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DNA unwinding by Viral Protein R Initializes Complicated Cellular Responses in HIV-1 Infection: Defining the Viper's First Bite

Kenta Iijima, and Yukihito Ishizaka*

Department of Intractable Diseases, National Center for Global health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo, 162-8655, Japan

***Corresponding author:** Yukihito Ishizaka, Department of Intractable Diseases, National Center for Global health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo, 162-8655, Japan, **E-mail:** zakay@ri.ncgm.go.jp

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Abstract

Vpr, an accessory gene of human immunodeficiency virus (HIV) type-1 that encodes a virion-associated protein, induces multiple cellular responses, *e.g.* transcriptional regulation, chromatin modulation and the DNA damage response with cell-cycle checkpoint activation. Vpr may promote HIV-1 infection of quiescent cells, including resting macrophages, but it remained elusive how Vpr contributes to viral infection into these cells. With the object of clarifying the role of Vpr, we first summarize the pleiotropic function of Vpr in the HIV-1 life cycle and subsequently the recently identified mode of Vpr-induced DNA damage triggered by unwinding of DNA in association with viral DNA integration. Finally, we discuss the role of Vpr in HIV-associated diseases, which are important issues for HIV-1–infected patients in the post-antiretroviral therapy era.

Keywords: HIV-1, Vpr, DNA damage, Integration, Macrophage

Introduction

Combined antiretroviral therapy (cART) for human immunodeficiency virus (HIV)-1 can suppress viral replication, such that viral DNA is at a non-detectable level in the blood of HIV-1–positive patients, and can prevent the immunodeficiency caused by T-cell depletion. After interruption of the cART regimen, however, viral replication readily resumes in long-lived reservoir cells [1]. Because macrophages are the major cell type involved in the formation of viral reservoirs, it is important to understand the mode of viral infection of resting macrophages [2,3].

Viral protein R (*Vpr*), an accessory gene of HIV-1, encodes a virion-associated nuclear protein of 96 amino acids and is proposed to facilitate viral infection of macrophages [4-8]. Structural analyses revealed that Vpr contains three α-helices with self-dimerisation/oligomerisation activity and a flexible carboxyl (C)-terminal region that includes a stretch of basic amino acids with DNA-binding activity [9-11]. Vpr possesses pleiotropic functions for stimulating viral replication in multiple steps [12,13]. Following viral entry into a target cell, the viral RNA is reverse transcribed into linear double-stranded DNA (dsDNA) in the cytoplasm [14]. The synthesized viral genomic DNA (vDNA) is wrapped in viral proteins to form a pre-integration complex (PIC), subsequently translocated to the nucleus and integrated into the host chromosome. The integration step of the vDNA is catalyzed by integrase (IN), which assembles at both ends of vDNA to form a strand–transfer complex, which facilitates establishment of a lasting infection [15,16]. The integrated proviral DNA serves as a template for the transcription of viral genes and is propagated along with the host genome.

Vpr induces a cell-cycle abnormality during the G_2/M phase as well as a variety of DNA damage responses (DDR) involving phosphorylation of histone H2AX and focus formation by multiple DNA repair proteins, and activation of ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR); central kinases response to DNA double-strand break (DSB) and single-strand break (SSB)/replication stress, respectively [17-23]. Regarding to the mechanism by which Vpr-induced G_2/M arrest promotes viral infection, it was proposed that Vpr provides a cellular environment suitable for viral gene transcription and/or delays cell death during viral replication [12,13,24]. In contrast, the biological importance of Vpr in resting (G_0/G_1 phase) macrophages is unclear. Smith *et al.* and others reported that DSBs promote HIV-1 infection and/or integration of vDNA [8,25,26]. We also demonstrated that Vpr-induced DSBs provide sites for vDNA integration [27]. Although the mode of Vpr-induced DSBs has been unknown, we recently discovered that Vpr alters DNA structure and activates both the ATM and ATR pathways [27]. The findings revealed that unwinding of dsDNA by Vpr leads to supercoiling of DNA, accumulation of topoisomerase 1 (Topo1) and DSBs. Of note, unwinding of dsDNA recruits replication protein A (RPA) 70, a single-stranded DNA (ssDNA)-binding subunit of the RPA complex, which activates ATR-dependent DDR [28]. Because DSBs may induce multiple cellular responses, including apoptosis and immune activation, it is likely that Vpr-induced DSB is the molecular basis of multiple acquired immunodeficiency syndrome (AIDS)-related symptoms.

Here we summarize the biological functions of Vpr related to DSBs and discuss the potential of Vpr as a target of candidate anti–HIV-1 compounds for treating HIV-1–positive patients.

