HIV-1 Vpr Protein Induces Proteasomal Degradation of Chromatin-associated Class I HDACs to Overcome Latent Infection of Macrophages

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Running Title: Vpr downregulates class I HDACs to overcome latency in MDMs

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Abstract: Mechanisms underlying HIV-1 latency remain among the most crucial questions that need to be answered in order to adopt strategies for purging the latent viral reservoirs. Here we show that HIV-1 accessory protein Vpr induces depletion of class I HDACs, including HDAC1, 2, 3, and 8, to overcome latency in macrophages. We found that Vpr binds and depletes chromatin-associated class I HDACs through a VprBP-dependent mechanism, with HDAC3 as the most affected class I HDAC. De novo expression of Vpr in infected macrophages induced depletion of HDAC1 and 3 on the HIV-1 LTR that was associated with hyperacetylation of histones on the HIV-1 LTR. As a result of hyperacetylation of histones on HIV-1 promotor, the virus established an active promotor and this contributed to the acute infection of macrophages. Collectively, HIV-1 Vpr downregulates class I HDACs on chromatin to counteract latent infections of macrophages.

HIV-1 genome encodes 4 accessory proteins, including Nef, Vif, Vpu and Vpr. These proteins are known to downregulate cellular proteins through different mechanisms to facilitate various stages of viral infection. For instance, Nef downregulates surface expression of CD4 and MHC-I (1); Vif downregulates APOBEC3G and APOBEC3F (2), and Vpu downregulates surface CD4 and tetherin (3). Recent studies also show a growing list of cellular proteins whose degradation is induced by Vpr while the outcomes of their degradation remain to be elucidated.

HIV-1 Vpr is well-documented to interact with the Cul4-DDB1[VprBP] E3 ubiquitin ligase to direct proteasomal degradation of a number of cellular proteins and induce G2/M cell cycle arrest (4-7). Vpr enhances proteasomal degradation of the natural substrates of the Cul4-DDB1[VprBP] E3 ubiquitin ligase (8-10). For instance, Vpr has been shown to enhance proteasomal degradation of UNG2 and dicer (11,12). These proteins are...
recruited by Vpr-binding protein (VprBP) to the E3 ubiquitin ligase complexes as their natural substrates. By binding to VprBP, Vpr enhances degradation of the natural substrates recruited by VprBP (10,13). Furthermore, minichromosome maintenance complex component 10 (MCM10) was recently shown to be the natural substrate of the Cul4-DDB1[VprBP] E3 ubiquitin ligase (14) and our lab showed that proteasomal degradation of MCM10, is also enhanced by Vpr (10).

It is believed that Vpr induces most of its functions by interacting with VprBP. In order to investigate the biological roles of Vpr, we investigated proteins that interact with VprBP. It was recently shown that VprBP directly interacts with nucleosomes by recognizing the unacetylated N-terminal tail of histone H3 (15). In this complex, VprBP was also shown to bind histone deacetylase 1 (HDAC1). HDACs are a class of enzymes that remove acetyl groups from ε-N-acetyl lysine amino-acids of histones and other proteins and also regulate a number of key cellular processes, including gene expression, protein complex formation, and protein stability and localization (16-19). In mammals, 18 HDACs have been described and they are classified into 4 classes based on their phylogeny and function (20,21). There is a high level of homology among the members of class I HDACs, which comprises HDAC1, 2, 3 and 8. Class I HDACs localize to the nucleus, except for HDAC3, which can be found both in the cytoplasm and the nucleus (22,23). In this study, we found that Vpr binds and depletes chromatin-associated class I HDACs, enabling the virus to overcome latent infection in primary macrophages.

**Experimental Procedures**

**Cell lines and Primary Cells**—HEK293T and HeLa cells were obtained from ATCC. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program. Doxycycline inducible HeLa cell lines (HeLa-iFlag-Vpr, HeLa-iFlag-Q65R) and the control cell line (HeLa-iMock) were described previously (10). Expression of Flag-Vpr was induced by adding 1 μg/ml doxycycline. HeLa and the inducible HeLa cell lines were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml of streptomycin. To isolate monocyte derived macrophages (MDMs), hepatitis B virus and HIV-1-seronegative adult donors that had signed a consent form, provided peripheral blood samples. PBMCs were isolated from whole blood using Ficoll-density gradient centrifugation. Monocytes were primarily isolated by plastic adherence. For differentiation into mature macrophages, monocytes were cultured in RPMI supplemented with 50 ng/ml macrophage-colony stimulating factor M-CSF, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. After 7 days, cells were washed with PBS to rinse off the nonadherent cells. Purity of MDMs was verified by flow cytometry using antibodies against CD14 and CD16.

**Antibodies and Reagents**—HDAC1 (ab7028), HDAC2 (ab7029), HDAC3 (ab32369), HDAC8 (ab18968), histone H3 (ab70550), DDB1 (ab124672), p24 (ab9071), SIRT3 (ab86671), and HDAC4 (ab11968) antibodies were purchased from Abcam. GFP antibody (G6539), Flag antibody (F3165), Monoclonal Anti-HA-Agarose Beads, and ANTI-FLAG M2 affinity gel were from Sigma-Aldrich. GAPDH (14C10) and rabbit IgG isotype control (2729) were from Cell Signalling Technology. VprBP (A301-888A) antibody was from Bethyl Laboratories. Vpr (NP_057852) antibody was from Proteintech. Ace H3K9 (39917) and Ace H4K5 (39699) antibodies were from active motif. PE-Texas Red anti-human CD14 (MHCD1417) was from Life Technologies. APC/Cy7 anti-human CD16 antibody (302018) was from BioLegend. Mouse and rabbit HRP-conjugated antibodies were from Abcam. Protein A sepharose beads were from Amersham. Benzonase nuclease was from Novagen. Flag peptides, caffeine, etidium bromide, doxycycline, SAHA and DMSO were from Sigma-Aldrich. MG132 was from Millipore. Protease inhibitor cocktail was from Roche. VprBP and non-targeting siRNAs were from Dharmacon. HDAC1 siRNA was from QIAGEN. HDAC3 siRNA was from Santa Cruz Biotechnology. SYBR Select Master Mix was from Life Technologies.

