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Lydia R. Studdard Date

Characterization of the *C. elegans* Ortholog of SAMHD1
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Characterization of the *C. elegans* Ortholog of SAMHD1

By

Lydia R. Studdard
B.S., Georgia State University, 2014

Advisor: Baek Kim, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Abstract

Characterization of the *C. elegans* Ortholog of SAMHD1
By Lydia R. Studdard

Aicardi-Goutières Syndrome (AGS) is a genetic disorder that mimics in-utero viral infection. The defining characteristic of AGS is the presence of increased levels of interferon alpha in the cerebral spinal fluid in the absence of any infection. It is thought that AGS is a result of defects in responses to aberrant nucleic acids. Mutations thought to lead to AGS are found in genes coding for proteins involved in regulating cellular responses to these nucleic acids. One such protein is sterile alpha motif and HD domain containing protein 1 (SAMHD1) which is responsible for degrading dNTPs in non-cycling cells. The dNTPase activity of SAMHD1 allows it to restrict HIV-1 infection in nondividing cells. Currently a mouse model is used to study SAMHD1 linked AGS, however this model has not been shown to reproduce the clinical phenotype of AGS. In this thesis, *C. elegans* and its ortholog of SAMHD1 are studied as a possible alternative animal model.

Total RNA derived from *C. elegans* was used to generate a cDNA clone of the *C. elegans* ortholog of SAMHD1 (ceSAMHD1). This cDNA was cloned into a bacterial expression plasmid and a lentiviral expression plasmid. The protein was produced and purified from bacteria and used in several biochemical assays. ceSAMHD1 was also expressed in a monocytic cell line lacking human SAMHD1 and its effect on dNTP levels as well as HIV-1 restriction was assayed. Finally, ceSAMHD1 was knocked down in *C. elegans* and the animals were observed for developmental defects.

This work determined that ceSAMHD1 is capable of degrading dNTPs and is regulated in a similar manner as human SAMHD1in biochemical assays. In human cells, ceSAMHD1 was not capable of degrading dNTPs or restricting HIV-1 infection. Finally, worms treated with ceSAMHD1 RNAi did not show any developmental defects. In conclusion, *C. elegans* does not provide a more clinically relevant animal model than the mouse model used for studying SAMHD1 related AGS.

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Chapter I: Introduction

(A) Aicardi-Goutières syndrome

a. Clinical Phenotype

In 1984 Aicardi and Goutières were the first to describe a genetic disorder affecting infants characterized by microcephaly, intracranial calcifications, and increased levels of white blood cells in cerebral spinal fluid (CSF) (1). It was noted that the symptoms where similar to those found in patients with viral infections and were suggestive of an inflammatory process (1). Four years later it was shown that this disorder, named after the discoverers, Aicardi-Goutières Syndrome (AGS), was found to be associated with increased levels of type I interferon in the CSF of patients (2). AGS has since been associated with other symptoms such as the presence of chilblains (a type of skin lesion) and recurrent fevers without evidence of infection; however, the defining symptoms is still considered to be raised levels of type I interferon present in the CSF without concurrent infection. AGS is now grouped with other disorders under the category 'type I interferonopathies' (3). It is thought that the increased level of type I interferon is directly related to causing the other symptoms (3).

b. Implicated Genes

The AGS phenotype may be caused by an abnormal cellular response to nucleic acids. This hypothesis is supported by the fact that AGS is associated with mutations in seven genes, all of which play some part in regulating or sensing nucleic acids. Three of the genes implicated in AGS encode for the three subunits of RNaseH2. RNaseH2 recognizes RNA-DNA hybrids and removes ribonucleotides embedded in the DNA portion (4). Another gene codes for the protein MDA5 or IFIH1 which is responsible for

sensing dsRNA found in the cytosol (5,6). ADAR is a protein that deaminates adenosines found in dsRNA and is found to be mutated in some AGS patients (7). TREX1 is a DNA endonuclease that has been implicated in AGS (8). Finally, AGS associated mutations are found in the gene coding for SAMHD1 (9,10). SAMHD1 is a protein that is capable of degrading cellular dNTPs.

c. Mouse Model of SAMHD1 associated AGS

There are two murine isoforms of SAMHD1, one which correlates with the full length human protein and one which is truncated as a result of alternative splicing. Two groups of researchers have created SAMHD1 deficient mice which lack both forms of murine SAMHD1 (11,12). Both studies showed that the lack of SAMHD1 lead to an increase in intracellular dNTP levels. These mice showed increased levels of interferon stimulated genes (ISGs), however an increase in type I interferon levels was not observed. Significantly, both groups of mice were healthy, fertile, and showed no signs of developmental defects. Since the clinical phenotype of SAMHD1 associated AGS has not to date been shown in mice, use of another animal model to study this disease could provide insights that the current model is not capable of producing.

(B) SAMHD1

a. Effect of SAMHD1 on HIV-1 infection

SAMHD1 gained notoriety when it was found to be a HIV-1 restriction factor (13). As a retrovirus when HIV-1 infects a cell it requires dNTPs to convert its RNA genome into proviral DNA through reverse transcription. The HIV-1 DNA may then be integrated into the host genome (which also requires a small amount of dNTPs). HIV-1 infection of macrophages results in a kinetic delay of replication (compared to infection

of activated T cells) due to low intracellular levels of dNTPs caused by SAMHD1 expression (14,15).

b. dNTPase Function and Regulation

Sterile Alpha Motif (SAM) and Histidine/Aspartate (HD) domain containing protein 1 (SAMHD1) limits the levels of available dNTPs in nondividing human cells through triphosphohydrolase activity. SAMHD1 degrades dNTPs by cleaving them into deoxynucleosides and triphosphates (15,16). This enzymatic activity is carried out by the active sight found in the HD domain of the protein (17). No function has yet been assigned to the SAM domain, although it is interesting to note that SAM domains are traditionally found to play a part in protein-protein and protein-RNA interactions (18).

SAMHD1's triphosphohydrolase activity is regulated in many ways. The SAMHD1 HD domain contains two allosteric sites. These two allosteric sites must be bound to either dGTP or GTP for SAMHD1 to oligomerize into the active tetramer form (19-23). Additionally, SAMHD1 is regulated by phosphorylation of threonine 592. Initially, phosphorylated SAMHD1 was thought to retain its ability to restrict HIV-1 infection but lose its ability to degrade dNTPs (24-26). It is now known that phosphorylation results in a loss of ability to form tetramers which negatively impacts both HIV-1 restriction and dNTP degradation (25-28).

c. Other Proposed Functions

In addition to degrading dNTPs, SAMHD1 has also been reported to be a nucleic acid binding protein (17,29,30). This function is both understandable, given that SAM domains are usually correlated with protein-nucleic acid binding (18), and interesting when considering that AGS is linked to mutations in known nucleases such as TREX1

and RNase H2. Building upon the observation that SAMHD1 can bind to nucleic acids, SAMHD1 has been described as having nuclease activities. There has been one report of SAMHD1 degrading both DNA and RNA (31) while another group has only observed RNase and not DNase activity (32-34). SAMHD1 RNase activity is hotly contested in the field with several groups having failed to reproduce the data shown in previous papers (35,36). More definitive data is needed to clear up whether or not SAMHD1 has nuclease activity. It will be interesting to see how this influences thinking surrounding AGS and how SAMHD1 mutations relate to the disease.

