**Supporting Information**

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**SI Materials and Methods**

**Cells and Reagents.** Peripheral blood mononuclear cells (PBMCs) from healthy blood donors were purified by Ficoll-Hypaque gradient centrifugation (11). Resting CD4 T cells were isolated from PBMCs by negative selection with the RosetteSep Human CD4 T Cells Enrichment Mixture (StemCell Technologies). Resting CD4 T cells were cultured at a density of 2 × 10⁶ cells/mL in RPMI-1640 medium (Gibco) supplemented with 10% (vol/vol) heat-inactivated FCS, glutamine (2 mM), and antibiotics (100 units/mL penicillin, 100 mg/mL streptomycin). For T-cell activation, phytohemagglutinin-P (PHA-P) (5 μg/mL) (Sigma-Aldrich) was added to the culture medium for 2–3 days together with 100 IU/mL IL2 (Bionord). Monocytic-macrophage-derived macrophages were isolated from PBMCs by negative selection with Monoxyte Isolation Kit II (Miltenyi Biotec) and separated by the autoMACS Pro Separator. Monocytes were differentiated in coated 24-well plates (Greiner) containing DMEM (Gibco) supplemented with 10% (vol/vol) heat-inactivated FCS, glutamine (2 mM), antibiotics (100 units/mL penicillin, 100 mg/mL streptomycin), and 10% (vol/vol) human AB-serum (Sigma-Aldrich). The human cell lines THP-1 and 293T were cultivated as reported (28). The following fluorochrome-conjugated antibodies were used: anti-human CD25 (clone M-A251, from healthy blood donors were purified by Ficoll-Hypaque sedimentation. The overall SAMHD1 phosphorylation pattern, lysates of YFP* cells were subjected to SDS-PAGE on phos-tag acrylamide (Wako Chemicals) gels according to the manufacturer’s instructions.

**PCR Analyses.** TaqMan-based quantification of HIV-1 2-LTR circles in DNA extracts from infected resting CD4 T cells and primary monocyte-derived macrophages was performed as described (38). The following quantitative PCRs were designed and validated based on the primer regions chosen by Zack et al. (6). Quantification of U3 region: forward primer M665 (5’-TCT ACC CTA TGA GCC AGC AT-3’), reverse primer M666 (5’-AGG CCA CAC CTC CCT GGA-3’), and fluorescent-labeled probe M665 (FAM-AGA GCT GCA TCC GGA GTA CAA AGA CTT GT-3’), reverse primer AA55 (5’-TGC TAG AGA TTT TCG CCT ACA CTG ACC AA-3’), and fluorescence-labeled probe M667 (FAM-TTG AGT CAA GTG YTG TTC CCG TCT GTT-TAMRA). Cycling conditions for U5 region were: 50 °C 2 min, 95 °C 10 min, 40 cycles with a denaturation step at 95 °C for 15 s, and annealing and elongation step at 62 °C for 1 min. Quantification of R5 region is as follows: forward primer M667 (5’-CAG CAC TGT GAA CCC ACT CCT GTA A-3’), reverse primer AA55 (5’-TGC TAG AGA TTT TCG CCT ACA CTG ACC AA-3’), and fluorescence-labeled probe M667 (FAM-TTG AGT CAA GTG YTG TTC CCG CCG GTT TAMRA). Cycling conditions were similar to R5-gag (11). Quantification of R5-PBS region is as follows: forward primer M667, reverse primer BB301 (5’-TTG CGC TTT GTA GGT CTC CCT GGT GGC ATT-3’), and fluorescence-labeled probe M667. Cycling conditions were already described (11). Quantification of gag is as follows: forward primer LA8 (5’-CAG GAC TCG TGC TGT GGA A-3’), reverse primer LA9 (5’-CTC GCA CCC ATC TCT CTC CTT-3’), and fluorescence-labeled probe LA8 (FAM-TCC AGT CCA AAT TTT TGG CGT ACT CAC TAMRA). Cycling conditions were default settings with an annealing and elongation step at 60 °C. Quantification of tat/rev was as follows: forward primer LA45 (5’-GAC AAA AGC CTT AGG CAT CTA CCA TTA-3’), reverse primer LA64 (5’-ATA GTG TGC ATT ACA TGT ACT ACT TGG T-3’), and fluorescence-labeled probe LA45 (FAM-TGA GTC TGA CTT TGC TTA GCA GTT CTT GGT CTT TAMRA). Cycling conditions were default settings with an annealing and elongation step at 60 °C. RNaseP quantification served as endogenous control. To account for residual plasmid DNA contaminations in virus preparations and to quantify only de novo synthesized HIV-1 cDNA, cells were treated with the RT inhibitor efavirenz, infected and measured in parallel. Data sets presented in Figs. 2 and 4 show normalized values, where input DNA contamination accounted for at maximum 30% of the absolute signal, and these values were subtracted from the values of cells in the absence of inhibitor.