**Vectors and Virus Constructs**—The lentiviral vectors pWPI, pWPI-Flag-Vpr, pWPI-Flag-Q65R, pWPI-Flag-R80A and their packaging plasmids pCMV-VSV-G and psPax2 were described.
previously (10). pWPI-Flag-Vpx was constructed by cloning of Vpx from simian immunodeficiency virus (clone pPBj 1.9) of sooty mangabeys into pWPI using a strategy previously described (10). To generate an expression vector for HIV-1 Vpr, the vpr gene of HIV Gag-iGFP was cloned into pcDNA3.1. pRK5-HA-Ubiquitin-WT, pcDNA3.1-HDAC1-Flag, and pcDNA3.1-HDAC3-Flag were obtained from Addgene. pNL(AD8), pNL4-3-deltaE-EGFP, and pNL4.3.Luc.Env- were obtained from the NIH AIDS Research and Reference Reagent Program. The R5-tropic clone of HIV-1, pNL4.3.AD8, IRES_GFP_Nef-, was generated by cutting a 1.7 kbp fragment between the KpnI and BsmI site in the envelope coding region from the R5-tropic clone pNL(AD8) and replacing this fragment for the corresponding region of pNL4.3_IRES_GFP_Nef-. The ΔVpr, Q65R, and R80A mutants were generated in the viral constructs using site-directed mutagenesis.

Lentiviral Vector and Virus Production—All viral and lentiviral vectors were produced in HEK293T cells using the standard calcium phosphate transfection method. Viral and lentiviral particles were collected 48 h and 72 h post-transfection by ultracentrifugation at 35000 rpm for 2 h. Briefly, lentiviral vectors were produced by cotransfection of pWPI, pWPI-Flag-Vpr, pWPI-Flag-Vpr(Q65R), pWPI-Flag-Vpx, and the packaging plasmids, pCMV-VSV-G and psPax2. Lentiviral vectors were titrated in HEK293T cells using GFP signal. The VSV-G-pseudotyped pNL4.3.Luc.Env-(WT/ΔVpr) and pNL4-3-deltaE-EGFP (WT/ΔVpr/Q65R/R80A) were produced by cotransfection of the proviral plasmids with pCMV-VSV-G and psPax2. Lentiviral vectors were titrated in HEK293T cells using GFP signal. The VSV-G-pseudotyped pNL4.3.Luc.Env-(WT/ΔVpr) and pNL4-3-deltaE-EGFP (WT/ΔVpr/Q65R/R80A) were produced by cotransfection of the proviral plasmids with pCMV-VSV-G. pNL4.3.AD8. IRES_GFP_Nef- and its mutants (ΔVpr/Q65R/R80A) were produced by transfection of the proviral plasmids in HEK293T cells. pNL4.3.Luc.Env-(WT/ΔVpr) and pNL4.3.AD8. IRES_GFP_Nef- derived viruses were titrated in TZM-bl cells. p24 of the concentrated viral stock for pNL4-3-deltaE-EGFP (WT/ΔVpr/Q65R/R80A) was titrated using ELISA.

Lentiviral Transduction and Viral Infection—HeLa cells were transduced with lentiviral vectors at an MOI of 2.0. Primary MDMs were infected at an MOI of 1.0 with fully replicative viruses or with 1 ng of p24 per 10^5 cells when infecting with single cycle VSV-G pseudotyped viruses.

siRNA Transfection—VprBP, HDAC1, HDAC3 and nontargeting siRNAs were transfected into inducible HeLa cell lines and/or primary MDMs using Lipofectamine RNAiMax Reagent according to the manufacturer’s instructions.

Cell Fractionation—In order to fractionate HeLa and inducible HeLa cell lines, cells were lysed in 1 ml of 0.5% Triton lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton, and protease inhibitor cocktail). Cells were incubated for 10 min with mild agitation at 4°C and then centrifuged at 6000 rpm for 10 min at 4°C to pellet chromatin and other large insoluble debris. Supernatant was collected as the soluble fraction and the pellet was resuspended in 2 ml of Benzonase buffer (50 mM Tris pH 8, 150 mM NaCl, 1.5 mM MgCl2, 0.1 mg/ml BSA, and protease inhibitor cocktail). The resuspended pellet was centrifuged and supernatant was discarded. One µl of Benzonase enzyme (25U/µl) was added to 1 ml of Benzonase buffer and this was used to resuspend the pellet and incubate it on ice for 60 min. The Benzonase treated pellet was centrifuged at 13000 rpm for 10 min at 4°C. Supernatant was collected as the fraction containing chromatin-bound proteins. Purity of the fractions was confirmed by the presence of GAPDH in soluble protein fraction and histone H3 in chromatin bound protein fraction.

Western Blotting and Immunoprecipitation—Anti-Flag and anti-HA immunoprecipitations were performed using 40 µl of commercial antibodies conjugated to agarose beads. For anti-VprBP, HDAC1, HDAC2, HDAC3, and HDAC8 immunoprecipitations, antibodies (2 µg per immunoprecipitation) were incubated overnight at 4°C with 50 µl protein A sepharose beads in 1 ml PBS supplemented with 5% FBS. All the immunoprecipitations were performed in the presence of 150 mM NaCl and 0.5% Triton X-100 for 2 h at 4°C. For VprBP pull-down, 25 µg/ml ethidium bromide was also added to the solution to avoid the non-specific pull-down of chromatin-bound proteins through DNA bridges. After thorough washes in 0.5% Triton lysis buffer, the
anti-Flag immunoprecipitated proteins were eluted by adding 100 µg/ml Flag peptides. Immunoprecipitations using protein A sepharose beads and Monoclonal Anti-HA-Agarose Beads were released by treating the beads with 0.1 M Glycine, pH 2.0, for 10 min on ice. Immunoprecipitated proteins and cellular fractions (30 µg) were resuspended in Laemmli buffer, heat-denatured for 5 min, and separated on 12% SDS-PAGE gels. Western blots were performed as described previously(10).

**ELISA**—Supernatants of the infected cells were collected, inactivated with Triton X-100 (1% final concentration), and stored at -80˚C until analyzed. Levels of HIV-1 p24 were determined using HIV-1 p24 Antigen Capture assay (ABL Inc.) according to the manufacturer’s instructions.

**Luciferase Assay**—Primary MDMs were infected at an MOI of 1.0 with single cycle VSV-G pseudotyped pNL4.3.Luc.Env-(WT/ΔVpr) that expresses luciferase in the infected cells. Seven days after infection, cells were washed with PBS and lysed directly in the tissue culture plates using Triton X-100 (1% final concentration). Luciferase was measured in the cell lysates using luciferase assay system (Promega).

