Mouse SAMHD1 restricts HIV-1 and murine leukemia virus infections independently of its phosphorylation at threonine 634

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Abstract

Background: The dNTPase SAMHD1 functions as an HIV-1 restriction factor in non-dividing cells by limiting intracellular dNTP levels required to complete viral reverse transcription. Phosphorylation of human SAMHD1 (hSAMHD1) at residue threonine (T) 592 by cyclin dependent kinase 1 (CDK1) and CDK2 impairs its restriction of HIV-1 infection. Mouse SAMHD1 (mSAMHD1) shares 72-74% protein sequence identity with hSAMHD1, and also restricts HIV-1 infection in non-dividing cells. However, it is unknown whether phosphorylation of mSAMHD1 regulates its restriction of retroviral infection.

Results: We identified and confirmed that T634 is a phosphosite of mSAMHD1 in dividing cells by mass spectrometry and using a phospho-specific antibody. Using specific inhibitors of CDK1 and CDK2 decreased the level of T634 phosphorylated mSAMHD1 in dividing cells. We generated human U937 and mouse NIH3T3 cell lines stably expressing mSAMHD1 wild-type (WT), phospho-ablative (T634A) or phospho-mimetic (T634D) mutants to examine the effect of T634 phosphorylation on mSAMHD1-mediated restriction of HIV-1 or murine leukemia virus (MLV), respectively. In differentiated U937 cells, overexpression of mSAMHD1 WT or the mutants significantly restricted HIV-1 infection and reduced the intracellular dNTP levels. In dividing NIH3T3 cells, mSAMHD1 WT or the mutants modestly reduced MLV infection.

Conclusions: CDK1 and CDK2 phosphorylate T634 of mSAMHD1 in dividing cells. Restriction of HIV-1 infection in non-dividing cells and MLV infection in dividing cells by mSAMHD1 is independent of its T634 phosphorylation. Our results suggest different mechanisms of regulating retroviral restriction by hSAMHD1 and mSAMHD1.

Background

Sterile α motif (SAM) and HD domain containing protein 1 (SAMHD1) is a deoxynucleotide (dNTP) triphosphohydrolase [1, 2] and exonuclease [3, 4], and functions as an HIV-1 restriction factor in non-dividing myeloid cells and resting CD4^{+} T cells [5-7]. Human SAMHD1 (hSAMHD1) inhibits the infection of a wide range of retroviruses including HIV-1 and murine leukemia virus (MLV) [8] in human macrophages [9], by depleting the intracellular dNTPs to below the levels required for retroviral replication [10].

SAMHD1 is a highly conserved protein in humans and mice [11]. Alternative splicing of the mSAMHD1 pre-mRNA results in two isoforms of the mSAMHD1 protein (isoform 1 and 2), which share 72% and 74% protein sequence identities with hSAMHD1. Both isoforms possess the dNTP triphosphohydrolase activity [2, 10, 12], and SAMHD1 isoform 1 mRNA is more abundantly expressed than isoform 2 mRNA in various mouse tissues [12]. Phosphorylation of hSAMHD1 at T592 by CDK1 and CDK2 diminishes its restriction of HIV-1 infection [13-17] and may also contribute to virus restriction by impairing its nuclease activity in cells [3]. These studies indicate that phosphorylation of T592 plays a critical role in regulating the functions of hSAMHD1. However, there is currently no evidence linking the phosphorylation of mSAMHD1 to its retroviral restriction function. It is also unknown whether mSAMHD1 and hSAMHD1 restricts HIV-1 and MLV through the same mechanism.

Here, we aim to elucidate the role of mSAMHD1 phosphorylation in regulating its retroviral restriction function in cells. We identified that T634 as a phosphosite of mSAMHD1 isoform 1 and demonstrated that CDK1 and CDK2 phosphorylate mSAMHD1 at T634 in dividing cells. We found that, irrespective of phosphorylation of T634, overexpression of mSAMHD1 wild-type (WT), phospho-ablative, or phospho-mimetic mutants restricts HIV-1 infection in differentiated human U937 cells and MLV infection in dividing mouse NIH3T3 cells. Overall, our results suggest differential mechanisms of post-translational regulation for retroviral restriction mediated by hSAMHD1 and mSAMHD1.