**dNTP Assay.** dNTPs from primary cells were extracted and quantified using the protocol previously described by Diamond et al. (41). Briefly, this assay uses a 5’ P²³ radiolabeled 23-mer primer annealed to one of four distinct 24-mer templates. The single nucleotide
overhang on the 24-mer template (A, C, G, or T) determines
the dNTP to be measured upon the dNTP incorporation. The
template/primer was incubated with extracted cellular dNTPs
and purified HIV-1 RT for 5 min at 37 °C and then quenched
with 40 mM EDTA and 99% (vol/vol) formamide at 95 °C for
2 min. The reactions were resolved on a 20% (wt/vol) urea-
PAGE gel (American Bio Sequel NE reagent) and imaged
using Pharos FX molecular imager (Bio-Rad Laboratories).
The increase in radiolabeled 24-mer product indicates that the
dNTP specific for the template has been incorporated, and the
primer extension amounts were used to measure the dNTP
levels in the samples.

Fig. S1. (A) Immunoblotting of X4 HIV-1*GFP virions for incorporated epitope-tagged Vpx proteins from SIVmac239 (WT and Q76A mutant; myc), SIVmd-2 or SIVrcm (WT and corresponding H72A and Q75A mutant, respectively; both flag). HIV-1 p24CA served as a loading control. The use of different tags pre-
cluded quantitative comparisons of virion incorporation of these Vpx variants. myc-Vpx–tagged and flag-Vpx–tagged virions were run on the same membrane, respectively, and unrelated samples are not shown. (B) Quantification of physical particles via p24CA ELISA, RT activity via SG-PERT, and determination of the
infectious titer on TZM-bl reporter cells via blue cell assay of sucrose-pelleted virions. Depicted is the mean ± SEM of two (p24CA ELISA) or three (RT activity and
blue cell assay) independently produced virus stocks.