**Chromatin Immunoprecipitation (ChIP)** —To analyze enrichment of class I HDACs and acetylated histones on the HIV-1 LTR, 5 x 10^5 MDMs were infected with pNL4.3.AD8. IRES_GFP_Nef- (WT and ΔVpr) at an MOI of 1.0. After 7 days, cells were looked under a fluorescent microscope for GFP-positive infected cells. If percentage of the infected cells was higher than 5%, we proceeded with the experiment. Cells were then fixed with 0.5% formaldehyde. After 5 min, fixation was quenched by adding glycin to a final concentration of 125 mM for 5 minutes and cells were then washed with PBS and harvested. Cells were lysed in TpA (0.25% Triton, 10 mM Tris ph8, 10 mM EDTA, 0.5 mM EGTA, protease inhibitor) for 5 min on ice and centrifuged. This would disrupt the cellular membrane and release the non-integrated proviruses. Cell nuclei were then washed with TpB (200 mM NaCl, 10 mM Tris ph8, 1 mM EDTA, 0.5 mM EGTA, protease inhibitor) and lysed in TpS (0.5% SDS, 0.5% Triton, 10 mM Tris ph8, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, protease inhibitor). Chromatin was sheared using Bioruptor XL sonicator (Diagenode) to obtain DNA fragments ranging from 200 to 800 bps.

For each immunoprecipitation, 2 µg antibody was conjugated to 10 µl of protein A and protein G Dynabeads (1:1 ratio) by overnight incubation of the beads/antibody in 500 µl of ChIP dilution buffer (1% Triton, 10 mM Tris pHe8, 150 mM NaCl, 2 mM EDTA) at 4˚C. ChIPs were performed by adding the sonicated DNA to the antibody conjugated Dynabeads and incubation at 4˚C for 4 h. Following extensive washes, the DNA of ChIP samples and nonimmunoprecipitated chromatin (input) were decross-linked in TpE (0.3% SDS, 50 mM Tris ph8, 10 mM EDTA, 0.4 M NaCl) by overnight incubation at 65˚C and extracted using the standard phenol-chloroform method. The DNA was then subjected to quantitative PCR.

**Quantitative PCR**—Occupancy of HIV-1 genome by class I HDACs and acetylated histones was analyzed using quantitative PCR. Briefly, the DNA that was pulled down using ChIP and the corresponding input DNA was subjected to PCR using the following primers: nu0-F 5´-CCACACACAAGGCTACTTCCCT-3´, nu0-R 5´-CAACTGGTACTAACTTAGCAAGCA-3´, nu1-F 5´-GTCCTCCTGTTAGACCAGA-3´, nu1-R 5´-TACTTTGAGCACTCAAAGCA-3´, nu2-F 5´-AAAAATTGGACTGCGGAGGCT-3´, nu2-R 5´-CCTAACCAGAATTTTTCAGCA-3´, ChIP-GAPDH-F 5´-CCTTCCCCCTAGTCCCCAGAA-3´, ChIP-GAPDH-R 5´-AGCGCGAAAGGAAAGAAAGC-3´. A pair of primers were also designed against an intergenic region (gene accession number: AF254641.1), where no hyperacetylation of histones is expected, as follows: INT-F 5´-GTAGAGGCAAGCGATCTGGGA-3´, INT-R 5´-CAAGGGCCACTCTCGGCTCT-3´. Amplification of the target sequences was detected using SYBR Select Master Mix and the CT values were normalized to the inputs and the intergenic region.

To assess whether Vpr affects class I HDACs at transcription level, HeLa-iFlag-Vpr and HeLa-iMock were induced by adding 1 µg/ml
doxycycline. After 30 h, cells were harvested and total RNA was extracted from 10⁶ cells using RNeasy Mini Kit (QIAGEN, Maryland, USA) according to the manufacturer’s instructions. Using SuperScript II Reverse Transcriptase (Invitrogen) and oligo(dT)₁₂₋₁₈ Primers (Invitrogen), RNA was reverse transcribed into cDNA. The cDNA was diluted 100-fold with DNase free water. Quantitative PCR was performed on the diluted cDNA using SYBR Select Master Mix. Primers used in qPCR include: HDAC1-F 5’-TACGACGGGGATGTTGGAAA-3’, HDAC1-R 5’-ATTGGCTTTGTGAGGGCGAT-3’, HDAC2-F 5’-CCATGGCGTACAGTCAAGGA-3’, HDAC2-R 5’-TCATTTCCTGCGCAGTGGCT-3’, HDAC3-F 5’-GCCAGAGGCCCTCAAACTTCT-3’, HDAC3-R 5’-GCCAGAGGCCCTCAAACTTCT-3’, HDAC8-F 5’-TCTCCAGAAGGTAGCCAAGA-3’, HDAC8-R 5’-TCTTTGCATGATGCCACCCT-3’, GAPDH-F 5’-GAGAAGGCTGGGGCTCATTT-3’, GAPDH-R 5’-GCAGTGATGGCATGGACTGT-3’. Data were normalized to GAPDH and calculated using the 2⁻(ΔΔCT) method.

To analyze expression levels of HIV-1 mRNA in infected MDMs, total mRNA was extracted using Ultrasens Viral RNA kit (QIAGEN, Maryland, USA) and reverse transcribed using SuperScript II Reverse Transcriptase and oligo(dT)₁₂₋₁₈ Primers. cDNA was then subjected to quantitative PCR using nuc-2-F and nuc-2-R primers.

Quantitative Alu-PCR—To compare integration of HIV-1 provirus in the presence or absence of Vpr, genome of infected MDMs was analyzed using Alu-PCR. Briefly, primary MDMs were infected with the single cycle VSV-G pseudotyped pNL4.3Env-.IRES.GFP WT and ΔVpr viruses. Four days post-infection, GFP-positive and negative cells were sorted and the genomic DNA was extracted from 20,000 sorted and unsorted cells using DNeasy Blood & Tissue Kit (QIAGen). DNA was amplified using the forward primer Alu-F 5’-GCCTCCCAAAGTGTGGGATTACG-3’ that binds human Alu gene and the reverse primer HIV-LTR-outer-R 5’-TGCTAGAGATTTCACACTGA-3’ that binds HIV-1 LTR. One microliter of the amplified DNA was used for qPCR using HIV-LTR-F 5’-CTGGCTAACTAGGGAAACCCT-3’ and HIV-LTR-inner-R 5’-CTCAATAAAGCCTTGCTAGTGCTC-3’ primers. To amplify the total HIV-1 provirus (integrated and non-integrated), DNA was amplified using HIV-LTR-F and HIV-LTR-inner-R. Amplification of a specific region on HIV-1 LTR was detected using the probe LTR-Probe 5’-(FAM)-CTCAATAAAGCCTTGCTAGTGCTC-( TAMRA)-3’. Results of Alu-PCR were normalized to the total HIV-1 provirus.

