Chromatin modifications by CARM1/PRMT4 and p300/KAT3B in the regulation of gene expression, probed by specific small molecule inhibitors

A Thesis Submitted for the Degree of

Doctor of Philosophy

By

Ruthrotha Selvi B

To

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research.

(Deemed University)

Jakkur, Bangalore 560 064

MAY 2010

DECLARATION

I hereby declare that this thesis entitled "**Chromatin modifications by CARM1/PRMT4 and p300/KAT3B in the regulation of gene expression, probed by specific small molecule inhibitors**", is an authentic record of research work carried out by me under the supervision of Prof. Tapas K Kundu at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, and that this work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described has been based on the findings of other investigators'. Any omission which might have occurred by oversight or misjudgement is regretted.

Ruthrotha Selvi B

Date: May 8 2010

Bangalore 64

Prof. Tapas K. Kundu,

Transcription and Disease Laboratory,

Molecular Biology and Genetics Unit,

Jawaharlal Nehru Centre for Advanced Scientific Research,

Jakkur, Bangalore 560 064

Certificate

This is to certify that the work described in this thesis entitled, "**Chromatin modifications by CARM1/PRMT4 and p300/KAT3B in the regulation of gene expression, probed by specific small molecule inhibitors**", is the result of the investigations carried out by Ms. Ruthrotha Selvi B in the Molecular Biology and Genetics Unit, Jawaharlal Nehru for Advanced Scientific Research (Deemed University), Bangalore, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Date: May 8 2010 Prof. Tapas K Kundu

Acknowledgements

I would like to express my heartfelt gratitude to my mentor, Prof. Tapas K Kundu, for his constant guidance, support all through my thesis work. His scientific inputs and his encouragement has helped me a great deal in gaining confidence and the ability to think innovatively. The amount of trust and belief that he has placed even during hardships has seen me through my thesis. Most importantly, his optimistic approach, useful discussions and the aim-high attitude has infused a lot of scientific temperament into me.

I would also like to acknowledge the support and encouragement provided by president, Prof. M.R.S. Rao, which has helped me greatly during my thesis work. The useful suggestions given by him have indeed been of great help. I would also like to thank ,chairman, MBGU, Prof. Anand for his encouraging comments. Many thanks are due to Prof. Ranga Uday Kumar, who has taken active interest in my work with due suggestions which have helped immensely. I would also like to thank, the MBGU faculty members. Prof. Hemalatha Balaram and Prof.Maneesha Inamdar for their useful suggestions and the course work, which has really put me on track with respect to these courses. I would also like to acknowledge, Prof. Namita Surolia and Dr.Kaustuv Sanyal for their encouraging comments.

I would like to acknowledge the help and support provided by Transcription and Disease Lab members. Many thanks are due to Mune Gowda, who has been a great helping hand all throughout. My seniors Dr.Swaminathan, Dr.Chandrima, Dr.Kiran, Dr.Kishore, Dr.Limgu, Dr.Shrikanth, Dr.Arif and Dr.Ravindra have not just helped in gaining experimental knowledge and scientific abilities but have made me feel at home, providing me a very congenial environment. My sincere thanks to Dr.Arif for being a very helpful and supportive senior. Thanks are due to Radhika, Ashok, Sailaja, Jayasha, Shankar, Dr.Mohan for providing me useful scientific inputs and helping me with the experiments. I would also like to acknowledge the help, support and encouragement provide by my juniors, Sujata, Dr.Karthigeyan, Jyoti and Yogesh. Thanks are due to Dr.Nagashayana, Parijat, Gowsica, Divya, Kranthi, Sukanya, Ramesh, Dr.Benaka, Dr.Thimme Gowda, Dr.Basha, Ramesh, Ramachandran for their help and support. The help and technical support received from Prof. Dipak

Dasguptan, Suman, Prof. Chandrabhas Narayana, Dr. Pavan, Dr. Eshwaramoorthy, Dr.Dinesh and Prof.Lorraine Young and Maria is duly acknowledged.

I also acknowledge my batchmates, Bharath, Mangai, Surbhi, Mamta, Nishtha, Priyanka for their help and support. Many thanks are due to my seniors, Pradeep, Gayatri, Vinay, Javaid, Anand, Rinky and my juniors Mahesh, Jeetu and Mukti for all the help including reagents, advice, experimental help). I would also like to acknowledge the efforts of Suma madam (confocal) as well as Anita madam (sequencing), Dr.Suresh, Dr.Prakash (animal facility) and Roopa for help with mass spec facility. I would also like to greatly acknowledge the support provided by my friends Meghna, Sambhav, Khyati and Dinesh.

I greatly acknowledge JNCASR, CSIR for funding and fellowship. I would also like to thank DST and DBT for financial assistance towards the research and the travel support for attending the Keystone conference in Victoria, British Columbia. My heartfelt thanks to the JNCASR facilities, computerlab, library, hostel and the non academic staff in admin, academic, library, accounts for their prompt and efficient help.

All this would have not been possible without the constant support, strength and encouragement of my family.

Table of Contents

Chapter 1

INTRODUCTION

This chapter describes the significance of the different chromatin modifications in regulating gene expression with special emphasis on the epigenetic language of arginine methylation and acetylation. It also argues in favor of an inhibitor based approach to understand the biological roles of the enzymes involved in these modifications.

Chapter outline:

1.1. CHROMATIN MODIFICATIONS

1.1.1. Chromatin

Chromatin is the complex nucleoprotein structure of DNA wrapped around the histones. This structure undergoes several hierarchical and distinct organizational states, aided by not only histones, but also nonhistone proteins and small RNAs, finally resulting into the compact entity packed into the tiny confines of the nucleus. The higher order chromatin is a deterrent to the essential cellular processes like transcription, replication and repair. Hence, there exists a dynamicity wherein based on the signals, the chromatin

undergoes decompaction, thereby exposing the DNA for the above mentioned processes. Once the requirement is met, the chromatin once again compacts (Kouzarides, 2007). This is probably, the reason for chromatin being considered as a 'relay platform of different signals'. However, this dynamicity is not a global phenomena, rather is a local process involving only certain chromatin territories. The eukaryotic genome is arranged into several chromatin territories interspersed with empty spaces, leading to a three dimensional fluid representation of chromatin. Thus, any cellular process is an event recognized by specific chromatin territories (Meaburn and Misteli, 2007) which interact with other regions if essential by processes called gene looping and thereby lead to gene expression.

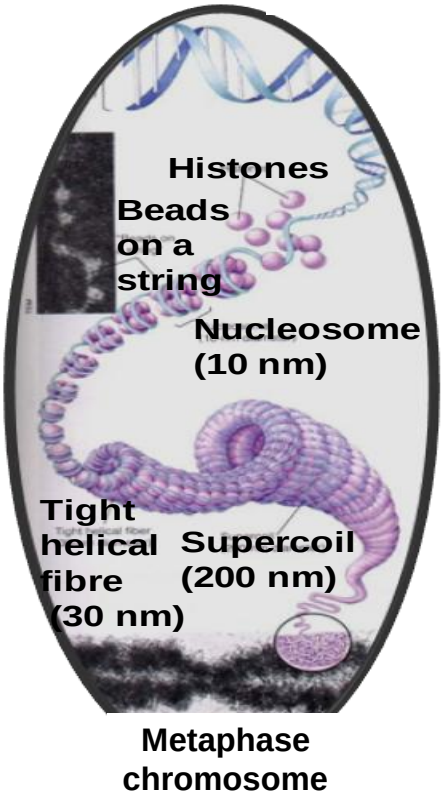


Figure 1.1. ***Hierarchical organization of chromatin.*** *The double stranded DNA is wrapped around the histones resulting in the formation of the nucleosome. This finally folds into 30nm and subsequently the 200nm supercoil which aids in the final packing into the compact metaphase chromosome.*

The nuclear processes have been shown to be organized in a highly compartmentalized manner (Sexton et al., 2007). There are sites (transcription factories)

which are dedicated to specific events such as transcription. There are other mechanisms that also modulate the transcriptional outcome. For example, the silent/repressed genes are found tethered to the lamin structure (Marshall et al., 1996) which functions by acting as a barrier to the transcription machineries. On the other hand, the genes poised for active transcription are found closer to the nuclear pore complexes (NPCs), which facilitates mRNA export (Dieppois et al., 2006) and efficient transcription.

The chromatin dynamicity is a reversible process regulated by several important processes like post translational chromatin modifications, ATP dependent remodeling events and nonhistone protein mediated regulation (Li et al., 2007). Of these various processes, the former two are catalyzed by enzymatic machineries wherein the latter can bring about effects both by its own involvement and interaction as well as by undergoing enzymatic modification. Reversible posttranslational modifications such as acetylation, phosphorylation and methylation function in a concerted manner to set the competence of chromatin template for the cellular processes like transcription, repair and recombination (Berger, 2007). A brief overview of the different cellular factors that mediate the process of chromatin dynamicity is provided below.

(a) ATP dependent remodeling factors:

The ATP dependent remodeling complexes are an integral part of the chromatin dynamicity regulation process since they utilize energy in the form of ATP hydrolysis for the remodeling activity thus facilitating the condensation or decondensation of chromatin. Broadly, there are three different group of ATP-dependent chromatin-remodeling complexes. All these complexes are characterized by an ATPase subunit that belongs to the SNF2 superfamily of proteins. Such proteins are further classified into the SWI2/SNF2 group and the imitation SWI (ISWI) group (Eisen et al., 1995). The final group of ATP dependent remodeling complex possesses deacetylase activity alongwith the Snf2-like ATPase activity. The Swi/Snf family of proteins are conserved across yeast (Swi/Snf, RSC), mouse (BRG1), drosophila (Brahma) and human (BRM and BRG1 complex). These complexes also have a bromodomain apart from the conserved ATPase subunit. The second group of remodeling complexes belong to the ISWI family and consist of fewer subunits compared to the SWI/SNF family. The most well characterized members of this family are from drosophila, namely ACF (ATP-utilizing chromatin assembly and remodeling factor), NURF

(nucleosome-remodeling factor), and CHRAC (chromatin accessibility complex). (Ito et al., 1997, Tsukiyama and Wu, 1995, Varga-Weisz, 1997).

The third and one of the important group of remodeling complex that is also coupled with deacetylase activity are the Mi-2 group of complexes (Wade et al., 1998). The representative member of this group is the NURD complex. The different components of the complex include the histone deacetylases, HDAC1 and -2, the retinoblastoma protein (Rb)-associated proteins RbAp46 and -48, and the Swi2/Snf2 ATPase homologue CHD4, also known as Mi-2β. Thus, the presence of the deacetylase activity alongwith this complex implicates the cross talk that exists between the chromatin modifications and the remodeling machineries. This is further strengthened by the presence of bromodomains in the remodeling complexes. The bromodomains are the recognition modules that bind to the acetylated proteins.

Thus, the chromatin remodeling process can be considered to be a three step process, wherein the bromodomain of the complex recognizes the acetylated lysine, thus recruiting the chromatin remodeling machinery. The second step is the binding of the remodeling complex that is independent of ATP and finally the third step which is the ATP dependent remodeling step. It is considered that the presence of ATP might lead to change in the conformation of nucleosome due to the altered histone-DNA interactions. The exact consequence of remodeling is likely to be dependent on the exact context of nucleosomes at a given promoter and can lead to either (i) activation of transcription or (ii) repression (reviewed in Vignali et al., 2000). The mechanism of the remodeling reaction and the subsequent organizational intricacies are still being worked out. The SNF2 and ISWI remodeling machinery exhibit common activities such as repositioning of nucleosomes in *cis*, generation of superhelical torsion, and DNA translocase activity (Havas et al., 2000; Lia et al., 2006, Zhang et al., 2006). Subsequently, there is an activation of ATPase activity. The presently accepted model of remodeling suggests a DNA loop formation on the nucleosome surface that further allows the sliding of the histone octamer (Zofall et al., 2006; Langst and Becker, 2001) and facilitates the nucleosome sliding (Zhang et al., 2006).

(b) Nonhistone chromatin associated proteins:

Apart from histones, there are several other nonhistone chromatin associated proteins which also modulate the chromatin dynamicity and thereby the chromatin function. One of the major component of this nonhistone protein group are the chromatin modifying enzymes.

Apart from these the high mobility group proteins, and the heterochromatin proteins are other important group of proteins that mediate the structural organization of chromatin (reviewed in McBryant et al., 2006). Several transcription factors are also found to be closely associated with chromatin mediating the chromatin function based on the cellular and physiological signals. There are also other chromatin associated proteins called as the scaffold proteins which comprise of the insulators, domain boundary elements and cellular memory modules.

The chromatin organization is mediated by several proteins including High Mobility Group (HMG) proteins, HP1 proteins, PC4, Histone H1 etc. Each of these proteins have been characterized separately as well as in combinations for their chromatin organizing property. Considering the fact that majority of the genome exists in a transcriptionally repressive state, the contribution of these proteins is indeed major in terms of maintaining the chromatin architecture.

The transcription factors are another major component and approximately 2600 proteins encoded by the human genome are considered to possess DNA binding domains and are hence classified as transcription factors. These proteins are considered to be the mediators of the information contained in the genome. They do so by different mechanisms, which includes stabilizing or blocking the RNA polymerase activity, aiding in the chromatin modifications and finally acting as recruiters of co-activators or co-repressors (Gill, 2001, Narlikar et al., 2002, Xu et al., 1999). All these processes are carried out by binding to either the promoter or enhancers of genes. The transcription factors are involved in several biological functions that include:

(i) Basal transcription mediated by the general transcription factors that are a part of the pre-initiation complex.

(ii) Enhancer binding transcription factors that fine tune the process of transcription towards a spatiotemporal regulation.

(iii) The transcription factors are important mediators of development as in the case of Hox genes.

(iv) Transcription factors also act as signal sensors, cell cycle regulators, as well as mediators of important cellular processes.

(c) Histone chaperones: The histone chaperones are slowly being recognized as important regulators of chromatin function which are associated with the chromatin at different stages of the chromatin organization. This concept has been completely revolutionized by evidences implicating histone chaperones as important escort proteins of histones during transcription, replication and DNA repair (reviewed in De Koning et al., 2007). The main role of histone chaperones has been in avoiding aggregation of histones during storage as well as provide a constant sink of histones. However, there are histone chaperones which play important role in histone deposition and eviction (reviewed in Park and Luger, 2008), thus indicating an important role corroborant with the ATP dependent remodeling complexes for remodeling processes. One of the major discovery in the field of histone chaperone has also linked its role in transcriptional activation (Swaminathan et al., 2005), thus indicating that indeed these proteins are not just mere escort proteins, rather are bonafide component of chromatin with distinct functions.

(d) Small RNAs:

For a long time, the direct contribution of RNA towards chromatin architecture was a much debated topic. Although, there were indications such as chromatin induced gene silencing and dosage compensation linking the role of RNA to chromatin function (Lippman and Martienssen, 2004, Kelley and Kuroda, 2000), no direct evidence was available. However, one of the early lead was the observation that the heterochromatin associated HP1 foci were sensitive to RNase treatment which could be subsequently recovered by addition of RNA (Maison et al., 2002) indicated that this nucleic acid might also have a role in chromatin function. The most direct evidence was provided when the chromatin fractions were analyzed systematically to identify a 2-5% RNA component. It was also shown that this RNA belonged to the noncoding class of RNA molecule (Camos and Azorin, 2007). There are several functional attributes conferred onto the presence of RNA in chromatin. One of the most important speculation is that RNA might be helping in stabilizing the binding of structural non histone proteins to chromatin and thereby facilitate chromatin organization. This could be due to the RNA binding property of chromatin organizing proteins or through the chromatin looping mechanisms.

1.1.2. Different chromatin modifications

The most important regulators of chromatin dynamicity and thereby modulate chromatin function are the chromatin modifications. The chromatin modifications occur on the amino acid residues of the histone tails and nonhistone protein substrates except in the case of DNA methylation. (**Table 1**). The most commonly modified residue is lysine which undergoes acetylation, methylation, biotinylation, ubiquitination, SUMOylation etc. (reviewed in Berger, 2007).

Table 1: Amino acid residues that undergo modification.

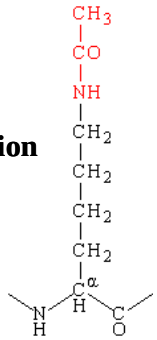
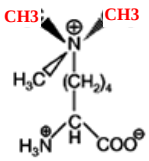
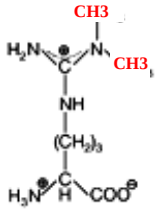
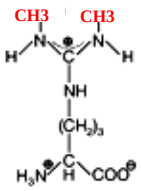
S.No.	Residue	Modification
1.	Lysine	Acetylation, Methylation, Ubiqutination, Sumoylation, Biotinylation
2.	Arginine	Methylation, ADP ribosylation
3.	Serine, Threonine, Tyrosine	Phosphorylation

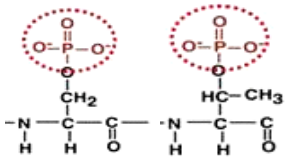
Among the modifications (**Table 2**), the most well studied is the acetylation and deacetylation of histones and nonhistone proteins (Li et al., 2007). Recently, several significant discoveries in the field of epigenetics, have turned the limelight onto methylation as an important regulator of transcription regulation and other cellular functions. Unlike acetylation that occurs only on lysine residues, methylation occurs on lysine and arginine residues (Kouzarides, 2007; Zhang and Reinberg, 2001). Based on the residue that gets modified, the methyltransferases are classified into lysine- and arginine- methyltransferases.

Lysine methylation can be mono, di or trimethylation based on the number of methyl groups added. Arginine methyltransferases catalyze two different types of modification: symmetric and asymmetric arginine methylation. As a result of these multiple modifications, a high level of regulation exists for methylation leading to a fine tuning at various steps. For a long time, histone methylation was considered to be a stable modification, but the recent

discoveries of demethylases and deiminase has shown that methylation too is reversible however, not as dynamic as acetylation.

Table 2: Chromatin modifications

MODIFICATIONS	SUBSTRATE	ENZYME MACHINERY	PHYSIOLOGICAL OUTCOME
Lysine Acetylation 	Lysine residue of: Histones H3, H4, H2A, H2B p53, Rb, STAT1, Cohesin, tubulin, p300, NF-κB,	Lysine acetyltransferases (KATs /HATs) Lysine deacetylases (KDACs/HDACs)	Transcriptional activation, DNA repair, Replication, Cell cycle progression,
Methylation **DNA methylation** 	Cytosine in CG dinucleotides	DNA methyltransferases (DNMTs)	Transcriptional repression, Ealry embryonic development
Lysine methylation 	Lysine residues of: Histone H3, p53,	Lysine methyltransferases (KMTs) Lysine demethylases	Transcriptional activation, elongation, repression
Arginine methylation  **Asymmetric dimethylarginine**	Arginine residues on Histone H3, H4, MRE11, CBP, STAT1, PABP1,	Protein arginine methyltransferases (PRMTs) Peptidyl arginine deiminase (PAD) Jumonji domain containing demthylase (JMJD)	Transcriptional activation, RNA processing, splicing, DNA repair, signal transduction
 **Symmetric dimethylarginine**	Arginine residues of Histone H3 (R8), H4(R3),		Transcriptional repression

MODIFICATIONS	SUBSTRATE	ENZYME MACHINERY	PHYSIOLOGICAL OUTCOME
Phosphorylation  Serine Threonine	Serine, Threonine , of Histone H3, H2B, Kinases , p53, Rb,	Kinases , Phosphatases	Signal transduction, transcriptional activation/repression, DNA repair, replication,
Ubiquitination polypeptide chain	Lysine resdiues on Histone H2A, H2B, nonhistone proteins	Ubiqutin ligases	Protein stability, Transcriptional activation, repression
Sumoylation small ubiquitin like modifier	Lysine residues on nonhistone proteins	SUMO ligases	Stability, Altered interacting proteomes
ADP ribosylation ADP ribose unit	Arginine residues on histones	NAD:arginine ADP - ribosyltransferase	Chromatin struvture
Biotinylation biotin	Lysine residues on histones	Biotinidase	Crosstalk of modifications

Lysine methylation has been shown to be closely linked with the reversible acetylation and thereby play a pivotal role in transcriptional activation or repression (Sims et al., 2003). Among lysine and arginine methylation, the former has been more extensively studied and reviewed. Arginine methylation, inspite of being identified five decades before, has gained attention only in the recent years. The majority of this modification is linked with transcriptional activation whereas there are also reports indicating its involvement with transcriptional repression in conjunction with DNA methylation.

The acetylation and methylation modifications very intricately regulate gene expression along with another important modification, phosphorylation. This is brought about by kinases and it is a reversible reaction, wherein the removal of the phosphate groups is catalyzed by the phosphatases. Phosphorylation is one of the most well characterized modification especially in the process of signal transduction. Chromatin dynamics, various cell cycle associated events are regulated by this modification. Distinct residues on histone tails and nonhistone proteins undergo either serine, threonine or in few cases tyrosine

phosphorylation. All the above modifications and others enumerated in **Table 2**, together help in the establishment of the transcriptionally competent state of the chromatin. With respect to regulation of gene expression, one of the most important modification is acetylation.

1.1.3. Chromatin acetylation

Acetylation is a well-characterized modification with important consequences on the chromatin function. It is a reversible reaction catalysed by the acetyltransferases and deacetylases. Lysine residue is the most preferred modification site. Acetyl CoA acts as the donor for the acetyl group. This modification deposits a partial negative charge on the lysine residue which leads to loosening of the histone-DNA contacts. In a global perspective, a wide spread reorganization occurs concomitant with the acetyl group introduction. The acetyltransferases and deacetylases exist usually in a complex consisting of remodeling factors etc. The yeast SAGA complex consisting Gcn5 acetyltransferase , human STAGA complex containing GCN5L acetyltransferase, human NUMAC complex containing CARM1 are complexes facilitating activation whereas human NCoR consisting of HDAC3, drosophila sin3 complex containing rpd3 deacetylase are repressor complexes. The acetyl group introduction on the histone tails is read by reader modules like the bromo domain present on the remodellers, which lead to a structural alteration in response to signals.

1.1.3.1. Histone acetylation:

The seminal discovery of histone acetylation associated with transcriptional activity was made by Vincent Allfrey in the early 1960s. Following this there was a spurt of research activities in this field which led to the identification of acetylated core histones as important components of transcriptionally active chromatin. This observation was further validated by other experimental evidences linking the possible conformational changes in the nucleosome (Bode et al., 1980; Bertrand et al., 1984) and enhanced accessibility to cellular machineries (Lee et al., 1993), subsequent to acetylation and hence histone acetylation came to be considered as a major determinant of the transcriptional competence of chromatin. However, these findings did not provide a mechanistic insight. Further research in this area was dominated by the mutant based analysis wherein the sites of modification were mutated and this showed conclusively the need for acetylation and transcription. The use of deacetylase inhibitors also compounded

this theory further. Finally, the identification of the various acetyltransferases and deacetylases provided the much awaited mechanistic and molecular details of the link between acetylation and transcriptional activation. Several of these acetyltransferases were found to exist in complexes with remodeling factors thus providing the explanation for the conformational changes associated with acetylation (reviewed in Marmorstein and Roth, 2001, Lee and Workman, 2007). Furthermore, these enzymes also had transcriptional co-activation property, as well as interacted with other transcription factors and hence could facilitate efficient transcription.

Table 3: Lysine acetyltransferases (KATs/HATs)

HAT family	Members
Type A HAT	**GNAT family**: GCN5/KAT2A, PCAF/KAT2B, ELP3, **MYST family**: Sas2, Sas3, Esa1, MOF, MOZ, HBO1, TIP60 **p300/CBP family**: CBP/KAT3A P300/KAT3B **Transcription factor related:** ATF1, TAF2, TFIIIC complex **Nuclear Hormone related:** SRC4, ACTR
Type B HAT	HAT1, HAT2

Histone acetylation is catalyzed by the histone acetyltransferases, (HATs), recently referred to as lysine acetyltransferases (KATs) due to the increased evidences of nonhistone protein acetylation. The HATs/KATs are broadly divided into two classes on the basis of their localization and are numbered from KAT1 to KAT13D (reviewed in Allis et al., 2007). The most abundantly present class is the type A HAT/KAT which are nuclear and hence act on histones and nonhistone chromatin proteins. On the other hand the cytoplasmic variant is called as type B HAT. The function of type B HATs is restricted to the

modification of newly synthesized histones as well as other cytosolic proteins (Qin and Parthun, 2002). The cytosolic HATs, HAT1 and 2 were first identified in *Saccharomyces cerevisiae* (reviewed in Parthun, 2007), subsequently homologs in other species were also identified. On the other hand, the nuclear HATs are many in number, with further subdivision into five main classes based on their functional characteristics (**Table 3**).

GNAT (Gcn5) family members were the first to be identified, the founding member of this family is Gcn5. This enzyme mostly acetylates histones with differential activities on the free form of histones against the nucleosomal form. Later on PCAF (p300/CBP associated factor) was also identified which has a similar active site structure as the above member. It has several nonhistone substrates apart from acetylating the histone tails. The second family is the p300/CREB-binding protein family, these proteins are commonly referred to as the master regulators of gene expression with well documented effects in various cellular processes such as differentiation, disease, transcription, replication, repair, apoptosis, cell survival and cell death. The third important class of HATs/KATs are the MYST family. This group of enzymes have a major role in the DNA damage and repair pathway, telomere maintenance. Majority of the H4 acetylation is brought about by this group of enzymes. The members of the MYST family are TIP60, MOZ, MOF,MORF, and HBO1. Each of these have been implicated in differentiation and development. These enzymes can form fusion proteins resulting in cancers. An interesting class of HATs are the transcription factor family, wherein the GTFs have been shown to possess acetyltransferase activity, thereby regulating transcription directly. The important enzymes belonging to this class are ATF2, TAF1, and TFIIIC90. The growth receptors and the hormone receptors are one of the main areas of active transcription based on extracellular signals. For a longtime, these were considered to be activated by the co-activators like p300 and PCAF. However, few nuclear hormone associated activators like ACTR, SRC2 have been shown to have acetyltransferase activity, thus in combination with the other HATs, these facilitate better signal integration and downstream target activation. Yet another unclassified group of acetyltransferases like CIITA, CDYL also exist (reviewed in Selvi and Kundu, 2009).

Table 4: Histone acetylation

HAT/HDAC	Modification site	Functional Outcome
GCN5/KAT2A , General Control Nonderepressible, acetyl transferase	Histone H2BK11, K16, H3K9, K14, K18, K23, K27, H4K8, K16	Transcriptional activation, histone deposition, repair,
PCAF/KAT2B, p300/CBP associated factor	Histone H3K14, H4 K8	Transcriptional activation, DNA repair
CBP/KAT3A, CREB Binding Protein	H2AK5, H2BK12, K15, H3K18, K23,	Transcriptional activation, DNA repair
p300/KAT3B	H2AK5, H2BK5, K12, K15, K20, H3K14, K18, K23, H4K5, K8, K12	Transcriptional activation, DNA replication and repair
Tip60 Tat Interacting Protein	Histone H2AK5, H3K14, H4K5, K8, K12, K16	Transcriptional activation, DNA repair, telomeric sliencing,
ATF2 Activating transcription factor 2	Histone H2BK5, K12, K15, H4K5, K8, K16	Transcriptional activation
Esa1/KAT5, essential SAS family acetyltransferase	Histone H2AK4, H2BK16, H3K4, K14, H4K5, K8, K12, K16	Transcriptional activation, histone deposition, repair,

Histone H3 and H4 lysines are the most acetylated by p300/CBP, PCAF and GCN5 at specific residues as depicted in **Table 4.** Most of these have a common functional outcome, i.e. transcriptional activation, but few have specific significance. Especially, the H4 acetylation of K16 by TIP60 is a mark associated with DNA repair. The histone H3K56 acetylation is yet another important epigenetic mark involved in histone deposition and chaperone function. Few residues of histone H2A and H2B also get acetylated, majorly by p300. The specific epigenetic language of each of these modifications in conjunction with methylation and phopshorylation will be discussed in the section on the cross talk established by these modifications.

Mechanism of histone acetylation:

The histone acetylation reaction is essentially a bi-susbtrate reaction involving the transfer of the acetyl group from the pseudo substrate, acetyl-CoA onto the ε-amino group of the lysine residue on histone/nonhistone protein substrates. Recent evidences suggest a possible autoacetylation reaction wherein the acetyltransferase itself undergoes acetylation, which is followed by the acetylation of the substrates as represented in **Figure 1.2.** Inspite of the presence of multiple acetyltransferase complexes, the well characterized are the enzymes that belong to the three families, namely, GNAT family, MYST family and the p300/CBP family. In the case of GNAT family, it has been shown that the reaction is an ordered sequential bi-bi kinetic mechanism (Tanner et al., 2000), A glutamate residue present in the active site has been shown to act as a general base, activating the ε- amino group of lysine for a nucleophilic attack forming a tetrahedral intermediate which finally results in the formation of the acetylated lysine residue and CoA (Tanner et al., 1999).

On the other hand, there have been two different mechanisms proposed for the MYST family of enzymes. It was earlier considered that the MYST family enzymes also follow a similar mechanism involving the glutamate residue as the catalytic base (Yan et al., 2000). Subsequent work led to the identification of a cysteine residue functioning as an acetylated intermediate leading to a ping-pong mechanism of catalysis (Yan et al., 2002). Several other attempts led to the conclusion that the acetylated cysteine although an important residue is not a catalytic residue and hence the mechanism of enzyme action for the MYST family is also a sequential bi-bi mechanism. The p300/CBP family showed a sequential mechanism similar to the other families of acetyltransferase (Sagar et al., 2004). However, the use of dead end inhibitors led to the identification of a ping-pong mechanism of action (Thompson et al, 2001). The final proof for the mechanism of action of p300 was obtained by the crystal structure data supplemented with the biochemical evidences (Liu et al., 2008) which clearly suggested a Theorell-Chance mechanism, which is indeed a sequential bi-bi mechanism, but the ternary complex has a very short life-time. Therefore, it can be concluded that all characterized HAT families follow an ordered sequential bi-bi kinetic mechanism where differences between families may affect substrate specificity but not the overall mechanism of catalysis (reviewed in Smith and Denu, 2010).

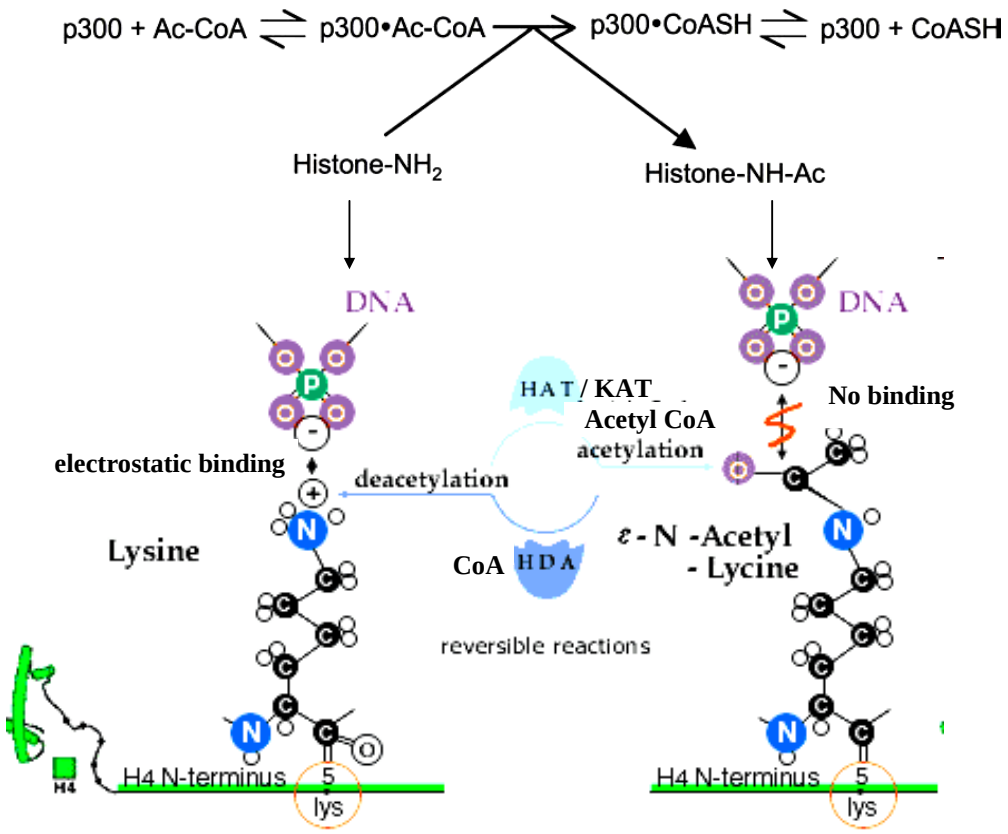


Figure 1.2. Mechanism of histone acetylation by p300 acetyltransferase. *p300 undergoes autoacetylation wherein multiple sites on the acetyltransferase are acetylated. Subsequent to this autoactivation, p300 transfers the acetyl group from acetyl CoA onto the lysine residue (histone H4K5 in this case). This modification occurs on the ε-aminogroup of lysine residue which loosens the interaction between DNA and histones. This reaction is a reversible reaction, wherein the deacetylase removes the acetyl group*

1.1.3.2. Nonhistone protein acetylation:

The field of nonhistone protein acetylation has grown at a very rapid pace, necessitating the change in the nomenclature of the acetyltransferases from HATs to KATs. Around 100 nonhistone proteins have been identified with functional implications and several more exist as observed by the recent acetylome data. The first non histone protein acetylation with functional significance was shown for the tumor suppressor p53 (Gu and

Roeder, 1997). The modified p53 was seen to have better DNA binding and transcriptional activation ability. Different acetylation sites have different cellular outcomes thus increasing the level of regulation brought about by a single modification on a single protein. The acetyltransferases themselves undergo acetylation, a mechanism akin to phosphorylation events. Apart from this acetylation of several signaling associated molecules like STAT1 (Kramer et al., 2006), RIP140 (Vo et al., 2001), SRC, PGC1α (Rodgers et al., 2005), as well as of several kinases has led to this modification regulating key signaling events.

Acetylation is an important modification for transcriptional activation, this is not through histone acetylation but also by acetylation of other proteins like p65, RelA of NF-κB (Perkins, 2006), c-myc, Tat etc. Furthermore, the important class of proteins, histone chaperones (NAP1, NPM1, Asf1) have also been shown to get acetylated with distinct functional outcomes (reviewed in Batta et al., 2007). Several metabolic enzymes have also shown to be regulated by acetylation. Another major finding in the field of acetylation was the cytosolic protein acetylation. α-tubulin was the first member in this category to be identified (Westermann and Weber, 2003). Although the exact acetyltransferase responsible for this has not been identified, a specific deacetylase, HDAC6 has been implicated in regulating the acetylation status of this protein. HDAC6 is a deacetylase that has also been linked with other cytosolic protein deacetylation including cortactin (Zhang et al., 2007), Hsp90 (Yu et al., 2002).

Thus, acetylation has a multifaceted means of regulating gene expression. On one hand, the promoter histone acetylation modulates the local chromatin environment so as to facilitate access to cellular machineries, and alongside, acetylation of nonhistone proteins facilitates better functioning with respect to signaling, protein-protein and protein-DNA interactions, thereby leading to a global alteration in the state of the chromatin and thus influencing gene expression (reviewed in Yang and Seto, 2008). This modification does not function alone, rather is influenced by several others which will be discussed in the subsequent sections.

