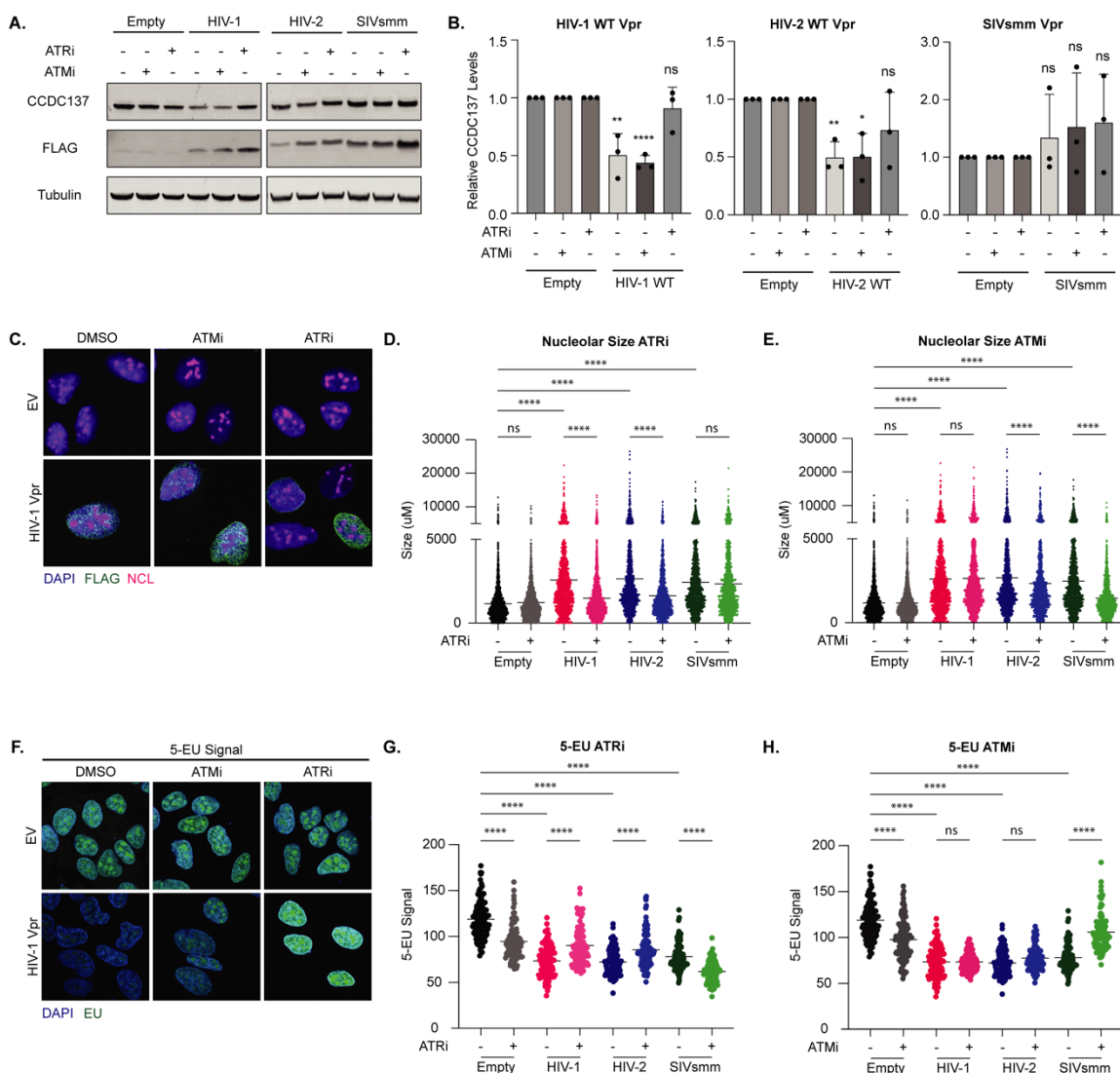


Figure 8. Vpr induces ATR-dependent nucleolar stress thereby depleting CCDC137. (A) Representative western blots of CCDC137 levels in the presence of the indicated Vpr orthologs and DMSO, 10 μ M ATMi, or 10 μ M ATRi following a 24hr incubation. Western blots were probed and (B) quantified as in Figure 1 ($n=3$). Unpaired t-tests, error bars represent $\pm$ standard deviation,

and asterisks indicate statistical significance from empty vector control; ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$. **(C)** Representative IF images of nucleolar size experiments probed for nucleolin (NCL) as described in Figure 7 (C). Images were taken at 100X. **(D-E)** Quantification of nucleolar size experiments performed as in (C) in ATRi (D) or ATMi (E) treated cells. Nucleolar area was measured for approximately 100 cells per condition. Representative quantification of two independent experiments ($n=2$). **(F)** Representative IF images of U2OS cells expressing HIV-1 WT Vpr or empty vector and stained for EU. Images were taken at 63X. **(G-H)** Quantification of total MFI in EU pulsed cells of the indicated conditions. MFI of EU signal was measured for approximately 100 cells per condition. Representative quantification of two independent experiments ($n=2$). Results in (D-E) and (G-H) were analyzed with a one-way ANOVA with Šidák's multiple comparison test. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from empty vector control or cells subject to the same treatment conditions without inhibitor as indicated; ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$.

DISCUSSION

Vpr-mediated CCDC137 depletion represents a powerful example of how indirect host targets can reveal novel lentiviral accessory protein functions and help to define novel roles for cellular proteins. In this study, we establish CCDC137 as a non-canonical, but evolutionarily important, Vpr target that is part of a broader cellular response to nucleolar stress. By leveraging a set of HIV-1 Vpr mutants and genotoxic agents that do not cause G2/M arrest, as well as CRISPR-Cas9-mediated CCDC137 depletion, we show that CCDC137 degradation is neither a direct cause or consequence of Vpr-induced cell cycle arrest. Further, we show that degradation is not mediated through canonical Vpr recruitment of the CRL4A^{DCAF1} ubiquitin ligase complex, and that Vpr and CCDC137 neither directly interact nor occupy the same nuclear compartments. By assaying degradation among Vpr and CCDC137 orthologs representative of the broader HIV and SIV diversity, we show that CCDC137 depletion is conserved among the HIV-1 but not HIV-2 lineage, suggesting it may constitute, or be a component of, an important function of Vpr from the pandemic HIV-1 lineage. CCDC137 depletion is also species-specific and CCDC137 harbors signatures of rapid evolution, consistent with a possible evolutionary arms-race. In defining how Vpr leads to degradation of CCDC137, we show that CCDC137 is depleted by Vpr orthologs and genotoxic agents that activate an ATR-mediated nucleolar stress response. We characterize CCDC137 depletion as one component of this nucleolar stress response and show that it occurs alongside other Vpr-induced nucleolar insults, including 1) modulated nucleolar pathways, 2)

altered nucleolar morphology, 3) nucleolar DNA damage, and 4) downregulated rRNA transcription. When nucleolar stress is not accompanied by CCDC137 degradation, as in the case of SIVsmm Vpr, it is ATM dependent. Together, we identify a current model where Vpr activates a nucleolar-specific ATR signaling pathway that triggers nucleolar stress and, with it, CCDC137 degradation.

Nucleolar stress may bridge Vpr-induced DNA damage and G2/M arrest. Here, we clarify that CCDC137 depletion is neither a direct cause nor consequence of cell cycle arrest. Yet, there is an apparent, albeit complex, relationship between these two phenotypes, and the temporal order of CCDC137 degradation, arrest, and nucleolar stress remains unresolved. Our results favor a process in which CCDC137 depletion precedes arrest: Vpr causes ATR-dependent nucleolar stress, of which CCDC137 degradation is a component, and this eventually triggers G2/M arrest. Given that CCDC137 depletion is not sufficient to trigger arrest, one possibility is that Vpr-induced nucleolar insults need to be severe enough to stall cell cycle progression. In support of this, the nucleolar stress response can activate an ATR-Chk1-mediated G2 arrest once a certain threshold of stress has been reached (92). The signatures of nucleolar stress we observe are not solely consequences of arrest, given that we see NPM1 redistribution and decreased rRNA transcription among conditions with varying arrest phenotypes. Moreover, through bioinformatic analyses, we also determine that downregulation of ribosome biogenesis and other nucleolar pathways is a specific cellular consequence of Vpr but not Vif, despite both proteins causing similar cell cycle arrest (93). In contrast to our model, others have shown that CCDC137 degradation can be caused by cell cycle arrest (63), though this may be due to the intertwined nature of arrest and nucleolar disruption, as ribosome biogenesis both regulates and is regulated by cell cycle progression. Such sensitivity of the nucleolus to disruptions may also explain why some Vpr orthologs are able to degrade only certain CCDC137 proteins in the same system. Overall, future work will be needed to establish a clearer temporal order of CCDC137 degradation, nucleolar disruption, and arrest. By comparing the effects of Vpr and different genotoxic agents on CCDC137 levels and nucleolar integrity, our data also provide insight into how Vpr engages the DDR. Previous studies from our lab and others have shown that Vpr causes DNA damage and simultaneously activates and