1. Vpr regulates Viral Infection at Multiple Steps

1a. Reverse transcription

HIV particles enclose viral proteins that include the nucleocapsid (NC), matrix (MA), IN, reverse transcriptase (RT), Vif, Vpr and Nef proteins [12,13]. Vpr is incorporated into viral particles through its association with the p6 domain of Gag [29]. After entry into a target cell, the viral RNA is reverse-transcribed in the cytoplasm into a linear dsDNA by the reverse transcription complex, which contains two copies of viral genomic RNA, as well as the RT, IN, NCp7, MA, Vpr and cellular proteins [15]. Vpr associates with the reverse transcription complex and modulates the quantity and quality of HIV-1 RT, an error-prone RNA-dependent DNA polymerase [15]. Without Vpr, the newly synthesized vDNA has a high frequency of mutations [30,31]. Quality control by Vpr is in part dependent on uracil DNA glycosylase (UNG) 2, which excises uracil bases from DNA [32] (Figure 1). UNG2 is required for vDNA synthesis, because uracil bases in DNA can result from misincorporation of dUTP or deamination of cytosine. Notably, Vpr associates with and incorporates UNG2 into viral particles. The involvement of UNG2 in Vpr-dependent mutation suppression was demonstrated using a VprW54R-UNG2 fusion protein [32]. The tryptophan residue of Vpr at amino acid 54 is essential for the interaction with UNG2 and its incorporation into viral particles. When the VprW54R-UNG2 fusion protein was incorporated into viral particles, the mutation rate of vDNA was decreased with comparable to that of Vpr wild-type virus. Interestingly, however, a catalytically inactive mutant of UNG2 fused to VprW54R also suppressed mutation in cells infected with the Vpr-defective virus. Further analysis revealed that p32, a subunit of the RPA complex that associates with UNG2, prevents mutation of vDNA by a mechanism different from that of UNG2 [32]. Although the precise role of p32 is unclear, it may protect vDNA from host nucleases.

In contrast, UNG2 suppresses vDNA synthesis [33-35]. UNG2 converts APOBEC3G-induced deaminated cytosines to a basic site, leading to cleavage by apurinic/apyrimidinic endonuclease. Thus, Vpr induces proteasomal degradation of UNG2. The roles of UNG2 in viral replication are controversial, but Vpr contributes to vDNA synthesis by modulating both functions of UNG2.

Gleenberg *et al.* reported that Vpr interferes with reverse transcription by directly interacting with RT [36], whereas Lyonnais *et al.* observed that Vpr induced the bridging and condensation of synthesized DNA to promote its nuclear import [37]. Although the biological importance of Vpr and RT is not fully understood, the close functional associations among Vpr, RT and IN may link reverse transcription and nuclear import, followed by chromosomal integration.

1b. Nuclear import and transactivation of the long terminal repeat

Vpr may promote HIV-1 replication in resting macrophages, but it is dispensable for virus replication in proliferating cells [4-8]. In resting macrophages, vDNA must be imported into the nucleus independently of cell division and without disrupting the nuclear membrane. vDNA associates with both viral and host proteins to form the PIC. Among the four viral proteins present in the PIC (RT, IN, MA and Vpr), Vpr reportedly facilitates nuclear import of the PIC by directly binding to importin-α [38,39] (Figure 1). Alternatively, Vpr may shuttle between the cytoplasm and nucleus using an exportin-1-dependent nuclear export signal [40]. Nucleocytoplasmic shuttling by Vpr is required not only for incorporation of Vpr in newly produced virions but also for efficient replication of HIV-1 in tissue macrophages. Vpr associates with the nuclear pore complex, particularly the nucleoporins p54, p58 and hCG1, and accumulates in the nuclear envelope [41,42]. Interestingly, integration of HIV-1 DNA occurs in the nucleus close to nuclear pores, where a series of cellular genes are transcriptionally active [43,44]. It is important to determine whether Vpr regulates vDNA integration into transcriptionally active chromatin by interacting with the nuclear pore complex, because the chromatin status at the time of viral integration is a critical determinant of the life cycle of HIV-1 [45].

Vpr transactivates the long terminal repeat (LTR) of HIV-1, which promotes viral replication and pathogenesis [12,13,46] (Figure 1). Vpr was initially reported to reactivate latent proviral DNA, and several activating elements—NF-AT, glucocorticoid response elements (GREs), NF-κB, Sp1, transactivation response element and a TATA box—were subsequently identified in the U3 region of the HIV-1 LTR [46]. Vpr interacts with the Sp1–DNA complex and may transactivate the Sp1-bound promoter by stabilising Sp1–promoter complexes [47]. Sawaya *et al.* reported that Vpr transactivates LTR in an Sp1-dependent manner, whereas p53 suppresses transactivation of the LTR [48]. Moreover, Vpr inhibited the transcriptional activity of p53, which was proposed to promote replication of HIV-1–infected cells without inducing apoptosis [48]. Vpr binds and augments the activity of several transcription factors, *e.g.* glucocorticoid receptor (GR), transcription factor IIB and p300 [12,13,46]. Vpr complexes with GR and transactivates the HIV-1 LTR and GRE-containing promoters in a manner dependent on the LXXLL GR-binding motif at amino acids 64–68 (helix-2) of Vpr [49]. Additionally, association of Vpr with p300 and TFIIH cooperatively induces GRE activation [50]. Furthermore, Vpr forms a tertiary complex of Vpr–GR–poly (ADP-ribose) polymerase-1 (PARP-1), and suppresses NF-κB by preventing the nuclear localisation of PARP-1, a co-activator of NF-κB [51]. However, Vpr reportedly activates NF-κB by enhancing the phosphorylation of TAK1 and IKK. These findings imply that Vpr modulates NF-κB activity to promote viral replication [52,53].