LINE-1 and Alu are some of the endogenous retroelements found in the human genome. These retroelements are capable of 'jumping': making new insertions in the human genome. This process is made capable by a reverse transcriptase encoded by LINE-1. SAMHD1 has been reported to inhibit activation of endogenous retroelements (37). Mutations that eliminate the dNTPase activity of SAMHD1 were shown to still allow SAMHD1 to restrict LINE-1 activation. It was also shown that certain AGS associated mutations limited LINE-1 restriction but did not affect dNTPase activity (37). While SAMHD1 restricts retrovirus infection in nondividing cells through its dNTPase activity, SAMHD1 restricts LINE-1 retrotransposition in dividing cells. SAMHD1 was discovered to promote stress granule formation (38). The stress granules are capable of sequestering LINE-1 and preventing retrotransposition (38). It is not currently clear how SAMHD1 promotes this.

d. *C. elegans* as a model to study SAMHD1

C. elegans is an ideal model for studying developmental disorders and AGS in particular. The developmental pathway for all cells in an adult worm is extraordinarily

well characterized. The *C. elegans* ortholog of SAMHD1 is predicted based on sequence analysis and is an uncharacterized protein, ZK177.8 (ceSAMHD1). Knockdown of ceSAMHD1 with RNAi has been reported to result in maternal sterility indicating that ceSAMHD1 plays a role in the developmental process of *C. elegans*, (39) which is reminiscent of the developmental delays encountered in AGS patients. Although ceSAMHD1 has 30% sequence identity with hSAMHD1, ceSAMHD1 is predicted to only contain an HD domain and not a SAM domain. Therefore any functions of SAMHD1 that are not conserved with ceSAMHD1 may be examined in the future as candidate SAM domain activities. Adult *C. elegans* have an invariant number of somatic cells which will be helpful for standardization when determining intracellular dNTP levels, a common assay used to study SAMHD1 dNTPase activity. Additionally, examination of the *C. elegans* genome shows only one complete, active retrotransposon, *cer-1* (40-42) which would allow for simple screening for the ability of ceSAMHD1 to restrict retrotransposon activation.

Chapter two focuses on the work done to characterize the SAMHD1 ortholog found in *C. elegans*. First, the canonical dNTPase activity of ceSAMHD1 was assessed. Next regulation of ceSAMHD1 dNTPase activity was explored. Finally, the ability of ceSAMHD1 to regulate dNTP levels and restrict HIV-1 infection of human cells was assayed.

Chapter II:

Biochemical Characterization of the SAMHD1 Ortholog in *Caenorhabditis elegans*, ZK177.8

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Running Title: The *C. elegans* ortholog of SAMHD1 degrades dNTPs

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ABSTRACT

SAM domain and HD domain containing protein 1 (SAMHD1) was identified as a myeloid specific host restriction factor against HIV-1. The dNTP triphosphohydrolase (dNTPase) activity of SAMHD1, which depletes cellular dNTPs, kinetically suppresses viral reverse transcription particularly in non-dividing macrophages. Mutations in the human SAMHD1 gene were identified in a neuro-developmental genetic disorder, Aicardi-Goutières Syndrome (AGS). *Caenorhabditis elegans* (*C. elegans*) encodes a SAMHD1 ortholog, ceSAMHD1, which reportedly also induces developmental defects upon gene knockdown. Here we demonstrated that ceSAMHD1 is an allosterically regulated dNTPase. ceSAMHD1 recognized both the 3'OH and the triphosphate of dNTP substrates for dNTP hydrolysis, and the allosteric activator dGTP induces the oligomerization of ceSAMHD1. However, ceSAMHD1 was ineffective at reducing dNTP levels or restricting HIV-1 infection of a human monocytic cell line. In conclusion, this study suggests that *C. elegans* SAMHD1 can serve as a model studying SAMHD1 biochemically but cannot functionally replace human SAMHD1.

INTRODUCTION

A series of mutations in the human sterile alpha motif (SAM) domain and histidine-aspartate (HD) domain containing protein 1 (SAMHD1) gene were identified in Aicardi-Goutières Syndrome (AGS) (9). AGS is a neurodevelopmental genetic disorder with abnormally elevated innate immune functions including interferon and interferon-related gene expression in the absence of any detectable infection, ultimately leading to abnormal brain development. Genes mutated in AGS such as SAMHD1, RNase H2, and TREX1 are involved in various cellular nucleic acid metabolism functions, suggesting that interrupted nucleic acid metabolism may trigger cellular nucleic acid sensing, which potentially induces the activation of the cellular innate immune responses without any infection events (43).

SAMHD1 was also identified as a host restriction factor against lentiviruses such as human immunodeficiency virus type 1 (HIV-1), type 2 (HIV-2), and simian immunodeficiency viruses (SIV) (13,15,23,44,45). This anti-lentiviral activity of SAMHD1 is observed particularly in non-dividing viral target cell types including macrophages (13,15), dendritic cells (46), and resting T cells (47,48), which abundantly express SAMHD1. SAMHD1 harbors deoxynucleotide triphosphohydrolase (dNTPase) activity that hydrolyzes deoxynucleoside triphosphate (dNTP) into dN and triphosphate (16). We previously reported that human primary monocyte derived non-dividing macrophages harbor extremely limited cellular dNTP concentrations (20-40nM), which are approximately 50-200 times lower than the dNTP concentrations found in activated/dividing CD4+ T cells (2-5uM) (14). This limited cellular dNTP availability in non-dividing cells kinetically restricts the reverse transcription step of HIV-1, which

consumes cellular dNTPs for proviral DNA synthesis (49). Indeed, we reported that the high level of SAMHD1 protein in terminally differentiated macrophages is responsible for the extremely low cellular dNTP concentration, which restricts lentivirus infection in this non-dividing viral target cell type (50). However, unlike HIV-1, HIV-2 and many SIVs encode an accessary protein, viral protein X (Vpx), which proteasomally degrades SAMHD1, leading to the elevation of the cellular dNTP levels and ultimately to the enhancement of viral infectivity in the nondividing myeloid cells.