Results

Identification of phosphorylation sites of mSAMHD1 protein

Mouse SAMHD1 was identified as a phosphoprotein in previous large-scale analyses of phosphorylated proteins [18-20]. The C-terminal protein sequences of human and mouse SAMHD1 are highly conserved [11], and contain the residue T592 in hSAMHD1 and a predicted phosphosite at position T634 in mSAMHD1 isoform 1 [15, 18]. Phosphorylation of hSAMHD1 at T592 impairs its restriction of HIV-1 infection [13-15]. These results indicate that phosphorylation may be a regulation mechanism for mSAMHD1-mediated restriction of retroviral infection. Given that mSAMHD1 isoform 1 is more abundantly expressed compared to isoform 2 in multiple tissues [12], we focused our study on mSAMHD1 isoform 1 (referred to as mSAMHD1 from this point).

To identify the phosphosites of mSAMHD 1, phosphorylation modification was analyzed by using tandem mass spectrometry (MS/MS) after immunoprecipitation of full-length mSAMHD1 [16]. The data showed that T634 is a phosphosite of mSAMHD1 in cells (Figure 1). Interestingly, based on sequence alignment of mSAMHD1 and hSAMHD1 (Figure 2A), T634 is homologous to the known T592 phosphosite of hSAMHD1 that negatively regulates hSAMHD1-mediated HIV-1 restriction [13-16]. To confirm the phosphorylation status of mSAMHD1 at T634 in cells, we generated a phospho-specific SAMHD1 antibody, and two mSAMHD1 mutants with a replacement of the threonine by the phospho-ablative residue alanine (T634A) or phospho-mimetic residue aspartic acid (T634D). After transient expression of mSAMHD1 WT, T634A or T634D in human embryonic kidney 293T (HEK293T) cells, total hSAMHD1 was detected at 72 kDa by immunoblotting, while mSAMHD1 WT and mutants were detected at 76 kDa (Figure 2B). Phosphorylated hSAMHD1 (T592) and mSAMHD1 (T634) were specifically

detected by the phospho-specific SAMHD1 antibody in cells that overexpress WT human or mouse SAMHD1. However, phospho-specific antibody did not recognize the mSAMHD1 mutants (Figure 2B), confirming that T634 of mSAMHD1 is a phosphosite in dividing cells.

CDK1 and CDK2 contribute to T634 phosphorylation of mSAMHD1 in cells

CDK1 and CDK2 phosphorylate hSAMHD1 at position T592, which impairs its ability to restrict HIV-1 infection in non-dividing cells [14-17]. CDK1 and CDK2 are highly conserved in human and mouse [21], and share 97% and 85% amino acid identity, respectively. Our previous results showed that both hSAMHD1 and mSAMHD1 interact with CDK1 and CDK2 in HEK293T cells [16]. Similar to T592 in hSAMHD1, T634 of mSAMHD1 is located within a predicted CDK-binding motif, TPXK [15] (Figure 2A), suggesting that CDK1 and CDK2 could be responsible for the phosphorylation of mSAMHD1 at T634.

To test whether T634 of mSAMHD1 is phosphorylated by CDK1 and CDK2 in cells, small molecule inhibitors specific to CDK1 or CDK2 were used to block the kinase activities of CDK1 and CDK2. After 6 hr pretreatment with inhibitors, HEK293T cells were transfected to transiently express mSAMHD1 WT protein, and cell lysates were collected 24 hr after the transfection for immunoblotting. Compared to control cells treated with dimethyl sulfoxide (DMSO), individual and combined treatments with inhibitors to CKD1 and CDK2 reduced the relative levels of phosphorylated mSAMHD1 (T634) by 62% ($p < 0.01$), 34% ($p < 0.05$), and 66% ($p < 0.01$), respectively (Figure 3A and 3B). These results suggest that both CDK1 and CDK2 phosphorylate mSAMHD1 at T634 in human cells.

To examine whether endogenous CDK1 and CDK2 phosphorylate mSAMHD1 at T634 in mouse cells, murine fibroblast NIH3T3 cells stably expressing mSAMHD1 were generated and treated with specific inhibitors to CDK1 or CDK2. After 24 hour treatment with inhibitors, cell lysates were collected for immunoblotting. Individual and combined treatments with CDK1 and CDK2 inhibitors at 0.1 µM reduced the relative levels of mSAMHD1 T634 phosphorylation by approximately 50% compared to control cells treated with DMSO, although the differences were not statistically significant (Figure 4A and 4B, $p > 0.05$). Compared to the control cells, individual and combined treatments with the inhibitors at 0.5 µM reduced the relative levels of mSAMHD1 T634 phosphorylation by 45% ($p > 0.05$), 78% ($p < 0.05$), and 83% ($p < 0.05$), respectively (Figure 4A and 4B). These results suggest that endogenous CDK1 and CDK2 contribute to T634 phosphorylation of mSAMHD1 in mouse cells.