Acetylation is a reversible process and the reverse reaction (removal of acetyl group is performed by the histone deacetylases/lysine deacetylases. There are four classes of HDACs; Class I (HDAC1-3,8), II (HDAC4-7, 9-10) and IV (HDAC 11) which possess an active-site metal dependent catalytic mechanism (Gregoretti et al., 2004). Class III HDACs

(or sirtuins) utilize a distinct nicotinamide adenine dinucleotide (NAD+) dependent catalytic mechanism and are conserved from bacteria to humans with seven human homologs (Sirt1-7) (Frye, 2000).

1.1.4. Chromatin methylation

Chromatin methylation has been considered to be a very important regulator of gene expression. For a long time it was looked upon as a stable epigenetic mark since the demethylating machinery were not discovered. However, the recent discovery of several demethylases specific towards specific modification sites has led to a re-defining of the field of chromatin methylation. Chromatin methylation has multiple levels of regulation on gene expression based on the residue and the modification status having implications in both activation as well as repression (reviewed in Martin and Zhang, 2005). The key components that undergo chromatin methylation are DNA, lysine and arginine methylation. DNA methylation is brought about the DNA methyltransferases which catalyze the transfer of the methyl group from the methyl cofactor donor, Adomet to the cytosine residues on the 5th carbon position. This modification has important consequences for the silencing of gene expression and heterochromatinization. DNA demethylation machinery have still been elusive. DNA methylation has been closely linked to the lysine methylation of histones especially in setting up the silent chromatin. Lysine methylation is brought about by the lysine methyltransferases on both histones and nonhistone proteins. The effector modules that recognize this modification are the plant homeo domain (PHD) and the chromo and the chromoshadow domain (CD and CSD), (reviewed in Taverna et al., 2007). DNA methylation has been closely linked with transcriptional repression whereas lysine methylation has been associated with both transcriptional activation and repression based on its modification status, asymmetric arginine dimethylation is a mark linked with transcriptional activation whereas the symmetric dimethylation of the arginine residue is associated with repression.

1.1.4.1. DNA methylation:

DNA methylation involves the transfer of the methyl group from Adomet to the cytosine residues (**Figure 1.3**) present in a CG dinucleotide motif (reviewed in Klose and Bird, 2006). There are 4 bonafide DNA methyltransferases (DNMTs) and an activator which is an inactive methyltransferase. The DNMTs are classified into two important groups, the

de novo methyltransferases (DNMT3A, DNMT3B) and the maintenance methyltransferase DNMT1 based on the differences in their substrate preference and activity. DNMT2 has weak methyltransferase activities. Although the catalytic domains are highly conserved, both across different enzymes as well as across different species, the enzymes differ in their activities. DNMT3A and 3B act on both methylated and hemimethylated DNA and are involved in methylation at early stages of development, hence referred to as de novo methyltransferases. On the other hand, the maintenance methyltransferase, DNMT1 can methylate only hemimethylated DNA and hence has important consequences on replication. The other member which shares the similar domains but lacks the methyltransferase activity is DNMT3L. This protein is present in the complex with DNMT3A and 3B and activates their function. The process of DNA demethylation is so far not been linked with any enzyme activity. The proposed hypothetical mechanisms of active DNA demethylation are: (a) the direct removal of the methyl group by enzymatic activity (very few reports indicate the presence of such enzyme), (b) removal of methylated base directly or by a two step process, wherein cytosine deamination occurs and then the thymine is removed, followed by the action of the base excision repair machinery. (c) the removal of a DNA stretch and strand filling by the nucleotide excision repair machinery.

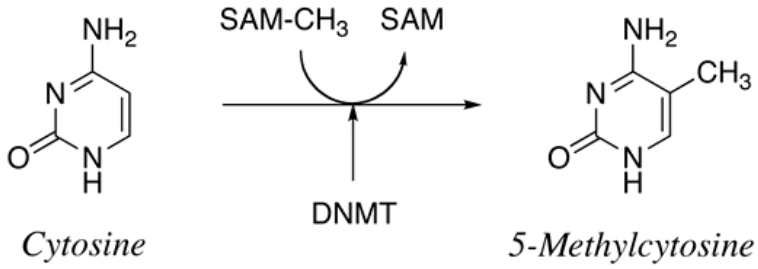


Figure 1.3.DNA methylation reaction. *The cytosine residue undergoes methylation at the 5th carbon position by the addition of methyl group from the methyl cofactor donor, SAM, catalyzed by the DNA methyltransferases.*

The field of epigenetics, gained momentum due to the discovery of this modification. Since, DNA methylation occurs on the promoters, it is a true candidate for epigenetic regulation of gene expression (reviewed in Miranda and Jones, 2007). The presence of methylated DNA acts as a signal for the recruitment of the other repressor complexes, thereby signalling for a closed chromatin state. The (methyl group binding domain) MBD

proteins such as MeCP recognize the methylated cytosine. These proteins are usually present in complexes consisting of H3K9 methyltransferases and deacetylase (Marhold et al., 2004). The above recognition leads to the activation of the deacetylase that removes the acetyl group from the H3K9 residue which is followed by the methylation. These complexes are also shown to contain important remodelling proteins such as CAF-1 and hence the entire event is a well co- ordinated process with distinct changes in the chromatin architecture. This modification also regulates the important cellular processes like genomic imprinting and X chromosome inactivation. Apart from such an important role in maintaining the transcriptionally competent state of the chromatin, DNA methylation has also been closely linked to disease manifestation with well documented evidences in cancer.

1.1.4.2. Lysine methylation:

Lysine methylation of chromatin is a dual functional mark, with effect on both transcriptional activation and repression. It was considered to be a stable mark but the recent identification of different classes of demethylases has changes this concept. However, in comparison with the reversible acetylation, the dynamicity of this modification is much less. The identification of the enzyme responsible for this modification took more than a decade following the observation of methylated lysines. The first methyltransferase to be identified was SUV39H1 (Melcher et al., 2000). This is responsible for the silencing associated epigenetic mark, H3K9 trimethylation. Lysine methylation can be mono, di or tri. The mono and di modifications exist on chromatin that can flip towards activation or repression. But, the trimethylation is a mark strictly associated with a single outcome. For example, H3K4 trimethylation is a mark of active chromatin, whereas H3K9 trimethylation is a mark of repressive chromatin (reviewed in Martin and Zhang, 2005). Few of the important representative members that catalyze this modifications on histones and their subsequent functional significance is enlisted in **Table 5.** Thus, it is clear that the SET 1-9 methyltransferases are the major lysine methyltransferases, since the different enzymes bring about the different modifications, such as H3K4, 36 and H4K20 methylation with distinct physiological outcomes. All lysine methyltransferases are characterized by the SET (Suppressor of Variegation, Enhancer of Zeste, Trithorax) domain, except for DOT1L. This enzyme methylates the H3K79 residue (Feng et al., 2002) with implications in telomere silencing, thus changing the concept that modifications occur only on the N-terminal tails.

The MLL methyltransferase is a multisubunit protein with several cellular functions, but, it is one of the most worked out fusion protein with oncogenic potential (Krivtsov and Armstrong, 2007).

Table 5: Histone Lysine Methylation

Enzyme	Modification site	Functional outcome
MLL (1-5) /KMT2A-2E	H3K4	Transcfriptional activation
hSET1A-1B/ KMT2F,2G	H3K4	Transcriptional activation
ASH1/KMT2H	H3K4	Transcriptional activation
SUV39H1/H2/ KMT1A/1B	H3K9	Heterochromatinization
G9a/GLP/KMT1C/1D	H3K9	Transcriptional repression
ESET/KMT1E	H3K9	Transcriptional repression
RIZ1/KMT8	H3K9	Transcriptional repression
Ezh2/KMT6	H3K27	Transcriptional repression
SET2/KMT3A	H3K36	Transcriptional elongation
DOT1L/KMT4	H3K79	Transcriptional activation
SET8/KMT4A	H4K20	Gene silencing

Lysine methylation can be partly considered to be a centrally present modification in the epigenetic network. This is probably the only modification with direct interactions with DNA methylation, lysine acetylation, phosphorylation, ubiquitination and arginine methylation. DNA methylation and repressive lysine methylation are coordinated and recognized by the MBD proteins and the heterochromatin protein HP1. These together establish the silent chromatin. H3K4 methylation marks the transcription initiation alongwith the H3, H4 lysine acetylation. H3S10 phosphorylation and H3K9 methylation are known to hinder with each other's outcomes. H2B ubiquitination signals for the lysine methylation. One reason for this multivalent action is probably due to the different effectors

which recognizes this mark, the chromo domain, chromoshadow domain, PHD domain, tudor domain etc.

Mechanism of histone lysine methylation

Lysine methyltransferases catalyze mono-, di-, and trimethylation of the lysine ε-amino group in an S-adenosyl-L-methionine (AdoMet) dependent manner (**Figure 1.4**) Histone lysine methyltransferases consist of two main classes, the SET domain containing family and the DOT1 family.

There are about 60 SET domain containing proteins in humans, but not all are methyltransferases. It is the size and bonding patterns of the active-site residues which determine whether the enzyme carries out mono-, di-, or trimethylation of its target lysine residue. SET domain methyltransferases catalyze a sequential bi-bi kinetic mechanism in which both substrate association and product release occur in a random fashion (Chin et al., 2006, Patnaik et al., 2004, Guo and Guo, 2007)..

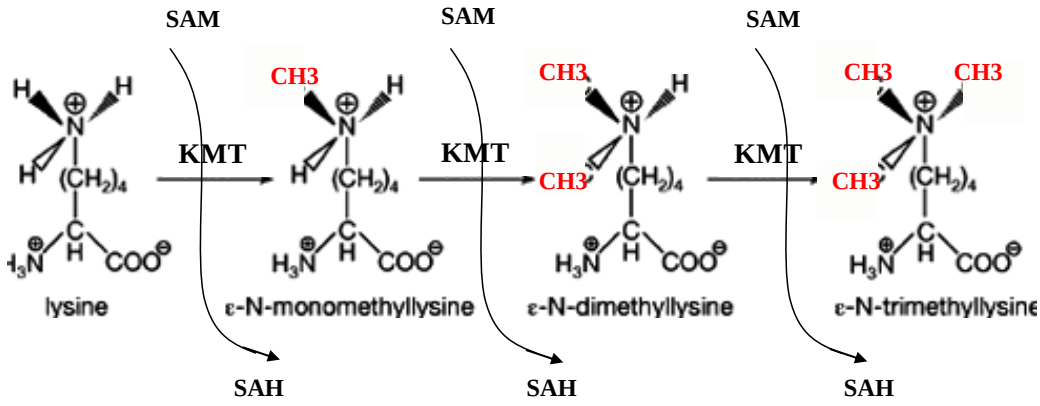


Figure 1.4. Lysine methylation reaction. *The lysine methyltransferases catalyze the transfer of methyl group from SAM to the lysine residue, which can sequentially undergo mono, di and tri-methylation. There are distinct enzymes that catalyze these modifications which have distinct functional outcomes. KMT refers to the lysine methyltransferase, SAM is S-adenosyl methionine and SAH refers to S-adenosyl homocysteine.*

Within the active site, a conserved aspartate or glutamate hydrogen bonds to the two-ribose hydroxyls of AdoMet. In addition, a conserved arginine or lysine forms a salt bridge with the carboxylate of AdoMet. These interactions position AdoMet in a U-shaped conformation that places the methylsulfonium group at the base of a hydrophobic channel in which the lysine substrate binds. The AdoMet methyl group is positioned and activated by

C–H…O hydrogen bonds between the methyl group and the side chain hydroxyl groups. Although, this bond is weak as compared to the N-O bond, the presence of the C–H…O hydrogen bonds in the folded proteins leads to greater contribution due to desolvation properties. After one round of methyl transfer, it is hypothesized that C–H…O hydrogen-bonding between the methylammonium group and active-site residues orient monomethyl- or dimethyl-lysine either for subsequent methylation events or to disfavor subsequent methyl transfer (Couture et al., 2006).

The discovery of several demethylases opened up a new view towards lysine methylation. The first member to be identified was Lysine specific demethylase (LSD1). Initially, it was thought to be specific to the H3K4 mark but was later found to demethylate the H3K9 mark as well. Following this, was a spate of several jumonji domain containing proteins with different specificities. Recent evidences link these demethylases to early embryonic development and differentiation (reviewed in Nottke et al., 2009).

Lysine methylation of nonhistone proteins:

Methylation of nonhistone proteins at lysine residues is quite rare as compared to acetylation. This is also true in the case of histones wherein the number of modification sites are also lesser than the acetylation sites. Probably, this is due to the more stable nature of methylation. There are a few important nonhistone protein substrates which get methylated at lysine residues with functional outcomes. The most well characterized is p53 methylation. G9a/GLP are known to modify p53 lysine residues (Hunag et al., 2010). The methylation by SET9 regulates the stability of p53 (Kurash et al., 2008). On the other hand, SMYD2 mediated p53 methylation decreases its DNA binding ability (Huang et al., 2006). LSD1 demethylates p53 which also leads to dissociation of the p53 transcription complex (Hunag et al., 2007). VEGF is methylated by SMYD3 (Kunizaki et al., 2007) which enhances its kinase activity. Besides, these substrates, few general transcription factors have also been identified as substrates of lysine methyltransferases, however no functional implication has been identified so far. As compared to lysine methylation of nonhistone proteins, the arginine methylation is quite predominant and regulates several essential cellular processes.

1.1.4.3. Arginine methylation:

Protein arginine methylation was identified almost five decades earlier (Liu and Dreyfuss, 1995; Kim et al., 1998) but histone arginine methylation (Lee et al., 2004) was

discovered only a decade ago. Protein arginine methyltransferases (PRMTs) catalyze the addition of methyl group from the methyl cofactor, S-adenosyl methionine to the guanidino nitrogen of the arginine residue on proteins (**Figure 1.5**). In humans, there are 9 PRMTs identified so far, which are classified into symmetric and asymmetric arginine methyltransferases (reviewed in Bedford et al., 2005).

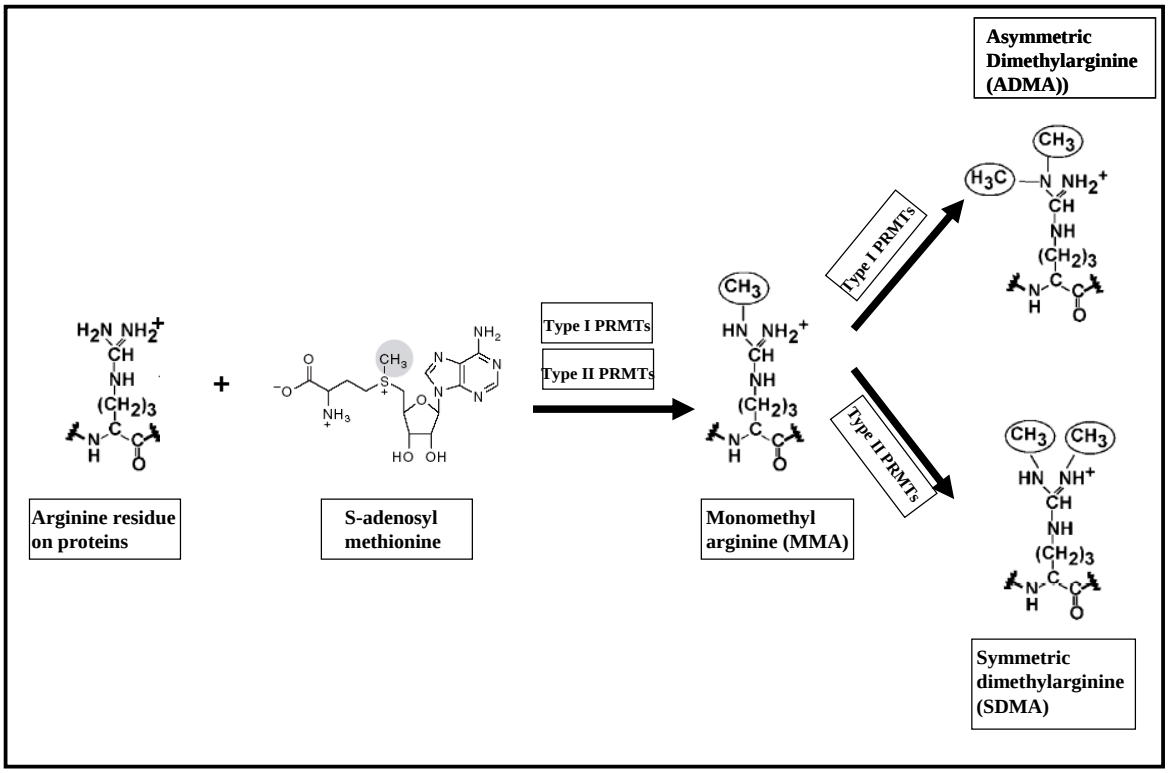


Figure 1.5. Arginine methylation reaction. *Arginine methylation involves the transfer of the methyl group from S-adenosyl methionine (SAM) to arginine residue on proteins by type I PRMTs to give asymmetric dimethyl arginine (ADMA) or by type II PRMTs to give symmetric dimethylarginine (SDMA).*

The arginine methyltransferases, PRMT5/JBP1, PRMT7 and PRMT9 (Lee et al., 2005; Cook et al., 2006; Gonsalvez et al., 2007) are known to bring about symmetric modification which leads to transcriptional repression. In contrast to these, the other PRMTs, PRMT1-4 and PRMT6, 8 are asymmetric dimethylarginne methyltransferases which are predominantly involved in transcriptional activation (reviewed in Bedford et al., 2005). There have been reports of PRMT10 and PRMT11 as well, however, their substrate specificities have not been identified so far.

Table 6: Protein Arginine Methylation

ENZYME	SUBSTRATE	FUNCTIONAL OUTCOME
PRMT1	Histone H4, nucleolin, Sam68, hnRNPA1,A2,R,K,Q,U, 53BP1, FXR, PGC1α, HNF4. TAFII68, RNA helicase A,DNA polβ Mre11 STAT1,STAT3, iSMADs, NIP45, RIP140, Ki-1/57 EBNA1, Adenoviral 100k protein, sHDAg,	TRANSCRIPTION REPLICATION REPAIR CELL SIGNALLING PATHOGENESIS
PRMT2	Histone H3 Receptor methylation	Transcription ER and AR coactivator
PRMT3	Sam68, PABP-N1, ribosomal protein S2	Transcription, ribosomal function
PRMT4	Histone H3, PABP1, CBP, HuR, SmB/B' TARPP, MyoD, PARP1	Transcription RNA processing Differentiation
PRMT5	Histone H4, histone H2A, histone H3, FCp1,SPT5 Sm proteins	Transcription RNA processing
PRMT6	HIV Tat, HIV nucleocapsid, HMGA1a, HMGA1b	Viral pathogenesis Gene expression
PRMT7	Sm proteins	RNA procesing
PRMT8	EWS	unknown

PRMT1, PRMT4/CARM1 and PRMT6 are arginine methyltransferases that have both histones and nonhistone substrates. PRMT1 is responsible for 80% methylation of all protein methylation in the cellular system. PRMT1 and other PRMTs except PRMT4, recognize glycine arginine rich motifs (Najbauer et al., 1993) for methylation whereas PRMT4 recognizes XXP/RR/PXX motif for methylation (Lee et al., 2002). The arginine methyltransferases thus have other nonhistone protein substrates. All these modifications result in various functional outcomes, outlined in **Table 6**. Protein arginine methylation has been reported to be involved in several processes like, RNA processing, DNA damage and repair, cell signaling and most importantly in transcription. In the case of RNA processing, the RNA splicing machinery is known to be regulated by arginine methylation of the splicing machinery. In a few instances, deregulation of this function has been reported to be involved in disease manifestation.

All PRMTs do not act on histone substrates, rather majority of them act on nonhistone targets. PRMT1 acts on histone H4R3 (Strahl et al., 2001), PRMT4/CARM1 solely methylates H3R17 and R26 *in vitro* as well as *in vivo* whereas H3R2 is an *in vitro* substrate (Schurter et al., 2001), PRMT6 methylates H3R2 (Guccione et al., 2007).

The site-directed methylation of histones H3 and H4 have direct functional significance in the process of transcriptional activation. Interestingly, PRMT1 and PRMT4 function cooperatively, with other modification (eg. acetylation) in specific transcription pathways (An et al., 2004, Hassa et al., 2008, Kleinschmidt et al., 2008). Protein arginine methylation of histones and non histone proteins together regulates several key cellular processes like RNA processing, signal transduction, DNA repair and transcriptional activation as depicted in **Figure 1.6**. Several RNA binding proteins such as hnRNPs are methylated by PRMT1. The splicing machinery is also regulated by protein arginine methylation. Sam68 is one of the preferred substrate of PRMT1 (reviewed in Bedford et al., 2005). This intricate network between the methylation machinery and the RNA processing factors indicates a significant contribution by this modification in transcription and translation. The repair pathway is also modulated by arginine methylation, wherein the helicase MRE11 function has been shown to be increased upon the modification. Most importantly, in the process of signal transduction, STAT1 and NFAT get methylated by PRMT1, which facilitates the dimerization and better downstream activation. It is important

to note here that STAT1 is not only an arginine methylation substrate but is also modified by acetylation. The interplay of arginine methylation and acetylation in regulating STAT1 function is not known.

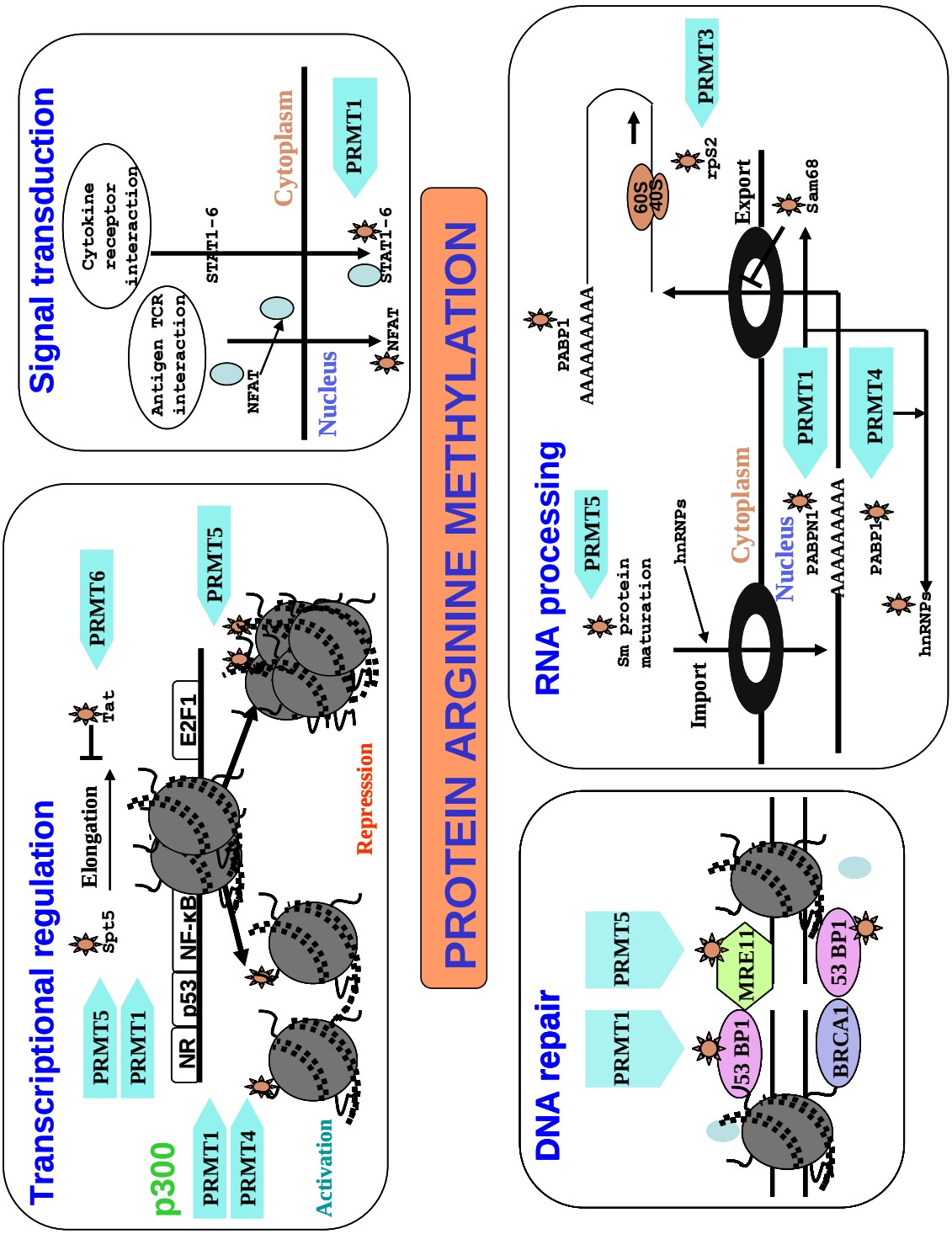


Figure 1.6. Functional implications of protein arginine methylation. *The arginine methylation of histones and non histone proteins regulates the functional outcomes of various cellular processes like transcription, signal transduction, DNA repair and RNA processing. The brown star implicates arginine methylation. The blue arrow heads depict the enzyme that brings about the arginine modification. In the case of transcriptional regulation, histone arginine methylation by PRMT1/PRMT4 leads to transcriptional activation whereas the modification by PRMT5 leads to transcriptional repression. Apart from this, the arginine methylation of proteins like tat, Spt5 have distinct transcriptional outcomes. The arginine methylation of STAT1 and NFAT have implications in signal transduction. In DNA repair, various repair associated proteins such as MRE11, 53BP1 undergo arginine methylation which enhances their functional outcome. Most importantly, arginine methylation is involved in the RNA processing machinery by modifying important export proteins, splicing factors as well as hnRNPs.*

Mechanism of arginine methylation:

Structural evidence suggest that PRMT enzymes catalyze an ordered sequential bi-bi kinetic mechanism in which AdoMet binds prior to the arginine substrate (Yue et al., 2007). A conserved glutamate and arginine residue interacts with the two ribose hydroxyls and carboxylate of AdoMet, respectively.

Similar to histone lysine methyltransferases, the methylsulfonium group of AdoMet is positioned at the base of a channel in which the arginine substrate binds. Two invariant glutamate residues hydrogen bond to and position the guanidinium side chain of the arginine substrate. These hydrogen bonds are hypothesized to localize the positive charge to one terminal nitrogen of the guanidinium side chain leaving a lone pair on the other nitrogen to attack the methylsulfonium of AdoMet (Zhang et al., 2000). Similar to histone lysine methyltransferases, methyl transfer proceeds through an SN2 mechanism. The proton elimination step after methyl transfer is hypothesized to occur through a His-Asp proton relay system. However, a common characteristic of the arginine methyltransferases is their existence as oligomers for proper functioning. The deletion of the dimerization domain leads to a loss in the methyltransferase activity. This alludes towards the possibility of a multiple modification possibility at the same time.

PRMTs play an important role in transcriptional activation. Although, the exact molecular mechanisms involved in transcription regulation have not been identified so far,

PRMT1 and PRMT4 mediated histone arginine methylation has been very closely linked to the nuclear hormone receptor associated transcriptional activation. The significance of these two enzymes has also been shown for the p53 dependent (An et al., 2004) and NF-κB dependent gene expression (Covic et al., 2005; Miao et al., 2006). One of the main observation by researchers is the co existence of arginine methylation and acetylation in several transcription associated complexes, but the question that still remains to be answered is whether this coexistence is due to physical proximity of these enzymes or is it a node in the epigenetic network. Preliminary evidences seem to suggest both these possibilities. This cross talk of modifications and their functional significance will be subsequently discussed.

1.1.5. Other chromatin modifications:

Few other important chromatin modifications include ubiquitination, ADP ribosylation, sumoylation and recently reported formylation, propionylation etc. have also been identified by mass spectrometric detection. Ubiquitination not only regulates protein degradation but also influences transcription (Weake and Workman, 2008). The ubiquitin ligases have been identified in several multienzyme complexes and furthermore, important acetyltransferases like p300 (Grossman et al., 2003) and PCAF (Linares et al., 2007) have also been shown to possess additional ubiquitination activity. Histone H2A and H2B monoubiquitination at residues in the globular domain have been shown to be important signals for subsequent methylation by COMPASS and DOT1. The related modification, SUMOylation (small-ubiquitin like modifier) has distinct functional outcomes (reviewed in Denuc and Marfany, 2010). This modification has been discovered recently, with only about a decade of research on it. However, the targets of this enzyme have been shown to be vast with enormous cellular outcomes. This is a reversible modification which influences the interacting proteome thereby affecting the localization, as well as functionality of the modified proteins. Yet another important modification is the ADP ribosylation, brought about by the ADP-ribosyltransferases. This reaction involves the transfer of ADP-ribose from NAD+ (Nicotinamide Adenine Dinucleotide) to arginine, glutamate or aspartate on substrates. Histones are modified by the NAD:arginine ADP-ribosyltransferase which is a mono modification. This is a reversible reaction. Poly AdP ribosylation (PARP) is also a

common modification present only in eukaryotes (reviewed in Kim et al., 2005). This has important implications in structure, DNA repair and telomere maintenance.

The other modifications that have been recently identified include formylation, propionylation and butyrylation. These modifications are not yet properly characterized and hence not much functional significance has been attributed to them. The N^6-formylation of lysine has been identified on histones and other nuclear proteins. This modification is possibly caused by the formyl group that arises from the oxidation of deoxyribose in DNA, adds onto the N^6-amino groups of lysine side chains (Jiang et al., 2007). This modified residue bears similarity to the acetylated lysine form, hence it is hypothesized to have negative regulatory effects on the known modifications such as acetylation and methylation, especially during oxidative stress. Histone propionylation and butyrylation are the most recently identified modifications occuring on the lysine residues (Chen et al., 2007). So far, no functional significance has been found, but the interesting observation is the identification of the enzymes p300/CBP and PCAF, responsible for this modification. These enzymes are important regulators of gene expression and they are not just acetyltransferases but also possess ubiquitination, propionylation and butyrylation activities, indicating the possibilty of a multifaceted regulation.

A vitamin – histone conjugate is also a commonly found histone modification. This refers to the biotinylation wherein biotin is covalently attached to the lysine residue by a mutlistep enzymatic process, mainly by biotinidase. Although the enzyme levels were not found to change across different cell cycle stages, it has been observed that biotinylated histone levels are more in proliferating cells. Moreover, this is also a reversible reaction Hassan and Zempleni, 2008). The major sites of biotinylation on histones are H2A K9, K13 and K129, H3 K4, K9 and K18, H4 K8 and K12. This modification is inhibited by adjacent lysine acetylation, however arginine dimethylation enhances the biotinylation.

1.2. CHROMATIN MODIFICATIONS AS IMPORTANT REGULATORS OF GENE EXPRESSION

The above section clearly states the important role of the chromatin modifications in different cellular processes. The expression of genes can be broadly divided into two main categories, the constitutive gene expression, which maintains the house keeping genes

constantly expressed, however, majority of the genes are expressed in response to various signals. The latter category has been more extensively worked out, and the role of chromatin and its modifications is more explicit in this signal associated changes and the subsequent gene expression. An individual's gene expression begins at a single cell stage which undergoes distinct developmental patterns. Thus, the initial gene expression is in a pluripotent state of the cell, wherein the chromatin is in a less compact euchromatin form. There exists a plasticity (fluidity) which aids in following any lineage based on the differentiation pathway. Thus, as the cells proceed from a pluripotent to a differentiated stage, it is accompanied by an overall change in its transcriptional competence, as evidenced by the difference in the chromatin architecture. An elegant work by Eric Lander's group on embryonic stem cells revealed a bivalent chromatin marks on important genes involved in development. The presence of both activation associated H3K4 methylation and repression associated H3K27 methylation indicated that the genes are poised to proceed towards either pathways based on the signals (Bernstein et al., 2006). This work also revealed that H3K4 methylation is an epigenetic mark of activation in the early developmental pathway. Since, the concept of gene expression seems to proceed from a predominantly active state to a repressed state alongside the developmental pathway, the process of gene expression can be considered to start from transcriptional activation. Histone H3, H4 acetylation and H3K4 trimethylation are marks associated with the transcription start site (**Figure 1.7**). The enzymes responsible for these modifications such as p300/CBP, PCAF, GCN5 and MLL are known to be present in the RNA polymerase complexes either directly or through interactions with other GTFs. In the case of specific events such as nuclear hormone receptor activation, the above complex is also characterized by the presence of the arginine methyltransferases, PRMT1 and PRMT4. This is followed by a gradual decrease in the acetylation and trimethylation mark, with a concomitant increase in the H3K36 methylation, which is a mark associated with elongation. Prior to the transcription elongation process, the RNA polymerase carboxy-terminal domain (CTD) undergoes phosphorylation indicating the essentiality of yet another important modification in the process of gene expression. The CTD phosphorylation status is an indication of the stage of the transcription.

The process of RNA processing is also significantly regulated by arginine methylation of the processing machinery including Sam68, PABPN1 as well as splicing

factors. PRMT1 methylates several hnRNPs which possesses the GAR motif (reviewed in Bedford et al., 2005). In a specialized scenario such as the SRC3/AIB1 complex, methylation of SRC3 by CARM1 has been shown to regulate the assembly and disassembly of the co-activator complex (Feng et al., 2006).

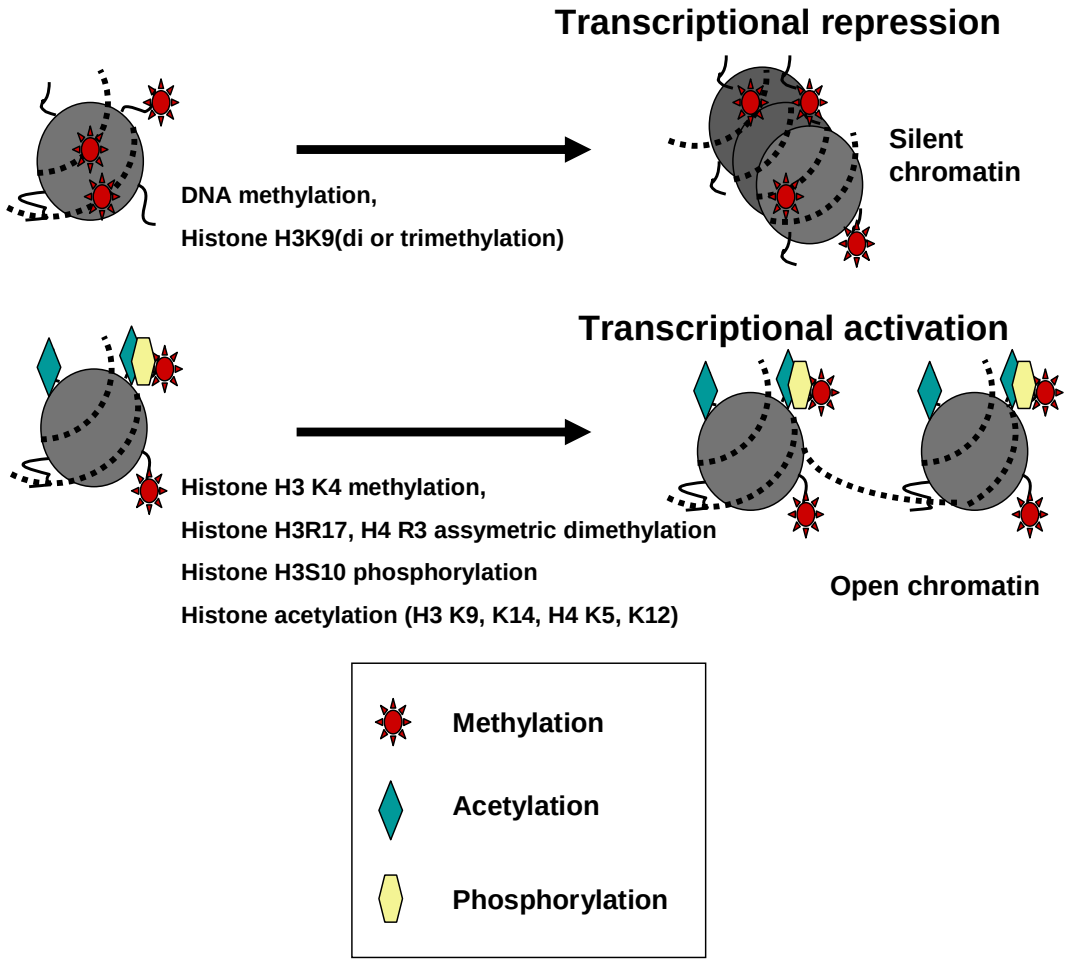


Figure 1.7. Chromatin modifications regulate transcriptional competence and thereby gene expression. *Epigenetic marks such as acetylation, histone H3K4 methylation, histone H3R17 methylation and H3S10 phosphorylation are marks associated with transcriptional activation. Transcriptional repression is generally characterized by DNA methylation and H3K9 methylation.*

It is evident that the process of gene expression right from transcription initiation is modulated by chromatin modifications. The process of repression or gene silencing is regulated by another set of epigenetic marks, namely DNA methylation, H3K9 and H3K27

methylation. These marks are recognized by important effector modules in proteins such as HP1 which by virtue of their various interactomes, mediate heterochromatinization.

