약학박사학위논문

Role of SAMHD1 Acetylation in Its dNTPase Activity and Cancer Cell Proliferation

SAMHD1 아세틸화가 뉴클레오티드 3 인산 분해능과 암세포 분열에 미치는 영향 연구

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ABSTRACT

Role of SAMHD1 acetylation in its dNTPase activity and cancer cell proliferation

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SAMHD1 is a deoxynucleotide triphosphohydrolase (dNTPase) which inhibits retroviruses by depleting intracellular deoxynucleotide triphosphates (dNTPs) in noncycling myeloid cells. Although SAMHD1 is expressed ubiquitously throughout the human body, the molecular mechanism to directly regulate its enzymatic activity and its function in non-immune cells are relatively unexplored. Here, I demonstrate that the dNTPase activity of

SAMHD1 is regulated by acetylation, which promotes cell cycle progression in cancer cells. SAMHD1 was acetylated at residue K405 by ARD1, an acetyltransferase, which directly enhanced its dNTPase activity in vitro. When SAMHD1 wildtype and non-acetylated K405R mutant overexpressing stable cells were constructed in cancer cells, K405R mutant expressing cells showed decreased G1/S transition and slower proliferation over wildtype cells. SAMHD1 acetylation level was strongest during the G1 phase, implicating its role during G1 phase. Collectively, these findings suggest that SAMHD1 acetylation enhances its dNTPase activity and promotes cancer cell proliferation.

Keywords: **SAMHD1/ acetylation/ dNTPase/ ARD1/ cell cycle/ cancer**

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LIST OF ABBRIVIATIONS

AGS	Aicardi-Goutières syndrome
AR	Androgen receptor
ARD1	Arrest defective 1

CDK1	Cyclin-dependent kinase 1
CTD	C-terminal domain
dATP	Deoxyadenosine triphosphate
DCIP	Dendritic cell-derived IFN-γ induced protein
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dNTPase	Deoxynucleotide triphosphohydrolase
dTTP	Deoxyribonucleotide triphosphate
HD	Histidine-aspartic
HIV-1	human immunodeficiency virus-1
HSP70	Heat shock protein 70
IFNβ-2a	Interferon β-2a
NAT	Nα-terminal acetyltransferases
PCNA	proliferating cell nuclear antigen
PMA	Phorbol-12-Myristate-13-Acetate
PTM	Posttranslational modification

RNR	Ribonucleotide reductases
Runx2	Runt-related transcription factor 2
SAM	Sterile alpha motif
SAMHD1	SAM domain and HD domain containing protein 1

INTRODUCTION

I. dNTPs

Deoxyribonucleotide triphosphate (dNTP) is a molecule which contains a phosphate group, a deoxyribose sugar and a nitrogenous base (Figure 1). 4 types of dNTPs exist depending on the nitrogenous base they contain; dATP, (deoxyadenosine triphosphate), and dGTP, (deoxyguanine triphosphate), dTTP, (deoxythymidine triphosphate), and dCTP, (deoxycytidine triphosphate). They are necessary for life, as they are the precursors of DNA synthesis. According to base pairing rules (A with T, and C with G), the nitrogenous bases of the two separate dNTPs are bound together with hydrogen bonds to build double-stranded DNA.

A strict balance in dNTP pool sizes is critical for proper DNA replication and repair in eukaryotic cells (Reichard, 1988). When a cell proliferates, dNTP pool sizes fluctuate with the largest pools seen during S phase and the smallest in G1; which control the initiation of DNA replication (Chabes and

Stillman, 2007) (Figure 2). This fluctuation needs to be within a range optimal for chromosomal replication, as imbalances can impede DNA replication and repair resulting in DNA damage, cell cycle arrest or cell death (Mathews, 2006). Thus eukaryotes use diverse mechanisms to strictly regulate the supply of dNTPs (Pai and Kearsey, 2017). Ribonucleotide reductases (RNR) are the major contributors to the pool as they synthesize the dNTPs from ribonucleoside diphosphates (Figure 3) (Nordlund and Reichard, 2006). RNRs have long been recognized as an attractive target for cancer treatment, for their dysregulated activity is associated with genomic instability, malignant transformation and cancer development (Mathews, 2015).

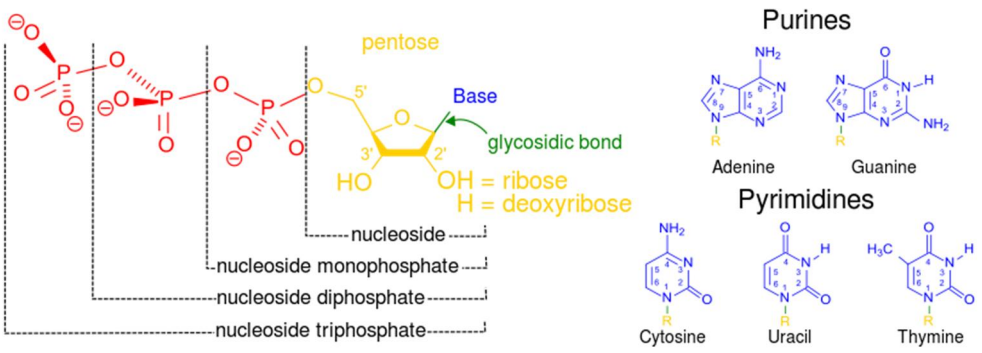

Figure 1. Structural elements of three nucleotides. Each in turn is attached to the nucleoside (in yellow, blue, green) at center: 1st, the nucleotide termed as a *nucleoside monophosphate nucleotide* is formed by adding a phosphate group (in red); 2nd, adding a second phosphate group forms a *nucleoside diphosphate nucleotide*; 3rd, adding a third phosphate group results in a *nucleoside triphosphate nucleotide*. + The nitrogenous base (nucleobase) is indicated by "Base" and "glycosidic bond" (sugar bond). All five primary, or canonical, bases—the purines and pyrimidines—are sketched at right (in blue).

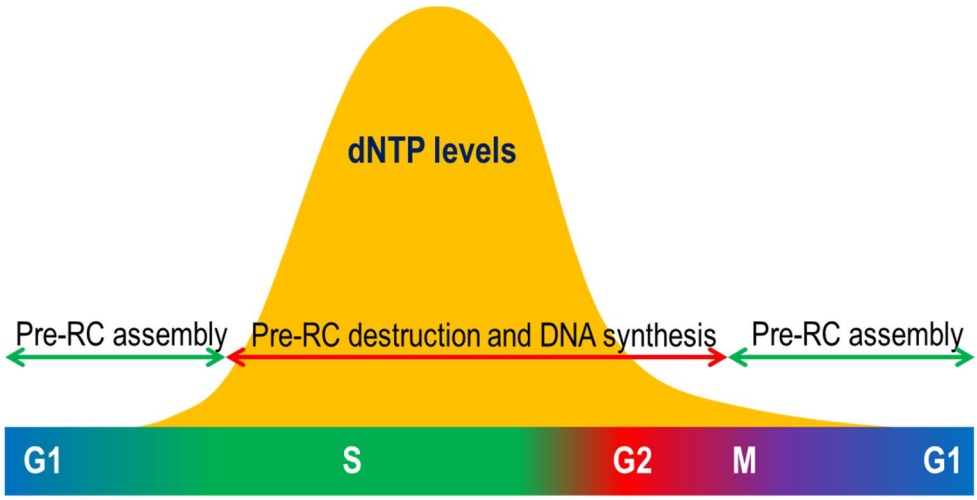

Figure 2. Schematic representation of dNTP levels throughout cell cycle progression. Highest dNTP levels are seen during S phase wheras lowest are during G1 phase. The Low G1-phase dNTP levels contribute to the G1 checkpoint. (Franzolin et al., 2013)

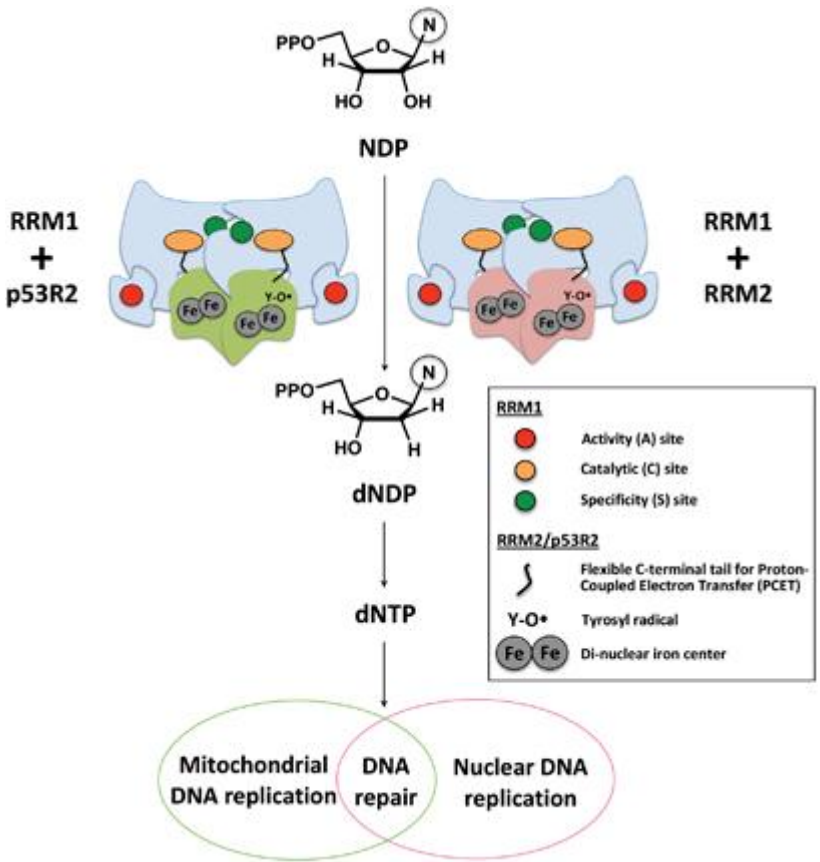

Figure 3. RNR and de novo dNTP biosynthesis. Mammalian RNR enzymes function to reduce the 2' carbon of NDPs to generate dNDPs that are subsequently phosphorylated by nucleoside diphosphate kinase, yielding dNTPs for nuclear and mitochondrial DNA replication and repair. (Aye et al., 2015)

II. SAMHD1

1. SAMHD1 is a dNTPase

An additional mode of dNTP pool control was discovered in 2011 as two groups reported a novel enzyme SAMHD1, Sterile alpha motif (SAM) domain and Histidine-aspartic (HD) domain containing protein 1. It is expressed ubiquitously throughout human body, and mainly in the nucleus at cell level. It is the first deoxynucleotide triphosphohydrolase (dNTPase) discovered in mammalian cells, as it depletes the cellular dNTP levels by cleaving them to respective deoxyribonucleosides plus tripolyphosphate (Figure 4) (Goldstone et al., 2011, Powell et al., 2011). This activity of SAMHD1 resides in its histidine-aspartate (HD) domain, while the N-terminal sterile alpha motif (SAM) domain exhibit other activities including substrate-binding. dGTP is reported to be its cofactor, as it binds to the allosteric sites of SAMHD1 and promotes tetramerization (Figure 5). Unlike inactive monomers or dimers, SAMHD1 tetramers show high enzymatic activities and facilitate various biological activities. In addition to the

dNTPase activity, SAMHD1 is reported to have RNase and nuclease activities, although further validations are required.

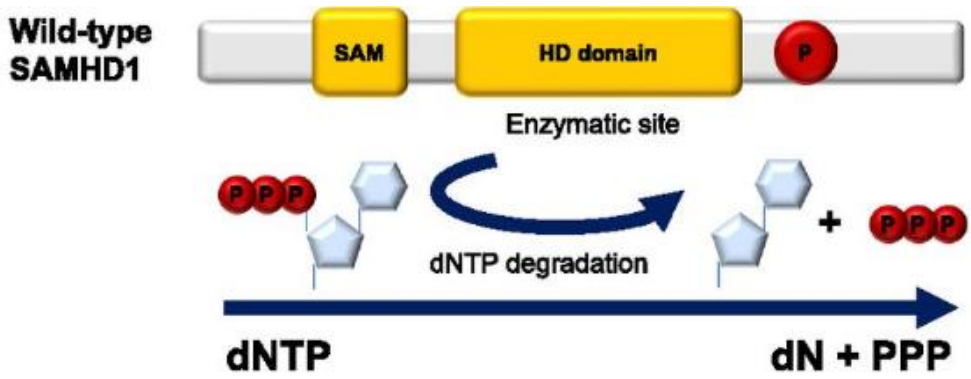

Figure 4. Representative schematic of the important domains of SAMHD1. The enzymatic activity of SAMHD1 is promoted by the HD domain. SAMHD1 degrades dNTPs into deoxynucleosides (dN) and inorganic triphosphates (PPP). (Rossi, 2014)

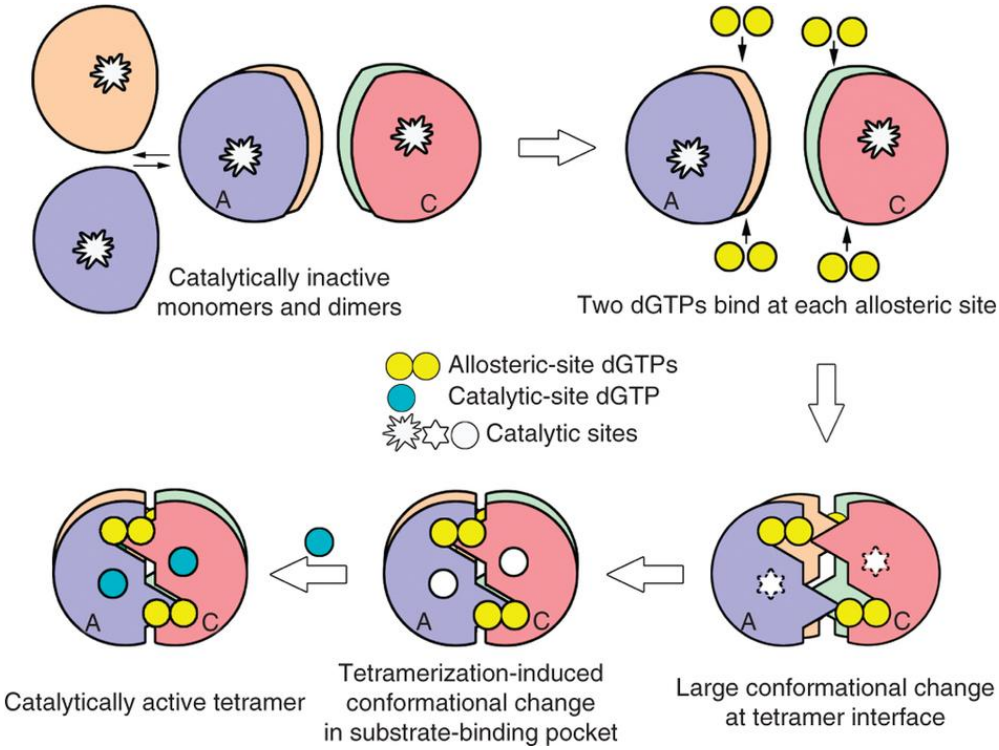

Figure 5. Schematic illustration of the mechanism of SAMHD1 activation by allosteric dGTP-induced tetramerization. Subunits of SAMHD1 are labeled A, B, C and D, and the catalytically inactive monomers and dimers are shown with empty substrate-binding pockets. Binding of four dGTP pairs at the allosteric sites promotes tetramerization and induces large conformational changes at the tetramer interface (AC and BD). This in turn causes conformational changes around the catalytic sites, enabling dNTP substrate binding in a catalytically productive conformation. (Ji et al., 2013)

2. SAMHD1 and innate immunity

Originally discovered as a component of the human innate immune system, SAMHD1 was first named as dendritic cell-derived IFN-γ induced protein (DCIP) as its levels were increased in response to IFN-γ treatment in dendritic cells (Li et al., 2000). Later studies revealed that mutations in SAMHD1 causes Aicardi-Goutières syndrome (AGS), a genetic encephalopathy whose symptoms mimic congenital infection (Rice et al., 2009). However, the most widely-known function of SAMHD1 is its anti-HIV-1 restriction activity. Observed in myeloid cells as well as in quiescent CD4+ T cells, SAMHD1 depletes the level of cellular dNTPs to the levels insufficient for reverse transcription, therefore results in blockage of early-stage retroviral replication (Figure 6) (Laguette et al., 2011, Hrecka et al., 2011, Baldauf et al., 2012). Together, these findings suggest new ways to interfere with the immune evasion and immune cell pathology of pandemic HIV-1.

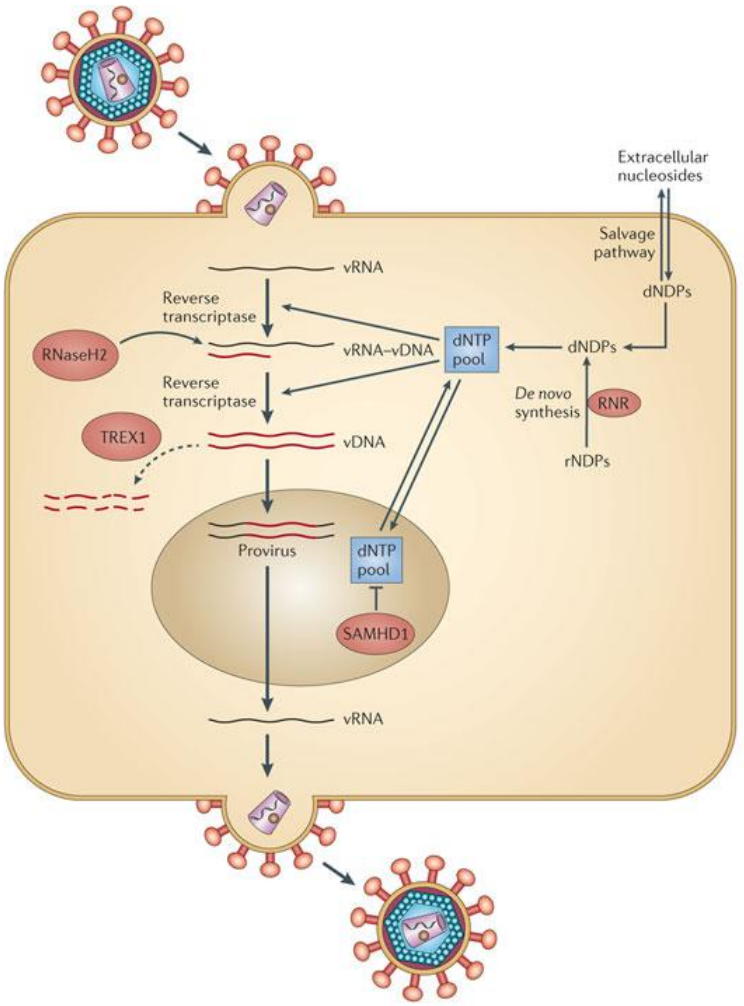

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Figure 6. Effect of SAMHD1 on HIV-1 replication. After entry into the target host cell, viral RNA (vRNA) is converted into viral DNA (vDNA) by the viral reverse transcriptase, and the resulting provirus is integrated into the host nucleus. HIV-1 relies on cellular dNTPs for reverse transcription. Various cellular enzymes that target nucleic acids either positively or negatively affect the early steps of viral replication. SAMHD1, which is located in the nucleus, hydrolyses dNTPs in non-cycling cells, thereby limiting HIV-1 replication. In the presence of SAMHD1, HIV-1 reverse transcription is severely impaired and levels of replication are very low. (Ayinde et al., 2012)

3. SAMHD1 and cell growth

Like RNR, which controls cell cycle progression by contributing to dNTP pools, SAMHD1 also plays role in cell cycle progression. SAMHD1 stimulates cell proliferation in lung fibroblasts and THP-1 cells (Franzolin et al., 2013, Bonifati et al., 2016). SAMHD1 was variably expressed during the cell cycle, maximally during quiescence and minimally during S phase (Figure 7). Low dNTP levels during G1 phase caused by high levels of SAMHD1 facilitated G1/S transition and resulted in increased cell proliferation in fibroblasts.