Phosphorylation of mSAMHD1 at T634 does not affect its restriction of HIV-1 infection in differentiated U937 cells

Previous studies reported that mSAMHD1 restricts HIV-1 infection in non-dividing cells [10, 22]. Whether phosphorylation regulation is important for mSAMHD1-mediated viral restriction remains unknown. To examine the importance of T634 phosphorylation in regulating mSAMHD1-mediated HIV-1 restriction, we generated human U937 cell lines that stably express mSAMHD1 WT or the mutants using lentiviral vectors [16, 22]. The hSAMHD1-expressing U937 cell line was used as a positive control for HIV-1 restriction [16]. After PMA differentiation of the cells, the expression of total WT hSAMHD1, mSAMHD1, and mSAMHD1 mutants was confirmed by immunoblotting (Figure 5A). Of note, T592-phosphorylated hSAMHD1 was detected in PMA-differentiated U937 cells, while phosphorylation of

mSAMHD1 WT was not detected (Figure 5A). It is possible that phosphorylation of mSAMHD1 might be regulated differently in human non-dividing cells.

To determine the effect of mSAMHD1 T634 phosphorylation on HIV-1 infection, PMA-differentiated U937 cells were infected with a single-cycle luciferase reporter HIV-1 and viral infection was measured 24 hr post infection by luciferase activity. Our results showed that HIV-1 infection were significantly reduced in U937 cells expressing WT mSAMHD1 or mutants, or WT hSAMHD1 compared to vector control cells (Figure 5A and 5B), indicating that HIV-1 restriction by mSAMHD1 is independent of its T634 phosphorylation.

To verify whether HIV-1 restriction by mSAMHD1 WT or mutants in PMA-differentiated U937 cells was due to reduced dNTP pool, the intracellular dNTP levels were measured using the single nucleotide incorporation assay [23]. Compared to PMA-differentiated vector control U937 cells, the intracellular dNTP levels in cells expressing comparable high levels of mSAMHD1 WT or mutants were reduced 50~67% ($p < 0.0001$) (Figure 5C). These results indicate that mSAMHD1 WT and mutants have the dNTPase activity, which is not regulated T634 phosphorylation in PMA-differentiated U937 cells. This observation is consistent with that the dNTPase activity of hSAMHD1 is not regulated by its phosphorylation at T592 [14]. In contrast to impaired HIV-1 restriction by T592 phosphorylation of hSAMHD1 in PMA-differentiated U937 cells [13, 14], T634 phosphorylation of mSAMHD1 does not affect its HIV-1 restriction function in non-dividing cells. Overall, these results suggest that hSAMHD1 and mSAMHD1 may have different mechanisms regulating their restriction of HIV-1 in non-dividing human cells.

MLV infection in NIH3T3 cells is reduced by mSAMHD1 independently of its T634 phosphorylation

The effect of mSAMHD1 on MLV infection is not consistent among studies using SAMHD1 knockout mice and knockdown mouse macrophage cells [12, 24, 25]. Since MLV only infects proliferating cells [26], we used a mouse fibroblast cell line (NIH3T3) as a model to determine whether MLV infection was blocked in cells overexpressing mSAMHD1 WT or mutants. To examine the effects of phosphorylation of hSAMHD1 or mSAMHD1 on their functions to restrict MLV infection, we generated stable NIH3T3 cell lines overexpressing WT hSAMHD1 or mSAMHD1, phospho-ablative hSAMHD1 mutant (T592A) or mSAMHD1 mutants (T634A, T634D) and then performed single-cycle MLV infection assays. The expression levels of WT or mutants of hSAMHD1 and mSAMHD1 in dividing NIH3T3 cells were confirmed by immunoblotting (Figure 6A). Interestingly, MLV infection was reduced by 36~52% in NIH3T3 cells expressing WT or mutants of hSAMHD1 and mSAMHD1 ($p < 0.0001$) compared to that in control cells (Figure 6B).