The dNTPase activity of human SAMHD1 is regulated by two allosteric sites, A1 and A2 (51,52): dGTP/GTP bind to the A1 site, and dNTPs bind to the A2 site. The binding of these nucleotides triggers the formation of the enzymatically active SAMHD1 tetramers (53-55). The structure of the full-length SAMHD1 protein is not currently available, while the HD domain structures of human SAMHD1 revealed regulatory coordination between allosteric sites and the dNTPase active site in the enzymatically active tetramer form (55). Human SAMHD1 is also regulated by phosphorylation at residue T592 near its C-T end, and SAMHD1 mainly stays in the phosphorylated form in dividing cells (56,57). Current models suggest that the phosphorylation at T592 prevents the formation of the enzymatically active SAMHD1 tetramers (28). Several previous studies also reported that SAMHD1 has RNase and RNA binding activities (29,31,58) however the role of the RNase/RNA binding activity of SAMHD1 needs to be further investigated.

SAMHD1 homologs and orthologs can be found in many species. However, two independent studies with SAMHD1 knockout mice failed to observe the AGS phenotypes even though these knockout mice exhibited elevations of both interferon-response gene

expression and cellular dNTP levels in various tissues (12,59,60). In zebra fish, SAMHD1 is reportedly involved in the brain developmental process (61). A recent study demonstrated that SAMHD1 restricts LINE-1 retrotransposon by sequestering retrotransposon ribonucleoprotein complex into stress granules in human cells (38). A previous genome-wide survey of *C. elegans* with RNA knockdown technology reported that reduced expression of the SAMHD1 ortholog, ceSAMHD1, led to maternal sterility, implying that ceSAMHD1 is also involved in host development. However, ceSAMHD1 contains only the C-T dNTPase related HD domain without the N-T SAM domain. Here, we report that ceSAMHD1 is a dGTP-regulated dNTPase that is capable of forming tetramers, which supports the idea that ceSAMHD1 harbors very similar enzymological properties with human SAMHD1.

RESULTS

Cloning and purification of C. elegans SAMHD1:

ZK177.8 encodes a SAMHD1 ortholog, ceSAMHD1. (http://www.wormbase.org/species/c_elegans/gene/WBGene00022673#0-9g-3). A wide gene knockdown study of *C. elegans* reported that reduction of ZK177.8 expression leads to maternal sterility (62), which is a developmental defect failure in producing eggs. As shown in Figure 1A, ceSAMHD1 contains the dNTPase related HD domain that consists of a series of highly conserved amino acid residues found in two separate allosteric regulatory sites (A1 and A2) as well as the dNTPase catalytic site (C) of human SAMHD1 (Figure 1B). However, ceSAMHD1 lacks the SAM domain that is found in the N-T end of human SAMHD1 and appears to be dispensable for the dNTPase activity of human SAMHD1 (63). However, ceSAMHD1 encodes a conserved threonine residue at its C-T end (Figure 1B), which is known to be a phosphorylation site for the negative regulation of the dNTPase activity in human SAMHD1. The full-length ZK177.8 gene was amplified by RT-PCR from cellular RNAs prepared from adult *C. elegans*. The amplified gene was cloned to pGEX 5x-3 for overexpressing a N-T GST-fused ceSAMHD1 protein in *E. coli*. The overexpressed GST-fused ceSAMHD1was initially applied to a GST affinity column, and the GST-free ceSAMHD1 was eluted from the GST column after the treatment with Factor Xa protease that cleaves the sequence between GST tag and ceSAMHD1. The GST-free ceSAMHD1 was further purified by gel filtration (Figure 1C); the ceSAMHD1 predominantly eluted as dimers, based on the molecular weight comparison in the gel filtration. The dimer fractions were pooled for various biochemical analyses in this study.

Test for dNTPase activity of ceSAMHD1: Next, we tested whether the purified ceSAMHD1 acts as a dNTPase by using two different assays. First, we used an HPLC-based dNTPase assay that detects the dN product generated from the hydrolysis of dNTP. dATP substrate (1mM) was incubated with ceSAMHD1 (400uM) in the presence and absence of a lower concentration of dGTP (50uM) to test whether ceSAMHD1 uses dGTP as an allosteric activator, and then applied to HPLC. As shown in Figure 2A, the dA product was detected in the reaction only with both dGTP and dATP. The incubation with only dATP did not produce dA, as is also observed with human SAMHD1 protein. The reactions with no enzyme failed to generate dA even in the presence of dGTP (Figure 2A). To further confirm the dNTP triphosphohydrolase activity of ceSAMHD1, which produces dN and triphosphate (TP), we also employed a TLC-based dNTPase assay. This assay employs alpha-^{32}P labeled dTTP substrate, generating the radiolabeled triphosphate (TP) product after hydrolysis, which can be separated and detected in TLC. As shown in Figure 2B, the incubation of the radiolabeled dTTP with ceSAMHD1 (and human SAMHD1) generated high levels of the radioactive TP product, compared to control reactions without the enzyme which showed only a baseline TP level. These two dNTPase assays demonstrate that ceSAMHD1 harbors dNTP triphosphohydrolase activity and this activity is allosterically regulated by dGTP as observed with human SAMHD1 protein (52).

Substrate specificity of ceSAMHD1: Human SAMHD1 also uses GTP, which is much more abundant than dGTP in cells (51), as an allosteric activator for the A1 site, which

initiates the formation of the enzymatically active tetramers (51,52,54,64). We tested whether ceSAMHD1can also use GTP as an allosteric activator. As shown in the HPLC-based dNTPase assay (Figure 3A), ceSAMHD1 was able to hydrolyze dATP substrate in the presence of GTP, supporting the idea that ceSAMHD1 also uses GTP as an allosteric activator, and that the A1 site of ceSAMHD1 does not recognize the 2' OH of the activator. It was previously reported that human SAMHD1 requires the 3' OH on the sugar moiety and all three phosphates at the phosphate moiety of dNTP substrate (51). As shown in Figure 3B, ceSAMHD1 was not able to hydrolyze various nucleotide substrates lacking 3' OH (ddGTP, AZTTP, ddITP, ddTTP), suggesting that ceSAMHD1 also requires 3' OH for its dNTPase activity. Finally, when dAMP and dADP were incubated with ceSAMHD1 in the presence of GTP activator, little dA product was detected (Figure 3C). This could be due to the failure of dAMP and dADT to bind to the active site and/or A2 sites of ceSAMHD1. The data shown in Figure 3 demonstrates that the activator/substrate specificity of ceSAMHD1 is similar with that of human SAMHD1.

Oligomerization of ceSAMHD1: The dGTP binding to both A1 and A2 sites forms the enzymatically active tetramers of human SAMHD1 (54,55). Next, we tested the tetramer formation of ceSAMHD1 by dGTP. For this test, we conducted formaldehyde induced crosslinking of ceSAMHD1 in the presence and absence of dGTP. As shown in Figure 4, ceSAMHD1 protein formed tetramers only in the presence of dGTP as also observed with human SAMHD1. This data supports that dGTP binding to the two allosteric sites of ceSAMHD1 forms tetramers which is the enzymatically active form for dNTP hydrolysis. Interestingly, a larger portion of the crosslinked ceSAMHD1 dimers was

observed, which is consistent with the gel filtration data (Figure 1C) that showed that ceSAMHD1 predominantly exists as dimers in solution, whereas human SAMHD1 exists as both monomer and dimers in solution (Figure 4).