Vpr activates LTR throughout viral infection and replication. Hoshino *et al.* showed that extracellular Vpr activates NF-κB in a TLR4/MyD88-dependent manner and induces IL-6 production with subsequent reactivation of latent HIV-1 [54]. Vpr induces reactivation of LTR in latently infected cells by promoting proteasomal degradation of histone deacetylases 1 and 3, which leads to silencing of viral genes [55,56].

Additionally, Vpr in cooperation with Tat transactivates the HIV-1 LTR by concomitantly occupying the multiple binding sites within the LTR [57]. Notably, Vpr but not Tat enhances Nef expression from unintegrated vDNA, indicating a role for Vpr during the early stage of viral infection [58].

1c. Induction of apoptosis

Vpr disrupts mitochondrial function and activates the intrinsic apoptosis pathway by associating via its C-terminus with two candidate factors of the permeability transition pore complex (PTPC): adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC) [59,60] (Figure 1). Jacotot *et al.* reported that synthetic Vpr induced mitochondrial membrane permeabilisation by the PTPC [59]. Furthermore, the C-terminus of Vpr cooperates with Bax to increase the permeability of ANT-containing liposomes. The Vpr-induced mitochondrial membrane permeabilisation leads to release of cytochrome c (cyt c) from the intermembrane space. The resulting apoptosome composed of cyt c, dATP, Apaf-1 and procaspse-9 activates procaspase-9 and -3, which mediate apoptotic

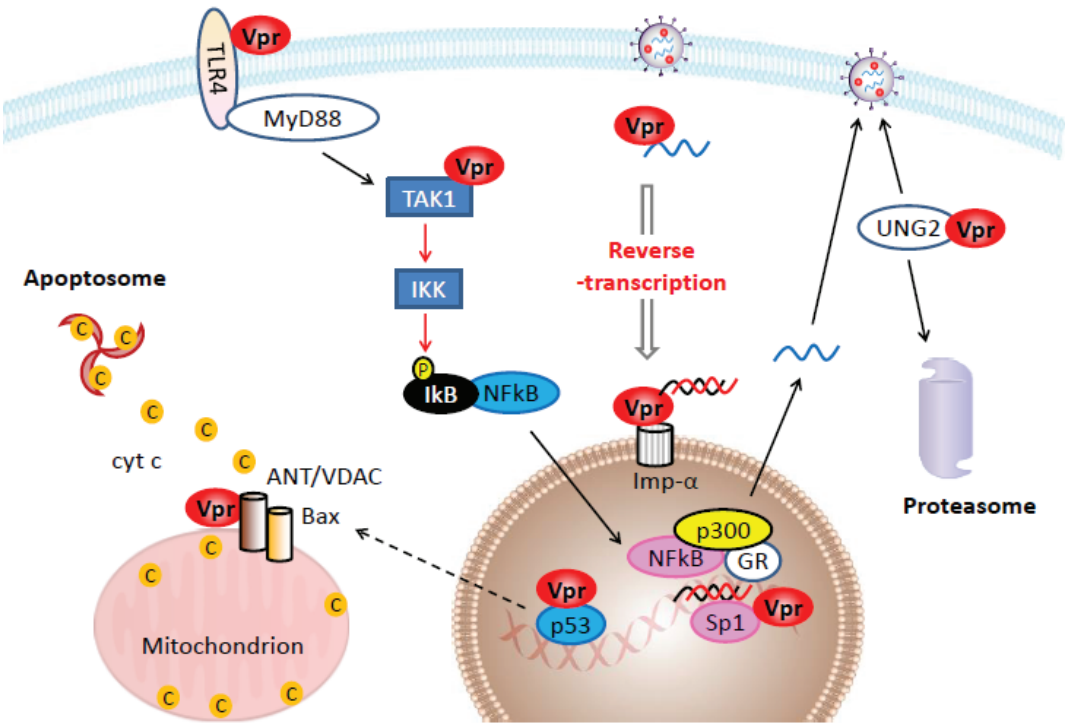

Figure 1: Involvement of Vpr in viral replication at multiple steps.