Discussion

In this study, we identified and confirmed that mSAMHD1 T634 is phosphorylated by CDK1 and CDK2 in cells. Our results indicate that T634 phosphorylation of mSAMHD1 does not affect its dNTPase function in non-dividing cells, nor regulates its restriction of HIV-1 infection in non-dividing cells or MLV infection in dividing cells.

Consistent with observations that CDK1 and CDK2 are the main cellular kinases that phosphorylate hSAMHD1 at T592 [14-17], we showed that both CDK1 and CDK2 contribute to T634 phosphorylation of mSAMHD1. Our data showed that residual phosphorylated hSAMHD1 (T592) was detectable in PMA-differentiated non-dividing U937 cells, possibly due to expression of CDK2, which unlike CDK1, does not decrease upon PMA-differentiation [16]. In contrast, T634-phosphorylated mSAMHD1 was undetected in non-dividing U937 cells, suggesting that phosphorylation of mSAMHD1 (T634) and hSAMHD1 (T592) might be regulated differently in non-dividing U937 cells. We recently reported that S-phase kinase-associated protein 2 interacts with hSAMHD1, but not mSAMHD1; while cyclin B1 interacts with mSAMHD1, but not hSAMHD1 in HEK 293T cells [16]. These results support the notion that the phosphorylation of human and mouse SAMHD1 may be regulated differently by cell cycle-related proteins in human cells.

WT and the phospho-ablative mutant of hSAMHD1 both restrict HIV-1 infection in PMA-differentiated U937 cells, while a phospho-mimetic mutant of hSAMHD1 loses the restriction of HIV-1 infection [13, 14]. We found that high levels of mSAMHD1 WT, the phospho-ablative or the phospho-mimetic mutants efficiently blocked HIV-1 infection in PMA-differentiated U937 cells. Previous studies indicated that both mSAMHD1 isoform 1 and 2 restrict HIV-1 infection in non-dividing cells [10, 22]. Due to the C-terminal amino acid differences between the two isoforms that results from alternative splicing, isoform 2 does not contain residue T634 [12]. Our results are consistent with the previous observations, suggesting that mSAMHD1 restricts HIV-1 infection independently of it T634 phosphorylation. The fact that phosphorylated hSAMHD1

(T592) and mSAMHD1 (T634) have different effects on HIV-1 restriction in non-dividing cells suggests the different regulatory mechanism of hSAMHD1 and mSAMHD1.

Interestingly, we observed that hSAMHD1 and mSAMHD1 modestly blocked MLV infection in dividing NIH3T3 cells irrespective of SAMHD1 phosphorylation status, while the late reverse transcript products of MLV were not affected by SAMHD1. Furthermore, we found that the levels of MLV 2-LTR circles were reduced by overexpressed SAMHD1 in infected NIH3T3 cells, suggesting that SAMHD1 could impair nuclear import of MLV cDNA in dividing mouse cells. However, the precise mechanism underlying SAMHD1-inhibited nuclear import of MLV cDNA remains to be studied. Our previous studies demonstrated that overexpression of hSAMHD1 in dividing human cell lines (HeLa and HEK293T) modestly reduced the intracellular dNTP levels, but does not restrict HIV-1 infection [23]. Other studies reported that MLV restriction by hSAMHD1 [8] and HIV-1 restriction by mSAMHD1 [10, 22] occur in PMA-differentiated U937 cells. Our new findings of MLV restriction by hSAMHD1 and mSAMHD1 in dividing mouse cells suggest potentially different mechanisms in regulating retroviral restriction function of SAMHD1 in human and mouse cells.

Conclusions

We identified and demonstrated that CDK1 and CDK2 phosphorylate T634 of mSAMHD1 in dividing cells. Restriction of HIV-1 infection in non-dividing cells and MLV infection in dividing cells by mSAMHD1 is independent of T634 phosphorylation, suggesting different mechanisms of regulating retroviral restriction by hSAMHD1 and mSAMHD1.

Methods

Plasmids

The plasmids encoding hemagglutinin (HA)-hSAMHD1, HA-mSAMHD1 isoform 1 and empty

vectors [10] were kind gifts from Dr. Nathaniel Landau (New York University). Plasmids

expressing HA-tagged mutant mSAMHD1 isoform 1 were generated using a Quickchange

mutagenesis kit (Agilent Technologies) according to manufacturer's protocol. To generate

mSAMHD1 T634A and T634D mutants, the following two primers were used, respectively: 5'-

GCT CCA CTC ATA GCC CCT CTG AAA TGG -3' and 5'- GCT CCA CTC ATA GAC CCT

CTG AAATGG -3'. The plasmids expressing HA-tagged WT or DN mutant of CDK1 or CDK2

[27] were purchased from Addgene.