Overall, as SAMHD1 controls the size of dNTP pool and cell cycle progression, there is a high possibility of its connection to cancer, similarly to RNRs. Indeed, SAMHD1 is reported to be frequently mutated in chronic lymphocytic leukemia and colon cancers (Clifford et al., 2014, Rentoft et al., 2016). However, little has been investigated about its molecular mechanisms in cancer proliferation.

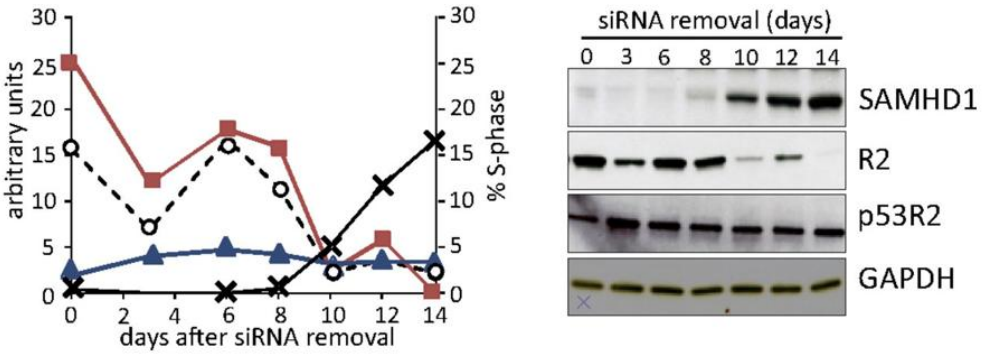


Figure 7. SAMHD1 expression change during cell proliferation. Abundance of SAMHD1 (x), and two subunits of RNR, R2 (filled square), and p53R2 (filled triangle) at the indicated days of culture without siRNA. Left reports in aribitrary units the quantification of the immunoblots on the right. Notice the parallelism between R2 expression and frequency of S-phase cells (open circle) in the cultures. (Franzolin et al., 2013)

4. Posttranslational modifications of SAMHD1

Posttranslational modifications (PTMs) are the chemical modifications of a protein after its translation, which highly increases the functional diversity of the protein (Khoury et al., 2011). A variety of PTMs exist in mammalian systems, including phosphorylation, glycosylation and acetylation. In 2013, two groups independently reported the phosphorylation of SAMHD1. In cycling immune cells, CDK1 (cyclin-dependent kinase 1) and cyclin A2 phosphorylates SAMHD1 at its T592 residue, which keeps SAMHD1 inactive (Cribier et al., 2013, White et al., 2013). However, in non-cycling cells, SAMHD1 is dephosphorylated and shows anti-viral restriction activity towards HIV-1 virus (Figure 8). However, this phosphorylation had no direct influence on the ability of SAMHD1 to decrease dNTP levels *in vitro*. These results raises the possibility that unknown forms of PTMs or non-cycling cell-specific cofactors may exist. Yet, other PTMs or molecular mechanisms that affects the dNTPase activity directly has not been discovered.

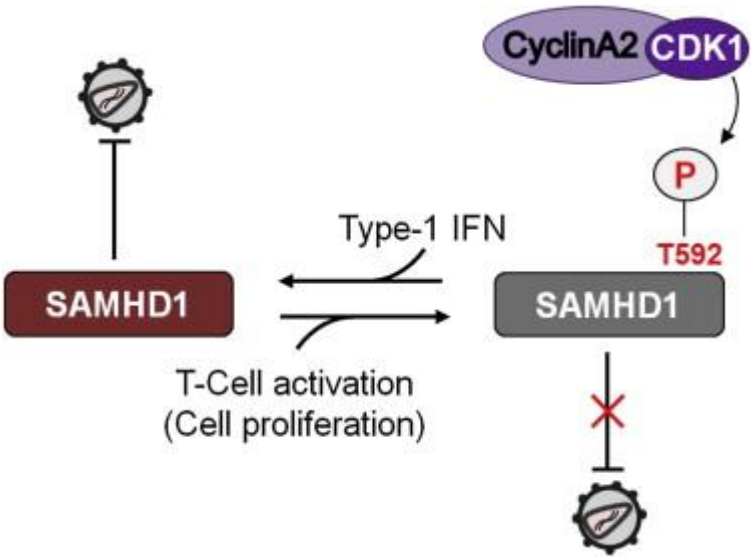


Figure 8. Model for the regulation of SAMHD1 restriction activity by phosphorylation at the Thr592 residue. SAMHD1 phosphorylation occurs at T592 residue by CDK1 and cyclin A2 in proliferative T-cells. Phosphorylated SAMHD1 shows no anti-HIV-1 activity (Cribier et al., 2013).

III. ARD1

1. Protein acetylation

Protein acetylation is one of the major PTMs in cell signaling and metabolism in eukaryotes as its regulation is crucial for various important cellular processes (Drazic et al., 2016, Choudhary et al., 2009, Zhao et al., 2010). It is catalyzed by acetyltransferases, which transfer acetyl groups from the donor acetyl-CoA to the target proteins. Two types of acetylation have been reported, N^{α}-terminal acetylation and N^{ε}-lysine acetylation (Figure 9). N^{α}-terminal acetylation is mediated by NATs (N^{α}-terminal acetyltransferases), as they acetylate the α-amino group of N-terminal residues of proteins. This is one of the most common protein modifications in eukaryotes, which occurs to about 80–90% of the cytosolic proteins in mammals and approximately 50% of proteins in yeast (Persson et al., 1985;

Lee et al., 1989). N^{α}-terminal acetylation is known to be co-translational and irreversible, contributing to protein stability and localization. NATs associated to the ribosome acetylates the N-terminal α-amino group of the nascent polypeptide newly synthesized and extruded from the ribosome. (Driessen et al., 1985).

N^{ε}-lysine acetylation, on the other hand, is less common, but a very important form of protein acetylation which occurs post-translationally on the ε-amino group of lysines. When an acetyl group is added onto lysines, the positive charges on its amino group is neutralized, which results in a significant change in the electrostatic properties of the protein. This type of acetylation is crucial for regulating diverse functions of proteins, such as protein-protein interaction or protein stability (Glozak et al., 2005). Unlike the N^{α}-terminal acetylation, this form of acetylation is reversible; therefore the acetylation/ deacetylation of a specific residues are reported to act as important switches in the regulation of gene transcription, cellular growth and differentiation.

Many acetyltransferases can regulate their catalytic activity by acetylating themselves (auto-acetylation). For example, p300, an acetyltransferase, is highly auto-acetylated in its activation loop, which results in its enhanced transcriptional activity (Karanam et al., 2006).

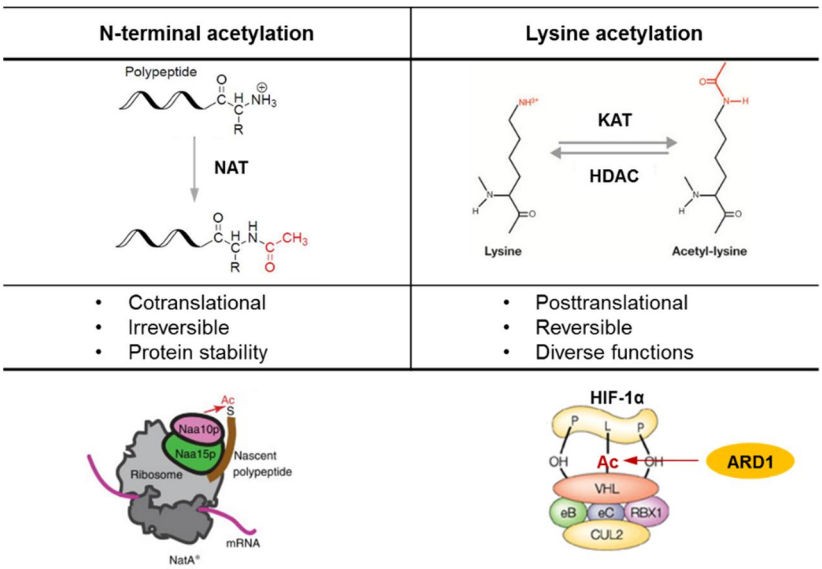


Figure 9. Comparison of two types of protein acetylation. N-terminal acetylation and lysine acetylation are compared. ARD1 shows both of the acetylation activities. (Jeong et al., 2002; Starheimm KK et al., 2012; Sharp FR et al., 2004)

2. ARD1 is an acetyltransferase

Arrest defective 1 (ARD1) is an acetyltransferase. Interestingly, it is known to have both N^{α}-terminal acetylation and Lysine-acetylation activities (ref) (Figure 9). First discovered in Saccharomyces cerevisiae (Lee et al, 1989; Park and Szostak et al, 1990), ARD1 associates with NAT1 to form NAT complex, and acts as a catalytic component in yeast (Park and Szostak et al, 1992).

Later studies have discovered various substrates of ARD1. Runt-related transcription factor 2 (Runx2) is known to transactivate many genes required for osteoblast differentiation. ARD1 acetylates this protein to block this transactivation, controlling balanced osteogenesis (Yoon et al., 2014). Heat shock protein 70 (HSP70) is also a recently-discovered substrate of ARD1. HSP70 acetylation acts as a functional switch to alter its activity from protein refolding to degradation (Figure 10) (Seo et al., 2016). Moreover, ARD1 is also auto-acetylated, which promotes the acetyltransferase activity of itself (Seo et al., 2010). Altogether, these reports demonstrate that ARD1-mediated acetylation acts as an important switch of various substrates to alter their

modes of action.

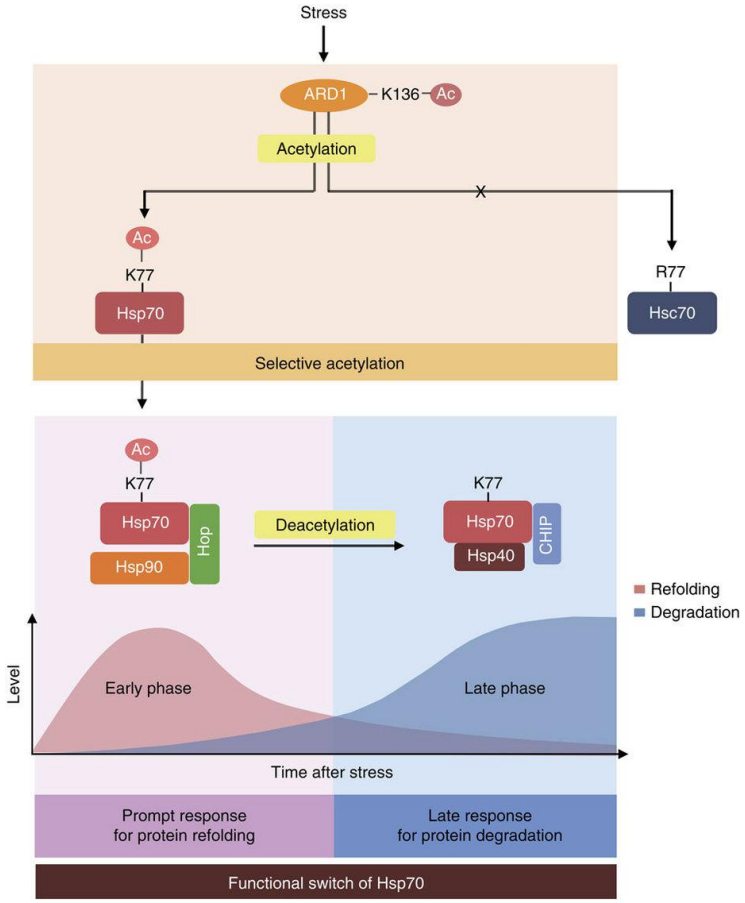


Figure 10. Schematics for functional switch of Hsp70 regulated by ARD1-mediated acetylation. After stress, ARD1 and Hsp70 are sequentially activated by an acetylation cascade. ARD1 selectively acetylates Hsp70 but not Hsc70. In the early phase after stress, Hsp70 is rapidly activated by acetylation to support protein refolding. In the late phase,

deacetylated Hsp70 contributes to protein degradation. (Seo et al., 2016)

3. ARD1 is oncogenic

When initially identified in yeast, ARD1 was reported as a mating-type switch that controls the mitotic cell cycle and alternative development (Whiteway and Szostak, 1985; Whiteway et al., 1987). Later, many studies have reported ARD1 to be oncogenic. It is highly expressed in several types of cancers including breast (Yu et al., 2009, Wang et al., 2011), prostate (Wang et al., 2012), lung and colorectal (Xu et al., 2012). Moreover, various molecular mechanisms of ARD1 were discovered to promote tumorigenesis. For example, ARD1 is proposed to promote the lung cancer cell proliferation by acetylating β-catenin (Lim et al., 2006; Lim et al, 2008). In prostate cancer, ARD1 activates androgen receptor (AR) by acetylation to promote prostate tumorigenesis (Wang et al., 2012).

Recent study has demonstrated that the nuclear translocation of ARD1 is required for cell cycle progression (Figure 11) (Park et al., 2014). In proliferating cells, ARD1 is imported to the nuclei especially during S phase, contributing to proper cell proliferation in HeLa and A549 cancer cell lines.

Altogether, these growing evidence suggest that ARD1 contributes to cancer proliferation.

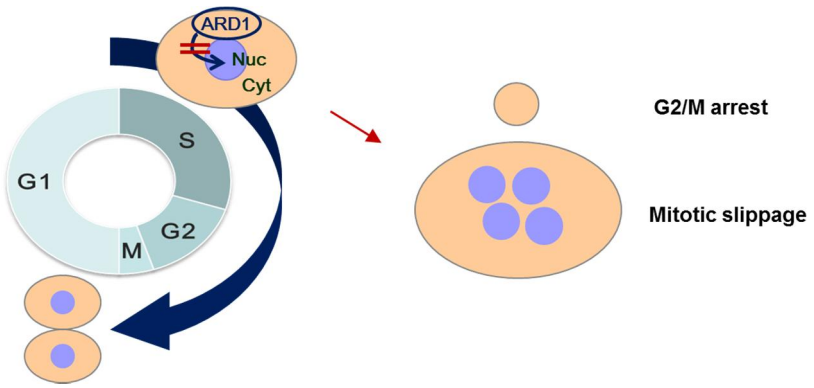


Figure 11. Schematic diagram showing the contribution of ARD1 in cell cycle progression. (Park et al., 2014)

PURPOSE OF THIS STUDY

SAMHD1 is the first dNTPase discovered in human cells which contributes to anti-retroviral defense by lowering the cellular dNTP pool levels in non-cycling myeloid cells. This restrictive activity is not shown in cycling cells, implying the existence of posttranslational modifications or cofactors specifically expressed in non-cycling cells. However, molecular mechanisms that directly regulates its enzymatic activity has not been reported, as its phosphorylation did not alter its dNTPase activity *in vitro*. Moreover, previous studies were mainly limited to its functions in immune cells, despite its ubiquitous expression throughout the body. SAMHD1 was reported to facilitate G1-to-S phase progression in monocytes and fibroblasts, yet further studies on functions of SAMHD1 in other cell types are required.

In this study, I present SAMHD1 as a novel acetylation substrate of ARD1. As protein acetylation can modulate the activities of the substrate proteins, I hypothesized that ARD1 may acetylate SAMHD1 to alter its dNTPase activity. Moreover, as ARD1 is reported to be oncogenic and the enzymatic activity of SAMHD1 is relevant to the cell cycle regulation, I investigated

whether the ARD1-mediated SAMHD1 acetylation contribute to cancer cell proliferation. By identifying the specific acetylation site of SAMHD1 and mutating it to block its acetylation, I aimed to discover a novel function of SAMHD1 acetylation in cancer cell growth.

MATERIALS AND METHODS

1. Human liver tissue samples

Hepatocarcinoma tissues and surrounding non-tumorous liver samples were obtained from patients at Asan Hospital (Seoul, Republic of Korea). Clinicopathological characteristics of hepatocarcinoma patients are summarized in Table S1. This study was approved by Institutional Review Board of Asan Hospital, and written informed consent was obtained from all the patients.

2. Cell culture and synchronization

HeLa, A549, Hep3B, MCF-7 and HEK293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 5% CO2 humidified atmosphere at 37°C. For serum starvation, cells were incubated in DMEM without serum for 48 h and re-stimulated with DMEM containing 10% FBS. A double thymidine block

was performed to synchronize cell populations in S phase. Cells were treated with 2 mM thymidine (Sigma-Aldrich) for 18 h, cultured for 9 h in normal growth medium, and then retreated with thymidine for another 16 h. After removing the thymidine, cells were released to normal medium and harvested at the indicated times.

3. *in vitro* acetylation assay

Acetylation assay was performed as described previously [19]. Briefly, 1 μg of purified recombinants or precipitated cellular proteins were incubated in the reaction mixture containing 50 mM Tris–HCl (pH 8), 0.1 mM EDTA, 1 mM DTT, 10% glycerol and 10 mM acetyl-CoA at 37 °C for 1.5 h. Reaction products were separated by SDS–PAGE and were analyzed by western blot using anti-Lys-Ac antibody. Input proteins were visually quantified using Coomassie brilliant blue staining or Ponceau S staining.

4. Mass spectrometric analysis

Mass spectrometric analysis was performed as previously described [30]. For enzymatic digestion of recombinant SAMHD1, two GST-SAMHD1 samples

used in the in vitro acetylation assay (incubated with ±ARD1) were immediately denatured using 6 M GuHCl and were reduced with 5 mM DTT for 30 min at room temperature, then alkylated with 25 mM iodoacetamide for 30 min. Glu-C digestions were carried out for 6 h at 25 °C then were quenched by 5% formic acid. Micro RPLC–MS/MS analysis was performed as previously described [22].