After the entry of HIV-1, viral RNA (blue wavy line) associates with Vpr is reverse-transcribed in double strand vDNA (black and red wavy lines). Vpr facilitates the incorporation of vDNA through interaction with importin-α (Imp-α). Vpr promotes the viral genes (or genomic RNA) transcription from LTR by utilizing cellular transcriptional factors. Transcribed genomic RNA is incorporated to new virions. At this time, Vpr transfers UNG2 (and RPA32) to new virions, which suppresses frequency of mutation rate in subsequent reverse-transcription. On the contrary, Vpr promotes proteasome-mediated degradation of UNG2. Extracellular Vpr stimulates TLR4/MyD88 pathway, which leads to activation of IKK, followed by NFκB nuclear translocation. Besides to association with TLR4, Vpr directly activates by interacting with TAK1, leading to NFκB activation. By binding to ANT/VDAC, Vpr induces the release of cyt c from mitochondrial inter-membrane space to cytoplasm. Complex of Apaf-1 and cyt c (apoptosome) activates capspase-9,-3 dependent apoptotic cell death. This intrinsic apoptosis induction is facilitated by Bax expression, while Vpr is known to downregulated Bax by inhibiting p53 activity. A part of figure was generated through the use of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

DNA fragmentation. Although a clear apoptotic cascade was proposed based on *in vitro* data [59], Vpr may play different roles *in vivo* because Vpr-induced apoptosis was prevented by preincubation of Vpr with DNA or RNA [59]. Anderson *et al.* reported that Vpr-induced apoptotic cell death is dependent on Bax, but not on ANT [60]. Moreover, Bax activation requires DDR activation, which involves ATR-mediated phosphorylation of BRCA1 and subsequent upregulation of GADD45α [61]. However, whether Bax-mediated apoptosis is coupled to Vpr-induced DDR activation is controversial. In contrast, Conti *et al.* reported that low levels of Vpr exerted an anti-apoptotic effect in CD4+ T-cell lines by upregulating Bcl-2 and downregulating Bax [62]. Thus, the role of Vpr in different cell types warrants further investigation.

1d. Vpr modulates the immune response

Vpr modulates the immune response directly and indirectly. Exogenous Vpr secreted by infected cells inhibits the immune response by inducing apoptosis and cell-cycle arrest [12,13]. Regarding the indirect effects, Vpr increases the expression of TNF-α in dendritic cells, which leads to induction of apoptosis in CD8+ T-cells [63]. Additionally, Vpr upregulates unique-long 16 binding proteins 1 and 2 (ULBP1/2), ligands for natural killer group 2 member D (NKG2D), in CD4+ T cells with concomitant activation of ATR kinase [64-66] (Figure 2). Upregulation of ULBP1/2 increases the cytolytic activity of NK cells and promotes clearance of infected cells. In contrast, the increased and decreased TGF-β and IL-12 production, respectively, in Vpr-affected CD4+ T cells suppresses the activity of NK cells by downregulating CD107α and interferon (IFN)-γ [67,68] (Figure 2). Vpr exerts complex effects on the immune system, and its influence on clearance of infected cells should be further investigated to understand the role of Vpr in T-cell depletion.

Transcriptomic analyses of monocyte-derived macrophages and dendritic cells revealed that Vpr affects the cellular immune response by upregulating IFN-stimulatory genes (ISGs): *MX1, MX2, ISG15, ISG20, IFIT1, IFIT2, IFIT3, IFI27, IFI44L* and *TNFSF10* [69,70] (Figure 2). Importantly, in CD4+ cells, Vpr increased the expression of type I IFNs and ISGs induced by cGAS-dependent sensing of HIV-1 DNA, which could contribute to hyperimmune activation and the progression of AIDS [71]. Interestingly, treatment of mouse macrophage-like cells with recombinant Vpr induced IFN-β production by activating the cGAS/STING pathway and mobilizing long interspersed element-1

(LINE-1), an abundant endogenous retrotransposon [72]. Proteomic analysis of Vpr-regulated proteins in monocyte-derived macrophages revealed that Vpr disturbs glucose and glutamate metabolism [73]. Vpr modulates the glycolytic pathway by increasing the expression of pyruvate kinase muscle type 2, which phosphorylates tyrosine 705 of STAT3, which can subsequently upregulate the HIV-1 LTR [73]. Notably, dysregulation of glutamate metabolism, including downregulation of glutamate dehydrogenase 2 and upregulation of glutaminase C, may be linked to HIV-associated neurocognitive disorder (HAND) via the neurotoxic effects of glutamate [74] (Figure 2). Together with its pro-apoptotic effect on neurons, these results suggest Vpr to be a target for novel therapeutic strategies for HAND [75-80].

