Sterile alpha motif domain- and HD domain-containing protein 1 (SAMHD1) is a deoxynucleoside triphosphohydrolase that restricts the replication of lentiviruses in myeloid cells by hydrolyzing the cellular deoxynucleotide triphosphates to a level below that which is required for reverse transcription. Human immunodeficiency virus type 2 (HIV-2) and some simian immunodeficiency viruses (SIVs) encode the accessory protein viral protein X (Vpx) that counteracts SAMHD1. Vpx recruits SAMHD1 to a cullin4A-RING E3 ubiquitin ligase (CRL4), which targets the enzyme for proteasomal degradation. Vpx and SAMHD1 both localize to the nucleus of the cell. We identified the nuclear localization sequence (NLS) of SAMHD1 as a conserved KRPR sequence at amino acid residues 11 to 14. SAMHD1 lacking a functional NLS localized to the cytoplasm but retained its triphosphohydrolase and antiviral activities. However, cytoplasmic SAMHD1 was resistant to Vpx-induced degradation, and its antiviral activity was not counteracted by Vpx. Cytoplasmic SAMHD1 interacted with Vpx and retained it in the cytoplasm. The inhibition of nuclear export with leptomycin B did not impair the ability of Vpx to degrade SAMHD1. These findings suggest that SAMHD1 is targeted by Vpx for ubiquitination and degradation in the nucleus.

Human cells express a variety of different proteins that restrict lentiviral replication, such as members of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) protein family of cytidine deaminases, tripartite motif protein 5 alpha (TRIM5α), and tetherin (22). To replicate successfully, lentiviruses have evolved to contain accessory proteins that counteract the antiviral activities of these restriction factors. One such antiviral mechanism is mediated by sterile alpha motif domain and HD domain-containing protein 1 (SAMHD1), a cellular deoxynucleoside triphosphohydrolase that is expressed in myeloid cells, where it blocks viral replication at the level of reverse transcription (18, 26). Lentiviruses, such as human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus (SIV) of sooty mangabeys, encode the Vpx accessory protein to counteract SAMHD1-mediated restriction. In SIVmus (SIV infecting mus-tached monkeys) and SIVdeb (SIV infecting De Brazza’s monkeys), this function is served by the related accessory protein viral protein R (Vpr) (25, 30). HIV-1 does not encode a Vpx protein, and its Vpr does not counteract SAMHD1; as a result, it remains sensitive to SAMHD1-mediated restriction.

SAMHD1, which was first identified as a gamma interferon (IFN-γ)-inducible mouse gene (24, 29), belongs to a family of HD domain–containing enzymes. The protein is a homodimer consisting of a short amino-terminal domain followed by a sterile alpha motif (SAM) domain and an HD domain. The SAM domain is thought to be involved in protein–protein or protein–RNA interactions (38), while the HD domain mediates catalytic activity (3). The HD domain catalyzes triphosphohydrolase activity, converting deoxynucleoside triphosphates to the deoxynucleoside and inorganic triphosphates (12, 34), when dGTP binds to the allosteric binding site in SAMHD1. Mutations in the HD domain of SAMHD1 that prevent hydrolysis activity result in the loss of antiviral function. In humans, polymorphisms in the SAMHD1 gene are associated with Aicardi-Goutières syndrome (AGS), a rare early-onset neurological syndrome associated with the overproduction of type I interferons (36). Several such polymorphisms that introduce missense mutations or premature terminations of the open reading frame and result in a lack of catalytic activity have been identified.

The triphosphohydrolase activity of SAMHD1 suggests that its antiviral activity may be mediated by the hydrolysis of deoxynucleoside triphosphates in the cell. In support of this mechanism, the expression of a transduced SAMHD1 gene in differentiated U937 cells causes deoxynucleoside triphosphate (dNTP) levels to fall as much as 100-fold, a level that is below the Km of reverse transcriptase and is insufficient to support reverse transcription (21, 27). Further supporting this mechanism is the fact that the addition of extracellular deoxynucleosides (dN) to monocyte-derived macrophage (MDM) cultures partially rescues lentiviral infection. In addition, the treatment of SupT1 cells that express a transduced SAMHD1 gene with hydroxyurea, an inhibitor of ribonucleoside reductase (32), accentuates the restriction to HIV-1 infection.

SAMHD1 is counteracted by the lentiviral accessory protein Vpx and, for some primary SIVs, by Vpr. Both accessory proteins are packaged into lentiviral virions, allowing them to act in the newly infected host cell prior to proviral integration. They are packaged through an interaction with amino motifs in the car-

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boxy-terminal p6 region of the Gag precursor polypeptide (p6\textsuperscript{poe}). Transfer of the motif, which in SIV infecting macaques (SIVmac) is located at amino acids 17 to 26 of p6 (1), into the corresponding location of HIV-1 Gag results in HIV-1 virions that package Vpx (39). Such virions are much more infectious on MDM and dendritic cell (DC) cultures than wild-type HIV-1. Vpx can also be provided in trans to myeloid cells by virus-like particles (VLPs). The pretreatment of DC with Vpx-containing VLPs enhances ∆vpx SIV and HIV-1 infections by as much as 100-fold (7, 14, 18, 26).