5. *in vitro* dNTPase assay

An enzymatic assay based on thin-layer chromatography was performed as described previously [9]. The purified recombinant or immunoprecipated cellular protein (1 μg) was incubated in reaction mixture composed of 50 mM Tris-Cl (pH 8.0), 20 mM KCl, 5 mM MgCl2, 0.1 μCi [α-^{32}P]dTTP, and 200 μM ice-cold dGTP for 3 h at 37 °C. The reactions were stopped by heat inactivation at 70 °C for 10 min. Mixtures were blotted onto polyethyleneimine (PEI)-cellulose plates (Sigma-Aldrich) and subsequently separated using a mobile phase of 1.2 M LiCl. After separation, the α-^{32}P-labeled reaction products were visualized using a phosphorimager.

6. siRNA and plasmid construction

The siRNA sequence targeting human SAMHD1 corresponds to GAUUCAUUGUGGCCAUAUA as previously described [9]. Full-length of cDNAs for human SAMHD1 (Genbank: NM_015474) and human ARD1 (Genbank: NM_003491.3) were obtained from PCR and subcloned into pCDNA3.1 (FLAG-SAMHD1), pCMV-tag2b (FLAG-ARD1), pEGFP-C3 (GFP-ARD1) and pCS2+ (MYC-ARD1) vectors for cellular expression or pGEX-4T (GST-SAMHD1) and pET28a (His-ARD1) vectors for the bacterial induction of recombinant proteins. Deletion mutants of GST-SAMHD1 were constructed from pGEX-4T-SAMHD1 plasmid. cDNAs of SAMHD1 corresponding to 34–113 aa, 160–339 aa and 334–626 aa were amplified by PCR and inserted into pGEX-4T-1. For construction of stable cell lines, cDNAs of SAMHD1 was co-inserted into pMX-IRES-BlasticidinR vector with PCR-amplified FLAG. Point mutations in SAMHD1 (K405R and K580R) and ARD1 (K136R and DN: R82A/Y122F) were generated using the Muta-Direct Site Directed Mutagenesis kit (Intron) according to the manufacturer's instructions. The following primers and its reverse-complement for each point mutation (mutated based in lower case bold):

SAMHD1 K405R: TACAGGTGCTGGAGGcggAAAGTATCGCATTTC

SAMHD1 K580R: GACAGAAATTTCACCcgGCCGCAGGATGGCGAT

ARD1 K136R: AGTGAAGTGGAGCCCAgATACTATGCAGATGGG

ARD1 R82A: TGTGAAGCGTTCCCACgcGCGCCTCGGTCTGGCT

ARD1 Y122F: GCCGCCCTGCACCTCTtTTCCAACACCCTCAAC

7. Transfection and Stable Cell Line Construction

Transfection was performed as described previously [22]. I transfected HEK293T cell with polyethyleneimine (PEI) at a ratio of 3:1 (μl PEI/μg plasmid DAN) in basal media overnight. siRNA targeting SAMHD1 was transfected with HiPerFect reagent (Quiagen) according to the manufacture's instruction.

For the establishment of stable cells, pIRES-FLAG-SAMHD1 plasmids were transfected into Platinum A cells and retroviral supernatants were harvested 72 h after transfection. Collected supernatants were used to transduce target cell lines (HeLa, A549, Hep3B and MCF-7) with polybrene (10 μg ml−1). Transduced cells were selected using blasticidin (10 μg ml−1). Expression of

FLAG-SAMHD1 was quantified by western blot.

8. Immunoblotting and immunoprecipitation

Cellular proteins were extracted using cell lysis buffer composed of 20 mM Tris-CL (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100 and a protease inhibitor cocktail (Roche). 20 ~50 μg of cell extracts were used for immunoblotting. For immunoprecipitation, 1 mg of proteins were incubated with corresponding primary antibody conjugated to A or G bead (Upstate) or Anti-DDDDK-tag mAb-Magnetic Agarose (MBL) overnight at 4 °C. Beads were washed three times with washing buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl and 0.1 mM EDTA. After SDS–PAGE, membranes were immunoblotted using the corresponding primary antibody overnight at 4 °C. HRP-conjugated secondary antibodies were incubated with the membranes for 1 h at room temperature. Visualization was performed using ECL Plus (Intron) and LAS-4000 (GE Healthcare).

9. Screening of binding proteins

The protein-protein interaction assay was performed as reported previously

[22]. HEK293T cells were transiently transfected with FLAG-HA tagged ARD1 and were lysed in lysis buffer consisting 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100 and a protease inhibitor cocktail (Roche). Cell lysates were incubated with anti-M2 resin (Sigma) for 2 h at 4 °C, then resins were collected by centrifugation and washed three times with washing buffer, composed of 20 mM Tris-Cl (pH 7.5), 150 mM NaCl and 0.1 mM EDTA. Bound proteins were eluted by 3 × FLAG-peptide and were immunoprecipitated again using anti-HA-antibody for 2 h at 4 °C. The bound proteins were analyzed by SDS–PAGE and silver staining.

10. Recombinant protein preparation

BL21 cells were transformed with plasmids pGEX-4T-1-SAMHD1 or pET28a-ARD1 and were grown to and OD600 of 0.6–0.8. 1 mM IPTG was added to induce GST-tagged SAMHD1 or His-tagged ARD1, then cells were grown overnight at 20 ℃. Cells were collected and proteins were extracted with a lysis buffer consisting 50 mM Tris–HCl (pH 8), 250 mM NaCl, 2.5 mM EDTA and 1% Triton X-100. GST-tagged and His-tagged proteins were purified with Glutathione Sepharose 4B (GE Healthcare) and TALON® Metal Affinity Resins (Clontech), respectively. Resin-bound proteins were

eluted with an elution buffer containing 50 mM Tris–HCl (pH 8) and 10 mM reduced glutathione (GSH).

11. Protein sequence alignment

SAMHD1 multiple protein sequence alignment was conducted by Constraint-based Multiple Alignment Tool provided from NCBI.

12. Flow cytometry analysis

For a flow cytometry assay, cells were collected, fixed in 1% PFA, and stored at 4°C for 30 min. Cells were then washed with PBS and stained with propidium iodide (PI, 30 μg ml−1) mixed with RNase A (1 μg ml−1) for 15 min at 37℃. The DNA content of cells was assessed with a FACS-Verse system (BD Biosciences), and cell cycle profiles were analyzed with the BD FACSuite software. At least 30,000 cells in each sample were analyzed to obtain a measurable signal, using the same instrument setting.

13. Cell proliferation assay

The proliferation rates were measured using a Cell Proliferation Assay kit (Promega) following the manufacturer's instructions. Briefly, 2×103 cells/well were seeded onto 96-well plates and were allowed to grow. At indicated time points, 20 μL of substrate solution was added to the cells and were incubated for 1 h for color development. The absorbance at 492 nm was measured to indicate the number of proliferating cells.

14. Colony formation assay

Colony formation assays were performed as described previously [31]. For anchorage dependent colony formation, cells were seeded at a density of 100 cells/well onto 6-well plates and were allowed to grow for 2 weeks. Cells were then fixed with 100% methanol, stained with 0.005% crystal violet and the number of colonies were counted. For anchorage independent colony formation, cells were resuspended in 0.5 mL DMEM containing 0.4% agar and were seeded onto a layer of 1% agar in 12-well plates. After 2 weeks of incubation, colonies were stained with 0.005% crystal violet and counted.

15. Fluorescence microscopy

For the analysis of PCNA by flourescence microscopy, cells were seeded onto the glass coverslips in 24-well plates. After synchronization, cells were fixed in 2% PFA for 20 min and were permeabilized in 1% triton X-100 in PBS 10 min at room temperature. Then, cells were incubated with PCNA antibody (Cell signaling) and visualized with Alexa 488-conjugated igG (Molecular Probes). Nucleus staining was performed with Hoechst 33342 (Molecular Probes). The immunofluorescence was visualized using a confocal microscopy (Carl Zeiss, LSM700).

16. Statistical analysis

Results are expressed as the mean's±s.d's or±s.e.m's. P values were calculated by applying the two-tailed Student's t test or one-way ANOVA. A difference was considered statistically significant at a value of $P<0.05$.

RESULTS

1. SAMHD1 is a novel acetylation substrate of ARD1

As ARD1 is an acetyltransferase, I first aimed to find its novel substrates to study the functions of ARD1. For this I have screened for ARD1-binding proteins using affinity purification combined with mass spectrometry. FLAG/HA-tagged ARD1 proteins were overexpressed in HEK293T cells, then immunoprecipitated overnight. The resin-bound proteins were separated by SDS-PAGE and the gel was silver stained for protein identification. Around 72 kDa, two strong bands were observed; one of them was identified to be HSP70 protein as previously reported (Seo et al., 2016). The other anonymous band was subjected to mass spectrometry for identification, which was revealed to be endogenous SAMHD1 protein (Figure 12). To confirm this result, FLAG mock or ARD1 expressing vectors were transfected to HEK293T cells and the lysates were immunoprecipitated using

FLAG-specific resin. When precipitates were separated by SDS-PAGE and immunoblotted by SAMHD1 specific antibody, the endogenous SAMHD1 proteins were found to be co-immunoprecipitated, in accordance to the previous result (Figure 13). These data demonstrates that SAMHD1 is a novel binding partner of ARD1.

Next, as ARD1 is an acetyltransferase, I investigated whether it could acetylate SAMHD1. Recombinant GST-SAMHD1 proteins were induced and purified from *E. coli*, then subjected to an *in vitro* acetylation assay with or without His-ARD1 in the presence of Acetyl-CoA. The protein was found to be acetylated when His-ARD1 was present, demonstrating that ARD1 directly acetylates SAMHD1 *in vitro* (Figure 14). To test this acetylation *in vivo*, GFP-mock or ARD1 vectors were overexpressed in HEK293T cells. The lysed proteins were incubated with Lys-Acetylation specific antibody to selectively immunoprecipitate Lys-Acetylated proteins. When the products were separated and blotted with SAMHD1 antibody, the intensity of endogenous SAMHD1 acetylated forms was observed to be stronger in GFP-ARD1 overexpressing cells than the mock cells, confirming the *in vitro* data (Figure 15). Together, these data indicate that SAMHD1 is acetylated by ARD1.

Our lab has previously reported that ARD1 is autoacetylated at K136 residue to enhance its catalytic activity (Seo et al., 2010). To test whether this affects SAMHD1 acetylation, I overexpressed K136R mutant FLAG- ARD1, which cannot be autoacetylated therefore shows less acetyltransferase activity. In addition, dominant negative (DN) mutants of ARD1 which does not exhibit any enzymatic activity at all were also expressed in HEK293T cells. When the Lys-acetylation intensity of endogenous SAMHD1 from these cells were evaluated, they were significantly decreased compared to the ARD1 wildtype expressing cells (Figure 16). This result indicates that the two ARD1 mutants blocked SAMHD1 acetylation; therefore it is suggested that ARD1-autoacetylation is required for SAMHD1 acetylation as an up-stream signal.

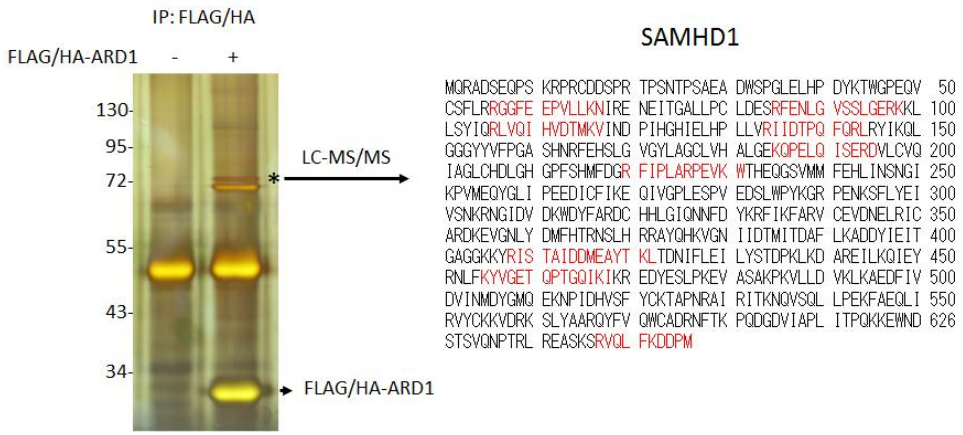


Figure 12. LC-MS/MS analysis reveals SAMHD1 as a novel binding partner of ARD1. HEK293T cells were transfected with FLAG/HA-ARD1 and lysed proteins were immunoprecipitated, separated by SDS-PAGE and silver stained. * refers to SAMHD1.

Figure 13. Co-immunoprecipitation of endogenous SAMHD1 and FLAG-ARD1. FLAG-ARD1 was immunoprecipitated from HEK293T cells, and co-precipitation of endogenous SAMHD1 was analyzed by western blot.

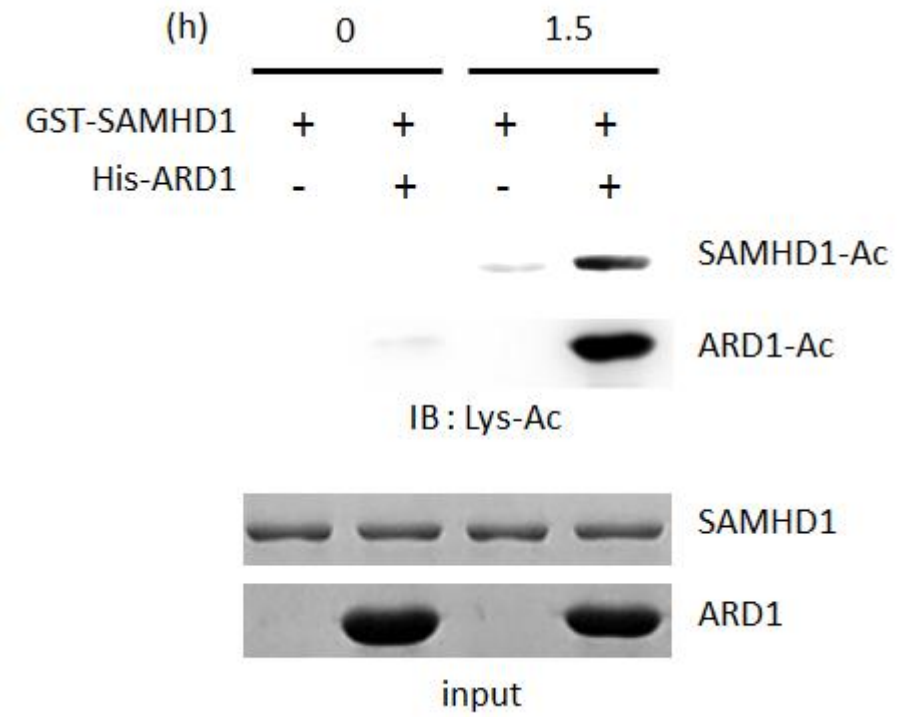


Figure 14. Recombinant SAMHD1 protein is acetylated by ARD1 *in vitro*.

Recombinant GST-SAMHD1 proteins were subjected to in vitro acetylation

assay with ±His-ARD1. The acetylation level was determined with anti-Lys-Ac antibody.

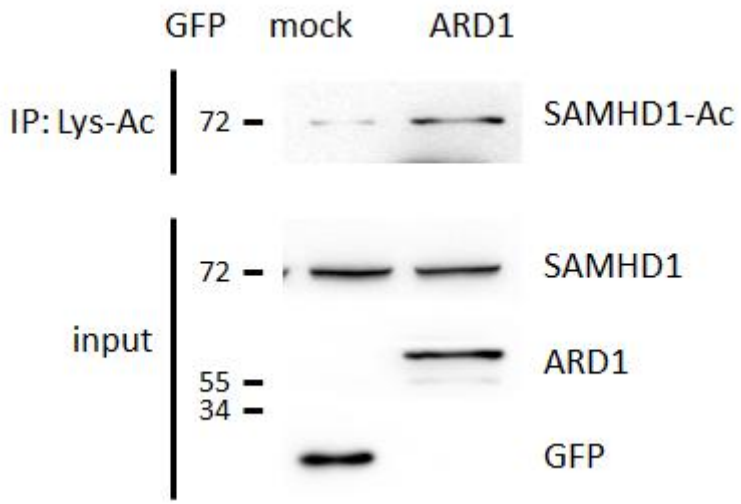


Figure 15. Endogenous SAMHD1 protein is acetylated by ARD1 *in vivo*. Lys-acetylated proteins were precipitated from GFP-mock or ARD1 expressing HEK293T cells and were blotted with anti-SAMHD1 antibody.

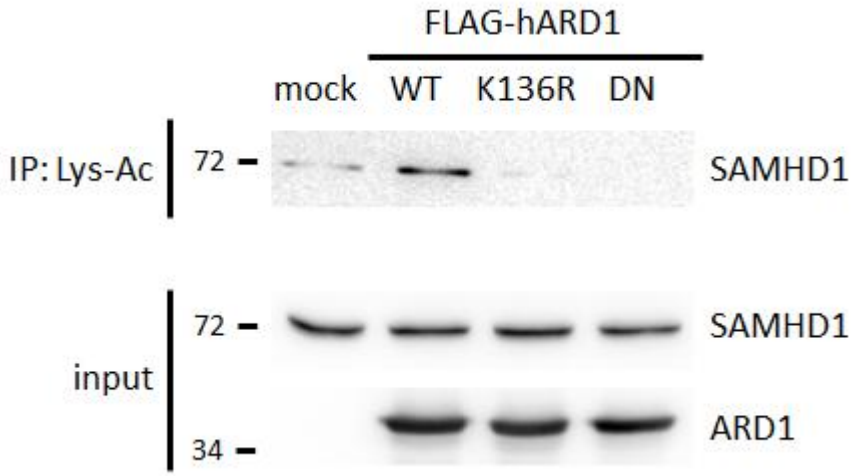


Figure 16. Endogenous SAMHD1 protein is less acetylated by ARD1 mutants *in vivo*. FLAG-ARD1 wildtype, K136R and DN mutants were overexpressed in HEK293T. Lysed proteins were precipitated with anti-Lys-Ac antibody and were blotted with anti-SAMHD1 antibody.

2. K405 residue of SAMHD1 is acetylated by ARD1.

Next, to identify the target site of acetylation, I constructed three types of deletion mutants of SAMHD1 according to their functional motifs; SAM domain (SAM), HD domain (HD) and C-terminal domain (CTD) (Figure 17). These vectors were transformed in to *E.coli* for induction and purification of corresponding recombinant proteins. When these proteins were subjected to *in vitro* acetylation assay in the presence of His-ARD1, only CTD was found to be acetylated, indicating that CTD is the major acetylated domain (Figure 18). To find the specific amino acid residue of acetylation, acetylated and non-acetylated GST-CTD were prepared by incubating with or without His-ARD1. Then, these products were digested into peptides and subjected to micro-liquid chromatography-tandem mass spectrometry (LC–MS/MS). When the acetylated sites were compared between the two, 2 potential acetylation sites were discovered; K405 and K580 (Figure 19). To verify the

critical site between the two and to establish the causality of this relationship, I mutated K405 and K580 of recombinant SAMHD1 to Arg 405 (K405R) and Arg 580 (K580R) residue respectively, to block their acetylation. When these mutants were subjected to *in vitro* acetylation, only K405R mutant showed decreased acetylation level, indicating that K405 is the main target site for acetylation and K580 as the minor site (Figure 20). To confirm this *in vivo*, FLAG-SAMHD1 K405R and K580R were constructed and overexpressed in HEK293T cells. When their acetylation levels were assessed by Lys-Ac antibody, K405R SAMHD1 showed a decreased acetylation level compared to wildtype or K580R expressing cells (Figure 21). Overall, these result demonstrates that ARD1-mediated SAMHD1 acetylation occurs at the K405 residue.