Flow Cytometry and Sorting—To analyze the cell cycle profile of inducible HeLa cell lines, they were induced by adding doxycycline and after 30 h their DNA content was labeled by propidium iodide staining and cells were analyzed as previously described (10). To examine purity and differentiation of MDMs, their surface markers were labeled using CD14 Mouse Anti-Human mAb, PE-Texas Red conjugate and APC/Cy7 anti-human CD16 for 30 min on ice. Cells were washed 3 times and analyzed using flow cytometry. To analyze infectivity of the single cycle HIV-1 in MDMs, they were infected with VSV-G-pseudotyped NL4-3-deltaE-EGFP (WT, ΔVpr, Q65R, and R80A). Four days post-infection, MDMs were analyzed for expression of GFP. All the analyses were performed using Cyan ADP Analyzer.

In order to analyze acute versus latent infections, primary MDMs were infected with single cycle VSV-G pseudotyped GFP reporter WT/ΔVpr viruses for 4 days. The GFP-positive and -negative cells were sorted in PBS using an Influx cell sorter (BD Biosciences) and analyzed using Alu-PCR or Western blot. In order to analyze viral expression in macrophages, primary MDMs were infected with GFP reporter WT/ΔVpr viruses for 7 days. The GFP-positive cells were then sorted using an Influx cell sorter (BD Biosciences) and analysed using Western blot.

Statistical Analysis—Student’s t-test or Analysis of variance (ANOVA) with Bonferroni’s multiple comparison test was used for statistical analysis using GraphPad Prism 6.0. A value of P < 0.05 was considered statistically significant. Results were expressed as mean±s.e.m. or mean±s.d., and
represent data from a minimum of three independent experiments unless otherwise stated.

Results

HIV-1 Vpr induces depletion of class I HDACs on chromatin—Since VprBP was reported to interact with HDAC1 on chromatin, we developed a fractionation method to examine the impact of Vpr on this interaction in a localized manner. Our fractions included soluble and chromatin bound protein fractions, in which the soluble fraction contained both cytoplasmic proteins and soluble nuclear proteins, while the chromatin bound protein fraction contained nuclear proteins released by treatment of the chromatin pellet with Benzonase DNase.

A doxycycline-inducible cell line, HeLa-iFlag-Vpr cells, was used as previously described (10). Expression of Flag-tagged HIV-1 Vpr was induced by adding doxycycline to HeLa-iFlag-Vpr cells and the quantities of class I HDACs were monitored in a time course experiment (Fig. 1A). A significant depletion of HDAC1 was found after 10 h in chromatin fraction followed by its depletion in the soluble fraction only after 30 h. Depletion of HDAC2 was only found significant in chromatin fraction 30 h post-induction with no significant depletion in the soluble fraction. Depletion of HDAC3 was initiated as early as 5 h post-induction in the chromatin fraction and then followed by its depletion in the soluble fraction 20 h post-induction. Depletion of HDAC8, which was only found in chromatin fraction, appeared significant after 20 h. Our results indicated that depletion of class I HDACs was more drastic on chromatin as the chromatin associated class I HDACs were depleted before the soluble class I HDACs. Chromatin depletion of HDAC3 was found to occur before other HDACs, suggesting more sensitivity of HDAC3 to expression of Vpr while HDAC2 was found the least sensitive member of class I HDACs.

Expression of Vpr did not seem to affect the mRNA levels of class I HDACs (Fig. 1B) suggesting the downregulation of HDACs occurs at protein level. Furthermore, we tested the effect of Vpr on other classes of HDACs (Fig. 1C). Expression of Vpr did not affect the protein levels of other classes of HDACs.

Vpr-induced depletion of class I HDACs is not resulted from G\textsubscript{2}/M arrest—Vpr is well-documented to induce a cell cycle arrest at G\textsubscript{2}/M phase through activation of ATR pathway (24). The cell cycle arrest could potentially change protein contents of the cells. To examine whether the depletion of class I HDACs is a by-product of G\textsubscript{2}/M cell cycle arrest we inhibited Vpr-induced G2/M cell cycle arrest by caffeine, an ATR inhibitor. As shown in Fig. 2A, treatment of induced HeLa-iFlag-Vpr cells with caffeine efficiently inhibited Vpr-induced G2/M arrest. However, inhibition of G2/M arrest did not affect the ability of Vpr for depletion of class I HDACs.

We also induced G2/M cell cycle arrest by treatment of HeLa cells with nocodazole. Despite the potent G2/M arrest induced by Nocodazole, the cell cycle arrest did not induce depletion of HDACs (Fig. 2B).

Vpr-induced depletion of class I HDACs is proteasomal dependent—To determine whether Vpr usurps a proteasomal pathway for degradation of class I HDACs, we used transduction to avoid the interference between proteasomal inhibitor and doxycycline induction. Normal HeLa cells were transduced with lentiviral vectors for expression of the Flag-tagged Vpr or Q65R and R80A mutants that have been previously reported defective for depletion of proteasomal targets of Vpr (10,12,13). HeLa cells were also transduced with lentiviral vectors for expression of simian immunodeficiency virus Vpx or empty lentiviral vectors. The transduced cells were treated with the proteasomal inhibitor, MG132, or DMSO 10 h before harvesting (Fig. 3A). Western blot analysis of the fractionated cells indicated that the wild-type Vpr was able to efficiently deplete all class I HDACs in the chromatin fraction of the cells treated with DMSO. A milder effect of Vpr on HDAC1 and HDAC3 was also observed in the soluble fraction that was only found significant for HDAC3. However, treatment of the cells with MG132 restored HDACs in both fractions. Q65R and R80A mutants as well as Vpx were not able to deplete HDACs in the presence or absence of MG132. Deficiency of the mutants for depletion of
HDACs, especially Q65R that is defective for interaction with VprBP, provided further support for proteasomal dependency of depletion of HDACs.

To provide direct evidence demonstrating that the Vpr-induced depletion of HDACs is in fact proteasomal dependent, we cotransfected HEK293T cells with expression vectors for HA-ubiquitin, Vpr and C-terminally Flag-tagged HDAC1 or HDAC3. Cells were fractionated and pulled down using anti-HA antibodies (Fig. 3B and C). Western blot analysis of the eluates indicated that ubiquitination of HDAC1 and 3 only occurred on chromatin. Interestingly, expression of Vpr increased the basal ubiquitination of HDAC1 and HDAC3 to 2.2 and 3.4 fold, respectively.