Upon infection of a target cell, virion-packaged Vpx molecules are thought to be released from the virion and to associate with the cullin4A-RING E3 ubiquitin ligase (CRL4) (2, 6), a multisubunit complex that consists of DNA damage binding protein 1 (DDB1), RING box protein 1 (RBX1), cullin ubiquitin ligase 4A (Cul4A), and DDB1- and Cul4A-associated factor 1 (DCAF1) (19, 28). Vpx binds to the carboxy-terminal domain of SAMHD1 and to DCAF1, loading SAMHD1 onto the complex. The interaction results in the proteasomal degradation of the SAMHD1 protein and an increase in dNTP levels approximately 8 h later (21). The binding of Vpx to SAMHD1 is both species and virus specific (2, 25, 30). Amino acids in the Vpx interaction domains at the amino and carboxy termini of SAMHD1 are under strong positive selection. The selective pressure suggests that SAMHD1 evolution is driven by lentiviruses and points to the importance of SAMHD1-mediated restriction on virus transmission and replication.

The Vpx and SAMHD1 proteins both localize to the nucleus of the cell. While the amino acid residues of Vpx that cause its nuclear localization have been characterized previously (5, 31, 33, 35), the determinants that direct SAMHD1 nuclear localization are not known, and the significance of SAMHD1 nuclear localization in lentiviral restriction and nucleotide pool depletion is also not known. In this study, we identified the nuclear localization signal (NLS) of SAMHD1 and found that when the NLS was mutated, SAMHD1 relocated to the cytoplasm but retained catalytic activity and antiviral function. The cytoplasmic SAMHD1 protein interacted with Vpx but was resistant to Vpx-induced degradation. Moreover, when SAMHD1 with the mutated NLS was coexpressed with Vpx, it redirected Vpx to the cytoplasm. In addition, the nuclear export inhibitor leptomycin B did not interfere with the Vpx-induced degradation of SAMHD1. Our findings strongly suggest that Vpx induces SAMHD1 ubiquitination and degradation in the nucleus.

**MATERIALS AND METHODS**

**Cell culture.** 293T and HeLa cell lines were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (FBS). U937 and THP1 cells were grown in RPMI 1640–10% FBS. U937 and THP1 cells were differentiated for 20 h with 30 ng/ml phorbol 12-myristate 13-acetate (PMA).

**Plasmids.** Hemagglutinin (HA)-tagged human and mouse SAMHD1 constructs were generated by PCR using Phusion Hot Start II high-fidelity polymerase (Finnzymes) as described previously (27). The amplicons were cleaved with BamHI and XhoI and then ligated into BamHI- and XhoI-digested pLenti (Addgene). Truncated SAMHD1 fragments were generated using the SAMHD1 construct as a template with the primers SAMHD1-F and SAMHD1ΔCterm-R for the SAMHD1 (residues 1 to 354) construct and the primers SAMHD1-R and SAMHD1ΔNterm-F for the SAMHD1 (residues 37 to 626) construct. Point mutations were introduced into the SAMHD1 NLS by using overlapping PCR. GFP-SAMHD1 constructs were generated by fusing the green fluorescent protein (GFP) fragment amplified from pLenti to the N terminus of the SAMHD1 protein or ΔNLS.SAMHD1 fragment amplified from the pLenti SAMHD1 purycin resistance gene (purin) through overlapping PCR. The fusion product was cleaved with BamHI and SalI and then ligated into BamHI- and SalI-digested pLenti. All plasmids were confirmed by nucleotide sequencing, and expression was confirmed by immunoblot analysis. Plasmids expressing FLAG-tagged Vpx from HIV-2 7312a and SIV infecting red-capped mangabeys (SIVrcm) isolate NG411 or those expressing FLAG-tagged Vpr from SIVmus isolate CM1239 and SIVdeb isolate CM5 have been described previously (30). The Myc-His-tagged Vpx (SIVmac239) plasmid has been described previously (39).

SAMHD1-targeted short hairpin RNA (shRNA) (reagent TRCR0000145408) in the pLKO.1 puro lentiviral vector was from the MISSION shRNA library (Sigma). A lentiviral vector that expressed an shRNA-resistant (shRNA*) SAMHD1 was generated by removing the purycin resistance gene of pLenti.puro and replacing it with a neomycin resistance (neo) cassette that had been amplified by PCR from pcDNA3.1 (Invitrogen). The ampiclon was cloned into the NsiI and XhoI sites to generate pLenti-GFP-neo. The shRNA target site was altered from the wild-type SAMHD1 sequence (GGGAGTATATAGAGATT) to the shRNA* sequence (GGGGAGTATATAGAGATT) by overlapping PCR and cloned into pLenti-neo at the BamHI and SalI sites to generate pLenti-SAMHD1-shRNA*-neo.