Proteins with key biological functions commonly have homologues from many different tissues and organisms. Therefore multiple sequence alignment of the protein from various species can highlight the conserved residues critical for its common function and key structure (Livingstone and Barton, 1993). Therefore to estimate the importance of K405 residue-specific acetylation, I investigated whether K405 residue is conserved among various species. Alignment of SAMHD1 amino acid sequence from various species

were carried out using BLAST (Basic Local Alignment Search Tool), which revealed that K405 residue is highly conserved especially in mammals, from human to *Mus Musculus*. Also, K405 was conserved in amphibians such as *Xenopus*, but not in fish such as *Danio Rerio*. Together, these result indicate that this acetylation site may be critical for its biological functions or protein structure in many species (Figure 22). To identify the structural importance of K405 acetylation, structural analysis was carried out using PyMOL software. The location of K405 was distant from the catalytically critical sites such as allosteric sites or substrate binding regions, but further studies are required to investigate its structural importance (Figure 23). In summary, ARD1-mediated SAMHD1 acetylation occurs at K405 residue, which is highly conserved among various species.

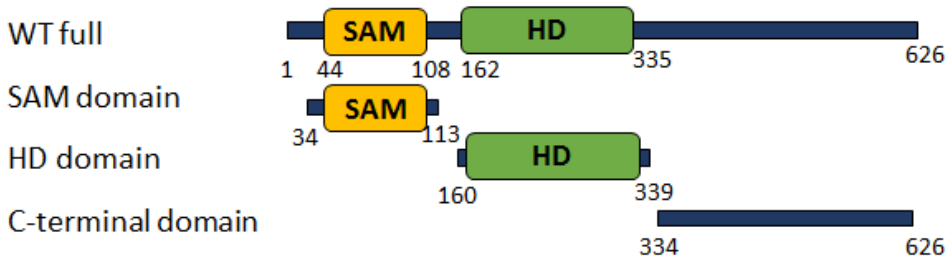


Figure 17. Construction of SAMHD1 deletion mutants. SAMHD1 deletion mutants were constructed according to their functional motifs. SAM; SAM domain, HD; HD domain, CTD; C-terminal domain.

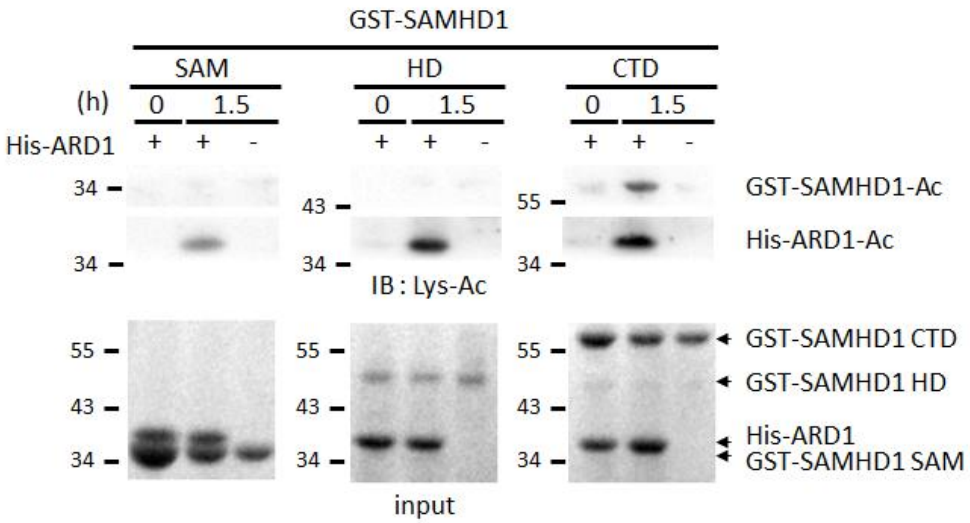
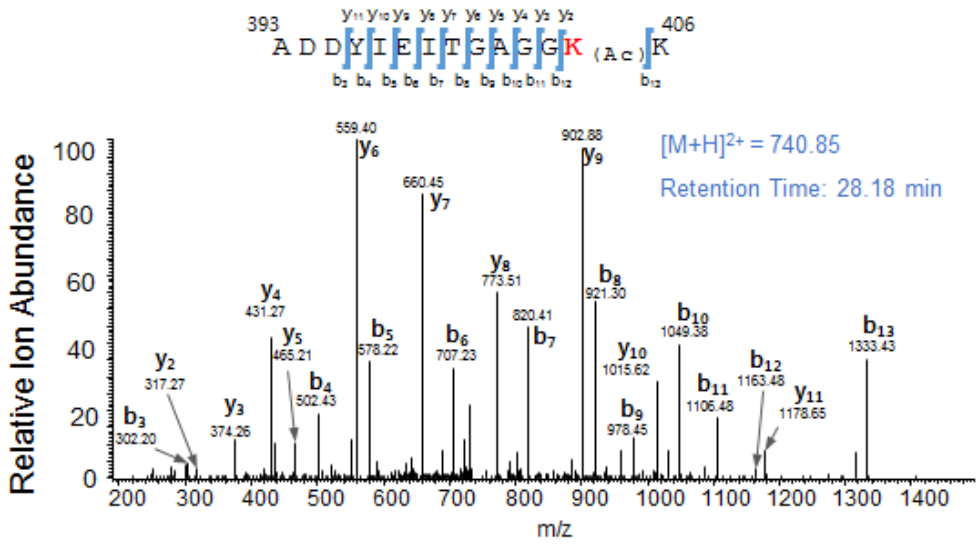


Figure 18. Recombinant CTD protein of SAMHD1 was mainly acetylated by ARD1 *in vitro*. Deletion mutants of GST-SAMHD1 were subjected to in vitro acetylation assays with ±His-ARD1 and blotted with anti-Lys-Ac antibody. SAM; SAM domain, HD; HD domain, CTD; C-

terminal domain.

A. K405



B. K580

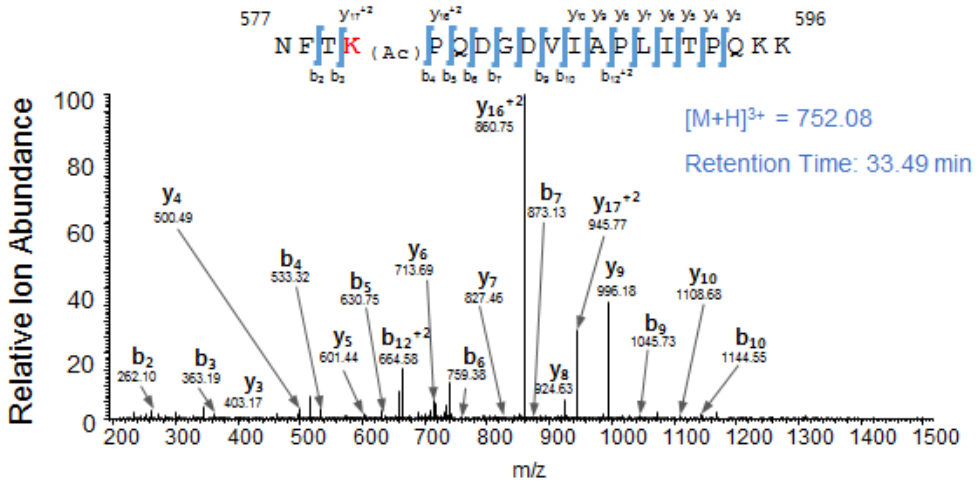


Figure 19. Acetylated sites of GST-SAMHD1 CTD. Recombinant GST-CTD were acetylated *in vitro* in the presence of recombinant ARD1 protein and subjected to LC–MS/MS to identify the acetylated sites. (A) K405 and relative ion abundance (B) K580 and relative ion abundance

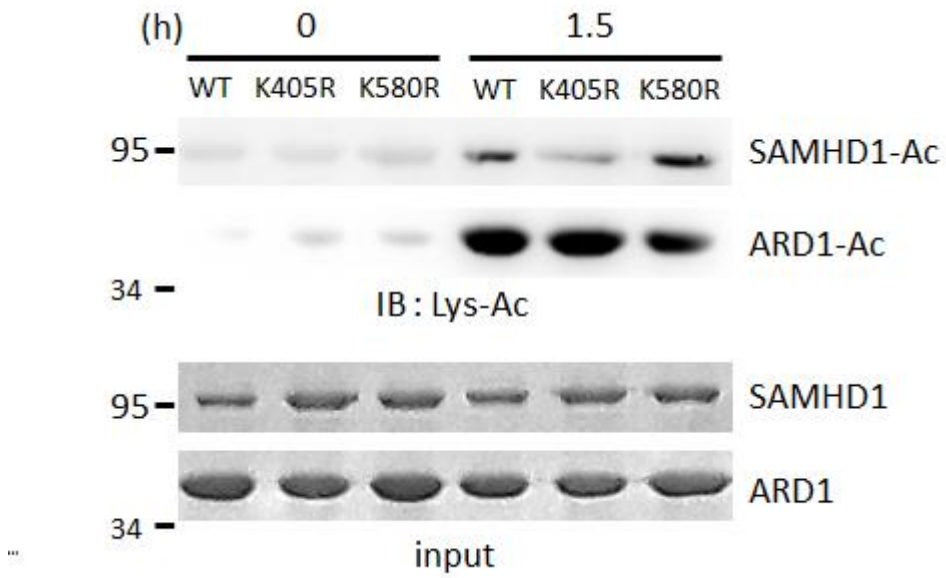


Figure 20. K405 is the major site of ARD1-mediated acetylation of SAMHD1 *in vitro*. GST-SAMHD1 recombinants were subjected to *in vitro* acetylation assays with His-ARD1. Acetylation levels were determined with an anti-Lys-Ac antibody.

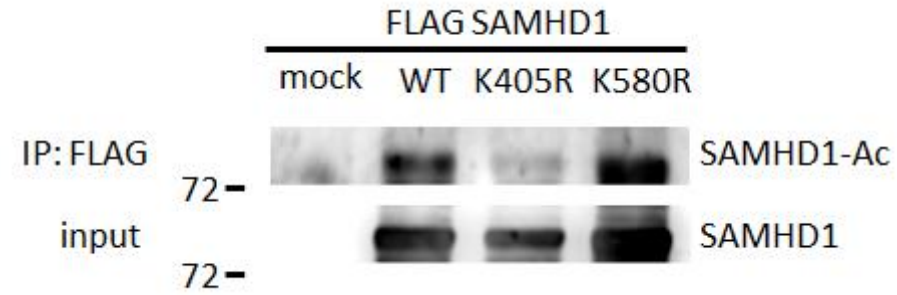


Figure 21. K405 is the major site of ARD1-mediated acetylation of SAMHD1 *in vivo*. FLAG-SAMHD1 variants were immnunoprecipitated from HEK293T cells, and their acetylation levels in vivo were analyzed using an anti-Lys-Ac antibody.

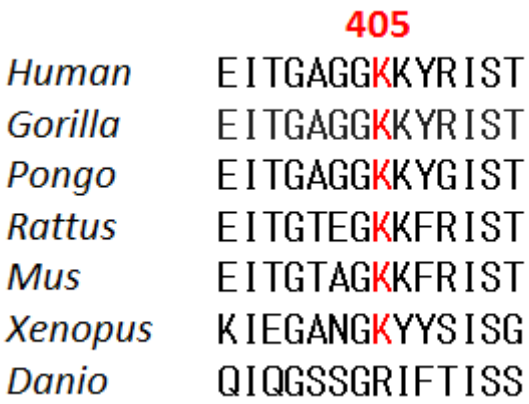


Figure 22. Sequence alignment of SAMHD1 K405 residue in various species.

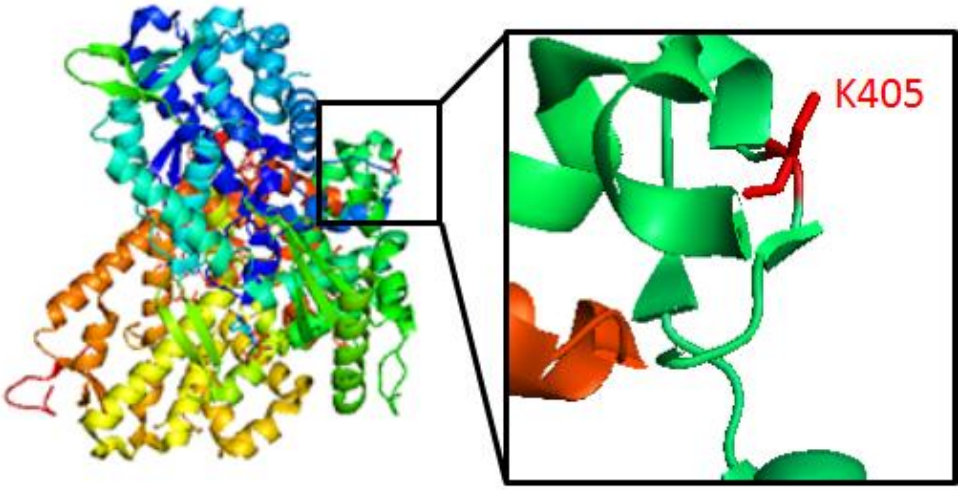


Figure 23. Location of K405 residue in SAMHD1 structure. Blue, orange, yellow and green colors represent each SAMHD1 monomers. Red indicates Lys 405 residue.

3. ARD1-mediated SAMHD1 acetylation enhances its dNTPase activity

As SAMHD1 is a dNTPase, I investigated whether SAMHD1 acetylation

could regulate this activity directly. By using *in vitro* dNTPase assay, I tested the ability of recombinant acetylated and non-acetylated SAMHD1 to hydrolyze α-^{32}P labeled thymidine triphosphate (α-[^{32}P]TTP) to deoxythymidine (dT) and α-[^{32}P]PP (Figure 24). In this assay, the released amount of α-[^{32}P]PP is considered as the marker for dNTPase activity; the more α-[^{32}P]PP is released, the stronger the enzymatic activity is. First, the GST-SAMHD1 proteins were subjected to *in vitro* acetylation assay with or without His-ARD1 to produce acetylated and non-acetylated SAMHD1 proteins, respectively. Then the products were incubated with α-[^{32}P]TTP and separated using thin layer chromatography to compare the amount of released α-[^{32}P]PP. The acetylated SAMHD1 released more α-[^{32}P]PP, indicating its higher enzymatic activity over the non-acetylated protein (Figure 25). To confirm this result, the K405R mutant SAMHD1 were also subjected to acetylation and dNTPase assay. As a result, the increase of activity found in wild type SAMHD1 was diminished in K405R mutant, as it could not be acetylated (Figure 26). Together, these results demonstrates that ARD1-mediated SAMHD1 acetylation directly enhances its dNTPase activity *in vitro*.

To confirm the phenomena *in vivo*, FLAG-SAMHD1 wildtype and K405R

mutant proteins were overexpressed and purified from HEK293T cells. When these proteins were subjected to dNTPase activity, SAMHD1 wildtype showed enhanced enzymatic activity over the K405R mutant, similarly to *in vitro* results (Figure 27). To test whether this was affected by ARD1, MYC-ARD1 were co-expressed with FLAG-SAMHD1 wildtype or K405R in HEK293T cells. When these SAMHD1 proteins were purified from these cells and their dNTPase activities were assessed, the amount of released α-[^{32}P]PP were increased in FLAG-SAMHD1 wildtype and MYC-ARD1 co-expressing cells compared to MYC-mock expressing cells (Figure 28). However, SAMHD1 K405R mutant proteins showed no difference whether co-expressed with ARD1 or not. Overall, these data suggest that the ARD1-mediated SAMHD1 acetylation enhances its dNTPase activity *in vitro* and *in vivo*.

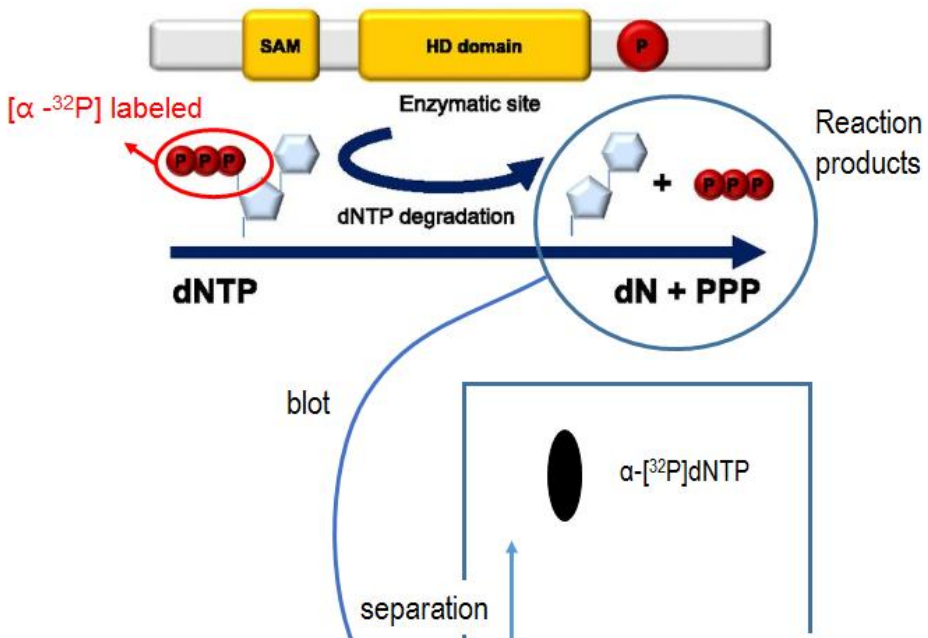


Figure 24. Schematic representation of *in vitro* dNTPase assay.