All these evidences strongly suggest that the chromatin modifications are not just chemical covalent tags, rather are flags that mark the region for a certain state of chromatin, which is read and recognized by the effector modules acting as platforms facilitating the downstream effects. It is clear that these marks are not individual signals rather are sequential nodes in a network leading to the complex language of chromatin characterized by the crosstalk among different modifications (**Figure 1.8**)

1.3. CROSS TALK OF CHROMATIN MODIFICATIONS, THE EPIGENETIC LANGUAGE

Epigenetics is defined as mitotically and meiotically heritable changes in gene expression that do not involve a change in the DNA sequence (Egger et al., 2004). The field of epigenetics has undergone a face lift due to the increasing literature connecting it with key processes in gene expression over the past few decades. The recent whole genome studies in different contexts have revealed characteristic signatures for distinct events, thus unraveling the complex language of epigenetic modifications. There seems to be an intricate network of chromatin modifications orchestrating the process of gene expression by involving various chromatin proteins and other components such as small RNAs. The elucidation of this network, warrants an investigation of the nodes of the network which is formed by a cassette of modifications. These can be broadly divided into those that regulate another modification positively or those that have a negative mode of regulation. A further level of classification divides these into *cis*-acting and *trans*-acting cross talk based on modifications on the same histone tail or another histone. The different cross talk that exist between the histone H3 and H4 tail are represented in **Figure 1.8.**

1.3.1. Modifications with a positive regulatory effect:

The most well characterized and probably, the earliest identified cross talk is between DNA methylation and H3K9 methylation. Several studies indicate the positive regulatory effects of the above modifications on each other. This has been evident by treatment of the DNA methylation inhibitor 5-aza dc, which apart from decreasing DNA methylation also reduces the H3K9 methylation (Zhang et al., 2007). Several knockout

studies of DNMTs, H3K9 methyltransferases in MEFs, ES cells and mammalian cells has revealed a global decrease in both DNA methylation and H3K9 methylation indicating the existence of a feed forward loop mechanism amongst these modifications. This regulation is further fine-tuned by the deacetylation process, since in the case of H3K9, deacetylation of this residue is a prior requisite for H3K9 methylation to occur.

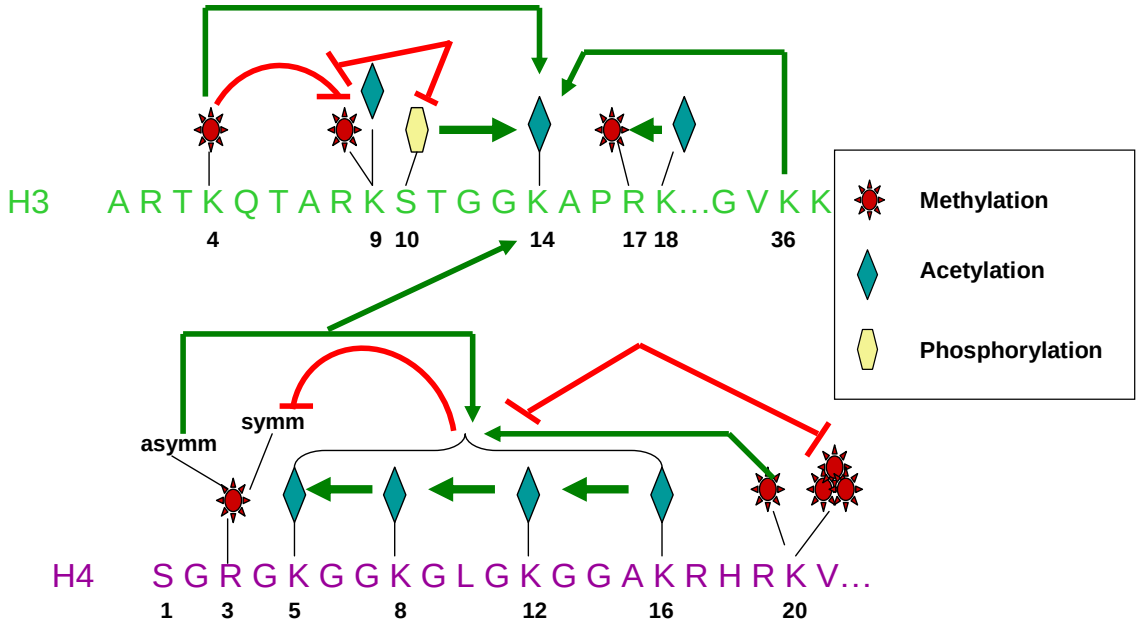


Figure 1.8. Cross talk of histone H3 and H4 modifications. *The amino acid residues and their corresponding positions of the histone H3 and H4 N-terminal residues are shown. The positive regulatory modifications are represented by the green arrow, whereas the red lines indicate the negative modulatory effects, i.e. antagonizing effects. With respect to histone H3, there are two antagonizing cross talk, H3K4 methylation inhibits H3K9 methylation and H3S10 phosphorylation inhibits H3K9 methylation. There exist several positive regulatory modifications like, H3K4 methylation and H3K14 acetylation, H3K14 acetylation and H3R17 methylation, H3S10 phosphorylation and H3K14 acetylation as well as H3K36 methylation and H3K14 acetylation. The histone H4 represents four major acetylation sites, H4K5, K8, K12 and K16 which sequentially regulate each other. H4R3 asymmetric methylation and H4K20 monomethylation positively regulates H4 acetylation. H4R3 symmetric methylation is negatively regulated by H4 acetylation. H4K20 trimethylation is a mark of silencing and hence inhibits H4 acetylation. All these examples are of cis-modulation. One example of trans-modulation is also shown where, H4R3 asymmetric methylation regulates H3K14 acetylation.*

There are evidences suggesting the co-existence of the DNA methyltransferases and histone deacetylases in a complex (Poleshko et al., 2010). Thus, it can be easily visualized that a transcriptional repression/gene silencing state, gets initiated by DNA methylation, which brings the deacetylase complex in close contact of the histones which deacetylates H3K9. This acts as a signal for the H3K9 methyltransferase to methylate and this is recognized by the chromodomain of heterochromatin proteins, which finally results in gene silencing. Another level of regulation has been recently introduced into this system by the discovery of the demethylases, since demethylases and deacetylases have also been found to coexist in complexes like the NURD, CoREST complex.

Early examples of such positive influential networks that act in *cis* are the H3S10 phosphorylation that enhances H3K14 acetylation, H3K18 and K23 acetylation regulating H3R17 methylation for estrogen responsive gene expression (reviewed in Berger et al., 2007). H3K36 methylation signals for the recruitment of deacetylase for removing the acetylation marks (Li et al., 2007). H3K4 methylation mark facilitates H3K14 acetylation. The best worked out epigenetic cascade is on the H4 tail, wherein H4K20 dimethylation signals for the acetylation of H4K16, which subsequently leads to a chain of events characterized by H4K12 acetylation, followed by H4K8 acetylation and finally H4K5 acetylation (Scharf et al., 2009). It is important to note here that all these modifications are not brought about by the same enzyme, rather there is an ordered recruitment of different enzymes in a cascade.

Trans modulation involving modifications on histone H3 and H4 are also known, one of the important modification being PRMT1 mediated H4R3 facilitating p300 mediated acetylation of H3 and H4 tails (An et al., 2004). H3K4 methylation by MLL is a signal for MOF mediated acetylation of H4K12 (Dou et al., 2005). An intriguing *trans* modulation with a positive regulatory effect is the H2BK123 monoubiquitination regulating the H3K4 and K79 methylation, since generally ubiquitination is a signal for degradation or repression (Nakanishi et al., 2009).

1.3.2. Modifications with a negative regulatory effect:

This event is more well established in the *cis* regulatory mode, especially when the same residue undergoes different modifications. So, H3K9 methylation inhibits its acetylation and vice versa.

An interesting cross talk is the inhibitory effect of H3S10 phosphorylation on H3K9 methylation (Duan et al., 2008). However, the presence of H3K9 methylation and H3K14 acetylation on the same histone is a much debated topic, since few organisms show the presence of these two marks at the same time, but in few such as mammalian systems these are exclusive of each other. In the case of H4, H4K12 biotinylation is a mark of repressive chromatin (Wijeratne et al., 2010) whereas the acetylation of this residue is a signature for activation. Hence, these two modifications antagonize each other. Yet another conflicting observation is the H4 acetylation antagonizing the PRMT1 mediated H4R3 methylation. If the methylation occurs first, it promotes acetylation but the vice versa does not hold true. The level of complexity in these cases increases further due to the involvement of other chromatin proteins in various modified forms.

An interesting but unanswered cross talk is the arginine methylation cross talk with other modifications, especially acetylation. Both for PRMT1 and PRMT4 activities, p300 mediated acetylation has been shown to be important, but in well defined specific systems. This leads to a speculation about this cassette of arginine methylation with respect to its mechanism which will be briefly discussed in the following section.

1.3.3. Crosstalk of acetylation and Arginine methylation

The protein arginine methylation which has been shown to have such enormous cellular functions, has been very closely associated with another important modification, i.e. acetylation.. Since, the bulk of arginine methylation is a mark corresponding to transcriptional activation, the epigenetic cassette for this chromatin state, is considered to be acetylation (**Figure 1.9**). However, the transcriptional repression brought about by the PRMT5 mediated symmetric methylation also exists in a cassette along with DNA methylation and deacetylation (Pal et al., 2003, Zhao et al., 2009) and thereby indirectly alongwith lysine methylation. The most well studied crosstalk within the above reference frame, is the histone modifications brought about by p300 mediated acetylation and CARM1 mediated arginine methylation. The first evidence of such a network was identified when CBP, the acetyltransferase itself was found to be a substrate of CARM1, wherein the

modified form of CBP had better acetyltransferase activity (Chevillard-Briet et al., 2002). An elegant study by An W et al., showed the existence of this cooperativity in the DNA damage associated p53 transcriptional activation (An et al., 2004). This work illustrated the co-operative, ordered but mutually exclusive effect of PRMT mediated H4 methylation, p300 mediated H3 and H4 acetylation followed by CARM1 mediated H3 methylation on p53 downstream target (*GADD45*) activation. This was the first arginine methylation associated epigenetic cassette identified, albeit in an *in vitro* system. Subsequently, the existence of such a network, especially for PRMT1 and CARM1 mediated modification was also shown for nuclear hormone receptor activation, CITED II activation as well as NF-κB activation (Covic et al., 2005; Miao et al., 2006).

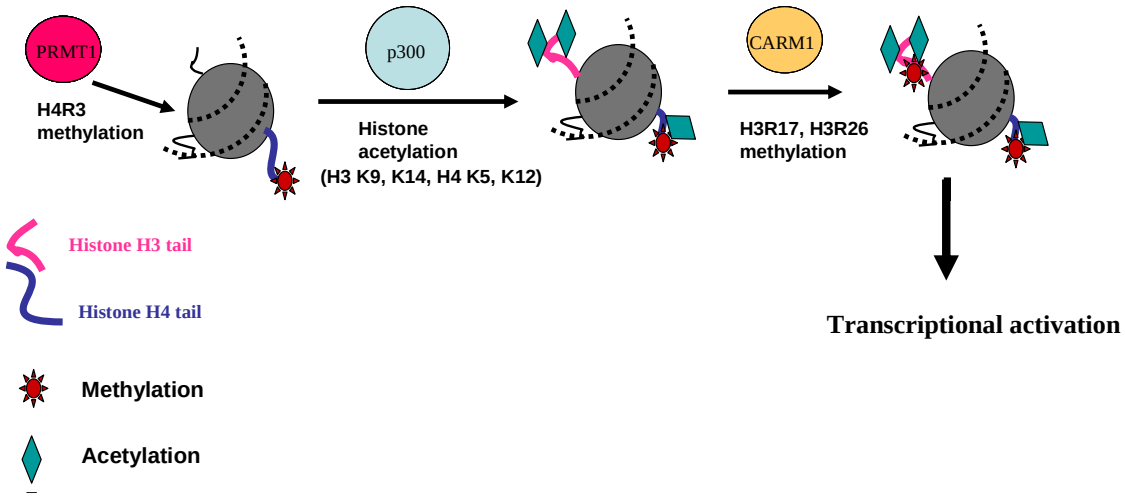


Figure 1.9. Acetylation and arginine methylation cross talk. *Methylation of H4R3 by PRMT1 facilitates the H3/H4 acetylation by p300 which in turn acts as a favorable substrate for CARM1 mediated methylation of H3 R2, R17 and R26*

Recent reports have shown an antagonizing effect of H4R3 methylation and H4 acetylation. A careful analysis has shown that this H4R3 methylation is a symmetric modification catalyzed by PRMT5 and hence is antagonized by the activation mark, acetylation. PRMT5 is a component of the mSin3a/HDAC as well as NURD complex (pal et al., 2003; Le Guezennec et al., 2006). Thus, a single residue modification by either PRMT1 or PRMT5 changes the transcriptional machinery associated at the modification site, leading to opposing effects on gene expression. The PRMT5, deacetylase complex is a repressive

complex and hence the subsequent inactivation of gene expression can be explained. In a similar context, since p300/CBP and CARM1 exist in co-activator complexes (SRC, p160 family), the possibility of this activation associated epigenetic cassette regulating the efficiency of transcription not just by coactivation but also by enzymatic activity is a fact that cannot be ruled out. To understand this, it becomes essential to look into the enzymatic activities of these co-activators.

1.4. CHROMATIN MODIFYING ENZYMES AS TRANSCRIPTIONAL COACTIVATORS

1.4.1. General introduction

There are several proteins which assist the process of transcription in several ways such as 'scaffold proteins' that connect different transcription factors, or as mediators facilitating better interaction of DNA and the transcriptional factors. These proteins are generally termed as transcriptional co-activators, because they aid in better transcription. However, there are few co-activators which not only have these abilities, but also have enzymatic activities. The most well known enzyme activity associated transcriptional co-activators are the histone acetyltransferase p300/KAT3B and the arginine methyltransferase CARM1/PRMT4. Such additional activities makes the process of transcription and gene expression very efficient since these proteins not only enhance interactions between the different transcription factors, but also bring about modifications of the histones, and transcription factors as well as other proteins in the local region, thereby leading to effective functionality.

1.4.2. Arginine methyltransferase CARM1/PRMT4:

As the name suggests CARM1 is co-activator associated arginine methyltransferase, so named because it was first identified as a nuclear receptor co-activator (Chen et al., 1999), following which its methyltransferase activity was identified. CARM1 has highly conserved protein arginine methyltransferase domains (Teyssier et al., 2002). Structurally, the domains have distinct functions and these in turn regulates its functionality. The enzyme has two substrate binding sites, one of which is an adomet binding pocket, and the other is an arginine binding pocket where the arginine residue of the target protein binds. It also has a dimer interface. Unlike, the other PRMTs it does not recognize the GAR rich motifs, rather

it has been indicated that it prefers XXPRX or a XXRPX motif, but as such there is no consensus recognition motif (Kim et al., 2004). The protein also exhibits functional domains which overlap. The N term portion is not much active functionally. The activation domain is in the C term of the protein. The homo-oligomerization domain and the GRIP1 binding domain are the same. The methyltransferase domain is in the centre. And the full length protein is required for its co-activator function. The domain organization is represented in **Figure 1.10.**

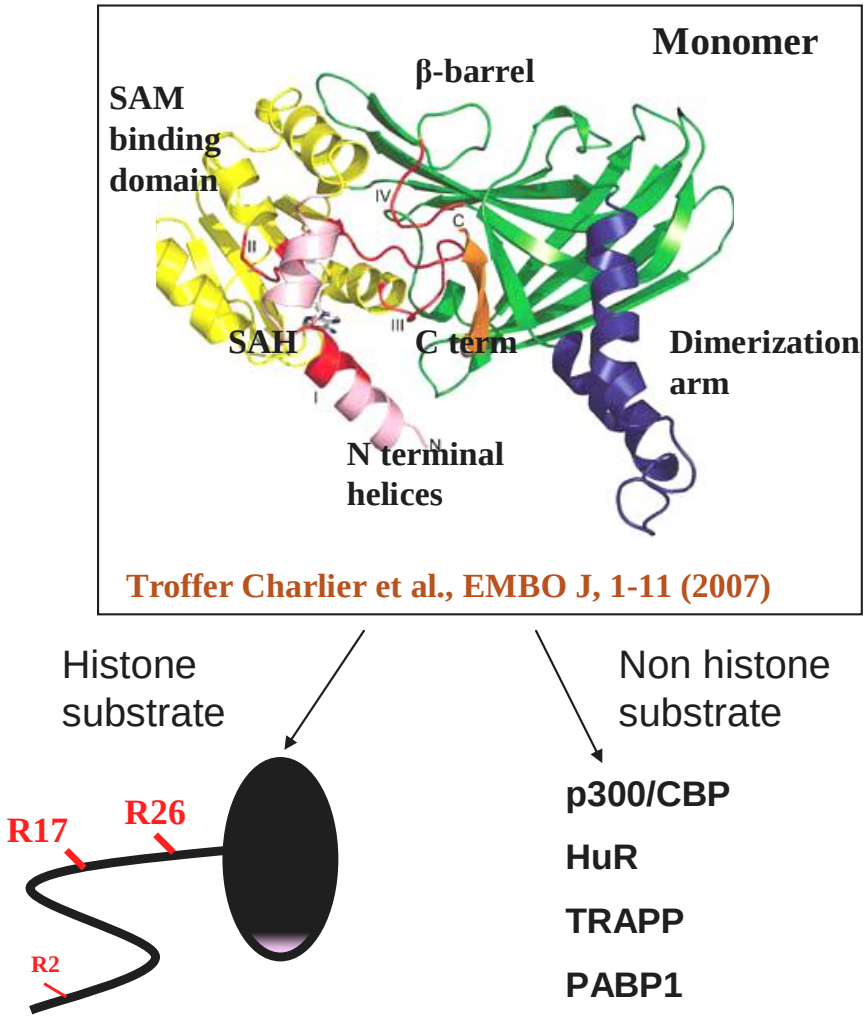


Figure 1.10. CARM1-SAH structure: *The structure of SAH-CARM1140–480. Monomeric form of CARM1bound by SAH (stick model), the SAH bound region is represented in yellow. The N-terminal helices are represented in pink and the β-barrel is shown in green. The dimerization arm is represented in blue and the terminal strand is shown in orange. The PRMT motifs are represented in red. The different substrates of CARM1 are also enlisted.*

The enzyme functions as a dimer. It methylates two major residues on the H3 tail, R17 and R26 both *in vitro* and *in vivo* (Bauer et al., 2002). Another modification site is H3R2 which is also methylated by PRMT6. Apart from histones, PRMT4 also methylates few nonhistone proteins namely, acetyltransferase CBP, HuR, TRAPP and PABP1 (**discussed in chapter 3**).

The methylation of these proteins regulates their functions, eg: TRAPP methylation is essential for thymocyte proliferation. This was identified in a screen for PRMT4 targets. Further validation was provided when the CARM1 null mice were found to have compromised T cell formation (Kim et al., 2004). Another interesting observation from these knockout mice were the defect in chondrocyte formation (Ito et al., 2009).

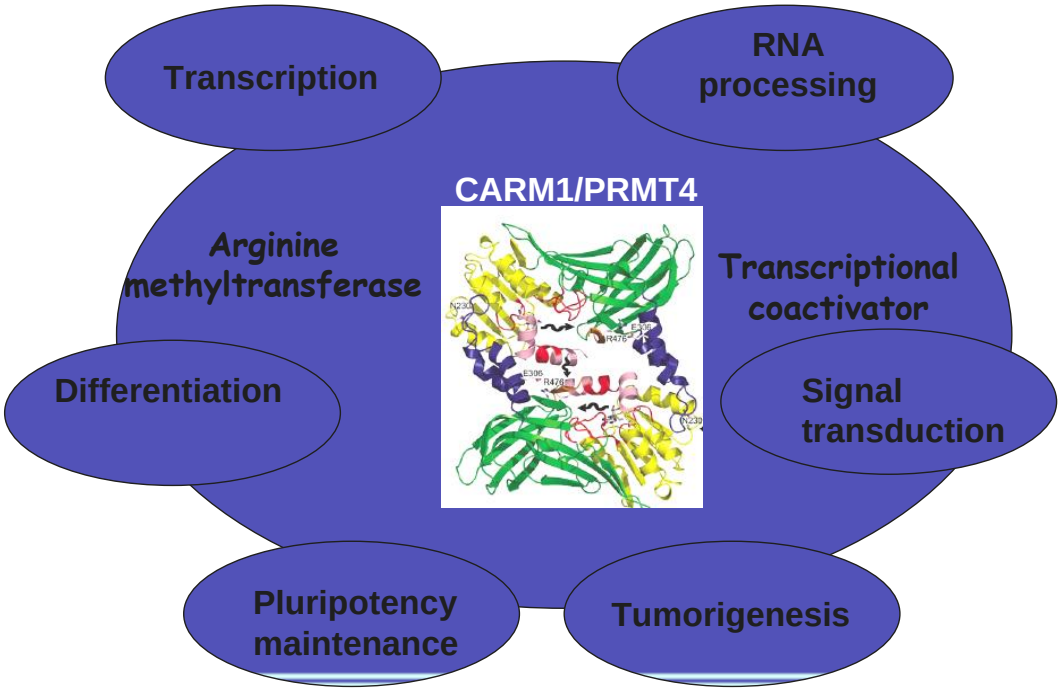


Figure 1.11. Physiological function of CARM1. *The different biological roles of CARM1 are due to its arginine methyltransferase as well as transcriptional co-activator properties. CARM1 has been shown to regulate RNA processing, transcription, differentiation, pluripotency maintenance, signal transduction as well as tumorigenesis.*

A detailed characterization led to the identification of MEF2 as the target of CARM1. It was also seen that the co-activation property of CARM1 regulates MEF2 (Chen et al., 2002) thereby implicating this enzyme in muscle differentiation. The co-activation of

PPARγ by CARM1 is essential for adipogenesis (Yadav et al., 2008). All these observations seemed to suggest an important role of CARM1 in differentiation. Ironically, the work on ES cells, silencing of CARM1 indicated that this enzyme is essential for pluripotency maintenance (Wu et al., 2009), leading to an important question regarding its contradictory roles on undifferentiated against committed cell types. Thus, CARM1 regulates important cellular processes (**Figure 1.11**) both by its enzymatic as well as co-activation property.

It is a co-activator for nuclear hormone receptors such as estrogen and androgen receptor. The recent literature shows compelling evidences for the PRMTs in not just hormone receptor activation but also for other transcription factors including p53, YY1, NF-kB, PPARg, RUNX1, and E2F1 (Yadav et al., 2008; Zhao et al., 2008). This leads to the speculation that there might be a general role of these enzymes in transcription. However, the effector modules or the mechanistic details have not yet been worked out for arginine modification as it is for acetylation inspite of their cross talk.

1.4.2. Lysine acetyltransferase p300/KAT3B:

p300/CBP is commonly referred to as the master regulator of gene expression, due to its wide range of histone and nonhistone protein substrates. It is a 2414 amino acid protein with distinct domains as shown in **Figure 1.12.**

It is characterized by three Zinc finger domains. It has a bromo domain, and most importantly the acetyltransferase domain is active without the full length form, thus enabling the crystallization. The lysyl CoA (p300 HAT specific synthetic inhibitor) bound form represents a very similar structure as the other HATs but with characteristic differences (**Figure 1.12, 1.13**). It has an autoacetylation loop which is made of abut 12 lysine residues which get sequentially acetylated giving rise to a hyperactive p300 (Thompson et al., 2005).

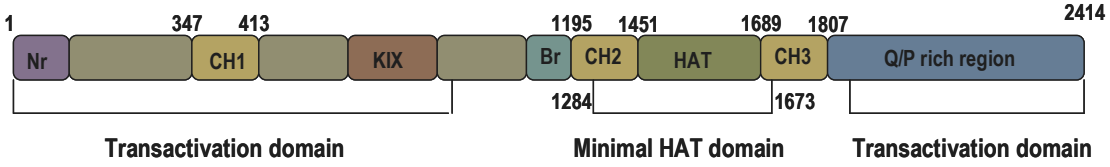
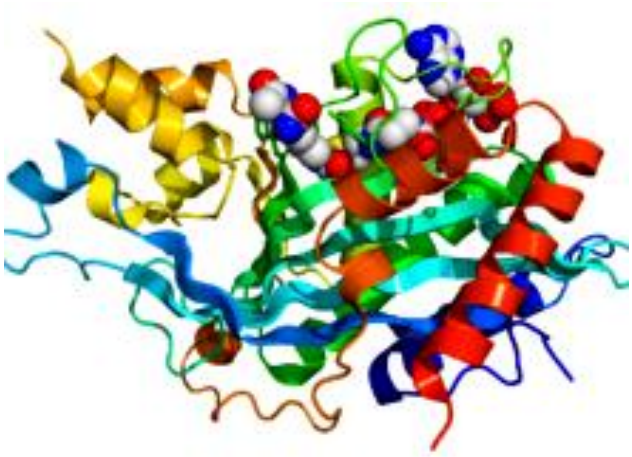


Figure 1.12. p300 domain architecture*. Domains of histone acetyltransferase p300. Apart from the minimal HAT domain (1284-1673), three CH domains, a single bromodomain and the KIX domain are present.*

Acetylation has in recent years emerged as a protein modification matching phosphorylation in versatility and importance. p300 and the closely related CBP were among the first vertebrate proteins to be recognized as acetyltransferases (Ogryzko et al., 1996; Bannister and Kouzarides, 1996).

The acetyltransferase p300 is a well known transcriptional co-activator of a large number of transcription factors. It is probably the only enzyme that acetylates all four histones. Thus, via acetylation p300 can modulate both the activity of transcription factors and the chromatin status of their target genes. However, p300 shows additional biochemical functions including E4 ubiquitin ligase activity (Grossman et al., 2003) and a SUMO-regulated transcriptional repression domain (Girdwood 2003) indicating that p300 has acetyltransferase-independent functions. The recent propionylation, butyrylation and formylation have also been hypothesized to be brought about by p300.



Adapted from Liu et al., 2008

Figure 1.13. p300 crystal structure. *Overall structure of the p300 HAT domain with N and C terminus coloured in blue and red, respectively. Lys-CoA is shown as a space filled model. carbon = white, oxygen = red, nitrogen = blue, phosphorous = orange)*

p300 has been shown to have about 100 different substrates with distinct functional outcomes ranging from transcription, replication, repair (**Figure 1.14**). p300 not only acts as a co-activator, acetyltransferase but also as a bridging molecule and as a scaffold (Chan et al., 2001) in transcription reactions. Its role in learning and memory (Oliviera et al., 2007) has added a new insight to the field of neuroscience and epigenetics. p300 has been implicated in regulating differentiation as well. Due to its role in such diverse cellular

processes, the dysfunction of this enzyme has been causally related to several disease manifestation including cancer. p300/CBP mutations have been mapped in several cancers including the Rubeinstein-taybi syndrome (reviewed in Iyer et al., 2004). If hypocetylation is a cause in these diseases, hyperacetylation and increased activity has also been implicated in inflammatory diseases especially of the cardiovascular origin. Thus indicating that it is the homeostatic balance of acetylation that regulates gene expression. Indeed, p300-deficient mice die at day E9.5 of embryonic development (Yao et al., 1998).

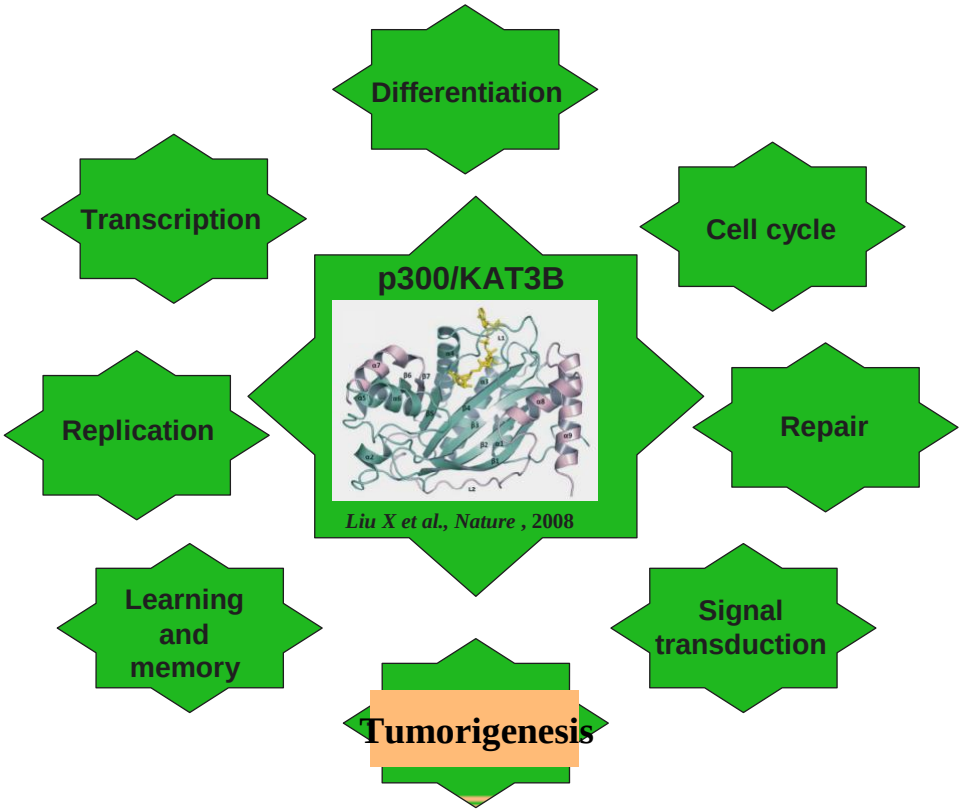


Figure 1.14. Physiological role of p300: *The different physiological role of p300 are represented in the figure.*

The role of p300 is exemplified both by its acetyltransferase as well as co-activation property. Unlike, arginine methylation, the different effector modules for this modification and the exact mechanistic details have been well worked out. However, it is well understood that any attempt towards understanding arginine methylation in transcription would be futile unless supported by the acetylation interplay.

1.5. ARGININE METHYLATION AND ACETYLATION IN TRANSCRIPTIONAL REGULATION:

Arginine methylation of both histones and nonhistone proteins have been linked to several cellular processes ranging from signal transduction, DNA repair, RNA processing to transcriptional regulation. Majority of these functions is possible due to the nonhistone protein methylation which constitutes about 80% of total arginine methylation in the cell. Much of this methylation is brought about by the type I PRMT, PRMT1, with a minor contribution (25%) by another type I PRMT, PRMT4/CARM1. The other members such as PRMT5 and 6 have been shown to modify few substrates. Inspite of the low level of histone arginine methylation, it is a key player in the transcription regulation function. One possibility for this important role could be its association with the other transcriptional competence regulating modification, i.e. acetylation. Indeed, there exists a co-operativity between these two modifications in regulating the process of gene expression. The earlier understanding was that these modifications act together for nuclear hormone receptor associated gene expression, but the recent findings have shed light on the possibility of these modifications acting together in the general transcription process as well. A brief overview of the various transcription events where this co operativity has been shown is highlighted below.

1.5.1. Arginine methylation and acetylation regulate nuclear hormone receptor dependent gene expression:

The nuclear hormone receptor associated transcriptional activation is one of the most extensively studied molecular system that emphasizes the need for a cooperative effect of acetylation and arginine methylation. The co-activators CARM1 and p300 which have been the focus of this study have been shown to be secondary co-activators for most of the NR associated transcription. The NR system includes the steroid receptors, estrogen receptor, androgen receptor, glucocorticoid receptor, thyroid receptors. Each of these receptors are mediators of signal dependent gene expression by specific receptor-ligand interactions. Since, these events are associated with transcriptional activation followed by gene expression, they are also associated with chromatin remodeling as well as the involvement of chromatin modifying machineries. Apart from the general transcriptional activators, these events also use secondary co-activators such as p300/KAT3B and CARM1/PRMT4. This is

possible because of the co-activation property as well as the enzymatic property of these enzymes. The various domain mapping led to the identification of the exact interaction regions wherein p300 interacts with the AD1 region of p160 activator GRIP. The AD2 region was found to be the primary site of interaction with the enzyme CARM1. Another independent study showed the existence of yet another activation domain in GRIP, i.e. AD3 which interacted specifically with another arginine methyltransferase PRMT1. The existence of all these co-activators led to several fold induction in transcription (Chen et al., 2000; Koh et al., 2001)

Additionally, the ability of CARM1 to interact with the chromatin remodeling factors in the Swi/Snf complex, such as Brg1/hBrm, also helps in a direct recruitment of the remodeling complexes (Xu et al., 2004) onto the already assembled co-activator complex thus facilitating, the transcription. However, all these mechanisms lead to a co operative effect

Each of these enzymes p300, PRMT1 and PRMT4 are all capable of interacting with the primary co-activators and thus can influence the transcriptional outcome individually. However, their coexistence increases the transcription efficiency several fold indicating a cascade of signaling events ranging from external signals evident as phosphorylation events, followed by ordered recruitment of primary and secondary co-activators, finally leading to chromatin remodeling events and transcriptional activation which is represented in the **Figure 1.15**. Although there are several lysine methyltransferases such as MLL and SET7/9 which are involved in transcriptional activation, the need for acetyltransferase and arginine methyltransferase for the NR associated gene expression signifies the existence of specific regulatory networks for specific transcription events.

