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Site Directed Mutagenesis, Expression and Enzymatic Studies of the 60 kDa Human HIV-TAT 1 Interactive Protein, TIP60

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SITE DIRECTED MUTAGENESIS, EXPRESSION AND ENZYMATIC STUDIES OF THE 60 kDa HUMAN HIV-TAT 1 INTERACTIVE PROTEIN, TIP60

by

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Under the Direction of Dr. Yujun George Zheng Ph.D.

ABSTRACT

Tip60 is a 60 kDa nuclear protein which exists in three isoforms, belongs to the MYST/HAT family of proteins and was discovered after its interaction with the Human HIV-1 Tat. As a nuclear protein, Tip60 can act as a coactivator or repressor. To understand the HAT action of Tip60, two possible catalytic models exist; the ping-pong and the ternary complex formation models. In correlation with the exploration of HAT catalytic action, mutations of a Cys to Ala and a Glu to Gln on Esa1 (yeast homolog of Tip60 and MYST/HAT prototype), was reported to show wild type-like and decreased acetylating properties, respectively. In this work, Tip60 HAT action was explored. In Tip60, the Cys in the active site is important for acetylation of the H4(1-20) substrate and the Glu showed semi loss in acetylating the H4(1-20) peptide substrate. These data highlight a unique mechanism of Tip60 catalysis.

INDEX WORDS: Tip60, Site-directed mutagenesis, HATs, MYST family HATs, Histones, Chromatin, Chromatin modification, Post-translational modification, Tip60 catalysis, HAT assay, His-tagged Protein Expression, Histone acetylation and HAT inhibitors

SITE DIRECTED MUTAGENESIS, EXPRESSION, AND STEADY-STATE KINETIC STUDIES OF THE HUMAN HIV-TAT 1 INTERACTIVE PROTEIN, TIP60

by

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DEDICATION

I dedicate this thesis to my parents, my late mom, Christine Singui Elouti, who passed away on June 21st, 2005 and to my father whom I'll forever be thankful. They both instilled in me the spirit of perseverance, positivity and hard work. To my sisters, Mary, Patricia, Susana and Johana (AKA Joey) and my brother Irenous Elangwe, thank you for your encouragement and support both physically and spiritually. To MohammedAli Khokhar, thank you for your support.

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CHAPTER 1

INTRODUCTION

Site directed mutagenesis is a widely used scientific technique first described in 1978 by Michael Smith (Hutschison, *et al.*, 1978, Smith, 1993). This method allows for the introduction or induction of point directed mutations to a known oligonucleotide (wild type) in order to understand the effects of the mutation(s) on a particular biomolecule. Also, this point directed mutation at a defined site of an oligonucleotide requires a circular biomolecule called a plasmid inserted in a vector with the features of interest. The mutations could either be done by insertion/substitution of an amino acid or by the deletion of a sequence of interest (Carter, P., 1986). The most common technique for this mutation is the PCR based site-directed mutagenesis. In this technique, an oligonucleotide complementary to part of a single stranded DNA template, but containing an internal mismatch, is used to direct the mutation during the thermal cycle (Carter, P, 1986). Before site directed mutagenesis becomes an option during research, it is imperative to understand the DNA structure.

DNA is a 6 feet long polymer of repeating nucleic acid bases found in the nucleus of most eukaryotes. This structure was referred to as a salt with ‘an angle of 36° between adjacent residues in the same chain, so that the structure repeats after 10 residues on each chain, that is after 34 Å (Watson and Crick, 1953). DNA is a double stranded biomolecule which contains the genetic material of most organisms. It exists in many forms of which three are commonly referred to (A-DNA, B-DNA and Z-DNA forms). The B-form DNA is the most common under normal cellular conditions. Within cells, DNA is organized into structures known as chromosomes. Chromosomes are nuclear structures that consist of DNA and proteins. Chromosomes contain many genes; regulatory elements like the proteins bound to DNA that

control DNA function and other nucleotide sequences. In humans and other eukaryotes, chromosomes are packaged by proteins into a structure known as chromatin.

Chromatin is a packaged structure of about 6 feet long of DNA material found in the nucleus of most eukaryotic genomes. As mentioned earlier, it is composed of DNA and proteins in which the proteins are mainly histones. Histones are small proteins that contain a high amount of basic amino acids, mainly arginine and lysine, and facilitate the packaging of DNA in the nucleus (Kuo and Allis, 1998; Wolffe, A.P., 2001). After numerous researches and studies, it is believed that chromatin structure plays a role in most nuclear processes (gene regulation) which include transcription, DNA replication and repair, mitosis and apoptosis (Cheung *et al.,* 2000). An understanding of the gene regulation role of chromatin could give major leads in therapeutic drug targets against modern killers like cancer and HIV. Also, after intensive research, it was reported that most of the proteins contained on chromatin are histones. These histones can be modified in many different ways in order to suit the purpose of the research which leads to regulation of chromatin activity. Taken together, chromatin bears pivotal epigenetic information for development and maintenance of genomic integrity (Fischle, W., 2008).

So what are histones and what are they made of? Histones are the main protein components of chromatin on which DNA is wrapped around that play major roles in chromatin functioning and gene regulation. There are six different histones that have been identified: H1, H2A, H2B, H3, H4 and H5, all bundled up into the histone core (H2A, H2B, H3 and H4, which are highly conserved in most eukaryotes) and a linker (H1 and H5) (Jasencakova *et al.*, 2000). Two of the core histones, H2B and H4, assemble in the form of an octomer to constitute a main eukaryotic nuclear structure known as a nucleosome. These histones are a main target for chromatin regulation because their N-terminal is rich with lysines (K) and arginines (R) which are

positively charged amino acids and enable DNA to bind very tightly to it. Since DNA is wrapped around these histones with their N-terminal (amino-terminal) tails hanging outside of the structure, they make easy targets for post-translational modifications and provide easy access to DNA (Silverman *et al.*, 1973; Robin *et al.*, 2007). These arginine or lysine side chains could be modified in five different ways; acetylation, methylation, ubiquination, sumoylation and phosphorylation. The most studied post-translational modifications of histones are acetylation of the ε-lysine residue, methylation of both arginine and lysine residues and phosphorylation mostly on serine and threonine residues (Robin *et al.*, 2007). Amongst the three most studied histone post-translational modifications, the best studied is acetylation. Acetylation is a reversible process that neutralizes the positive charge of the lysine residue and can occur on all four core histone tails (see figure 2). The reverse process of acetylation is known as deacetylation and is done by histone deacetylases (HDACs). Understanding how these HDACs influence chromatin regulation is an important issue towards understanding the effects of HATs on chromatin regulation (Holbert and Marmorstein, 2005). Histone acetyltransferases (HATs) are an important class of epigenetic enzymes involved in chromatin restructuring and transcriptional regulation.

Post-translational changes are best achieved using the concept of epigenetics. Epigenetics is defined as the biology of heritable changes in gene expression without changes in the DNA sequence. The beauty of this approach is the fact that the genes can be modified without alterations of the coding sequence (Wu *et al.*, 2008). Not surprisingly, the deregulation of epigenetic mechanisms is linked to cancer, aging, hereditary and neurodegenerative diseases.

1.0 Post-translational modification of histones by histone acetylransferases (HATs)

After the RNA-polymerase involved in DNA transcription has completed making a copy of one strand of the DNA is released, this strand (known as the mRNA strand) is released from

the nucleus through nuclear pores and into the cytoplasm. Once in the cytoplasm, this mRNA strand binds to a ribosome to begin the process of translation. This translation process is the decoding of the nuclear coding to the amino acid coding which constitute a protein. The protein created through translation then undergoes post-translational modifications where the protein folds into its functional conformation (Campbell, Neil and Reece, Jane. Biology, 7th edition). The processes of transcription, mRNA processing and translation lead to the outward expression of a gene which is the protein. The protein can then carry out its function whether it is as a structural protein, a transport protein, or an enzyme; this completes the process of gene expression (Campbell, Neil and Reece, Jane. Biology, 7th edition). One of these proteins could be a histone. Histones, mostly found on chromatin, are structural modules to which DNA winds on and therefore play an important role in gene regulation. Since histones are bound to DNA and can be modified, it is possible that certain processes involving DNA can either be promoted or inhibited by specialized enzymes in the body. As early as the 1950s, histones were reported by Stedman and Stedman to have an inhibitory role on nucleic acid synthesis, particularly with RNA (Stedman and Stedman, 1951). This inhibitory aspect raised an enormous interest amongst scientists back then, so much so that, Huang and Bonner proved that histones, which protected DNA from thermal denaturation, also blocked DNAs action as a sequencing initiator (Huang and Bonner, 1962). All these histone modifications, particularly acetylation, are achieved with the help of enzymes such as HATs.

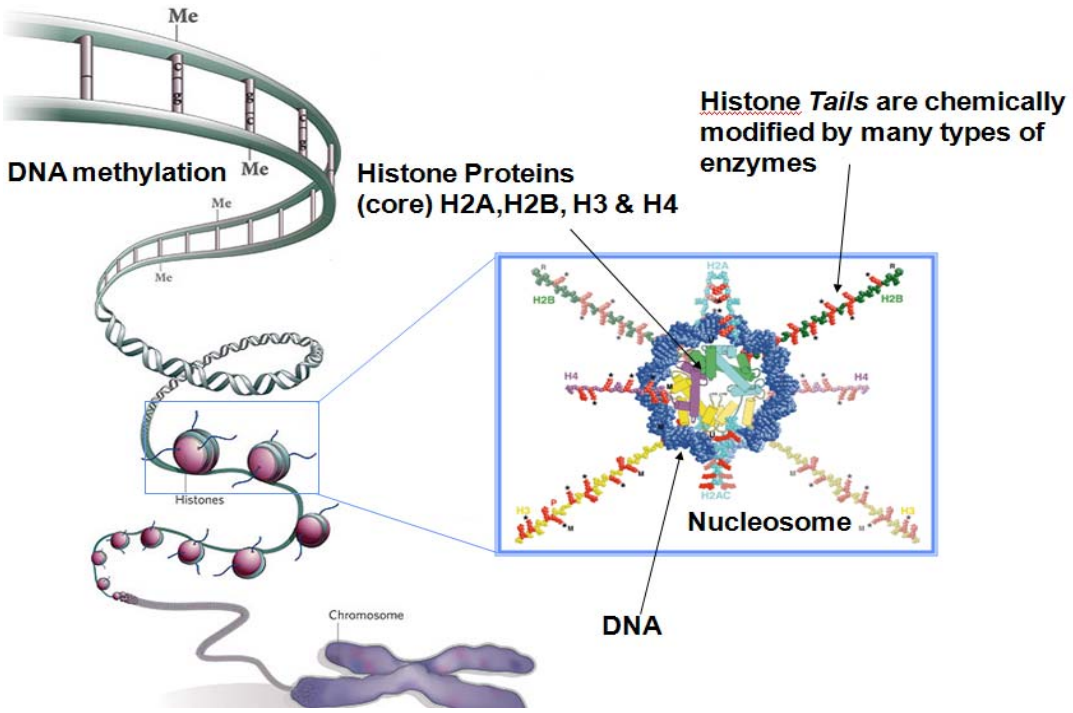


Figure 1: The epigenetic control of cell function

HATs are essential chromatin modifying enzymes that participate in multiple cellular processes which include but are not limited to transcription regulation through acetylation. HAT is an important class of epigenetic enzymes involved in transcriptional regulation. Transcriptional regulation, which is after the polypeptide has formed (post-translational) allows for chromatin modification by acetylating the histone substrate or by self-acetylating (Allfrey *et al.,* 1964). Depending on the acetylating enzyme, certain lysine residues on the side chains of core histones in the nucleosome could be acetylated using acetyl-coenzyme A (acetyl-CoA) for this process. By acetylating the lysine residue (s), DNA would bind less to the histones thus allowing for access to the chromatin and facilitating modifications and control. Since any nuclear process is highly regulated, it was reported by Bird *et al.* that in order for DNA to be repaired, it is imperative that the damaged DNA be acetylated (Bird *et al.*, 2002). This aspect of histone acetylation by HAT enzymes is of much interest that many HATs have been identified and

characterized. We are interested in studying and characterizing the MYST family of HATs.