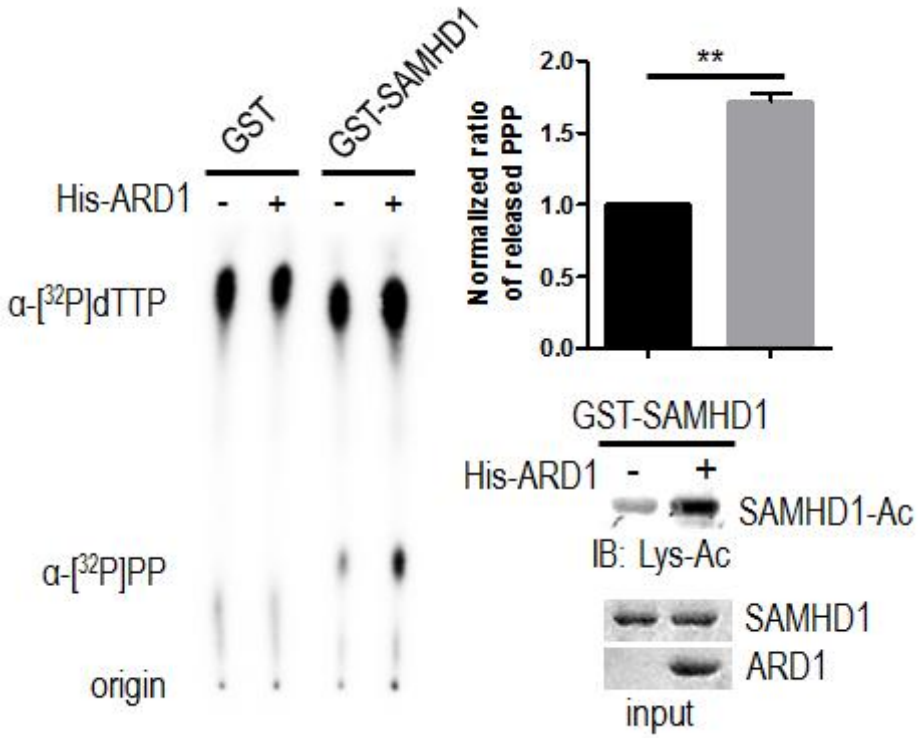


Figure 25. SAMHD1 acetylation upregulates its dNTPase activity *in vitro*.

Recombinant GST proteins and GST-SAMHD1 proteins were acetylated *in vitro* with ±His-ARD1 and blotted with anti-Lys-Ac antibody (lower right). Products were subjected to *in vitro* dNTPase assay and imaged using a phosphorimager (Left). The released amount of α-[32P]PP were measured and presented as mean ±S.D. (n=3) (upper right). **P<0.01; *t*-test

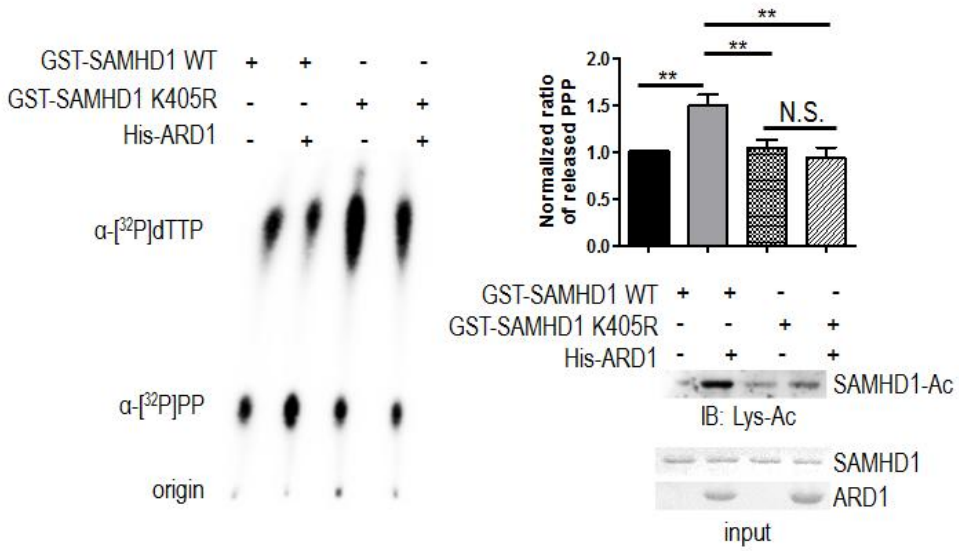


Figure 26. SAMHD1 K405R mutant does not show upregulated dNTPase activity in the presence of ARD1 *in vitro*. Recombinant GST-SAMHD1 WT and K405R proteins were acetylated *in vitro* with ±His-ARD1 and blotted with anti-Lys-Ac antibody (lower right). Products were subjected

to *in vitro* dNTPase assay and imaged using a phosphorimager (Left). The released amount of α-[32P]PP were measured and presented as mean ±S.D. (n=3) (upper right). **P<0.01; *t*-test

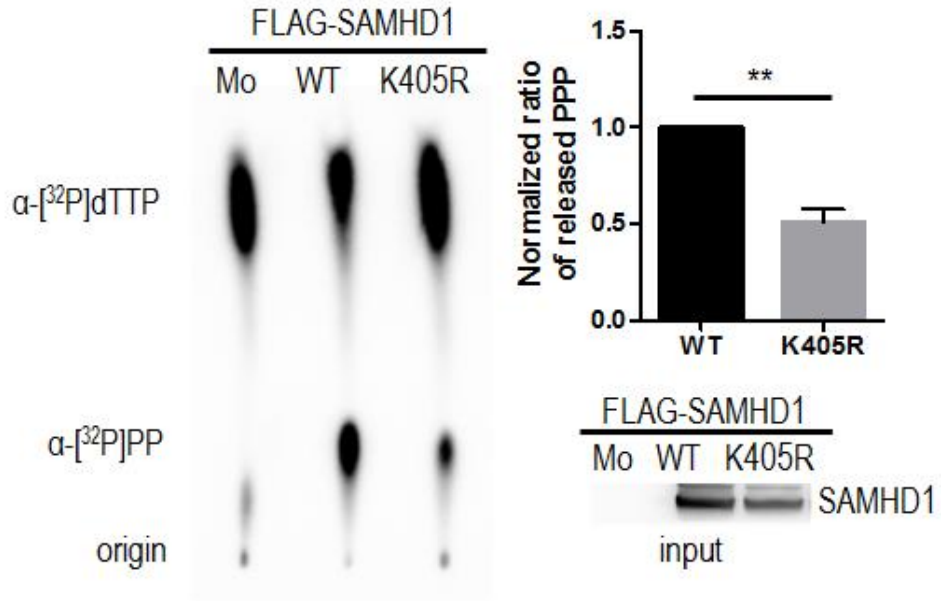


Figure 27. SAMHD1 WT shows stronger dNTPase activity over K405R mutant *in vivo*. FLAG-mock, SAMHD1 wildtype or K405R mutants were

overexpressed and immunoprecipitated from HEK293T (lower right). Precipitated proteins were subjected to *in vitro* dNTPase assay (left) and were quantified (upper right). Shown are mean ±S.D. (n=3). **P<0.01; *t*- test

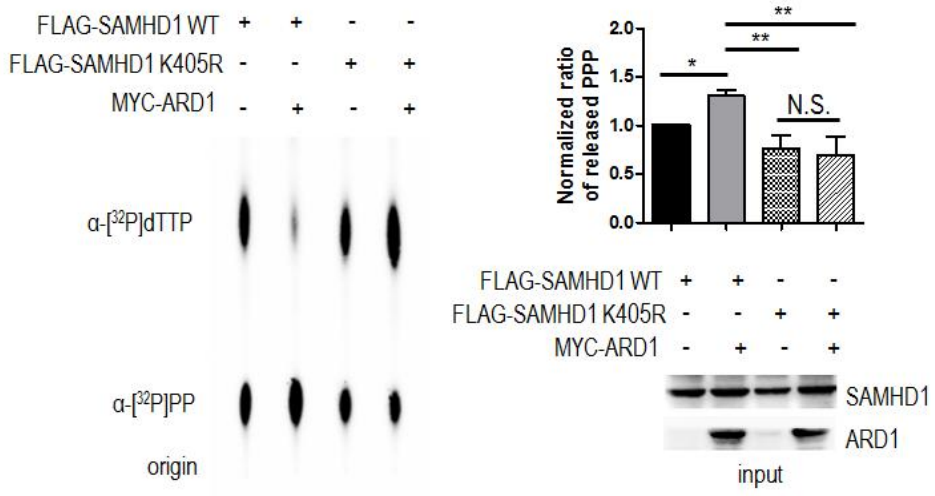


Figure 28. SAMHD1 WT shows stronger dNTPase activity in the presence of ARD1 *in vivo*. FLAG- SAMHD1 WT or K405R mutants were overexpressed with ±MYC-ARD1, then FLAG-tagged proteins were immunoprecipitated from HEK293T (lower right). Precipitated proteins were

subjected to hydrolysis (left) and were quantified (upper right). Shown are mean ±S.D. (n=3). *P<0.05; **P<0.01; *t*- test, N.S.; not significant

4. SAMHD1 acetylation is important for cell proliferation in various cancer cells

In proliferating cell populations, the supply of dNTPs needs to be strictly balanced for proper DNA replication and repair. Indeed, SAMHD1 is reported to control intracellular pool of dNTP via its dNTPase activity and promotes cell cycle progression in monocytes and fibroblasts. Since ARD1 is oncogenic and ARD1-mediated SAMHD1 acetylation enhances its enzymatic activity, I hypothesized that SAMHD1 acetylation may also regulate cell proliferation in cancer. However, little has been investigated about the relationship between SAMHD1 expression and tumorigenesis; thus I first aimed to establish the connection between the two. To investigate the SAMHD1 expression level changes between tumor and normal tissues, the hepatocarcinoma tissues (tumor tissues) and surrounding normal tissues

(non-tumor tissues) were collected from 7 patients; their clinicopathological characteristics are listed in table 1. Then SAMHD1 expression levels were analyzed by western blot, which revealed an increase of SAMHD1 levels in the tumor tissues than non-tumor tissues; statistical analysis confirmed the significance of this increase (Figure 29). This result suggests that SAMHD1 may be related to hepatocellular tumorigenesis. Moreover, the level of ARD1 was also elevated in tumor tissues as previously reported (Shim et al., 2012), implying the possibility that ARD1-mediated SAMHD1 acetylation level may have been upregulated in hepatocarcinoma, and that combined action of the two enzymes may be positively linked to cell proliferation in tumor cells

Next, to investigate whether SAMHD1 shows tumorigenic activity in cancer cell lines *in vitro*, SAMHD1 was silenced in various cancer cell lines by transfecting SAMHD1-specific siRNA (Figure 30). When siCtrl and siSAMHD1 transfected HeLa cells were subjected to MTS assay to assess their growth rate, SAMHD1-downregulated HeLa showed relatively slow growth compared to the control cells (Figure 31). In accordance, control and SAMHD1-silenced MCF7 cells were subjected to anchorage-dependent colony formation assays, which resulted in decreased number of colonies formed in SAMHD1-silenced cells (Figure 32). These results indicate that

SAMHD1 expression promotes cancer cell growth.

I next investigated whether SAMHD1 acetylation can affect this phenomena. First, stable cell lines expressing SAMHD1 wildtype and K405R mutant were constructed in HeLa, MCF7, A549 and Hep3B cells, which are derived from cervical cancer, breast adenocarcinoma, lung cancer and hepatocarcinoma, respectively (Figure 33). When the growth rate of stable HeLa cells were analyzed by MTS assay, overexpression of SAMHD1 wildtype resulted in increased growth rate over the mock cells, whereas K405R mutant could not (Figure 34). Moreover, wildtype expressing MCF7 cells exhibited increased number of colonies over mock or K405R expressing cells when subjected to anchorage dependent colony formation assay (Figure 35). This result was also confirmed in A549 and Hep3B cells, as they too showed increased colony forming abilities only in the wildtype expressing cells (Figure 36, Figure 37).

Anchorage-independent colony formation assay, or the soft-agar assay is a common method to measure the ability of transformed cells to grow independently of a solid surface; a hallmark of carcinogenesis. (Borowicz et al., 2014) To investigate the role of SAMHD1 acetylation in malignant transformation, stable HeLa cells were subjected to soft agar assay.

SAMHD1 wildtype expressing cells showed increased growth over mock or K405R expressing cells, indicating that SAMHD1 acetylation may contribute to the malignancy of the cancer cells (Figure 38). Taken altogether, these data demonstrate that SAMHD1 acetylation increases its ability to promote cancer cell proliferation.

Table 1. Clinicopathological characteristics of 7 hepatocarcinoma patients.

Patient No.	Age	Gender	Etiology	Tumor size, max (cm)	Procedure	Edmonson grade	Cirrhosis
1	42	M	HBV	1.8	Surgical resection	3	No
2	74	M	NBNC	10.5	Surgical resection	4	No
3	48	M	HBV	3.5	Surgical resection	2	Yes
4	43	M	HBV	3.8	Surgical resection	4	No
5	45	M	HCV	2.5	Surgical resection	3	No

6	**70**	**F**	**HBV**	**5.3**	**Surgical resection**	**3**	**Yes**
7	**56**	**M**	**HBV**	**2.2**	**Surgical resection**	**3**	**No**

Abbreviations: M, male; F, female; HBV, hepatitis B virus; NBNC, non-B non-C; HCV, hepatitis C virus

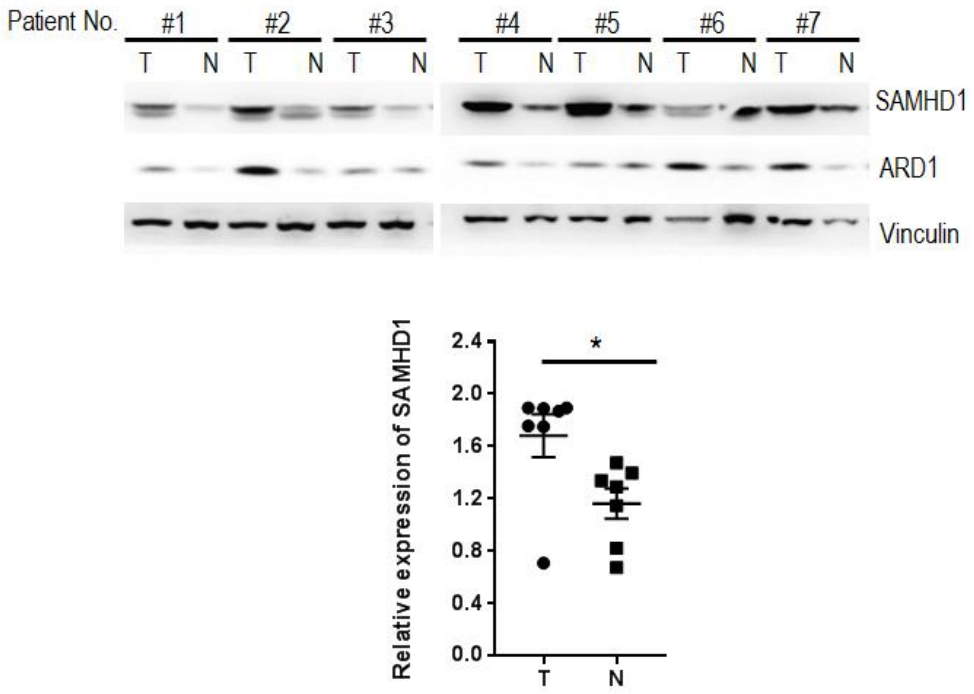


Figure 29. Expression of SAMHD1 in hepatocarcinoma tissues and surrounding normal tissues. Tumor and non-tumor tissues from 7 hepatocarcinoma patients were lysed and extracted. Protein expression levels were analyzed by western blot and presented as mean ±S.E.M. (n=7). T, Tumor tissues; N, Non-tumor surrounding tissues;*P<0.05; *t*- test

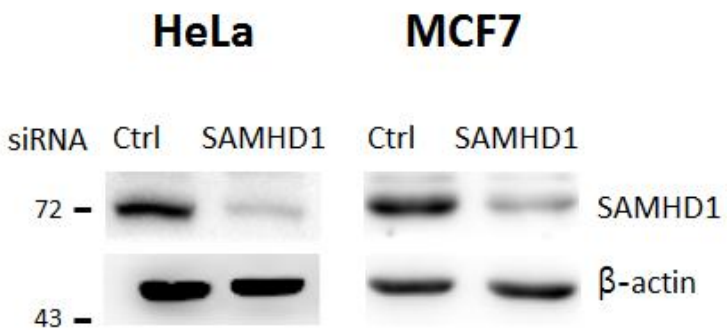


Figure 30. Establishment of SAMHD1-downregulated cancer cells. HeLa

and MCF7 cells were transfected with siCtrl or siSAMHD1 and were lysed after 48~72h. The level of proteins were assessed by western blot.

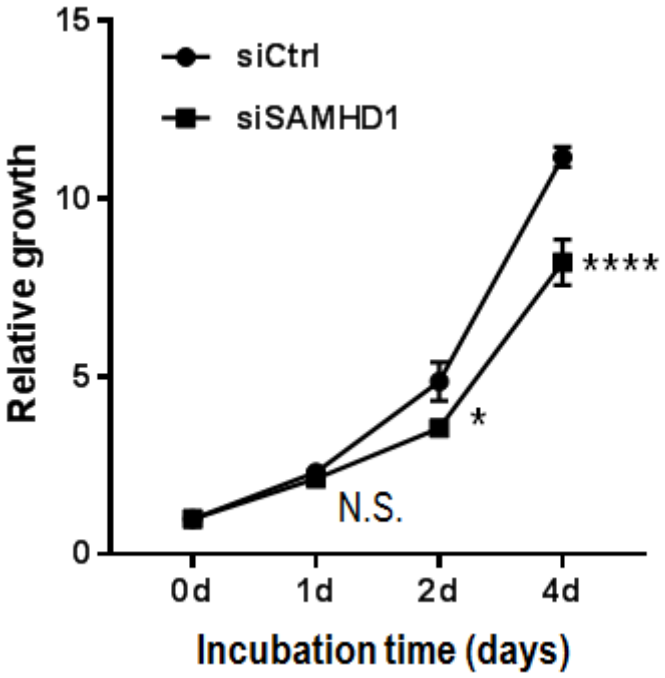


Figure 31. Relative cell growth of SAMHD1-downregulated HeLa cells. Cell growth of siCtrl or siSAMHD1 treated HeLa cells were monitored over time by MTS assay. Shown are mean ±S.E.M. (n=5). *P<0.05; ****P<0.0001; t test; N.S.; not significant

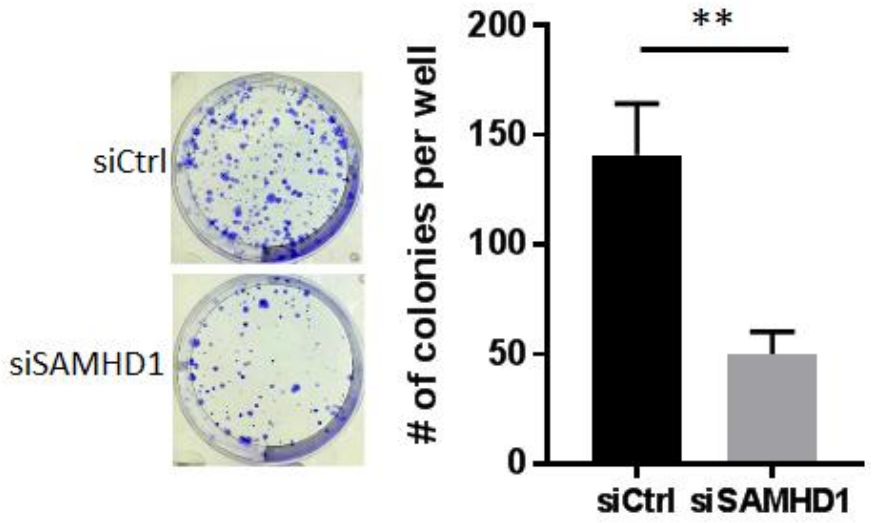


Figure 32. SAMHD1-downregulated MCF7 cells show decreased anchorage-dependent colony formation. siCtrl or siSAMHD1 treated MCF7 cells were subjected to anchorage-dependent colony formation assays. Number of colonies were presented as mean ±S.D. (n=3) with representative well pictures. **P<0.01; t test