<table>
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<tr>
<th>Vpx</th>
<th>p24CA ELISA (ng/ml)</th>
<th>RT activity (U/ml)</th>
<th>Blue cell assay (IU/ml)</th>
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<tr>
<td>no Vpx</td>
<td>28089 ± 9194</td>
<td>1.5x10^10 ± 1.3x10^10</td>
<td>1.5x10^7 ± 1x10^7</td>
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<tr>
<td>Vpx mac239</td>
<td>20501 ± 13595</td>
<td>8.9x10^10 ± 7.8x10^10</td>
<td>2.5x10^7 ± 1.3x10^7</td>
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<tr>
<td>Vpx md-2</td>
<td>33258 ± 21840</td>
<td>3.5x10^11 ± 3.4x10^11</td>
<td>1.9x10^7 ± 9.4x10^6</td>
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<tr>
<td>Vpx rcm</td>
<td>9824 ± 33</td>
<td>2.9x10^10 ± 7.9x10^10</td>
<td>3.9x10^6 ± 1.4x10^6</td>
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**Fig. S2.** (A) Quantification of GFP expression levels 3 d postinfection of activated CD4 T cells challenged with equal TZM-bl infectious units of X4 HIV-1*GFP* reporter virus carrying no Vpx or the indicated Vpx variants. Depicted is the mean ± SE of mean of seven donors, each measured in technical triplicates. (B) Time course of SAMHD1 expression in resting CD4 T cells after challenge with HIV-1*GFP* with incorporated Vpx mac239 (n = 4 donors), Vpx mnd-2 (n = 4 donors), or Vpx rcm (n = 2 donors). Data points mark the mean percentages (±SD) of cells with high SAMHD1 expression levels.
Fig. S3. (A–C) Resting CD4 T cells were loaded with Cell Trace Dye (A) or BrdU (B) 1 d before infection and analyzed 3 d postinfection for Cell Trace-FarRed and BrdU expression levels, respectively. Alternatively, cells were stained for CD25/CD69 at the day of analysis (C). Depicted is one representative donor out of eight. Activated T cells served as positive control.
Fig. S4. (A) Schematic of HIV-1 reverse transcription to illustrate the six different early to late HIV-1 RT products that were quantitatively detected by real-time PCR. (B) Positions of the different primer pairs are indicated and numbers refer to the order of detection shown in Fig. 2.
Fig. S5. (A) Sequence alignment generated with ClustalW of Vpx proteins from SIVrcm, SIVmnd-2, SIVmac239, HIV-2 GH-1, and HIV-2 ROD9. Boxes highlight helical structures. Arrows indicate positions for site-directed mutagenesis in SIVmac239 Vpx. Note that for Vpx rcm, mnd-2, and Rod9, the N-terminal flag tag and linker sequences are included in the alignment. (B) Summary of functional analyses of Vpx mutants and variants in resting CD4 T cells and monocyte-derived macrophages (MDMs). + or − delineate the ability or inability, respectively, of the depicted virion-incorporated Vpx mutants or variants to lead to dNTP elevation, SAMHD1 degradation, and HIV-1*GFP infection enhancement. n.d., not done.
MDMs were infected with VSV-G pseudotyped HIV-1*GFP with incorporated SIVmac239 WT or the depicted mutants using identical TZM-bl infectious units. (A and B) Primary FACS dot plots for SAMHD1 expression and GFP of MDMs from one donor 20 h postinfection (A) and 72 h postinfection (B). (C) Quantification of the percentage of GFP⁹ cells 72 h postinfection. Depicted is the arithmetic mean + SEM of four donors, each measured in technical duplicates.

Fig. S6.
Fig. S7. (A) Resting CD4 T cells were challenged with X4 HIV-1*GFP carrying the indicated Vpx WT variants and mutants, the latter incapable of interaction with the proteasome complex. Cells were challenged with identical TZM-bl infectious units and analyzed for GFP expression 3 d postinfection. Primary dot plots for GFP expression are shown for one representative donor. (B) Quantification of the percentage of GFP+ cells shown in A. Depicted are arithmetic means ± SEM of analyses of eight (SIVmnd-2WT and SIVmnd-2H72A) and four donors (SIVrcmWT and SIVrcmQ75A), respectively, each measured in technical triplicates.
Fig. S8. Proposed model for HIV restrictions in primary resting CD4+ T cells and counteraction by SIV Vpx variants. (A) Both SAMHD1 (RT block 1) and an unknown factor (RT block 2) are able to restrict HIV at the level of reverse transcription. Downstream, an unknown factor limits nuclear import of the preintegration complex (NI block 1). (B) SIVmac239 Vpx WT targets SAMHD1 for degradation to overcome RT block 1. In the presence of SAMHD1 and RT block 2, SAMHD1 is the preferred target of SIVmac239 Vpx WT but in the absence of SAMHD1, RT block 2 is targeted. SAMHD1 degradation-deficient mutants of SIVmac239 Vpx target RT block 2 similarly to SIVmnd-2 and SIVrcm Vpx through a mechanism that likely involves proteasomal degradation. (C) SIVmnd-2 and SIVrcm Vpx are unable to target SAMHD1 for degradation but apparently overcome the major restriction at the level of reverse transcription by targeting RT block 2. Despite highly efficient reverse transcription, levels of 2-LTR circles are similar to those observed with SIVmac239 Vpx, indicating that also under these conditions, nuclear import is restricted by NI block 1.