**Virus and VLP preparation.** Viruses were produced in 293T cells cotransfected with reporter virus plasmid and pSV-G (which expresses the G glycoprotein of the vesicular stomatitis virus) using calcium phosphate. An HIV-1 cytokemovirus (CMV)-GFP reporter virus was pseudotyped with pSV-G by transfection at a mass ratio of 3:1. The infectious titer of the virus was determined using 293T cells as the number of GFP-positive cells per ml of virus stock. Vpx-containing and control VLPs were produced by the transfection of 293T cells with pSI3\textsuperscript{V} (VLP Vpx\textsuperscript{V}) or pSI3\textsuperscript{V} + ∆vpx (VLP Vpx\textsuperscript{V}) (14), respectively. VLP packaging of different Vpx and Vpr proteins from SIV and HIV-2 was produced by the cotransfection of 293T cells with pSI3\textsuperscript{V} (VLP Vpx\textsuperscript{V}) (15) and their respective FLAG-tagged Vpx or Vpr expression plasmids. The VLPs were pelleted through 20% sucrose by ultracentrifugation at 30,000 rpm for 90 min at 4°C and lysed in 1% NP-40 buffer containing a protease inhibitor. The lysates were analyzed on an immunoblot probed with an anti-FLAG M2 monoclonal antibody (MAB) (Sigma), an anti-myc MAb (Covance), or an anti-SIVmac p27 MAb (clone 55-2F12) obtained from the NIH AIDS Research and Reference Reagent Program (17).

Lentiviral expression vector stocks were generated by the cotransfection of 293T cells with pLenti.SAMHD1, pRSV-Rev, pMDG gag-pol, and pVSV-G using calcium phosphate. Polyclonal U937 or HeLa cell lines that stably expressed SAMHD1 were established by infection with the lentiviral expression vectors and selection in 1 μg/ml puromycin. The clones U937 cell lines were generated by limiting dilution. SAMHD1 expression in the single-cell clones was determined by immunoblot analyses. Lentiviral shRNA expression vector stocks were produced by the cotransfection of 293T cells with pLKO.1-shRNA scramble or anti-SAMHD1, pΔN8,2, and pSV-G at a mass ratio of 3:1:1 using calcium phosphate. THP1 cells were infected with the vectors and selected in 1.0 μg/ml puromycin. The THP1 cell lines transduced with SAMHD1 shRNA were complemented by infection with the pLenti-neo vector encoding an shRNA-resistant cDNA for SAMHD1 and then selected with 1.0 μg/ml puromycin and 0.4 mg/ml G418 ([geneticin, O-2-amino-2,7-dideoxy-o-glycero-α-L-rutheo-hexopyranosyl-(1→4)-O-(3-deoxy-4-C-methyl-3-(methylamino)-B-L-arabinopyranosyl-(1→6)]-o-streptamine).

**Fluorescence microscopy.** HeLa cells (5.0 × 10\textsuperscript{5}) were transfected with 4.0 μg of HA-tagged SAMHD1 expression vector, a Myc-tagged Vpx expression vector, or pRhe1.4-GFP-nuclear export signal (NES) (20) using Lipofectamine 2000 (Invitrogen). Alternatively HeLa cells stably expressing GFP tagged SAMHD1 were transfected with a myc-tagged Vpx.
expression vector. The next day, the cells were transferred to a 35-mm glass-bottom culture dish (MatTek). The following day, the Vpx-transfected cells were treated with 20 μM proteasome inhibitor MG132 for 5 h, fixed with 4% paraformaldehyde, and permeabilized with 1% Triton X-100. The cells were stained with the anti-influenza hemagglutinin (HA) epitope tag monoclonal antibody (MAB) HA.11 (Covance) or the anti-Myc MAB 9E10 (Covance) followed by Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody (Invitrogen). The nuclei were counterstained with Hoechst 33342 (Molecular Probes). The cells were imaged on an LSM 710 laser scanning microscope (Zeiss), and the images were analyzed using ImageJ software. HeLa cells transfected with pVpx-GFP-NES were treated with 0, 1.0, or 10.0 ng/ml of leptomycin B for 3 h and fixed. The cells were counterstained with Hoechst 33342 and visualized using an Eos fluorescence microscope (Advanced Microscopy Group).