Effect of ceSAMHD1 expression on cellular dNTP levels in human non-dividing macrophage THP-1 cell line: Next, we tested whether ceSAMHD1 affects cellular dNTP levels in human cells. For this test, we employed a human monocytic SAMHD1 knockout THP-1 cell line that we recently established using lentiviral vector based CRISPR/Cas9 technology (65). Upon differentiation to nondividing macrophages by PMA treatment, the dNTP levels in the SAMHD1 knockout THP-1 cells increased, compared to both parental THP-1 cells and control cells transduced with the empty knockout control vector, suggesting that the loss of human SAMHD1 elevates cellular dNTP levels (65). We expressed ceSAMHD1 in the THP-1 SAMHD1 KO cells using the pLVX-IRES-mCherry lentiviral vector system expressing both HA-tagged ceSAMHD1 and mCherry protein or only mCherry protein (control). The mCherry + cells were FACS sorted and propagated. Three independent cell populations were analyzed for the ceSAMHD1 expression by western analysis with HA antibody after PMA differentiation. As shown in Figure 5A, ceSAMHD1 was expressed in the SAMHD1 KO transduced with the pLVX vector expressing both ceSAMHD1 and mCherry protein, but not in the control cells transduced with the empty pLVX vector expressing only mCherry protein. Next, we determined the dNTP levels in the PMA treated SAMHD1 KO cells expressing both ceSAMHD1 and mCherry protein or only mCherry protein. As shown in Figure 5B, the SAMHD1 KO cells expressing ceSAMHD1 did not display reduced dATP levels,

compared to the SAMHD1 KO cells that express only mCherry protein. This result suggests that ceSAMHD1 is ineffective at hydrolyzing dNTPs within this human cell line.

HIV-1 restriction by ceSAMHD1 in THP-1 cells: The reduction of cellular dNTP level by SAMHD1 restricts HIV-1 infection in nondividing cells (46,66). Therefore, we tested if ceSAMHD1 expression, which did not reduce cellular dNTP levels, is able to restrict HIV-1 infection in the THP-1 cell model. For this test, we employed HIV D3-GFP which is an NL4-3 based HIV-1 vector encoding the entire HIV-1 genome except env and nef, which was replaced with eGFP (14). The PMA-treated SAMHD1 KO THP-1 cells with and without ceSAMHD1 expression were transduced with HIV D3-GFP, and we determined the GFP + cells at different time points post transduction by FACS. As shown in Figure 5C, the ceSAMHD1 expressing SAMHD1 KO THP-1 cells did not show delayed HIV-1 transduction kinetics, compared to the control SAMHD1 THP-1 cells.

DISCUSSION

Most AGS linked proteins including SAMHD1, TREX1 and RNase H2 are involved in cellular nucleic acid metabolism. Interruptions of these gene functions proactively induce innate immune activation, including interferon and interferon-induced genes even in the absence of any detectable infection, leading to neurodevelopmental defects (43,67). However, two independent SAMHD1 knockout mouse models have failed to exhibit AGS phenotypes even though both interferon responses and cellular dNTP levels were elevated in these KO mice. Interestingly, a recent SAMHD1

knockdown study in zebra fish demonstrated the AGS-like phenotype in this knockdown model (61).

A genome wide gene knockdown survey in *C. elegans* reported that the reduction of the SAMHD1 ortholog, ceSAMHD1, led to maternal sterility: a strong indication for the host developmental role of ceSAMHD1, which supports its potential as an AGS model. Indeed, our biochemical data confirm that ceSAMHD1 displays very similar enzymatic properties with human SAMHD1, including the dGTP-mediated activation of dNTPase activity, chemical recognition of substrates and regulators, and oligomerization. However, ceSAMHD1 was unable to functionally substitute for human SAMHD1 in SAMHD KO THP-1 cells with respect to the reduction of cellular dNTP levels and HIV-1 restriction. These data suggest that specific activities and regulation of SAMHD1 proteins may have diverged over evolutionary time.

Lastly, dNTP biosynthesis, which includes various cell cycle regulated enzymes such as ribonucleotide reductase and thymidine kinase, has been extensively studied. Pharmacological drugs targeting these dNTP biosynthesis enzymes have been developed as anti-cancer agents (68-72). However, dNTP degradation has not been considered as a key dNTP regulatory mechanism until SAMHD1 was identified as a nondividing cell specific anti-HIV-1 restriction factor: SAMHD1 suppresses the viral reverse transcription step which consumes cellular dNTPs, particularly in nondividing myeloid cells including macrophages and DCs. While the dNTPase activity of SAMHD1 is directly involved in HIV-1 restriction, whether and how this activity induces a AGS phenotype remains unclear. Some AGS SAMHD1 mutations reportedly affect its exclusive nuclear localization (29), but no AGS mutations have been shown to generate significant loss of

biochemical dNTPase activity. In fact, in addition to dNTPase activity, SAMHD1 was initially reported to have RNase activity (32,33) while a recent study demonstrated SAMHD1 is not RNase, but does bind to RNA (29,58). Both detection and roles of these RNA related activities remain to be further investigated.

An animal model is needed to further study SAMHD1 and mutations in SAMHD1 lead to the AGS phenotype seen in patients. This study demonstrates that *C. elegans* ZK177.8 codes for an ortholog of human SAMHD1. ceSAMHD1 is an allosterically regulated dNTPase and displays very similar enzymatic properties with human SAMHD1. However, ceSAMHD1 cannot serve as a functional substitute for human SAMHD1 in human cells to reduce of cellular dNTP levels or restriction HIV-1 infection. This data suggests that *C. elegans* is not an improved animal model over the current mouse model for studying AGS