represses DDR pathways (16). Here, we have demonstrated that Vpr activates a nucleolar-specific ATR pathway that is distinct from ATR triggered by general DNA damage, as all of the genotoxic agents we tested activate classical ATR (80–83), but few disrupt the nucleolus. Yet, it remains to be seen whether activation of this pathway demonstrates Vpr-mediated ATR dysregulation or represents one of numerous ATR signaling branches engaged by Vpr. Our data show that Vpr activates markers of ATR activation in rDNA, though the precise locations and nature of these foci, as well as a causal link to other ATR-dependent nucleolar stress phenotypes, will require further investigation. Moreover, it is also possible that the nucleolar ATR pathway is not activated by DNA damage but by independent nucleolar insults. What these insults may be and how they could differentially activate ATR remains to be identified. We further identify multiple phenotypes shared by HIV-1 Vpr and the Topo I inhibitor camptothecin, including robust CCDC137 degradation, and activation of ATR-dependent changes in nucleolar size and rRNA transcription. Past work has shown that Vpr induces double-stranded DNA breaks by structurally altering DNA in a manner that is Topo I and ATR dependent (98), supporting a mechanism in which Vpr might inhibit Topo I activity to activate ATR signaling. In addition, camptothecin has immediate effects on rRNA processing (72), corroborating a role for CCDC137 in this pathway, though it remains unclear whether which, if either, camptothecin mechanism Vpr phenocopies.

CCDC137 may act as a sensor of nucleolar disruption with implications for cancer progression. CCDC137 has been classified as an oncogenic RNA binding protein, but its cellular functions and precise roles in cancer progression are only beginning to be understood. Our results reveal that CCDC137 is depleted as a consequence of ATR-dependent nucleolar stress. Depletion of CCDC137 itself, however, does not result in cell cycle arrest, DNA damage, or nucleolar stress. Identifying the exact nucleolar pathways in which CCDC137 participates is the next outstanding question. Considering that the nucleolus is a cellular stress sensor, it is possible that CCDC137 degradation, like NPM1 redistribution, represents an early step in this stress response. However, it is also possible CCDC137 degradation is a consequence of specific nucleolar pathways being disrupted. Our proteomic and STRING network analyses suggest that CCDC137 may be involved in rRNA processing, the inhibition of which has been identified as a stress-dependent regulatory event in

the maintenance of nucleolar integrity (99). In line with this, other nucleolar proteins depleted by Vpr – RRP36 and UTP11 – are involved in pre-rRNA processing. This would support a model in which CCDC137 acts as a nucleolar stress sensor, is depleted in response to specific ATR-dependent stress signaling, and contributes to a pause in rRNA processing that triggers the eventual downregulation of other ribosome biogenesis pathways. Given that unprocessed rRNA is stored in the nucleolus (99), this may explain why we see increased nucleolar size with Vpr and other agents that degrade CCDC137. This storage mechanism allows for the resolution of nucleolar stress and eventual return to normal function; if the nucleolus cannot coordinate this pause in rRNA processing with other steps of ribosome biogenesis, however, it can enter a state of irreversible stress that leads to cell cycle arrest and death (100). It is this disconnect in the ability to resume versus not recover homeostasis that may explain why some Vpr proteins are able to degrade CCDC137 without further downstream consequences, such as cell cycle arrest. Such a disconnect would also have important implications in cancer. For instance, if CCDC137 is acting as a sensor of nucleolar stress that helps dampen nucleolar processes when depleted, it is plausible that CCDC137 overexpression would prevent this ATR-dependent nucleolar inhibition. In line with this, ribosome biogenesis is hyperactivated in cancer and has been causally associated with malignant transformation and cancer progression (101). Although there is much exploration to be done in eliciting the exact pathways involved, this study supports that CCDC137 may be a potential therapeutic target whose depletion may slow oncogenesis. Moreover, it clarifies the DNA damage pathways that influence the nucleolus and recognizes various genotoxic agents that may be used to target CCDC137 and impede nucleolar processes.

Vpr is one of many diverse viral proteins that may hijack the nucleolus to enhance viral replication. Finally, our findings raise the question of how Vpr-induced nucleolar stress might favor viral replication. Viral disruption of the nucleolus is a broad phenomenon, and numerous proteins from both plant and animal viruses localize to the nucleolus, redistribute nucleolar proteins for use in viral replication, and either enhance or inhibit ribosome biogenesis (27, 102). For instance, in herpes simplex virus infection (HSV1), nucleolar proteins localize to viral replication centers and co-localize with the viral ICP8 protein to enhance viral replication (103). Similarly, the infectious

bronchitis coronavirus (IBV) N protein localizes to the nucleolus (104), and IBV results in nucleolar enlargement similar to that seen with Vpr (105).

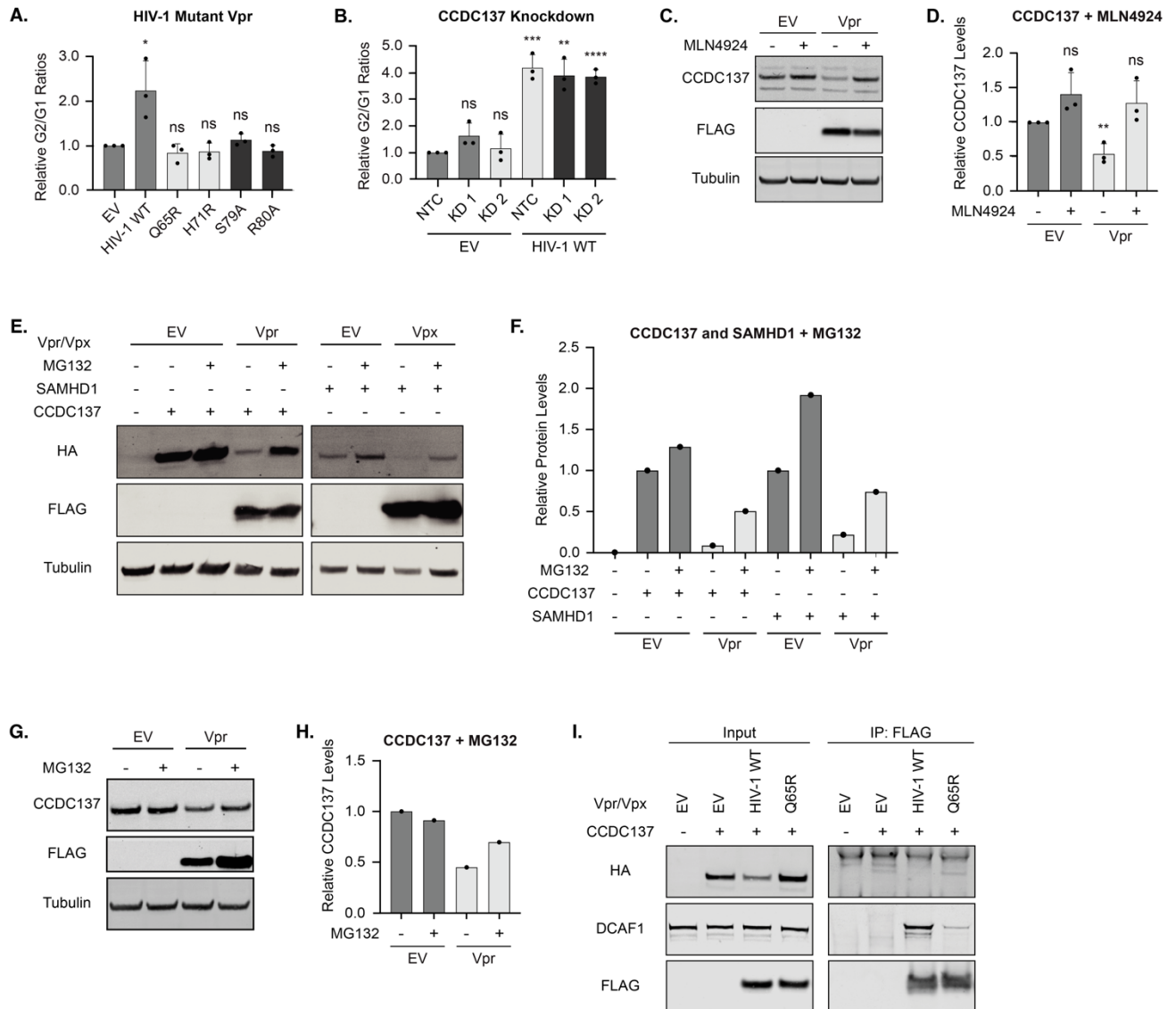
In the context of HIV, the nucleolar localizations of both trans-activating proteins Tat and Rev are crucial to HIV-1 replication (106–108). Within this subnuclear compartment, Tat impairs rRNA processing (109) and remodels the nucleolar proteome (110), and Rev facilitates the export of un- and partially spliced viral RNA (111). How the effects of Vpr on the nucleolus coordinate with these functions is unknown, though previous studies connecting Vpr to translational impairment may provide insight into such an interplay. Vpr is reported to repress cellular translation via a mechanism that does not impede the translation of viral mRNAs (112), and in doing so may ready nucleolar processes for optimal Tat and Rev exploitation. In addition, NPM1 mediates the nucleolar retention of Tat and prevents its association with the super elongation complex (113), and it is conceivable that Vpr-induced NPM1 redistribution may increase Tat nucleoplasm localization to enhance transcriptional elongation. This is consistent with the proposed role of CCDC137 depletion in HIV-1 transcription (17), which may occur as part of the stress response that releases Tat. Future work will be necessary to dissect how Vpr-induced nucleolar stress influences other HIV processes and impacts viral replication.