In vitro dNTP hydrolysis assay. 293T cells were transfected with SAMHD1 or ∆NLS.SAMHD1 expression vectors using Lipofectamine 2000. After 2 days, cells lysates were prepared and precleared for 1 h at 4°C with 30 μl protein G-Sepharose (GE Healthcare). SAMHD1 was immunoprecipitated for 1 h at 4°C with 2 μg of the anti-HA MAB followed by 1 h with 50 μl of protein G-Sepharose. The beads were washed three times with lysis buffer (50 mM HEPES, 150 mM KCl, 2 mM EDTA, 0.5% NP-40) and once with an assay buffer (250 mM Tris [pH 8.0], 250 mM KCl, 25 mM MgCl₂, 0.5% Triton X-100). Half of the beads were used to elute the bound protein by heating them to 95°C in gel loading buffer. The eluted protein was analyzed by immunoblot analysis. The residual beads were incubated with 0.2 μCi [γ-32P]ATP in the presence of 0.4 mM unlabeled dATP and 0.4 mM unlabeled dGTP for 1 h at 37°C in the assay buffer. The enzyme was inactivated at 70°C for 5 min. The reaction products were separated on a cellulose 300 polyethylenimine thin-layer chromatography (TLC) plate (Sorbert Technologies) in TLC running buffer (1 M LiCl, 0.5 M formic acid) and visualized using autoradiography on Hyperfilm (GE Healthcare).

Quantification of cellular dNTP pools. U937 cells that stably expressed SAMHD1 (2.0 × 10⁶) were differentiated with PMA for 20 h and lysed, and the levels of dNTPs in the lysate were determined by the single nucleotide extension assay as described previously (10).

U937 cell infection assay. U937 cell lines were plated at 5.0 × 10⁴ per well in a 96-well dish and cultured overnight in the presence of 30 ng/ml PMA. Vpx-containing control VLPs were added, and the cells were infected with the HIV-1 CMV-GFP reporter virus. The cells were infected in triplicate, and mock-infected controls were included. After 2 days, the cells were harvested, and the number of GFP-positive cells was quantified by flow cytometry on an LSRII (BD Biosciences). The data were analyzed using FlowJo software.

THP1 infection assay. SAMHD1-silenced THP1 cell lines (5.0 × 10⁶) expressing SAMHD1 or ΔNLS.SAMHD1 were differentiated with PMA for 20 h in a 96-well plate. The cells were treated with Vpx-containing and control VLPs for 2 h and then infected with the VSV-G-pseudotyped NL4-3.E⁺ luciferase reporter virus (9). After 3 days, the luciferase activity was measured using Steady Light Plus reagent (PerkinElmer).

SAMHD1 degradation assay. U937 cells stably expressing human wild-type or mutant SAMHD1 were differentiated for 20 h with 30 ng/ml PMA and then incubated with Vpx- or Vpr-containing and control VLPs for 10 h. The amount of Vpx-containing VLPs used was the amount required to induce the complete degradation of SAMHD1 in U937 cell lines that stably expressed SAMHD1. The control VLPs were used with a corresponding amount of p27 MAB. The cells were lysed in lysis buffer (50 mM HEPES, 150 mM KCl, 2 mM EDTA, 0.5% NP-40) containing Halt protease inhibitor (ThermoScientific), and the SAMHD1 was detected by immunoblot analysis. Stably transfected HeLa cells were seeded in a 24-well plate (5.0 × 10⁴ cells per plate). The next day, Vpx-containing and control VLPs were added together with 10.0 ng/ml leptomycin B (Sigma). After 4 h, MG132 (10 μM) was added. After an additional 6 h of incubation, the cells were lysed in NP-40 lysis buffer containing the protease inhibitor, and SAMHD1 was detected by immunoblot analysis. The immunoblots were visualized using the Odyssey Fc dual-mode imaging system (Li-Cor) and quantified with Image Studio software (Li-Cor).

Coimmunoprecipitation. 293T cells were cotransfected with pcDNA6-based expression plasmids encoding wild-type or mutant HA.SAMHD1 and Vpx.myc-His using Lipofectamine 2000. Two days posttransfection, 20 μM MG132 was added, and after 5 h, cell lysates were prepared using lysis buffer (50 mM HEPES, 150 mM KCl, 2 mM EDTA, 0.5% NP-40). The lysates were precleared for 1 h with protein G-Sepharose and then incubated with the anti-Myc MAB 9E12 (Covance) for 1 h on ice. The complexes were collected by the addition of 50 μl of protein G-Sepharose and analyzed on an immunoblot.

RESULTS
Identification of a nuclear localization signal in SAMHD1. SAMHD1 is a nuclear-localized protein, but the determinants that specify nuclear localization have not been identified. An analysis of the amino acid sequence using NLS prediction software (23) identified a consensus KRPR NLS amino terminal to the SAM domain (Fig. 1A) that is conserved in the human, rhesus, and mouse homologues. To determine whether the motif specifies the nuclear localization of SAMHD1, we generated expression vectors for mutated forms of the protein. The vectors expressed SAMHD1 with a deletion of amino acids 1 to 36 (SAMHD1[residues 37 to 626]), a mutation of the three amino acids of the consensus motif to alanine (∆NLS.SAMHD1), or a truncation of the 272 carboxy-terminal amino acids (SAMHD1[residues 1 to 354]). To determine the cellular localization of the mutated SAMHD1 proteins, we transfected HeLa cells with each of the mutated expression vectors or a wild-type control and visualized the protein by indirect immunofluorescence. We found that human and mouse isoform 2 (mu iso2) SAMHD1, the major splice variant expressed in mouse cells, localized to the nucleus, as did the C-terminal-truncated SAMHD1(residues 1 to 354). In contrast, SAMHD1(residues 37 to 626) and ∆NLS.SAMHD1 were cytoplasmic (Fig. 1B). We have concluded that the KRPR motif determines the nuclear localization of SAMHD1.