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FIGURES

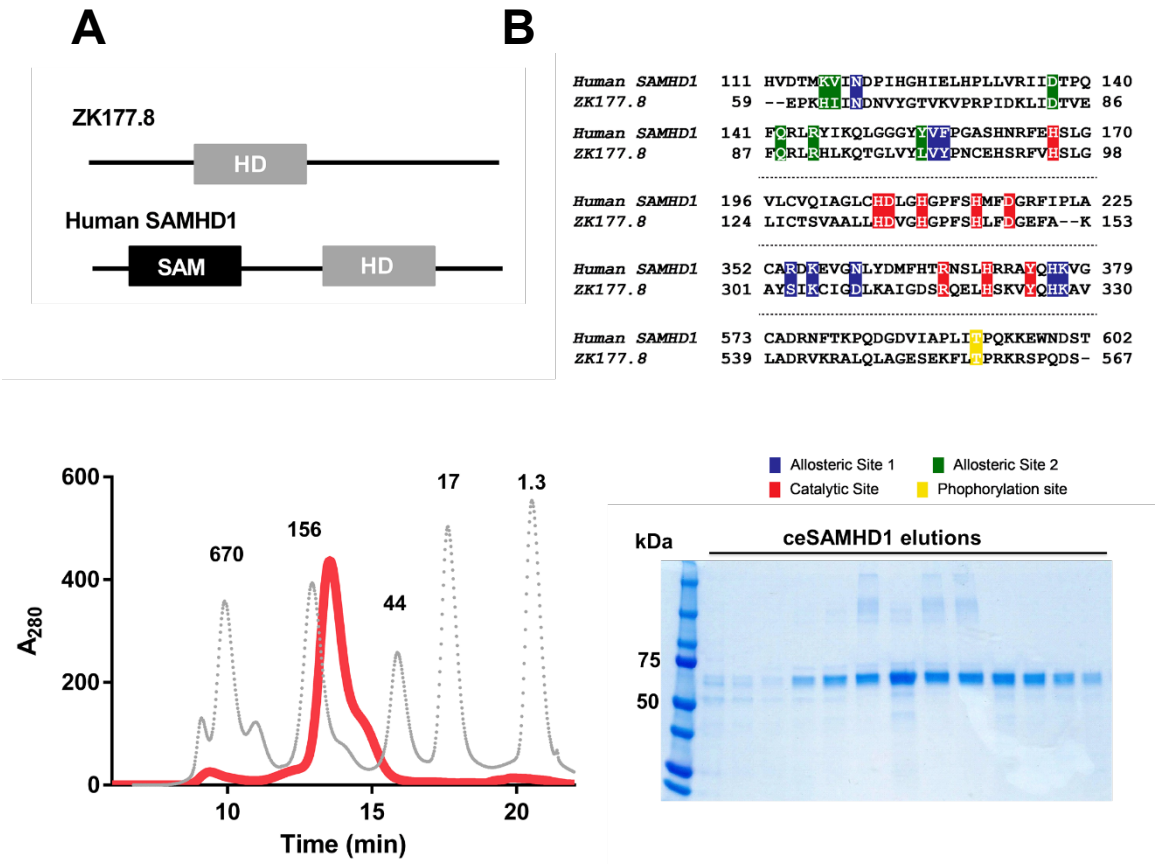

Figure 1: Cloning, overexpression and purification of *C. elegans* ZK177.8 protein, ceSAMHD1: (A) domain comparison of ceSAMHD1 and human SAMHD1 protein. ceSAMHD1 encodes only the dNTPase active HD domain (white), but not the SAM domain (grey). **(B)** The residues known to be important for allosteric regulation in A1 and A2 sites, dNTPase catalysis, and regulatory phosphorylation (Thr592) of human SAMHD1 were compared with those in ceSAMHD1. **(C)** Overexpression and purification of ceSAMHD1. Chromatogram of ceSAMHD1 elution following gel filtration is shown overlaid with a gel filtration standard. The overexpressed ceSAMHD1 protein after GST affinity column purification, digestion to remove GST tag, and gel

filtration is shown. ceSAMHD1 is predicted to be 62 kDA.

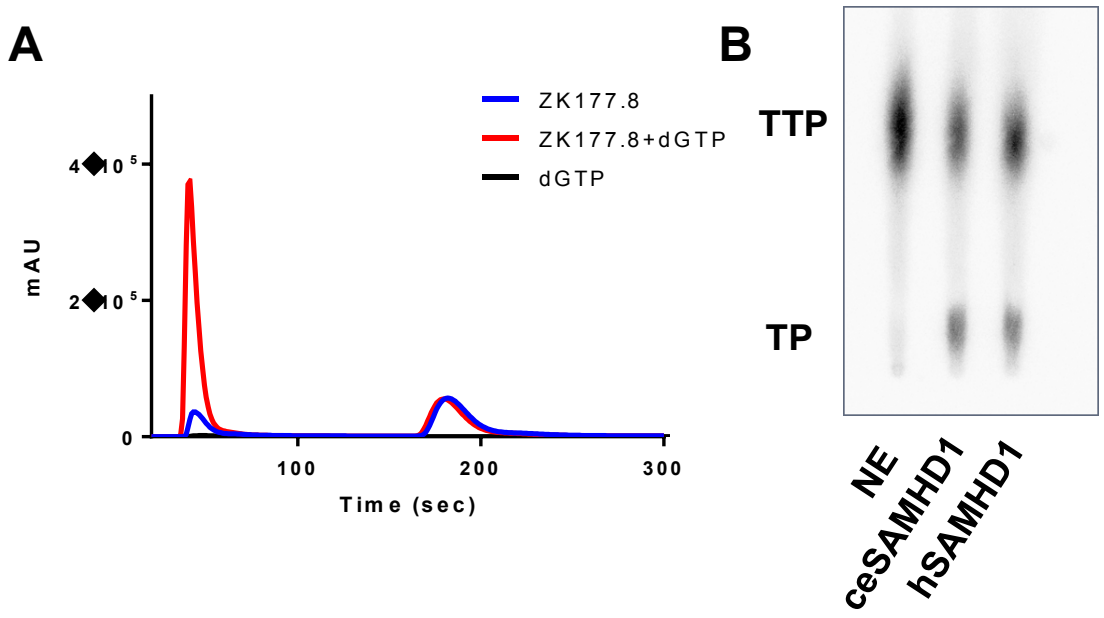

Figure 2: dNTPase activity of ceSAMHD1: (A) HPLC-based dNTPase assay with purified ceSAMHD1. The elution profile of the dA product from HPLC after incubation of dATP substrate (1MM) with purified ceSAMHD1 (400uM) in the presence (red) and absence (blue) of dGTP (50uM) at 22C for 60min . dCMP (~200sec) was used for the internal loading control. The same reaction was repeated without ceSAMHD1 protein in the presence of dGTP (black). **(B)** TLC-based dNTPase assay for ceSAMHD1. alpha-^{32}P TTP was incubated with purified ceSAMHD1 and human SAMHD1 under the standard assay condition, and applied to TLC for detecting triphosphate (TP) product. NE: no enzyme control.