Cell culture and generating stable cell lines expressing SAMHD1

HEK293T cells and a HIV-1 reporter cell line GHOST/X4/R5 have been described [28, 29].

Monocytic U937 cells lines were maintained as described [30, 31]. U937 cells were treated with

100 ng/mL PMA for 24 hr, then media changed and cells rested for a further 24 hr and were

differentiated into non-cycling, macrophages-like cells [6]. U937 cells stably expressing WT

hSAMHD1, WT mSAMHD1, or mutant mSAMHD1 (T634A or T634D) were generated by

spinoculation of U937 cells with concentrated lentiviral vectors, and then cultured with 0.8

μg/mL puromycin containing media for selection as described [16, 22]. NIH3T3 cells were

transduced with lentiviral vectors expressing hSAMHD1, mSAMHD1 WT or mutants for 2 hr,

and then cultured with 2 μg/mL puromycin containing media (DMEM with 10% fetal bovine

serum, and 1% penicillin and streptomycin) 48 hr after the transduction.

Transfections of HEK293T cells

To produce lenti-viral vectors containing hSAMHD1, mSAMHD1 WT, mSAMHD1 mutants or vector control, HEK293T cells were seeded at 3×10^5 cells/well in 6-well plates. After 24 hr, cells were transfected using a calcium phosphate method as described [16].

Immunoblotting and antibodies

Cells were harvested at 24 hr after transfection, and lysed with cell lysis buffer (Cell Signaling) containing protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were used for immunoblotting as described [32]. Membranes were probed with antibodies specific against to HA (Covance, Ha.11 clone 16B12), GAPDH (AbD serotec), CDK1 (Cell signaling, #9112), Cdk2 (Santa Cruz, sc-163). Rabbit antibodies specific for phospho-SAMHD1 were generated by ProSci Inc. using phosphorylated hSAMHD1 peptide [15]: FTKPQDGDVIAPLI(**Tp**)PQKKE. The phospho-specific antibody was sequentially affinity-purified from the rabbit sera with a non-phospho-peptide conjugated column and a phospho-peptide conjugated column. Immunoblotting images were obtained and analyzed using Luminescent Image analyzer (LAS 4000) and Mutil-Gauge V3.0 software (Fuji Film) as described [23].

Mass Spectrometry

HEK293T cells were transfected to express HA-tagged mSAMHD1 and prepared for mass spectrometry as described [16]. Peptides were analyzed at the Ohio State University Mass Spectrometry Core Facility using an LTQ-Orbitrap mass spectrometer. Tandem mass spectrometry samples were analyzed using Mascot (Matrix Science, version 2.4.1). Mascot was set up to search the SwissProt_ID_2013_02 database (selected for *Mus musculus*) assuming the

digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20 PPM. Phosphorylation of serine, threonine and tyrosine were specified in Mascot as variable modifications. Analysis of identified peptides and phosphorylated residues was performed using Scaffold software with a minimum protein probability of 99.0% and minimum peptide probability of 95% (Proteome Software Inc.).

Treatment of HEK293T cells with CDK1 and CDK2 inhibitors

HEK293T cells were pre-treated with specific inhibitors to CDK1 [15] (CGP74513A, EMD Millipore), CDK2 [33] (inhibitor II, Santa Cruz Biotech) at 1 μM, or DMSO (Sigma-Aldrich) for 6 hr. Cells were transfected with plenti-HA-mSAHMD1 to express mSAMHD1 WT. The inhibitors were present during transfection, and at 12 hr post-transfection fresh media with inhibitors were added. Cells were harvested at 24 hr post-transfection, and processed for immunoblotting.