While there is still much to understand about the roles of CCDC137, Vpr, and the nucleolus in viral replication and cellular homeostasis, our data begin to unravel these complex connections. Moreover, we have characterized CCDC137 as an early sensor of nucleolar insults, and, in doing so, have opened new avenues for discovering further CCDC137 functions and pathways involved in the nucleolar stress response.

DATA AVAILABILITY

The CCDC137 alignment data underlying this article are available in FigShare at <https://figshare.com/> and can be accessed with <https://doi.org/10.6084/m9.figshare.26841640> and <https://doi.org/10.6084/m9.figshare.26841634>.

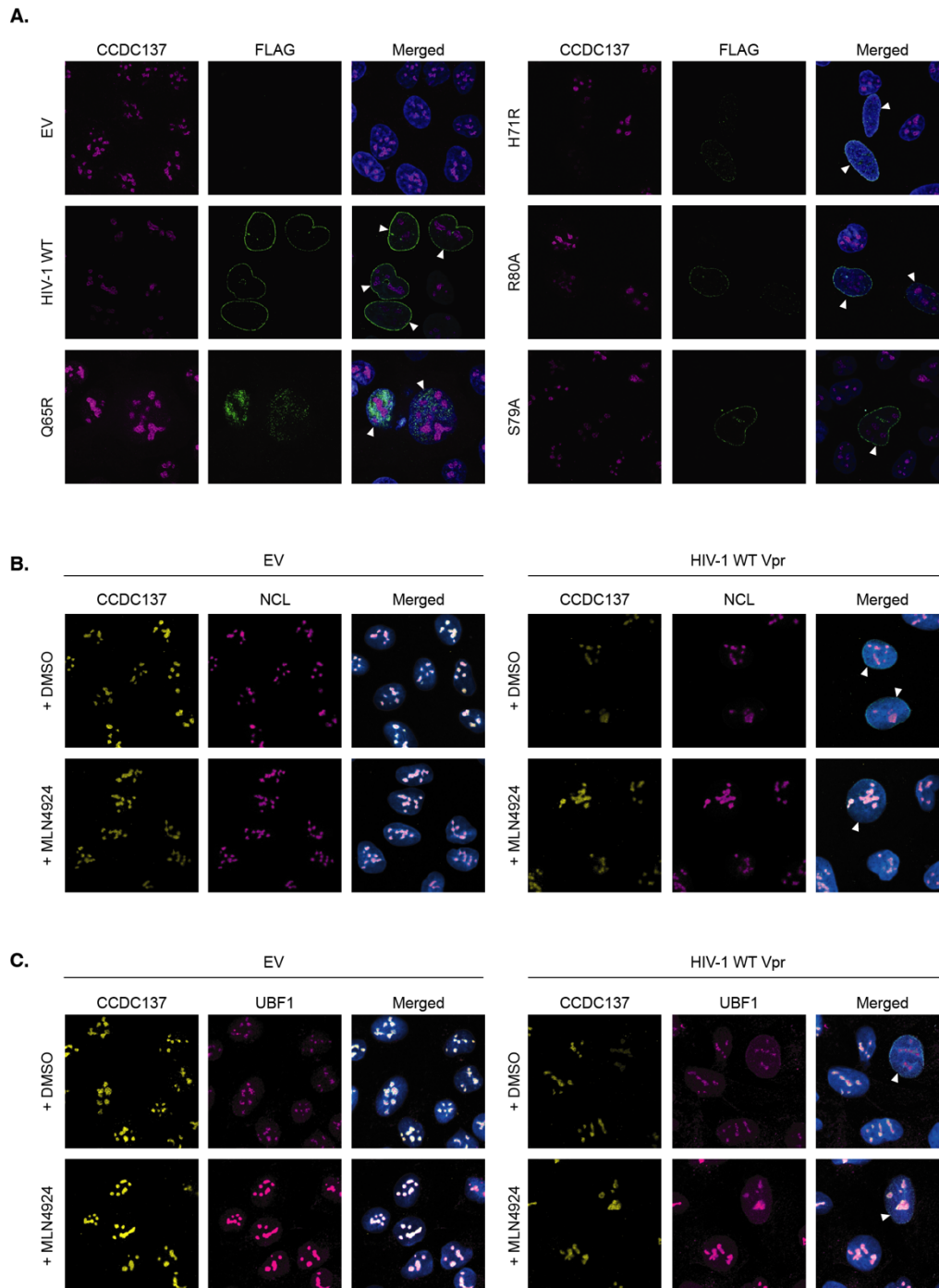
SUPPLEMENTARY DATA



Supplement Figure 1. CCDC137 degradation is independent of G2/M arrest and CRL4-E3-ubiquitin ligase complex co-option.

(A) Quantification of G2/G1 values of U2OS cells infected with the indicated rAAV WT or mutant Vpr calculated as in Figure 1 (A). Data from 3 biological replicates are shown. (B) Quantification of G2/G1 values for the indicated U2OS knockdown cell lines infected with EV or WT Vpr as in (A). (C) Representative western blot of endogenous CCDC137 levels in U2OS cells expressing EV or WT Vpr and treated with 10 μ M MLN4924 or DMSO. (D) Quantification of (C) as described for Figure 2 (B) ($n=3$). (E) Western blot showing HA-CCDC137 or HA-SAMHD1 levels following transient co-transfection with HIV-1 Q23-17 Vpr or HIV-2 Rod9 Vpx, respectively, or empty vector control in the presence of 10 μ M MG132 or DMSO. (F) HA-CCDC137 and HA-SAMHD1 levels in (E) quantified and normalized as in Figure 2 (B). (G) Representative western blot and (H) quantification