Cytoplasmic SAMHD1 restricts HIV-1 infection. To investigate the role of nuclear localization in the function of SAMHD1, we compared the properties of the nucleus-localized SAMHD1 with those of the mutated NLS of SAMHD1. To determine whether the mutated cytoplasmic protein retained catalytic activity, we tested it for triphosphohydrolase activity in an in vitro assay. For this, we transfected 293T cells with expression vectors for SAMHD1, ∆NLS.SAMHD1, or catalytically inactive SAMHD1 (H233A). After 2 days, we lysed the cells, immunoprecipitated the HA-tagged SAMHD1 with the anti-HA MAB, and tested its catalytic activity in an in vitro phosphohydrolase assay. Unlabeled dGTP was included in the assay because the mammalian cell-expressed SAMHD1 is dGTP dependent (see Fig. S1 in the supplemental material), as is the case for an Escherichia coli-produced recombinant protein (12, 34). As shown by the production of inorganic triphosphate (PPP), both SAMHD1 and the ∆NLS.SAMHD1 mutant were catalytically active (Fig. 2A). The catalytically inactive SAMHD1(H233A) produced no detectable PPP, P, was not detected, demonstrating the absence of contaminating phosphatas. We have concluded that the cytoplasmic SAMHD1 protein retained its enzymatic activity.

The ability of ∆NLS.SAMHD1 to hydrolyze dNTP suggests that it retains the ability to deplete the cellular dNTP pools of...
myeloid cells. To determine whether this was the case, we generated U937 cell lines that stably expressed the mutant and wild-type SAMHD1 proteins. U937 cells do not express endogenous SAMHD1, but when transduced with expression vectors and differentiated with PMA, they support SAMHD1-mediated restriction. We differentiated the cells with PMA and quantified the intracellular dNTPs using the single nucleotide extension assay (10). The cells that expressed SAMHD1 or \( \text{H}9004 \text{NLS.SAMHD1} \) had a 5- to 6-fold lower level of dATP and dTTP than a mock-transduced cell line or a cell line expressing a catalytically inactive SAMHD1 with alanine mutations in the HD domain \( \text{SAMHD1(HD-AA)} \) (Fig. 2B; see also Fig. S2 in the supplemental material). This result showed that \( \text{H}9004 \text{NLS.SAMHD1} \) retained its ability to deplete the intracellular dNTP pool.

To determine whether the nuclear localization of SAMHD1 is required for antiviral activity, we infected PMA-differentiated U937 cells stably expressing human SAMHD1, mouse SAMHD1, SAMHD1(residues 37 to 626), and \( \text{H}9004 \text{NLS} \) with the HIV.GFP reporter virus. The results showed that SAMHD1(residues 37 to 626) and \( \text{H}9004 \text{NLS} \) had the highest restriction activity. These findings demonstrate that the cytoplasmic forms of SAMHD1 retain their antiviral activities and suggest that the protein may have increased antiviral activity when localized to the cytoplasm.

**Cytoplasmic SAMHD1 is resistant to Vpx.** To determine whether the antiviral activity of cytoplasmic SAMHD1 was sensitive to Vpx, we incubated the U937 cell lines that stably expressed SAMHD1 with Vpx-containing or control VLPs and then infected them with the HIV.GFP reporter virus. We found that Vpx from SIVmac251 relieved the block to the infection of cells expressing SAMHD1 but not those expressing \( \text{H}9004 \text{NLS} \) (Fig. 2E). To test whether this was the case in another cell line, we generated a clonal THP1 cell line in which the endogenous SAMHD1 was knocked down by a targeted shRNA and was complemented by shRNA-resistant SAMHD1 or \( \text{H}9004 \text{NLS} \). The infection of these cells after the addition of VLPs confirmed the resistance of