1.5.2. Arginine methylation and acetylation regulate other transcriptional events:

The major role of arginine methylation in transcriptional regulation is due to its histone modifying as well as co-activation ability. Various transcription factors are known to be substrates for arginine methyltransferases and this is known to play a role in transcriptional regulation as well. In case of signal transduction, arginine methylation regulates the interaction mediated by SH3 domains, but WW and Tudor domain interactions are unaffected or are enhanced by arginine methylation. There are several pathways that have been shown to utilize arginine methylation as a tag for signal transduction downstream

of the interferon receptor, T cell receptor, cytokine receptors and Nerve growth receptors. Few reports also suggest the role of arginine methylation in DNA damage. Asymmetric dimethyl arginine methylation has been reported to be involved in the establishment of transcriptional activation and most often this involves the histone acetyltransferase recruitment, or the methylation of the HAT itself. This indicates that there is a functional interplay between the histone modifications, more so with respect to PRMTs and HATs in the establishment of transcriptional activation.

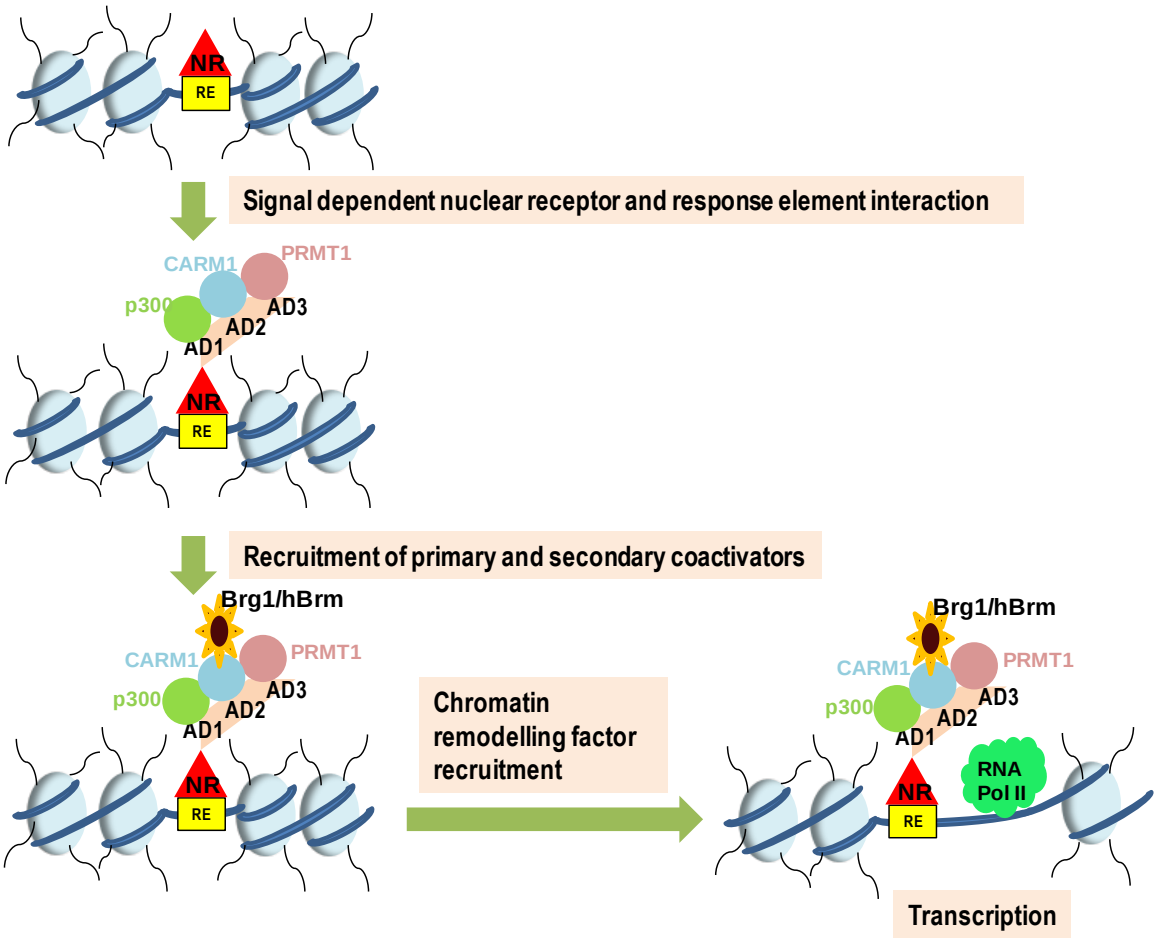


Figure 1.15. Nuclear hormone receptor associated transcription activation. *The first step involves a signal dependent interaction of the nuclear receptor with the respective response element concomitant with a receptor-ligand interaction. This is followed by the recruitment of the primary and secondary co-activators. The p160 family of co-activator is a most common primary co-activator which has three activation domains, AD1, AD2 and AD3. The histone acetyltransferase p300 interacts with AD1, PRMT4/CARM1 interacts with AD2. PRMT1 interacts with AD3. CARM1 then recruits the chromatin remodeling complex via interaction with*

Brg1/hBrm which leads to a remodeling event. This is followed by the recruitment of the transcription machinery resulting in transcription.

The molecular dissection of the exact co-operativity has not been very clear. Arginine methylation mediated by PRMT1 and PRMT4 are emerging as regulators of several transcription factors ranging from p53, YY1, NF-kB, PPARγ, RUNX1 and E2F1. The histone acetylation mediated regulation of all the above transcription factor associated activation is a well documented evidence. Although, a direct role of the arginine methylation and acetylation together has not been shown for all these factors, it has been worked out very elegantly for p53 using a reconstituted chromatin transcription system which was taken as the model system to study the effect of the modulators on the crosstalk of these two modifications which is described in **chapter 5**.

1.6. ARGININE METHYLATION AND ACETYLATION IN DEVELOPMENT:

It is indeed clear that arginine methylation by CARM1 and acetylation by p300 are part of an epigenetic cassette associated with transcriptional activation. Although, it is not clear of how universal this epigenetic cassette is, there are few transcriptional events where the involvement of both these co-activators has been shown, and in few cases their enzymatic activities have also been shown to be linked, which has been discussed in the previous sections. Recent investigations, indicate another transcriptional system which might be associated with these modifications, which is the embryonic stem cell associated pluripotency, differentiation and finally development. Independent studies have shown the expression of p300 and CARM1 in distinct developmental stages. Furthermore, in mouse embryonic stem cells, p300 and CARM1 have been shown to be essential for the maintenance of the pluripotent state (Wu et al., 2009; Bhattacherjee et al., 2009). However, it is not clear whether, it is only the co-activation property or the enzymatic property that regulates these effects.

Notably, during ES cell differentiation, the level of H3K9 methylation, a modification essential for heterochromatin formation, is increased. Thus, global transcription in ES cells becomes more restricted upon differentiation, with concomitant decreases in histone H3 acetylation and H3K4 methylation and increases in silent and heterochromatic regions.

Moreover, pluripotency can be reestablished in somatic cells by high levels of histone H3 acetylation and H3K4 methylation. A recent study showed that the arginine methylation of histone H3 is maximal in cells that will form the inner cell mass (the cells that will give rise to the fetus) and the polar trophectoderm (extraembryonic tissue adjacent to the inner cell mass at the embryonic pole of the embryo), and minimal in cells that are destined to contribute to the mural trophectoderm (extraembryonic tissue at the opposite, abembryonic pole). Injecting mRNA for the arginine methyltransferase CARM1 in one blastomere at the two-cell stage leads to an increased proportion of progeny cells that will form the inner cell mass (Torres-Padilla et al., 2007). Even in the case of differentiation, CARM1 which has been shown to be essential for regulating muscle differentiation and adipocyte differentiation is probably assisted with p300 as well. p300 has been earlier shown to regulate muscle differentiation since it is acetylates MEF2 and increases its DNA binding ability (Ma et al., 2005). Thus, it is indeed clear that CARM1 and p300 have significant roles in pluripotency, differentiation and development which will be further discussed in **chapter 5.**

1.7. ARGININE METHYLATION AND ACETYLATION IN DISEASE MANIFESTATION:

Arginine methylation is a modification which although discovered five decades back, has gained significance only in the past decade. Hence, the exact role of this modification in disease manifestation or progression is very limited. However, there are implications of the modification and the corresponding methyltransferase in cancers like prostate and breast cancer (Hong et al., 2004; Frietze et al., 2008). Since, CARM1 is a secondary co-activator of the hormone receptors, its involvement in the above two cancers were not surprising. The exact molecular details of the role of CARM1 in cancer manifestation is currently being worked out. The role of acetylation in cancer is a much studied topic. Few cancers such as hepatocellular carcinoma (Bai et al., 2008) and oral cancer (Shandilya et al., 2009) have been causally linked with hyperacetylation whereas prostate and breast cancer have been shown to have a hypoacetylation. Apart from cancer, CARM1 has been shown to regulate splicing of SMN protein (Tadesse et al, 2008) and the dysregulation of this splicing event has been linked to spinal muscular atrophy. p300 has

also been shown to cause neurodegenerative disorders (Rouaux et al., 2003). Yet another important disease that is causally linked to the arginine methylation is cardiovascular disorder. All the Class I PRMTs are involved in the formation of asymmetric dimethyl arginine (ADMA), which is a competitive inhibitor of nitric oxide synthase (NOS), and thus decreases Nitric oxide (NO) availability in the cells. Decreased NO levels is a hallmark of cardiovascular diseases. Thus, all the Class I enzymes including CARM1 are responsible for the oxidative stress induced disease manifestation via the ADMA intermediate (reviewed in Beltowski et al., 2006). The role of p300/CBP in various cardiovascular disorders including cardiac hypertrophy is a well studied area (reviewed in Selvi and Kundu, 2009). Thus, there is a clear link between arginine methylation and acetylation in disease manifestation, however, there is no evidence to suggest if there is any co-operativity. But, what is clear is that the modulators of these enzymes, surely have a role as therapeutic agents which will be discussed in the final chapter.

1.8. UNDERSTANDING THE ENZYMATIC ROLES OF THESE TRANSCRIPTIONAL COACTIVATORS:

It is indeed clear that the arginine methyltransferase CARM1 and the acetyltransferase p300 regulate key cellular processes, not only by their respective enzymatic activity but also by participating in the process of transcription as coactivators. Although, p300 was considered to be a more generalized co-activator, CARM1 was implicated in specific nuclear hormone receptor associated processes. But the recent observations speculate the possibility of arginine methylation by CARM1 also to be a general co-activator atleast in defined contexts. One of the easiest method to understand the role of a protein is by exogenously modulating that protein levels in the system. This can be achieved by overexpression or knockdown studies, and most of the information available about these enzymes is due to such studies. However, the possible flaw in this approach is the alteration of the interacting proteome on modulating the protein level. Thus, the outcome would essentially be a downstream effect and not a direct modulatory effect. Although the further validations such as examining the important promoters for these proteins or pulldowns, can resolve this dispute, another efficient way to understand the enzyme function is an inhibitor based approach.

The field of CARM1 methylation is still arguing about the involvement of its co-activation versus enzymatic property in its functional outcomes. The use of catalytically inactive mutant led to the conclusion that few of the physiological functions is solely due to its co-activation property (Jayne et al., 2009). On the other hand, the use of a knockdown model which was knocked in with the same catalytically inactive mutant strongly suggested the role of the enzymatic activity for all its in vivo functions (Kim et al., 2010). These conflicting reports allude towards the need for a specific inhibitor of this enzyme to understand the mechanistic and functional involvement of this enzyme in the various cellular processes.

1.8.1. Source of inhibitors

Having established the necessity for an inhibitor based approach to understand the enzyme function, it also becomes essential to decide the methodology for obtaining these inhibitors. The most efficient method is to design substrate analogs which would compete for the binding site and thereby inhibit the enzyme. Unfortunately, this methodology did not succeed for CARM1 (Cheng et al., 2004). Although the methyltransferase domain is conserved across the different PRMTs, CARM1 is distinctly different due to its inability to methylate the GAR or RGG motifs, that are the methylation motifs for other PRMTs. This signifies the difference in the substrate binding pockets. It was more than a decade after these substrate analogs were tried that the structure of CARM1 was reported (Troffer Charlier et al., 2007; Yue et al., 2007). This clearly indicated that there is a significant difference indeed in the substrate binding pockets although the adomet binding region was conserved. Following this, few chemical modulators for CARM1 have been reported which were not properly characterized (Wan et al., 2009; Therrien et al., 2009) and were verified only in the *in vitro* reactions.

This clearly indicates that the synthetic route towards the inhibitor does not seem to be the right option and possibly it is essential to also look into the naturally occurring small molecules. This approach is further strengthened by the fact that most of the important histone acetyltransferase inhibitors are from natural source, namely anacardic acid from cashewnut shell liquid, garcinol from *Garcinia indica*, Curcumin from *Curcuma longa*. The recent spurt in the field of histone deacetylase inhibitors, (HDACi) from natural sources,

such as Sulphorophane from cruciferous vegetables (Ho et al., 2009) is a further validation to the fact that plant origin molecules are an attractive target for the search of inhibitors.

1.8.2. The small molecule approach to understand enzyme function

Small molecule modulators (inhibitors/activators), of enzymes have been used very successfully to unravel the mechanistic details of enzyme function. The best characterized example is the electron transport chain. The inhibitors of each of the complexes in the ETC helped in deciphering the entire process. Most of the important crystal structures have been reported with their inhibitors including the recent noble prize awarded ribosome structure. In the field of chromatin, the identification of lysyl CoA as a p300 HAT specific inhibitor opened up the entire field of p300 HAT biology. This is further supported by the fact that more than a decade later the p300 HAT domain structure could be reported with the same specific inhibitor. The p300 HAT specific inhibitor, curcumin has been used in transcription experiments to study the role of p300 in pre-initiation complex (PIC) formation (Black et al., 2006). The role of p300 was solely considered to be in the process of transcriptional activation with very few reports indicating the possibility of p300 also acting as a repressor. The gene expression analysis using p300 specific inhibitor, LTK14 sealed this fact that indeed there are about 5% of genes that are negatively regulated by p300 (Mantelingu and Reddy et al., 2007). The role of histone acetylation in early stages of mesenchymal stem cell differentiation was studied using another HAT inhibitor, anacardic acid (Muscari et al., 2008). The field of phosphorylation, has been heavily influenced by specific inhibitors which have helped in understanding the process of phosphorylation so extensively.

These different inhibitors have not just helped in understanding the enzyme function but also greatly influenced the field of therapeutics which will be discussed later in the future perspective.

1.9. RESEARCH FOCUS:

The area of chromatin research has advanced by leaps and bounds in the past two decades due to the increased evidences linking chromatin as an important entity regulating gene expression. This has been possible because of the various whole genome studies as well as different chromatin immuno-pulldown data indicating the importance of chromatin

modifications for the various cellular processes. It is slowly being realized that these modifications are not functional as single marks rather they exist in a network which is modulated by different environmental and physiological cues. The universality of these cross talk is still in debate, since for every modification network, there seems to exist even more exceptions. However, a common thread that is observed is the epigenetic cassette for activation and repression. Based on the different transcription complexes, there are fine changes in the chromatin modifiers that get associated, but as a general rule, acetylation of H3, H4, trimethylation of H3K4, H3S10 phosphorylation are the marks associated with transcriptional activation (reviewed in Berger et al., 2007). A very recent addition into this cassette is the arginine methylation of H3 and H4. This necessitates an investigation into this arginine methylation associated cassette. The components of the arginine methylation network, so far seem to be PRMT1, p300 and PRMT4. PRMT1 is an enzyme with a major nonhistone protein modification profile. Furthermore, the H4R3 modification by PRMT1 is also a substrate for PRMT5 (leading to transcriptional repression). Hence, to rule out any ambiguous interpretations, we decided to investigate the physiological role of arginine methylation and the associated epigenetic mark, acetylation in the global perspective of gene regulation. PRMT4/CARM1 had other interesting features which have not been clearly understood. These are enlisted below.

(1) PRMT4 is the only arginine methyltransferase that does not recognize the GAR motifs in substrates.

(2) PRMT4 is an arginine methyltransferase with multiple cellular functions which are partly due to its transcriptional co-activation property as well as due to its enzymatic property. However, a clear delineation of which cellular function is regulated by which of these two abilities has still been not possible, thereby alluding towards the need for an enzyme specific inhibitor.

(3) Although, the expression data of PRMT4 suggests almost ubiquitous expression, the cellular functions seem to suggest specific locations within the cell (eg. myocytes, chondrocytes, neural crest etc.)

(4) The role of PRMT4 was considered to be only for specialized (steroid receptor) transcriptional activation cassettes. However, recent work has started showing the essentiality of this enzyme in a multitude of transcriptional events ranging from p53

dependent to NF-κB dependent gene expression. This led to the speculation that PRMT4 might also be a part of the general transcriptional activation system.

(5) One of the intriguing observations was the presence of PRMT4 in transcriptionally repressive states, implicating that it may not be just a transcriptional co-activator, rather a master regulator of gene expression (atleast for few events, regulated by ER signallling).

(4) Most importantly, although the enzymatic activity was identified more than a decade back, the exact mechanism of enzyme action has still not been elucidated. There are two major histone modification sites that have been identified for this enzyme, histone H3 R17 and H3 R26. The crystallographic data suggests the possibility of different substrate binding pockets for the modification of these two residues. One of the question that has remained unanswered till date is whether these two residues get modified at the same time or whether there exists some sequential modification cascade?

(5) The enzyme PRMT4 has been linked to physiological functions from differentiation to disease, but there have been no selective, well characterized inhibitor of this enzyme.

Of the several approaches available to understand these modifications, we decided to adopt an inhibitor based methodology to understand the above mentioned arginine methylation cassette. Since, the synthetic route for inhibitor designing, has not been very successful for CARM1 inhibition, we decided to search the naturally occurring plant sources for the inhibitors. Although, few specific inhibitors of p300 are available, even with cell permeability (Curcumin, LTK14, EGCG) it has become necessary to increase the library of naturally occurring HAT inhibitors. One of the main reasons is to identify an easier scaffold since all the other examples are large structures. We resorted to a similar screening for p300 HAT inhibitor from natural sources.

One of the main unanswered question with respect to CARM1 is whether there is a generality to its transcriptional activation property or is it present only on specific promoters. To understand this, we decided to look at the effect of CARM1 mediated modification on the global gene expression profile. Once, this question is partly answered, we decided to delineate pathways that require the CARM1 mediated arginine methylation and p300 mediated acetylation network, thereby proving the essentiality of these modifications by the enzymes in different cellular processes especially in transcription and differentiation.

1.10. AIM AND SCOPE OF STUDY:

The research focus of this study is towards understanding the physiological role of arginine methylation and the associated epigenetic mark, acetylation in the regulation of gene expression. The main objectives are enlisted below.

As discussed in the introduction, the arginine methyltransferase CARM1 is an important chromatin modifying enzyme which methylates histone H3 and several nonhistone proteins with distinct functional consequences. It is also a transcriptional co-activator. **The exact contribution of the methyltransferase activity and the co-activation ability towards its biological functions, is poorly understood. Moreover, CARM1 methylates two residues on histone H3, R17 and R26 *in vivo* and the exact contribution of each of these modifications is also not known.** We decided to address these aspects by a small molecule approach. Indeed, it was possible through the identification of TBBD, a CARM1 specific inhibitor which specifically inhibits H3R17 methylation.

The histone arginine methylation and histone lysine acetylation function in a co-operative manner for transcriptional activation, possibly through the establishment of an epigenetic language. Small molecule modulators could also be used to address this co-operativity. Although, there are known HAT inhibitors, there is an active effort for identifying new p300 specific inhibitors from natural sources, to probe the biological function of p300. In accordance, with this objective, we have identified a p300 HAT specific inhibitor from the natural source which has led to the identification of a novel inhibitory scaffold as well as the chemical entity responsible for HAT inhibition.

By employing, these two enzyme specific inhibitors, we have attempted to elucidate the significance of H3R17 methylation and histone lysine acetylation in transcription regulation and gene expression. Based on the results, it is clear that these two modifications regulate p53 dependent gene expression in response to genotoxic insult. The role of R17 methylation and lysine acetylation in the pluripotency marker gene expression in human embryonic stem cells has also been addressed with the help of these enzyme specific inhibitors.

Chapter 2

MATERIALS AND METHODS

This chapter is a brief note on the experimental procedures and also consists information on the different reagents used for the study reported in this thesis.

Chapter outline:

2.1. General Methods

2.2. Cloning and Protein Expression and Purification

2.3. Histone modifying enzyme assays

2.4. Biophysical experiments

2.5. Molecular docking studies

2.6. Histone/Protein modification analysis

2.7. Gene expression studies

2.8. Human Embryonic stem cell related experiments

2.9. Isolation of active components from the plant material

2.10. Miscellaneous

2.1. General Methods

2.1.1. Reagent information:

The molecular biology reagents were purchased from SIGMA, USB or Invitrogen for the different experiments as indicated alongside. Buffers for routine use were prepared from Qualigenes AR grade chemicals. Bacterial culture requirements were purchased from Himedia. Mammalian culture requirements were purchased from SIGMA or Invitrogen.

Insect cells requirements were purchased from SIGMA and the human embryonic stem cell requirements were purchased from GlobalStem USA. Tissue culture plasticware used were from NUNC, Primary antibodies used were essentially from Upstate or Abcam whereas the secondary antibodies were purchased from Bangalore Genei or Invitrogen.

2.1.2. Competent cell preparation:

E. coli strains BL21 DE3 and DH5α were the mainly used competent cells. These were grown overnight in 5 ml of Luria Bertani (LB) medium containing 10 gm/L tryptone, 5 gm/L yeast extract and 10 gm/L NaCl (from Himedia) at 37°C without any antibiotics. The overnight grown culture was streaked on a LB agar plate without antibiotic and grown in a 37°C incubator. A single colony was inoculated in 5 ml of LB medium and cultured overnight at 37^{o}C. Secondary inoculation was done with overnight culture in to 500 ml of medium A (500 ml LB containing 10 mM $MgSO_4$ and 11 mM glucose) and grown till the OD_{600} reached to 0.3. The culture was precooled prior to centrifugation at 4°C, 2000 rpm for 10 mins. The pellet was resuspended in medium A at 4°C. The cells were pelleted again and resuspended in 25 ml of storage buffer B (36% Glycerol, 12 mM $MgCl_2$, 12 gm of PEG 8000 in 100 ml) aliquoted and stored at -80°C.

2.1.3. Transformation:

Frozen aliquot of competent cells was thawed on ice for 5 mins prior to addition of 100 ng of DNA to be transformed followed by incubation on ice for 30 mins. Heat shock was given for 90 seconds at 42°C and the cells were immediately incubated on ice for 5 mins.1 ml of LB was added to the cells and cells were grown for 45 mins at 37°C in a shaker incubator, followed by centrifugation of cells. The cells were then plated on LB agar plate containing the appropriate antibiotic for selection and grown overnight at 37°C.

2.1.4. DNA purification:

DNA was isolated from *E. coli.* DH5α cells that were transformed with individual plasmid or clone constructs, using Mini prep kits according to the protocol supplied by manufacturers (QIAGEN). PCR products were purified using QIAGEN-PCR purification kit

according to the manufacturer's protocol. DNA was also gel eluted from agarose gels using QIAGEN gel elution kit according to the manufacturer's protocol.

2.1.5. RNA isolation:

Cells after culturing at different conditions were scraped/trypsinized, centrifuged at 2000 rpm, 4ºC for 10 mins. The pellet was washed with 1X PBS. The pellet was resuspended by vortexing in TRIZOL (Invitrogen), reagent at a ratio of 1ml per 10 million cells, followed by centrifugation at 12000 rpm, 4ºC for 10 minutes. The supernatant was subjected to chloroform extraction thrice. RNA was precipitated using equal volume of isopropanol, following which the pellet was resuspended in 70 µl DEPC treated water. Reprecipitation was done using 3 M sodium acetate, pH 5.2 and equal volume isopropanol. Pellet was washed with water, allowed to air dry and finally was resuspended in water.

2.1.6. cDNA synthesis:

3 µg of total RNA was used for synthesis of 20 µl cDNA. 3 µg RNA was taken in a 12 µl reaction containing 40 picomoles of oligo dT and incubated at 70°C for 10 mins followed by immediate incubation on ice for 10 mins. Reaction was initiated by the addition of 10 mM DTT, 0.5 mM dNTPs, 4 µl of 5X First strand synthesis buffer and 1 µl superscript RT (Invitrogen). The reaction was incubated for 60 mins at 42°C followed by heat inactivation at 70°C for 10 mins. The cDNA was then used for real time or normal PCR analysis using specific set of primers. Alternatively, cDNA synthesis was also prepared by SIGMA MMLV RT, wherein the RNA template was incubated with 40 picomoles of oligo dT and 1 mM dNTPs in a 10 µl reaction volume at 70°C for 10 mins followed by immediate incubation on ice for 5 mins. The reaction was initiated by addition of the MMLV RT enzyme and the buffer making up the volume to 20 µl, and incubation at 37°C for 50 minutes. Finally the RT was inactivated by incubating the reaction mixture at 90°C for 10 minutes. The cDNA synthesized by this method was essentially used for the gene expression analysis by real time PCR.

2.1.7. Estimation of nucleic acids and proteins:

a) Nucleic acids concentration was estimated spectrophotometrically by measuring the absorbance of DNA solution diluted in 10 mM Tris, 1 mM EDTA at 260 nm wavelength (A_{260}). The concentration was calculated according to Beer-Lamberts law.

$$C = A260 \text{ X } \theta$$

C is the concentration of nucleic acid in ng/µl. For DNA θ is 50 ng/µl. In case of oligonucleotide θ is 33 ng/µl. In case of RNA θ is 40 ng/µl.

b) Estimation of proteins: The concentration of protein in cell lysates was estimated with Bio-rad protein estimation reagent according to the protocol supplied by manufacturer using BSA as standard. Recombinant purified proteins were estimated by running different concentrations of BSA along with protein of interest on a SDS-PAGE gel.

2.1.8. Agarose gel electrophoresis:

Agarose gel electrophoresis was carried out to visualize/analyze DNA/RNA samples. Indicated percentage of agarose was added to 1X TBE (0.09 M Tris-Borate and 0.002 M EDTA) /0.5X TBE and dissolved by melting in microwave oven. Samples were prepared in 1X loading buffer (0.25 % Bromophenol blue, 0.25% Xylene cyanol in 40% sucrose) and were elecrophoresed at different voltages for varying time periods as indicated, in 1X TBE/0.5X TBE. Gels were stained with EtBr (10 µg/100 ml H_2O) with gentle rocking. Nucleic acids were visualized on U.V. lamp in gel documentation system (Bio-rad).

2.1.9. Poly Acrylamide Gel Electrophoresis (PAGE):

SDS-PAGE: SDS-PAGE was performed to separate the proteins according to molecular weight and also to analyze the purity of the protein samples. Separating gels were made in various percentages of acrylamide (Stock: 30%, Acrylamide: Bis acrylamide 29:1) along with 0.375 M Tris pH 8.8, 0.1% SDS, 0.1% APS and 8% TEMED. Stacking gels were made with 5% acrylamide along with 0.375 M Tris pH 6.8, 0.1% SDS, 0.1% APS and 8% TEMED above the separating gel. Protein samples were made in 1X sample buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 0.1% bromophenol blue, 10% glycerol) heated at 90°C for 10 mins and subjected to electrophoresis using SDS, Tris-Glycine buffer (25 mM Tris, 250 mM Glycine pH 8.3, 0.1% SDS). Gels were visualized by staining with coomassie (45%

methanol, 10% Acetic acid, 0.25 % bromophenol blue) followed by destaining with destaining solution (30% methanol, 10% acetic acid in H_2O).

2.1.10. Western blot analysis:

The separated proteins on a 12% SDS-PAGE gel were blotted to PVDF/Nitrocellulose membrane using semidry western apparatus. Initially the gel was equilibrated with transfer buffer (25 mM Tris, 192 mM glycine, 0.038% SDS, 20% methanol v/v) for 30 mins on a rocker. The PVDF membrane was activated by soaking in methanol for 1 min followed by washes with transfer buffer. The proteins were transferred to the membrane at 25V for appropriate time period according to the size and nature of the protein. The nonspecific sites were blocked using 5% skimmed milk or 1% BSA at 4°C overnight or at room temperature for 3 hours. The blot was then incubated with primary antibody in 2.5% skimmed milk or 1% BSA in PBS for 3 hours or overnight at 4°C depending on the antibody. The blot was washed with wash buffer according to standardized conditions for each primary antibody. Further the blot was incubated with appropriate secondary antibody conjugated with HRP in 2.5% skimmed milk or 1% BSA in PBS for 3 hours at 4°C or at room temperature for 1.5 hours. The membrane was washed and the blot was developed using Pierce Super Signal West Pico chemiluminiscent kit as described by the manufacturer. The blot was exposed to TMS (Kodak) films for different time points and developed using GBX-Developer-Fixer solutions (Premier Kodak reagents).

2.1.11. Mammalian Cell culture:

HeLa, H1299, MCF7, HBL100, HepG2 and HEK293T cells were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and appropriate antibiotics in 5% CO2 incubator. For cell storage, approximately 2 million cells were suspended in 1 ml of 90% FBS and 10% DMSO. The temperature of the cells was brought down gradually to -80°C with the help of thermo cooler and finally cells were stored in liquid nitrogen for long term storage. Cell revival was done by keeping the cells at 37°C for 3 mins followed by washing with 10 ml of DMEM to remove DMSO. Cells were further seeded in 25 mm flask for maintenance. On attaining confluency, the cells were trypsinized using 0.05% Trypsin EDTA solution (Invitrogen) at 37°C for 1-2 mins based on

the cell type, followed by immediate neutralization with serum containing media. The cells were centrifuged at 700 rpm for 5 mins and seeded in flasks or dishes as per the experimental requirements.

2.1.12. Sf21 insect cell culture:

The *Spodoptera frugiperda* ovarian epithelial cell line Sf21 was cultured in the commercial TC100 medium (Sigma), supplemented with 0.1% Pluronic F-68 solution (Sigma), 10µg/ml Gentamycin (Sigma) and 10% FBS (Invitrogen) at 27ºC in a BOD incubator. After attaining confluency, the cells were dislodged from the substratum using a cell scraper and subcultured in a 1:3 ratio. For cell storage, approximately 2 million cells were suspended in 1 ml of 90% FBS and 10% DMSO. The temperature of the cells was brought down gradually to -80°C with the help of thermo cooler and finally cells were stored in liquid nitrogen for long term storage. Cell revival was done by keeping the cells at 37°C for 3 mins followed by washing with 10 ml of TC100 to remove DMSO. Cells were further seeded in 25 mm flask for maintenance.

2.1.13. Human Embryonic stem cell culture maintenance:

a) Matrigel coating:

Matrigel (BD Biosciences 354277) solution was prepared in DMEM media (Invitrogen) in the ratio of 1ml matrigel per 10 ml of media. Tissue culture coated flasks and culture dishes were coated with matrigel and left for a minimum of 45 mins at room temperature. Prior to use, the matrigel solution was discarded and the tissue culture plate/dish/flask was rinsed with 1X PBS. Care was taken so as to not let the coating dry.

b) Feeder free culturing of cells:

BG01V cells were purchased from Global stem (GSC-1103). The frozen cells were thawed at 37ºC, and transferred to DMEM media containing knockout serum (KSR, Invitrogen) followed by centrifugation at 700 rpm at room temperature for 5 mins. The cells were then revived in MEF (mouse embryonic fibroblast) conditioned medium, (GLOBALSTEM) containing 4ng/ml bFGF (basic fibroblast growth factor, Invitrogen). After three days, a media change using MEF CM + bFGF was given. Colonies were observed after five days of culturing. Confluent cultures were trypsinized using 0.05% trypsin EDTA (Invitrogen) for 2

mins at 37ºC, followed by neutralization using DMEM + KSR and centrifuged at 700 rpm for 5 mins at room temperature. Cell count was routinely performed during each trypsinization and seeded as per experimental requirements.

Alternatively, HUES7 cells thawed from the frozen stocks were maintained as mentioned above and were primarily used for the cytotoxicity assays.

2.2. Cloning, Protein Expression and Purification:

(a) Protein purifications:

2.2.1. Core histone purification from HeLa nuclear pellet:

HeLa nuclear pellet was homogenized in 0.1 M potassium phosphate buffer (pH 6.7), containing 0.1 mM EDTA, 10% glycerol, 0.1 mM PMSF, 0.1 mM DTT with 630 mM salt concentration and the supernatant was kept for binding with Hydroxyapatite. Following washes with 630 mM salt over a prolonged period (12 hours), the histone octamer was eluted with 2M salt and was dialyzed against BC100 (20 mM Tris, pH 7.9, 100 mM KCl, 20% glycerol, 0.1 mM DTT). Protein concentration was estimated using Bio-rad protein reagent assay and electrophoresed on 15% SDS PAGE **(Figure 2.1.A).** Aliquots were flash frozen in liquid nitrogen and stored in -80ºC.

2.2.2. Histone H3 purification from inclusion bodies:

Recombinant core histones (*Xenopus*) H3 DNA was transformed into the *E. coli* BL21 strain, which was then grown in LB medium containing 100 μg/ml ampicillin and grown for 12 hours at 37ºC followed by induction at 0.8 OD_{600} with 0.4 mM IPTG for 3 hours. The cells were pelleted and resuspended in wash buffer (50 mM Tris HCl, pH 7.5, 100 mM NaCl, 1 mM benzamidine, 1 mM β-mercapto ethanol), was sonicated and the lysate was clarified by centrifugation. The pellet was washed with wash buffer containing Triton X 100. The detergent was removed by washes with wash buffer lacking detergent. The pellet containing inclusion bodies was soaked in DMSO for 30 minutes at 22ºC. 6 M Guanidium hydrochloride containing 20 mM Sodium acetate pH 5.2, 1 mM DTT was added slowly and the unfolding was allowed to proceed for 1 hour at 22ºC. The solubilized inclusion bodies were centrifuged to remove the particulate materials and the supernatant was subjected to

extensive dialysis for refolding in the refolding buffer containing water and β-mercaptoethanol. The soluble fraction was separated and checked out on 15% SDS PAGE **(Figure 2.1.B).**

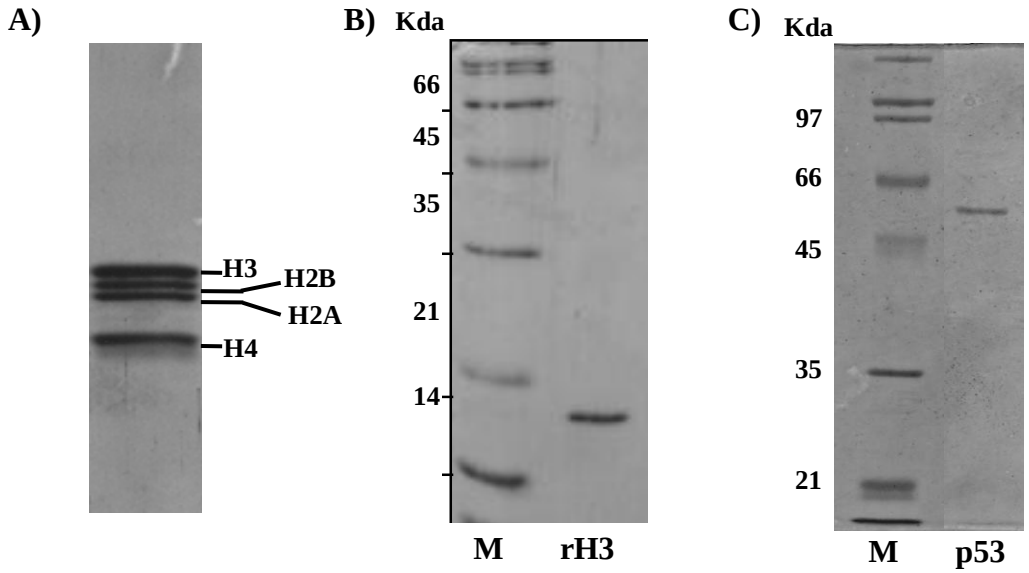


Figure 2.1. Protein substrates (A) *The core histone octamer profile on a 15% SDS-PAGE representing all the four histones, H3, H2B, H2A and H4.* ***(B)*** *The protein profile of recombinant histone H3 in the second lane.* ***(C)*** *The protein profile of bacterial expressed and purified FLAG-tagged p53. Lane 1 represents the protein molecular weight marker, lane 2 represents p53 protein.*

2.2.3. FLAG tagged p53 purification from *E.coli*

FLAG tagged p53 protein was expressed and purified by transforming the p53 containing expression plasmid in *E.coli.* The primary inoculum was grown at 37ºC overnight from a single colony. The secondary inoculum and the subsequent induction was done at 30ºC, wherein the culture of 0.4 OD_{600} was induced with 0.4 mM IPTG for 3 hours. The culture was centrifuged and the cells were homogenized in BC300 buffer containing, 300 mM KCl, followed by sonication at amplitude 32% with a pulse on period of 3 seconds and pulse off period of 5 seconds for a total time of 3 minutes repeated thrice and centrifuged at 16000 rpm for 30 minutes at 4ºC. The supernatant was kept for binding with FLAG-M2 agarose beads (SIGMA) for 3 hours in cold room. The beads were washed initially with BC300 buffer followed by washes with BC100 buffer containing 100 mM salt. The protein

was eluted using FLAG peptide (SIGMA). Aliquots of the protein were flash frozen and stored in -80ºC. The protein was electrophoresed on 12% SDS PAGE **(Figure 2.1.C)** and the concentration was estimated using the Bio-rad reagent.