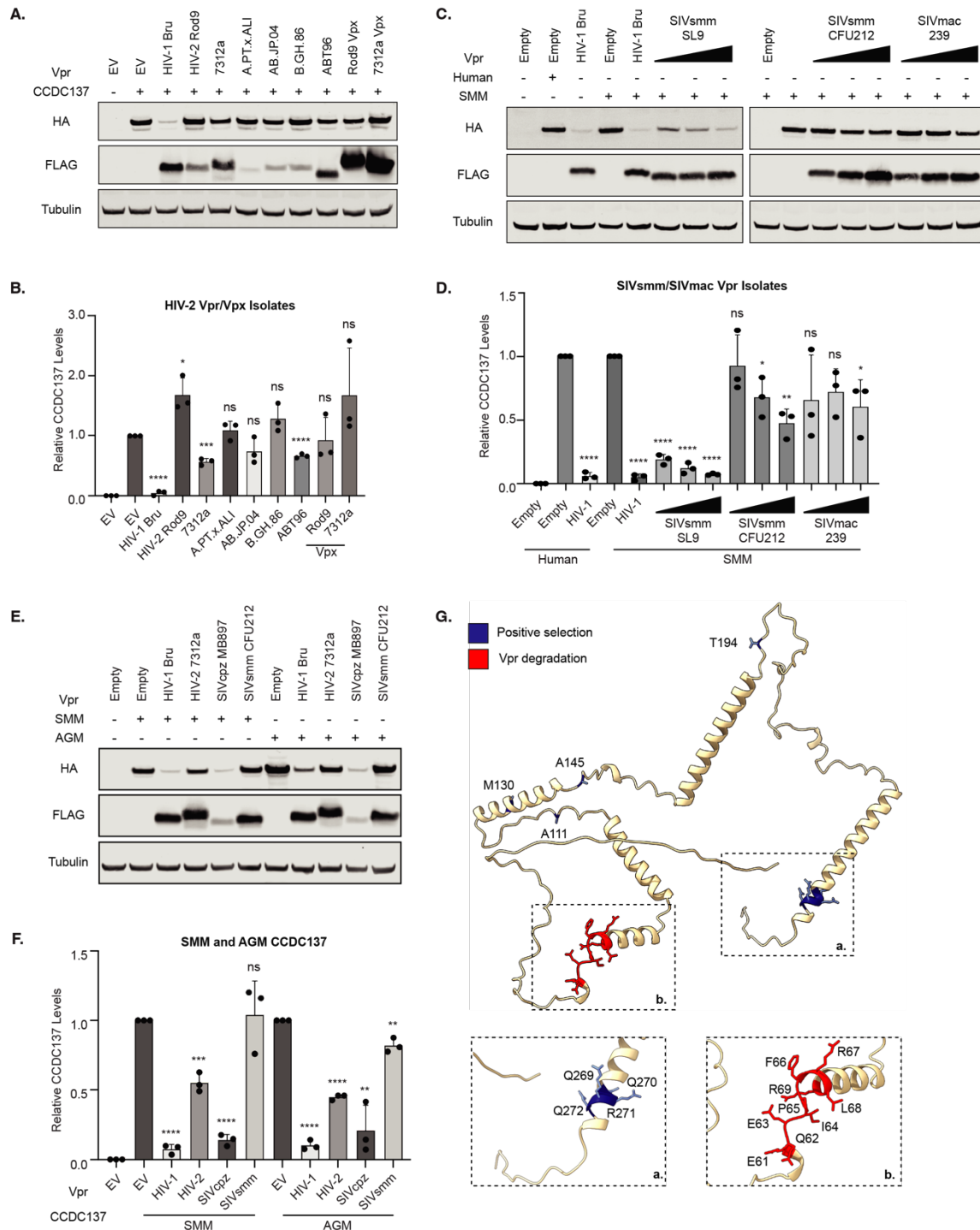
of endogenous CCDC137 levels in the presence of EV or WT Vpr in cells treated with 10 μ M MG132 or DMSO. (I) Co-immunoprecipitation assays against 3X-FLAG Vpr in U2OS cells treated with 10 μ M MLN4924, probed for HA, DCAF1 or 3X FLAG Vpr. All results were analyzed by unpaired t-tests. Asterisks indicate statistical significance from empty vector control; error bars represent $\pm$ standard deviation (ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$). EV=empty vector. (A-B) are related to Figure 1. (C-I) are related to Figure 2.



Supplement Figure 2. CCDC137 localizes to the nucleolar exterior.

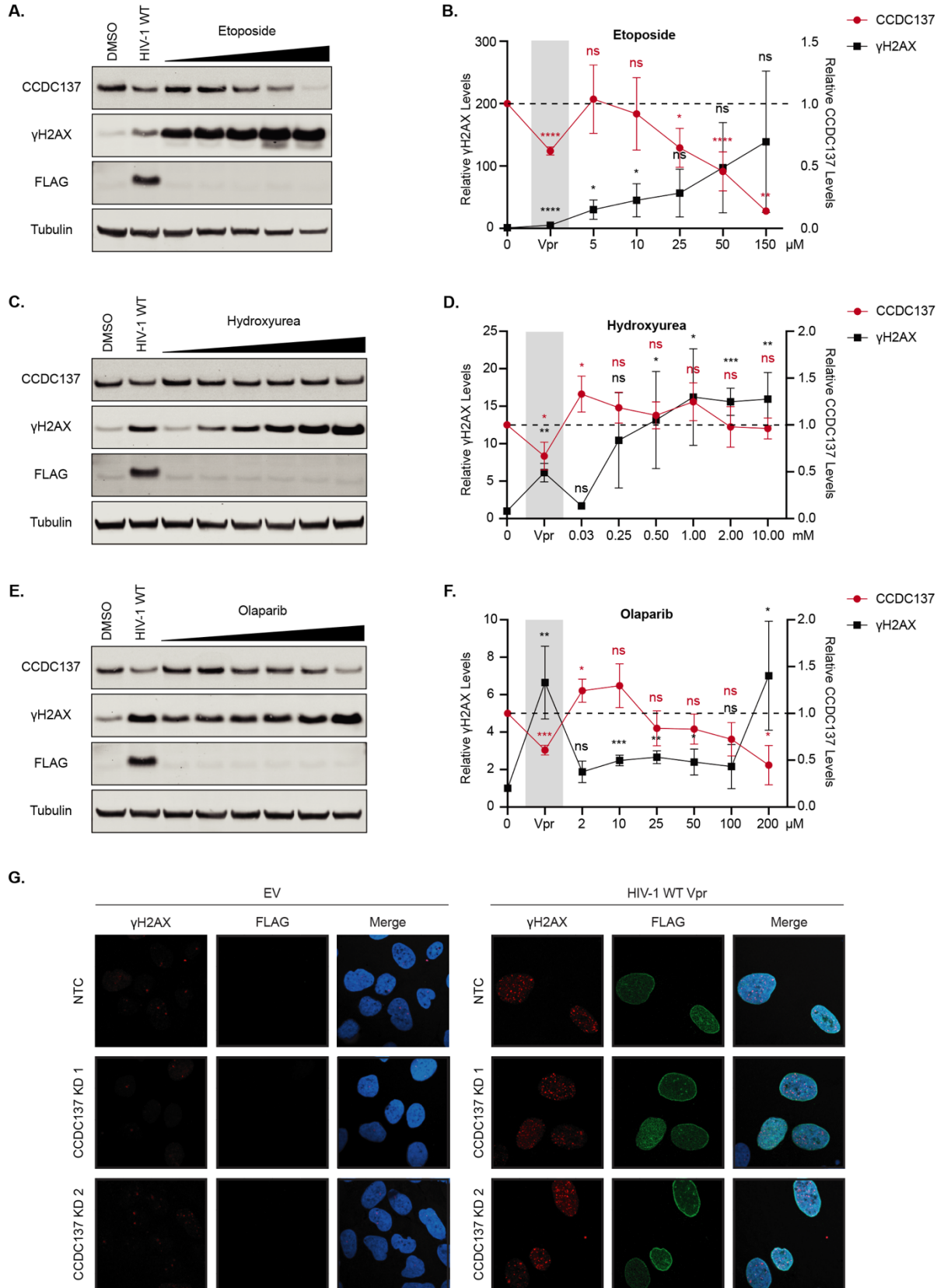
(A) IF images of U2OS cells infected with rAAV expressing the indicated Vpr or mutants. Cells were permeabilized prior to fixation at 28hpi among all conditions except for the Q65R mutant, for which these steps were performed in reverse due to this mutant's exclusive presence in the soluble fraction. Cells were probed for endogenous CCDC137 (magenta), 3X-FLAG (green) and DAPI (blue) and imaged at 63X. **(B)** IF images of U2OS cells infected with EV or HIV-1 WT Vpr rAAV in the

presence of 10 μ M MLN4924 or DMSO. Cells were permeabilized prior to fixation at 28hpi and probed for CCDC137 (yellow), NCL (magenta), 3X-FLAG (green) and Hoechst (blue) and imaged at 63X. **(C)** IFs performed as in (B) and probed for CCDC137 (yellow), UBF1 (magenta), 3X FLAG (green) and Hoechst (blue) and imaged at 63X. For (A-C), Vpr-expressing cells are indicated by white triangles in the merged images. Related to Figure 2.