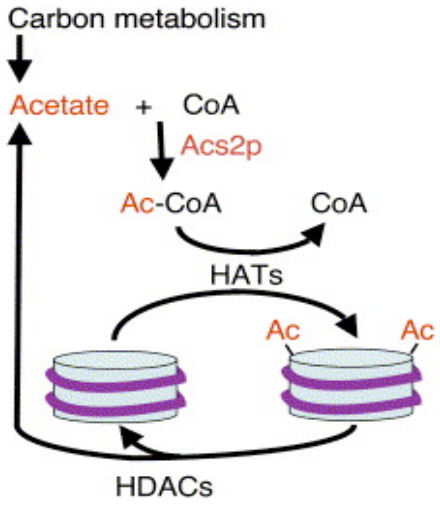


Figure 2: The reversible acetylation mechanism of the nucleosome

1.1 MYST family HATs

The MYST domain of HATs is a 370 amino acid residues region which is highly conserved in all MYST family member proteins. As can be seen in Figure 5, the highly conserved MYST domain in all of its family members contains an acetyl-CoA binding motif, zinc-binding domain within the HAT regions and an N-terminal chromodomains. The name MYST derived from its four founding members from both yeast and mammals, MOZ, Ybf2/Sas3, Sas2 and Tip60. Now, this family includes four human HATs (not counting Tip60 since it had been mentioned before); Tip60, MOZ, MORF, HBO and MOF (Kimura *et al.*, 1998; Goyon *et al.*, 2007). Most of what is known today about MYST family functions is based on studies of model organisms, for example yeast. Because MYST family proteins have certain subunits in common with other HATs and nuclear proteins, they are capable of modifying chromatin which in turn regulates cell growth (Lafon *et al.,* 2007; Goyon *et al.*, 2007). These members possibly utilize a cysteine present in the active site for their catalytic activity.

Esa1 is a HAT catalytic subunit of the native multisubunit complex (NuA4; nucleosome acetyltransferase 4). Esa1 stands for essential sas2-related acetyltransferase 1. As a result of the resolved crystal structure of the yeast homolog of Tip60, Esa1 and other model MYST proteins including mouse and *Drosophila,* most of the catalytic characteristics of MYST members are known (Hodawadekar *et al.,* 2007.). After the resolved structure of Esa1 and several experiments carried out, it was established that the target of all H4 residues by Esa1 for acetylation was necessary for yeast cell growth (Pillus *et al.*, 2008). Because of this acetylating property and a MYST/HAT family member, this mechanism was determined to be of commonality with the other members thus relating it to DNA double-strand break repairs (Bird *et al.*, 2002; Berndsen *et al*., 2007).

MOZ (monocytic leukaemia zinc-finger protein also known as MYST3)/MORF (MOZ-related factor also known as MYST4) are oncogene regulatory proteins with structural and functional similarities (Doyon *et al.*, 2006). MORF and MOZ function as co-activators of Runx1- and 2-dependent transcription. Since MORF was discovered three years later after MOZ was identified by Borrow *et al.*, it was reported to be the first fusion associator of the CREB-binding protein (CBP/p300 HATs) during a study on acute myeloid leukaemia (AML) (Borrow *et al*., 1996; Utley *et al.*, 2003, Thompson *et al.*, 2004, Avvakumov *et al*., 2007). MOZ/MORF was found to form a complex with ING5 for H3 targeting in cells. ING (INhibitor of Growth) is a group of type II tumor suppressor proteins which are involved in many cellular processes such as apoptosis, DNA repair and tumorigenesis. They are of five categories, ING1-ING5, and also play an essential role in acetylation of chromatin substrates during DNA repair (Gordon *et al*., 2008). The role of MOZ and MORF in the maintenance and development of hematopoietic stem

cells in humans and neutral stem cells in rat, respectively have been reported (Katumoto *et al.,* 2006; Thomas *et al.,* 2006).

HBO1 (histone acetyltransferase binding to ORC1 (origin recognition complex), also known as MYST2), is one of the recently discovered human MYST/HAT family proteins which contribute to oncogenic transformation and is required by ING4 for the normal progression of cell growth through the S phase and the majority of H4 histone acetylation in vivo (Doyon *et al.,* 2006). It has also been reported to positively regulate pre-RC assembly and initiate DNA replication. Two hybrid screening by Georgiakaki *et al.* reported that a correlation between the MYST protein, HBO1 with progesterone receptor (a member of the steroid receptors family) required the steroid receptor co-activator SRC-1 to enhance PR-dependent transcription (Georgiakaki *et al.,* 2006). This is an interesting study because steroid receptors and their coactivators play key regulatory roles in gene expression (Lonard and O'Malley, 2006) and participate in signaling pathways which are susceptible to misregulation in cancer cells (Lafon *et al.,* 2007).

The MYST/HAT family member of interest in this research is the human interactive Hat-1 protein, Tip60.

1.2 Tip60 as a MYST family HAT

Tip60 is a 60 kDa intrinsic nuclear protein which was first identified as an acetyltransferase protein which interacts with HIV-1 Tat. It contains intrinsic acetyl transferase activity which indicates that it has potential in chromatin remodeling and gene regulation. It is located at chromosome 11q13.1 and consists of 14 exons which if spliced otherwise will lead to the expression of its most common variants: isoform 1, isoform 2 and isoform 3 (Sapountzi *et al*., 2006). Isoform 2, also referred to as Tip60α (domain structure shown in Figure 5; and referred to

as Tip60 in this entire paper unless otherwise stated), is the most studied and characterized full length form of Tip60 which was used for this experiment (Sapountzi *et al.,* 2006). As can be seen in Figure 3, isoform 2 encodes 513 amino acids, an N-terminal chromo-domain, N-terminal zinc-finger like region and a C-terminal conserved MYST domain (Red: alternatively spliced intron 1, Yellow: exon 5, Green: other amino acid residues and Cyan: MYST domain (http://atlasgeneticsoncology.org/Genes/HTATIPID40893ch11q13.html)).

The chromodomain is the chromatin modifier region, the zinc-finger like domain which was not too long ago reported to be a target by HDAC7 for de-acetylation is essential for acetyl transferase activity and for protein-protein interaction (Xiao *et al.,* 2003; Sapountzi *et al.*, 2006). The MYST domain defines Tip60s membership to the MYST family of histone acetyl transferases (HATs).

Although the HAT activity of Tip60 is well known, it is still unclear whether that activity is necessary during the process of DNA transcription or is it before or after the process of transcription. Knowing that Tip60 is capable of affecting DNA transcription, Tip60 was also thought to affect that process by either acting as a co-regulator, a co-repressor, or as a co-activator. However, whether its acetyl transferase activity is required in DNA transcription still needs to be understood (Kim *et al.*, 2006). This co-regulator and co-activator functions increase the already diversified function of Tip60 to participate in many processes which include but not limited to cellular signaling, DNA damage repair, cell cycle and with the introduction of checkpoint control and apoptosis (Kusch *et al.*, 2004, Latrasse *et al.*, 2008).

To support the fact that Tip60 could act as a transcription enhancer and co-activator, recent data by Beekum *et al.* suggested that Tip60 acts as a positive regulator of PPARγ (peroxisome proliferator-activated receptor γ) (Beekum *et al.,* 2008). This adipogenic

transcriptor (PPARγ) functions by recruiting Tip60 in the cell, which relies on the N-terminal activation function 1 of PPARγ, and by using chimeric proteins Beekum *et al*. confirmed that Tip60 acted as a co-activator. PPARγ is a family of transcriptional activators which regulate lipid and glucose metabolism in adipocytes.

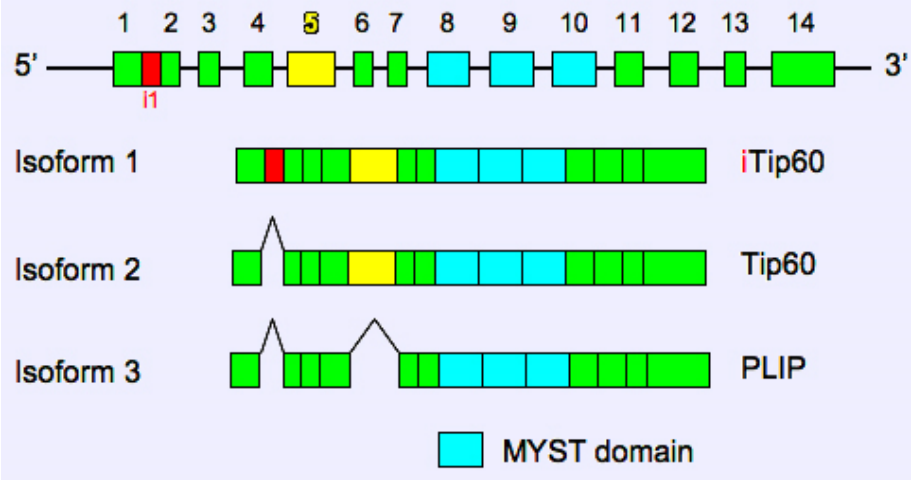


Figure 3: The various isoforms of Tip60 that have been identified

1.3 Involvement of Tip60 in nuclear receptor activity and prostate cancer

Depending on the cell type or cellular process, Tip60 forms stable complexes with the appropriate binding partners and can either up-regulate or down-regulate the process of transcription by its HAT activity (Dohmesen, 2006, Brady *et al*., 1999; Creaven *et al*., 1999; Hlubek *et al.,* 2001). Also, Tip60 has been reported to act as both an activator and an inhibitor of certain cellular activities. Sun *et al.* reported that Tip60 acted as an activator by forming a complex with ATM (ataxia telangiectasia mutant) thus activating it (Sun *et al.*, 2005). However, it is worth noting that ATM's kinase activity is not initiated by the complex formation of Tip60-ATM, but that DNA damage activates the complex formation. ATM is a protein kinase which regulates cell response to DNA damage (Sun *et al.,* 2005, Nunnari *et al.*, 2008). Also, Tip60 uses its extreme C-terminus (see figure 5; blue region from 390 – 513) which contains an NR box to mediate interaction with androgen receptors (AR; estrogen and progesterone) (Gaughan *et al*.,

2001; Brady *et al.*, 1999). Although Tip60 is reported to act as a mediator to enhance and up-regulate the transactivation of these ARs, it is limited to class I NRs which include estrogen receptors α and β (ERα and ERβ) and glucocorticoid receptor (GR).

Nuclear receptors (NRs) are ligand-activated transcription factors which are subdivided into 7 subfamilies based on their amino acid sequence. Tip60 at low ligand concentration or in the absence of the ligand influences the development of androgen escape by activating the AR (Edwards and Bartlett, 2005). Since NRs are highly conserved across species, studying them could facilitate research. So far, there are about 48 identified NRs in the human genome. In order for these NRs to function, they must bind to receptor sites to elucidate their functions. Their binding to receptors could either be inhibited or activated by certain enzymes. Earlier reports suggested that for NRs to modulate their specific targets and perform their transcriptional activation functions efficiently, help from certain factors was needed. This resulted in the identification of common NR cofactors (Conneely *et al.,* 1989; Tasset *et al.*, 1990). One of the NR co-activator enzymes that have been identified is Tip60. Brady *et al.* have reported that Tip60 acts as a nuclear receptor co-activator for AR by direct interactions through the LBD (Ligand binding domain) of the AR (Brady *et al*., 1999). In the same sense that Tip60 acts as an NR co-activator, Frank *et al.* reported that Tip60 is recruited by the transcription factor MYC to target genes for acetylation in response to mitogenic signals (Gorrini *et al,* 2007, Frank *et al*., 2003, Taubert *et al.*, 2004). MYC is a transcription factor which induces histone acetylation after binding to DNA and is expressed in proliferating and mitogen-stimulated cells (Bouchard *et al.,* 2001; Fernandez *et al.*, 2003; Frank *et al.*, 2001). Figure 4 shows the oncogenic connections of Tip60.

The AR signaling pathway is necessary for the development of prostate cancer and since Tip60 has been reported to act as a co-activator of AR, Tip60 is being investigated for the purpose of finding potential cures for prostate cancer. Using immunohistochemical evaluation, it was determined by Ruizeveld De Winter *et al.* that AR protein is present in primary, metastatic and hormone refractory prostate cancer (HRPC) regardless of the tumor stage. Despite the fact that functional signaling of androgen receptor is necessary for the development of prostate cancer, its exact role in this process is not well known (Ruizeveld De Winter *et al.*, 1991; Taplin *et al.*, 2007). The development of new strategies and new drugs that more effectively terminate AR signaling will probably result in important clinical benefits. Co-regulatory proteins, like Tip60, can act as potential drug targets when an imbalance between AR co-activator and co-repressor which result in the advantageous growth of prostate cancer cells occurs. Indeed, Halkidou *et al.* studied Tip60 and reported that 87 % of HRPC specimens showed nuclear accumulation of Tip60 compared to a more diffuse cellular distribution in noncancerous or androgen-dependent cancer (Halkidou *et al.*, 2003).