Vpr-induced depletion of class I HDACs is VprBP-dependent—To characterise composition of the complex involved in the Vpr-induced depletion of HDACs, we decided to test involvement of VprBP. We transfected inducible HeLa cell lines with non-targeting siRNA or siRNA against VprBP. Expression of Flag-Vpr or Flag-Q65R was induced by doxycycline treatment. Analysis of the fractionated cell lysates demonstrated that the wild-type Vpr was able to deplete class I HDACs on chromatin only in the presence of VprBP since depletion of VprBP abolished the Vpr ability to induce depletion of class I HDACs (Fig. 4A). The Q65R mutant, which is defective for binding to VprBP, was not able to induce depletion of HDACs in the presence or absence of VprBP, providing further support for the key role of VprBP in Vpr-induced depletion of HDACs. Immunoprecipitation of Vpr indicated that the wild-type Vpr was able to interact with class I HDACs only in the presence of VprBP and only in the chromatin fraction. Depletion of VprBP abolished the Vpr ability to bind HDACs, suggesting Vpr indirectly binds HDACs through VprBP on the chromatin. The Q65R mutant was not able to interact with HDACs and VprBP, however, Q65R was also found defective for efficient localization on chromatin (Fig. 4A, compare lanes 9 and 10 with lanes 11 and 12). We previously found that MG132 treatment stabilized the Q65R mutant on DNA in order to provide a bona fide control that can be pulled down in chromatin fraction (Fig. 4B). Pull down of Q65R at quantities comparable with those of the wild-type Vpr indicated that Q65R was not able to efficiently interact with VprBP and therefore it was not able to pull down class I HDACs. Of note, expression of Vpr did not seem to affect the localization or expression levels of VprBP in any of the fractions (Fig 4A and B).

We then tested interaction of VprBP with class I HDACs by immunoprecipitation of the endogenous VprBP. Ethidium bromide was also used in the immunoprecipitations to dissociate chromatin-bound proteins from bridging DNA fragments. VprBP was able to pull down class I HDACs only in the chromatin fraction (Fig. 4C). This interaction was not due to nonspecific pull-down through DNA bridges since it also occurred in the presence of ethidium bromide. Individual pull-down of class I HDACs showed that HDACs bind VprBP and DDB1 only in the chromatin fraction and these bindings occur independently of Vpr (Fig. 4D).

Vpr enhances HIV-1 infection in macrophages through a VprBP-dependent mechanism—Vpr is known to enhance HIV-1 replication in macrophages. In order to examine whether this effect of Vpr is related to depletion of HDACs, we depleted VprBP in MDMs and infected them with the wild-type or Vpr-defective (ΔVpr) viruses. Replication kinetics of the viruses was measured by quantification of p24 in the supernatant of the infected cells (Fig. 5A) and it was shown that in the presence of VprBP, the WT virus infected MDMs more efficiently than the ΔVpr virus. Interestingly, depletion of VprBP abolished the effect of Vpr such that there was no significant difference between the WT and ΔVpr viruses. We also showed that the Q65R and R80A mutants did not increase infection of MDMs at levels comparable to the WT virus (Fig. 5B).

Since class I HDACs are known to suppress gene expression, we tested whether Vpr-induced depletion of HDACs increases the HIV-1 gene expression. Primary MDMs were infected with the WT and ΔVpr viruses that had a luciferase gene as an indicator of active transcription of the viral
genome. Measuring the luciferase reporter indicated that transcription of the WT virus was 3.58 fold higher than that of the ΔVpr virus (Fig. 5C). We also compared infectivity of the single cycle VSV-G pseudotyped GFP-reporter WT HIV-1 or the corresponding ΔVpr, Q65R, and R80A mutants. As shown in Fig. 5D and E, the ΔVpr, Q65R, and R80A viruses infected MDMs at comparable levels. But the WT virus showed ~3 fold increase in the efficiency of the infection, suggesting the efficient infection of MDMs by the WT virus is dependent on Vpr and its interaction with VprBP. Conversely, when we compared the expression of p24 and GFP in equal numbers of sorted cells, we did not observe a significant difference between the WT and ΔVpr infected MDMs (Fig. 5F). The mRNA level of HIV-1 was also compared in total population of WT and ΔVpr infected MDMs (Fig. 5G). Additionally, GFP-positive infected MDMs were sorted and their HIV-1 mRNA was compared (Fig. 5H). Although the viral mRNA had increased in the total population, when equal numbers of individual infected cells were compared, no significant difference was observed in the presence or absence of Vpr. This suggested that Vpr increases the total number of infected MDMs but not the viral expression in individual infected cells and opened new venues for new models and further investigations as discussed in the following sections.

Vpr depletes class I HDACs on the HIV-1 LTR of infected macrophages—Fractionation requires a large number of cells that is not feasible in the case of primary MDMs. Therefore, we performed chromatin immunoprecipitations (ChIPs) to examine whether Vpr induces depletion of HDACs on the HIV-1 LTR of infected MDMs. HIV-1 has two conserved nucleosomes on its LTR, known as nuc-0 and nuc-1 (25,26). We designed primers to test depletion of class I HDACs and hyperacetylation of histones on nuc-0 and nuc-1 as well as nuc-2 that is located downstream of LTR (Fig. 6A). Primary MDMs were infected with the WT and ΔVpr viruses. After 7 days, cells were treated with MG132 or DMSO for 20 h and then we performed ChIPs against individual class I HDACs. As shown in Fig. 6B, the WT virus depleted 50-60% of HDAC1 on the HIV-1 LTR of the DMSO-treated cells. Interestingly, treatment with MG132 restored the levels of HDAC1 on HIV-1 genome. The levels of HDAC2 were 10-20% lower in the WT HIV-1 LTR but this was not statistically significant. HDAC3, however, showed about 80% depletion on the HIV-1 LTR and, similarly to HDAC1, it was restored after treatment with MG132. We could not find an enrichment of HDAC8 on the HIV-1 LTR.

We also examined the effect of Vpr on gapdh coding region. Unlike LTR that is only found in infected cells, gapdh is found in both infected and uninfected macrophages. To ensure that our ChIP assay only analyzes gapdh in the infected cells, GFP-positive infected cells were sorted before ChIP (Fig. 6C). Unlike the HIV-1 LTR, Vpr did not affect enrichment of HDAC1 and HDAC3 on gapdh gene, suggesting that Vpr degrades HDACs in a localized manner that does not affect all cellular genes.