2. Cell-Cycle Abnormality during the G_2/M Phase and Activation of the DNA Damage Response

Cells are equipped with sophisticated system responding to DNA damages to counteract cytotoxic effects by exogenous and endogenous DNA damages, including DNA single- or double-strand breaks, chemical modifications and replication stress. Among enormous numbers of players in DDR, ATM, ATR and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) function as the most upstream kinases and activate the downstream events including repair of DNA damages and activation of cell-cycle checkpoint [23]. The association between retroviral integration and DDR was initially investigated by Daniel *et al.* [81,82]. IN catalyses vDNA integration by transferring the 3'-terminus of vDNA to nicked host genomic DNA, because the IN-induced flanking nicks on both strands of DNA are recognised as DSBs by the cellular DNA repair machinery [15,81,82]. Given that the ligation of vDNA to host DNA requires processing of DNA ends, retroviral DNA integration must involve the host DNA repair pathway, as observed in the process of removing flapped DNA strands by FEN1 [83]. Additionally, numbers of evidence indicate the indispensable role of cellular DSB repair pathway. DSB repair by DNA-PK-dependent non-homologous end joining is required for vDNA integration and the survival of infected cells [84]. Lau *et al.* reported that the activity of ATM is required for survival of HIV-1–infected CD4+ T cells [85]. Data indicate that microhomology-mediated end joining (*i.e.* using short patch of homologous DNA sequences at the ends of DSBs) is involved in vDNA integration or the repair of IN-generated DSBs [86].

Importantly, DNA damage induced by external factors increases viral infectivity, particularly in quiescent cells including resting macrophages [8,25,26]. The HIV-1 LTR is more active during the G_2 phase, implying that Vpr-induced cell-cycle arrest optimizes the cellular environment for viral replication [12,13,24]. However, Vif, an

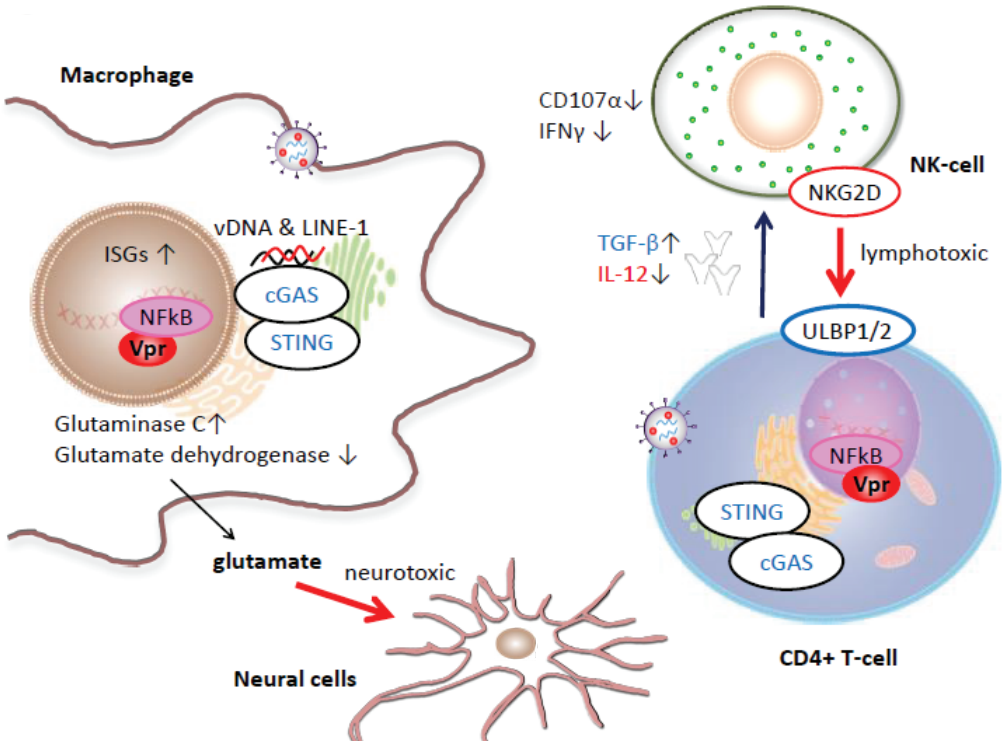

Figure 2: Vpr modulates both intra- and extra-cellular environment

HIV-1 infection to macrophages evokes cGAS/STING and NFκB activation, which is enhanced by Vpr. Resulting upregulation of ISGs facilities tissue inflammation. Furthermore, Vpr induces dysregulation of glucose and glutamate metabolism. Increased concentration of extracellular glutamate shows neurotoxic effect and induces neural cell death. By the infection of HIV-1 to CD4+ T-cells, ATR activity is elevated in Vpr dependent manner. ATR activation induces upregulation of ULBP1/2, ligands of NKG2D, by which cytotoxic activity of NK-cells is increased, while Vpr dysregulates the production of cytokines from HIV-1–infected CD4+ T-cells. Thus, Vpr suppresses the activity of NK-cells, which is characterized by the reduced expression of CD107α and IFNγ. TGF-β, an immune suppressive cytokine; IL-12, an immune stimulative cytokine. A part of figure was generated through the use of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

HIV-1 accessory protein, induces G_2/M cell-cycle arrest, suggesting that promotion of viral infection via induction of DDR is not unique to Vpr [87]. Additionally, the biological importance of Vpr-induced DDR in resting macrophages is unclear, because these cells are in G_0/G_1 phase and express low levels of ATR and Chk1 [21]. Smith *et al.* and others reported that DSBs promote HIV-1 infection and/or integration of vDNA [8,25,26]. We also reported that DNA damaging agents promote viral infection of resting cells [8,27]. Moreover, DSB sites could facilitate vDNA integration by providing a platform for vDNA integration [8,27]. Notably, even the IN-D64A mutant virus, which is deficient in IN activity, could integrate into DSB sites. Although DSB-directed viral integration is a small population of the whole viral infection, it may hamper complete virus elimination by cART [8,27].