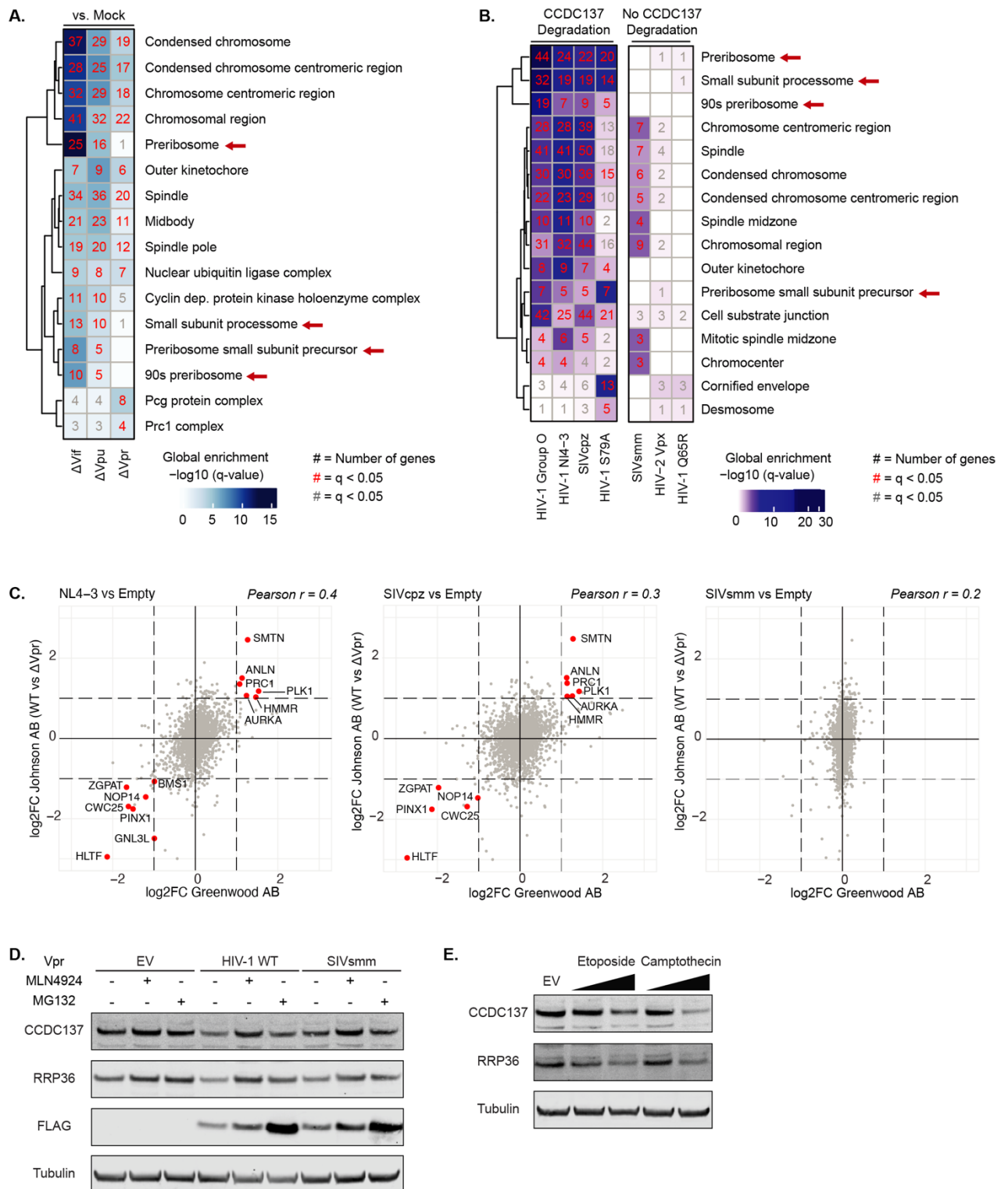
Supplement Figure 3. CCDC137 exhibits signatures of longstanding evolutionary antagonism.

(A) Representative western blots of human HA-CCDC137 degradation by HIV-2 Vpr isolates probed and (B) quantified as in Figure 3. (C) Representative western blots and (D) quantification of human or sooty mangabey HA-CCDC137 degradation by SIVsmm or SIVmac Vpr isolates. (E) Representative western blot and (F) quantification of sooty mangabey (SMM) and African Green monkey (AGM) HA-CCDC137 degradation by a panel of HIV-1 Bru, HIV-2 7312a, SIVcpz MB897 and SIVsmm CFU212 Vpr orthologs. (G) Alphafold predicted protein structure of CCDC137 labeled with PS marks (blue) and a Vpr binding site (red). Insets represent the variable C-terminus (a.) and a Vpr binding site (b.). All results were analyzed by unpaired t-tests. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from empty vector control; ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$. (A-D) Related to Figure 3. (E-G) Related to Figure 4.



Supplement Figure 4. CCDC137 depletion is a consequence, but not a driver, of specific genomic insults.

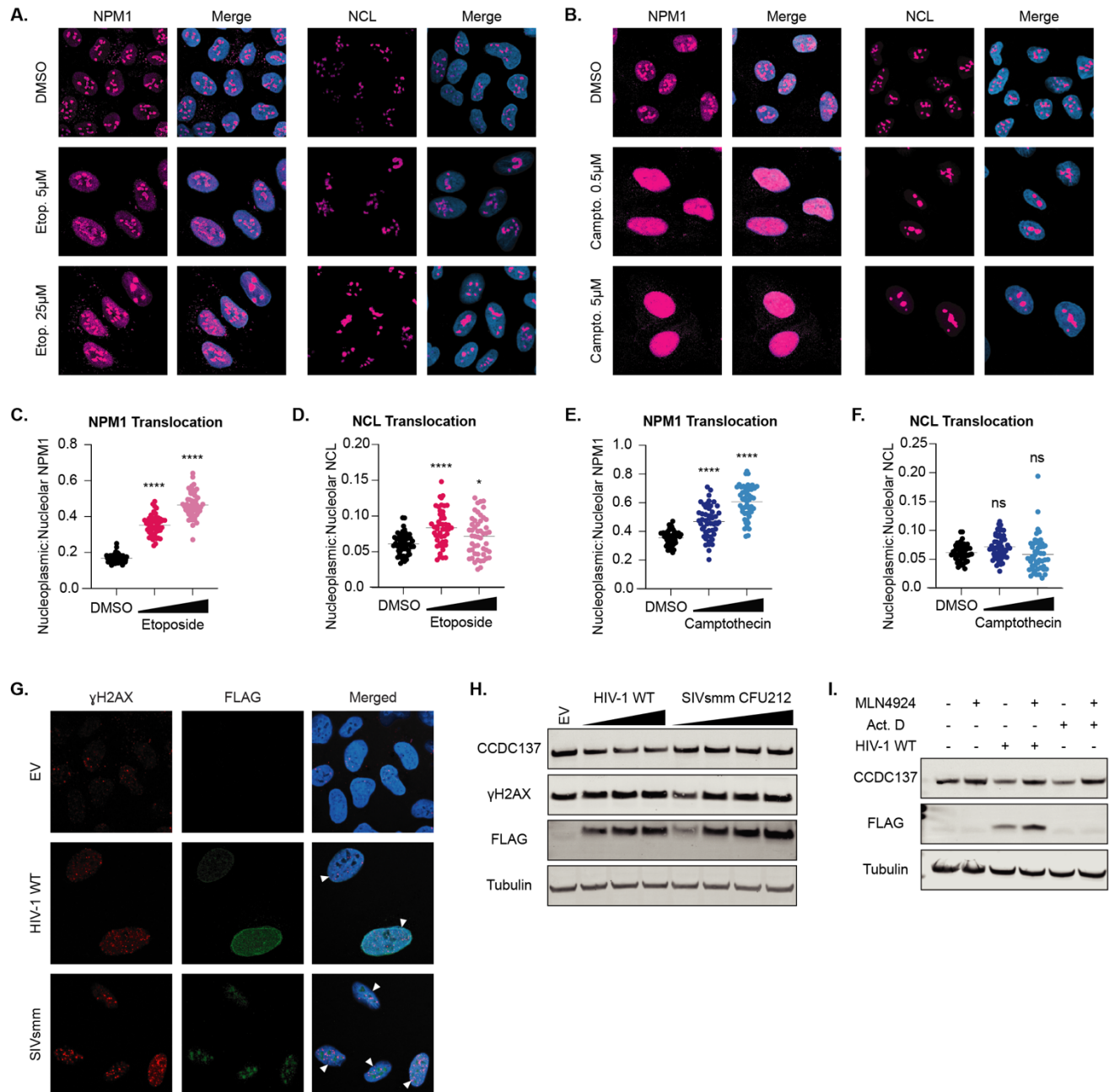
Representative western blots and quantification of endogenous CCDC137 and γ H2AX levels following 24hr of treatment with DMSO, HIV-1 WT Vpr or titrating amounts of etoposide (**A-B**), hydroxyurea (**C-D**), or olaparib (**E-F**). Western blots were probed and quantified as in Figure 5 (A-D). Quantification is representative of three separate experiments ($n=3$). (**G**) IF images of the indicated CCDC137 knockdown U2OS cell lines infected with EV or HIV-1 WT Vpr rAAV. Cells were permeabilized prior to fixation, probed for γ H2AX (red), 3X-FLAG (green) and Hoechst (blue), and imaged at 63X. All results were analyzed by unpaired t-tests. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from empty vector control; ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$. Related to Figure 5.



Supplement Figure 5. Vpr depletes proteins involved in nucleolar processes.