Vpr induces hyperacetylation of core histones on the HIV-1 LTR—Class I HDACs remove acetyl groups from the lysine residues of histones and therefore depletion of HDACs is expected to induce hyperacetylation of histones. To examine whether the Vpr-induced depletion of HDACs is followed by hyperacetylation of histones, we infected MDMs with the WT and ΔVpr viruses. After 7 days, cells were ChIPed against acetylated histone H3 (Ace H3K9) and acetylated histone H4 (Ace H4K5) (Fig. 7A and B). Comparison of MDMs infected with the WT and ΔVpr viruses indicated that Ace H3K9 was enriched on nuc-0, nuc-1, and nuc-2 of the WT viruses, 3.26, 3.82, and 3.45 fold, respectively. Similarly, Ace H4K5 was enriched on nuc-0, nuc-1, and nuc-2 of the WT viruses, 2.98, 3.07, and 2.34 fold, respectively. Interestingly, there was no significant difference between the gapdh gene of the WT and ΔVpr infected MDMs. As a negative control, we examined enrichment of Ace H3K9 and H4K5 on an intergenic region, where no enrichment of hyperacetylated histones is expected, and our results showed that there was no significant enrichment on this region. In order to test whether hyperacetylation of histones follows the same pattern as depletion of HDACs we examined if the hyperacetylation was proteasomal dependent. We
infected MDMs with the WT and ΔVpr viruses and after 7 days we treated cells with MG132 or DMSO for 20 h. Using ChIP assay, cells were then analyzed for the enrichment of acetylated histones (Ace H3K9 and Ace H4K5) on nuc-0 (Fig. 7C and D). Comparison of the WT versus ΔVpr infected MDMs, indicated that expression of Vpr by the WT virus induced about 3-fold enrichment of both Ace H3K9 and Ace H4K5 on nuc-0, while MG132 treatment significantly abolished the ability of the Vpr such that there was no significant difference between the WT and ΔVpr infected MDMs.

We included an additional control against total histone H3 to ensure that there is no significant difference in localization of histones between the WT and ΔVpr infected viruses (Fig. 7E). Although there was a good enrichment of histones on nuc-0, nuc-1, and nuc-2, there was no significant difference between MDMs infected with the WT and ΔVpr viruses.

Vpr overcomes latent infections of macrophages—We found that Vpr induces depletion of HDAC1 and HDAC3 on the HIV-1 LTR of infected MDMs and this depletion was associated with hyperacetylation of histones in this region. Hyperacetylation is normally associated with active transcription which is consistent with our finding in total population of infected MDMs (Fig. 5A-C). However, we did not observe an increase in the expression of p24 or GFP when we compared equal numbers of infected cells (Fig. 5F). We hypothesized that by hyperacetylation of histones on the HIV-1 LTR, Vpr may overcome latent infections in macrophages to increase the number of acutely infected cells, as consistent with our findings (Fig. 5D and E). To test our hypothesis, we infected primary MDMs with the single cycle VSV-G pseudotyped GFP-reporter WT or ΔVpr HIV-1. After 4 days, cells were harvested and Alu-PCR was performed on the total population (Fig. 8A) or cells were sorted and Alu-PCR was performed on equal numbers of the GFP-positive and -negative cells, separately (Fig. 8B). Alu-PCR showed no significant difference in the integration of HIV-1 in the total population of unsorted cells, in the presence or absence of Vpr (Fig. 8A). Alu-PCR did not also show a significant difference when integrated DNA was compared in equal numbers of GFP-positive cells. Interestingly, when GFP-negative cells were compared, MDMs infected with ΔVpr virus had a significantly higher occurrence of integrated HIV-1 genome, suggesting the presence of more latent infections in the absence of Vpr (Fig. 8B).

Since Vpr was found to inhibit HDACs in macrophages, we decided to test whether the lack of Vpr could be complemented in ΔVpr viruses using an HDAC inhibitor. In order to test our hypothesis, we infected primary MDMs with the single cycle VSV-G pseudotyped GFP-reporter WT or ΔVpr virus. After 3 days, cells were treated with SAHA or DMSO for 24 h. Four days after infection, the GFP-positive and -negative cells were sorted and analyzed using Alu-PCR (Fig. 8C). Consistent with our previous results (Fig. 8B), when cells treated with DMSO, we found more HIV-1 DNA integrated in the genome of MDMs infected with the ΔVpr virus. However, when cells treated with SAHA, relatively equal copies of integrated HIV-1 genome were found in the MDMs infected with the ΔVpr or WT viruses, suggesting SAHA has reactivated the latent proviruses in MDMs infected with the ΔVpr virus. Interestingly, SAHA significantly increased infectivity of the ΔVpr virus from 1.2% to 3.9% but had a very slight effect on the WT virus (3.5% in DMSO treated cells and 4.0% in SAHA treated cells). Taken together, de novo expression of Vpr or treatment with SAHA helped the virus counteract latent infections in macrophages.

In order to investigate the effects of Vpr-induced depletion of HDACs, we also examined the impact of depletion of class I HDACs on infection of MDMs. Primary MDMs were infected with the single cycle VSV-G pseudotyped GFP-reporter WT HIV-1 or the corresponding ΔVpr virus. After 2 days, HDAC1 and HDAC3 were depleted in the infected cells. Four days post-infection, cells were analyzed for GFP expression (Fig. 8 D). While in the cells transfected with the non-targeting siRNA, the ΔVpr virus infected 1.06% of the cells, depletion of HDAC1 and HDAC3 increased infectivity of the ΔVpr virus to 2.98% and 3.03%, respectively. Interestingly, siRNA transfection of MDMs with non-targeting siRNA resulted in 3.42% infection with the WT virus but siRNA depletion of HDAC1 and HDAC3 did not
significantly change infectivity of the WT virus (3.58% and 3.38%, respectively).

Discussion

HIV-1 Vpr has been well documented to enhance proteasomal degradation of a number of proteins by interacting with the Cul4-DDB1[VprBP] E3 ubiquitin ligase (4,27). Nonetheless, degradation of none of these proteins fully explains the effect of Vpr in increasing viral infectivity in macrophages. Here we report novel protein targets for Vpr that explain the previously described functions of this protein. We showed that overexpression of Vpr resulted in depletion of all the four members of class I HDACs with no significant effect on other classes of HDACs. The depletion was more drastic on chromatin and when tested in time course and dose-response experiments, depletion of chromatin-associated HDACs preceded that of soluble HDACs. VprBP and DDB1 were found to bind class I HDACs and we showed that VprBP plays a crucial role in Vpr-induced depletion of HDACs. Our results suggest targeted proteasomal depletion of class I HDACs by Vpr through an E3 ubiquitin ligase on chromatin that contains both VprBP and DDB1 (Fig. 9, upper panels). However, even in an overexpressed system, the chromatin depletion of class I HDACs was never complete, suggesting a specific mechanism that does not deplete the entire pool of the chromatin-associated class I HDACs. This suggests that Vpr may target a fraction of chromatin-associated class I HDACs on specific regions of the genome.