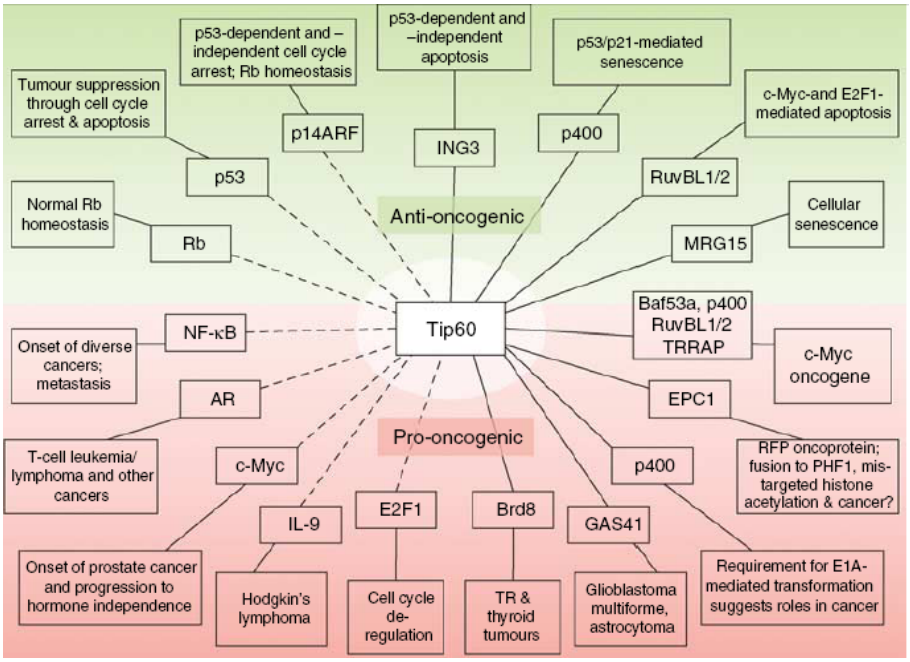


Figure 4: The anti-oncogenic (green) and pro-oncogenic (pink) connections of Tip60

1.4 Structure and catalytic activity of Tip60

As mentioned earlier, Tip60 is a member of the MYST family of HAT proteins in which the MYST domain is well conserved from yeast to mammals. This short sequence binds to acetyl coenzyme A and substrates (for example, H4(1-20) peptide) and allows for substrate acetylation. Also, as described in the above section, Tip60 is capable of playing a role in damaged DNA repair and apoptosis. The mechanism by which Tip60 does this is yet to be fully understood. Although the catalytic mechanism of two prototypes of the MYST family (Gcn5 and Esa1) has been studied and reported, the acetylating mechanism of Tip60 is still uncertain because not to long ago, it was reported that Tip60 used the formation of a ternary complex to catalyze its substrate(s) contradictory to an earlier report that Tip60 used a double displacement mechanism for its catalytic activity. All these contradictory reports were based on an active fragment on the crystal structure of the yeast homologue of Tip60, Esa1 (Essential sas2-related acetyltransferase 1), which favored a ping pong mechanism involving residues Cys304 and Glu338 for catalysis (Decker *et al.*, 2008). Because of this contradiction, the catalytic activity of Tip60 is uncertain (Yan *et al.*, 2002; Berndsen *et al.*, 2007).

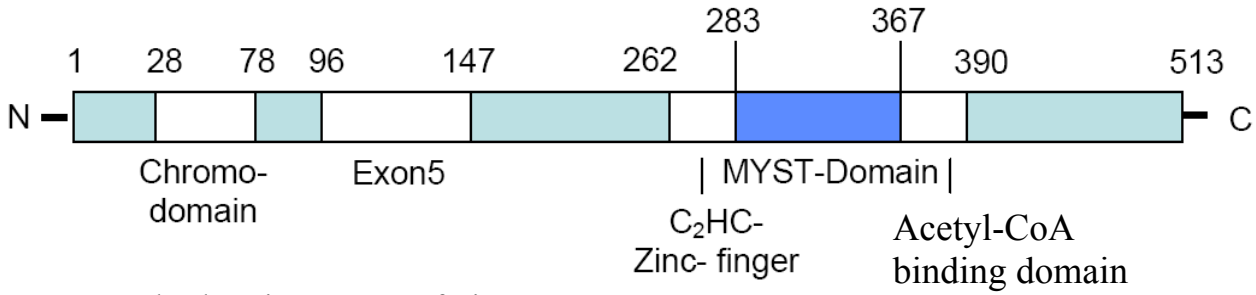


Figure 5: The domain structure of Tip60

The catalytic mechanism that has been suggested by Berndsen *et al*., is the formation of a ternary complex between the enzyme and the H4 histone substrate. In this mechanism, acetyl-CoA binds first releasing CoA as a by-product. In a classical ternary complex, two substrates bind to the enzyme at the same time forming the complex (Figure 6). In the scheme shown

below, whether the acetyl-CoA binds first or the histone binds first leaves room for further investigation, if not the binding is referred to as random.

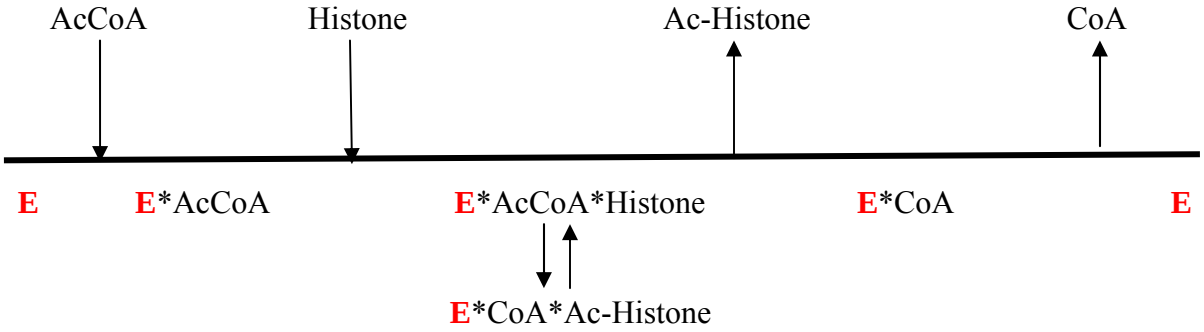


Figure 6: Catalytic mechanism by ternary complex formation

The use of ping pong mechanism by MYST/HATs was reported by Yan *et al.* (Yan *et al.*, 2002). This mechanism is also referred to as a double displacement mechanism because after the first substrate binds, the enzyme conformation is changed (Figure 7). This changed enzyme is known as the intermediate. The first substrate has to leave before the second substrate can bind and react with the altered enzyme while releasing the original enzyme. In the ping pong mechanism reported by Yan *et al*., the first step in the Esa1 HAT reaction is the deprotonation of Cys304 by Glu338 and nucleophilic attack transferring the acetyl moiety from acetyl-coenzyme A (AcCoA) to Cys304, creating an acetyl-Cys304 enzyme intermediate. Subsequently, Glu338 deprotonates the histone lysine residue, mediating the transfer of the acetyl group from acetyl-Cys304 to the ε-amino group of the lysine substrate (Yan *et al.,* 2002; Decker *et al.*, 2008). A scheme of this process is demonstrated below.

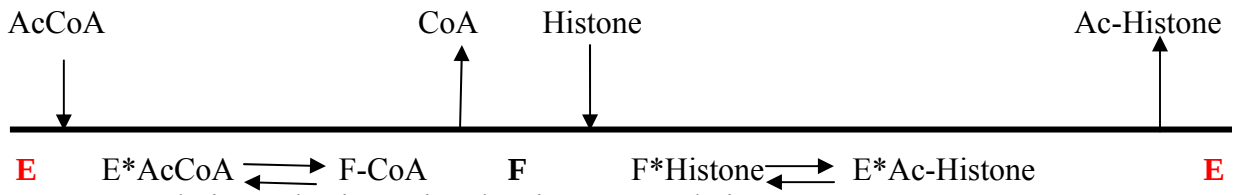


Figure 7: Catalytic mechanism using the ping pong technique

Understanding how HATs perform their substrate acetylation properties is essential for drug design and discovery. In this work, site-directed mutagenesis, protein expression and HAT radioactive catalytic assays were performed in order to characterize the residues present in the MYST/HAT Tip60 active site.

1.5 Questions to be asked about this work

The following questions should be answered in the course of this work:

1. What role does Tip60 play in post-translational modification?
2. Which domain(s) of Tip60 is required for catalysis?
3. What role does Cys play in the catalytic process?
4. What role does Glu play in the catalytic process?

CHAPTER 2

MATERIALS AND METHODS

The 513 amino acid Tip60 isoform 2 in a pET15b vector was used for this experiment.

2.0 Chemicals and abbreviations

Acetic acid (Glacial)

Acrylamide

Agarose electrophoresis grade

Ampicillin (AMP)

Ammonium persulfate (APS)

1,4-Dithiothreitol (DTT)

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

β - mercaptoethanol

Bovine serum albumin (BSA)

Coomassie brilliant blue

DNA polymerase

dNTP-mix

Ethanol

Ethidium bromide (EB)

Ethylene diamine tetraacetic acid (EDTA)

Ethylene glycol

Glycerol

Imidazole

Isopropyl β-D-1-thiogalactopyranoside (IPTG)

Magnesium chloride

Magnesium sulfate

Ni-NTA magnetic agarose beads

Phenylmethanesulphonyl fluoride (PMSF)

Sodium chloride

Sodium dodecyl sulfate (SDS)

Trisacetate buffer (TAE)

N, N, N', N'- tetramethylethylenediamine (TEMED)

Tris (hydroxyl methyl) aminomethane (Tris/HCl)

Triton-X 100

2.1 Enzymes

Native pfu DNA polymerase

2.2 Kit

A *Wizard Plus* SV Miniprep DNA purification system from Promega was used to extract the mutated DNA plasmid from bacteria cells.

2.3 Cell culture media and supplements

LB- Media	10 g Tryptone
	5 g Yeast extract
	5 g NaCl
	1 mL of 1 N NaOH
	Fill up to 1000 mL
NZY Media	1 mL LB media

12.5 µL 1M $MgSO_4$

12.5 µL 1M $MgCl_2$

20 µL 20 % Glucose

2.4 Bacteria cells

XL1 Blue competent cells	Stratagene
BL21(DE3) competent cells	Stratagene

2.5 Oligonucleotides

After designing the targeted mutation oligonucleotide, using both the protein sequence part to be mutated and the matching sequence on the plasmid, the 34-35 base pairs long oligonucleotides were obtained from IDT website.

TipCys369Ala fwd: 5'- GACTACAATGTGGCCGCCATCCTAACCCTGCCTC-3'

TipCys369Ala rev: 5'- GAGGCAGGGTTAGGATGGCGGCCACAT TGTAGTC-3'

TipGlu403Gln fwd: 5'- CAAGAGTTATTCCCACAGCCTGTGTCTTTTGGCC-3'

TipGlu403Gln rev: 5'- GGCCAAAAGACACAGGCTGTGGGAATAACTCTTG-3'

2.6 Buffers

Cell Lysis buffer	25 mM Na-HEPES, pH 7.0
	150 mM NaCl
	1 mM $MgSO_4$
	5 % Ethylene glycol
	5 % Glycerol
	1 mM PMSF
Column buffer	25 mM HEPES, pH 8.0

	500 mM NaCl
	1 mM PMSF
	30 mM Imidazole
	10 % Glycerol
Wash buffer	25 mM HEPES, pH 8.0
	300 mM NaCl
	1 mM PMSF
	70 mM Imidazole
	10 % Glycerol
Elution buffer	25 mM HEPES, pH 7.0
	300 mM NaCl
	1 mM PMSF
	10 % Glycerol
	1 M Imidazole
Dialysis buffer	25 mM HEPES, pH 7.0
	250 mM NaCl
	1 mM EDTA
	10 % Glycerol
	10 mM DTT

12 % SDS-PAGE Stacking gel (10 mL total volume)

30 % acrylamide 1.7 mL

dd H_2O 6.8 mL

1.5 M Tris-HCL pH 6.8 1.25 mL

10 % SDS 0.1 mL

10 % APS (ammonium persulfate) 0.1 mL

TEMED 0.01 mL

12 % SDS-PAGE Resolving gel (20 mL total volume)

30 % acrylamide 8.0 mL

dd H_2O 1.7 mL

1.5 M Tris-HCL pH 8.8 1.25 mL

10 % SDS 0.2 mL

10 % APS (ammonium persulfate) 0.2 mL

TEMED 0.008 mL

2XRB

100 mM HEPES pH 8.0

200 mM EDTA

100 μg/ml BSA

1 mM DTT.

2.7 Instruments

An MJ Mini personal thermal cycle from BIORAD was used for the PCR reaction. A PowerPac Basics from Biorad voltage provided was used for electrophoresis.

FRENCH® Press system from ThermoScientific was used.

Tj-25 Centrifuge from Beckman-Coulter and a 5415 D centrifuge from eppendorf.

UV-1700 PharmaSpec UV-Vis spectrophotometer from SHIMADZU.