2.2.4. Full length FLAG tagged CARM1 purification from Sf21 insect cells infected with baculovirus of recombinant CARM1 virus:

Sf21 insect cells (5 million cells per 150 mm plate) were infected with the recombinant CARM1 baculovirus. Following 60 hours of infection when all the cells show distinct infected morphology and detachment properties from the surface, the cells were scraped and lysed in homogenization buffer (20 mM Tris pH 7.4, 500 mM NaCl, 4 mM $MgCl_2$, 0.4 mM EDTA, 2 mM DTT, 20 mM β-glycerophosphate, 20% glycerol, 0.4 mM PMSF and protease inhibitors). The supernatant was kept for binding with M2-agarose beads (SIGMA) followed by washes with buffer consisting of 20 mM Tris pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 15% glycerol, 0.01% NP-40, 0.2 mM PMSF and protease inhibitors. Protein was eluted using FLAG peptide (SIGMA) in BC100 buffer. The protein aliquots were flash frozen in liquid nitrogen and stored in -80ºC. The protein concentration was estimated using the Bio-rad reagent, and was checked on 12% SDS PAGE **(Figure 2.2.A).** The activity of the protein was verified by filterbinding assay and fluorography gel assay. In case of biophysical experiments, since the FLAG peptide hinders with the experimental procedures, the protein was eluted by pH elution with 0.1 M glycine, pH 3.5 followed by neutralization with Tris-NaCl buffer (0.5 M Tris pH 7.4, 1.5 M NaCl). For performing SERS, the protein was dialyzed against Tris-NaCl buffer extensively so as to remove glycine.

2.2.5. Full length hexa histidine tagged G9a purification from Sf21 insect cells infected with baculovirus of recombinant G9a virus:

Sf21 insect cells (5 million cells per 150 mm plate) were infected with the recombinant G9a baculovirus. Following 60 hours of infection when all the cells show distinct infected morphology and detachment properties from the surface, the cells were scraped and lysed in homogenization buffer (10 mM Tris, pH 7.4, 500 mM NaCl, 0.1% NP-40, 15 mM Imidazole, 2 mM PMSF, 2 mM β-mercaptoethanol and protease inhibitors). The

supernatant was kept for binding with Ni-NTA beads (Novagen) followed by washes with buffer consisting of 10 mM Tris, pH7.4, 300 mM NaCl, 10% glycerol, 15 mM Imidazole, 0.2% NP-40, 2 mM PMSF and 2 mM β-mercaptoethanol. Protein was eluted using elution buffer consisting of 250 mM Imidazole. The protein aliquots were flash frozen in liquid nitrogen and stored in -80ºC. The protein concentration was estimated using the BIORAD reagent, and was checked on 10% SDS PAGE **(Figure 2.2.B).** The activity of the protein was verified by filterbinding assay and fluorography gel assay.

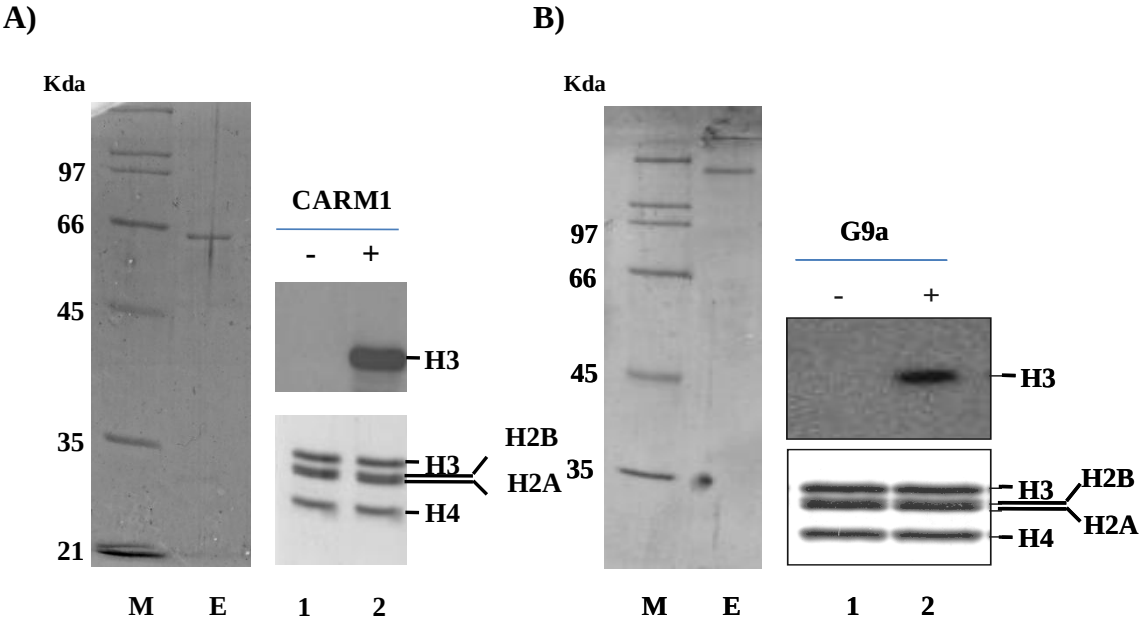


Figure 2.2. Recombinant histone methyltransferases*. Protein profile of* ***(A)*** *full length CARM1 arginine methyltransferase,* ***(B)*** *full length G9a lysine methyltransferase purified from respective recombinant baculo virus infected Sf21 cells. The activity of the enzyme was verified by the gel fluorography assay as represented in lane 1 versus lane 2. Lane 1, histones without enzyme: lane 2, histones with enzyme. The upper panel represents the autoradiogram profile whereas the lower panel is the coomassie stained gel.*

2.2.6. Full length hexa histidine tagged p300 purification from Sf21 insect cells infected with baculovirus of recombinant p300 virus:

Sf21 insect cells (5 million cells per 150 mm plate) were infected with the recombinant p300 baculovirus. Following 60 hours of infection when all the cells show

distinct infected morphology and detachment properties from the surface, the cells were scraped and lysed in homogenization buffer (10 mM Tris pH 7.4, 500 mM NaCl, 0.1% NP-40, 15 mM Imidazole, 2 mM PMSF, 2 mM β-mercaptoethanol and protease inhibitors).

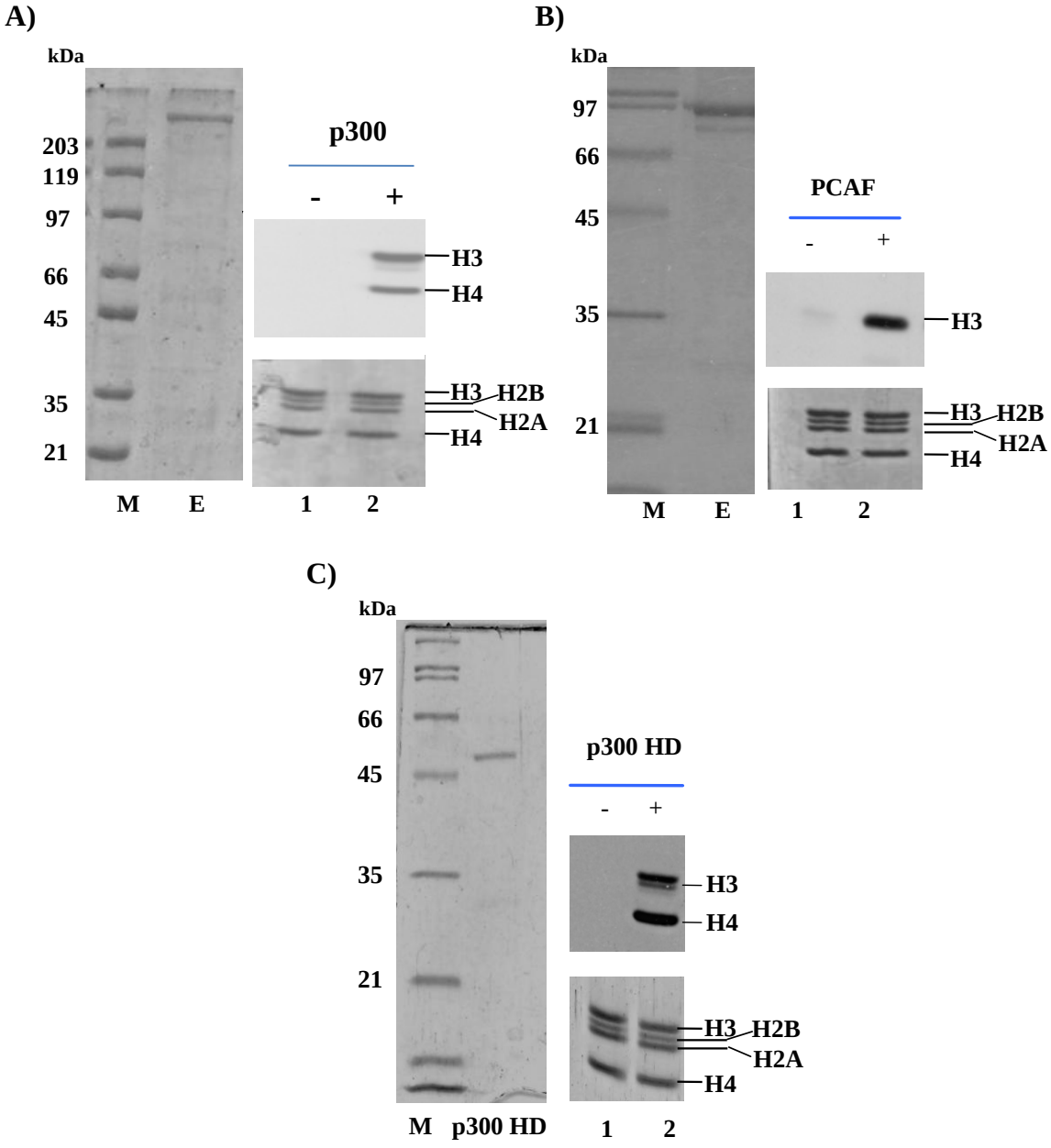


Figure 2.3. Recombinant histone acetyltransferases. *Protein profile of* ***(A)*** *full length p300,* ***(B)*** *full length PCAF purified from respective recombinant baculo virus infected Sf21 cells.* ***(C)*** *bacterial expressed p300 minimal HAT domain. The activity of the enzyme was verified by the gel fluorography assay as represented in lane 1 versus lane 2. Lane 1, histones without enzyme: lane 2, histones with enzyme. The upper panel represents the autoradiogram profile whereas the lower panel is the coomassie stained gel.*

The supernatant was kept for binding with Ni-NTA beads (Novagen) followed by washes with buffer consisting of 10 mM Tris, pH 7.4, 300 mM NaCl, 10% glycerol, 15 mM Imidazole, 0.2% NP-40, 2 mM PMSF and 2 mM β-mercaptoethanol. Protein was eluted using elution buffer consisting of 250 mM Imidazole. The protein aliquots were flash frozen in liquid nitrogen and stored in -80ºC. The protein concentration was estimated using the BIORAD reagent, and was checked on 8% SDS PAGE **(Figure 2.3.A).** The activity of the protein was verified by filterbinding assay and fluorography gel assay.

2.2.7. Full length FLAG tagged PCAF purification from Sf21 insect cells infected with baculovirus of recombinant PCAF virus:

Sf21 insect cells (5 million cells per 150 mm plate) were infected with the recombinant PCAF baculovirus. Following 60 hours of infection when all the cells show distinct infected morphology and detachment properties from the surface, the cells were scraped and lysed in homogenization buffer (20 mM Tris, pH 7.4, 500 mM NaCl, 4 mM $MgCl_2$, 0.4 mM EDTA, 2mM DTT, 20 mM β-glycerophosphate, 20% glycerol, 0.4 mM PMSF and protease inhibitors). The supernatant was kept for binding with M2-agarose beads (SIGMA) followed by washes with buffer consisting of 20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 15% glycerol, 0.01% NP-40, 0.2 mM PMSF and protease inhibitors. Protein was eluted using FLAG peptide (SIGMA) in BC100 buffer. The protein aliquots were flash frozen in liquid nitrogen and stored in -80ºC. The protein concentration was estimated using the BIORAD reagent, and was checked on 12% SDS PAGE **(Figure 2.3.B).** The activity of the protein was verified by filterbinding assay and fluorography gel assay.

2.2.8. p300 minimal HAT domain purification from *E.coli*

p300 minimal HAT domain was purified from *E.coli* BL21 strain by co transformation of hexa histidine tagged p300 HAT domain in pET28b (kan^r) and GST tagged SIRT2 in pGEX-CD (amp^r) expression plasmids as described in (Arif M et al., 2007). The protein expression was done by growing the co-transformed colony in LB medium containing 100 µg/l ampicillin- and 50 µg/l kanamycin at 37ºC followed by induction with 0.4 mM IPTG at 0.5 OD_{600} for three hours at 37ºC.

The cell pellet was homogenized in buffer containing 0.02 M Tris, pH 7.4, 20% glycerol, 0.4 mM EDTA, 2 mM PMSF, 0.3 M KCl, 0.1% NP-40, 20 mM Imidazole and β-mercaptoethanol, followed by sonication at amplitude 32% with a pulse on period of 3 seconds and pulse off period of 5 seconds for a total time of 3 mins repeated thrice. The sonicated suspension was centrifuged at 16000 rpm for 30 minutes at 4ºC and the supernatant was kept for binding with Ni-NTA beads (Novagen), for 3 hours at 4ºC. After the binding step the beads are washed with buffer containing 40 mM Imidazole. The protein was then eluted with buffer containing 250 mM Imidazole. The protein aliquots were flash frozen to be stored in -80ºC. The protein concentration was estimated by using the Bio-rad reagent, and the protein profile was visualized by electrophoresing the sample on 12% SDS PAGE **(Figure 2.3.C).** Activity was verified by filterbinding and gel fluorography assays.
For biophysical experiments like isothermal titration calorimetry and fluorescence titration, the HAT domain was dialyzed against BC100 buffer containing 0.2 M Tris, pH 7.4, 0.2 mM EDTA and 100 mM KCl, extensively and the protein concentration was estimated again.

(b) Site-directed mutagenesis (SDM):

2.2.9. Generation of point mutants of histone H3, A25P and P16A:

The forward and reverse primers were designed with the sequence coding for the desired mutation in the centre flanked by atleast five codons on either side. Histone H3 expression clone (*Xenopus*) was used as the template and the mutagenesis was done by using a Stratagene SDM kit. Briefly, the first step was the synthesis of the mutant strand of a 10 ng dsDNA template (Histone H3 plasmid) using 125 ng of both forward and reverse primers, by the *pfu* ultra HF DNA polymerase (2.5U). The cycling parameters were decided based on the length of the DNA template to be amplified for a total of 18 cycles. The amplified mutant product was then subjected to Dpn1 (10U) digestion at 37ºC for 1 hour to digest the parental (non-mutated) supercoiled DNA. 2 µl of the Dpn1 digested reaction was then directly transformed into XL-10 gold ultracompetent cells using NZY+ broth by the standard transformation protocol described in the general methods section. The selection marker used was ampicillin and the colonies were screened by sequencing for positive clones. Sequencing was carried out with the T7 polymerase forward and reverse primers **(Figure 2.4.A).** Positive clones were sequenced and transformed into the BL21 strain of

E.coli. The expression and purification was carried out similar to the wildtype protein **(2.2.2).** The soluble fraction was separated and checked out on 15% SDS PAGE **(Figure 2.4.B).**

PRIMER details:

1) A25P Forward: 5' CAGCTGGCCACCAAGGCACCCAGGAAGTCCGCTCCTGCTAC 3'

A25P reverse: 5' GTAGCAGGAGCGGACTTCCTGGGTGCCTTGGTGGCCAGCTG 3'

2) P16A Forward: 5' CACCGGAGGGAAGGCTGCCCGCAAGCAGCTG 3'

P16A reverse: 5' CAGCTGCTTGCGGGCAGCCTTCCCTCCGGTG 3'

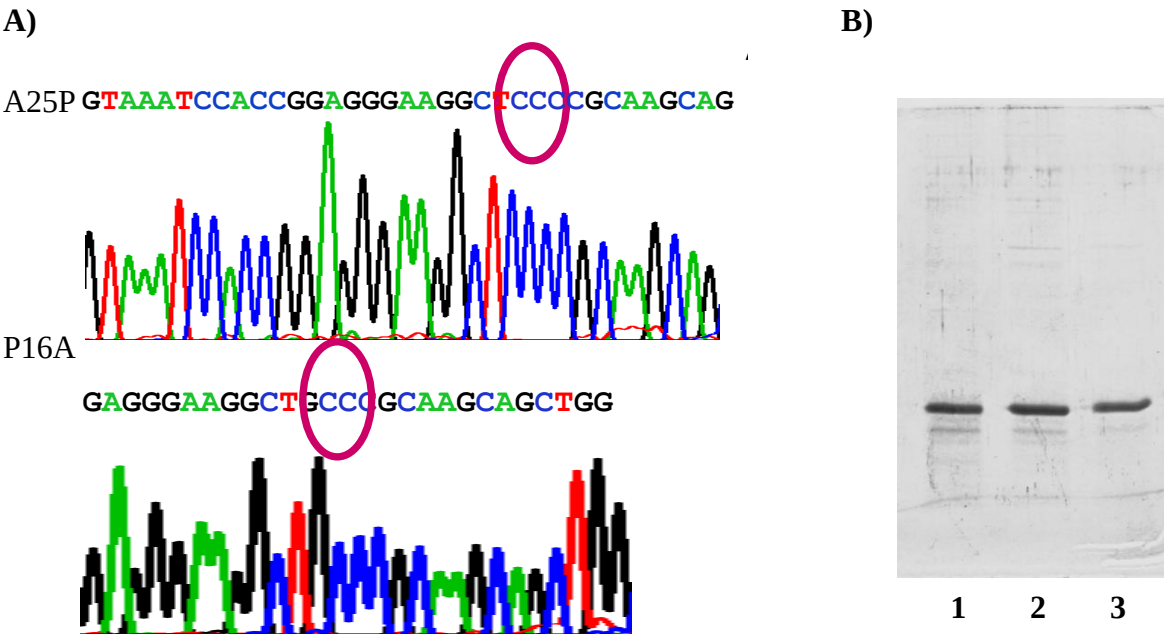


Figure 2.4. **Histone H3 point mutants, A25P and P16A.** ***(A)*** *The sequence chromatogram of the histone H3 mutants. The red circle denotes the base that has been modified.* ***(B)*** *The protein profile of bacterial expressed and purified recombinant histone H3 (lane 1), recombinant histone H3 mutant A25P (lane 2) and recombinant histone H3 mutant P16A (lane 3)*

2.2.10. Generation of point mutant of p300 minimal HAT domain K1358A.

The p300 HAT domain mutant K1358A was generated using the p300 HAT domain expression clone following the same experimental procedure as described in **section 2.2.8.** The positive clones were sequenced (**Figure 2.5.A**) and transformed into the BL21 *E.coli*

strain and expression, purification was same as the wild type protein. The protein concentration was estimated and electrophoresed on 12% SDS-PAGE (**Figure 2.5.B)**

PRIMER details:

HD K1358A Forward: 5' TTTCCATACCGAACCGCCCTCTTTGCCTTTG 3'

HD K1358A reverse: 5' CAAAGGCAAAGAGGGCTGCGGTTCGGTATGGAAA 3'

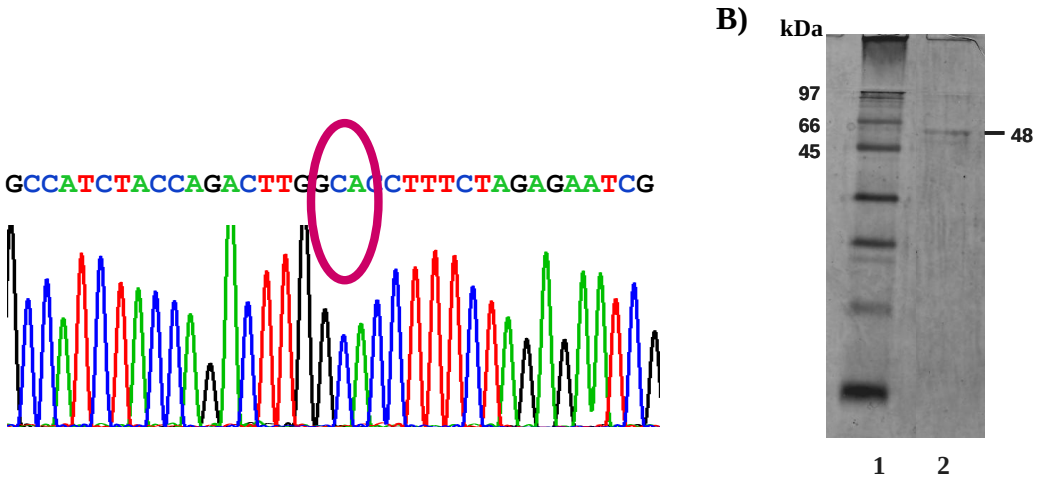


Figure 2.5. **p300 HAT domain mutant K1358A.** ***(A)*** *The sequence chromatogram of the HD K1358A mutant. The red circle denotes the base that has been modified.* ***(B)*** *The protein profile of bacterial expressed and purified mutant protein in lane 2. Lane 1 represents the protein molecular weight marker.*

2.3. Histone modifying enzyme assays:

2.3.1. Histone Methyltransferase Assays:

Histone methyltransferase assays were performed in a 30 µl reaction. The reaction mixture containing highly purified HeLa core histones, with or without histone methyltransferases in the HMT assay buffer 20 mM Tris, 4 mM EDTA, pH 8.0, 200 mM NaCl, along with varying concentrations of compound was incubated for 10 mins at 30 °C. After the initial incubation, 1 µl of 15 Ci/mmol ^{3}H-(S)-adenosyl methionine (Amersham) was added to the reaction mixtures, and the incubation continued for 15 mins. The reaction mixture was then blotted onto P-81 (Whatman) filter paper, and radioactive counts were

recorded on a PerkinElmer Wallac 1409 liquid scintillation counter. The reaction products were TCA-precipitated, resolved on 15% SDS-PAGE, and subjected to fluorography followed by autoradiography.

2.3.2. Histone Acetyltransferase (HAT) Assay:

HAT assays were performed using 2.4 µg of highly purified HeLa core histones, incubated in HAT assay buffer at 30ºC for 10 mins with or without baculovirus expressed recombinant p300 or PCAF in the presence or absence of compounds followed by addition of 1 µl of 3.6 Ci/mmol ^{3}H-acetyl CoA (NEN-PerkinElmer) and further incubated for another 10 mins in a 30 µl reaction at 30ºC. The reaction mixture was then blotted onto P-81 (Whatman) filter paper, radioactive counts were recorded on a Wallac 1409 liquid scintillation counter.

2.3.3. Kinetic characterization of TBBD-mediated inhibition

The histone methyltransferase reaction was carried out with CARM1 in the presence of three concentrations of TBBD (10, 20 and 50 µM). The reaction consisted of two substrates, histone H3 and the tritiated methyl group donor of SAM. In the first assay, the concentration of histone H3 was kept constant at 1.467 µM and ^{3}H-SAM was varied from 0.39 µM to 1.98 µM. In the second assay, the concentration of ^{3}H-SAM was kept constant at 1.98 µM and histone H3 was varied from 0.026 µM to 1.467 µM. The incorporation of radioactivity was taken as a measure of the reaction velocity, which was recorded as counts per minute (cpm). The obtained values were plotted on a Lineweaver-Burk plot using GraphPad Prism software.

2.3.4. Kinetic characterization of RTK1 mediated p300 inhibition:

The histone acetyltransferase reactions were carried out with the baculovirus expressed recombinant full length p300, in the presence of different concentrations of the inhibitor RTK1 (15, 20, 35 and 45 µM). The HAT reaction consists of two substrates, core histones and the acetyl group donor, ^{3}H-acetyl CoA. Therefore, the kinetic analysis was done in two different sets. In the first set, concentration of core histones was kept constant at 1.6 µM and ^{3}H-acetyl CoA was varied from 1.08 µM to 8.66 µM. In the second assay, ^{3}H-

acetyl CoA was kept constant at 2.78 µM and core histones were varied from 0.003 µM to 0.068 µM. The incorporation of the radioactivity was taken as a measure of the reaction velocity recorded as counts per minute (cpm). Each experiment was performed in triplicate, and the reproducibility was found to be within 15 % of the error range. Weighted averages of the values obtained were plotted as a lineweaver burk plot using Graphpad Prism software.

The kinetic analysis of RTK1 mediated inhibition of p300 acetyltransferase activity was also studied using p300 minimal HAT domain. (aa. 1284-1673) and two concentrations of the inhibitor RTK1 (0.5 µM and 3.5 µM) was assayed. All the other parameters were similar to the above experiment.

2.4. Biophysical experiments:

2.4.1. Isothermal titration calorimetry:

It is a biophysical technique used to determine the thermodynamic parameters of (biochemical) interactions. It is a quantitative technique that can directly measure the binding affinity (Ka), enthalpy changes (ΔH), and binding stoichiometry (n) of the interaction between two or more molecules in solution. From these initial measurements, Gibbs energy changes (ΔG), and entropy changes (ΔS), can be determined using the relationship: $\Delta G = -RT\ln K = \Delta H - T\Delta S$. A schematic of the instrument setup is represented in **Figure 2.6.**

2.4.2. Isothermal titration calorimetry (ITC) titration of histone H3 and mutants against TBBD.

ITC experiments were carried out in a VP-ITC system (Microcal LLC, Northampton, MA) at 25ºC. Samples were centrifuged and degassed prior to titration. Titration of TBBD against protein (histone H3/CARM1) was carried out by injecting 0.14 mM TBBD in HMTase assay buffer against 0.007 mM histone H3/ CARM1. A two-minute interval was allowed between injections for equilibration, sufficient for the return of the heat signal to baseline. A total of 35 injections were carried out to ensure complete titration. The protein concentration was chosen to achieve sufficiently high heat signals with a minimum enthalpy of dilution. To minimize the error associated with diffusion from the syringe during baseline

equilibration, the volume of the first injection was only 1 µl and the associated small heat change was not considered for data analysis.

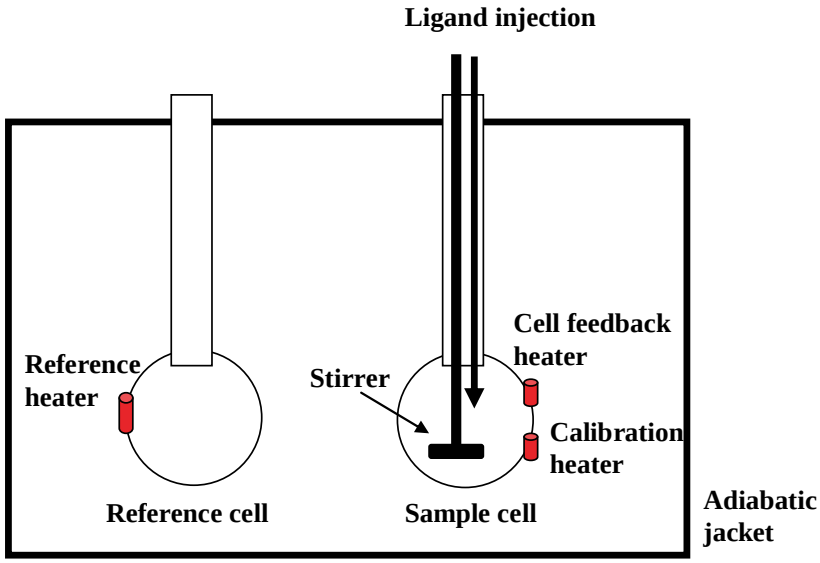


Figure 2.6. Isothermal titration calorimetry *set up representing the adiabatic jacket and the reference cell and the sample cell. The ligand is injected into the sample cell as represented. The different heat sensors that record the heat change are also shown.*

Blank experiments involving titration of TBBD against buffer as well as buffer against buffer were carried out and used to subtract the background heat change. The corrected heat change values were plotted against the molar ratio of the titrated products and analyzed by using the manufacturer's software which yielded the stoichiometry n (expressed in terms of the number of molecules of TBBD to protein) and equilibrium constant (Ka). From the relationship ΔG^o=-RT ln Ka and the Gibbs-Helmholtz equation, the free energy of binding and the entropy of association (ΔS^o) were calculated.

2.4.3. Isothermal titration calorimetry (ITC) titration of p300 HAT domain against RTK1 and RTK2.

ITC experiments were carried out in a VP-ITC system (Microcal LLC) at 25ºC. Samples were centrifuged and degassed prior to titration. Titration of RTK1/RTK2 against the protein HAT domain was carried out by injecting 0.2 mM RTK1/RTK2 in 20 mM Tris, pH 7.5, 0.2 mM EDTA, 100 mm KCl buffer against 0.0034 mM HAT domain. A two

minute interval was allowed between injections for equilibration. A total of 40 injections were carried out to ensure complete titration. The protein concentration was chosen to achieve sufficiently high heat signals with a minimum enthalpy of dilution. To minimize the error associated with diffusion from the syringe during baseline equilibration, the first injection was only 1 µl and the associated small heat change was not considered for data analysis. Blank titrations were carried out using buffer and DMSO with no ligand against HAT domain and used for subtraction of the background heat change. The corrected heat change were plotted against molar ratio of the titrated products and analyzed using manufacturer's software which yielded the stoichiometry n (in terms of number of molecule of RTK1/protein), equilibrium constant (Ka). From the relationship $\Delta G^{o} = -RT \ln Ka$ and the Gibbs-Helmholtz equation, the free energy of binding and the entropy of association (ΔS^{o}) were calculated.

2.4.4. SERS measurements with CARM1:

Ag nanoparticles were prepared by Lee and Meisel method (Lee and Meisel, 1982). The average diameter of the nanoparticle was around 50 nm, and had a plasmon band centered at 426 nm. CARM1 in buffer solution was mixed with nanoparticles in the ratio 10:70 by volume. After 5 minutes, the mixture was drop-coated over a microscope slide and place under a water immersion objective lens of a custom built Raman microscope, whose details can be found elsewhere13. A 532 nm, frequency double Nd-YAG laser was used as Raman excitation source. The typical accumulation time was around 40 to 60 seconds.

2.4.5. Fluorescence titration of p300 HAT domain with RTK1:

Fluorescence spectra of p300 HAT domain with RTK1 was recorded on a Hitachi F-2500 spectrofluorimeter. Spectra were recorded in buffer containing 10 mM Tris, pH7.4, 0.2 mM EDTA and 100 mM KCl, at a protein concentration of 1.8 µM. Protein samples were excited at 290 nm and emission monitored from 292–450 nm. Excitation and emission bandwidths were set to 5 nm. Baseline corrections were done by subtraction of the buffer spectrum.

2.5. Molecular docking studies:

2.5.1. Docking of TBBD with CARM1 and histone H3 (ES complex):

Atomic coordinates of histone H3 (chain A and E) from the X-ray structure of the nucleosome core particle complex (1KX5) were extracted from the PDB. The PDB ID of CARM1 structure used was 2V74. Polar hydrogens were added, nonpolar hydrogens were merged, AutoDock 4 atom types and Gasteiger charges were assigned to all atoms of the receptor molecule (i.e., histone H3). Rotatable bonds, AutoDock 4 atom types, and Gasteiger charges were also assigned to the ligand molecule (TBBD) according to AutoDock Tools (ADT). To target residues 14 to 18 (KAPRK) of the receptor, 'P' (proline 16) was assigned as the center of the grid, and an affinity grid box was made with default grid spacing 0.375Å using AutoGrid 4. A Lamarckian genetic algorithm was selected to evaluate the ligand binding energies with the receptor using AutoDock 4 with default parameters except the number of energy evaluations (ga_num_evals, 25000000).

2.5.2. Docking of RTK1/RTK2 with p300 HAT domain and RTK1 with the *in silico* generated mutant K1358A.

Crystal structure of p300 HAT domain was extracted from PDB (PDB id: 3BIY). Crystal structure of the inhibitor RTK1 and its inactive derivative RTK2 was obtained and solved (Bruker X8 APEX). The HAT domain was docked with the structure of RTK1 and RTK2 to find out their interaction sites on HAT domain. RTK1 has a hydroxyl group at the 5th carbon position and the derivative RTK2 has a methoxy group instead of the hydroxyl group.

Molecular simulation and the docking of HAT domain with RTK1 and RTK2 were performed using Hex 4.5 software. The docking calculations were done using 3D parametric functions of both the protein (HAT domain) and the chemical structures (RTK1/RTK2). These calculations were used to encode surface shape and electrostatic charge and potential distributions. The parametric functions are based on expansions of real orthogonal spherical polar basis functions. The docking was performed in full rotation mode; both domain and inhibitor were taken at 180 ranges for 20,000 solutions.

2.6. Histone/Protein modification analysis:

2.6.1. Immunofluorescence Analysis:

Cells were grown on cover slips coated with poly-Lysine at 37°C in a 5% CO_2 incubator. After the indicated treatment, cells were washed with PBS and fixed with 4% paraformaldehyde (in PBS) for 20 mins at room temperature. Cells were then permeablized using 1% Triton X-100 (in PBS) for 10 mins and subsequently washed with PBS for 10 mins, 3 times. Nonspecific sites were blocked using 5% FBS (in PBS) for 45 mins at 37°C. Immunostaining was done with primary antibody at appropriate dilutions for 1 hour at room temperature. The cells were washed with wash buffer (1% FBS in PBS) 4 times, 3 mins each. Primary antibody stained cells were incubated with secondary antibody tagged with fluorescent dye at appropriate dilutions for 1 hour at room temperature. After washes with wash buffer the nuclei were stained with Hoechst (1:10,000 dilution) for 20 mins. Two times PBS washed cover slips were inverted on to a microscopic slide over 2 µl of 70% glycerol (in PBS) and visualized using confocal microscopy.