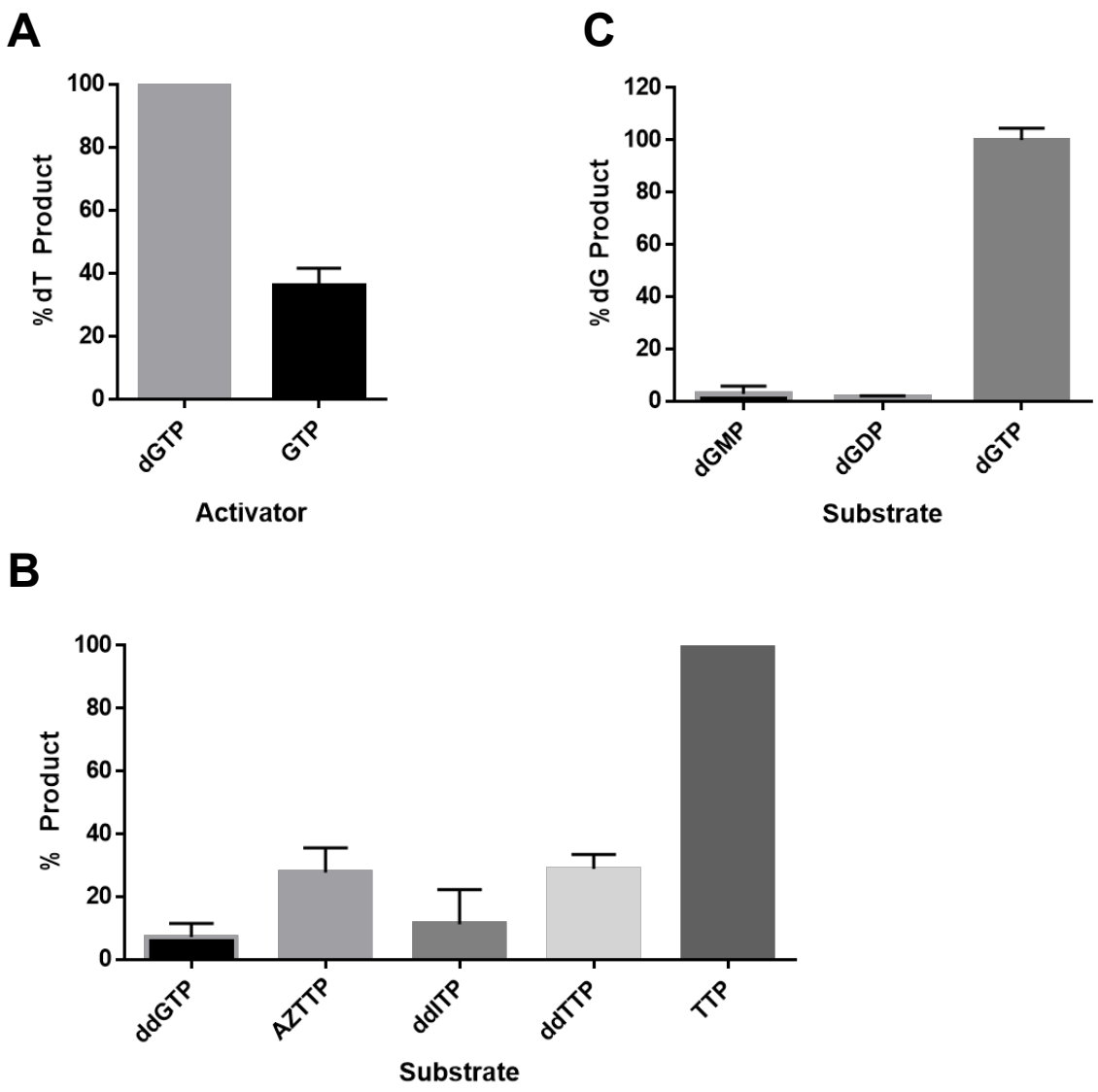

Figure 3: Substrate and activator specificity of ceSAMHD1. (A) GTP as activator. dTTP was incubated with ceSAMHD1 (400uM) in the presence and absence of GTP (50uM) and dGTP (100%), and the dT product was analyzed by HPLC-based assay. Human SAMHD1 (400uM) was used for control. **(B)** Hydrolysis of ddNTP chain terminators lacking 2-OH. AZTTP, ddATP, ddITP, and ddTTP (1mM) were incubated with ceSAMHD1 (400uM) in the presence and absence of dGTP (50uM), and the product was analyzed by HPLC. Normal dNTP counterparts (100%) of these chain terminators

(i.e. dATP for ddATP) were used for comparison**. (C)** dGTP, dGDP and dGMP hydrolysis by ceSAMHD1. An equal concentration of these three nucleotides (1mM) was incubated with ceSAMHD1(400uM) in the presence of GTP (50uM) and their corresponding products were analyzed by HPLC (dGTP for 100%). All reactions with ceSAMHD1 (400uM) were conducted at 22C for 60mins.

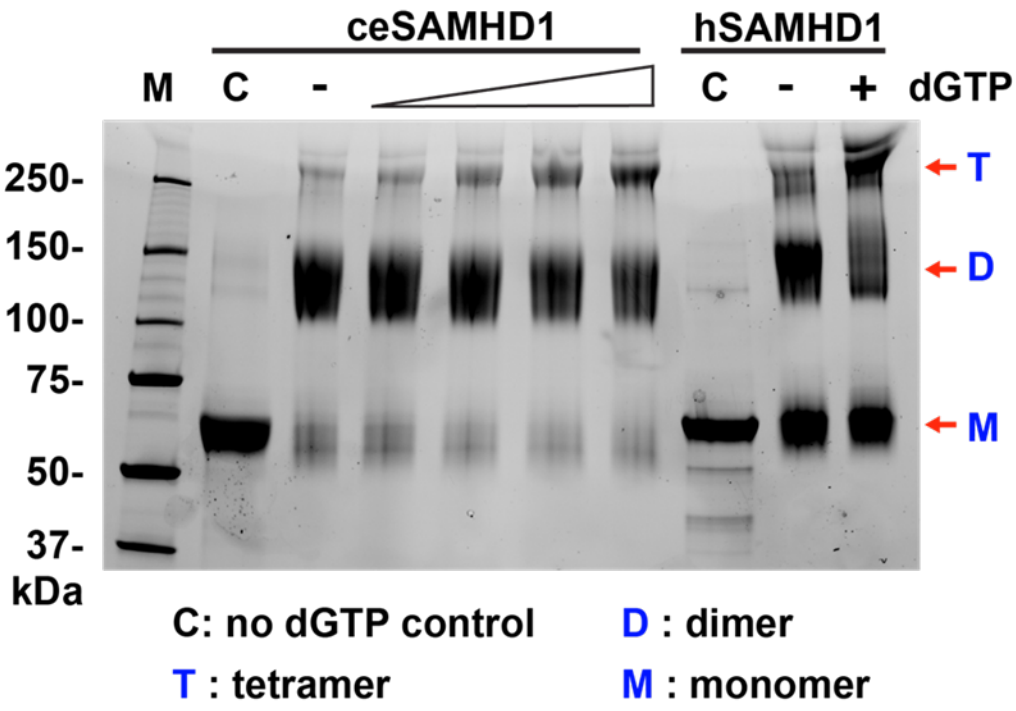

Figure 4: Tetramerization of ceSAMHD1. dGTP-mediated tetramerization of ceSAMHD1. ceSAMHD1 and human SAMHD1 (20uM) were cross-linked by formaldehyde (2%) in the presence or absence of dGTP (2mM). The oligomers for each protein were marked based on their expected molecular weights. M: monomer, D: dimer and T: tetramer.