Virus stocks and infection assays

HIV-1 and MLV stocks were generated by transfecting HEK293T cells as described [34]. Virus stocks were harvested at 48 hr post-transfection, and filtered through 0.45 μM filters. For luciferase reporter HIV-1 pseudotyped with vesicular stomatitis virus G protein (HIV-luc/VSV-G), 10 μg pNL4-3-luc E^-R^+ and 4 μg pMD2-G (VSV-G) were used for transfection [35]. For GFP reporter MLV (MLV-GFP/VSV-G), 10 μg pMIGR1, 5 μg pHIT60 and 4 μg pVSV-G were used [36]. HIV-luc/VSV-G and MLV-GFP/VSV-G stocks were titrated with GHOST/X4/R5 cells [28] and HEK293T cells, respectively.

For HIV-1 luc/VSV-G infection, cells (1×10^5 per well) of each U937 stable cell lines were seed in 96-well plates and were differentiated with PMA (100 ng/ml) for 48 hr. Cells were infected at a multiplicity of infection (MOI) of 1 as described [28, 37]. HIV-1 infection was determined by luciferase assays at 24 hr post-infection [16]. For MLV-GFP/VSV-G infections, 6×10^4 cells per well of each NIH3T3 stable cell lines were seeded in 24-well plates. Cells were infected at a MOI of 1. The infections were determined by detecting GFP-positive cells with flow cytometry (GAUVA mini 2.0) 48 hr post-infection. The flow cytometry data were analyzed by FlowJo.

Intracellular dNTP measurement

For dNTP analysis and quantification, cells were harvested and lysed in iced cold 65% methanol, and vigorously vortex for 2 minutes. Extracts were incubated at 95°C for 3 minutes. Supernatants were collected and dried in a speed vacuum. Samples were processed for the single nucleoside incorporation assay as described [38].

Statistical analysis

The data were analyzed by one-way ANOVA followed with Dunnett's test, or by *t*-test in Graphpad 5.0. The statistical significance was defined as $p < 0.05$.

Competing interests

The authors declare that they have no competing interests.