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**Figure 1** Identification of the SAMHD1 NLS. (A) The domain structure of SAMHD1 is shown at the top, and at the bottom is the ClustalW alignment of the first 36 amino acids in human (Homo sapiens), rhesus (Macaca mulatta), and mouse (Mus musculus) SAMHD1. A single NLS motif (boldface) was identified by analysis with cNLS mapper software (23). Identical residues are indicated by asterisks, strongly conserved residues by colons, weakly conserved residues by dots, and nonconservative changes by blank spaces. A \( \text{H}9004 \text{NLS} \) mutant was generated in which the K11, R12, and R14 residues were changed to alanine. (B) The images show confocal immunofluorescence of HeLa cells transfected with HA-tagged human SAMHD1 (wild type [WT]), \( \text{H}9004 \text{NLS} \), amino-terminal-deleted SAMHD1(residues 37 to 626), carboxy-terminal-truncated SAMHD1(residues 1 to 354), and mouse SAMHD1 isoform 2 (mu iso2) expression vectors. The transfected SAMHD1 was stained with an anti-HA antibody (red), and nuclei were counterstained with Hoechst (blue).
ΔNLS.SAMHD1 to Vpx (see Fig. S3 in the supplemental material). We noted in these cells that the ΔNLS.SAMHD1 was expressed at a higher abundance than the wild-type protein, as discussed below.

**Nuclear localization of SAMHD1 is required for Vpx-mediated degradation.** To determine the susceptibility of ΔNLS.SAMHD1 to Vpx, the U937 cell lines were incubated with dATP-containing or control VLPs. After 10 h, the SAMHD1 protein levels in the cells were quantified by immunoblot analysis. The results showed that Vpx-containing VLPs, but not control VLPs, caused a dose-dependent degradation of ΔNLS.SAMHD1 in the U937 cell lines expressing wild-type (WT), mouse isoform 2 (mu iso2), or the indicated mutated SAMHD1 protein (Fig. 3B). The ΔNLS.SAMHD1 proteins expressed in the U937 cell lines were shown on an immunoblot probed with an anti-HA MAb. GAPDH was detected as a loading control. In these cell lines, ΔNLS.SAMHD1 was resistant to Vpx-induced degradation when compared to wild-type SAMHD1 and ΔNLS.SAMHD1 (Fig. 3B). An analysis of Vpx-induced degradation of SAMHD1 in the clonal cell lines showed that ΔNLS.SAMHD1 remained resistant to Vpx.

To determine the susceptibility of ΔNLS.SAMHD1 to Vpx and Vpr from other viruses, we generated a panel of VLPs. These contained Vpx from SIVmac251, SIVmac239, SIVrcm, and HIV-2 7312a and Vpr from SIVmus and SIVdeb. We then analyzed the ability of the VLPs to induce the degradation of wild-type SAMHD1 and ΔNLS.SAMHD1 in the PMA-differentiated U937 cell clones. The results showed that SIVmac251, SIVmac239, and HIV-2 7312a Vpx and SIVmus and SIVdeb Vpr induced the degradation of wild-type SAMHD1 but not ΔNLS.SAMHD1. Subsequently, Vpx-induced degradation of ΔNLS.SAMHD1 was shown to be resistant to a variety of Vpr and Vpx proteins.

**ΔNLS.SAMHD1 binds to Vpx in the cell.** To determine whether ΔNLS.SAMHD1 binds to Vpx, we tested whether Vpx:ΔNLS.SAMHD1 complexes could be pulled-down from cells. To do this,
we cotransfected 293T cells with Myc-tagged Vpx and expression vectors for HA-tagged SAMHD1, ΔNLS.SAMHD1 (ΔNLS), and mouse isoform 2 (mu iso2) were differentiated with PMA. Vpx-containing or control VLPs were added. After 10 h, lysates were prepared, and SAMHD1 was visualized on an immunoblot probed with an anti-HA MAb. GAPDH was detected as a loading control. (B) Single-cell subclones derived from the polyclonal SAMHD1 cell lines that had similar levels of SAMHD1 were chosen. The susceptibility of the SAMHD1 proteins to Vpx-induced degradation was determined as described for panel A. SAMHD1 levels in cells treated with Vpx- or Vpr-containing VLPs were quantified relative to the cells treated with control VLPs (VLP X+). (D) The VLPs were pelleted by ultracentrifugation. They were solubilized, and the packaged Vpx or Vpr proteins were visualized on an immunoblot probed with an anti-Myc MAb (SIVmac239 Vpx) or an anti-FLAG MAb (Vpx from HIV-2 7312a and SIVrcm and Vpr from SIVmus and SIVdeb). SIV p27 was detected by probing with an anti-p27 MAb to confirm the presence of similar amounts of virions.