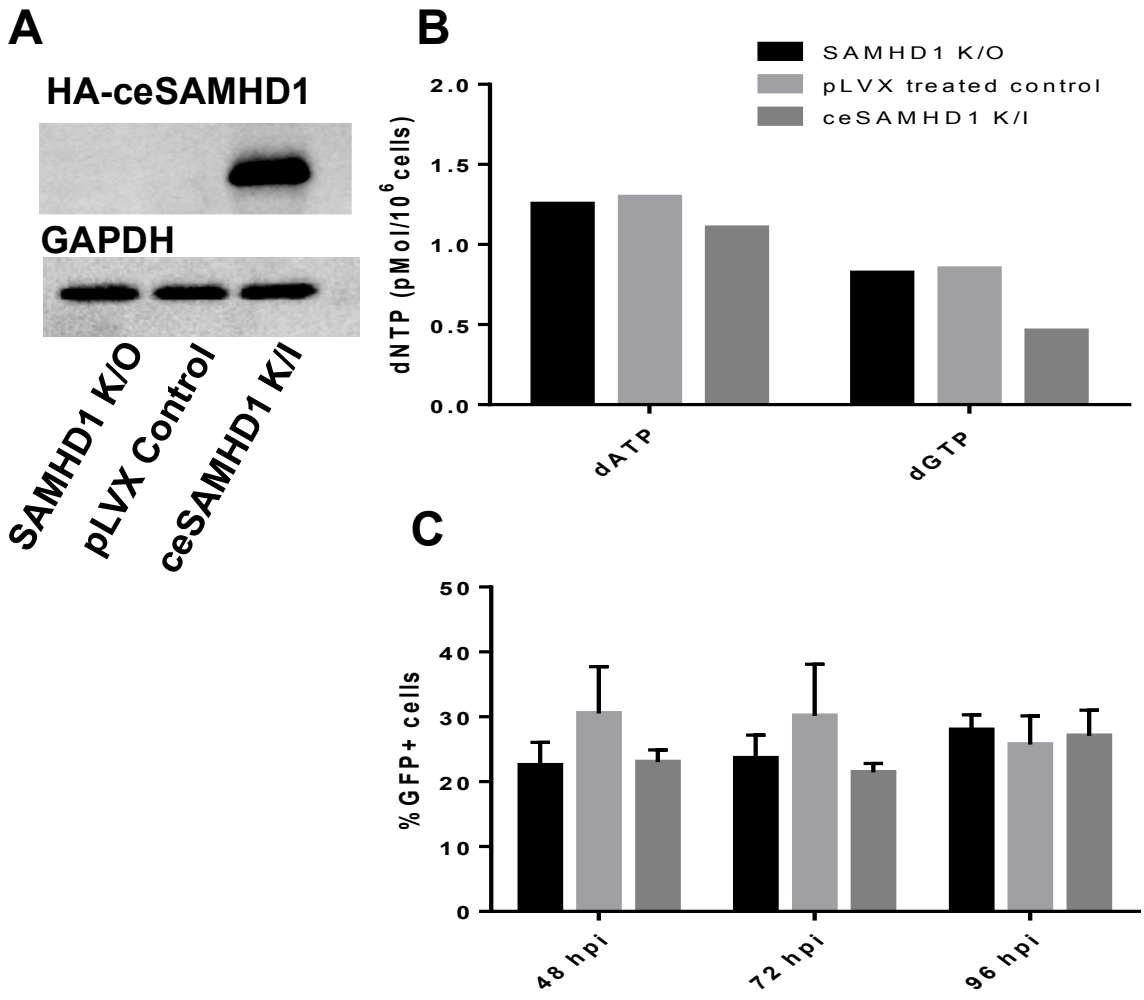

Figure 5: Effect of ceSAMHD1 expression on cellular dNTP levels and anti HIV-1 restriction activity of ceSAMHD1 in human THP-1 cell model. (A) Expression of HA-tagged ceSAMHD1 in THP-1 cells lacking its own SAMHD1 expression. The SAMHD1 knockout THP-1 cells, which we previously established (65), were transduced with pLVX-IRES-mCherry based lentiviral vector with (+) and without (-) HA-tagged ceSAMHD1 expression. The mCherry+ cells were FACS-sorted and the three independent clonal cells were propagated. The PMA-treated mCherry+ cells were then analyzed for ceSAMHD1 expression by western blots with HA tag antibody. **(B)** dNTP

levels in PMA-treated SAMHD1 KO cells with and without ceSAMHD1 expression. $2x10^6$ SAMHD1 KO cells with and without ceSAMHD1 expression were lysed for dNTP extraction, and the dNTP levels in these cells were determined by HIV-1 RT based dNTP assay (14). **(C)** Restriction of HIVD3-GFP vector by ceSAMHD1. The PMA-treated SAMHD1 KO THP-1 cells with and without ceSAMHD1 expression were transduced with an equal p24 level of HIVD3-GFP vector, and the GFP+/mCherry+ cells were determined by FACS at various time points post transduction.

EXPERIMENTAL PROCEDURES

Plasmids- Total C. elegans RNA was kindly provided by Dr. Bill Kelly (Emory University). ZK177.8 cDNA was reverse transcribed using SuperScript III Reverse Transcriptase (ThermoFischer) and primers listed in Table S1. The ZK177.8 cDNA was cloned into pGEX5x-3 (GE) and pLVX-IRES-mCherry (Clontech).

ceSAMHD1 Expression and Purification- The ZK177.8 gene product (ceSAMHD1) with an N-terminal GST tag was expressed in Rosetta DE3 cells (Novagen) by inducing with 0.2 mM IPTG at OD 0.5-0.8 for 48 hrs at 16 °C. Cells were harvested by centrifugation at 4,000 x g for 30min, followed by sonication in lysis buffer containing 40 mM Tris HCl at pH 7.5, 250 mM KCl, 5% glycerol, 0.1 % Triton X-100, 5 mM Beta mercaptoethanol (β-Me), 0.1mM PMSF and 0.5mM Benzamidine. Cleared lysate was obtained by centrifugation at 39,000 x g and applied to a GSTrap FF column (GE Healthcare) that had been equilibrated with binding buffer containing 50 mM Tris-HCl pH 7.5, 10 % glycerol, 250 mM KCl, and 5mM β-Me. The column was washed for 20 Column Volume (CV) with the binding buffer, followed by 5CV wash with increased KCl (1M final). The column was re-equilibrated for 5 CV with protease cleavage buffer containing 100mM NaCl and 2mM CaCl2, and Factor Xa (New England Biolabs) was added (50 U/CV) to the column and allowed to cleave overnight on-column at 4°C. The protein was then eluted with the protease cleavage buffer. Fractions containing ceSAMHD1 were combined and further purified on Superdex S200 10/300 with gel-filtration buffer containing 50mM Tris HCl at pH 7.5, 20% glycerol, 150mM KCl, 1mM β-Me, and 0.25mM EDTA and the fraction corresponding to the dimer of ceSAMHD1 (major product) were combined and flash frozen in liquid nitrogen and stored in -80°C

until use. Purity of the purified ceSAMHD1 is determined to be >95 %, as judged by SDS-PAGE (Fig. 1).

dNTPase Assay- Reactions were performed and analyzed as described previously (15) with the following modifications: the reactions were carried out at 21°C for varying time points up to 60 minutes and the TLC plate was analyzed using a phosphorimager (Pharos FX Plus Molecular Imager, Biorad). The percent of product formed was determined using densitometry analysis by Image Lab 5.2.1 (Biorad) and dividing the triphosphate product by the lane total.