**Authors' contributions**

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Figures and Figure legends
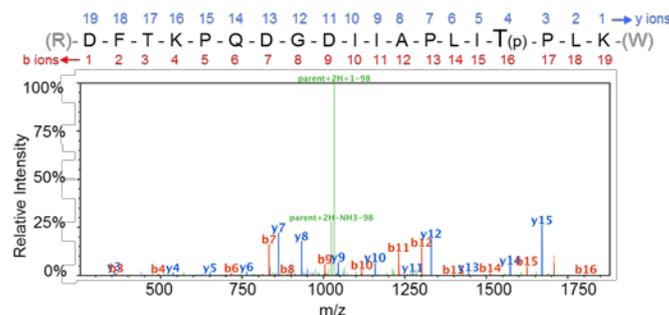
Figure 1. Residue T634 in the mSAMHD1 protein sequence is a phosphosite as identified by tandem mass spectrometry. Position T634 was identified as a phosphosite by MS/MS analysis, which is highlighted in the sequence and denoted by a "(p)". Assigned b ions are shown in red, and y ions are shown in blue. Green peaks represent a loss of a 98 Da fragment that corresponds to a loss of the phosphate group as indicated.
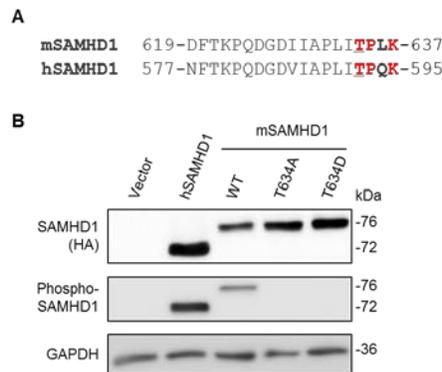
Figure 2. Confirmation of the phosphorylation of mSAMHD1 at position T634 using a phospho-specific antibody. (A) Partial protein sequence alignment of mSAMHD1 isoform 1 and human (hSAMHD1) depicting conserved phosphosites at residue positions T634 and T592 in mSAMHD1 and hSAMHD1 sequences, respectively. The phosphorylated residue threonine (T) is underlined and the residues encompassing a consensus sequence (TPXK) for CDK interaction are highlighted in red. **(B)** HEK293T cells were transiently transfected to overexpress hSAMHD1, mSAMHD1 wild type (WT), phospho-ablative mutant (T634A) or phospho-mimetic mutant (T634D). Immunoblotting was performed to confirm position T634 of mSAMHD1 is a phosphosite. Total SAMHD1 protein levels were probed using an anti-HA antibody (top panel), and phospho-SAMHD1 levels were detected using a phospho-specific SAMHD1 antibody (middle panel). GAPDH was used as a loading control. A representative immunoblotting result from 3 independent experiments is shown.
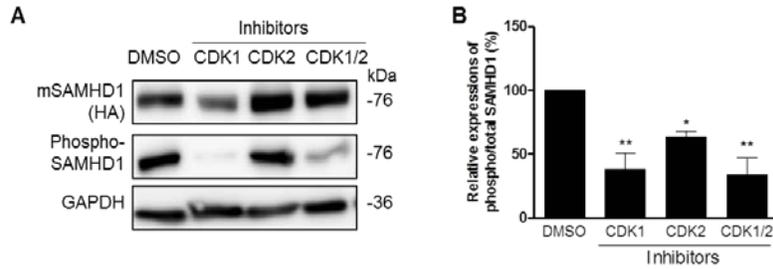
Figure 3. CDK1 and CDK2 contribute to T634 phosphorylation of mSAMHD1 in HEK293T cells. (A) Cells were pre-treated with DMSO or inhibitors specific to CDK1 or CDK2 at 1 µM for 6 hr and transiently transfected to over-express mSAMHD1. The effect of each inhibitor on the phosphorylation of mSAMHD1 at T634 was assessed at 24 hr post-transfection by immunoblotting with a phospho-specific antibody. The total SAMHD1 levels were detected using an HA-specific antibody. GAPDH was used as a loading control. **(B)** An average of 3 independent experiments described in (A). Based on the densitometry analysis, the levels of total mSAMHD1 and phospho-SAMHD1 were normalized to GAPDH, and then the ratio of phospho/total mSAMHD1 was calculated and the ratio of the vector group was set as 1. Results are shown as mean ± SD (n=3). The data was analyzed by one-way ANOVA with Dunnett's test (* is $p < 0.05$, ** is $p < 0.01$).
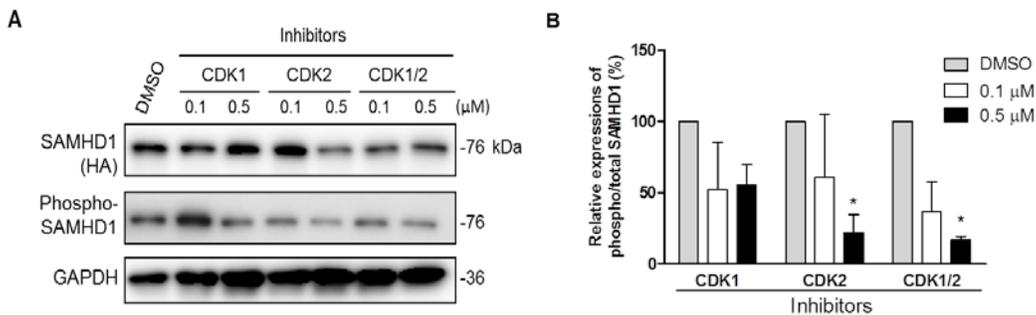

Figure 4. CDK1 and CDK2 phosphorylate mSAMHD1 at T634 in mouse fibroblast cells.
(A) NIH3T3 cells stably expressing mSAMHD1 were treated with inhibitors specific to CDK1

or CDK2 at the indicated concentrations. The effects of each inhibitor on the phosphorylation of

mSAMHD1 at T634 were assessed at 24 hr post-treatment by immunoblotting with a phospho-

specific antibody. **(B)** An average of three independent experiments. The protein levels were

quantified based on the band densities. The levels of total mSAMHD1 and phospho-SAMHD1

were normalized to GAPDH, and then the ratio of phospho/total mSAMHD1 was calculated and

the ratio in the vector group was set as 100%. Results are shown as mean $\pm$ SD (n=3). The data