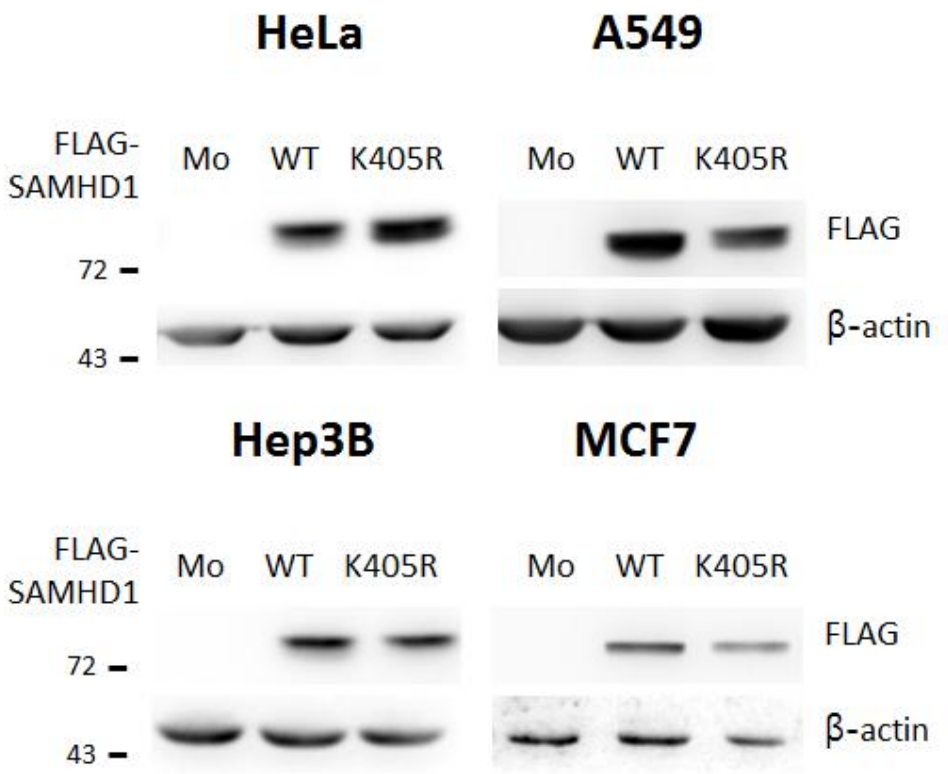


Figure 33. Establishment of FLAG-SAMHD1 WT or K405R overexpressing stable cancer cell lines. SAMHD1 wildtype or K405R expressing stable cell lines were constructed in HeLa, A549, Hep3B, and MCF7 cells and their protein levels were assessed by western blot.

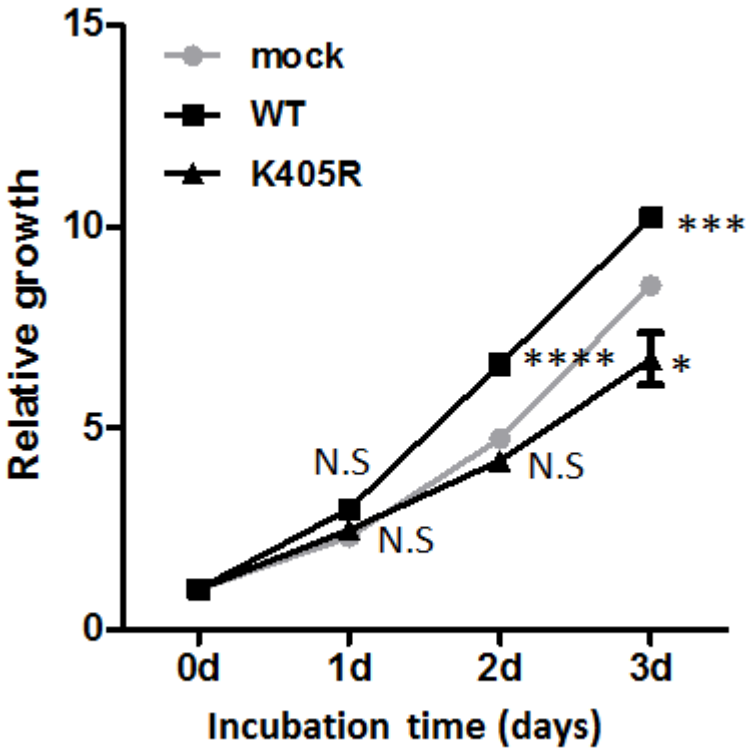


Figure 34. Relative cell growth of FLAG-SAMHD1 WT or K405R overexpressing HeLa cells. Cell growth of stable HeLa cells expressing SAMHD1 wildtype, K405R or empty vector were monitored over time by MTS assay. Shown are mean ±S.E.M. (n=5). *P<0.05; ***P<0.001; ****P<0.0001; *t*- test; N.S.; not significant

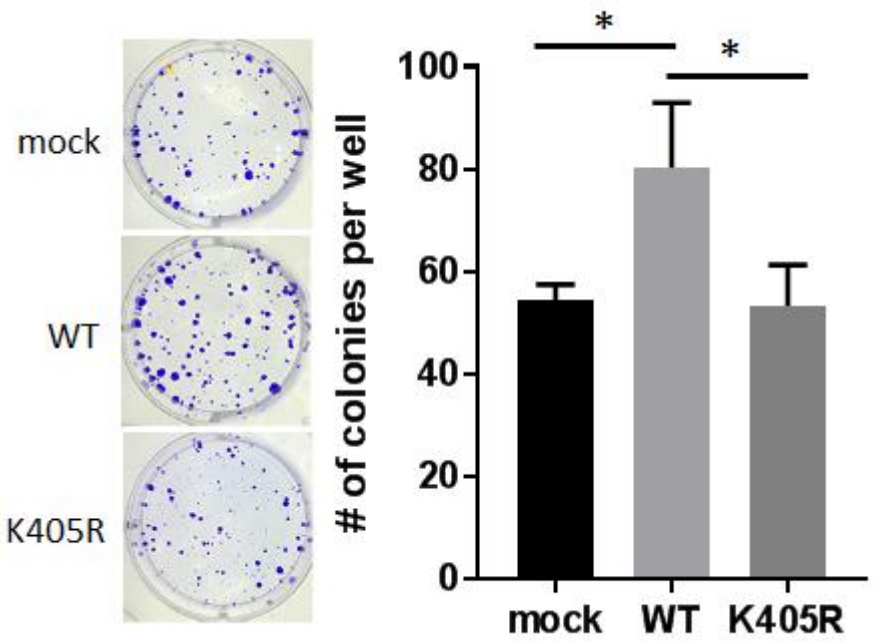


Figure 35. Anchorage-dependent colony formation of FLAG-SAMHD1 expressing stable MCF7 cells. Stable MCF7 cells expressing SAMHD1 wildtype, K405R or empty vector were subjected to anchorage-dependent colony formation assays. Number of colonies were presented as mean ±S.D. (n=3) with representative well pictures. *P<0.05; *t*- test

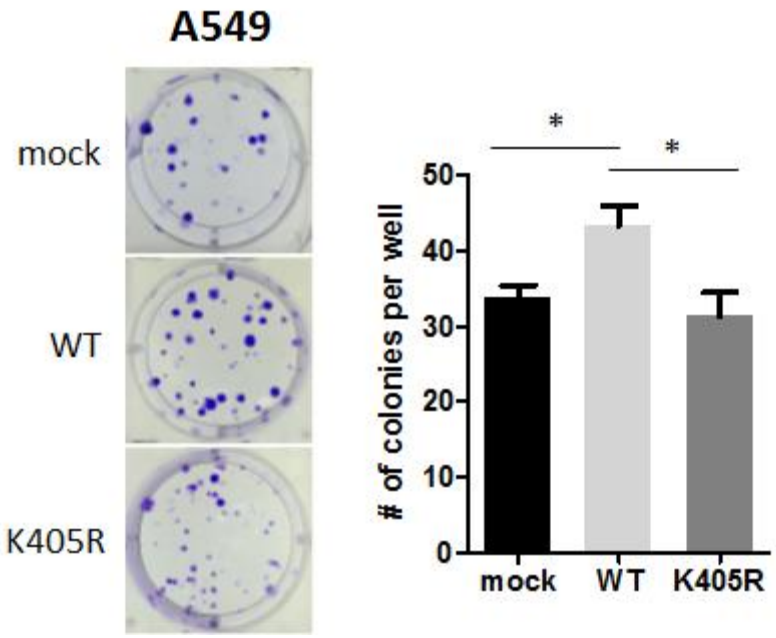


Figure 36. Anchorage-dependent colony formation of FLAG-SAMHD1 expressing stable A549 cells. Stable A549 cells expressing SAMHD1 wildtype, K405R or empty vector were subjected to anchorage-dependent colony formation assays. Number of colonies were presented as mean ±S.D. (n=3) with representative well pictures. *P<0.05; *t*- test

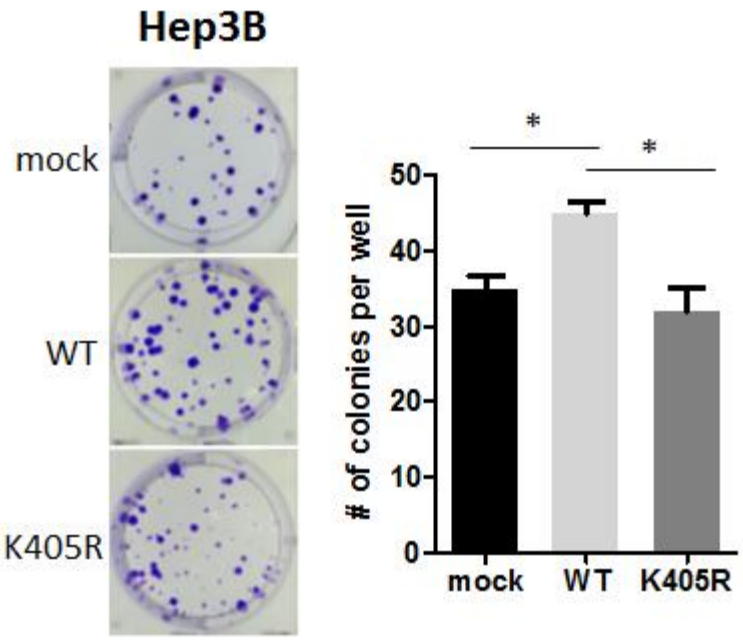


Figure 37. Anchorage-dependent colony formation of FLAG-SAMHD1 expressing stable Hep3B cells. Stable Hep3B cells expressing SAMHD1 wildtype, K405R or empty vector were subjected to anchorage-dependent colony formation assays. Number of colonies were presented as mean ±S.D. (n=3) with representative well pictures. *P<0.05; *t*- test

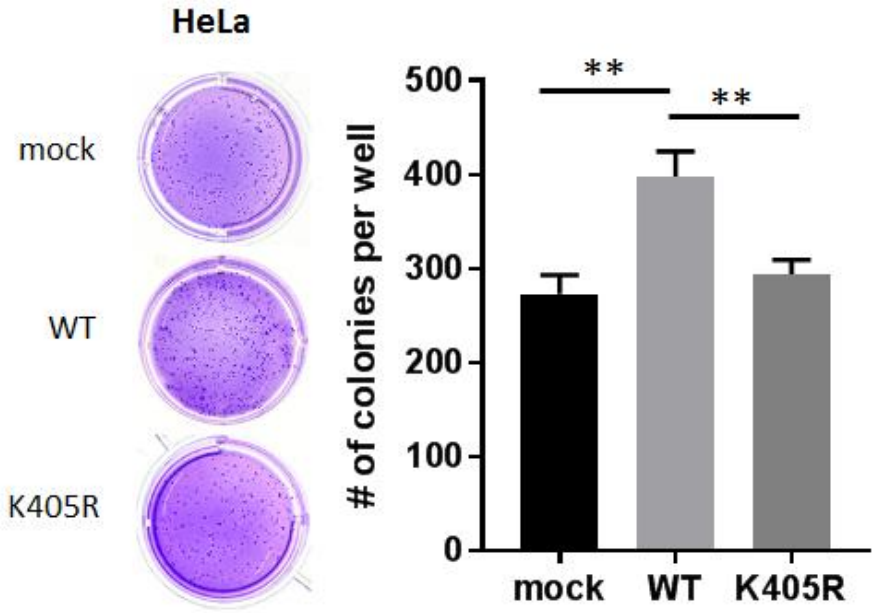


Figure 38. Anchorage-independent colony formation of FLAG-SAMHD1 expressing stable HeLa cells. Stable HeLa cells expressing SAMHD1 wildtype, K405R or empty vector were subjected to anchorage-independent colony formation assays. Number of colonies were presented as mean ±S.D. (n=3) with representative well pictures. **P<0.01; *t*- test

5. SAMHD1 acetylation promotes G1/S transition in cancer cells

SAMHD1 has been previously reported to control cell cycle status in mammalian cells by regulating DNA precursor pools (Franzolin et al., 2013, Bonifati et al., 2016). To determine whether cell cycle progression is altered by SAMHD1 in cancer cells, SAMHD1-silenced HeLa cells were synchronized by 48h of serum-starvation and re-stimulated for 24 h, then subjected to flow cytometry for cell cycle analysis. Depletion of SAMHD1 caused G1 arrest, as G1/G0 population increased whereas S and G2/M populations relatively decreased (Figure 39). Western blot analysis also revealed that the expression levels of cyclin D1 and B1, markers for S and G2/M phase respectively, were decreased in siSAMHD1 treated cells (Figure 40). Therefore, these data indicate that SAMHD1 contributes to G1/S transition in HeLa cells.

Next, to identify the role of SAMHD1 acetylation in G1/S transition, stable A549 cells expressing FLAG-mock, SAMHD1 wildtype or K405R were also serum-starved and re-stimulated for cell cycle analysis. Unlike the K405R mutant, SAMHD1 wildtype expressing cells exhibited increased G1-to-S phase progression (Figure 41). This result was also confirmed by the expression levels of cyclin D1 and B1 (Figure 42). The immunofluorescence

images of proliferating cell nuclear antigen (PCNA), which is mainly expressed during the S phase, also reflected the increased S phase in SAMHD1 wildtype expressing Hep3B cells, but not in K405R cells (Figure 43). Collectively, these data demonstrates that SAMHD1 acetylation contributes to G1/S transition in various cancer cell lines.

To validate the role of SAMHD1 acetylation in cell cycle progression in detail, I next checked the acetylation level during each phases. Using either thymidine-double block method or serum-starvation method, cancer cells stably expressing FLAG-SAMHD1 wildtype were synchronized to the specific cell phases. Then FLAG-tagged proteins were immunoprecipited and blotted with Lys-Ac antibody to analyze their levels of Lys-Acetylation. In A549 cells, the intensity of acetylated SAMHD1 was the strongest in the G1/G0 phase and weakest in S phase, indicating that SAMHD1 was mainly acetylated during G1 phase (Figure 44). This phenomena was also observed in HeLa cells (Figure 45). These data suggest the possibility that the dNTPase activity of SAMHD1 may be strongest during the G1/G0 phase.

Previous report has demonstrated SAMHD1 expression level fluctuate throughout the cell cycle, which contributed to dNTP level changes and thus facilitated proper cell cycle progression in fibroblasts (Franzolin et al., 2013).

To investigate whether this phenomena is also seen in cancer cells, various cancer cells were synchronized to specific cell cycle phases and their endogenous SAMHD1 expression levels were assessed by western blot analysis. However, their levels displayed no significant difference in Hela, Hep3B, A549 and MCF7 cells (Figure 46, Figure 47, Figure 48 and Figure 49). Collectively, these results indicate that the difference in cell cycle progression between SAMHD1 wildtype and mutant expressing cells is caused by its acetylation, not by its expression level change. Taken altogether, SAMHD1 acetylation mainly occurs during G1 phase and promotes tumor growth via facilitating G1/S cell cycle progression.

CDK1 and cyclin A2 binds and phosphorylates SAMHD1 and to block its anti-retroviral activity. To test whether SAMHD1 acetylation influences SAMHD1 phosphorylation, I investigated whetherSAMHD1 acetylation can alter the binding of SAMHD1 and cyclin A2. FLAG-SAMHD1 wildtype and K405R expressing HEK293T cells were lysed and the lysates were immunoprecipitaed with anti-FLAG resin overnight to selectively co-immunopreicipitate SAMHD1 binding proteins. When precipitants were blotted with anti-cyclin A2 antibody, the SAMHD1 wildtype and K405R showed no difference (Figure 50). This result suggests that SAMHD1

acetylation does not alter the binding of SAMHD1 and cyclin A2. However, additional research is needed to establish the relationship between SAMHD1 acetylation and phosphorylation, as variety of molecular mechanisms may reside in between the two.