Tetramer Formation Assay- Tetramer formation reactions were performed with 20uM protein and 2,000uM dGTP with a final concentration of 50mM KCl and 5mM MgCl2. The mixtures were incubated on ice for 30min and 5 min at room temperature. Formaldehyde at a concentration of 2% and 1M glycine were added and mixtures were incubated for 15min at 22°C. Reactions were analyzed by running on at 4-15% gradient SDS-PAGE gel.

ceSAMHD1 Activator Assay- Reactions were performed in buffer containing 50mM Tris-HCL pH 8.0, 50mM KCl, and 5 mM MgCl2. ceSAMHD1 at a concentration of 400uM was incubated with 1mM dNTP with or without 50uM of the indicated activator. Reactions were performed at 21°C for 60 minutes, stopped by heating at 70°C for 10 minutes and centrifuged at 14,000 x g for 5 minutes. Reactions were diluted 5-fold with acetonitrile (10%, final) and analyzed by anion exchange HPLC using a DNAPac PA100 Nucleic Acid Column (Thermo Scientific). The column was equilibrated with buffer containing 25mM Tris-HCl pH8.0 and 0.5% acetonitrile, and products were eluted with increasing amount of Ammonium chloride up to 250mM. Elution of the products was

monitored at 254 nm and peaks were quantified using 32 Karat software (Beckman Coulter).

Cell Lines- 293FT or THP-1 cells were cultured in DMEM or RPMI, respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Differentiation of THP-1 cells into non-dividing macrophage like cells was induced by treatment with 100ng/mL of PMA.

Virus Production- VSV pseudotyped lentivirus was produced by transfecting 293FT cells with 25ug pSPAX2, 25ug pLVX-IRES-ceSAMHD1-mCherry, and 10ug VSV-G encoding plasmid. Media was changed 16 hours post transfection. HIV-D3-GFP was produced as described previously (14) by transfection of 293FT cells with 25ug of D3 plasmid and 5ug of VSV-G encoding plasmid. Viruses were harvested 48hpi and concentrated by ultracentriguation at 22,000 rpm for 2h at 4°C.

Infection- Differentiated THP-1 cells were infected as described previously (65) and analyzed by flow cytomtery (Miltenyi MACSQuant) 72 hpi.

Western Blot Analysis- Whole-cell extracts were prepared from THP-1 cells with buffer containing 1% NP-40, 1.25% deoxycholate, 0.1% SDS, 0.1mM DTT, and 2.5mM PMSF. Mouse anti-HA antibody and rabbit anti-GAPDH antibody were purchased from Santa Cruz Technologies. Anti-rabbit IgG and anti-mouse IgG antibodies were purchased from GE Healthcare.

dNTP Extraction- dNTPs were extracted from THP-1 cells as described previously (14). Briefly, $2x10^6$ cells were washed twice with 1x Dulbecco's phosphate-buffered saline and resuspended in 200uL ice cold 65% methanol. Samples were vortexed, heated at 95°C for 3 minutes, and centrifuged for 3 minutes at 14,000 rpm. The supernatant was transferred

to a new tube and dried in a CentriVap Complete Vacuum Concentrator (Labconco). Samples were stored at -80°C. Prior to use, samples were resuspended in buffer containing 50mM Tris-HCl pH 8.0 and 10mM MgCl2.

Single Nucleotide Incorporation Assay- Concentrations of dNTPs in the dNTP extracts were determined using a single nucleotide incorporation assay described previously (73). Two microliters of dissolved dNTP sample were used in each reaction and the reaction products were analyzed on a 14% urea-PAGE gel. The gel was imaged using a phosphorimager (Bio-rad) and analyzed using Quantity One software (Bio-rad). Using the previously determined standard curves, the concentrations of dNTPs were determined.

Chapter III: Conclusion

Aicardi-Goutières Syndrome (AGS) is a genetic autoimmune disorder that causes severe development delays in those affected by it. AGS can be caused by mutations in several genes which are involved in cellular nucleic acid sensing and regulation. This thesis described my attempts to validate *C. elegans* as an animal model for studying AGS caused by mutations in Sterile Alpha Motif and HD domain containing protein 1 (SAMHD1), which is a dNTP triphosphohydrolase.

This study discovered that *C. elegans* encodes a SAMHD1 ortholog, ceSAMHD1. ceSAMHD1 is a dNTP triphosphohydrolase like its human counterpart. Further, ceSAMHD1 is activated by dGTP and GTP and forms tetramers similarly to human SAMHD1. SAMHD1 is unable to degrade nucleoside analogs that lack a 3'OH and this also holds true for ceSAMHD1. SAMHD1 is also capable of degrading dNTPs in nondividing human cells thus kinetically delaying HIV-1 infection. However, when ceSAMHD1 was introduced into nondividing human cells lacking SAMHD1 it was not capable of regulating dNTP levels nor delaying HIV-1 infection. It should be noted that all of the biochemical assays were carried out at 22°C to achieve maximum efficiency of ceSAMHD1; ceSAMHD1 was noted to be less efficient when reactions were carried out at 37°C.

It was previously reported that knockdown of the gene ZK177.8, which codes for ceSAMHD1 lead to maternal sterility (39). When these experiments were repeated in collaboration with the Kelly lab, no developmental defects, including maternal sterility, were observed. Analysis of the ZK177.8 knockdown efficiency using qRT-PCR were

inconclusive (data not shown). Further studies are needed to determine what role ceSAMHD1 plays *in vivo*. Does ceSAMHD1 control dNTP levels in *C. elegans*? Does ceSAMHD1 exhibit nucleic acid binding/ RNase activity? These are some of the questions that remain to be addressed. Additionally, it would be interesting to observe if knockdown of ceSAMHD1 effects activation of cer-1, a *C. elegans* retrotransposon. Chimeras containing the human SAM domain and the *C. elegans* HD domain would be useful in complementation studies for identifying functions of the SAM domain.

Based on these results, ceSAMHD1 could serve as a useful model for studying SAMHD1 functions *in vitro*, however no evidence was seen for ceSAMHD1 to serve as a model *in vivo*. Additional work is needed to determine an appropriate model for studying the clinical AGS phenotype *in vivo*.

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