An innova 2000 platform shaker from New Bunswick Scientific.

Typhoon 9400 Scanner from Amersham Biosciences.

2.8 Methods in molecular biology

Molecular biology is primarily involved in the understanding interactions as well as the regulation of those interactions that take place between various systems in a cell. These interactions include DNA, RNA and protein synthesis.

2.8a Site-directed mutagenesis using polymerase chain reaction (PCR)

The 34 (fwd) and 35 (rev) primer oligonucleotides shown below were designed by selecting the 34 oligonucleotides containing the position of (three oligonucleotides) to be mutated (CAG for Gln and GCC for Ala). These primers were then each used for the mutagenesis using PCR.

Primers:

TipCys369Ala fwd: 5'- GACTACAATGTGGCCGCCATCCTAACCCTGCCTC-3'

TipCys369Ala rev: 5'- GAGGCAGGGTTAGGATGGCGGCCACAT TGTAGTC-3'

TipGlu403Gln fwd: 5'- CAAGAGTTATTCCCACAGCCTGTGTCTTTTGGCC-3'

TipGlu403Gln rev: 5'- GGCCAAAAGACACAGGCTGTGGGAATAACTCTTG-3'

Table 2: PCR sample preparation for site directed mutagenesis

Samples	**1**	**2**	**3**
ddH_2O	36 µL	35 µL	32 µL
10X Native pfu buffer	5 µL	5 µL	5 µL
Tip60 in pET15b (20 ng/µL)	1 µL	2 µL	5 µL
Primer fwd (40	3 µL	3 µL	3 µL

ng/µL)			
Primer rev (40 ng/µL)	3 µL	3 µL	3 µL
10 mM dNTP	1 µL	1 µL	1 µL
Native pfu DNA polymerase	1 µL	1 µL	1 µL
Total volume	50 µL	50 µL	50 µL

Table 3: PCR program setup for site directed mutagenesis

Cycle	**T(°C)**	**Time**
1	95	30 sec
2	95	30sec
3	55	1 min
4	68	15 min
5	2-5	17 times
6	4	forever
7	end	

2.8b DNA amplification check in 1 % agarose gel electrophoresis at 200 V

For this assay, 50 mL of 1X TAE buffer was measured, 0.500 g of agarose powder weighed and microwaved for 2.5 minutes. The solution was then allowed to cool for a few minutes before 2 µL of EB was added. This solution was then poured in a gel plate and an 8 lane comb inserted in it to form the grooves/wells. The solution was then allowed to solidify at room temperature. The samples were then loaded at ran at 200 V in TAE buffer.

2.8c Transformation of XL1-Blue competent cells

XL1-Blue cells were obtained from Stratagene, 0.85 µL of the β-mercaptoethanol provided was then added to 50 µL of the cells, incubated on ice for 10 minutes, 3 µL of the DNA plasmid obtained after PCR quick change added, the tube was incubated on ice for another 30 minutes, heat pulsed at 42 °C (water bath) for 45 seconds, incubated on ice for 2 minutes again, 0.5 mL NZY solution to the tube later added and the tube incubated at 37 °C for 1 h (rotation speed 6). After the incubation, 50 µL of the solution was applied to one AMP (ampicillin) agar plate, and the rest was centrifuged at 6 rpm for 1 minute, and applied to another AMP agar plate.

The plates were then incubated at 37 °C overnight. The next day, a single colony from an agar plate was picked up, added to a culture tube containing 8 mL LB media, 8 µL of 250 mg/mL AMP added and allowed to rotate at 6 rpm at 37 °C overnight. The next day, the amplified DNA had to be extracted.

2.8d Isolation of DNA plasmid

The isolation of the Tip60 DNA plasmid in pET15b vector was performed using a Promega miniprep DNA systems kit. The cells after 8 mL inoculation in LB media and 250 mg/mL AMP, the cells were centrifuged for 10 minutes, at 3000 rpm 4 ºC.

Cell lysis: the cell pellets were re-suspended with 250 µL of cell re-suspension solution and transferred to a clean 1.5 mL eppendorf tube. 250 µL of cell lysis solution was then added to the tube, inverted 4 times to mix, next 10 µL of alkaline protease solution was added and mixed by inverting the tube 4 times. Next, the tube was incubated at room temperature for 5 minutes and 350 µL of neutralizing solution added. The tube and its contents were then centrifuged with a bench top centrifuge at room temperature, top speed for 1 minute.

Binding of plasmid DNA: a spin column was inserted into a collection tube; the clear lysant from the tube after centrifuge was poured into it and centrifuged at room temperature, at top speed for 1 minute. The clear lysate was discarded and the spin column re-inserted into the collection tube.

Washing: 750 µL of wash solution (with ethanol already added) was added to the spin column and centrifuged at room temperature, at top speed for 1 minute. The flow through was discarded, and another 250 µL of the wash solution added and centrifuged again this time for 2 minutes.

Elution: the spin column was then transferred to a clean 1.5 mL eppendorf tube and 40 µL of nuclease-free water added to the center, allowed to stand for 2 minutes and later centrifuged at top speed, room temperature for 1 minute. The flow through did contain the DNA plasmid thus it was collected and the DNA plasmid concentration calculated as below.

2.8e Measurement of DNA concentration

To 98 µL of ddH_2O, 2 µL of the extracted DNA plasmid was added and the absorbance measured at 260 nm with a UV-Vis instrument. The absorbance was obtained 3 times and averaged. This average was then used towards the calculation of the DNA concentration using the following formula: average absorbance * 0.05 * 50 mg/mL.

2.8f DNA sequencing

The extracted DNA plasmid whose concentration is now known was submitted for sequencing by preparing the sample as follows: the extracted DNA was diluted to a final concentration of 0.12 mg/mL with ddH_2O and submitted for DNA sequencing.

2.8g Transformation of BL21(DE3) competent cells

BL21(DE3) competent cells were obtained from Stratagene, 0.85 µL of the β-mercaptoethanol provided was then added to 50 µL of the cells, incubated on ice for 10 minutes, 3 µL of the DNA plasmid obtained after PCR quick change added, the tube was incubated on ice for another 30 minutes, heat pulsed at 42 °C (water bath) for 45 seconds, incubated on ice for 2 minutes again, 0.5 mL NZY solution to the tube later added and the tube incubated at 37 °C for 1 h (rotation speed 6). After the incubation, 50 µL of the solution was applied to one AMP (ampicillin) agar plate, and the rest was centrifuged at 6 rpm for 1 minute, and applied to another AMP agar plate. The plates were then incubated at 37 °C overnight.

2.8h Nickel-NTA Histidine-tagged protein expression & purification

8ml LB media inoculation: to 8 mL of LB media in culture tube(s), 4 µL of 2000x AMP was added along with a colony from the agar plates, this culture was incubated at 37°C overnight with 6rpm rotation speed.

IPTG induction: to 1 L of LB media, 0.5 mL of 250 mg/mL AMP was added, 1 culture tube from the 8 mL inoculation step was added, and the culture incubated while shaking at 37 °C for several hours until the O.D. value was in the range of 0.6 - 0.8. The culture was then cooled down until the incubated equilibrated to 16 °C. 100 mM IPTG was then added and the culture incubated overnight.

Cell harvesting and His.tagged protein expression using Ni-beads:

After IPTG induction, the cell culture was centrifuge for 10 minutes at 5000 rpm at 4°C. The cultured cells were re-suspended in cell lysis buffer (see section 2.6). After re-suspending the cells, they were French press twice and centrifuged immediately at 14,000 rpm, for 30 minutes at 4°C and the supernatant was collected.

Protein purification: 6 mL of Ni-beads was added to the column, washed two times with column buffer (section 2.6 for preparation) to equilibrate the column, the supernatant loaded to the column with the beads, shaken at 4°C for 1h. The supernatant was then allowed to flow through the column into a tube. The column was washed again 10 times with 40 mL wash buffer (see section 2.6 for preparation).

Protein elution and concentration: Elute protein from beads seven times with 7 mL each time with elution buffer (see section 2.6). The eluants were then combined and dialyzed with dialysis buffer for 48 hrs (see section 2.6). A 12 % SDS-PAGE gel (see preparation in section 2.6) was ran at 120 V.

2.8i Radioactive HAT assay

This assay was divided into two, testing the autoacetylation of Tip60 wt and the mutants and the other testing Tip60 acetylation of substrate H4(1-20) histone. The substrate and enzyme concentrations were held constant throughout the experiment.

Sample preparation:

Dilutions to obtain final enzyme concentration of 0.6 μM, H4(1-20) substrate concentration of 200 μM and ^{14}AcCoA concentration of 20 μM were made. For the enzyme dilutions, 2XRB was added for a final concentration of 1XRB and the appropriate amount of water was added as well to make a total volume of 20 μL.

Original concentration of Tip60 wild type (wt) 7.1 μM

Original concentration of Tip60Cys369Ala 6.9 μM

Original concentration of Tip60Glu403Gln 4.6 μM

Mix solution: 100 μL 2XRB, 15.5 μL ddH_2O and 4.5 μL ^{14}AcCoA.

For 2XRB preparation see Chapter 2 section 2.6 Buffers.

Table 4: Sample preparation for radioactive HAT assay

Sample	**0**	**1**	**2**	**3**	**4**	**5**	**6**
H4(1-20)	-	-	-	-	6 μL	6 μL	6 μL
Protein/enzyme	wt	wt	Tip369A	Tip403Q	wt	Tip369A	Tip403Q
^{14}AcCoA	2XRB	*	*	*	18 μL	18 μL	18 μL

- were replaced with 6 μL ddH_2O

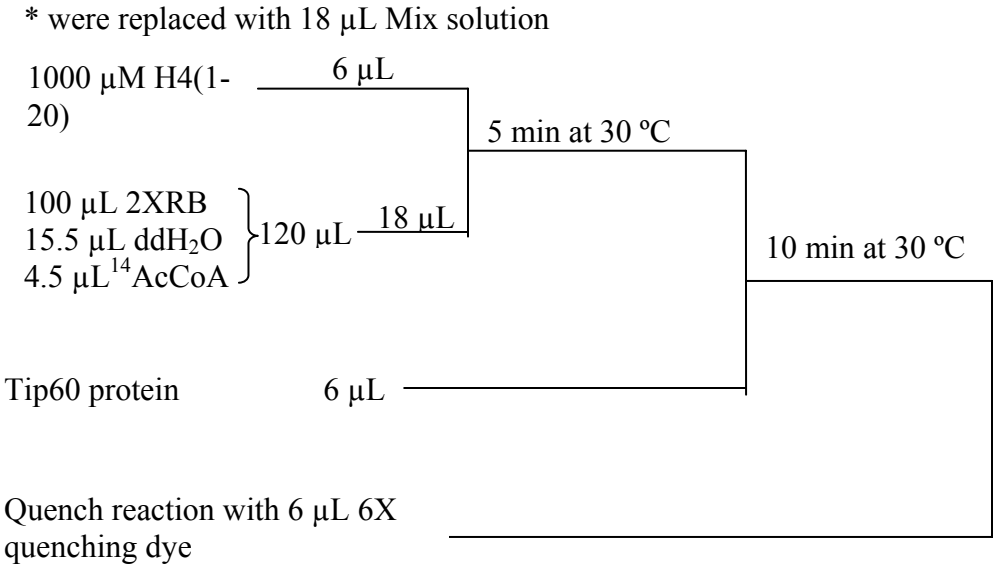


Figure 8: Scheme of HAT radioactive assay

Table 5: HAT assay reaction time

Sample	Add mixture at 30 ºC	Add enzyme at 30 ºC	Quench reaction with 6x dye
0	0	5	15
1	0.5	5.5	15.5
2	1	6	16
3	1.5	6.5	16.5
4	2	7	17
5	2.5	7.5	17.5
6	3	8	18

CHAPTER 3

SITE-DIRECTED MUTAGENESIS

3.0 Site-directed mutagenesis using polymerase chain reaction (PCR)

This is a widely used technique which was first introduced in the 1970s to study and localize the effects of mutations on genes (Smith, 1993). Before this process can be carried out, an appropriate mutagenic primer has to be designed. This designed primer determines the efficiency of the reaction because a 100 % base pairing at either ends of the target sequence is necessary. The method of site-directed mutagenesis used for this experiment is a quick-change PCR method using the quick-change site-directed mutagenesis kit from stratagene.