2.6.2. Acid extraction of histones:

Cells (3 million cells per 90-mm dish) were seeded overnight, and histones were extracted from the cells after 24 h of compound treatment. In all the cases serum was added after a period of 2 h of compound treatment. Cells were harvested, washed in ice-cold buffer A (150 mM KCl, 20 mM HEPES, pH 7.9, 0.1 mM EDTA, and 2.5 mM $MgCl_2$) and lysed in buffer A containing 250 mM sucrose and 1% (v/v) Triton X-100. Nuclei were recovered by centrifugation, washed, and proteins were extracted (on ice) for 1 hour using 0.25 M HCl. Chromosomal proteins were precipitated with 25% (w/v) trichloroacetic acid and sequentially washed with ice-cold acidified acetone (20 µl of 12 N HCl in 100 ml of acetone), and acetone, air-dried, and dissolved in the sample buffer (5.8 M urea, 0.9 M glacial acetic acid, 16% glycerol, and 4.8% β-mercaptoethanol). The protein was quantified using a protein assay reagent (Bio-Rad). This was later used for immunoblotting analysis to assay the *in vivo* histone modification status.

2.6.3. Immunohistochemistry:

Compound was treated Plumbagin/TBBD (25 mg/kg body weight) dissolved in 50 µl of DMSO was injected intra-peritoneally to 2 month old swiss albino mice. As controls, two mice were injected 50 µl of water and 50 µl of DMSO intra peritoneally as negative control and solvent control respectively. The experiment was done in triplicates. All mice were staged in animal cages for next six hours, anesthetized and the liver was collected for further protein, RNA and Immunohistochemical analysis. Liver samples stored in formalin were further dehydrated in alcohol, followed by xylene treatment and allowed to get impregnated in paraffin blocks. 3 to 4 micron thick paraffin-embedded sections were deparaffinized in xylene, followed by rehydration in decreasing concentration of ethanol solutions. For routine pathological examination, deparaffinized sections from all blocks were stained with hematoxylin and eosin stains (**Figure 2.7.A)**. Antigen retrieval was performed by micro waving in appropriate buffer. After washing the sections in 0.1 M phosphate buffer, blocking was done using 3% skimmed milk, following which the sections were incubated with the appropriate primary antibody for 3 hours in a humidification chamber. After 0.1 M phosphate buffer wash, the sections were incubated with link secondary antibody (DAKO LSAB +) for 3 hours in a humidification chamber. After washing, the sections were developed by 3´,3´-diaminobenzidine tetrahydrochloride (DAB; Sigma). Hematoxylin was used as counter stain to identify the unstained nucleus (**Figure 2.7.B)**.

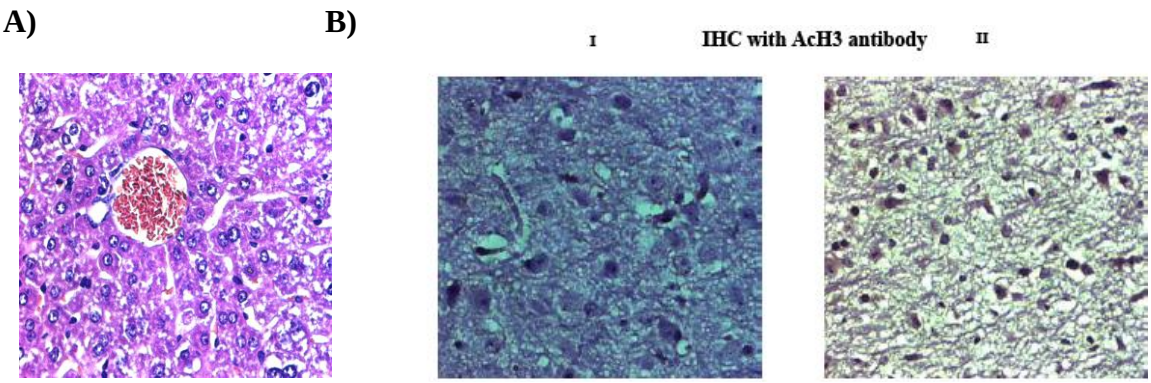


Figure 2.7. Staining of animal tissue section*. **(A)** Heamotoxylin and Eosin staining of mice liver represents the nuclei and cytoplasmic staining visualized as blue and pink respectively. **(B)** Immunohistochemical staining using anti acetylated histone H3 antibody of brain tissue from mice showing negative staining (panel I) and postitive staining (Panel II).*

2.6.4. Whole cell extract preparation:

Cells were harvested by scraping/trypsinization follwed by centrifugation at 6000 rpm for 5 mins at 4°C. Cell pellet was washed with cold PBS and resuspended 10 times packed volumes of cell pellet in RIPA (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml each of aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1 mM NaF) or TNN (50 mM Tris-HCl, pH 7.4; 100 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 µg pepstatin per ml, 200 µM PMSF, 0.5 mM dithiothreitol (DTT), 1 µg of leupeptin per ml) buffer. Homogenization was done using pipette tip and homogenate was incubated at 4°C for 30 mins- 3 hours. Lysates were cleared of debris by centrifuging at 13,000 rpm for 5 mins and the supernatant was used for further experiments.

2.7. GENE EXPRESSION STUDIES:

2.7.1. Transient transfection

The p53 null cell line H1299 was transfected with a mammalian expression construct of p53 (100 ng) using Lipofectamine reagent (Invitrogen), (twice the amount of DNA used) as recommended by the manufacturer. CARM1 mammalian expression construct (2.3 µg) was also transfected to check for the co-operativity of p53 transcriptional activation. Following 36 hours of transfection, the cells were lysed using reporter lysis buffer (Promega), and the lysate was checked for luciferase activity using the substrate. The luciferase activity was determined by recording the counts in a liquid scintillation counter and normalized to β-galactosidase activity. As a positive control, 1.5 µg of PC4 was also transfected. For transfection experiment followed by inhibitor treatment, after 12 h of transfection, the cells were treated with TBBD for 24 h. After the treatment, the cells were subjected to crosslinking for chromatin immunoprecipitation (ChIP) analysis.

2.7.2. Chromatin immmunoprecipitation assay (ChIP)

Chromatin immunoprecipitation was carried out as described in (Pokholok et al., 2005). Briefly, the cells were fixed with 4% para formaldehyde followed by neutralization with 0.25 M glycine. The pulldown was done using antibody against dimethyl histone H3R17 (Abcam ab8284), dimethyl histone H3R26 (Upstate 07-215), and the

immunoprecipitated samples were deproteinized and ethanol-precipitated to recover the DNA. PCR analysis was performed using primers for p53-responsive site on the *p21* promoter.

2.7.3. Endogenous gene expression assay

Human Embryonic Kidney cell line HEK293T was treated with doxorubicin to induce p53 levels and was later treated with TBBD for 24 h or left untreated. Following the stipulated treatment time, total RNA was isolated using Trizol reagent (Invitrogen). cDNA was synthesized with oligo dT (28 mer) (Invitrogen) and MMLV reverse transcriptase (SIGMA), and the expression analysis was carried out using iQTM SyBR green supermix (BioRad) and gene-specific primers of *p21 and actin*.

2.7.4. Microarray analysis:

HeLa cells were treated with 100 μM TBBD. DMSO-treated cells were used as a control. RNA was isolated after 24 h of treatment. The treated samples were hybridized on an Agilent human 44K whole-genome array. Hybridization was carried out in triplicate. Data analysis was done using GeneSpring software. Normalization was done with GeneSpring GX using the recommended per spot, per chip intensity dependent (lowess) normalization.

2.7.5. Statistical analysis:

All experiments were performed atleast in triplicates. The statistical significance of the difference in the means was evaluated by two-tailed paired student's t-test using Graph Pad Prism software, version 4.0. Means were considered significant if the p values of the paired t-test were less than 0.05.

2.8. Human Embryonic stem cell related experiments

2.8.1. Cytotoxicity assays:

HUES7 cells were seeded at a density of 25000 cells per well in a 24 well tissue culture plate precoated with matrigel as described earlier. An initial cytotxicity assay was done using inhibitors in DMSO solvent ranging from concentrations as low as 0.01 μM to

100 µM. Cell counts were taken after every 24 hours for a period of 4 days. Cells were trypsinized and counted using hemocytometer cell counter. Experiments were done in replicates and the cell count obtained was converted to percent cell viability and plotted using Microsoft excel worksheet.

2.8.2. Gene expression analysis:

Cells were cultured in 30 mm dishes pre coated with matrigel as described above. Following indicated time points of treatment with inhibitors, the cells were trypsinized (scraping was avoided so as not to disturb the matrigel coating), using 0.05% trypsin EDTA solution and the cells were centrifuged at 2000 rpm, 4ºC for 10 minutes. The cell pellet was washed with 1X PBS, and processed for RNA isolation and subsequent gene expression analysis as described in the general methods section, **2.1.5 and 2.1.6.**

2.8.3. Alkaline phosphatase staining:

Alkaline phosphatase staining was performed on ES cells to check for the pluripotency status. Cells that were in culture for a minimum of five days were used for the staining purpose. The staining was performed by the Alkaline phosphatase staining kit (Millipore) as per the manufacturer's instructions. The images were captured on a microscope and as per the experimental requirements were quantified.

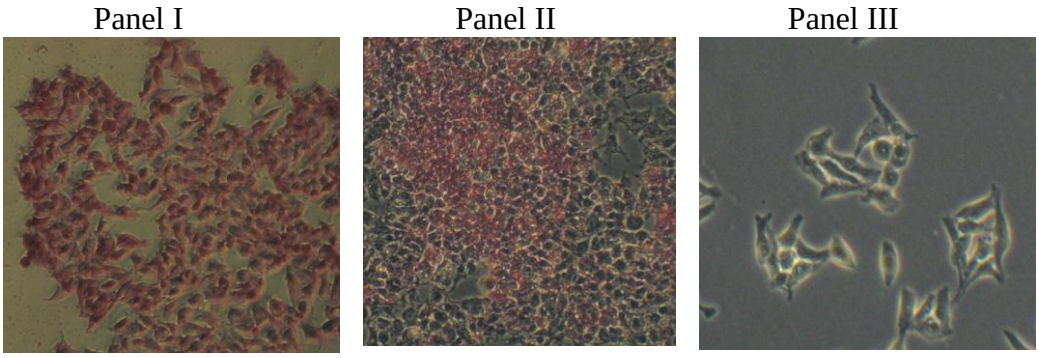


Figure 2.8. Alkaline phosphatase staining of human ES cell line, *BG01V (Panel I and Panel II), HeLa cells (Panel III). Panel I represents a typical ES cell colony with maximum undifferentiated cells, Panel II represents the hES culture subjected to stress, hence few cells donot show the staining (differentiating cells), and Panel III represents the completely differentiated cell type, HeLa, hence no staining is observed.*

2.9. Isolation of active components from the plant material:

2.9.1. Isolation of TBBD (ellagic acid) from pomegranate crude extract:

Pomegranate fruits were washed and cleaned to yield their husks which were separated from seeds and juice. The husks were dried in the sunlight and powdered by mixer. Pomegranate husk powder (250 g) was charged into the four neck one litre flask fitted with magnetic stirrer, followed by addition of one litre of water and stirred for 24 hours. The precipitate was filtered with the help of a buchner funnel and the collected filtrate was distilled up completely to give a semi solid brown coloured component. This semisolid extract was divided into 10 portions. Two portions were loaded into silica gel column of 180-200 mesh with water: methanol as the solvent system. The column was eluted with distilled water until the sugary pale yellow eluate was clear in colour. The crude product was eluted with 2:1 methanol:water system. The methanol and water was distilled up completely with rotary evaporation. The resultant crude product was purified on the Sephadex LH20 column. The sephadex LH20 column was pre-equilibrated with water:methanol (8:2) system. Crude product (3 g) was loaded into the column. The column was eluted with increasing amount of methanol. Different fractions were collected and spotted on TLC. Similar fractions were combined. Five different fractions were collected and named as fraction A, B, C, D, E. The fraction A was characterized as the molecule having inhibitory activity against the histone methyltransferase CARM1. The further characterization of the purified small molecule compound by mass spectrometric analysis and NMR spectroscopy suggest that it is TBBD (2,3,7,8-tetrahydroxy[1] benzopyrano [5,4,3(de)[1]benzopyran5,10-dione).

2.9.2. Isolation of Plumbagin from *Plumbago rosea* roots:

The dried and powdered roots of *Plumbago rosea* were extracted with ethyl acetate. The ethyl acetate extract after removal of the solvent under reduced pressure gave a dark brown semisolid, which was separated into phenolic and neutral fractions by treatment with 5% NaOH and subsequent acidification of the aqueous layer with 2 M HCl and extraction with diethyl ether. The phenolic fraction was subjected to flash chromatography (230-400 mesh) using increasing polarity. The fraction extracted with 4% ethyl acetate in hexane

mixture gave an orange colored solid, which on further recrystallization yielded plumbagin/ RTK1.

2.9.3. Derivatization of Plumbagin:

(a) RTK2: 5-Methoxy-2-methyl-1,4-naphthoquinone

The solution of RTK1 (100 mg, 0.53 mmol) in acetone, anhydrous potassium carbonate (146 mg, 1 mmol) was stirred for 10 mins and then methyl iodide (43 mg, 0.3 mmol) was added drop-wise and the stirring was continued for 2 hours. The reaction was monitored by TLC. Then solvent was evaporated to dryness and subjected to column chromatography which yielded the compound RTK2.

(b) RTK3: 5-Ethoxy-2-methyl-1,4-naphthoquinone

Ethyl iodide (55 mg, 0.35 mmol) was added drop wise to the solution mixture of RTK1 (100 mg, 0.53 mmol) and anhydrous potassium carbonate (146 mg, 1 mmol) in acetone for 20 mins and the reaction mixture was stirred for 2 hours. The solvent was evaporated to dryness and subjected to CC to yield RTK3.

(c) RTK4: 5-Isopropoxy-2-methyl-1,4-naphthoquinone

The mixture of RTK1 (100 mg, 0.53 mmol) and anhydrous potassium carbonate (146 mg, 1 mmol) was stirred for 10 mins in a two necked round bottom flask. Isopropyl iodide (63 mg, 0.37 mmol) was then added drop wise and stirring was continued for another two hours. The solvent was evaporated under reduced pressure and subjected to CC to yield compound RTK4.

(d) RTK5: 6-Methyl-5,8-dioxo-5,8-dihydronaphthalen-1-yl acetate

A solution of RTK1 (100 mg, 0.53 mmol) in dichloromethane was added to a cooled solution of triethylamine (148 mg, 1.4 mmol) in dichloromethane. The reaction mixture was stirred for 30 mins in 0-5^{o}C and then acetyl chloride (56 mg, 0.71 mmol) was slowly added into the reaction mixture for a period of 30 min. After stirring at 0-5^{o}C for 6 hours, the temperature was slowly increased to 20-25^{o}C and again stirred for 4 hours. The solvent was evaporated to dryness and purified by CC resulting in compound RTK5.

(e) RTK6: 6-Methyl-5,8-dioxo-5,8-dihydronaphthalen-1-yl methanesulfonate

RTK1 (100 mg, 0.53 mmol) was dissolved in dichloromethane (10 ml). Triethylamine (148 mg, 1.4 mmol) was added and the reaction mixture was cooled to 0-5 ^{o}C. Methyl sulfonyl

chloride was slowly added (61 mg, 0.538 mmol) in dichloromethane. The reaction mixture was stirred for four hours at 0-5^{o}C, the temperature was raised slowly to 20-25°C and stirred for 8-10 hours. The resulting reaction mixture was isolated and subjected to CC to yield RTK6.

(f) RTK7: 2-Methyl-5-(2-piperidin-1-ylethoxy)-1,4-naphthoquinone

RTK1 solution (100 mg, 0.53 mmol) in ethanol (20 ml), potassium hydroxide (59 mg, 1 mmol) was taken in two necked round bottom flask, stirred for 10 mins followed by addition of 1-(2-chloroethyl)piperidine hydrochloride (117 mg, 0.63 mmol) and the reaction mixture was heated upto 60^{o}C. After 2 hours, the reaction mixture was cooled to room temperature, poured into water, acidified with dilute HCl and extracted with ethyl acetate. The extract was evaporated to get residue, which was subjected to CC to get RTK7.

(g) RTK8: 2-Methyl-5-(2-morpholin-4-ylethoxy)-1,4-naphthoquinone

4-(2-chloroethyl)morpholine hydrochloride (118 mg, 0.63 mmol) was added into the reaction mixture of RTK1 (100 mg, 0.53 mmol) and potassium hydroxide (59 mg, 1 mmol) in ethanol. The reaction mixture was heated up to 60^{0}C over a period of 90 mins followed by cooling. The mixture was poured into ice cold water and acidified with dilute HCl to pH 7.0 and extracted with ethyl acetate. The extract was evaporated to get residue and subjected to CC to yield RTK8.

(h) RTK9: Ethyl [(6-methyl-5,8-dioxo-5,8-dihydronaphthalen-1-yl)oxy]acetate

The solution of RTK1 in acetone (100 mg, 0.53 mmol) and potassium carbonate (146mg, 1 mmol) were stirred in room temperature for 10 mins followed by addition of ethyl bromoacetate (76 mg, 0.46 mmol) and the temperature was slowly increased upto 50^{0}C. The reaction mixture was heated for 2 hours and poured into ice and acidified with dilute HCl (to pH 7) and then extracted with ethyl acetate. The extract was evaporated to get residue, which was subjected to CC to get RTK9.

(i) RTK10: 5-[2-(Dimethylamino)ethoxy]-2-methyl-1,4-naphthoquinone

RTK1 solution (100 mg, 0.53 mmol) in ethanol and potassium hydroxide (59 mg, 1 mmol) were taken in two necked round bottom flask, stirred for 10 mins followed by addition of 2-chloro-N,N-dimethylethanamine (92 mg, 0.63 mmol) and the reaction mixture was heated up to 60^{o}C. After 2 hours, the reaction mixture was cooled to room temperature, poured in

water and extracted with ethyl acetate. The extract was evaporated to get residue, which was subjected to CC to get compound RTK10.

CC- represents column chromatography

2.10. Miscellaneous:

2.10.1. FACS analysis of PI stained cells:

Briefly cells were harvested by mild trypsinization (0.25%) followed by centrifugation at 2000 rpm for 10 mins at 4ºC. Cells were washed with cold PBS by centrifugation at 2000 rpm for 10 mins at 4ºC. Cells were fixed in cold 70% ethanol which was added dropwise along with mild vortexing. Samples were left for 12 hrs, after which ethanol was removed followed by two washes in cold PBS. RNase (100 µg/ml) treatment was subsequently given at 37ºC/30 mins to ensure only DNA staining. 50 µg/ml Propidium Iodide was added for staining. Cells were sorted and analyzed by flow cytometry for the cell cycle distribution using inbuilt software of BD FACScalibur instrument. Analysis was done in FL2 channel.

2.10.2. MTT cytotoxicity assay:

HeLa cells (5000 cells) were seeded in 96 well plate. The cells were treated with the inhibitor for 24 hours. 5 hours prior to completion of the incubation, 20 µl MTT (5mg/ml stock) was added to the culture media and incubated at 37ºC for 5 hours. The media was aspirated with the help of a needle and 200 µl of DMSO was added to solubilize the crystals. After mixing by pipetting, the cells were incubated at 37ºC for 5 mins and the absorbance was recorded at 540 nm in an ELISA reader (VERSA Max microplate reader, Molecular Devices). The values were normalized with the untreated control and plotted. Error bar indicates the standard deviation.

2.10.3. Primer information:

1) Primer sequence of p53 RE1 on *p21* promoter:

Forward primer: 5’ CAGGCTGTGGCTCTGATTGG 3’

Reverse primer: 5’ TTCAGAGTAACAGGCAAGG 3’

2) Primer sequence of *p21* gene:

Forward primer: 5' CATGCCATGGGCTCAGAACCGGCTGGGGATG 3'

Reverse primer: 5' CCGCTCGAGGGGCTTCCTCTTGGAGAAG 3'

3) Primers used for gene expression validation:

MLLT1 Forward primer, 5' TCCGCGGCCCCGAGCAATGTGAC 3',
Reverse primer, 5' CGATGGGCATGATGAAGCCAG 3',

VEGF Forward primer, 5' CAAAATCACTGTGGATTTTGGAAAC 3',
Reverse primer, 5'GGTCACTCACTTTGCCCCTGTCGC 3',

DKKL1 Forward primer, 5' GCTCCTCTCTACCCTGGTGATC 3',
Reverse primer, 5' TCATGCCCCGAAGCAGGTTAC 3',

RPL35 Forward primer, 5' CGAGTCGTCCGGAAATCCATTG 3',
Reverse primer, 5' GCATGGCACGGTCTTCTTAG 3',

FOLR1 Forward primer, 5' ACTGAGCTTCTCAATGTCTGC 3',
Reverse primer, 5' GTAGGAAACATCCTTATGGG 3',

RPS13 Forward primer, 5' TTGCACAAGTACGTTTTGTGAC 3',
Reverse primer, 5' CCTTATCCTTTCTGTTCCTCTC, 3'

2.10.4. microRNA profiling assays

RNA isolation:

RNA isolation was performed as described in the general methods section by the TRIZOL method. RNA quality was determined by visual examination of agarose gels stained with ethidium bromide as well as nanodrop estimation to determine $A_{260}/_{280}$ ratio.

Northern blotting analysis:

a) Electrophoretic separation of RNA and transfer:

The RNA samples (40 μg) for the electrophoretic separation were prepared with equal volume of formamide and heated at 65ºC. The samples were then separated on 15% Urea PAGE (150 V) which was subjected to a pre-run with 0.5X TBE buffer. The samples were the transferred to Hybond N+ nylon membrane (Amersham), by a semi dry blotting apparatus using 0.5X TBE as transfer buffer for 1 hour at 20V. Prior to transfer, the gel was

thoroughly rinsed with water to remove urea and then equilibrated in the transfer buffer (0.5X TBE).

b) UV cross linking: After transfer, the gel was stained in ethidium bromide to visualize the equal loading and the membrane was allowed to dry and subjected to UV cross linking using a cross linker (Strategene) for 2 minutes with the auto crosslink option. The membrane was then stored at 4ºC till the oligo labeling procedure.

c) Labelling of oligonucleotide probe:

The oligonucleotide was reconstituted in water and used at a final concentration of 200 ng/µl for the end labeling procedure using polynucleotide kinase and γ^{32}P ATP in a kinase reaction at 37ºC for 30 minutes, followed by denaturation at 70ºC for 15 minutes. The labeled probe was then purified using Sephadex G-50 column. The probe labeling was verified by performing a scintillation count of 1 µl of the labeled reaction in a liquid scintillation counter (Wallac).

d) Hybridization:

The membrane was incubated in a prehybridization solution consisting of 5X SSC, 2X Denhardt's solution, 7% SDS, 0.2M sodium meta bisphosphate (pH 7.2), and herring sperm DNA, at 42ºC for 3 hours in a hybridization chamber. The labeled probe was then added to the solution and hybridization was allowed to proceed at 42ºC for 16 hours.

e) Post hybridization washes:

After hybridization the membrane was subjected to a low stringency wash at room temperature and a medium stringency wash at 42ºC using 2X SSC/1% SDS solution and 1X SSC/0.1% SDS solution respectively.

f) Visualization:

The membrane was dried and the hybridization was verified by examining the exposed film in phophorimager (BAS FUJIFILM)

Chapter 3

IDENTIFICATION OF A NOVEL MECHANISM OF ENZYME INHIBITION; ROLE OF A SPECIFIC INHIBITOR OF ARGININE METHYLTRANSFERASE CARM1/PRMT4

This chapter describes the identification and characterization of a specific inhibitor of arginine methyltransferase CARM1, The inhibitor, ellagic acid (TBBD) isolated from pomegranate crude extract possesses a novel mechanism of enzyme inhibition wherein the inhibitor binds to the enzyme-substrate complex and this interaction is determined by the substrate sequence thereby leading to residue specific inhibition. The chapter details the various biochemical and biophysical approaches used to establish this novel mechanism of enzyme inhibition.

Chapter outline:

3.1. Co-activator associated arginine methyltransferase (CARM1/PRMT4)

3.1.1. CARM1, an arginine methyltransferase:

Co-activator associated arginine methyltransferase CARM1/PRMT4 is a type I arginine methyltransferase (Schurter et al., 2001) that catalyzes the formation of monomethyl and asymmetric dimethyl arginine residues of histones and nonhistone proteins. It is different from the other known PRMTs since it does not recognize the Glycine-Arginine rich (GAR) motifs (Kim et al., 2004) which is the consensus sequence for the other PRMTs. However, the known CARM1 substrates indicate the possibility of alanine and proline residues in the vicinity of the arginine residue as probable recognition sequences. The CARM1 substrates suggest that the proline in ± 1 position or within five residues (Kim et al., 2004) could serve as a recognition site, whereas there are also evidences which suggest that the alanine residue at the -2 position as a consensus residue (Dagger et al., 2002). However, there is no unambiguous consensus motif reported so far. The substrates of CARM1 are fewer than PRMT1 (another important arginine methyltransferase). The CARM1 substrates are broadly classified into two types: (1) those associated with chromatin such as histone H3 (Schurter et al., 2002), acetyltransferase p300/CBP (Chevillard-Briet et al., 2002), the steroid receptor family member SRC3 (Feng et al., 2006) and (2) those associated with RNA binding activities like PABP1 (Lee and Bedford, 2002), HuR (Li et al., 2002), CA150, SmB, SAP49, U1C (Cheng et al., 2007). Another class of CARM1 substrates whose representative numbers are increasing steadily are transcription factors and molecules involved in development such as TARPP (Kim et al., 2004), MEF2 (Chen et al., 2002) and Sox9 (Ito et al., 2009). The histone methyltransferase activity of CARM1 was found to be primarily on histone H3R17 and R26 (Bauer et al., 2002). H3R2 and few other *in vitro* modification sites have also been identified. Recombinant CARM1 preferentially methylates free histones. Apparently in the *in vivo* condition, CARM1 is found as a component of the NUMAC complex (Xu et al., 2004) which methylates nucleosomal histones. This alteration is concomitant with an increased interaction of CARM1 with one of the Swi/Snf components, BRG1 which is a part of the NUMAC complex. Presumably, the histone methylation might be an event associated with the functional interaction of CARM1 and the Swi/Snf remodeling machinery. Although, the NUMAC complex has not been well characterized, it is possible that the co-operative role of Swi/Snf and CARM1 could be a

signal or tissue specific event as in the case of muscle differentiation. In the case of most of the acetyltransferases, one of the major regulatory role is mediated by autoactivation, i.e. autoacetylation. Although, there is no direct evidence of automethylation of CARM1 modulating its activity, a mass spectrometric approach identified automethylation in CARM1 (Kuhn et al., 2009), which has led to the speculation of an additional level of regulating the methyltransferase activity of CARM1. CARM1 mediated histone methylation exemplifies a histone modification crosstalk since p300/CBP mediated acetylation of H3K18 (Daujat et al., 2002, An et al., 2004) is considered a pre-requisite for methylation by CARM1 at H3R17. Yet another intriguing and interesting observation is related to the H3K4 point mutant which is defective for CARM1 methylation implicating a crosstalk of H3K4 methylation and H3R17 methylation (Lee et al., 2004). However, this observation has not been further validated and needs further investigation.

3.1.2. CARM1, a transcriptional co-activator:

Inspite of such enormous functional significance as an arginine methyltransferase, CARM1 is also a well established transcriptional co-activator. In fact, it was initially identified as a p160 interacting protein through a yeast two hybrid. CARM1 is a secondary co-activator and much of its co-activation property has been attributed to its interaction ability with the other known primary transcriptional co-activators such as GRIP1. It binds to the AD2 activation domain and thereby leads to co-activation on several nuclear receptors ranging from ER, AR, TR etc (reviewed in Bedford and Clarke, 2009). Initially, it was considered to be a specific co-activator of nuclear receptors but recently, it has been shown to function as a co-activator of non-nuclear receptor pathways including NF-κB (Covic et al., 2005; Miao et al., 2006), p53 (An et al., 2004), IFN-γ (Zika et al., 2005), MEF2C (Chen et al., 2002) as well as β-catenin (Koh et al., 2002). Each of these co-activation events have been shown to be distinctly different. In the case of nuclear receptor associated co-activation event, it was clear that the methyltransferase activity was not essential but not completely insignificant. The absence of the methyltransferase domain led to a decrease in the overall co-activation property and the interaction domain was found to be absolutely essential for its co-activation ability (Teyssier et al., 2002). The β-catenin associated co-activation ability of CARM1 is similar to the nuclear receptor transcriptional activation (Koh et al., 2002). β-catenin is not only involved in normal cellular functions through Wnt signaling but is also a

co-activator of androgen receptor associated signaling pathway (Chesire et al., 2002). Thus, CARM1 mediated co-activation might be one of the critical factors influencing the switch between normal developmental pathway and the disease associated pathway. Yet another AR associated co-activation by CARM1 has been reported with SRCAP (SNF-2 related-CBP activator protein) (Monroy et al., 2003), a co-activator which requires the CBP-GRIP1-CARM1 complex for the transcriptional activation. The p53 dependent DNA damage pathway has been shown to be greatly influenced by other co-activators such as p300/CBP, PRMT1 and CARM1. A sequential cooperative effect of these different co-activators has been demonstrated on the p53 responsive gene promoter *GADD45* (An et al., 2004).

Thus, the mechanism of co-activation involves the primary co-activator (GRIP1, β-catenin) which is recruited to the promoter followed by the recruitment of secondary co-activators (p300, CARM1) which by virtue of interaction and/or enzymatic activity regulate the transcriptional output. However, in the case of the transcription factor NF-κB, CARM1 seems to recruit p65 to the responsive promoters since, the CARM1 knockout MEFs were deficient in p65 recruitment to MIP-2 and IP-10 promoters. But, the IL-6 promoter showed concomitant recruitment of CARM1 and p65 thus, implicating a differential mechanism of co-activation at different promoters (Covic et al., 2005). Most of these transcriptional co-activation results were obtained from transfection experiments wherein the physiological concentrations were greatly exceeded. A titration of different concentrations revealed a dose dependent increase resulting in a saturation effect at higher concentrations. On the other hand, the actual concentrations within the cellular system are very low, hence the essential role of these co-activators in a physiological condition is mostly at minimal concentrations but with maximum transcriptional output.

3.1.3. Structural features of CARM1:

CARM1 is a 608 amino acid-protein with distinct functional and structural domains as depicted in **Figure 3.1.** The enzymatic activity of the protein is regulated by three domains: the two substrate binding domains (the SAM or AdoMet binding domain (145-250 aa) and the dimer interface (155-230, 313-332)) and the arginine binding pocket (258-267 aa). There is a dimer interface from 165-230, 313-332 amino acids. Apart from this domain organization, the enzyme also has been structurally demarcated into the following domains: the C-terminal activation domain (500-608 aa), the GRIP1 binding domain (120-460 aa), the

homo-oligomerization domain (120-460 aa), the methyltransferase domain (120-500 aa), and the co-activator domain, which is the full length protein (1-608 aa)., (Teyssier et al., 2002).

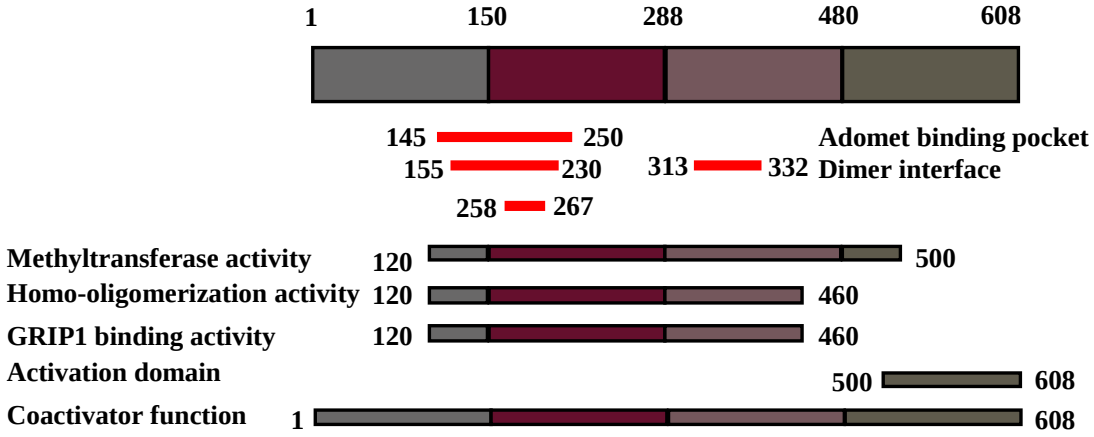


***Figure 3.1*. Structural functional domains of CARM1/PRMT4.** *Schematic diagram showing the structural and fuctional domain organization of CARM1/PRMT4: 1-608 represents the amino acids in the protein. Structurally, the protein has three distinct regions: the AdoMet binding pocket (145-250), the dimer interface (155-230, 313-332) and the arginine binding pocket (258-267). The functional domains are, as indicated, the methyltransferase domain (120-500), the homo-oligomerization domain (120-460), the GRIP1 binding domain (120-460), the activation domain (500-608) and the full length protein (1-500), which is required for the co-activator function.*

The elucidation of structural details of CARM1 has been attempted by two independent approaches. The first attempt was the Surface Enhanced Raman Spectroscopic method which provided insights into the secondary structural details of the human CARM1 (Kumar et al., 2007). However, the crystallographic data of mouse CARM1 reported subsequently provided information about the structural as well as the enzyme mechanism.

3.1.3.1. SERS studies of CARM1:

The spectroscopic techniques such as Raman spectroscopy act as a complimentary technique to the conventional X-ray crystallography for studying protein structures. Since the raman signal intensity has been a major hurdle and in an attempt to improve the signal intensity, we resorted to Surface Enhanced Raman Spectroscopy. Upon adsorption of molecules on coinage metallic nanosurfaces exhibiting atomic scale roughness, the Raman signal intensity of the molecule is enhanced by several orders of magnitude.