2a. ATR activation with Vpr-induced ubiquitination

Vpr was initially reported to inhibit the proliferation of HIV-1–infected T cells at the G_2/M phase by hyperphosphorylating CDC2, a cyclin-dependent kinase complexed with cyclin B [88]. Vpr-induced cell-cycle arrest is dependent on ATR kinase and accompanied by activation of a typical DDR, including phosphorylation of histone H2AX and formation of BRCA1, 53BP1, RAD51 and FANCD2 foci [17-22,89]. Although Vpr induced a DDR including focus formation of DSB repair proteins and accumulation of RPA [21,27], ATR has been mostly highlighted for approaching the mode of Vpr-induced DDR, because ATM is not essential for Vpr-induced G_2/M arrest [21].

Vpr-induced DDR requires ubiquitination of cellular protein(s) by Vpr-binding protein (VprBP)/ DNA damage-binding protein 1 (DDB1), components of the cullin E3-ubiquitin ligase complex [90-93]. Depletion of VprBP or DDB1 abrogates Vpr-induced DDR activation, and the Q65R mutant of Vpr, which is unable to bind to VprBP, did not induce a DDR [90,91]. Belzile *et al.* reported that Vpr-induced DDR required K48-linked ubiquitination of cellular proteins, indicating a pivotal role for proteasomal degradation [93]. Despite much effort, the factors responsible for Vpr-induced DDR and authentic targets of Vpr-dependent ubiquitination are not known. UNG2 was first identified as a DDR-associated factor that is degraded by Vpr-induced ubiquitination [94]. Because UNG2 mediates base-excision repair, Vpr-induced degradation of UNG2 may increase the magnitude of DNA damage [94]. This scenario fits to helicase-like transcription factor (HTLF), a translocase involved in repair of damaged replication forks [95]. Klockow *et al.* reported that Dicer, an endonuclease subunit of RNA-induced silencing complex (RISC) that may suppress viral replication, is a candidate target of Vpr-induced ubiquitination [96]. Small RNAs are generated by RISC immediately after DNA damage and activate the DDR [97-99]. Thus, DNA damage would be increased if the RISC system is impaired by Vpr. Additionally, telomerase reverse transcriptase, an RT subunit of telomerase, may be a target of Vpr-induced ubiquitination [100]. If so, Vpr may destabilize telomeric DNA. It is possible that such Vpr-induced telomeric DNA instability is linked to the chromosomal breakage/fusion/bridge cycle, as observed in Vpr-expressing cells [101].

2b. Involvement of the replication fork and SLX4 in the Vpr-induced DDR

Our understanding of Vpr function was recently advanced by the identification of SLX4 as a Vpr-binding factor [89]. SLX4 is a structure-specific endonuclease that complexes with Mus81/Eme1 and plays roles in resolving DNA replication and recombination intermediates [102,103]. Consequently, downregulation of SLX4 induces the DDR as a result of accumulation of DNA damage. In contrast, Laguette *et al.* proposed that the SLX4 complex is prematurely activated by Vpr and directly induces the DDR by cleaving DNA strands [89]. In addition to DDR activation, Vpr-activated SLX4 degrades excess vDNA to prevent the activation of cellular immune sensing, promoting viral replication [89].

DNA replication may be involved in the Vpr-induced DDR because the latter resembles the DDR caused by the DNA-synthesis inhibitors hydroxyurea and aphidicolin [21]. Indeed, Vpr-induced DDR depends on ATR, a kinase activated by RPA-coated ssDNA during DNA replication. Interestingly, Romani *et al.* identified minichromosome maintenance (MCM) 10, a component of the MCM complex with roles in DNA replication initiation and strand elongation, as a novel target of Vpr [104]. They demonstrated that Vpr induces proteasomal degradation of MCM10 as a required step of Vpr-induced cell-cycle arrest.

3. Integrated Mode of the Vpr-Induced DDR and DSBs

Vpr induces cell-cycle arrest at the G_2/M phase by ATR-dependent activation of the DDR [17,21], DDB1/VprBP-dependent ubiquitination [90-93], stalled replication folk [21], and recruitment of RPA and SLX4 [21,27,89]. However, the critical molecular event responsible for these cellular responses is unclear. We discovered that Vpr alters the structure of dsDNA, which may uncover the links among these factors [27]. An overview of our findings follows (Figure 3).