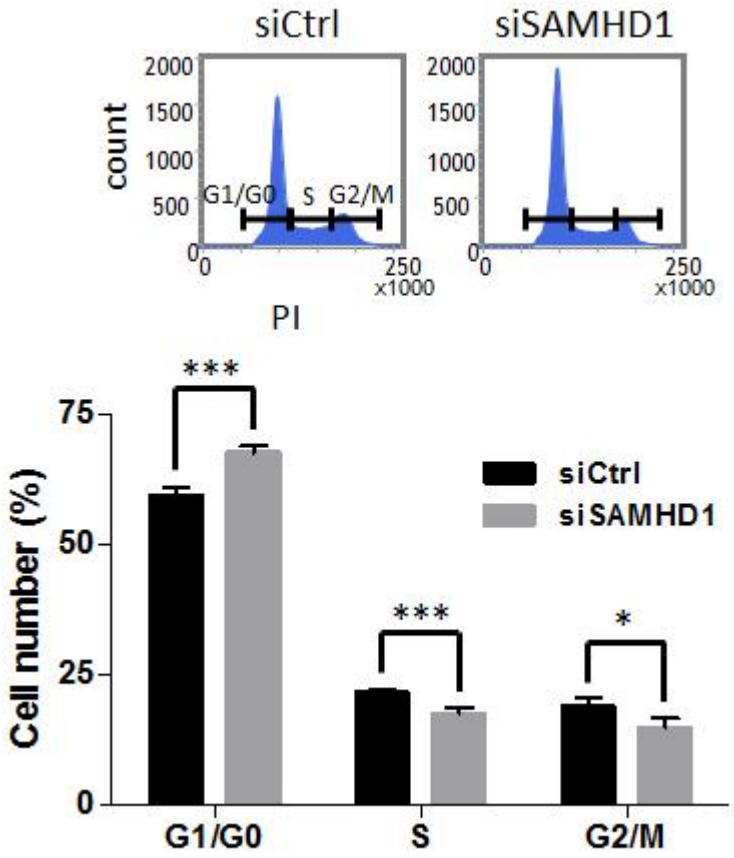


Figure 39. Cell cycle profiles of SAMHD1-downregulated HeLa cells. Cell cycle profiles of siCtrl or siSAMHD1 treated HeLa cells were determined by flow cytometry. Representative cell cycle profiles (top); the percentage of cells in each phase is presented as mean ±S.D. (n=3) (bottom) *P<0.05; ***P<0.001; *t*- test

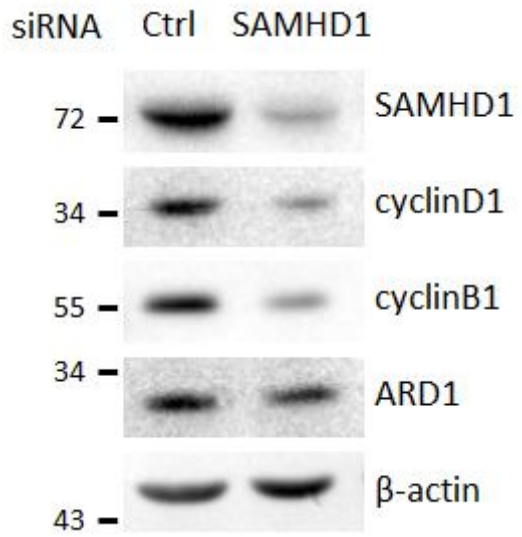


Figure 40. Expression levels of cyclins in SAMHD1-down regulated HeLa cells. Lysates were extracted from siCtrl or siSAMHD1 treated HeLa cells and the levels of cyclin D1 and cyclin B1 were immunoblotted by corresponding antibodies.

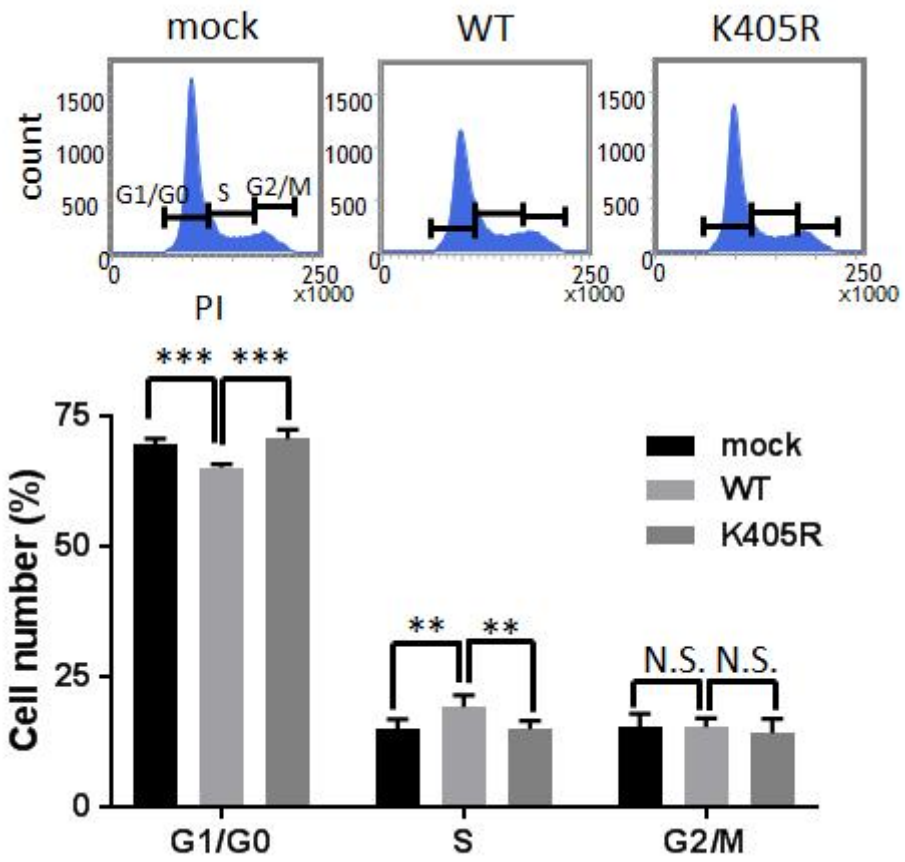


Figure 41. Cell cycle profiles of FLAG-SAMHD1 expressing stable A549 cells. Cell cycle profiles of stable A549 cells expressing SAMHD1 wildtype, K405R or empty vector were determined by flow cytometry. Representative cell cycle profiles (top); the percentage of cells in each phase is presented as mean ±S.D. (n=3) (bottom) *P<0.05; **P<0.01;***P<0.001; *t*- test; N.S.; not significant

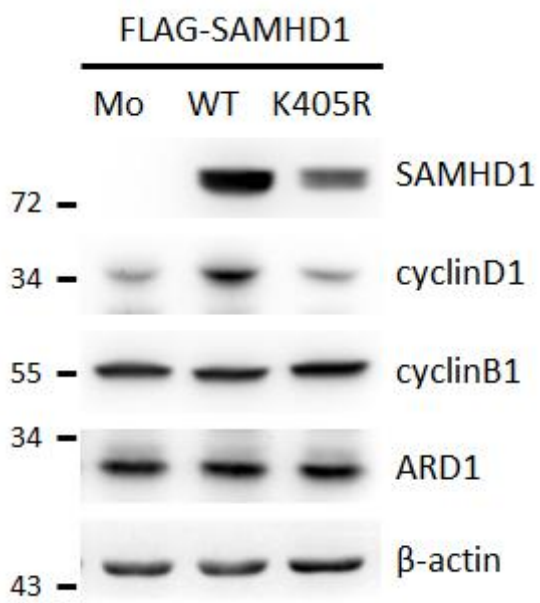


Figure 42. Expression levels of cyclins in FLAG-SAMHD1 overexpressing stable A549 cells. The levels of cyclin D1 and cyclin B1 were immunoblotted in the lysates extracted from stable A549 cells expressing SAMHD1 wildtype, K405R or empty vector.

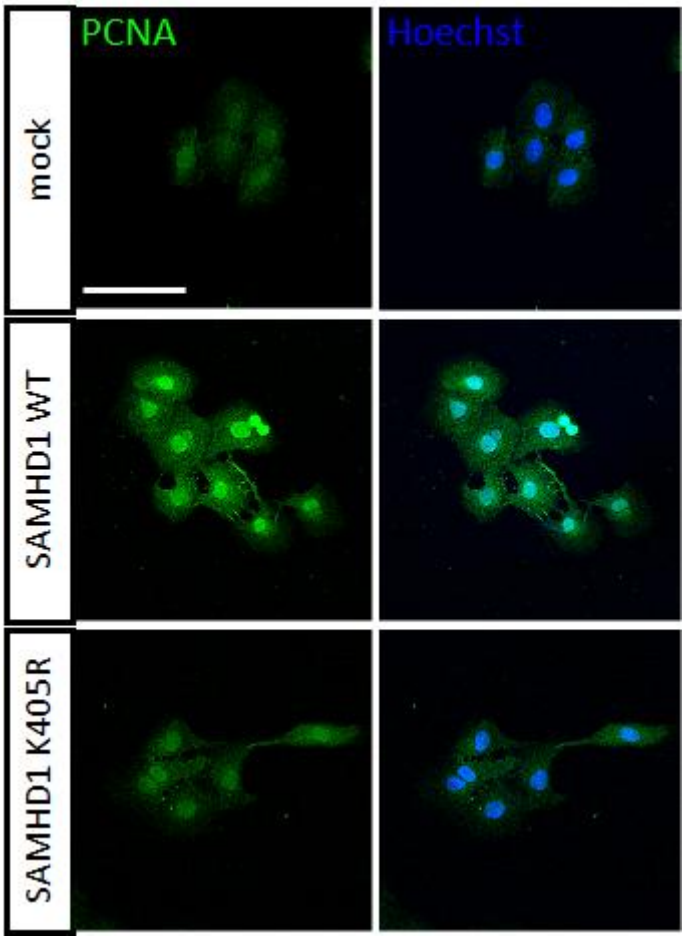

PCNA
Hoechst
mock
SAMHD1 WT
SAMHD1 K405R

Figure 43. PCNA expression of FLAG-SAMHD1 overexpressing stable Hep3B cells. Stable Hep3B cells expressing SAMHD1 wildtype, K405R or empty vector were serum-deprived for 48 h and were re-stimulated for 24 h, then the expressions of PCNA were monitored by confocal microscopy with immunofluorescence using the PCNA antibody. Hoechst was used for nuclei staining. PCNA, Proliferating cell nuclear antigen; Scale bar; 100 μm

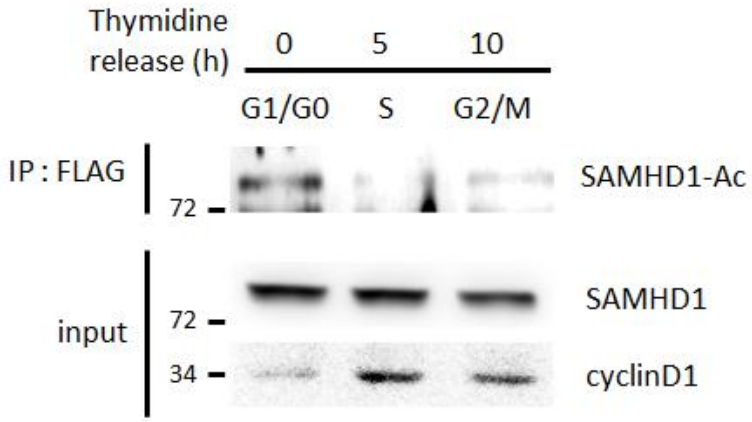


Figure 44. SAMHD1 acetylation levels during cell cycle progression of FLAG-SAMHD1 overexpressing stable A549 cells. FLAG-SAMHD1 wildtype expressing stable A549 cells were synchronized to specific cell cycle phases using thymidine-double block method. SAMHD1 was precipitated and blotted with an anti-Lys-Ac antibody.

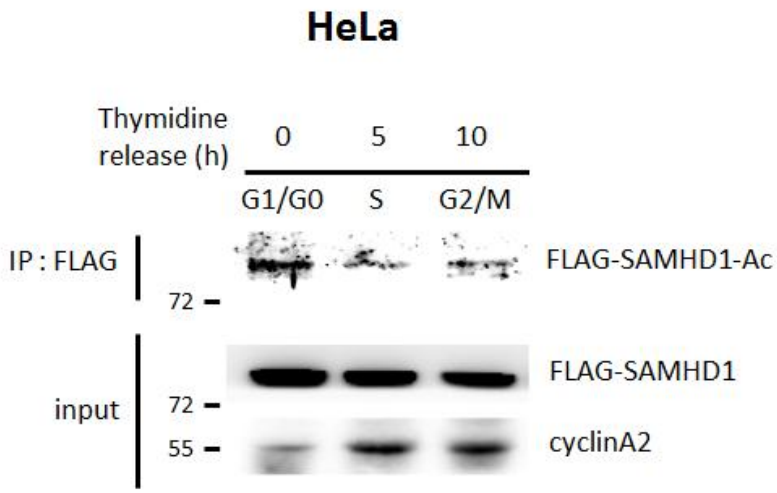


Figure 45. SAMHD1 acetylation levels during cell cycle progression of FLAG-SAMHD1 overexpressing stable HeLa cells. FLAG-SAMHD1 wildtype expressing stable HeLa cells were synchronized to specific cell cycle phases using thymidine-double block method. SAMHD1 was precipitated and blotted with an anti-Lys-Ac antibody.

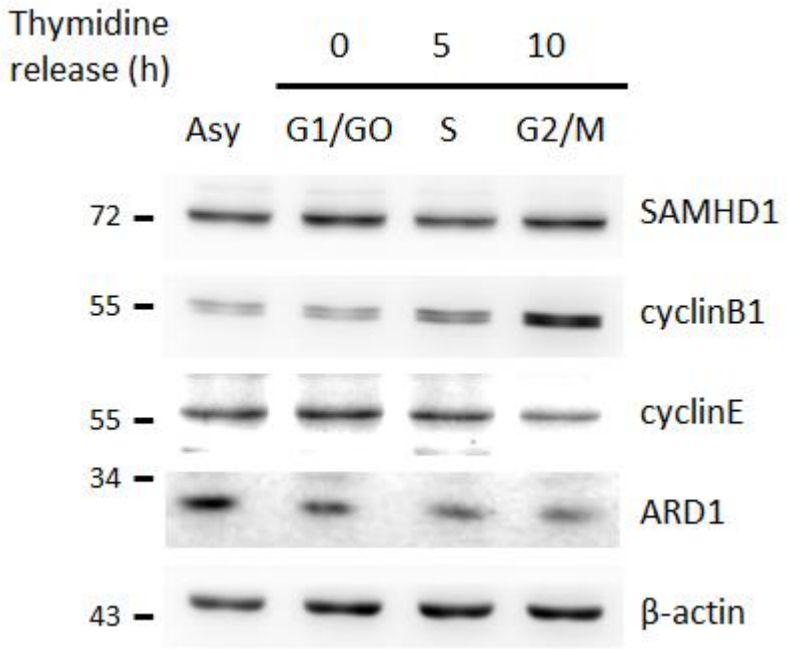


Figure 46. Expression levels of endogenous SAMHD1 during cell cycle progression of HeLa cells. HeLa cells were synchronized to specific cell cycle phases by thymidine double block method. The expression levels of SAMHD1 was assessed by western blotting.

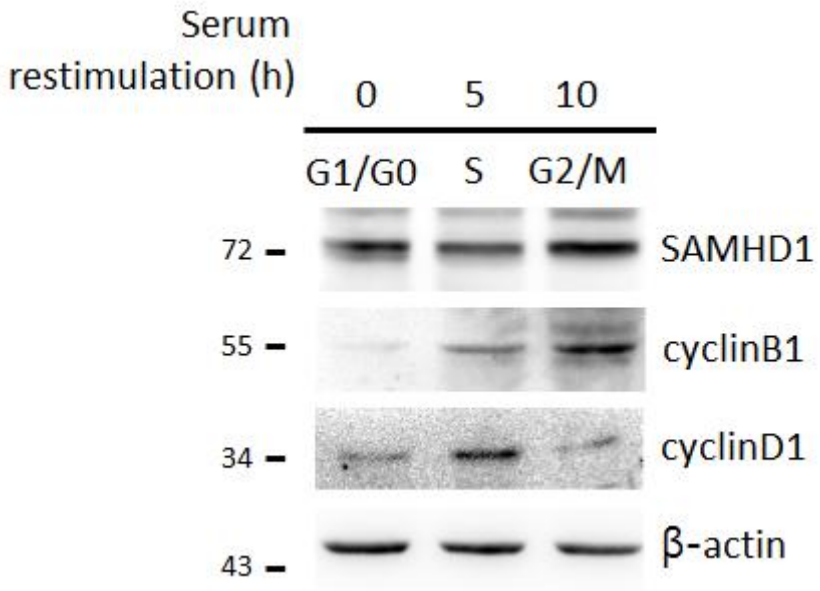


Figure 47. Expression levels of endogenous SAMHD1 during cell cycle progression of Hep3B cells. Hep3B cells were synchronized to specific cell cycle phases by thymidine double block method. The expression levels of SAMHD1 was assessed by western blotting.

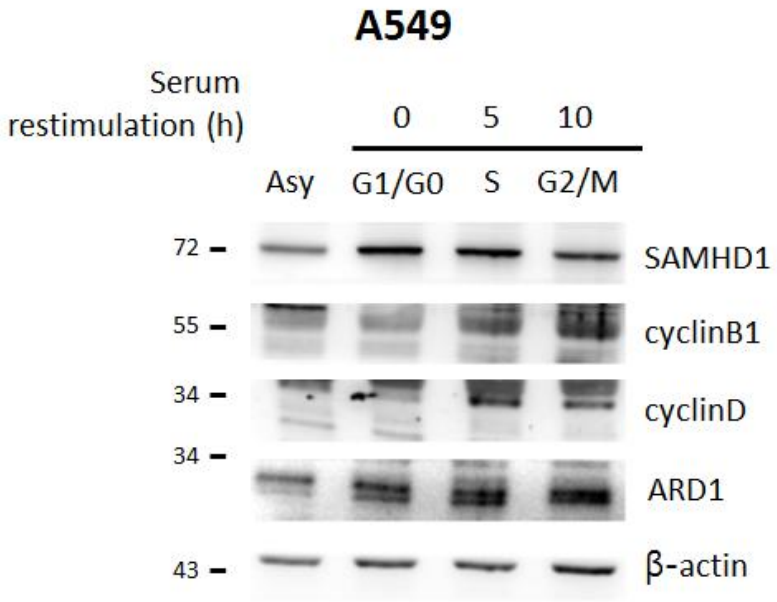


Figure 48. Expression levels of endogenous SAMHD1 during cell cycle progression of A549 cells. A549 cells were synchronized to specific cell cycle phases by thymidine double block method. The expression levels of SAMHD1 was assessed by western blotting.

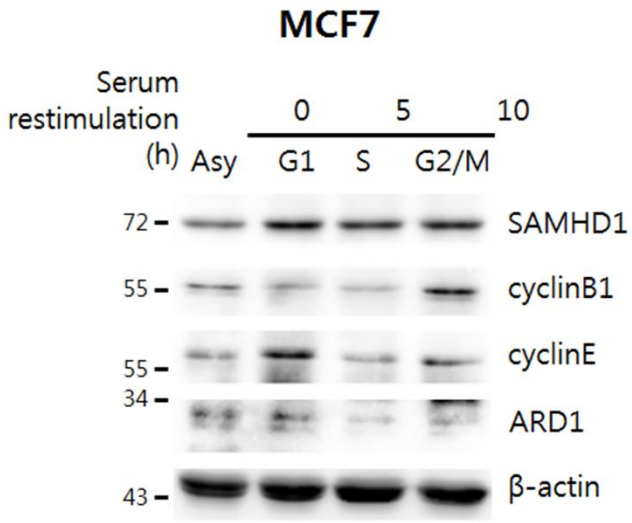


Figure 49. Expression levels of endogenous SAMHD1 during cell cycle progression of MCF7 cells. MCF7 cells were synchronized to specific cell cycle phases by thymidine double block method. The expression levels of SAMHD1 was assessed by western blotting.