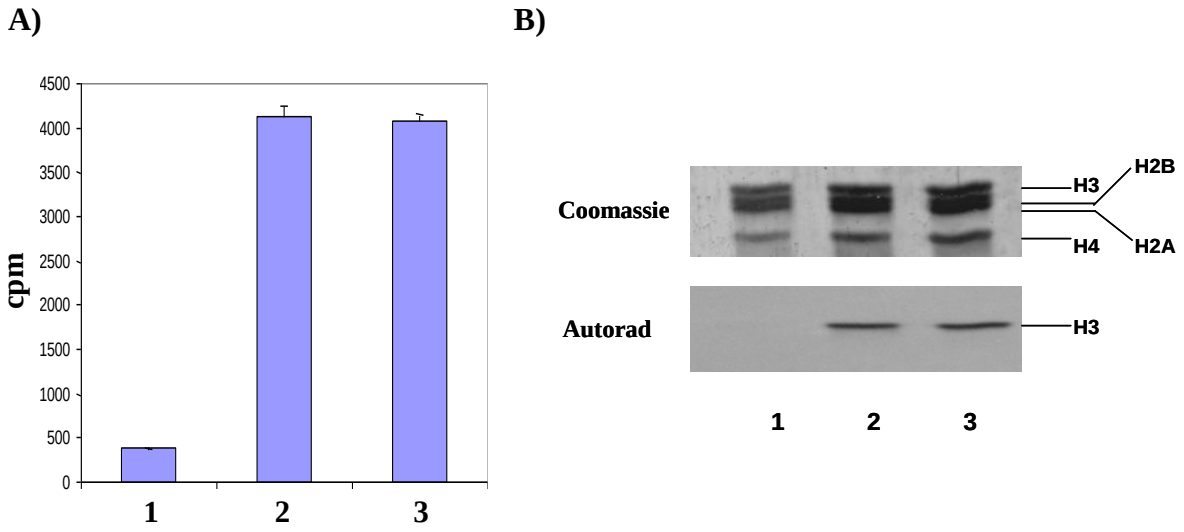


Figure 3.2. Effect of silver nanoparticles on CARM1 activity. (A) *Filterbinding Assay: HMTase assay was performed with CARM1 (20 ng) by using highly purified HeLa core histones (600 ng). Lane 1, core histones without enzyme; lane 2, histones with enzyme; lane 3, histones with CARM1 incubated with silver nanoparticles (1:5). The results are the mean values with the error bars (SD) of replicate reactions. (B) Fluorography: HMTase assay was performed with CARM1 (20 ng) by using highly purified HeLa core histones (3 μg). Lane 1, core histones without enzyme; lane 2, histones with enzyme; lane 3, histones with CARM1 incubated with silver nanoparticles (1:5). The radiolabeled histones were resolved on 15% SDS-polyacrylamide gel and visualized by coomassie blue staining followed by fluorography and autoradiography.*

In the past two decades, SERS has emerged as an effective tool for ultratrace analysis as well as for biomolecular detection (Kneipp et al., 2002). Yet another advantage of SERS is the ability to probe the protein secondary structure in solution phase. The SERS spectra of CARM1 was obtained by adsorbing the protein onto silver nanoparticle. The activity of the enzyme was unaffected by this adsorption as observed by filterbinding and fluorography gel assay (**Figure 3.2. A and B, lanes 2 and 3**).

Raman spectra of proteins are complex and is an average signal of the whole protein with contributions from various amino acids. However, in case of SERS, only specific modes of vibration are enhanced depending upon the orientation and distance of the molecule from the metallic surface. The dominant bands in SERS of proteins arise from aromatic amino acids like tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe) and histidine (His). This is due to a strong interaction between the π electrons in aromatic rings and the metallic surfaces (Podstawka et al., 2004).

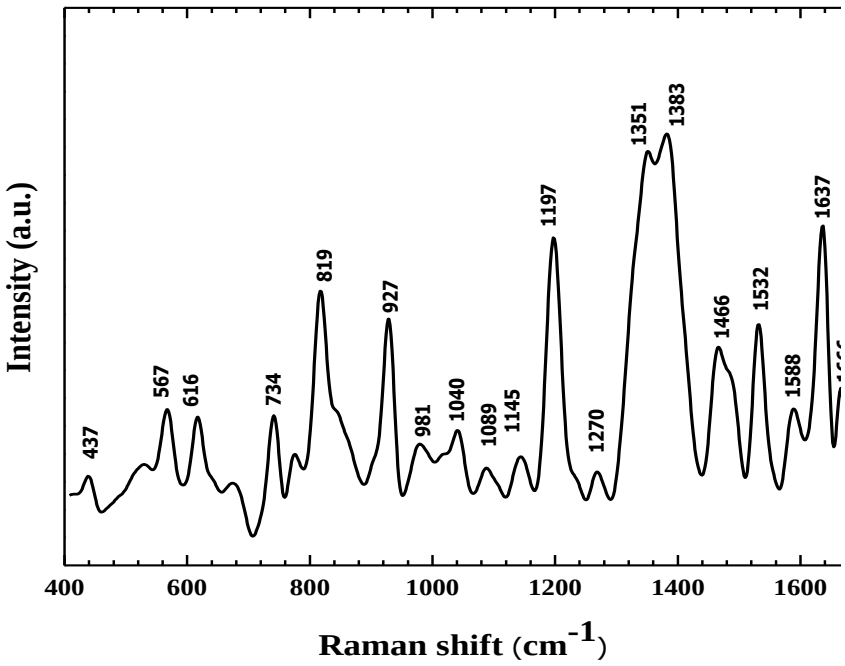


Figure 3.3. *SERS spectra of CARM1 protein. λ= 532 nm; signal accumulation time = 40 s.*

CARM1 has 56 aromatic amino acids (Tryptophan-5, Tyrosine-23, Phenylalanine-28). Therefore, the SERS of CARM1 was expected to be dominated by the aromatic amino acid modes. **Figure 3.3** shows the SERS of the CARM1 recorded in solution phase. The complete band assignment of the SERS spectra of CARM1 is shown in **Table 1** and some of the significant modes present in the SERS are discussed below.

SERS spectrum of CARM1 did show a number of modes arising due to aromatic amino acids (see **Table 1**). Significant contribution was observed from different substituted ring modes of Tyr (1588 cm-1, 1197 cm-1, 819 cm-1) Phe (1588 cm-1, 1197 cm-1, 616 cm-1) and Trp (1588 cm-1, 1351 cm-1). The amide bond vibrations are of three types, amide I vibration arising from C=O stretching with a small contribution from N–H bending. Amide II and amide III vibrations are due to NH2 scissoring and C-N stretching with N-H bending, respectively.

Table 1 - SERS band assignment of CARM1 protein

Raman shift (cm^{-1})	**Band assignment**
1666m	Amide I (random coil)
1637vs	Amide I (α helix)
1588m	Trp, Tyr and /or Phe (ν_{8a})
1532s	Amide II and/or Trp
1466s	$\delta(CH_2)$
1383vs	ν (COO-)
1351vs	Trp and/or δ (CH)
1270w	Amide III
1197vs	Tyr and/or Phe (ν_{9a})
1145w	ν_{as}(C α C N)
1089w	ν (C α N)
1040w	Gly
981w	Met
927s	ν(C-COO^-)
819s	Tyr and/or ν_{as}(C-S-C)
734m	Trp or His
616m	Phe (ν_{6b})
567m	Trp
437w	Pro

m-medium, vs- very strong, s-strong, w-weak; ν -symmetric stretch, ν_{as}- asymmetric stretch, δ - deformation

Generally, there is a one to one correspondence between the secondary structure of the protein and the amide vibrations. The normal, non-resonant Raman spectra of a protein exhibits amide I and amide III vibration, where as amide II vibration is generally a Raman inactive mode (Mayo et al., 2004). However, in SERS, the amide II vibration is also active. In CARM1, all the three amide vibrations were observed. Interestingly, two, distinguishable

amide I modes, which correspond to two different parts of the secondary structure of proteins were observed. At 1666 cm-1, we observe a band corresponding to random coil of the protein, and at 1637 cm-1, an intense band arises due to the α-helix. We observe a strong band at 1532 cm-1 and a weak band at 1270 cm-1, which was assigned to amide II and amide III vibrations, respectively. It should be noted here that amide II and III bands have overlap with bands arising from other amino acids. It is a common feature in SERS spectra of proteins to exhibit strong modes around 1380 cm^{-1} and 930 cm^{-1}, which corresponds to symmetric stretching of COO^- and stretching vibration of C–COO^- , respectively. These bands arise due to the adsorption of carboxylate groups pertaining to amino acids Asp, Glu, and/or C terminal groups. We observe asymmetric stretching mode of C□CN and symmetric stretching mode of C_αCN at 1145 cm^{-1} and 1089 cm^{-1}, respectively. The deformation modes of CH_2 and CH were observed at 1466 cm^{-1} and 1351 cm^{-1}, respectively. Both these modes have a strong overlap with vibrations of aromatic amino acids. However, all these secondary structural details are only complimentary to any structural detail obtained from the crystallographic data which is discussed in the subsequent section.

3.1.3.2. Crystallographic information of CARM1:

The functional differences in CARM1 with respect to other PRMTs is partly governed by the structural difference. Although, the core catalytic domain (methyltransferase domain) is conserved, the protein also has a unique N-terminal and C-terminal domain. The C-terminal domain has been attributed with the interaction and co-activation property. But structurally, not much is known about the N-terminal domain. The conserved methyltransferase domain is characterized by mixed α-helices and β-strands. The last β-strand has been shown to be important for the methyltransferase activity. Three highly conserved residues, valine-leucine-aspartate, or VLD have been identified as critical residues for the enzymatic activity. The VLD sequence has been mapped to the core methyltransferase domain and is considered essential for the formation of the tertiary structure and thereby the function of the enzyme. The methyltransferase domain exhibits distinct adomet binding pocket and substrate binding pocket. Hence, the arginine residue of the substrate has to bind in an acidic pocket composed of two glutamate residues. This binding is presumably through salt bridge formation between the guanidine group of the

nitrogen atoms of the substrate arginine residue. In CARM1, these conserved glutamate residues are located at amino acid positions 258 and 267. A mutation of the residue Glu-267 (**Figure 3.4.A**) to glutamine has no effect on the structure or the function of the protein This methyltransferase deficient CARM1 has been a widely used tool to understand the enzymatic role of CARM1 in biological systems.

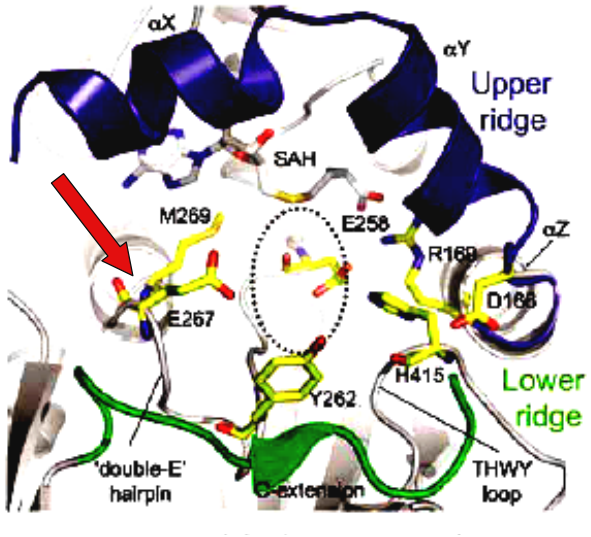
CARM1 also differs from other PRMTs in the dimerization arm. One of the unique features of this enzyme is the presence of the EVH1 domain of the superfamily 'pleckstrin homology domain' (PH domain) which is formed by two perpendicular antiparallel β-sheets followed by a C-terminal helix (Troffer-Charlier et al., 2007, Yue et al., 2007). These domains are shown to be important for recognition of proline rich sequences in different proteins. The observation that CARM1 substrates are characterized by the presence of proline residue in the vicinity of the arginine residue and since XXPRX is considered a probable consensus motif of CARM1, the presence of the PH domain implicates its possible role in substrate recognition. The PH-domain containing proteins are characterized by very low sequence similarity but high structural similarity which leads to functional differences ranging from signal transduction, cytoskeletal organization, nuclear transport to DNA repair (Blomberg et al, 1999; Ball et al, 2002; Lemmon et al, 2002; Gervais et al, 2004; She et al, 2004; Lemmon, 2007). The PH domain has been linked with multi-protein interactions characterized by low affinity yet high specificity which implicates that this could be a major determinant for the functional diversification of CARM1 unlike other PRMTs.

3.1.4. Biological functions associated with CARM1:

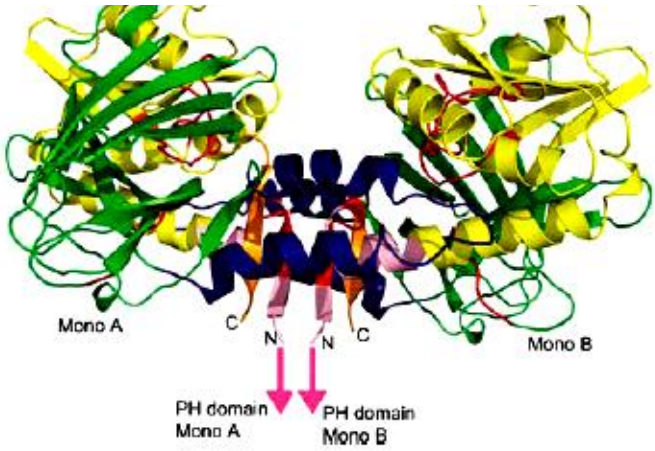
The functional relevance of CARM1 has been implicated in several biological processes from transcription to differentiation and development. The role of CARM1 in gene expression is signified at multiple levels. The transcription event is influenced by CARM1 both as a co-activator (nuclear receptor and non-nuclear receptor pathways) as well as a histone methyltransferase (increased H3R17 methylation at the above promoters). The arginine methylation of CBP/p300 at different regions with different functionalities is yet another mode of regulation at the transcription level. The SRC3 complex assembly (associated with transcriptional activation) and disassembly (resulting in transcriptional repression), which exhibits different transcriptional output based on the modified substrate is

an elegant mechanism indicating the importance of CARM1 as a co-activator as well as a methyltransferase.

A)



B)



Adapted from Troffer-Charlier et al., 2007

Figure 3.4. Structural details of CARM1. ***(A)*** *Conserved residues in the putative arginine pocket, the red arrow highlighting the E267 residue. The upper and lower ridges are coloured blue and green, respectively. This pocket can accommodate the side chain of the target arginine (position indicated by dotted circle).* ***(B)*** *Crystallographic CARM128–507 dimer indicating the putative global position of the PH domain. Monomer A of crystallographic CARM128–507 dimer and monomer A of noncrystallographic SAH-CARM1140–480 dimer have been superimposed and shown in the same orientation.*

The steroid receptor co-activator (SRC) complex is also regulated by acetylation. The recruitment of SRC3 leads to the recruitment of the secondary co-activators p300 and CARM1 which bring about the chromatin modifications (acetylation and H3R17 methylation) thereby leading to a transcriptionally active state. But, CARM1 also methylates SRC-3 and this methylation event has been shown to be responsible for the disassembly of the complex and transcriptional repression (Feng et al., 2006). Thus, the presence of CARM1 at the same promoter can lead to both transcriptional activation as well as repression based on the methylation substrate (H3 or SRC3).

The role of CARM1 is not just restricted to transcription but has also been shown to be essential for splicing (Tadesse et al., 2008) and RNA processing (Cheng et al., 2007). The fact that PABP1 is methylated by CARM1 indicates its essentiality in RNA processing. The process of transcription and downstream RNA processing are highly co-ordinated processes and there have been earlier indications of transcriptional co-activators being involved in transcription coupled splicing events (Lai et al., 1999, Monsalve et al., 2000, Dellaire et al., 2002). These events are linked to the RNA polymerase II C-terminal domain (CTD), since this is a scaffold for many RNA processing factors. CA150 is one such factor which is also methylated by CARM1. Evidences also suggest the significant role of CARM1 in RNA processing events such as splicing, exon skipping etc. The RNA binding protein HuR which is also a CARM1 substrate is an important RNA binding protein associated with stabilizing labile RNA. Thus, the role of CARM1 both as a co-activator and as a methyltransferase is evident at stages beginning from transcription to RNA processing and their stabilization.

Apart from its role in the transcription associated events, CARM1 has a well established role in differentiation and development. The knockout of CARM1 leads to perinatal death (Kim et al., 2004), indicating its role in the later stages of development. The expression pattern observed in mouse embryos, indicated the appearance of CARM1 from E8.5 in the anterior and posterior regions of the embryo. The transverse sections of these embryos revealed strong expression of CARM1 with a ubiquitous distribution throughout the neuroepithelium, surrounding mesenchyme, presumptive cardiac region, and first branchial arch. The E9.5 embryos showed cranial expression in the neuroepithelium around the otic vesicle, hindbrain and forebrain, and in the mesenchyme of the frontonasal region

and branchial arch which was later detected in other regions in a development associated pattern (Torres-Padilla et al., 2007). The elucidation of the exact role of the stage dependent appearance of CARM1 and its functional significance has not been attempted so far. However, a detailed study of CARM1 and its associated methyltransferase activity has been done in the mouse embryonic stem cells and this revealed the essential role of CARM1 and H3R17 and R26 methylation for pluripotency maintenance (Wu et al., 2009). The presence of CARM1 on the promoters of *Oct4* and *Nanog* seemed to resist differentiation and thus promote pluripotency. On the other hand, there have been evidences implicating CARM1 in promoting differentiation especially of the committed cell types. CARM1 acts as a co-activator of MEF2, facilitating myogenic differentiation (Cheng et al., 2007), which is entirely due to the co-activation property of CARM1 and not due to its enzymatic activity. A similar observation has also been highlighted for the role of CARM1 in adipocyte differentiation wherein it acts as a co-activator of PPARγ and thereby facilitates the adipogenesis (Yadav et al., 2008). But, the role of CARM1 in chondrocyte and thymocyte differentiation is due to its methyltransferase activity wherein the methylation of the substrates Sox2 (Ito et al., 2008) and TARPP (Kim et al., 2004) respectively, influences the process of differentiation. All these evidences indicate an important role of CARM1 in the different development associated pathways, but the contribution of the enzyme activity versus the co-activation activity towards these functions are still controversial (Jayne et al., 2009, Kim et al., 2010). Yet another intriguing observation is the need for CARM1 in two opposing events, pluripotency maintenance in undifferentiated cells as well as directing differentiation in committed cell types.

3.1.5. CARM1 and disease association:

There are very few evidences that link CARM1 with disease processes. The well established example is the role of CARM1 in the hormone dependent cancers. Prostate cancer (Hong et al., 2004) and breast cancer (Frietze et al., 2008) have been shown to be causally linked with increased CARM1 expression and increased methylation. Additionally, the co-activation ability also regulates the cancer manifestation. Surprisingly, although an increased recruitment of CARM1 was observed at ER promoter and enhancer concomitant with estrogen induction, the levels of H3R17 methylation did not show such a drastic

increase (Frietze et al., 2008). Furthermore, the promoter showed an increased methylation but the enhancer did not show such enhanced level indicating that CARM1 might have other regulatory roles at the enhancer and other regulatory elements. Another disease which has been shown to be indirectly regulated by CARM1 is Spinal Muscular Atrophy (SMA). CARM1 methylates the KH type splicing factor which regulates the splicing event leading to the generation of the functional Survival of Motor Neuron (SMN) gene (Tadesse et al., 2008). Any aberration in this splicing event is manifestated as the SMA disease.

3.1.6. Inhibitor based approach to understand the role of CARM1 in physiology and pathophysiology:

The different cellular processes regulated by CARM1 are also modulated by different signaling events as well as a multitude of protein complexes. Most importantly, these processes also exhibit tissue specificity. However, the question that remains to be answered is the exact contribution of the enzymatic versus the co-activation ability of CARM1 in the above described physiological processes. The use of the methyltransferase defective mutant E267Q was considered to be a useful method to understand the enzymatic contribution, and indeed it has helped in delineating the co-activation and the enzymatically regulated processes (Jayne et al., 2009). However, a recent study using a knock-out followed by a knock-in of the inactive mutant E267Q, identified most of the cellular functions (thymocyte development, interaction with CA150 etc.) to be regulated by the enzymatic activity (Kim et al., 2010). Since, the H3 arginine methylation constitutes only about 3-4% of the total modification and these modifications are very low represented modifications which exhibit a spatio-temporal abundance, the global analysis of such phenotypes might not be a right approach to understand the role of CARM1 methyltransferase. Rather tissue specific and signal specific investigations might help in understanding the role of the enzyme. The use of the inactive mutant not only abrogates its methylation ability but also decreases the co-activation ability. The complete knock-out compromises both of these abilities, hence warranting the need for a new approach to delineate these outcomes. Hence, it has become necessary to devise strategies to study these two effects in isolation. A very simple and efficient way to study the co-activation property is the use of peptides that can

block the protein interactions and the enzymatic contribution can be best addressed through a modulator (activator/inhibitor) based approach.

3.1.7. Arginine methylation inhibitors:

The field of arginine methylation inhibitors are relatively new with very few well-characterized inhibitors. It is indeed surprising that an active inhibitor (Sinefungin) was identified even before the enzymatic machineries were characterized (Amur et al., 1986). However, not much development has occurred in this area since then. There was an active search for arginine methylation inhibitors, but most of the identified molecules are substrate analogs of the methyl group donor Adomet, which incidentally is a common cofactor for DNA, Lysine and arginine methylation thereby leading to nonspecificity. An inhibitor AMI-1 (Cheng et al., 2004) was identified by a small molecule screen but was found to be specific to PRMT1 which was also true for RM-65. Recently, several *in vitro* studies related to CARM1 inhibitors have been reported. These have been designed based on few earlier non specific targets and various modeling associated data. These inhibitors are essentially the pyrazole inhibitors (Huynh et al., 2009), benzo(d)imidazole derivatives (Wan et al., 2009) as well as 1,2-diamine compounds (Therrien et al., 2009). All these reports are mostly *in vitro* studies with very less information about their *in vivo* targets and specificities in the physiological context.

3.2. Small molecule modulator selection

3.2.1. Strategy of small molecule selection:

There exist several strategies to select small molecule modulators of an enzyme. One of the most commonly used method is the homology modeling followed by lead optimization approach. In this case, the active site or important catalytic sites such as the substrate binding site is selected and followed by screening of substrate analogs or any known small molecule library for their interactions and this would give a list of the potential modulators which can be further biochemically validated. Another approach involves the synthetic route wherein previously reported inhibitors to similar enzymes could be derivatized and thereby new modulators could be generated. Yet another approach is the source of naturally available modulators from plant or animal sources which has actually

gained momentum recently. The latter strategy is currently been exploited successfully for the identification of several HAT inhibitors, anti cancer agents, kinase inhibitors, signaling disruptors etc. To use this methodology it was first necessary to define the target (i.e. pathophysiological condition associated with the enzyme CARM1) and then search for the possible modulator from natural source (natural cure or treatment components for the above pathophysiological conditions). Unlike, acetylation which has direct roles in cancer or inflammatory disorders, the role of arginine methylation in disease manifestation is very limited as per the available literature. However, two cancer conditions, prostate cancer and breast cancer have been shown to have an increased CARM1 level both in the cell line and patient sample level and there are indications that this might be contributing to cancer manifestation. Several natural sources have been tried against these cancers and the most effective has been pomegranate crude extract. In fact, several clinical trials were initiated for the therapaeutic potential of pomegranate against prostate cancer. With respect to breast cancer, there are multiple complexities due to the receptor type and status involved in the disease manifestation. Hence, it was possible that the crude extract of pomegranate might have an active component against CARM1.

3.2.2. Ellagic acid, TBBD:

The pomegranate crude extract comprises of several constituents (reviewed in Jurenka, 2008) as enlisted in **Table 2**. The screening process was initiated with the pomegranate fruit rind (pericarp) and this crude extract was initially tested for any chromatin modifying ability described in the next section. Since, this did show activity, the crude extract was further purified as described in the methods **section 2.8.1**. One of the major component that was isolated was ellagic acid, ((2,3,7,8-tetrahydroxy[1] benzopyrano [5,4,3-(di)] [1] benzopyran-5,10-dione) (TBBD).

Ellagic acid is also a well known anticancer compound shown to possess anti cancer effects on breast cancer, prostate cancer and ovarian cancer cell lines (McGovern et al., 2003, Losso et al., 2004). However, the exact molecular target for its anti cancer activity is not known. A single report indicated its effect on kinase (Cozza et al., 2006) with few structural informations as well as other prokaryotic enzymes. But, these investigations did not provide an answer for its anticancer property. Since, it is an antioxidant it might have

some pleotropic effects. However, the specificity of its anticancer activity towards the hormone dependent cancers such as breast and prostate cancer signify the role of some hormone dependent signaling events being perturbed and since CARM1 has been tightly linked with AR-dependent and ER-dependent transcriptional activation, TBBD seemed to be an attractive small molecule to investigate.

Table 2: Different components in pomegranate crude extracts

PLANT COMPONENT	CONSTITUENTS
Pomegranate juice	Anthocyanins, ascorbic acid, glucose, ellagica cid, gallic acid, caffeic acid, catechin, EGCG, quercetin
Pomegranate seed oil	Major component: Punicic acid , ellagic acid , fatty acids, sterols
Pomegranate pericarp (peel)	Phenolic punicalagins, Ellagitannins, gallic acid, catechin, EGCG, quercetin, flavones, flavanones
Pomegranate leaves	Tannins (punicalagin) and ellagic acid, flavones glycosides such as luteolin and apigenin

3.3. TBBD is a specific inhibitor of the arginine methyltransferase CARM1 in vitro:

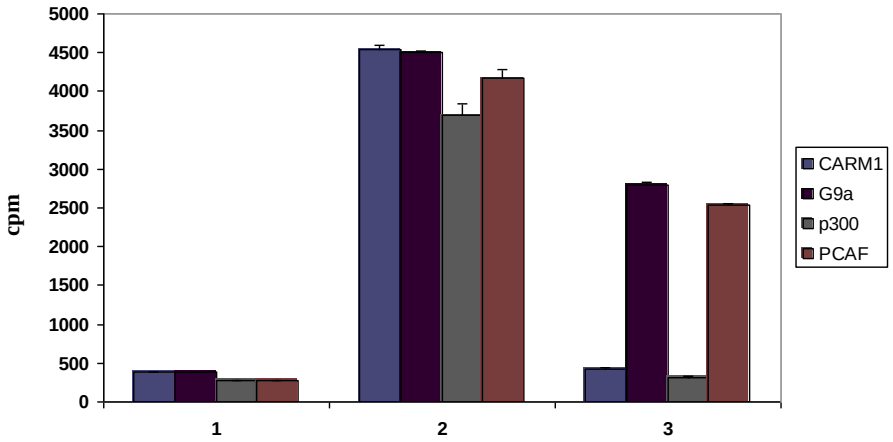
3.3.1. Crude extract screening:

Pomegranate (*Punica gratum*) extract was tested on the chromatin modifying enzymes in an *in vitro* assay. The crude extract of pomegranate fruit skin, was found to inhibit the histone arginine methyltransferase activity of CARM1/PRMT4 as well as the acetyltransferase activity of p300/KAT3B (**Figure 3.5.A, lane 2 vs lane 3**). Further purification of the active components from the fraction yielded a highly purified compound, TBBD (**Figure 3.5. B**).

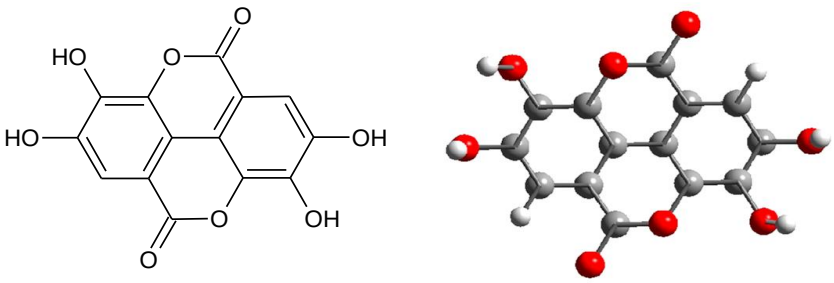
3.3.2. Purified component screening:

The purified component TBBD led to a dose dependent inhibition of CARM1 methyltransferase activity (**Figure 3.6.A, lanes 3-6**), as observed by a filterbinding assay. However, when a similar assay was performed with the histone acetyltransferase p300/KAT3B (which was inhibited by the crude extract), a minimal effect was observed even with increasing concentrations of TBBD (**Figure 3.6.A, lanes 3-6**). Furthermore, TBBD did not show any significant effect on the HMTase activity of lysine methyltransferase G9a, as well as on the HAT activity of PCAF (**Figure 3.6.A, lanes 3-6).**

A)



B)



$C_{14}H_6O_8$

Figure 3.5. Effect of pomegranate crude extract on histone modifying enzymes. (A) *Histone methyltransferase assays and histone acetyltransferase assays were performed as described in methods section, with the crude extract by using purified HeLa core histones as substrate and processed for filterbinding. Lane 1, Corehistones without enzyme; lane 2, histones with enzyme; lane 3, histones with enzyme in the presence of crude extract. Error bars represent the mean standard deviation of replicates.* ***(B)*** *Structure of TBBD. Structural formula representation (Panel I) and ball and stick model (Panel II).*

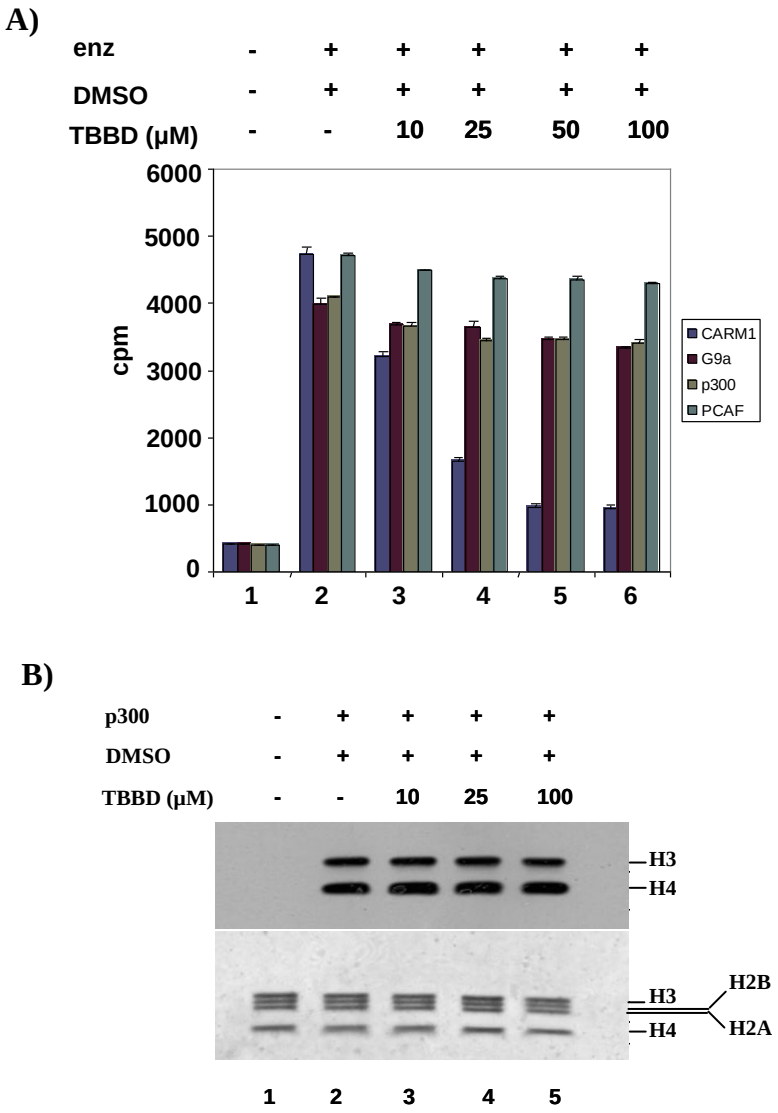


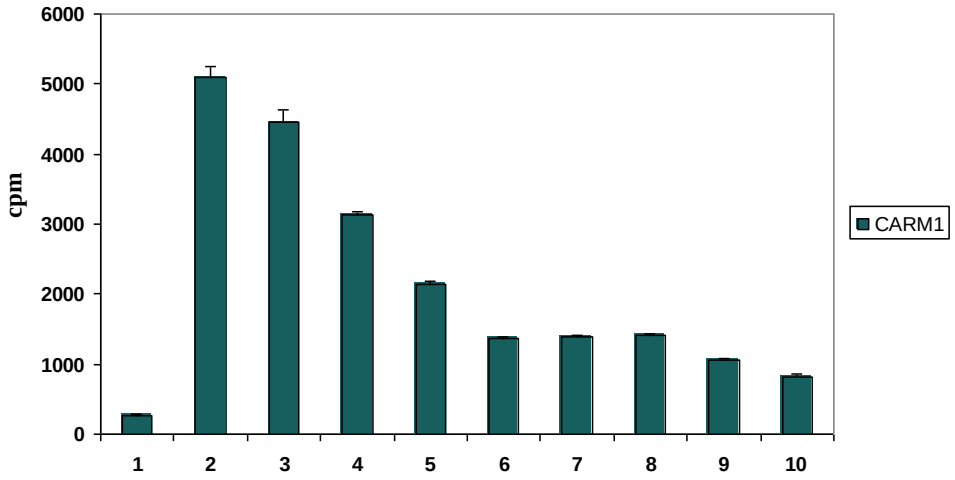
Figure 3.6. Effect of TBBD on histone modifying enzymes. *(A) Filter-binding assay for inhibition of histone modification. The HMTase assay was performed with CARM1 and G9a, and the HAT assay was performed with p300, PCAF in the presence or absence of TBBD by using highly purified HeLa core histones and processed for filter-binding assay. Lane 1, core histones without enzyme; lane 2, histones with enzyme in the presence of DMSO; lanes 3-6, histones with enzyme in the presence of 10 µM, 25 µM, 50µM or 100 µM TBBD. Error bars represent standard deviations of the means of duplicate reactions. (B) gel fluorography of the HAT assay was performed with p300 in the presence or absence of TBBD by using highly purified HeLa core histones and processed for filter-binding or fluorography assay. Lane 1, core histones without enzyme; lane 2, histones with enzyme in the presence of DMSO; lanes 3-5, histones with enzyme in the presence of 10 µM, 25 µM or 100 µM TBBD. The top panel is the autoradiogram and the bottom panel is the coomassie stained gel*

Although, the activity of all the four enzymes (CARM1, p300, G9a, PCAF) were normalized with the histone substrate (**Figure 3.6.A, lane 2**), the purified component, TBBD had significant inhibitory effect on the arginine methyltransferase CARM1 alone, indicating its specificity towards CARM1 in an *in vitro* reaction. The crude extract had shown potent inhibitory activity against the acetyltransferase p300, however the purified component had very minimal effect on the p300 activity. Hence, the data was further verified by performing a gel fluorography assay wherein there was a clear indication that this inhibitor had no effect on the p300 acetyltransferase activity (**Figure 3.6. B, lanes 3-5**).