3a. Unwinding of dsDNA is the initial step in the Vpr-induced DDR

First, atomic force microscopy and supercoiling 294 assays revealed that Vpr induces morphological changes in supercoiled DNA [27]. A mutational analysis indicated that the four arginines in the C-terminal stretch of Vpr are required for the topological changes. Moreover, Vpr induces loading of RPA70 onto dsDNA, by unwinding of dsDNA (Figure 3). These findings support our hypothesis that the structural alternation of dsDNA by Vpr triggers RPA-dependent activation of ATR.

To demonstrate that Vpr plays a similar role in cells, we used a LacO/LacI system in human cells [105,106]. Using this system, LacR-fused Vpr can be tethered to the chromosome region containing the LacO array [27]. We first analyzed whether Vpr unwinds the corresponding region using psoralen, a DNA-intercalating cross-linker that preferentially binds to negatively supercoiled DNA [107,108]. LacR-fused Vpr increased the unwinding of dsDNA at the LacO repeats in a C-terminal stretch-dependent manner. Next, we analyzed RPA70 accumulation on the LacO repeats by chromatin immunoprecipitation assay. The results showed that RPA70 accumulation is also dependent on the C-terminal stretch of Vpr. Because formation of RPA-coated ssDNA triggers ATR activation, Vpr-induced unwinding of dsDNA could lead to DDR activation.

3b. Mechanism of DSB induction by Vpr

In vitro, Vpr increased the supercoiling of DNA. Accumulation of supercoiled DNA recruits Topo1, which relaxes supercoiled DNA by cleavage/ligation cycle for regulating the DNA replication, transcription and repair [109]. Excess accumulation of Topo1 leads to stable formation of the covalent complex of Topo1 and DNA (Topo1-cc), which induces DSBs by colliding with the replication fork or transcriptional machinery [110]. In quiescent cells, DSBs are likely induced by the latter mechanism.

As expected, Topo1-cc accumulated in Vpr-expressing cells (Figure 3). Consistently, the number of DSBs was increased by Vpr expression in a Topo1-dependent manner [27]. Using the LacO/LacI

system, we demonstrated that Topo1-cc and DSBs accumulate at Vpr-tethered LacO repeats. Moreover, vDNA was integrated at Vpr-induced DSB sites, indicating a role for Vpr in providing sites for vDNA integration. The importance of Vpr-directed viral integration is unknown because it accounts for only small fraction of overall HIV-1 infection. Vpr-directed viral integration may influence the selection of integration sites. Thus, Vpr accumulation at centromeres through its interaction with HP1 is interesting [111], because the heterochromatic status of these regions likely contributes to latent viral infection [112,113].

Consistent with previous reports that Topo1 dysfunction leads to formation of DNA/RNA hybrids (R-loop) during transcription [114], Vpr expression promoted accumulation of R-loops [27]. Furthermore, the overexpression of RNaseH1, which degrades R-loops, reduced DDR activation by Vpr. Although the mechanism of Vpr-induced accumulation of Topo1-cc is unknown, spontaneous generation of Topo1-cc might be augmented by Vpr-induced negative supercoiling of DNA and/or production of reactive oxygen species due to mitochondrial dysfunction. Oxidative modification of DNA traps the Topo1–DNA complex at the ligation step [115].

3c. Histone H2B is a candidate target of Vpr-dependent ubiquitination

We analyzed the mobility of histone H2B in chromatin using GFP-tagged histone H2B by a fluorescence recovery after photobleaching assay [27,116]. Expression of wild-type, but not a ubiquitination-deficient mutant (Q65R), Vpr significantly increased the mobility of histone H2B (Figure 3). Interestingly, Q65R was defective in RPA70 loading, but competent in unwinding dsDNA. Consistently, Vpr induced ubiquitination of histone H2B at lysine 120, a target residue of DDB1/VprBP-dependent modification [27,117]. Finally, treatment with trichostatin A, an inducer of chromatin relaxation, recovered the defective loading of RPA70 in cells expressing the Vpr Q65R mutant [27,118]. These data suggest that histone H2B is a target of Vpr-dependent ubiquitination, and chromatin remodelling by ubiquitination of histone H2B facilitates RPA loading of chromatin.

3d. SXL4 is involved in Vpr-induced DSB, and Topo1 is required for vDNA integration in resting macrophages

Formation of Topo1-cc is followed by activation of SLX4, which resolves the stalled replication fork and removes Topo1-cc via its endonuclease activity [102,103] (Figure 3). The R-loops produced due to dysregulation of Topo1 induce DSBs and the ATM-dependent DDR via nucleotide excision repair [109,110,114,119]. This also occurs in resting macrophages, which do not express ATR/Chk1 [21]. Indeed, downregulation of Topo1 in monocyte-derived macrophages diminished the Vpr-mediated enhancement of viral infectivity [27]. These observations imply that Topo1-cc is related to the effects of Vpr in resting macrophages. Although Vpr induces DSBs and the ATM-dependent DDR [8,11,22,27,120], involvement of ATM activation has been ignored from Vpr-induced DDR, because ATR activity in Vpr-induced DDR is much more potent, and Vpr-induced cell-cycle abnormality is attenuated by downregulation of ATR [21]. Current data clearly indicate that DSBs and ATM activation are important for understanding the function of Vpr [27].