FIG 3 Cytoplasmic SAMHD1 is resistant to Vpx- and Vpr-induced degradation. (A) U937 polyclonal cell populations stably expressing SAMHD1 (WT), ΔNLS.SAMHD1 (ΔNLS), and mouse isoform 2 (mu iso2) were differentiated with PMA. Vpx-containing or control VLPs were added. After 10 h, lysates were prepared, and SAMHD1 was visualized on an immunoblot probed with an anti-HA MAb. GAPDH was detected as a loading control. (B) Single-cell subclones derived from the polyclonal SAMHD1 cell lines that had similar levels of SAMHD1 were chosen. The susceptibility of the SAMHD1 proteins to Vpx-induced degradation was determined as described for panel A. SAMHD1 levels in cells treated with Vpx- or Vpr-containing VLPs were quantified relative to the cells treated with control VLPs (VLP X+). (D) The VLPs were pelleted by ultracentrifugation. They were solubilized, and the packaged Vpx or Vpr proteins were visualized on an immunoblot probed with an anti-Myc MAb (SIVmac239 Vpx) or an anti-FLAG MAb (Vpx from HIV-2 7312a and SIVrcm and Vpr from SIVmus and SIVdeb). SIV p27 was detected by probing with an anti-p27 MAb to confirm the presence of similar amounts of virions.

we cotransfected 293T cells with Myc-tagged Vpx and expression vectors for HA-tagged SAMHD1, ΔNLS.SAMHD1, and SAMHD1 (residues 1 to 570), a truncation mutant that lacks the Vpx binding site. The proteasome inhibitor MG132 was added to the cultures, and the Myc-tagged Vpx was immunoprecipitated with an anti-Myc MAb. Analysis of the complexes on an immunoblot showed that Vpx pulled down SAMHD1, SAMHD1(residues 37 to 626), and ΔNLS.SAMHD1 but not SAMHD1(residues 1 to 570) or mouse SAMHD1 (Fig. 4A). We have concluded that ΔNLS.SAMHD1 associates with Vpx in the cell.

To determine whether Vpx and SAMHD1 colocalize in the cell, we generated lentiviral vectors that express SAMHD1 and ΔNLS.SAMHD1-GFP fusion proteins and used them to establish HeLa cell lines that stably expressed the two proteins. The visualization of the proteins by fluorescent confocal microscopy showed that GFP.SAMHD1 localized to the nucleus, while ΔNLS.SAMHD1-GFP localized to the cytoplasm. We then transfected the cell lines with Vpx expression vector and added MG132. We found that in cells that expressed SAMHD1, Vpx was in the cytoplasm and nucleus, but in cells that expressed ΔNLS.SAMHD1, Vpx accumulated in the cytoplasm and had reduced nuclear staining (Fig. 4B). These results suggest that ΔNLS.SAMHD1 prevents the nuclear localization of Vpx.

Vpx induces the degradation of SAMHD1 that is confined to the nucleus. It is possible that the SAMHD1 protein is targeted for degradation in the nucleus but then is exported to the cytoplasm for proteasomal degradation. To address this possibility, we tested the effect of the nuclear export inhibitor leptomycin B (LMB) on the Vpx-induced degradation of SAMHD1 (11). To do this, we incubated the SAMHD1- and ΔNLS.SAMHD1-expressing HeLa cell lines with Vpx-containing and control VLPs in the presence or absence of LMB, and after 5 h we detected the SAMHD1 proteins on an immunoblot. The results showed that the SAMHD1 was degraded to a similar extent in the presence and absence of LMB (Fig. 5A). LMB had no effect on ΔNLS.SAMHD1, and the protein maintained its resistance to Vpx-mediated degradation. We confirmed that the LMB was active by transfecting HeLa cells with pRev1.4-GFP-NES, a vector that expresses a Rev-GFP fusion pro-
tein linked to a nuclear export sequence (NES) (20). The addition of LMB at concentrations equal to or lower than what were used in the degradation experiments caused the Rev-GFP-NES fusion protein to relocalize from the cytoplasm to a compartment within the nucleus (Fig. 5B). The results suggest that the SAMHD1 protein is targeted and degraded in the nucleus.

DISCUSSION

We identified the NLS of SAMHD1 as the KRPR sequence at amino acids 11 to 14. A mutation of the sequence caused the protein to localize to the cytoplasm but had no effect on its catalytic activity, its ability to deplete the dNTP pool, or its ability to restrict lentivirus replication. Interestingly, cytoplasmic SAMHD1 was resistant to Vpx-mediated proteasomal degradation. Vpx and Vpr proteins derived from several SIV and HIV-2 isolates, which are capable of mediating the degradation of the SAMHD1 protein, did not induce the degradation of cytoplasmic SAMHD1. The cytoplasmic SAMHD1 bound Vpx in the cell and trapped it in the cytoplasm. These findings imply that Vpx cannot induce the degradation of SAMHD1 in the cytoplasm. LMB, which prevents exportin 1-mediated export of nuclear proteins (11), did not interfere with the Vpx-induced degradation of SAMHD1. This rules out the possibility that SAMHD1 is ubiquitinated in the nucleus and then is exported to the cytoplasm for degradation. Our findings support a model in which Vpx induces the ubiquitination and proteasomal degradation of SAMHD1 in the nucleus.