Many studies have been done on the MYST family prototype Esa1, so based on the results of those studies and the close relation of Esa1 to Tip60, our point mutations were determined to be at amino acids 369 and 403. Amino acid 369 which is a Cys was mutated to an Ala. Ala because it eliminates the concerns of a similar side chain while not affecting the conformation of the main-chain, i.e., we are interested in investigating the role the thiol in the Cys side chain plays during catalysis (Lefèvre *et al*, 1997). Also, amino acid 403 was mutated from a Glu to a Gln because Gln has a side chain that is structurally similar to glutamate but lacks the acidic character required for it to function in a catalytic capacity (Trievel *et al.*, 1999). Also using the full length gene sequence of Tip60α inserted in the pET15b vector between NcoI and BamH1 sites (map shown in figure 9); the point mutations were individually introduced using thermal cycling quick change PCR base site-directed mutagenesis technique. The pET15b vector was ideal for this experiment because it contains a His-tagged and a lac operon coding sequence which helped enhance the concentration of the protein during expression of both the Tip60 wild type and Tip60 mutants (Tip60Cys369Ala and Tip60Glu403Gln).

Quick change PCR based site-directed mutagenesis technique allows for DNA amplification using thermostable polymerases for nucleotide incorporation. The thermal cycles allow for the denaturation of the plasmid DNA to form single-stranded regions. The synthesized mutant oligonucleotide is then annealed to the target strand at which point the *pfu Turbo* DNA polymerase, which allows for high fidelity replication of the plasmid and extension of the mutant strand and keeping the mutant oligonucleotide in place. In other words, during this thermal cycle, the annealing of the oligonucleotides which carry the desired mutation (s) to one strand of the DNA of interest which is accomplished (Cosby *et al.*, 1997). The original strand of DNA serves as a primer to initiate DNA synthesis in this process. In other words, the designed mutated primer is introduced to the reaction, using the non-strand displacement characteristic of the *pfu Tubor* DNA polymerase the oligonucleotide strands are incorporated into the site of mutation and extended in a circular manner (Stratagene.com).

A digestive enzyme, *Dpn I* is then used to digest the parent DNA for 1 hour. This *Dpn I* digestive enzyme targets the parent DNA because the parent DNA is methylated where as the mutated DNA is not. The parent DNA is already methylated because it was obtained from the cell, and a methylated DNA sequence is essential for gene expression control in a cell due the fact that any alterations in methylation pattern could lead to serious forms of diseases such as cancer (Richardson and Yung, 1999). After this step, the mutated DNA plasmid is not completely circular, so it has to be transformed into an *E coli.* bacteria cell.

Following the protocol and thermal cycle described in section 3.1 of chapter 3, the results shown in Figure 10 were obtained. The top gel is that for Tip60Cys369Ala mutation and the bottom gel is for the Tip60Glu403Gln mutation. Using gel electrophoresis to confirm

amplification, as was expected the mutation using the PCR based technique for site directed mutagenesis was successful as indicated by the bright bands in lanes 4 and 7 shown in figure 10**.**

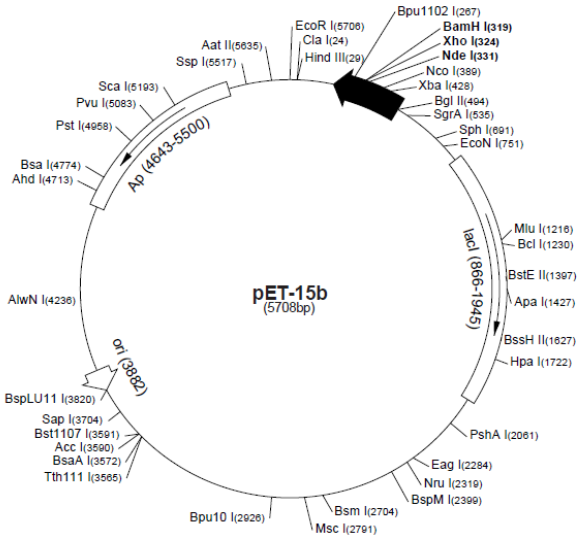
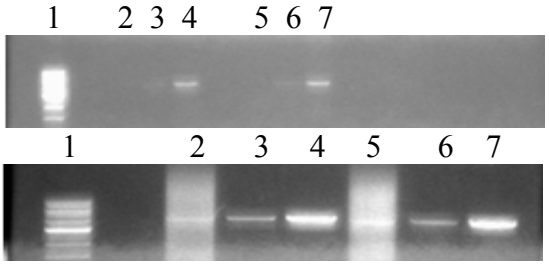


Figure 9: pET15b vector map (http://depts.washington.edu/bakerpg/plasmid_maps/pet15bm.pdf)

The success of the PCR product (sample in well # 7) was then transformed in XL1-Blue competent cells. XL1-blue cells are ideal because they are resistant to tetracycline, endonuclease deficient and recombinant deficient. These features allow for better quality and stability of insertion. After the growth of the cells on an ampicillin agar plate, the cells were cultured and the new non-methylated DNA plasmid extracted with the Promega mini-prep kit and submitted for DNA sequencing.



1) DNA ladder
2-4) PCR Samples 1-3 (from table 1.) before Dpn1 digest
5-7) PCR Samples 1-3 (from table 1.) after Dpn1 digest

Figure 10: Agarose gel-electrophoresis to confirm success of mutation after PCR

3.1 Measurement of DNA concentration

After successful transformation of the mutated DNA plasmids, the mutant proteins were extracted using a Promega miniprep kit. The protein absorbance was then measure with a

UV-Vis instrument at 260 nm and used towards the calculation of DNA concentration.

3.2 DNA sequencing

The sequence obtained after submitting the sample for DNA sequencing was successful as can be seen in figure 11((1) Tip60Cys369Ala mutation and 2) Tip60Glu403Gln mutation). The actual sequence of the new mutated Tip60 (isoform 2) is shown below with the targeted mutated amino acid as the 'Sbjct' and the sequencing aligned with the original full length Tip60α (isoform 2; Query) for comparison and confirmation reasons.

```
Query  470   CAACTCCA-GTGCCC-AGCGAGACAGCCCCGGCCT-CGGTTTTTCCCCAGAATGGAGCCG  526
             ||||||||| ||||||| |||||||||| ||||| || ||| ||||||||||||||||||||
Sbjct  5     CAACTCCAGGTGCCCAAGCGAGACAG-CCCGG-CTCCGG-TTTTCCCCAGAATGGAGCCG  61

Query  527   CCCGTAGGGCAGTGGCAGCCCAGCCAGGACGGAAGCGAAAATCGAATTGTTTGGG-CACT  585
              ||||||||||||| ||||||||| |||||| |||||||||||||| |||||||||| ||||
Sbjct  62    -CCGTAGGGCAGT-GCAGCCCAG-CAGGAC-GAAGCGAAAATCG-ATTGTTTGGGCCACT  116

Query  586   GATGAGGACT-CCCAGGACAGCTCTGATGGAATACCGTCAGCACCACGCATGACTGGCAG  644
             |||||||||| |||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  117   GATGAGGACTCCCCAGGACAGCTCTGATGGGATACCGTCAGCACCACGCATGACTGGCAG  176

Query  645   CCTGGTGTCTGATCGAAGCCACGACGACATCGTCACCCGGATGAAGAACATTGAGTGCAT  704
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177   CCTGGTGTCTGATCGAAGCCACGACGACATCGTCACCCGGATGAAGAACATTGAGTGCAT  236

Query  705   TGAGCTGGGCCGGCACCGCCTCAAGCCGTGGTACTTCTCCCCGTACCCACAGGAACTCAC  764
             |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  237   TGAGCTGGGCCGTCACCGCCTCAAGCCGTGGTACTTCTCCCCGTACCCACAGGAACTCAC  296

Query  765   CACATTGCCTGTCCTCTACCTGTGCGAGTTCTGCCTCAAGTACGGCCGTAGTCTCAAGTG  824
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297   CACATTGCCTGTCCTCTACCTGTGCGAGTTCTGCCTCAAGTACGGCCGTAGTCTCAAGTG  356

Query  825   TCTTCAGCGTCATTTGACCAAGTGTGACCTACGACATCCTCCAGGCAATGAGATTTACCG  884
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357   TCTTCAGCGTCATTTGACCAAGTGTGACCTACGACATCCTCCAGGCAATGAGATTTACCG  416

Query  885   CAAGGGCACCATCTCCTTCTTTGAGATTGATGGACGTAAGAACAAGAGTTATTCCCAGAA  944
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  417   CAAGGGCACCATCTCCTTCTTTGAGATTGATGGACGTAAGAACAAGAGTTATTCCCACAG  476

Query  945   CCTGTGTCTTTTGGCCAAGTGTTTCCTTGACCATAAGACACTGTACTATGACACAGACCC  1004
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  477   CCTGTGTCTTTTGGCCAAGTGTTTCCTTGACCATAAGACACTGTACTATGACACAGACCC  536

Query  1005  TTTCCTCTTCTACGTCATGACAGAGTATGACTGTAAGGGCTTCCACATCGTGGGCTACTT  1064
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  537   TTTCCTCTTCTACGTCATGACAGAGTATGACTGTAAGGGCTTCCACATCGTGGGCTACTT  596

Query  1065  CTCCAAGGAGAAAGAATCAACGGAAGACTACAATGTGGCCTGCATCCTAACCCTGCCTCC  1124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  597   CTCCAAGGAGAAAGAATCAACGGAAGACTACAATGTGGCCTGCATCCTAACCCTGCCTCC  656

Query  1125  CTACCAGCGCCGGGGCTACGGCAAGCTGCTGATCGAGTTCAGCTATGAACTCTCCAAAGT  1184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  657   CTACCAGCGCCGGGGCTACGGCAAGCTGCTGATCGAGTTCAGCTATGAACTCTCCAAAGT  716

Query  1185  GGAAGGGAAAACAGGGACCCCTGAGAAGCCCCTCTCAGACCTTGGCCTCCTATCCTATCG  1244
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  717   GGAAGGGAAAACAGGGACCCCTGAGAAGCCCCTCTCAGACCTTGGCCTCCTATCCTATCG  776

Query  1245  AAGCTACTGGTCCCAGACCATCCTGGAGATCCTGATGGGGCTGAAGTCGGAGAGCGGGGA  1304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  777   AAGCTACTGGTCCCAGACCATCCTGGAGATCCTGATGGGGCTGAAGTCGGAGAGCGGGGA  836

Query  1305  GAGGCCACAGATCACCATCAATGAGATTAGTGAAATCACCAGCATCAAGAAGGAGGATGT  1364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  837   GAGGCCACAGATCACCATCAATGAGATTAGTGAAATCACCAGCATCAAGAAGGAGGATGT  896
```

Mutated site

Figure 11: Result of the NCBI Blast alignment of the sequencing results obtained after the Tip60Glu403Gln and Tip60Cys369Ala mutations

CHAPTER 4

PROTEIN EXPRESSION

After DNA transcription in the nucleus, the synthesized mRNA migrates to the cytoplasm where it binds to a ribosome in preparation for the process of translation. During translation, the genetic information carried by the mRNA strand is translated into amino acids which later encode for the appropriate protein. The study of proteins in a biomolecular system is known as proteomics. Many methods have been used to isolate and characterize proteins after translation. Some of these methods include mass spectroscopy, chromatography and gel analysis. For this experiment, column chromatography using Nickel-NTA beads and later SDS-PAGE were used to analyze and separate the target protein from others. Gene expression in cells under a given set of experimental conditions could lead to the discovery of clues as to which proteins are involved in certain pathways and disease states.

4.0 IPTG induction for protein expression

Using *E. coli* as a host to the Tip60 protein will allow control on the amount of protein is obtained after protein expression. Through the use of AMP enhanced LB growth media, we were able to amplify the successfully mutated Tip60 proteins through the methods described in chapter 2. We were able to manipulate the expression of the target protein due to a host strain condition introduced by Williams *et al.* in 1998. Williams *et al.* created an *E. coli* host containing a conditionally essential gene under control of the *lac operato/promoter* region and a multicopy plasmid containing the *lac operator* (Williams *et al*, 1998; Baneyx, F.; 1999). Based on the results obtained by Williams *et al.* and others, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was found to be and ideal lactose analog along with an appropriate host to obtain better amounts of the target protein. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is the non-hydrolyzed and

commonly used *lac-type* operon (lactose metabolite reagent) that triggers/induces the transcription of a lac operon present on the gene insertion of interest (Chen *et al.*, 1997). The presence of a *lac repressor operator* system is important for gene regulation in *E. coli* (Yansura *et al.*, 1984). Depending on the target protein, as little as 0.1-0.5 mM IPTG can be used for induction during protein expression.

Using Bl21(DE3) as a host, Hur, *et al.* reported that produce a series of metabolic changes over time (Hur *et al.*, 2005). Also, according to their results, these metabolic changes could be due to the presence of the *T7/lac* promoter which is triggered by IPTG to produce the T7 RNA polymerase which subsequently clones the *lacZ* gene on the plasmid by the strong T7 polymerase (Studier *et al.*; 1986; Hur *et al.*, 2005).

During this study, BL21(DE3) competent cells from stratagene were used for IPTG induction for protein expression. This procedure is described in section 2.8h.