(A) Enriched GO terms by GSEA (adjusted p-value < 0.05) of differentially expressed genes in the Johnson *et al.* dataset ($\log_2\text{FC} > 0.5$). Top terms represent Cellular Components. All numbers refer

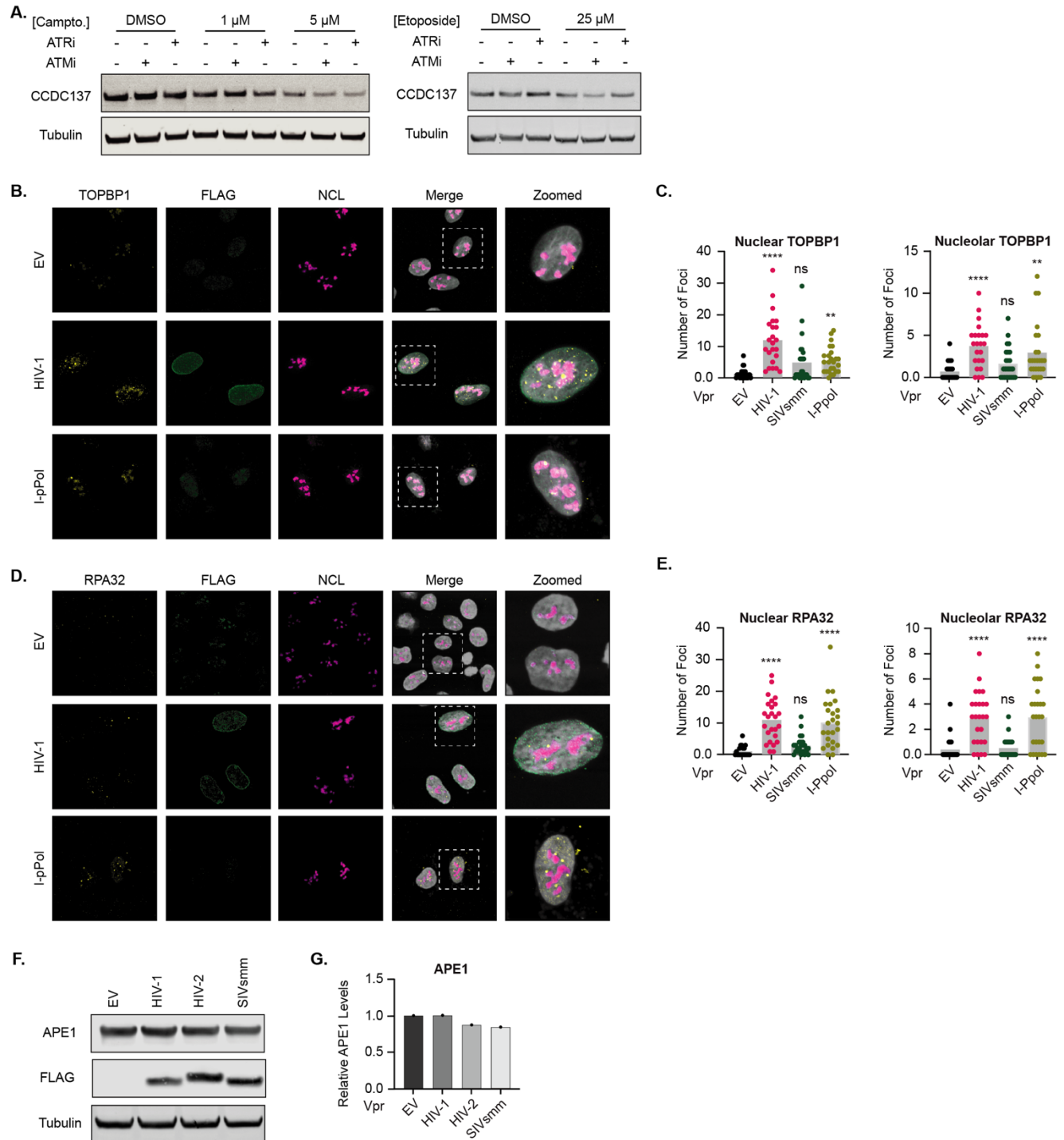
to the number of differentially expressed genes in a particular overrepresented GO term, and red numbers show gene sets with an adjusted p-value < 0.05. **(B)** Enriched GO terms by GSOA (adjusted p-value < 0.05) of differentially expressed genes in the Greenwood *et al.* dataset ($\log_2FC > 0.5$). Top terms represent Cellular Components. Numbers refer to the number of differentially expressed genes in a particular overrepresented GO term, and the red numbers show gene sets with an adjusted p-value < 0.05. **(C)** Up- or down-regulated proteins identified in both the Johnson *et al.* and Greenwood *et al.* abundance datasets. Jitter plots show comparisons between $\log_2FC$ values from HIV-1 NL4-3 vs Empty, SIVcpz vs Empty, or SIVsmm vs Empty (X-axis), and the $\log_2FC$ values from WT vs ΔVpr comparison (Y-axis). Red labeled dots represent genes that belong to the nucleolus term in the GO Cellular Component ontology and meet the following requirements: adjusted p-values ≤ 0.05 , and $\log_2FC > 1$ or $\log_2FC < -1$. Linear relationship between datasets was calculated using Pearson correlation coefficient. **(D)** Western blot images of CCDC137 and RRP36 levels in U2OS cells infected with EV, HIV-1 Vpr, or SIVsmm Vpr rAAV in the presence of 10 μ M MLN4924, 10 μ M MG132, or DMSO. **(E)** Western blot images of CCDC137 and RRP36 levels in U2OS cells treated with 5 μ M or 25 μ M etoposide or 0.5 μ M or 5 μ M camptothecin for 24hr. Related to Figure 6.



Supplement Figure 6. CCDC137 degradation is not correlated to total amount of DNA damage.

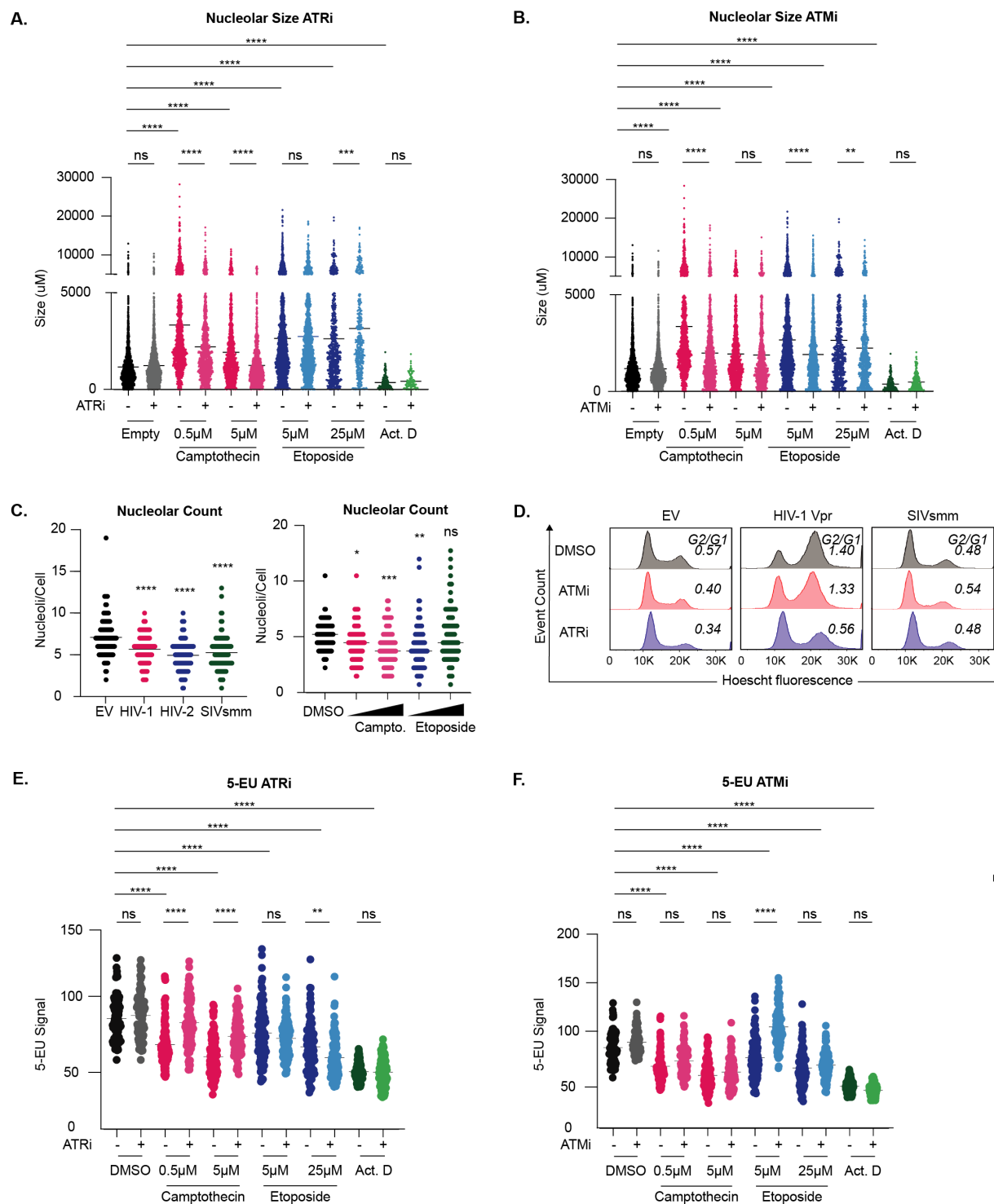
(A) Representative IF images of NPM1 and NCL localization in U2OS cells treated with 5 μ M or 25 μ M etoposide and (B) 0.5 μ M or 5 μ M camptothecin prepared as in Figure 6A. (C) Quantification of NPM1 or (D) NCL nucleoplasmic translocation displayed in (A) for etoposide treated cells and NPM1 (E) and NCL (F) nucleoplasmic translocation displayed in (B) for camptothecin treated cells as described in Figure 7B. (G) IF images of γ H2AX levels in U2OS cells treated with EV, HIV-1 WT, or SIVsmm Vpr. Cells were permeabilized prior to fixation and probed for γ H2AX (red), 3X-FLAG (green) and Hoescht (blue). Vpr-expressing cells are indicated by white triangles in the merged