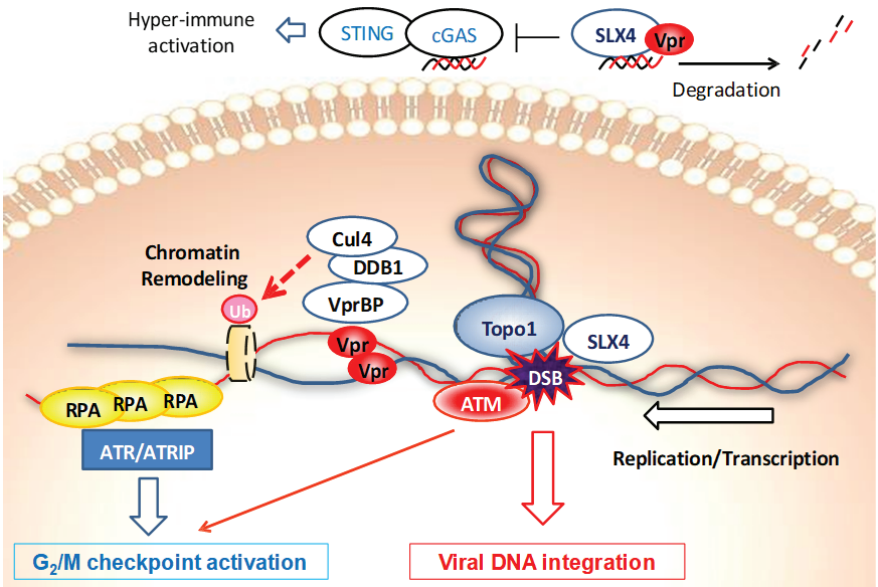

Figure 3: Mode of Vpr-induced DDR and DSB

At first, Vpr binds and unwinds dsDNA. Partial unwinding of dsDNA promotes loading of RPA70, which activates ATR-dependent DDR. Then, chromatin remodeling is provoked by Vpr-induced ubiquitination of histone H2B, and promotes the loading of RPA70, leading to full activation of ATR pathway. On the other hand, unwinding of dsDNA accumulates supercoiling of DNA, which recruits Topo1 for relaxation. Excess accumulation of Topo1 induces covalent complex of Topo1 and DNA, where replication and/or transcription machineries collide and DSB is induced. Vpr-induced DSB is targeted by vDNA integration. SLX4 is involved in the resolution of stalled replication fork and the excision Topo1-bound DNA, therefore causes DDR. Additionally, SLX4 is required for degradation of excess amounts of vDNA to escape from cGAS/STING-immune sensing. A part of figure was generated through the use of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Future Perspectives

In the post-ART era, the progression of AIDS can be suppressed, but HIV-1–infected individuals are still susceptible to related various complications, *e.g.* HAND and malignancies [121-123]. This is due to the existence of cART-refractory sanctuaries that include gut-associated lymphoid tissue, lymph nodes, tissue macrophages and the central nervous system [2,3]. In these sanctuaries, viral replication is abortive but sustained, and several viral proteins, including Vpr, might be continuously produced. Indeed, the serum of patients on cART reportedly contains up to hundreds ng/mL Vpr [124-126]. Notably, soluble Vpr in serum is biologically active and induces retrotransposition of LINE-1 [126]. Thus, Vpr induces DSBs in a manner involving Topo1, and soluble Vpr induces formation of Topo1-cc and DSBs [27].

Topo1-mediated DNA damage could be responsible for several neurodegenerative disorders [127], suggesting Vpr to be involved in HAND development. Additionally, HIV-1–positive patients have a high incidence of lymphomas [121]. Because Vpr in serum induces DSBs in blood cells, it may be a risk factor for the development of malignancies. Interestingly, expression of NKG2D ligands in CD4+ T cells was induced by Vpr [64-66]. Moreover, the DDR upregulates the expression of NKG2D ligands [128], implying that soluble Vpr is involved in perturbation of cell-mediated immunity. The expression of immune-checkpoint molecules, including PD-L1, is upregulated by DNA damage in a ATM/ATR/Chk1-dependent manner [129], supporting a role for Vpr in HIV-associated cancer development. Although Vpr is dispensable for viral integration in proliferating lymphocytes [5-8], its suppression of apoptosis and the immune response could prevent elimination of infected cells and lead to malignant transformation [12,13]. Therefore, Vpr is a target for development of novel anti–HIV-1 agents with the aim of improving the quality of life of HIV-infected individuals.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

K.I. and Y.I. wrote the paper. All authors read and approved the final manuscript.

Methodology Used to Search and Select Information

PubMed was almost used to search literatures including term of "Vpr".

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