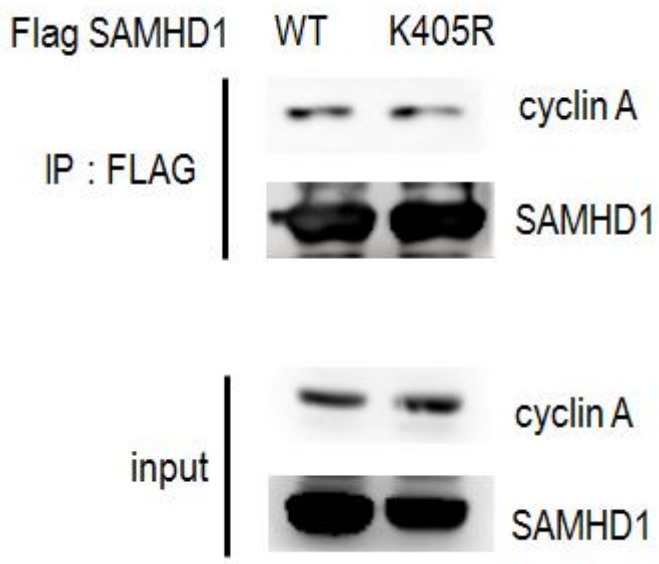


Figure 50. SAMHD1 acetylation does not alter SAMHD1-cyclin A2 binding. FLAG-SAMHD1 wildtype or K405R were over expressed in HEK293T cells and immunoprecipitated lysates were blotted with anti-cyclin A2 antibody.

6. SAMHD1 acetylation may play other undiscovered roles in various cell lines.

In an attempt to discover new roles of SAMHD1 acetylation, I have performed several additional experiments.

(1) Physiological conditions of SAMHD1 acetylation in THP-1 cells

The antiviral activity of SAMHD1 is observed only in non-cycling immune cells such as dendritic cells, macrophages, monocytes, and quiescent CD4+ T cells. Since this activity is mediated by dNTPase activity of SAMHD1, I hypothesized that different acetylation status in cycling/non-cycling cells may have resulted in such phenomena. THP-1 cells were cultured in normal RPMI media or treated with PMA (Phorbol-12-Myristate-13-Acetate) for differentiation and stop cycling. To assess the acetylation levels of SAMHD1 proteins from these cells, their cellular proteins were lysed, immunoprecipitated with Lys-Ac antibody and blotted with SAMHD1 anitbody. PMA-treated THP-1 cells showed higher levels of acetylation compared to the control group (Figure 51A), which demonstrates that

differentiated THP-1 cells may exhibit stronger dNTPase activity. This result is opposite to the phosphorylation status of SAMHD1, as phosphorylation levels were reduced when PMA was treated (Cribier et al., 2013).

Type I interferon is known to be a negative regulator of the antiviral activity of SAMHD1, reducing SAMHD1 T592 phosphorylation in various immune cells (Cribier et al., 2013). To investigate whether type I interferons also affect SAMHD1 acetylation, I have treated IFNβ-2a (Interferon β-2a) to THP-1 cells and checked SAMHD1 acetylation levels. The acetylation levels were reduced in IFNβ-2a treated cells (Figure 51B), opposite to the phosphorylation status reported in previous study (Cribier et al., 2013). Collectively, these results suggest the possibility that SAMHD1 acetylation may facilitate its antiviral activity, and that its functions may be opposite to SAMHD1 phosphorylation.

Figure 51. Physiological conditions of SAMHD1 acetylation in THP-1 cells. (A) Endogenous SAMHD1 proteins were immunoprecipitated from PMA-treated THP-1 cells and blotted with Lys-Ac antibody. (B) 24h after IFNβ-2a treatment, endogenous SAMHD1 proteins were immunoprecipitated from THP-1 cells and blotted with Lys-Ac antibody.

(2) SAMHD1 and autophagy

Previous study has reported that cellular dNTP levels directly affects the induction of autophagy (Chen et al., 2014). Since SAMHD1 is a dNTPase and depletes cellular dNTPs, I hypothesized that SAMHD1 may play a role in autophagy induction. To investigate the function of SAMHD1, I downregulated SAMHD1 levels in HEK293T cells by transfection of specific siRNA. When SAMHD1 was silenced, the level of LC3B-II, a marker for autophagy induction, was increased (Figure 52A). This result demonstrates that SAMHD1 may inhibit the induction of autophagy. Next, to study the role of SAMHD1 when autophagy is induced, the siCtrl or siSAMHD1 treated HEK293T cells were treated with 4h of EBSS (Earle's Balanced Salt Solution) to induce autophagy. When compared to cells grown in normal DMEM, SAMHD1 expression levels were increased, indicating that autophagy may upregulate the induction of SAMHD1 protein (Figure 52A). This result was also confirmed in HeLa cells (Figure 52B). The siSAMHD1-treated cells showed reduced LC3B-II levels compared to the siCtrl cells under EBSS treated condition, which suggests the possibility that SAMHD1 may enhance the autophagy status of the cell when autophagy is

induced; however this result conflicts with the previous results under normal condition. Considering the sophisticated effect of cellular dNTPs to the autophagy induction – low dNTP levels induce autophagy and continuous autophagy results in low dNTP levels, a reciprocal regulation – it is suggested that the relationship between SAMHD1 and autophagy may also be complicated depending on the specific conditions. However these data shows that SAMHD1 does affect autophagy, therefore further studies are needed.

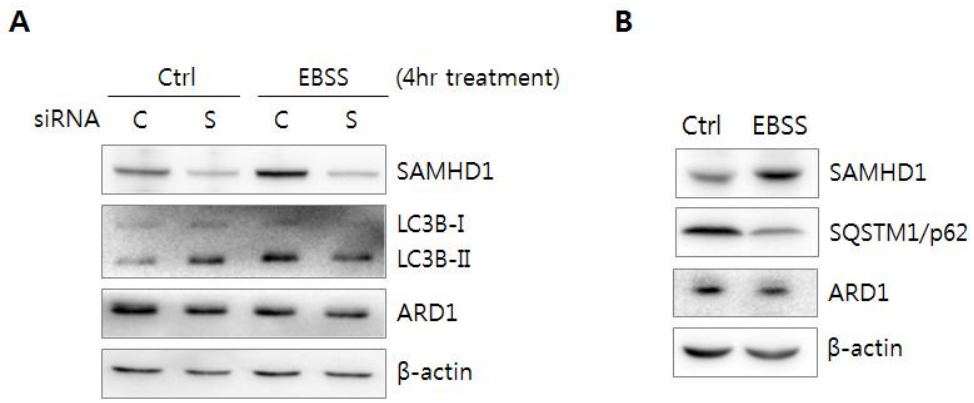


Figure 52. Relationship between SAMHD1 and autophagy. (A) HEK293T cells were transfected with siCtrl or siSAMHD1 then subjected to normal DMEM or EBSS for 4 hrs. Cellular proteins were lysed, separated by SDS-PAGE and blotted with respective antibodies. (B) HeLa cells were cultured in either normal DMEM or EBSS for 24 hrs, and their cellular proteins were lysed and analyzed by western blot. C, Ctrl; S, SAMHD1

(3) SAMHD1 acetylation and tight junction proteins

Tight junctions are the closely associated areas of two cells whose membranes join together forming a virtually impermeable barrier to fluid. Tight junction proteins such as ZO-1, occludin and E-cadherin are membrane proteins which cross-links to each other and forms the tight junctions. Expression of such proteins in cancer cells are especially important for their loss may result in invasion and ultimately to the metastasis of cancer cells; therefore these proteins are widely-used as a metastasis marker. To investigate whether SAMHD1 acetylation can affect the levels of these proteins, Hep3B, HeLa, and A540 cells stably expressing FLAG-mock, SAMHD1, WT or K405R mutant were analyzed by western blot. In SAMHD1 WT expressing cells, expression levels of ZO-1 in HeLa cells and occludin in A549 cells were decreased, which were not observed in mutant expressing cells (Figure 53). Moreover, the levels of N-cadherin, a negative tight junction marker which promotes transendothelial migration of cancer cells, were increased in SAMHD1 WT expressing Hep3B cells and

not in mutant expressing cells (Figure 53). Altogether, these data suggest that SAMHD1 acetylation weakens the tight junction in cancer cells and that it may promote metastic behavior of these cells.

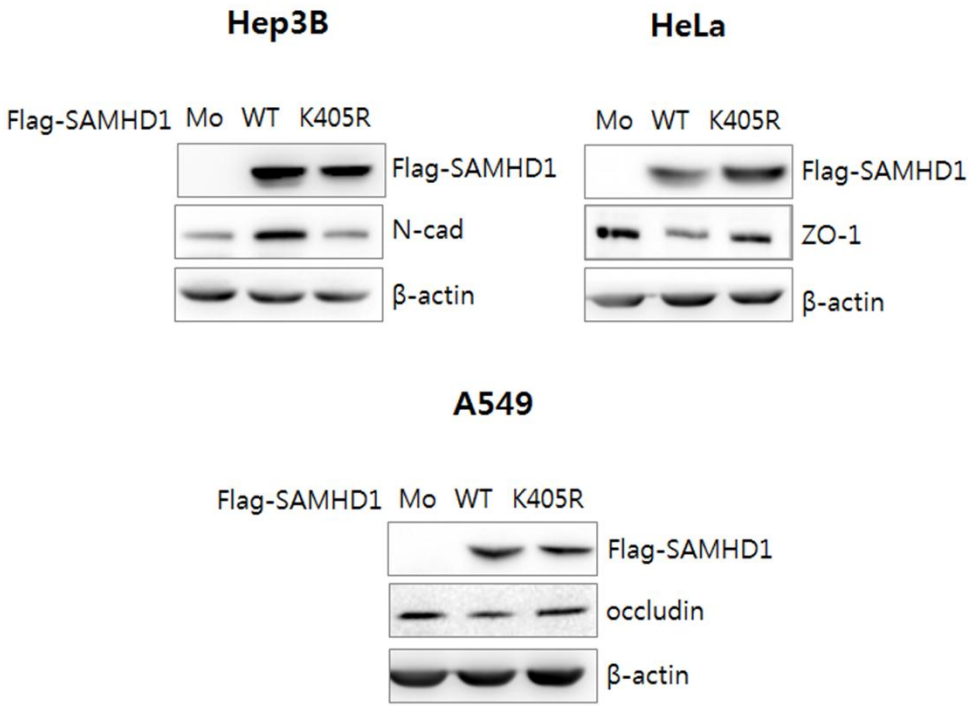


Figure 53. SAMHD1 acetylation alters the expression of junction proteins in various cancer cells. Stable Hep3B, HeLa, A549 cells expressing Flag-mock, SAMHD1 WT or K405R were lysed and their expression levels of junction proteins were analyzed by western-blot analysis.

DISCUSSION

Since SAMHD1 has been first reported to have anti-HIV-1 activity, many studies have mainly concentrated on its mechanism of antiviral action. However, the expression of SAMHD1 is not only limited to immune cells but is observed ubiquitously in human organs, implying the existence of molecular mechanisms to regulate its enzymatic activity and its additional functions in non-immune cells. Here, I demonstrate a novel PTM of SAMHD1, its acetylation, and its roles in cancer cell proliferation (Figure 54). SAMHD1 was acetylated by ARD1 at K405 residue, which directly enhanced its dNTPase activity in vitro. SAMHD1 acetylation level was found to be highest during G1 phase and facilitated G1/S transition, resulting in increased cell growth and colony forming ability in various cancer cells. I assume that the ability of SAMHD1 acetylation to promote G1-to-S phase is caused by its ability to deplete dNTPs, as K405R mutant, the acetylation-blocked variant which shows decreased dNTPase activity, could not reproduce such phenomena. dNTP levels are known to have a direct effect on

control of cell cycle progression. Indeed, constitutively high dNTP concentration delays entry into S phase (Chabes and Stillman, 2007), and SAMHD1 is reported to promote S phase progression by lowering cellular dNTP pool critical for G1 checkpoint (Franzolin et al., 2013, Bonifati et al., 2016). Hence, I suggest that the increased level of SAMHD1 acetylation during G1 phase contributes to low dNTP pool size sufficient for G1/S transition, which in turn promotes cancer cell proliferation (Figure 54). These findings broaden the understating of dNTP metabolism and cell cycle regulation, and provides SAMHD1 and its acetylation as a novel therapeutic target for cancer treatment.

LC/MS-MS analysis revealed 2 acetylation site candidates; K405 and K580 (Figure 19). Among them, K580 was found to be a minor site as its acetylation levels showed little difference in the presence of ARD1 *in vitro* or *in vivo* (Figure 20, Figure 21), and its mutation did not alter its dNTPase activity in vitro (data not shown). Thus K580-acetylation may not play a meaningful role in ARD1-mediated SAMHD1 activation. However, I will not exclude the possibility that K580 residue-acetylation may exhibit other molecular mechanisms related to SAMHD1.

SAMHD1 acetylation may contribute to its protein stability. While

overexpressing SAMHD1 wildtype and K405R mutant proteins in cell lines, I have noticed that SAMHD1 K405R expression levels tend to be lower than that of wildtypes (Figure 27, Figure 42). One hypothesis is that K405 acetylation might alter the oligomerization of the protein and thus altering its protein-stability. Although this phenomenon was not evident in all the cell lines I have tested, I believe that further studies on this matter may find interesting mechanisms of SAMHD1 protein stability and degradation.

SAMHD1 acetylation may not only affect the cell cycle progression but also the anti-viral activity in non-cycling immune cells, for the ability of SAMHD1 to deplete dNTP pools is critical for limiting lentiviral replication in non-cycling immune cells (Goldstone et al., 2011, Lahouassa et al., 2012, White et al., 2013). The sole expression of SAMHD1 itself was insufficient to induce this effect in proliferating immune cells, implicating the existence of PTMs and/or a cofactor expressed only in non-proliferating cells (Baldauf et al., 2012, Descours et al., 2012). Here, I have discovered that SAMHD1 acetylation mediated by ARD1 enhances its dNTPase function directly as acetylated SAMHD1 showed higher enzymatic activity over non-acetylated proteins and K405R mutants (Figure 2). Indeed, previous studies have reported that histone deacetylase inhibitors (HDACi) possess antiretroviral

activity that is SAMHD1-dependent in macrophages; which suggests that protein acetylation related to SAMHD1 plays role in viral defense (Mlcochova et al., 2017). Therefore, further investigations on acetylation status in cycling/non-cycling myeloid cells are needed for deep understanding of the role of SAMHD1 acetylation in immune defense.

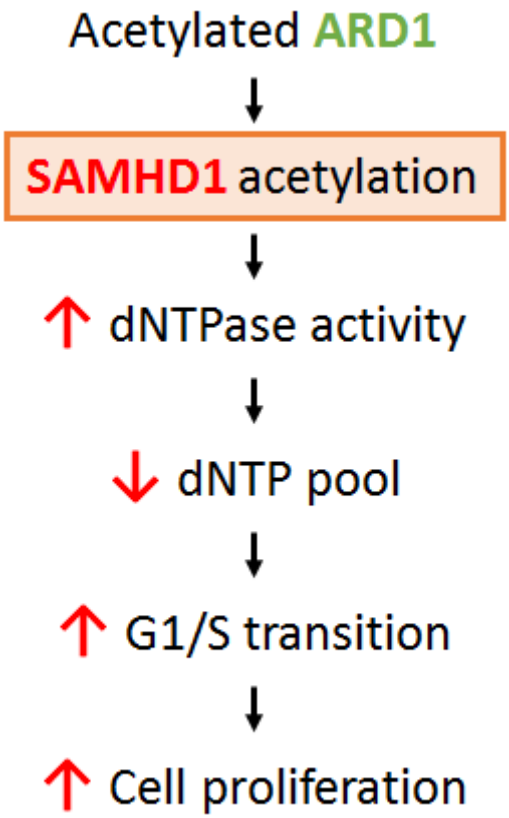


Figure 54. Schematic for the mechanism of ARD1-mediated SAMHD1 acetylation in cancer cell proliferation.

CONCLUSION

SAMHD1 is a dNTPase that contributes to cell cycle progression and innate immunity. However, molecular mechanisms that directly regulates the dNTPase activity of SAMHD1 has been unexplored. Here, I suggest a novel post-translational modification of SAMHD1, acetylation, which regulates its dNTPase activity. SAMHD1 bound to and were acetylated at its well-conserved K405 residue by ARD1, an acetyltransferase. Acetylated SAMHD1 showed higher dNTPase activity, whereas K405R mutants that cannot be acetylated did not. SAMHD1 was overexpressed in hepatocarcinoma tissues and its down regulation resulted in slow growth and less colony formation in cancer cell lines. SAMHD1 wildtype-overexpressing stable cell lines grew faster and formed more colonies over mock or K405 mutant expressing cells in various cancer cell lines. These differential growth were caused by altered cell cycle, as SAMHD1 wildtype expressing cells showed increased G1/S transition over mock or K405R expressing cells. Moreover, SAMHD1 acetylation mainly occurred during

G1 phase, whereas endogenous expression levels of SAMHD1 did not show significant changes throughout the cell cycle progression. Altogether, ARD1-mediated SAMHD1 enhances its dNTPase activity and contributes to cancer cell proliferation by facilitating G1/S transition.

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국문초록

SAMHD1은 뉴클레오티드 3인산 분해능을 갖는 효소로써 이를 통해 면역세포에서 항레트로바이러스 효과를 나타낸다. 그러나 SAMHD1의 효소작용을 직접적으로 조절하는 분자기전은 밝혀진 바가 없다. 또한 SAMHD1이 면역세포뿐만 아니라 인체 내 다양한 조직들에서 광범위하게 발현되므로 다른 세포에서도 특이적 기능을 가질 가능성이 크다. 본 연구자는 SAMHD1이 아세틸화됨으로써 그 분해능이 증가하고 이를 통해 암세포 증식이 촉진됨을 연구하였다. SAMHD1은 아세틸화효소인 ARD1과 결합함으로써 라이신 405 잔기에 아세틸화가 일어났는데, 이러한 아세틸화가 *in vitro* 상에서 SAMHD1의 뉴클레오티드 3인산 분해능을 강화시킴을 밝혔다. 아세틸화가 불가능하게 라이신 405를 아르기닌으로 치환시킨 변이형 SAMHD1 단백질은 아세틸화가 일어나지 않았

으며 분해능도 강화되지 않았다. 선행 연구결과에 의하면 뉴클레오티드 3인산의 양은 세포의 분열주기를 조절함으로써 세포증식에 영향을 미친다. SAMHD1 아세틸화가 암세포의 증식에 어떤 영향을 미치는지 확인하게 위해 변이형 SAMHD1을 각종 암세포에 과발현 시켰을 때 야생형보다 세포분열 및 군집생성능이 감소됨을 확인하였다. 야생형과 변이형 SAMHD1을 과발현 하는 암세포들의 각 세포분열시기별 조성을 분석한 결과 변이형에서는 G1에서 S시기로의 전이가 감소되어있음을 확인하였다. SAMHD1 아세틸화 정도를 세포분열시기 별로 확인해 본 결과 G1/G0 시기에서 가장 많이 일어나는 것을 발견하였다. 그러나 SAMHD1의 발현량 자체는 분열 시기별 변화가 미미하였으므로, SAMHD1의 양이 아닌 아세틸화가 세포분열주기에 영향을 미친 것으로 생각된다. 결론적으로, SAMHD1 아세틸화는 그 뉴클레오티드 3인산 분해능을 강화함으로써 암세포의 증식을 촉진시킨다.

주요어: SAMHD1, 아세틸화, ARD1, 3인산염 분해, 세포분열주기,

암세포 증식