images. **(H)** Western blot of endogenous CCDC137 and γ H2AX levels induced by SIVsmm and HIV-1 WT Vpr at 28hpi performed as in Figure 5. **(I)** Western blot of endogenous CCDC137 levels in cells treated with EV (- HIV-1 WT Vpr lanes) or HIV-1 Q23-17 Vpr in the presence of 5nM Actinomycin D (Act.D) and/or 10 μ M MLN4924. Campto. = camptothecin; Etop. = etoposide. Related to Figure 7.



Supplement Figure 7. Vpr induces nuclear and nucleolar markers of ATR activation.

(A) Western blots of endogenous CCDC137 levels in the presence of the indicated concentrations of camptothecin or etoposide incubated with DMSO, 10 μ M ATMi or 10 μ M ATRi following a 24hr incubation. (B) Representative immunofluorescence images of U2OS cells treated with the indicated conditions probed for TOPBP1 (yellow), 3X-FLAG (green), NCL (magenta) and Hoechst (grey). Images taken at 63X. Dashed white boxes indicate individual cells digitally magnified in the Zoomed panel. (C) Quantification of nuclear or nucleolar TOPBP1 foci in at least 25 cells prepared as in (B) using Fiji software. Each circle represents one cell. (D) IF images prepared as in (B) probed for RPA32 (yellow), 3X-FLAG (green), NCL (magenta) and Hoechst (grey). (E) Quantification of RPA32 foci in cells prepared as in (D) as described for TOPBP1 in (C). (F) Western blot images of APE1 levels in U2OS cells expressing the indicated Vpr orthologs harvested at 24hpi. (G) Quantification of APE1 levels in (F) as described in Figure 1C. Results in (C) and (E) were analyzed with a one-way ANOVA with Dunnett's multiple comparisons test. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from the empty vector control (ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$). EV = empty vector. Campto. = camptothecin. Related to Figure 8.



Supplement Figure 8. Camptothecin and Etoposide induced ATR and ATM dependent nucleolar stress phenotypes.

(A-B) Quantification of nucleolar size experiments in ATRi (A) or ATMi (B) treated cells. U2OS cells were treated as Figure 8A and nucleolar area was measured for 100-200 cells per condition. Each

dot represents a single cell. **(C)** Quantification of nucleolar number among U2OS cells incubated with the indicated Vpr or genotoxic agent as in Figure 8(D-E) and (A-B). The number of nucleoli/cell was measured for ~100 cells per condition. Results were analyzed with a one-way ANOVA with Dunnett's multiple comparison test. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from empty vector or DMSO control; ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$. **(D)** Representative flow cytometry plots of one cell cycle arrest experiment performed as in Figure 1A following 24hr incubation with the indicated Vpr and ATMi or ATRi. **(E-F)** Quantification of 5-EU experiments in ATRi (E) or ATMi (F) treated cells. Total MFI was measured for 100-200 cells per condition. Results were analyzed with a one-way ANOVA with Šidák's multiple comparison test. Error bars represent $\pm$ standard deviation, and asterisks indicate statistical significance from empty vector control or cells subject to the same treatment conditions without inhibitor (ns = not significant, * $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$, **** $P < 0.0001$). EV = empty vector; Campto. = camptothecin; Etop. = etoposide. Related to Figure 8.

File Supplement 1: Detailed primate CCDC137 evolutionary analysis. Statistical results for the Detection of Genetic INNovations (DGINN) pipeline applied to 23 simian CCDC137 sequences. Bold values indicate tests that pass statistical thresholds for the indicated analysis. Bold sites are those that passed multiple analyses for significance of positive selection (PS), as indicated in "Summary of sites under PS" column. See Methods section for further details on the individual analyses performed. Related to Figure 4.

AUTHOR CONTRIBUTIONS

Karly Nisson: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. Rishi S Patel: Formal analysis, Investigation, Writing – review & editing. Lucie Etienne: Conceptualization, Data curation, Formal analysis, Investigation, Resources, Writing – review & editing. Yennifer Delgado – Formal analysis, Investigation. Mehdi Bouhaddou – Formal analysis, Resources, Supervision. Oliver Fregoso: Conceptualization, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Chapter 4: Conclusion

Lentiviruses encode multiple factors that modulate the DDR, including the accessory gene Vpr, for which DDR engagement is a conserved phenotype (1). Despite the identification of several host DDR repair proteins degraded by Vpr, none can fully explain how Vpr engages the DDR at multiple, unique steps, which include: induction of DNA damage, activation of the DDR, repression of DSB repair and G2/M arrest (2, 3). Moreover, how Vpr-induced DNA damage alters the nuclear environment to favor viral replication remains unclear. In this dissertation, I explored how Vpr-mediated activation of ATM and ATR signaling leads to downstream cellular stress phenotypes that may enhance viral infectivity. In **Chapter 2**, I established that Vpr-induced DNA damage activates ATM-NEMO signaling to alter cellular transcription via NF-KB/RelA. In **Chapter 3**, I clarify the relationship between Vpr-mediated G2/M arrest and CCDC137 degradation and identify CCDC137 depletion as a signature of ATR-dependent nucleolar stress. These findings provide insight into 1) Vpr function, which has thus far remained elusive, 2) the mechanisms by which viruses activate the DDR and other stress pathways to create a cellular environment that supports lentiviral replication, 3) novel nucleolar biology and 4) the potential to leverage Vpr function to target HIV+ cells.

Vpr activates ATM-NEMO dependent NF-KB activation independent of G2/M arrest

Both ATM and ATR can be activated or repressed in response to viral infection depending on the distinct viral context. In HIV infection, Vpr has been shown to activate ATM through the induction of DSBs and markers associated with ATM signaling (2). Specifically, HIV-1 and HIV-2 Vpr activate gH2AX, an early marker of DNA damage, and 53BP1, a marker that is recruited to DSBs by gH2AX and is indicative of ATM signaling (4). Prior to the work I discuss in Chapter 2, whether Vpr activated canonical ATM signaling, or dysregulated this branch of the DDR remained an open question. Similarly, a link between Vpr-induced ATM activation and NF-KB signaling was unclear, and the literature contained – and still contains – conflicting reports as to whether Vpr activates or represses NF-KB signaling (5–8). In this Chapter we show that Vpr activates canonical ATM

signaling and does so independent of its ability to cause G2/M arrest. While we use live-cell imaging to show that DDR repair factors involved in ATM signaling accrue at sites of damage as expected, the timing of this lesion resolution remains unknown. Future work is necessary to define the temporal nature of Vpr-induced DNA damage, particularly in the context of Vpr-mediated repression of DSB repair.