In overexpressed systems, HDAC3 showed the highest sensitivity to expression of Vpr by being depleted at lower concentrations of Vpr while HDAC2 showed the lowest sensitivity by being depleted at higher concentrations of Vpr. De novo expression of Vpr in infected MDMs, however, resulted in depletion of HDAC1 and 3 on the HIV-1 LTR. We did not observe a significant depletion of HDAC2 on the HIV-1 LTR of infected MDMs. HDAC8 was not found to localize on HIV-1 LTR and therefore we did not observe its depletion in MDMs. Nonetheless, we cannot rule out the possibility of depletion of class I HDACs in other genomic regions, such as cellular genes, which were not addressed in this study.

It has previously been reported that depletion or inhibition of HDACs, especially HDAC3, reactivates latent HIV-1 infection (28-32). Interestingly, Vpr has been reported to activate HIV-1 in latently infected cells (33-35), as well as endogenous retroviruses, LINEs and SINEs (36,37). In this study, we showed that Vpr depletes HDAC1 and HDAC3 on the HIV-1 LTR of infected MDMs which was found in line with hyperacetylation of histones on this region. Hyperacetylation of histones is normally associated with active transcription and we showed that expression of Vpr was associated with active transcription and expression of the virus in a VprBP-dependent manner. However, Vpr did not enhance viral expression when equal numbers of WT and ΔVpr infected cells were compared. As depicted in Fig. 9 (lower panels), Vpr does not enhance integration of more proviral DNAs in the infected cells. Instead, Vpr counteracts latent infection of macrophages. Expression of Vpr maintains the HIV-1 LTR active, resulting in active expression of the viral genome. This function of Vpr increases the proportion of acutely infected macrophages. In the absence of Vpr, there are more latently infected macrophages and we showed that these cells carry a latent proviral genome integrated in their chromatin (Fig. 8A and B). As shown in Fig. 8C, the latent infections established in the absence of Vpr, can be reactivated by SAHA to restore a higher level of infectivity that is comparable with the infectivity of the wild-type virus. Interestingly, SAHA did not significantly reactivate viral infection in the wild-type infected macrophages, indicating that there is a lower prevalence of latently infected cells in this population.

We also showed that depletion of HDAC1 and HDAC3 using siRNA induces a similar effect as that of Vpr by activating the virus in the ΔVpr-infected MDMs. Taken together, our results suggest that Vpr depletes members of class I HDACs in a dose-dependent manner, with HDAC3 as the most affected HDAC, on chromatin of infected cells to maintain an active HIV-1 promoter.

A previous study has shown that components of Sin3a-HDAC1 complex are incorporated into HIV-1 virions. The study also showed that the
virion incorporation of HDAC1 plays a role in efficient early post-entry events (38). Since Vpr has already been shown to incorporate one of its target proteins, UNG2, into viral particles (39), it is worth examining whether Vpr plays a role in incorporation of HDACs into the virions or if there is a link between degradation and virion incorporation of HDACs.

In addition to the viral genome, Vpr-induced depletion of HDACs has the potential to activate or enhance expression of cellular genes that need to be further investigated. Maintaining an active LTR may be only one of the advantages of downregulation of HDACs. There may be other consequences in downregulation of HDACs on other genomic regions of the infected cells and even non-infected bystander cells that needs to be elucidated.

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Author Contributions: B.R., N.S.B., M.H.F., and E.A performed the experiments. E.A. and M.R.A supervised the study. E.A applied for grant. B.R. and N.S.B prepared the Figures. B.R. wrote the manuscript. All authors discussed the data and commented on the manuscript.

References


FIGURE 1. HIV-1 Vpr downregulates class I HDACs. A, HeLa-iFlag-Vpr cells were treated with doxycycline to express Flag-Vpr. Cells were harvested at 0, 2.5, 5, 10, 20, 30, and 40 h post-induction (h.p.i) and fractionated into soluble and chromatin-bound proteins. The experiment was repeated 4 times. B, HeLa-iFlag-Vpr (WT) and HeLa-iMock (Mock) cells were induced with doxycycline. After 30 h, cells were harvested and expression of class I HDACs was analyzed at mRNA and protein level. C, HeLa-iFlag-Vpr (WT) and HeLa-iMock (Mock) cells were induced with doxycycline. After 30 h, cells were fractionated and analyzed for depletion of class I HDACs, as well as SIRT3 (class III) and HDAC4 (class II). *P<0.05.

FIGURE 2. Vpr-induced depletion of class I HDACs is not resulted from G2/M arrest. A, HeLa-iFlag-Vpr and HeLa-iMock cells were induced using doxycycline. At the same time of doxycycline induction, 2.5 mM caffeine or vehicle control was also added to the media. Thirty hours post-induction, cells were harvested and 10^6 cells were spared for the cell cycle analysis using propidium iodide staining. The rest of the cells were fractionated and analyzed using Western blot. B, HeLa-iFlag-Vpr and HeLa-iMock cells were induced using doxycycline and normal HeLa cells were treated with 1 μM nocodazole. After 30 h, cells were harvested and the cell cycle and quantity of HDACs were analyzed.

FIGURE 3. Vpr induces depletion of class I HDACs through a proteasomal pathway. A, HeLa cells were transduced with lentiviral vectors expressing the Flag-tagged Vpr, Vpr mutants (Q65R and R80), SIVsmm Vpx, and empty vector at an MOI of 2.0. After 38 h, cells were treated with 10 μM MG132 or DMSO, and 10 h later (total of 48 h post-transduction), cells were fractionated and analysed. The experiment was repeated 4 times. B and C, HEK293T cells were cotransfected with vectors for expression of HA-ubiquitin (HA-Ub), Vpr and HDAC1-Flag or HDAC3-Flag. After 40 h, cells were treated with 10 μM MG132 or DMSO and after 8 h (total of 48 h post-transfection), cells were fractionated and immunoprecipitated using anti-HA antibody. Experiments shown in B and C were repeated two times and one representative experiment is shown.

FIGURE 4. Vpr binds and depletes class I HDACs through VprBP. A, HeLa-iFlag-Vpr (WT), HeLa-iFlag-Q65R (Q65R) and HeLa-iMock (M) cells were transfected with VprBP or non-targeting (NT) siRNAs. After 48 h, cells were induced with doxycycline. Cells were then fractionated 40 h post-induction and immunoprecipitated using αFlag antibodies. B, HeLa-iMock (M), HeLa-iFlag-Q65R (Q65R), and HeLa-iFlag-Vpr (WT) cells were induced using doxycycline. After 30 h, cells were treated with 10 μM MG132 or DMSO for 10 h. Cells were then harvested and fractionated. Fractions of the chromatin-bound proteins were immunoprecipitated using αFlag antibody. C, HeLa cells were fractionated and immunoprecipitated using VprBP or rabbit IgG antibodies in the presence of 25 μg/ml ethidium bromide. D, HeLa cells were fractionated and immunoprecipitated against class I HDACs (abbreviated to HD1, 2, 3, and 8) or rabbit IgG.