The role of CRL4<sup>DCAF1</sup> in the regulation of nuclear proteins may explain why Vpx evolved to use this specific E3 ubiquitin ligase to induce the degradation of SAMHD1 out of the many E3 ubiquitin ligases in the cell. The substrates of CRL4 are proteins that play roles in DNA replication, DNA damage response, transcription, histone methylation, and cell cycle control, all of which are nuclear functions. The complex is localized to the nucleus, and its constituents Cul4A and DCAF1 contain an NLS (16, 19, 28). As an E3 ubiquitin ligase that regulates nuclear proteins, the CRL4 complex may be particularly well suited to adopt SAMHD1 as a substrate. The requirement for Vpx to target SAMHD1 in the nucleus thus results from the nuclear function of the CRL4. As part of its role in cellular physiology, SAMHD1 may be subject to proteasomal regulation in the nucleus by CRL4 or another E3 ubiquitin ligase even in the absence of Vpx. In support of this possibility, we found that in the stably transduced cell lines, ∆NLS.SAMHD1 was reproducibly expressed at a higher level than...
the wild-type protein. Thus, the cytoplasmic protein may escape this nuclear regulation.

The conclusion that Vpx targets SAMHD1 in the nucleus suggests that virion-packaged Vpx must transit to the nucleus shortly after virus entry and uncoating. Consistent with this model, although Vpx does not contain a consensus NLS, it is karyophilic (5, 31, 33, 35). Virion-released Vpx could transit to the nucleus and associate there with SAMHD1, or it could associate with SAMHD1 in the cytoplasm and then, as a complex, transit to the nucleus for ubiquitination and degradation. Transit of Vpx from the virion to the nucleus must be an early event, as reverse transcription in myeloid cells cannot occur until the amount of SAMHD1 protein has been decreased and dNTP levels subsequently recover. Arhel et al. and Schaller et al. have proposed that the uncoating of the virion occurs at the nuclear pore (4, 37). This could provide a means for Vpx to enter the nucleus through the nuclear pore. However, it is difficult to reconcile such a model with the fact that Vpx must be released into the nucleus to degrade SAMHD1 prior to reverse transcription, which cannot proceed until dNTP levels are restored.

During the preparation of this article, Brandariz-Nunez et al. reported findings similar to ours (8). They identified the NLS and showed that point mutations in the motif cause the relocation of SAMHD1 to the cytoplasm and resistance to Vpx-induced degradation. However, their findings differed from ours with respect to the requirement for SAMHD1 nuclear localization. They found that the cotransfection of cells with the mutated NLS of SAMHD1 and the Vpx of HIV-2 B, a protein that they found localized mainly in the cytoplasm, induced the degradation of cytoplasmic SAMHD1. This finding led to the conclusion that SAMHD1 can be degraded in the cytoplasm. In contrast, we found that HIV-2 7312a Vpx, which is identical to HIV-2 B Vpx, failed to induce the degradation of ΔNLS.SAMHD1. We cannot explain this discrepancy, although in our experiments, the Vpx was introduced by VLPs to the cells that stably expressed SAMHD1, a scenario that mimics what happens in a natural infection. Brandariz-Nunez et al. expressed Vpx and SAMHD1 by cotransfection. Our findings are also inconsistent with those of Laguette et al., who found that LMB prevented the Vpx-induced degradation of SAMHD1 (25). That conclusion was drawn by microscopic visualization of the proteins, in contrast to our results based on biochemical analysis of the protein levels. Goncalves et al. reported that some of the SAMHD1 point mutants that cause AGS are the result of cytoplasmic mislocalization of the protein (13). This could be viewed as inconsistent with our results, as we found that mislocalized SAMHD1 remains active. However, we tested one of those proteins, I201N, and found that it is unstable, lacks detectable triphosphohydrolase activity, and localizes to the nucleus (see Fig. S4 in the supplemental material).

The roles of SAMHD1 in cellular physiology are not well understood, and the purpose of its nuclear localization is not clear. The NLS is well conserved, indicating the importance of this feature of the protein, yet the cytoplasmic mutant maintained the ability to regulate the dNTP pool. This is consistent with the known ability of nucleotides to diffuse throughout the cell (40). A possible explanation is that nuclear localization allows for the regulation of SAMHD1 by a nuclear E3 ubiquitin ligase. It is also possible that SAMHD1 affects the concentration of dNTP at the localized sites of DNA/RNA synthesis or DNA damage response within the nucleus. One caveat to the findings reported here is that they are based on cell lines and could differ in some respects from what occurs in primary myeloid cells.

It is remarkable that a virion-packaged accessory protein present at a low copy number in the virion can transit to the nucleus to induce the degradation of an abundant host protein. The degradation of a large number of SAMHD1 molecules would require that Vpx act through multiple rounds of SAMHD1 ubiquitination. It is also possible that the entry and uncoating of multiple viral particles act cooperatively to release a sufficient amount of Vpx proteins to decrease SAMHD1 levels. The evolution of a virion-packaged protein that targets a host nuclear protein prior to the synthesis of new virus proteins demonstrates the resourcefulness of lentiviruses in finding strategies to relieve the cellular host restrictions they encounter.

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