We further clarify that Vpr activates NF- κ B, specifically via the ATM-NEMO pathway in U2OS and primary human monocyte-derived-macrophages. Yet, how the activation of an inflammatory pathway might be advantageous for lentiviral infectivity is not fully understood. One possibility may lie in the HIV-1 5'LTR, which contains two overlapping NF- κ B binding sites that enhance proviral transcription when bound by NF- κ B (9). Such a model – in which Vpr enhances NF- κ B transcription to drive proviral gene expression – would temporally fit with Vpr-mediated DNA damage and ATM-NEMO signaling occurring early in the lentiviral lifecycle, prior to the expression of Tat. This timing is supported by our finding that Vpr packaged in the incoming virion, and thus active in early infection, is sufficient to drive these changes. In line with this, Vif, once sufficiently expressed from the viral promoter, inhibits NF- κ B signaling (10), perhaps to limit prolonged inflammatory signaling that would otherwise restrict viral replication. A more extensive characterization of NF- κ B chromatin binding, particularly at the HIV-1 5'LTR, via chromatin immunoprecipitation sequencing will be necessary to unravel whether NF- κ B is directly binding and activating HIV gene expression. Moreover, determining when in the viral lifecycle NF- κ B driven proviral transcription occurs, as well as the specific consequences of prolonged ATM-NEMO signaling on viral restriction, will be necessary subjects of future work.

Vpr activates an ATR-dependent nucleolar stress pathway to deplete CCDC137

Perhaps the most well-studied Vpr phenotype is its ability to cause ATR-dependent cell cycle arrest at the G2/M checkpoint (11). Yet, following decades of research, the host target(s) Vpr depletes to induce this arrest remain elusive. In Chapter 3 I investigate the relationship between

Vpr and a host nucleolar protein, CCDC137, whose depletion has been linked to this conserved Vpr phenotype. I demonstrate that, while associated with Vpr-mediated G2/M arrest, CCDC137 degradation is neither a direct cause or consequence of this arrest. Moreover, it is not depleted via canonical Vpr co-option of the E3 CRL4^{DCAF1} ubiquitin ligase complex. Yet, our evolutionary analyses of this degradation indicate that it is a conserved function of Vpr from the HIV-1 lineage – the pandemic-causing HIV – and is species specific. In line with this, CCDC137 exhibits signatures of longstanding evolutionary antagonism.

Our evolutionary work suggested that CCDC137 degradation was either itself important to the function of HIV-1 Vpr, or a consequence of another important HIV-1 Vpr phenotype. In exploring the latter possibility, I discovered that specific types of DNA damage – particularly those that induce nucleolar stress – are sufficient to induce CCDC137 depletion. I show for the first time that Vpr is sufficient to induce such nucleolar stress, and Vpr-mediated alterations to the nucleolar proteome are unique among the lentiviral accessory genes. Finally, I demonstrate that CCDC137 degradation is triggered by a nucleolar ATR pathway that appears to be unique from global ATR activation.

This work raises some fascinating questions about Vpr, CCDC137 and nucleolar biology, particularly in the context of ATR signaling. For instance, the particular advantages ATR-dependent nucleolar stress might confer to lentiviral replication remain unknown. It is possible that these advantages resemble those of Vpr-induced NF- κ B/ RelA signaling, in which Vpr may trigger an inflammatory response to enhance proviral transcription, though the precise mechanisms and factors that might underlie such a connection remain unclear. Moreover, whether this response might be mitigated later in infection, and how this may occur, is unknown. Vif-mediated inhibition of Vpr-induced ATM signaling does not extend to ATR signaling, for instance, suggesting either that there is no avenue for the curtailment of this phenotype, or that it is carried out by an alternate viral protein. Alternatively, Vpr-mediated nucleolar stress may dysregulate the

innate immune response to create nuclear conditions conducive to viral replication. In support of this, the nucleolus has been reported as the site for inflammatory RNA decay during infection (12), suggesting that Vpr-mediated nucleolar stress may interfere with the ability of the infected cell to maintain inflammatory gene expression integrity. Understanding whether the nucleolar perturbations induced by Vpr inhibit innate immune signaling will be exciting subjects of later studies.

Further, understanding the exact role of ATR signaling in Vpr-mediated nucleolar stress will be an important area of future work. While Vpr is known to activate ATR to induce ATR-dependent G2/M arrest, it is unclear whether the nucleolar ATR pathway underlying CCDC137 degradation is an extension of this pathway, an instance of Vpr-mediated ATR dysregulation, or a consequence of damage induced specifically within rDNA. Our findings that HIV-1 Vpr, but not a Vpr ortholog capable of depleting CCDC137, SIVsmm Vpr, induce significant numbers of nucleolar ATR foci suggests a link between nDDR activation and CCDC137 depletion. Whether this is the primary trigger of the ATR-dependent nucleolar stress response we observe, however, will require further investigation. Importantly, such investigations into the Vpr-induced nDDR would benefit from the application of super-resolution microscopy, which may help better distinguish damage foci that have been excluded from the nucleolus for repair.

Finally, this study draws important attention to the pathways that might define the oncogenic potential of CCDC137 and identifies CCDC137 as nucleolar stress sensor. CCDC137 overexpression is increasingly linked to tumorigenesis, though the mechanisms underlying such a connection are unclear. The work discussed in Chapter 3 connects CCDC137 to ATR-dependent nucleolar stress signaling and clarifies that CCDC137 is a component of, but not a driver of, this response. Like CCDC137, Vpr depletes other nucleolar proteins RRP36 and UTP11 via a cullin-dependent mechanism. Importantly, these proteins, as well as many of those depleted by Vpr in our proteomic analyses, are implicated in rRNA processing. The idea that this processing

step may sense and convey nucleolar stress is supported by the discovery that early rRNA processing is a stress-dependent regulatory event (13). Whether Vpr is causing defects in this processing, and whether such defects are linked to ATR activation, however, are unknown and will be important areas of future investigations.

Vpr-mediated DDR activation may directly enhance HIV transcription

A common theme underlying the questions raised by both Vpr-induced ATR and ATM activation is that of their relevance to lentiviral replication. Overall, inflammatory responses, including the DDR, can exacerbate viral replication (14, 15). While the advantages conferred by Vpr-induced DDR signaling are most likely complex, beneficial to multiple stages of the lentiviral lifecycle, and accompanied by antiviral effects, I will discuss here a potential model in which Vpr-induced DDR signaling directly augments proviral transcription.

A potential explanation for how Vpr-induced damage activates the 5' LTR may lie in the ability of DNA damage repair factors to function as transcriptional activators. For instance, DNA damage that occurs at gene promoters can influence transcriptional activation (16), and multiple mammalian cell types have been found to “schedule” DNA damage to achieve targeted transcription (17, 18), involving both ATM and ATR. Moreover, over 120 TFs, including those canonically regarded as “pioneer” factors, are recruited to damage foci (19), suggesting the exciting possibility that DNA repair foci may harbor the factors necessary to activate the initial, post-integration transcription of the HIV-1 LTR. Such foci have been found to form through rapid loop extrusion (20) that generates a topologically associated domain (TAD) that directs gH2AX spreading. This may help concentrate repair and transcription factors in a single “center” that may favor viral replication; a mechanism by which an integrated provirus associates with a DDR TAD in the 3D nuclear space might explain how HIV Vpr-induced damage promotes activation of the 5' LTR. Future studies considering this model will need to determine whether DDR repair factors

co-localize with the 5'LTR, and whether damage foci and/or the 5'LTR mobilize within the nuclear space.

Vpr-mediated DDR engagement hypersensitizes HIV-1 infected cells to genotoxic agents

Many of the efforts to develop HIV therapies have focused on specifically targeting different steps of the viral lifecycle as opposed to leveraging the effects of the virus on the host cell. In considering the latter approach, Vpr has been shown to hypersensitize cells to DNA damaging agents(2). This ability presents a promising opportunity to selectively clear infected cells expressing Vpr with genotoxic agents already approved for clinical use, though a better understanding of the pathways altered in Vpr-induced hypersensitivity will be necessary to further such a HIV therapy. This work clarifies the nature of these pathways and defines their downstream cellular effects, including NF- κ B activation and nucleolar stress, that might be attractive targets for future therapies. Importantly, this work also suggests that Vpr-induced ATR signaling induces phenotypes similar to those induced by the topoisomerase I inhibitor Camptothecin, indicating a potential mechanism by which Vpr induces DNA damage. Understanding how Vpr causes DNA damage, as well as how Vpr represses the DDR, will be imperative in optimizing HIV treatments that exploit Vpr function.

Overall, this dissertation clarifies the nature and consequences of Vpr-induced DDR engagement, specifically in the context of ATM and ATR activation. Moreover, it reveals a link between the oncogene CCDC137 and ATR-mediated nucleolar stress, furthering our understanding of nucleolar stress signaling and positioning CCDC137 as a novel nucleolar stress sensor. Importantly, these findings extend beyond our knowledge of Vpr, and even lentiviruses: they dually explore how viruses hijack the host DDR and uncover novel host biology, thus bringing us closer to novel antiviral and cancer therapeutics alike.

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