FIGURE 5. Vpr increases viral expression in MDMs through a VprBP-dependent mechanism. A, MDMs were transfected with VprBP or non-targeting siRNAs. After 48 h, MDMs were infected with the CCR5-tropic WT or Vpr-defective (∆Vpr) viruses. Supernatants of the infected cells were analyzed by p24 ELISA. Right: Western blot analysis of samples examined for depletion of VprBP. B, MDMs were infected with WT, ∆Vpr, and the mutant (Q65R and R80A) CCR5-tropic viruses and their supernatants were analyzed by p24 ELISA. C, MDMs were infected with the single cycle VSV-G-pseudotyped WT- or ∆Vpr-luciferase reporter viruses. After 7 days, luciferase activity was measured. D, MDMs from 8 donors were infected with the single cycle VSV-G pseudotyped WT, ∆Vpr, Q65R and R80A GFP marked viruses. After 4 days, cells were analysed for expression of GFP. E, One representative experiment as described in 5D. F, Primary MDMs were infected with the GFP-marked WT or ∆Vpr viruses at an MOI of 1.0. After 7 days, infected GFP-positive cells were sorted and equal numbers of cells were analysed using Western blot. G and H, MDMs were infected with the single cycle VSV-G pseudotyped WT and ∆Vpr GFP-marked viruses. After 4 days, GFP-positive and negative cells were sorted. To determine
levels of HIV-1 transcripts, quantitative PCR was performed on the total population (unsorted cells) or the sorted GFP-positive cells.

FIGURE 6. Vpr induces depletion of class I HDACs on the HIV-1 LTR in a proteasomal-dependent manner. A, Schematic presentation of the ChIP primers on the HIV-1 LTR. B, MDMs from 4 donors were infected with the GFP reporter WT or ΔVpr viruses. Seven days post-infection, cells were treated with 5 μM MG132, or DMSO for 20 h and the enrichment of class I HDACs on nuc-0, nuc-1, and nuc-2 was assessed by ChIP. C, MDMs were infected with the GFP reporter WT or ΔVpr viruses. Seven days after infection, the GFP-positive cells were sorted and enrichment of HDAC1 and HDAC3 on the gapdh gene was examined by ChIP.

FIGURE 7. Vpr induces hyperacetylation of histones on the HIV-1 LTR in a proteasomal-dependent manner. A and B, MDMs from 4 donors were infected with the GFP reporter WT or ΔVpr viruses. After 7 days, chromatin immunoprecipitations were performed against acetylated histone H3 (Ace H3K9) and acetylated histone H4 (Ace H4K5). Quantitative PCR was performed against nuc-0, nuc-1, and nuc-2, gapdh and an intergenic region (INT). C and D, MDMs were infected with the WT and ΔVpr viruses. After 7 days, cells were treated with 5 μM MG132 or DMSO for 20 h. Using ChIP, cells were then analysed for occupancy of acetylated histones (Ace H3K9 and Ace H4K5) on nuc-0. E, MDMs were infected with the WT and ΔVpr viruses. After 7 days, cells were ChIPed using antibody against total histone H3. The pulldown chromatin was analysed using quantitative PCR against nuc-0, nuc-1, nuc-2, and gapdh.

FIGURE 8. Vpr maintains an acute HIV-1 infection in MDMs. A and B, MDMs were infected with the single cycle VSV-G pseudotyped GFP-reporter WT and ΔVpr viruses. After 4 days, Alu-PCR was performed on the total populations (A). Alu-PCR was performed on sorted GFP-positive and negative cells (B). C, MDMs were infected with the single cycle VSV-G pseudotyped GFP-reporter WT and ΔVpr viruses. After 3 days, cells were treated with SAHA or DMSO and 24 h later (4 days post-infection), the GFP-positive and negative cells were sorted and analyzed using Alu-PCR. D, MDMs were infected with the single cycle VSV-G pseudotyped GFP-reporter WT and ΔVpr viruses and after 48 h, HDAC1 and HDAC3 were depleted using siRNA transfection. Four days post-infection, cells were analyzed for expression of GFP. Right: Western blot analysis of MDMs.

Figure 9. Vpr induces ubiquitination of class I HDACs on chromatin that results in a higher proportion of acutely infected macrophages versus latently infected macrophages. Vpr binds chromatin-associated class I HDACs through a VprBP-dependent mechanism and enhances ubiquitination and degradation of the targeted HDACs. This function of Vpr results in hyperacetylation of histones on certain regions including HIV-1 LTR. As a result, the wild-type virus counteracts latent infections and results in more acutely infected cells, despite the fact that the wild-type and ΔVpr HIV-1 virions infect equal numbers of macrophages in the first round of infection. There is a higher ratio of acute versus latent infections in the presence of Vpr but the total number of infected macrophages is not affected by Vpr in the first round of infection.
Figure 1
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A

Figure 2

B

Figure 2
### Figure 4

#### Table

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#### Figures

- **A**: Western blots showing the effect of NT siRNA and VprBP siRNA on soluble and chromatin-bound proteins. The proteins analyzed include VprBP, HDAC1, HDAC2, HDAC3, HDAC8, GAPDH, and His-H3.
- **B**: Western blots showing the effect of DMSO and MG132 on soluble and chromatin-bound proteins. The proteins analyzed include VprBP, HDAC1, HDAC2, HDAC3, HDAC8, iFlag-Vpr, His-H3, and IgG.
- **C**: Western blots showing the effect of input (4%) on soluble and chromatin-bound proteins.
- **D**: Western blots showing the effect of αHD1 IP, αVprBP IP, αHD2 IP, αHD3 IP, and αHD8 IP on soluble and chromatin-bound proteins.

**Legend**
- M: Control
- Q65R: Mutated form
- WT: Wild type
Figure 5
**Figure 6**

A. Diagram illustrating the mRNA expression and HDAC enrichment. The mRNA is shown as a horizontal line with three segments: U3, R, and U5. The GLS and gag regions are highlighted. The HDAC enrichment is represented by bars with different colors for ΔVpr and WT conditions. Stars denote significant differences.

B. Bar charts showing HDAC enrichment for nuc-0, nuc-1, and nuc-2 under DMSO and MG132 treatments. The ΔVpr and WT conditions are compared.

C. Additional bar charts with a y-axis labeled "HDAC enrichment."
Figure 7
Figure 8

A

B

C

D

Figure 8

A

B

C

D

Figure 8
Uninfected macrophage

Latently infected macrophage

Acutely infected macrophage

Infection with ΔVpr HIV-1

Infection with WT HIV-1

Components of the E3 ligase

VprBP

Histones

Ubiquitin

HDAC