was analyzed by one-way ANOVA with Dunnett's test (* is $p < 0.05$).
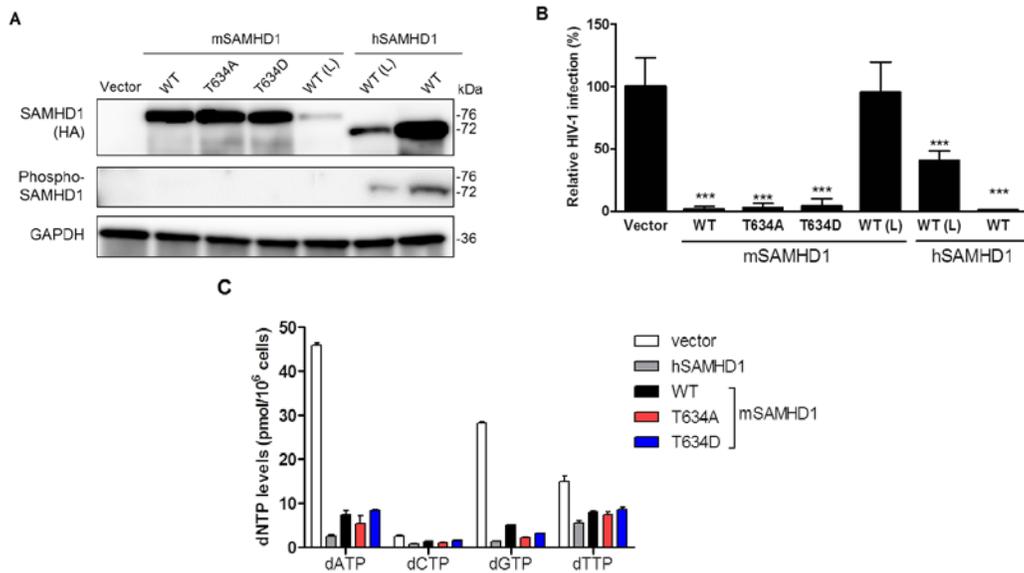
Figure 5. Restriction of HIV-1 infection by mSAMHD1 is independent of its T634

phosphorylation in PMA-differentiated U937 cells. (A) The expression levels of total and

phosphorylated mSAMHD1 (T634) were determined by immunoblotting with an anti-HA

antibody and phospho-specific SAMHD1 antibody, respectively. WT (L) indicates cells

expressing low level of wild type (WT) mSAMHD1 or hSAMHD1. GAPDH was used as a

loading control. **(B)** PMA-differentiated U937 cells expressing hSAMHD1 WT, mSAMHD1

WT, phospho-ablative mutant (T634A), phospho-mimetic mutant (T634D) or an empty vector

were infected with a single-cycle HIV-1 luc/VSV-G reporter virus at a multiplicity of infection (MOI=1). At 24 hr post-infection, cells were harvested for luciferase assay to measure HIV-1 infection. An average of 3 independent infection experiments carried out is presented. The infection level in the vector control group was set as 100% for normalization. Statistical analysis was performed by one-way ANOVA and Dunnett's test (***, $p < 0.0001$). **(C)** The dNTP levels in cells were determined following PMA treatment by a single-nucleotide incorporation assay. The results are shown as mean ± SD (n =2). The significance of difference was analyzed by one-way ANOVA with Dunnett's test for each dNTP. The levels of all four dNTPs were significantly decreased by hSAMHD1, mSAMHD1 WT or mutants compared to the vector control (***, $p < 0.0001$).
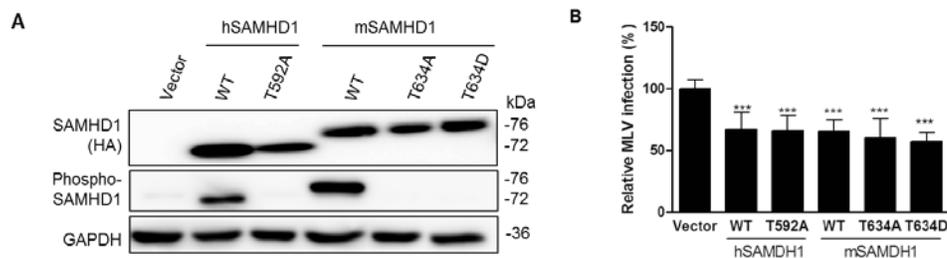
Figure 6. **MLV infection in NIH3T3 cells is reduced by mSAMHD1 independently of its T634 phosphorylation. (A)** The expression levels of mSAMHD1WT and T634 mutants in NIH3T3 cells were confirmed by immunoblotting using anti-HA antibody. The levels of phosphorylated mSAMHD1 (T634) were assessed using a phospho-specific antibody. **(B)** An average of MLV infection in NIH3T3 stable cells from three independent experiments. Cells were infected with a single-cycle MLV-GFP/VSV-G reporter virus (MOI=1). At 48 hr post-infection, cells were harvested for flow cytometry to determine the percentage of GFP-positive cells.