4.1 Nickel-NTA Histidine-tagged protein expression assay

This method of protein expression makes use of Ni^{2+} cations immobilized on the Ni-NTA (Ni-nitriloacetic acid) His-Binding to resins forming a metal chelation complex in an affinity chromatography. With this technique, the solid-phase NTA resin forms a four side chelating bond with the Ni^{2+} metal cations. After the protein has been extracted from the cells, this lysate is loaded to the chromatographic column where the protein binds to the Ni-resin. The unbound proteins are washed away with a washing buffer (section 2.6 shows the buffer used in this experiment). The target protein is then eluted with high concentrations of imidazole. Imidazole is preferable because it is similar in structure to His, so during the washing process, it replaces the His on the Ni^{2+} complex for the release of the protein.

This method allows for proteins to be purified under gentle and non-denaturing conditions. As can be seen in figure 12, the mutated proteins were successfully expressed and the concentrations calculated to be 4.6 µM for TipGlu403Gln and 6.9 µM for TipCys369Ala. In figure 11: 1) is the molecular weight marker (kDa), 2) Flow through after loading the supernatant on the Ni-beads 3) is the cell pellets 4) is the supernatant obtained after breaking the cells open 5) is the beads before cleavage for TipCys369Ala 6) Tip60Cys369Ala 7) Tip60Glu403Gln 8) Tip60 wt of the protein eluted with the elution buffer from section 2.6.

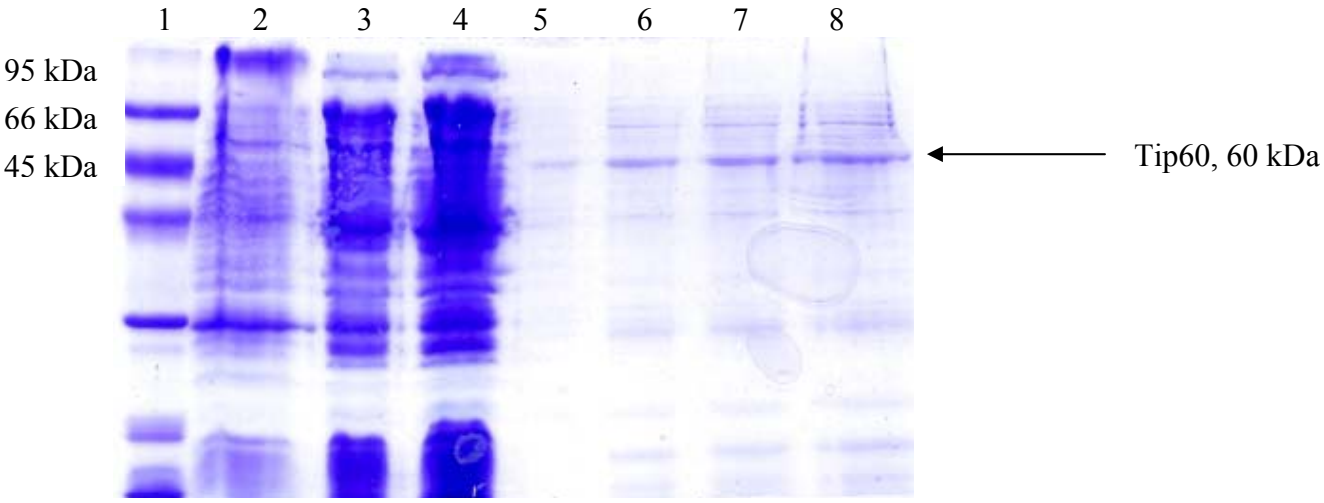


Figure 12: 12 % SDS-Page for wt, TipCys369Ala and TipGlu403Gln mutants

CHAPTER 5

ENZYMATIC STUDY

To understand how HATs function in regulating transcription/post-translation, it is important to carry out certain assays. Acetylation of a lysine residue has been reported to affect other post-translational modifications on a neighboring residue, particularly phosphorilation (Yang and Seto, 2007). Histone acetyltransferases (HATs) have been reported to play crucial roles in many cellular functions, such as gene transcription and cell proliferation. The method used in the acetylating activity analysis of Tip60 is an assay which makes use of the phosphoimaging technique where after the reaction of Tip60, Histone H4(1-20) and AcetylCoA over time, the results could be quantified using phosphoimaging. AcetylCoA is ideal because acetylCoA is independently produced and consumed by different organs and is a key intermediate in a number of metabolic pathways (Oliver *et al.*, 2009).

During this assay, Tip60, H4(1-20) and acetyl-CoA are incubated at 30 °C at different time intervals (see section 28i). After the incubation, the expected outcome is the release of the Tip60 (as is the case with enzymes), CoA and the acetylated H4(1-20).

With this easy phosphoimaging assay, whether the Tip60 mutants could acetylate the H4(1-20) histone substrate or themselves with ^{14}AcCoA was being investigated. As can be seen in figure 13, Tip60 can acetylate the H4(1-20) histone substrate; 1 shows Tip60Glu403Gln mutation, 2 shows Tip60Cys369Ala and 3 shows wild type Tip60. The wild-type showed more activity, followed by TipGlu403Gln and lastly TipCys369Ala. The fact that TipCys369Ala did not show much acetylating activity is indicative of the fact that the Cys present in its HAT active is essential for catalysis contrary to the earlier report using the MYST prototype Esa1. The

Tip60Glu403Gln on the other hand shows some activity which is indicative that the Glu does not play a major role in substrate acetylation.

5.0 Radioactive HAT assay

The characterization of HAT activity is crucial towards understanding their function during cellular processes. Two continuous non-radioactive processes, coupled enzyme systems with either α-ketoglutarate dehydrogenase or pyruvate dehydrogenase with CoASH, were described by Kim *et al.* towards minimizing hazards faced when using a radioactive assay (Kim *et al.*, 2000). However, for our experiment, the radioactive assay was suitable because it was quick and simple. Histone acetyl transferases catalyze the transfer of an acetyl-group from acetyl-CoA to the ε-amino terminal of a specific lysine residue within the core of a histone (H4(1-20) in this case). During this radioactive assay, the peptide gel was dried for 2 hours after staining and destaining and placed in a photoimager for 72 hours (for more on analysis by phosphorimaging see Poveda and Sendra, 2008). The resulting spot signals were quantified using a typhoon phosphoimager (shown in Figure 13).

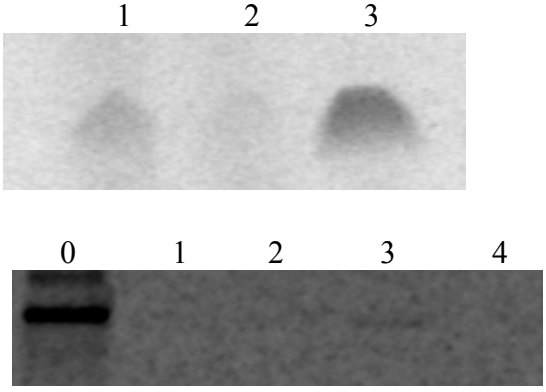


Figure 13: Phosphorimaging of Tip60 H4(1-20) histone acetylation and autoacetylation

i) Phosphorimage of substrate, H4(1-20), acetylation by Tip60 1) TipGlu403Gln 2) TipCys369Ala 3) wt. Tip60 (ii) Phosphorimage of autoacetylation by Tip60 prepared as shown in Table 5 (0 is the BSA standard).

Table 5: Sample preparation for Tip60 autoacetylation

Sample	**1**	**2**	**3**	**4**
ddH_2O	+	+	+	+
Mix	+	+	+	*
Tip60	Tip60Glu403Gln	Tip60Cys369Ala	wt	wt

* 2XRB added instead

5.1 Initial enzymatic characterization

Enzyme kinetics is a study of the time-dependent activity of enzymes with a goal of attaining equilibrium (Hans Bisswanger, Enzyme Kinetics: principles and methods, 2nd edition, *Wiley VCH* 2008). This study involves the behavior of an enzyme after binding with the substrate to form the enzyme-substrate complex and its conversion into product(s) (Hans Bisswanger, Enzyme Kinetics: principles and methods, 2nd edition, *Wiley VCH* 2008). In this study, the acetylating activity of Tip60 wild type, Tip60Cys369Ala and Tip60Glu403Gln mutants on H4(1-20) histone was individually evaluated over a period of time with a 3 minute interval between reaction quenching.

In our assay, radioactive acetylCoA, H4(1-20) histone and the three various Tip60 expressed proteins were prepared separately at 30 ºC. The set up was as shown in figure 8 with a 3 minute interval between the quenching of each mixture in a tube. The reaction times are shown in table 6 for both mutants. To show the steady state linearity and explore the acetylating activity of the Tip60 wt. and Tip60Glu403Gln was performed and stopped at 12 minutes (see table 7) for Tip60Cys369Ala, the reaction time was extended to 15 minutes. After the reactions for both the wt. Tip60 and the two mutants, the final enzyme concentration was 200 nM and 380 nM respectively. The H4(1-20) substrate concentration was 440 μM for both mutants and 400 μM for the wt. The acetylCoA concentrations were 10 μM for the wt. reaction and 17.86 μM for the mutants. As expected (shown in figure 14), the product formation increased with time and

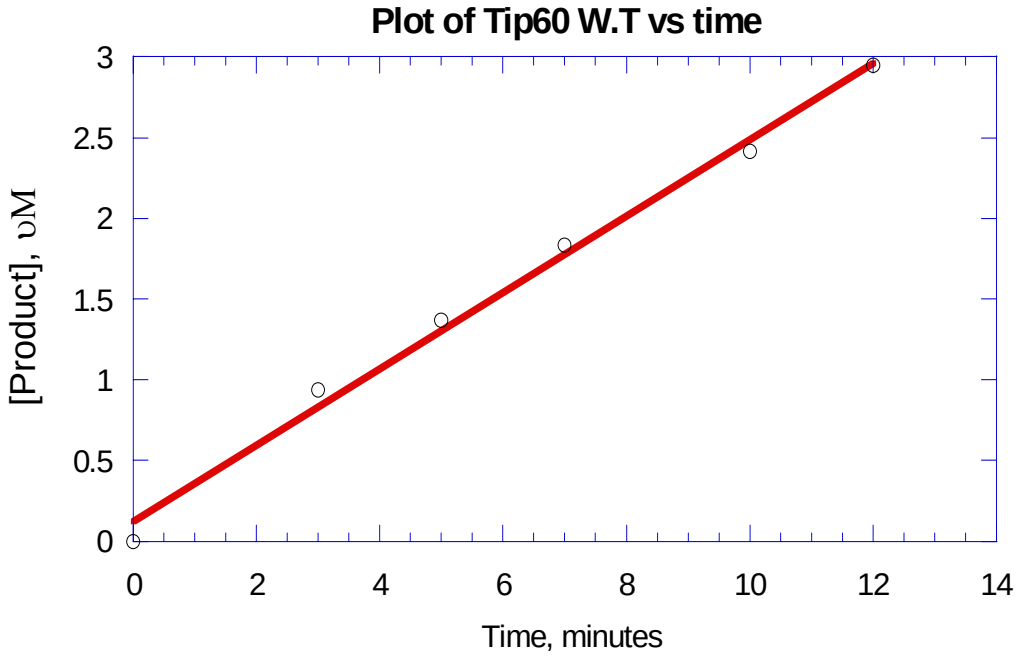
leveled off after 12 minutes. This suggests that the enzyme has reached its maximum activity at that time and the rate of product formation should remain the same.

Table 6: Tip60Cys369Ala enzyme kinetics reaction times

Sample	Add H4(1-20)	Add enzyme	Quench reaction on P81 paper
0	0	5	8
1	0.5	5.5	8.5
2	1	6	12
3	1.5	6.5	15.5
4	2	7	19
5	2.5	7.5	22.5

Table 7: Tip60 wt. and Tip60Glu403Gln enzyme kinetics reaction times

Sample	Add H4(1-20)	Add enzyme	Quench reaction on P81 paper
0	0	5	8
1	0.5	5.5	8.5
2	1	6	11
3	1.5	6.5	13.5
4	2	7	17
5	2.5	7.5	19.5



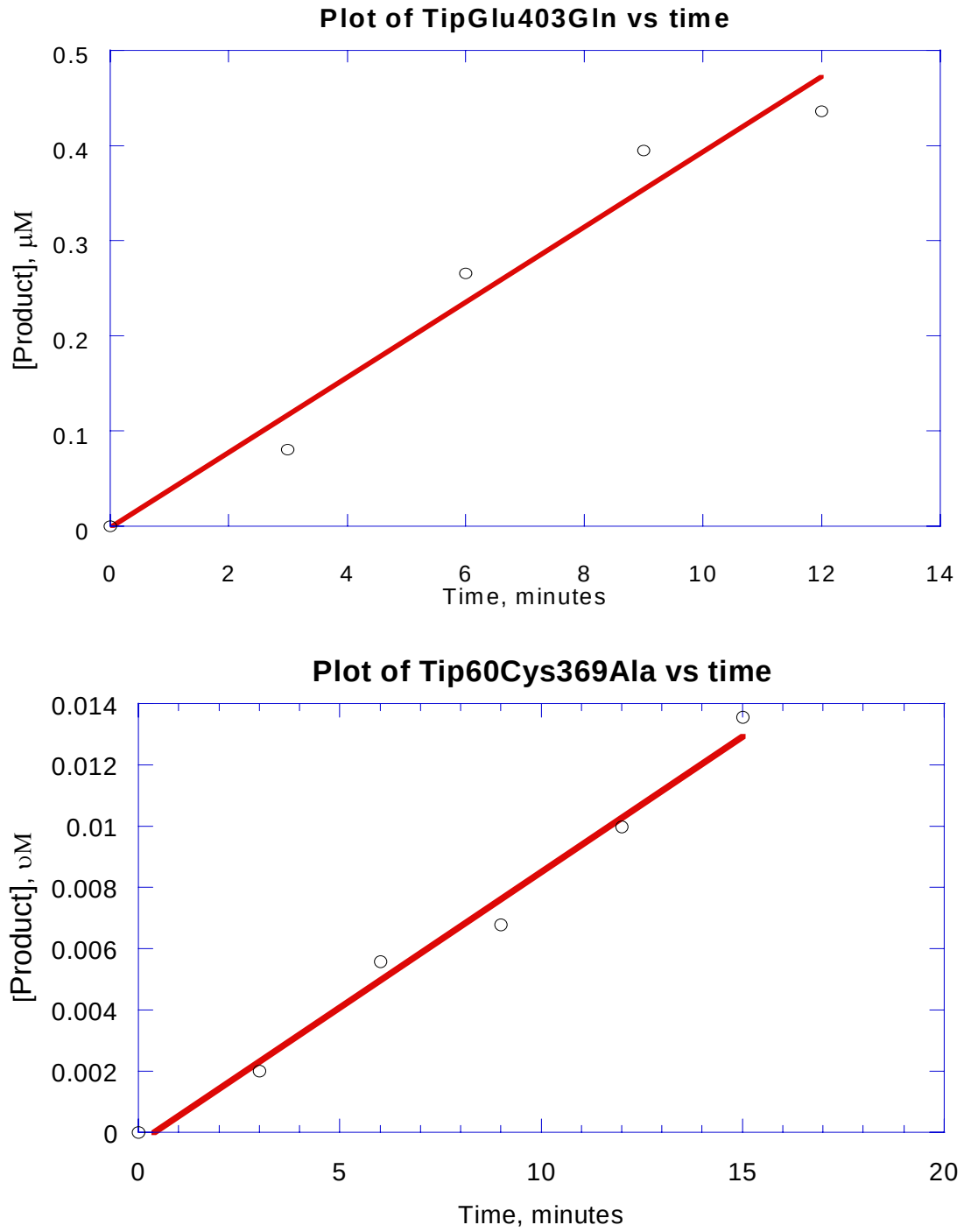


Figure 14: Plot of Product concentration, µM vs Time for Tip60 wild type, Tip60Cys369Ala and Tip60Glu403Gln

5.2 Future kinetic study of Tip60

Based on the results obtained above, we conclude that the enzyme is stable until 20 minutes also, the substrate/product yield is less than 10%. This value is within the acceptable

percent yield value. Our future work will involve an investigation towards understanding and characterizing the enzyme mechanism of Tip60. With this mechanism investigation, it could lead to a possible explanation as to what mechanism Tip60 uses during catalysis, i.e., whether Tip60 uses the ternary complex or the ping pong mechanism. Also, because Tip60 has been shown to autoacetylate, the role this autoacetylaiton places during catalysis will be investigated.

CHAPTER 6

A DUAL-MODE FLUORESCENCE STRATEGY FOR SCREENING HAT MODULATORS

Many, but limited, approaches have been explored to characterize and identify HAT inhibitors. An ideal method to characterize HAT catalytic activity which eventually leads to HAT modulating inhibitor identification is the classical labeling of enzymes with a radioactive element (Martinez *et al.,* 2006; Hardcastle *et al.,* 2005). This approach is a novel approach reported by Xie *et al.* as a means to screen HAT small molecule inhibitors. This method is a simple single-step assay that provides direct readout of acetylation products via fluorescent intensity changes.

6.0 Using the classical radioactive way to characterize HATs

This is the commonly used assay by most scientists who have the goal of characterizing HAT proteins/enzymes. In a radioactive method described by Turlais *et al.*, a mixture of radio-labeled acetylCoA and substrate (histone) were added together in one step followed by enzyme in another step to initiate the acetylation reaction. The results were measured by means of scintillating microtiter plates (Turlais, *et al.*, 2001). This method allows for HTS and the accurate identification of potential inhibitor leads for HATs.

6.1 Using immunoblotting to characterize HAT inhibitors

The immunoblotting technique involves the use of antibodies or other specific ligands to identify target proteins in form of antigen-antibody (or protein-ligand) specific reactions. This technique, when used in the study of HATs (acetylation or deacetylation), relies on the recognition of acetyl-lysines by specific antibodies and has been reported to be of potential in inhibitor screening studies (Hardcastle *et al.*, 2005; Martinez *et al.*, 2006; Stockwell *et al.*, 1999; Zhang *et al.*, 1998). Although this method involves the use of original/specific antibodies,

antibodies are expensive, the washing step is time consuming and a robust deconvolution scheme is needed to validate the action of hits identified in a cell-based screen. High throughput screening (HTS) and automation are the features preferred by most scientists when using certain assays in the quest to find the best inhibitor (s) or small molecule amongst a large pool of potential molecules. The immunoblotting technique is not ideal for HTS because of the absence of certain practical tools (Wegener *et al.*, 2003). Since HATs have been reported to HTS is beneficial in identifying small molecule inhibitors related to HATs functionality, fluorescence spectroscopy has been reported as a potential technique for these purposes.

6.2 Using FRET and anisotropy measurements to characterize HAT inhibitors

Over expression of HATs has been related to several diseases states because they have the capability to modify histones and if histones are modified, they grant easy access to chromatin (Timmermann *et al.*, 2001). The identification of small molecules that are involved in epigenetic regulation is important in normal and disease cell processes (Johnson *et al.,* 2008). Anacardic acid, garcinol, isothiazolone, curcumin and cinnamoyl compounds are the most recent small molecule HAT inhibitors. However, these small molecule inhibitors exhibit nonspecific inhibition and have limited usage in pharmacological settings (Wynne *et al.*, 2002, Wu *et al.*, 2008).

Fluorescence resonance energy transfer (FRET) is a distance-dependent technique. With this technique, the donor and acceptor must be in close proximity, the absorption spectrum of the acceptor must overlap with the emission spectrum of the donor and the donor acceptor transition dipole orientation must be paralleled. This use of this strategy to characterize potential inhibitors should provide renovating insights in the screening for new anticancer drugs that target the substrate interfaces of HAT targets, as well as in the mechanistic characterization of HATs.

In this new approach we reported, FRET and fluorescence anisotropy were used to identify and characterize HAT inhibitors (plots shown in Figures 18 & 19). We used Dabcyl (Dab) to label the HATs p300 which acted as a FRET acceptor. Methoxycoumarin (Mca) in conjugate with HAT substrate analogues functioned as fluorescent donors, specifically; LysCoAMca was used to interact with p300. In this assay, when a protein binds the ligand to form a complex, the fluorescence anisotropy increases and the fluorescence intensity of the donor fluorophore decreases due to FRET quenching by the Dab acceptor. Figure 15 shows a schematic diagram of the fluorescent reporter system (Xie *et al.*, 2009).

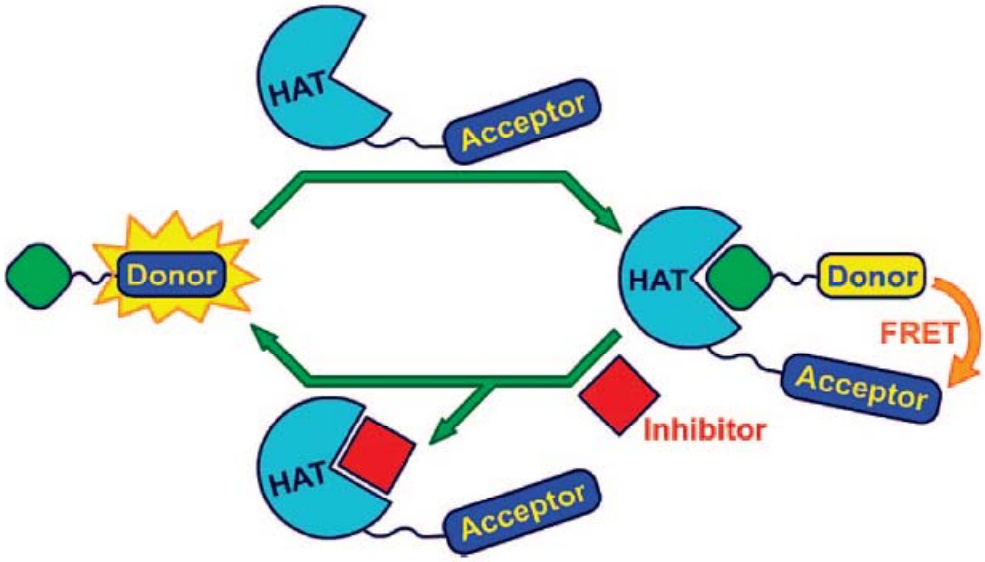


Figure 15: Schematic showing acetylation products via fluorescent intensity changes.

6.3 Expression of Dabcyl-labeled p300 protein

p300 protein is part of the HAT domain family and has been reported to perform several functions which include histone modification, regulation of cell growth, and gene expression. Labeling of p300 protein with Dabcyl was achieved using the EPL protocol essentially the same as in the literature (Thompson *et al.*, 2004). First, the DNA sequence encoding for the p300 HAT domain (aa 1287-1652) was subcloned into PTYB2 vector (a gift from Prof. Phillip Cote). BL-

21(DE3) Codonplus RIL competent cells were used for protein expression (Figure 16). The expressed p300-intein-CBP fusion protein was loaded to chitin beads. After extensive washing, 6 mg of the ligation peptide (CMLVELHTQSQDRFK(Dabcyl)-NH2; Molecular weight; 2086 kD) in 3 mL of ligation buffer (25 mM HEPES pH8.0, 250 mM NaCl, 1 mM EDTA, and 200 mM MESNA solution) was added to the beads. The ligation reaction was kept at room temperature for 48 h. The protein product was confirmed with 12% SDS-PAGE (Figure 16). The combined eluants were dialyzed against the storage buffer and then concentrated in a centrifuge filter tube. The concentration of labeled p300 protein (p300Dab) was determined by Bradford assay.

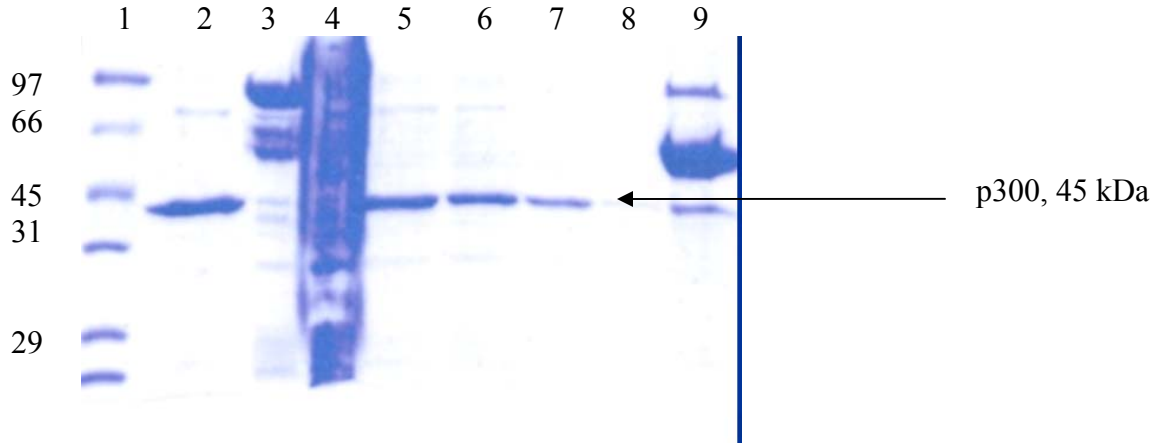


Figure 16: 12 % SDS-PAGE for p300 protein expression

In figure 15, (1) MW marker (kDa) (2) Eluant 1 (3) Beads before cleavage (4) Flow through (5-8) eluants 2-5 (9) Beads after cleavage.

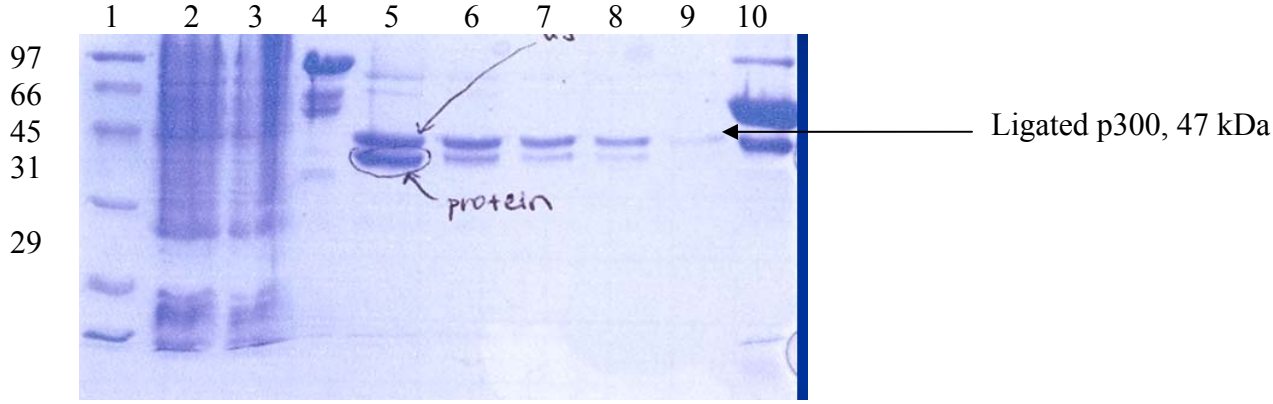


Figure 17: 12 % SDS-PAGE for Dabcyl-labeled p300

In figure 17, (1) MW marker (2) Flow Through (3) Protein supernatant (4) Beads before cleavage (5-9) eluants 1-5 (10) Beads after cleavage (kDa).

6.4 Fluorescence measurement

The fluorescence experiments were performed on a Fluoromax-4 instrument (Horiba Jobin Yvon, Edison, NJ) at 25 °C using a 0.5 mL cuvette in a buffer solution containing 50 mM Hepes (pH 8.0), 0.1 mM EDTA, 150 mM NaCl, and 1 mM DTT. The dissociation constants (K_d) were determined by measuring the fluorescence signals of Mca-labeled ligands (at fixed concentration) with increasing Dab-labeled protein concentrations.

Fraction bound = $(F - F_o)/(F_f - F_o)$

$$= \{(F_f - F_o)/2[Pr]_{tot}\} \{b(b^2 - 4[L]_{tot}[Pr]_{tot})^{1/2}\} + F_o \quad (1)$$

$b = K_d + [L]_{tot} + [PCAF]_{tot}$

Where F_0 and F_f are the initial and final fluorescence intensities, respectively, $[Pr]_{tot}$ is the total protein concentration, and $[L]_{tot}$ is the total concentration of Mca-labeled ligand. The inhibition constants (K_i) were determined by measuring the fluorescence signals of the binary mixture containing Mca-labeled ligand and Dab-labeled protein (at fixed concentrations) with increasing concentrations of respective inhibitors. Equation 2 was used for K_i value calculations (values not shown) (Nikolovska-Coleska *et al.*, 2004).

$$K_i = [I]_{50}/ ([L]_{50}/ K_d + [Pr]_o/K_d + 1) \quad (2)$$

Where $[I]_{50}$ denotes the concentration of the free inhibitor at 50% inhibition, $[L]_{50}$ is the concentration of the free Mca-labeled ligand at 50 % inhibition, $[Pr]_0$ is the concentration of the free protein at 0 % inhibition, and K_d is the dissociation constant of the protein-ligand complex.

The results obtained from the both the FRET studies and anisotropy are shown in figures 18 and 19. As anticipated from these results, upon addition of p300Dab, the fluorescent intensity

of LysCoAMca declined in a dose-dependent manner owing to FRET quenching interaction between Dab and Mca (Figure 18a,b). The fluorescence intensity was recovered when the nonfluorescent ligand LysCoA was added to the solution, competitively displacing LysCoAMca out of the binding pocket in p300 (Figure 18c,d). We also performed fluorescence anisotropy measurements of LysCoAMca (Figure 19). In this regard, the performance of LysCoAMca was analogous to that of H3CoA20Mca; the anisotropy value increased upon binding to p300Dab protein, and the change was reversed by increasing concentrations of the competitive inhibitor LysCoA. Therefore, both FRET and anisotropy signals were effective to probe and characterize the p300-inhibitor interactions.

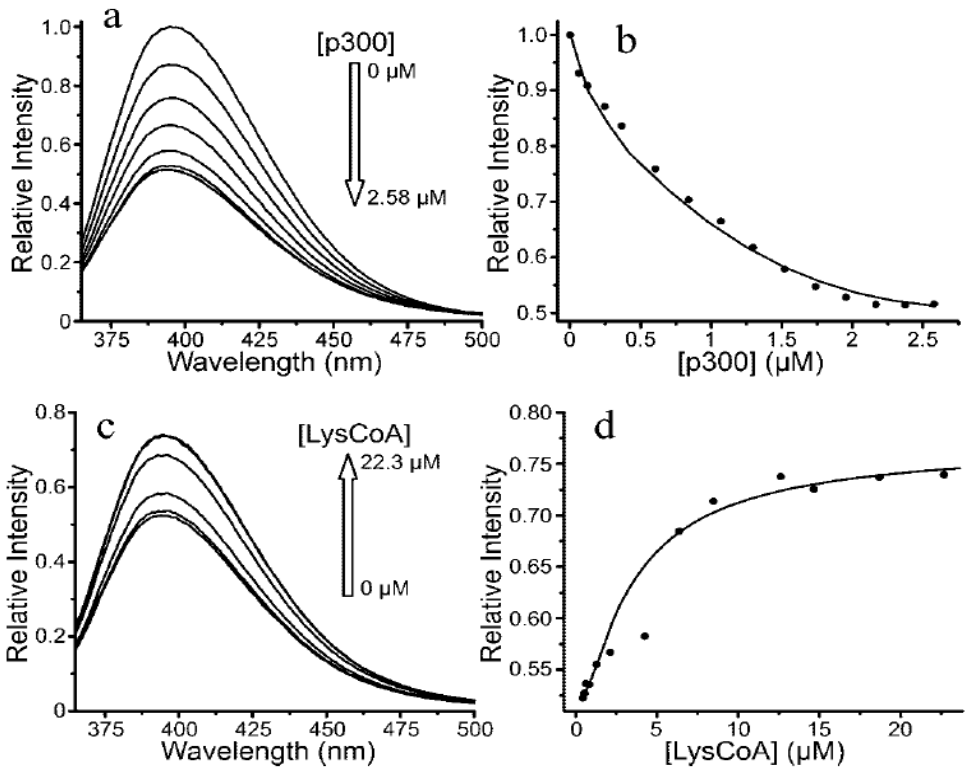


Figure 18: FRET changes of LysCoAMca

(a) The emission spectral changes of LysCoAMca (0.2 μM) at different concentrations of p300-Dabcyl (λex = 326 nm). (b) The titration curve of LysCoAMca (0.2 μM) emission at 396 nm vs the concentrations of p300-Dabcyl. *K*d is calculated to be 0.40 ± 0.06 μM. (c) The spectral

changes of LysCoAMca (0.2 μM) at increasing concentrations of the p300 inhibitor LysCoA while fixing the concentration of p300Dab at 2.2 μM (λex = 326 nm). (d) The fluorescent intensity changes of LysCoAMca (0.2 μM) at 396 nm vs the concentrations of LysCoA (the concentration of p300Dab is 2.2 μM). *K*i of LysCoA is calculated to be 0.57 ± 0.07 μM.

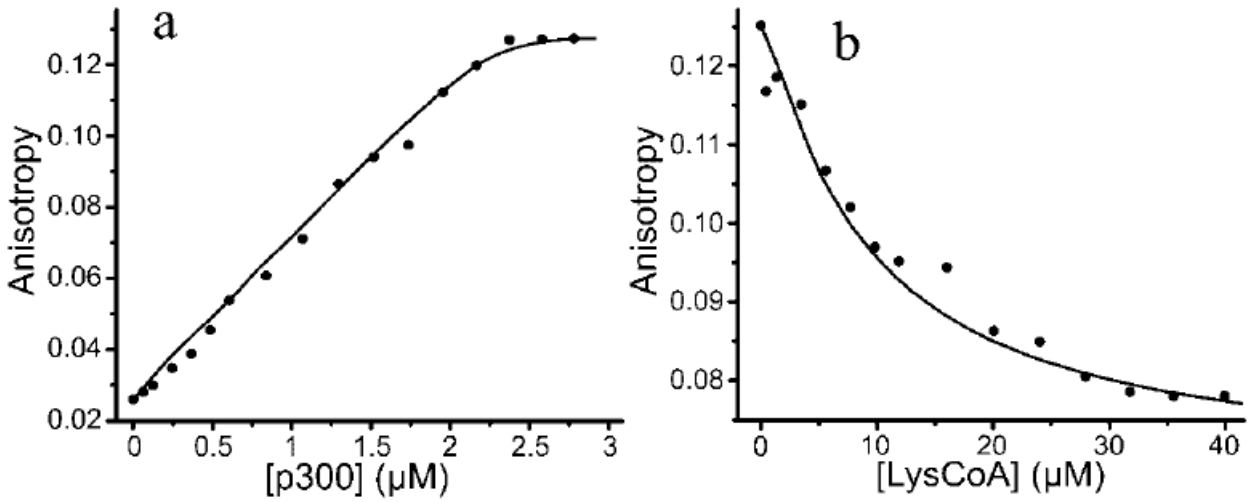


Figure 19: Fluorescence anisotropy changes of LysCoAMca

(a) Titration of LysCoAMca (0.2 μM) with p300-Dab by fluorescence anisotropy (λex = 326 nm, λem = 396 nm). K_d is calculated to be 0.73 ± 0.15 μM. (b) Titration of the binary mixture (0.2 μM of LysCoAMca and 2.2 μM of p300Dab) with HAT inhibitor LysCoA by fluorescence anisotropy (λex = 326 nm, λem = 396 nm). K_i of LysCoA is calculated to be 1.92 ± 0.29 μM.

No specific equations were used for the above calculations because this work is not intended to calculate Kd, but it is a proof-of-principle work of using fluorescence to create new assays for inhibitor screening.

CHAPTER 7

CONCLUSION

Our results are different from that published for the yeast analog of Tip60, Esa1. The results published stated that the Cys in the active site was not important for acetylating activity. Cysteine is essential for acetylating substrate. Here, the effect of a mutation by substituting the Cysteine on amino acid 369 with an Alanine and amino acid 403 a glutamate to glutamine is explored. Kamine *et al*., reported that a mutation to the essential cysteine residue at the N-terminal 31 of HIV-1 Tat terminates Tat's transcription promoter and elongation activity (Kamine *et al.,* 1996). To evaluate the longevity of the enzyme activity, and kinetic assay was carried out. This kinetic assay revealed that the enzyme activity is optimum up until 12 minutes of reaction time with the substrate. With these kinetic studies and other further studies, better understanding of the vast diversity of the MYST domain of Tip60 will be better obtained and used towards to discovery of cures for many degenerative diseases. Tip60 may promote acetylation of cellular proteins other than histones to regulate gene expression. For example, Tip60 enhances the activity of wild-type but not an acetylation-deficient mutant of androgen receptor, 'demonstrating that Tip60 acts as a co-activator by promoting the acetylation of androgen receptor but not histones' (Xiao *et al.,* 2003). Based on the kinetic studies results, we conclude that Cys369 plays a more vital role in the enzyme activity of Tip60 compared to Glu403.

The autoacetylation action of Tip60 by the wild-type and the acetylation of H4(1-20) substrate were investigated using a radioactive assay. It can be concluded from this radioactive study that both the Cys and the Glu in the active site are important for Tip60 to perform its post-translational property of HATs have been reported to control chromatin structure allowing or

blocking gene transcription. Because of this evidence, suggestions have been made that inhibition of HATs could result in antitumor effects (Turlais *et al.*, 2001). In correlation with this suggestion, we used both FRET and fluorescence anisotropy to characterize and identify HAT inhibitors. From this experiment, both FRET and anisotropy signals were effective to probe and characterize the p300-inhibitor interactions. Also, a displacement strategy for the study of HAT-inhibitor interactions was demonstrated. The developed strategy should be useful in the search of new anticancer drugs that target the substrate interfaces of the HAT targets, as well as find values in mechanistic study of HATs.

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