3.3.3. TBBD is a CARM1 inhibitor:

The inhibitory effect of TBBD on CARM1 was found to be partial, even at an inhibitor concentration tenfold above the IC_{50} of 25 µM (**Figure 3.7.A, lanes 4-10**). All these initial screening was carried out with core histones. Since, CARM1 methylates only histone H3 among the core histones, we checked the inhibitory efficiency using bacterially expressed and purified recombinant histone H3. TBBD could inhibit CARM1-mediated methylation of recombinant histone H3 similar to its effect on CARM1 (**Figure 3.7.B, lanes 4-8).**

A)



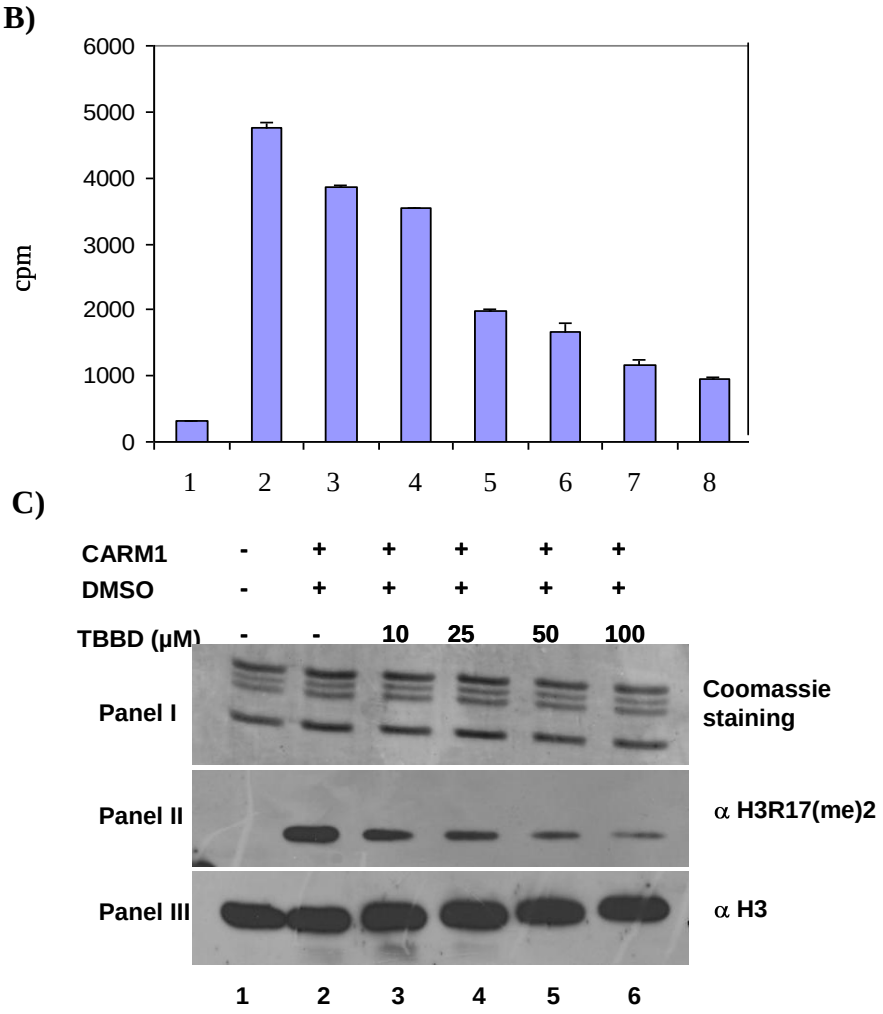


Figure 3.7. (A) *HMT assay was performed with CARM1 in the presence or absence of TBBD by using highly purified core histones as substrate and processed for filterbinding. Lane 1, Corehistones without CARM1; lane 2, histones with CARM1; lane 3, histones with CARM1 in the presence of DMSO; lanes 4- 10, histones with CARM1 in the presence of increasing concentration of TBBD (5μM, 10μM, 25μM, 50μM, 100μM, 200μM, 250μM). Error bars represent the mean standard deviation of replicates.* ***(B)*** *HMT assays were performed with CARM1 in the presence or absence of TBBD by using recombinant bacterially expressed histone H3 and processed for filterbinding. Lane 1, Histones without CARM1; lane 2, histones with CARM1; lane 3, histones with CARM1 in the presence of DMSO; lanes 4- 8, histones with CARM1 in the presence of increasing concentration of TBBD (5μM, 10μM, 25μM, 50μM, 100μM). Error bars represent the mean standard deviation of replicates.* ***(C)*** *HMTase assay with CARM1 using core histones as substrate in the presence or absence of TBBD, processed for immunoblotting analysis. Lane 1, core histones without enzyme; lane 2, histones with enzyme in the presence of DMSO; lanes 3-6, histones with enzyme in the presence of 10 μM, 25 μM, 50 μM or 100 μM TBBD. Panel I represents coomassie staining, Panel II represents immunoblotting with dimethylated H3R17 antibody. Panel III represents the histone loading control using histone H3 antibody.*

The specificity of the inhibitor was further confirmed by subjecting the *in vitro* reaction to immunoblotting with site specific histone H3R17 dimethylation antibody. This residue is one of the modification sites of CARM1 on histone H3 tail. A dose dependent inhibition of H3R17 methylation was observed with increasing concentration of TBBD (**Figure 3.7. C, lanes 3-6**). The same reaction was probed with antibody against histone H3, which indicated similar levels of histone H3 (**Figure 3.7.D, Panel III, lanes 3-6**), inspite of a drastic decrease in the H3R17 methylation levels. Thus, the small molecule TBBD, purified from pomegranate fruit skin crude extract, is a specific inhibitor of arginine methyltransferase CARM1 *in vitro*.

3.4. Characterization of the CARM1 inhibitor, TBBD

3.4.1. Kinetic characterization:

The characterization of the inhibitor with respect to its mechanism was essential to understand its interactions with the enzyme and the substrate either alone or as a complex. Hence, a detailed kinetic characterization of the inhibition was done using recombinant histone H3 as substrate. The methyltransferase reaction consists of two substrates, histone H3 and the methyl cofactor donor, S-adenosyl methionine (SAM). Hence, the kinetic analysis was done by performing two sets of reactions, wherein once histone H3 concentration was kept constant and SAM concentration was varied and vice versa for the second set. It was observed that TBBD-mediated inhibition of CARM1 activity led to a change in the Km as well as Vmax of both the reaction conditions (**Figure 3.8. A and B**).

Such a pattern of inhibition corresponds to the uncompetitive mode indicating that the inhibitor binds to the enzyme-substrate ES complex resulting in the formation of the ESI complex. This ternary complex results in a decrease in the velocity of the reaction and hence there is an alteration in the V_{max}. Since, there is an active sequestration of the ES complex by the inhibitor, the effective substrate concentration is also altered and hence there is a change in the Km value with increasing inhibitor concentration. Although the pattern of inhibition with increasing concentration of histone H3 (susbtrate) indicates a characteristic uncompetitive pattern. On the other hand, the inhibition with increasing concentration of the pseudo substrate Adomet (SAM) shows characteristics of both uncompetitive (at higher inhibitor concentration) and noncompetitive inhibition (at lower inhibitor concentration) indicating that this could be a mixed inhibition. On the other

hand, this could also be a technical limitation associated with a radioactivity based kinetic profiling.

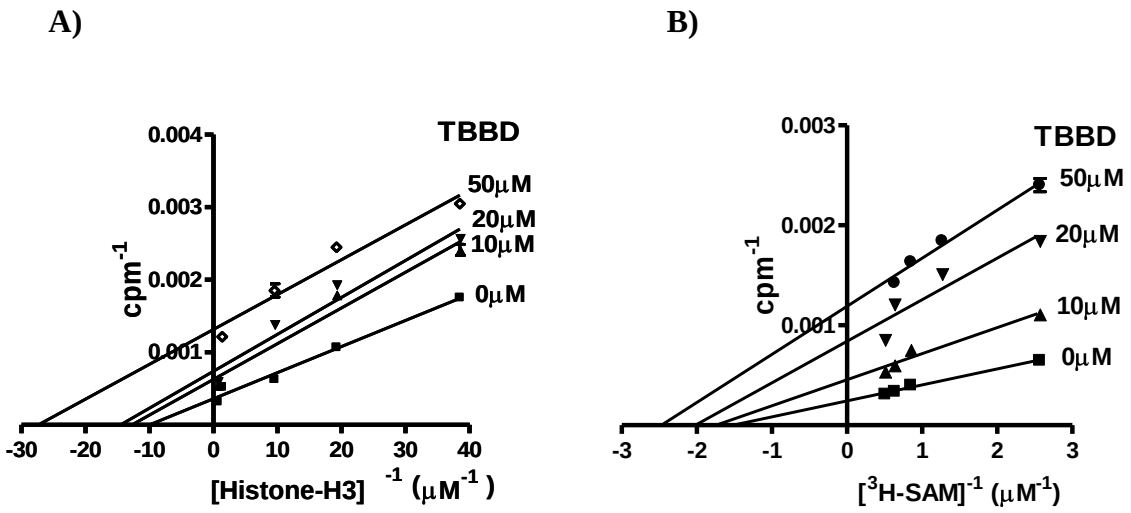


Figure 3.8. Lineweaver-Burk plot representation of TBBD's effect on CARM1 activity (A) *at a fixed concentration of (³H-SAM) (0.88 μM) and increasing concentrations of histone H3 in the presence (10, 20 or 50 μM) or absence of TBBD.* ***(B)*** *at a fixed concentration of histone H3 (1.467 μM) and increasing concentrations of (³H-SAM) in the presence (10, 20 or 50 μM) or absence of TBBD. The results were plotted using GraphPad Prism software.*

3.4.2. Biophysical characterization:

The kinetic characterization suggested the involvement of a functional interaction between TBBD and the enzyme–substrate (ES) complex in the process of inhibition. To understand the affinity of the inhibitor towards the two components of the ES complex, we investigated the nature of the interactions of TBBD with CARM1 and histone H3 by employing isothermal calorimetry (ITC) studies. The ITC measurements not only provide the thermodynamic parameters of a system but also provide an indication of the binding affinity. Any interaction results in a heat change which could be either absorbed or released. These heat changes are negated by the isothermal system to maintain the constant temperature and this energy spent by the system provides the thermodynamic details of the system. Titration of the ligand (TBBD) with histone H3 showed a significant heat change, as expected (**Figure 3.9.A).** The heat change due to the buffer and buffer–ligand contribution was appropriately subtracted. The resultant heat change could be fitted to a single binding

site (n = 1.43 ± 0.0372) which is enthalpy driven ((ΔH = -18.3 kcal/mole) and **Table 3**). Interestingly, the ITC studies showed very minimal interaction between TBBD and the enzyme CARM1 (**Figure 3.9.B).**

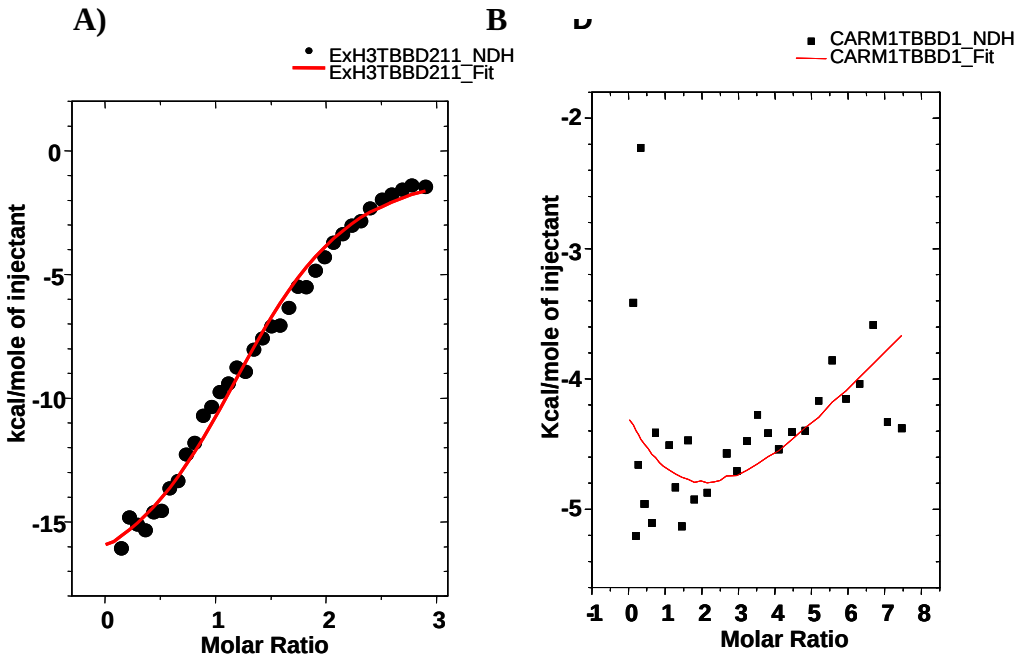


Figure 3.9. Isothermal calorimetric (ITC) titration (A) *was carried out by titrating histone H3 (7 µM) against the ligand TBBD (140 µM) at 25°C. The one-site binding model of the isotherm is shown.* ***(B)*** *was carried out by titrating CARM1 (7 µM) against the ligand TBBD (140 µM) at 25°C. The dots represent the heat change associated with the titration. The fit represents the curve fitting done by the software.*

It was essential to study the interaction of the inhibitor with the ES complex as a whole but this would lead to complex conditions since, the enzyme and substrate interaction itself would result in some heat changes and when there are two proteins together being titrated against a single ligand, it might be difficult to attribute the heat change to any specific interaction. However, the need for the ES complex for inhibition is evident from the kinetic characterization which was further validated by biochemical methods as discussed in the later part of this chapter. Together, the biophysical and kinetic data suggest that the partial inhibition of CARM1 by TBBD is mediated through its interaction with the ES complex, and predominantly through the substrate, histone H3 at a single site.

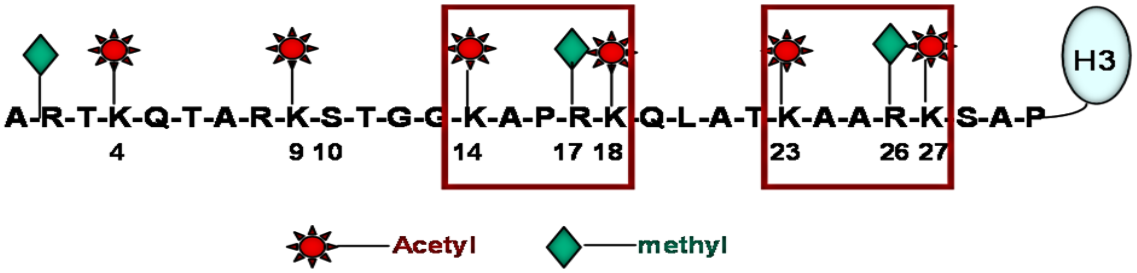
3.5. TBBD is a site specific inhibitor of CARM1

CARM1 can methylate histone H3 at three sites: R2, R17 and R26. Though R2 has so far been reported as an *in vitro* site of methylation by CARM1, the enzyme has been shown to methylate both R17 and R26 (**Figure 3.10. A**) *in vivo* with several functional consequences. TBBD-mediated inhibition of H3 methylation by CARM1 was found to be partial even at high concentrations of TBBD, and because the ITC results suggest that one site on histone H3 is favored for TBBD binding, we hypothesized that TBBD could be a site-specific inhibitor of CARM1. To investigate this phenomenon in the physiological condition, HeLa cells were treated with TBBD for 24 hours and the H3R17 methylation and H3R26 methylation status was examined by immunofluorescence analysis which indicated the site specific inhibition.

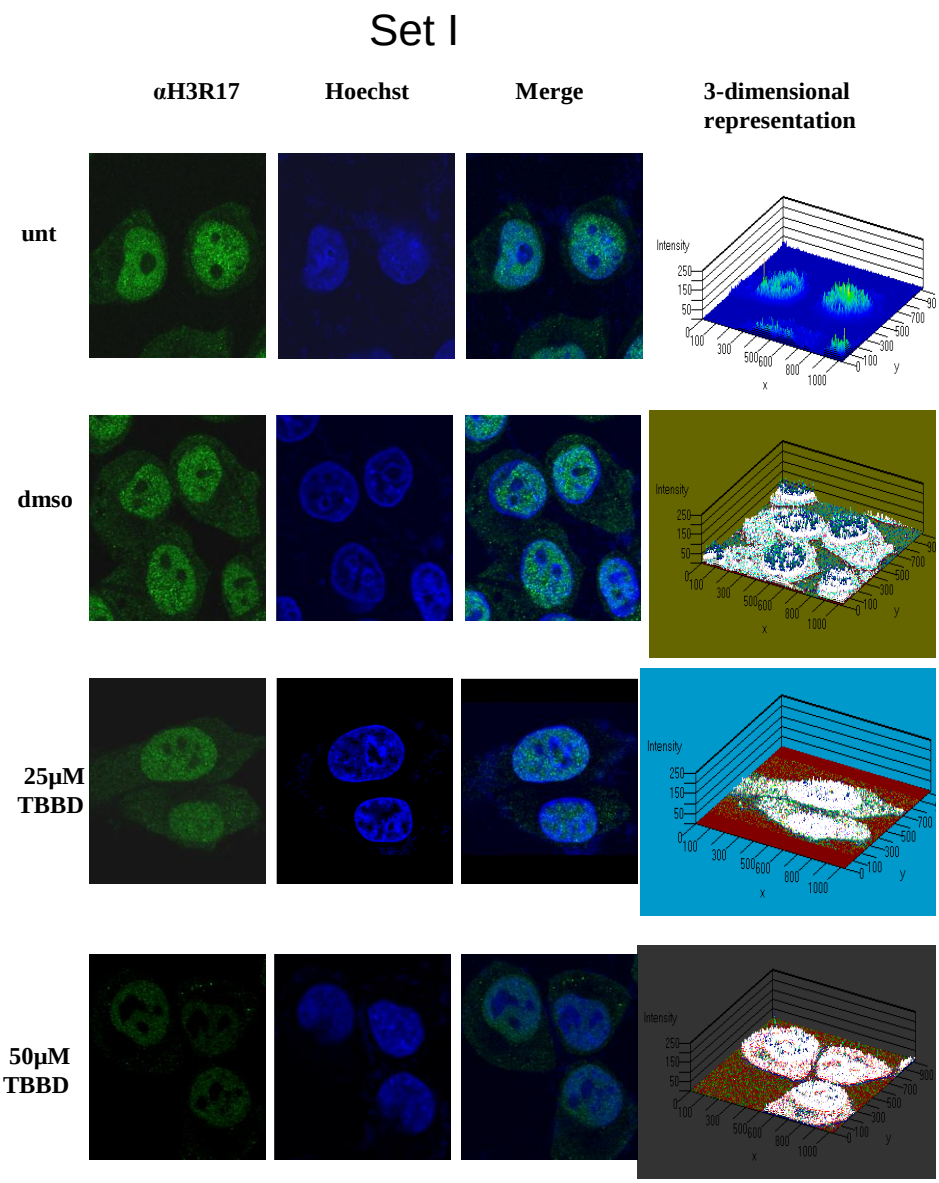
Since, the expression of CARM1 is reported to be high in breast cancer cell lines, we decided to check the effect of TBBD on cell lines exhibiting normal methylation levels and hence, HeLa cell line was used for this purpose. The solvent control seemed to increase the modification status slightly over the untreated. However, the subsequent inhibitor treatments show a dose dependent decrease for R17 methylation (**Figure 3.10.B**) with minimal effect on R26 methylation (**Figure 3.10.C)**.

Although, the intensity profiles of the immunofluorescence images exhibits a difference in the two antibodies used, the comparison is essentially across different concentrations of the same antibody and not across antibodies. These differences have been normalized in the immunoblotting analysis discussed subsequently. The acid-extracted histones from the cells were probed with specific antibodies against methylated H3R17 and H3R26. Significantly, it was observed that methylation of the H3R17 residue was inhibited by TBBD in a dose dependent manner (**Figure 3.11.A, panel I, lanes 3-5).** The lower concentration of 5 µM led to more than 50% reduction of H3R17 methylation. As the dose increased to 25 µM, a drastic reduction of almost 80% was observed.

A)


A-R-T-K-Q-T-A-R-K-S-T-G-G-K-A-P-R-K-Q-L-A-T-K-A-A-R-K-S-A-P
H3
4
9 10
14
17 18
23
26 27
Acetyl
methyl

B)


Set I
αH3R17
Hoechst
Merge
3-dimensional representation
unt
dmso
25µM TBBD
50µM TBBD
Intensity
x
y

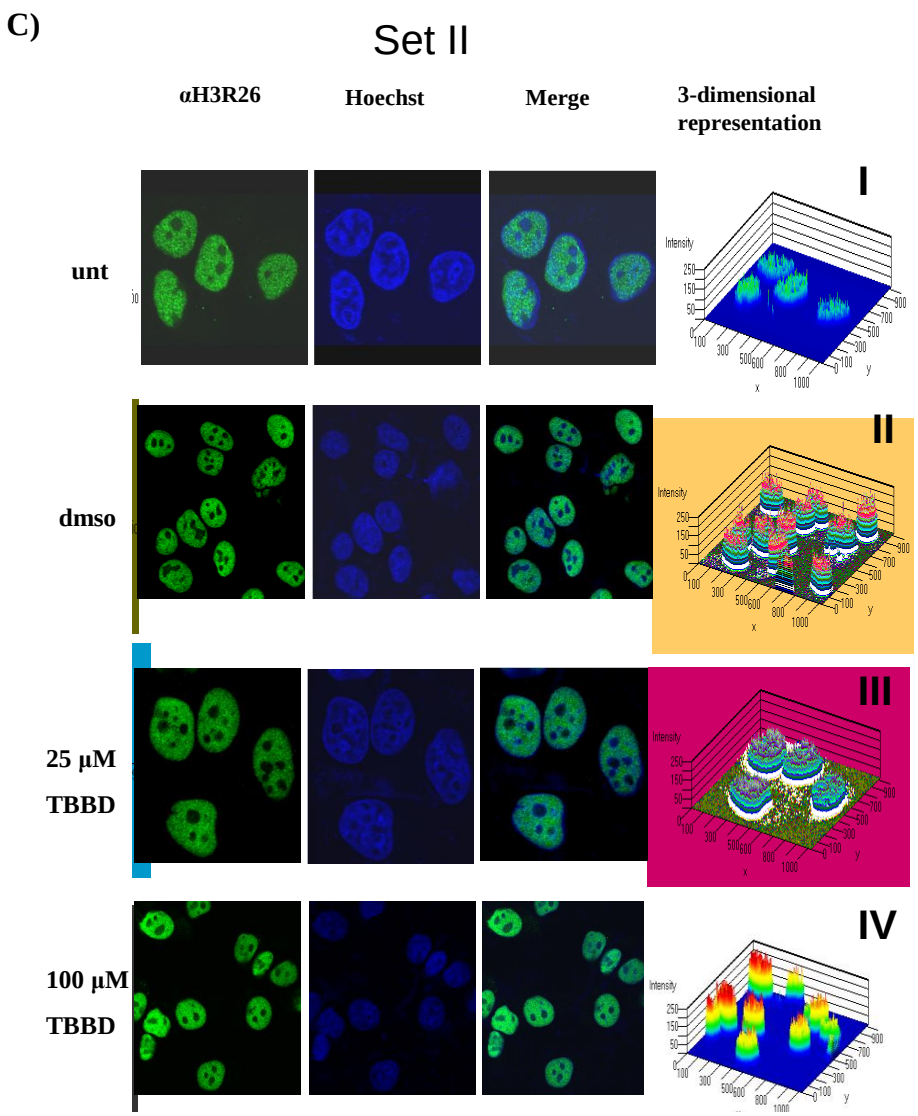


Figure 3.10. TBBD is a site-specific inhibitor of CARM1. ***(A)*** *The possible sites of acetylation and arginine methylation on histone H3 tail sequence are indicated. The immunofluorescence data represented has been done on HeLa cells treated with TBBD. The cells were immunostained with the specific antibodies, dimethylated H3R17* ***(B)*** *and dimethylated H3R26* ***(C).*** *The blue staining represents the Hoechst stained DNA and the three- dimensional representation of the fluorescence intensity arising from cells across different concentrations depicts the specific inhibition.*

At the higher concentration of 100 µM, there was about 95% reduction in the H3R17 methylation levels, whereas, H3R26 methylation was not affected even with 100 µM TBBD **(Figure 3.11.A, panel II, lanes 3-5).** Closer examination of the histone H3 tail sequence revealed a characteristic pentapeptide consensus sequence, KAXRK, at the two CARM1 methylation sites, H3R17 and H3R26 (**Figure 3.10.A**).

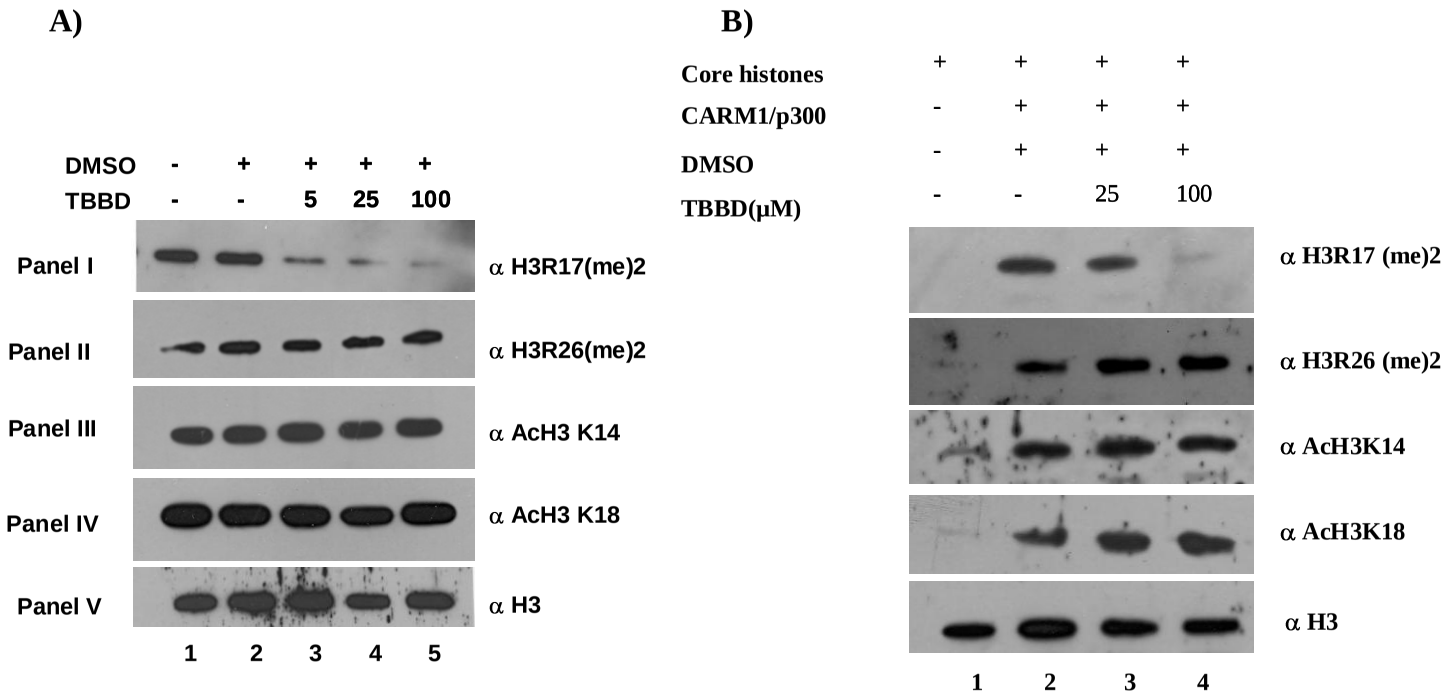


Figure 3.11. ***(A)*** *HeLa cells were treated as indicated for 24 h; histones isolated from untreated cells (lane 1); DMSO-treated cells (lane 2); TBBD-treated cells (lanes 3-5). Histone modifications were probed by western blotting using the indicated site specific antibodies.* ***(B)*** *Histone acetyltransferase and methyltransferase assays were performed with p300 and CARM1 respectively in the presence or absence of TBBD by using core histones as substrate and processed for immunoblotting with site specific modification antibodies.Panel I and II represent methylation reaction probed with antibody against H3R17 and H3R26 methylation respectively. Panel III and IV represent acetylation reaction probed with antibody against H3K14 and H3K18 acetylation. Lane 1, Histones without enzyme; lane 2, histones with enzyme in the presence of DMSO; lanes 3- 4, histones with enzyme in the presence of 25μM and 100μM TBBD.*

The pentapeptide motif differs by one amino acid between the two sites. In the case of the residue whose methylation is inhibited (R17), the amino acid (X) preceding R17 is P16. For residue R26 whose methylation is not affected by TBBD, R26 is preceded by A25. Because proline is known to be a conformational disrupter of polypeptide chains, we hypothesized that P16 may be acting as the docking site for TBBD and thus preventing methylation at R17. To confirm whether, the inhibition observed is a true enzyme inhibition or an artefact of nonspecific blocking of histone accessibility at a certain region, we examined the acetylation status of histone H3K14 and K18 which are also adjacent to the H3P16 site.

When histones from TBBD treated HeLa cells were probed with antibodies against acetylated histone H3K14 and K18, interestingly, the acetylation status of both these residues was not affected by TBBD treatment (**Figure 3.11.A, panel III, panel IV, lanes 4-5**). The *in vivo* acetylation status of acetylated H3 K14 and acetylated H3 K18 were monitored by checking the steady state levels of the total histones. To exclude the possibility of TBBD affecting the total acetylation status by modulating acetyltransferase and deacetylase activities, an *in vitro* acetylation reaction in the presence of TBBD, using p300/KAT3B was performed using core histones as substrate. These *in vitro* modified histones were processed for immunoblotting using the acetylated H3K14 and K18 antibodies. It was observed that the histone acetylation was unaffected by TBBD treatment (**Figure 3.11.B, panel III and IV, lanes 2-4**) although the H3R17 methylation was affected at similar concentrations (**Figure 3.11.B, panel I, lanes 2-4**). H3R26 methylation was unaffected (**Figure 3.11.B, panel II, lanes 2-4**) as observed earlier. These observations clearly indicate that TBBD is a specific inhibitor of CARM1 even in the *in vivo* context with preference towards H3R17 methylation.

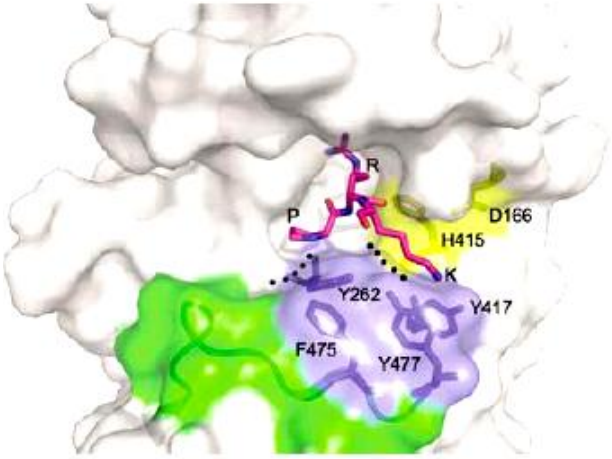
3.6. TBBD mediated CARM1 inhibition is directed by the proline residue of the substrate

3.6.1. Docking studies of TBBD on CARM1-Histone H3 complex :

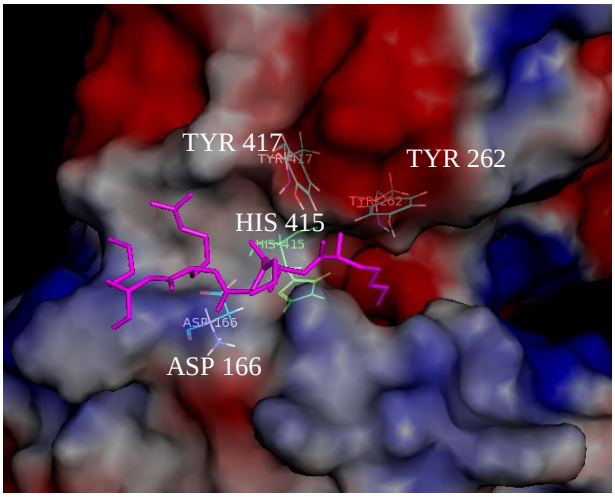
To verify any differential interaction of TBBD with the two motifs, KAPRK and KAARK, we resorted to molecular modeling and docking studies. We docked the

pentapeptide sequence from amino acids 14 to 18, KAPRK of histone H3 into the substrate binding site of CARM1 along with TBBD.

A)



B)

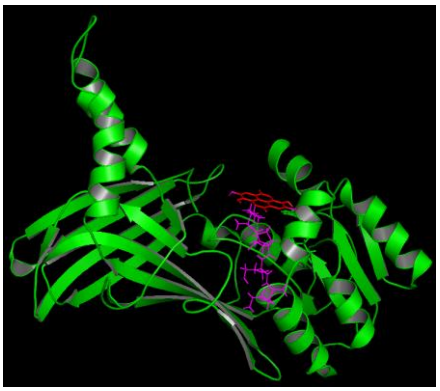


-adapted from Yue et al., 2007

Figure 3.12. Surface features of the CARM1 active site (A). *Features of the active site are coloured: the His415-Asp166 couple (yellow), C-extension (green) and aromatic residues from Tyr262 in the double-E hairpin and the PFFRY/THWY motifs (blue). A peptide sequence of histone H3 (Pro16-Arg17-Lys18) is modelled into the active site with reference to the structures of PRMT1 and PAD4.* ***(B)*** *H3 (P-R-L) peptide has already been modeled into CARM1 active site. The H3 pentapeptide KAPRK has been modeled in the active site of CARM1. The figure shows the H3 pentapeptide near the active site residues of CARM1 (TYR417, HIS415, ASP166 & TYR 262).*

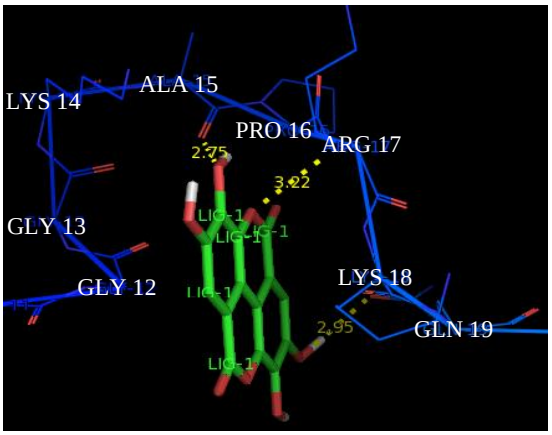
Since, the reported crystal structure was solved with only the three residues 'PRK' of the above motif (**Figure 3.12.A**) in the substrate binding site, the other two residues 'KA' were simulated in the susbtrate binding site (**Figure 3.12.B**). TBBD was found to interact with this ES complex and interestingly, this arrangement yields a ligand conformation with ΔG=-5.05 kcal/mol, which is the lowest free energy of binding among the analyzed structures (**Figure 3.13.A)**. However, to get a better understanding of TBBD interaction with the pentapeptide motif, the subsequent docking analysis was done using the pentapeptide motif and the inhibitor. As per the docking studies, TBBD forms three hydrogen bonds with A15, R17 and Q19 of histone H3, with ΔG=-5.36 kcal/mol (Figure 3.13. B, panels I and II). When the docking experiment was conducted by targeting the grid centered on the motif spanning amino acids 23 to 27, KAARK, of the same receptor molecule (histone H3), a different ligand conformation was obtained with a higher ΔG (Figure 3.13.C, panels I and II).

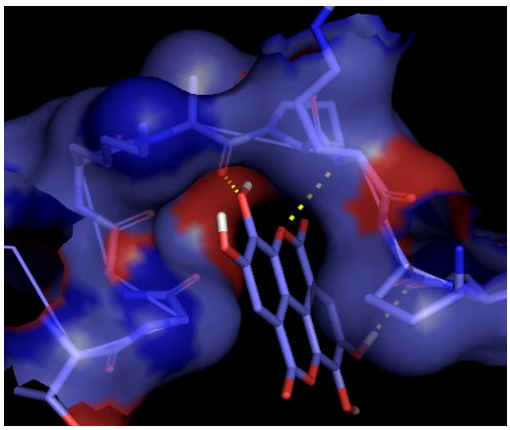
A)



B)

Panel I **Panel II**





C)

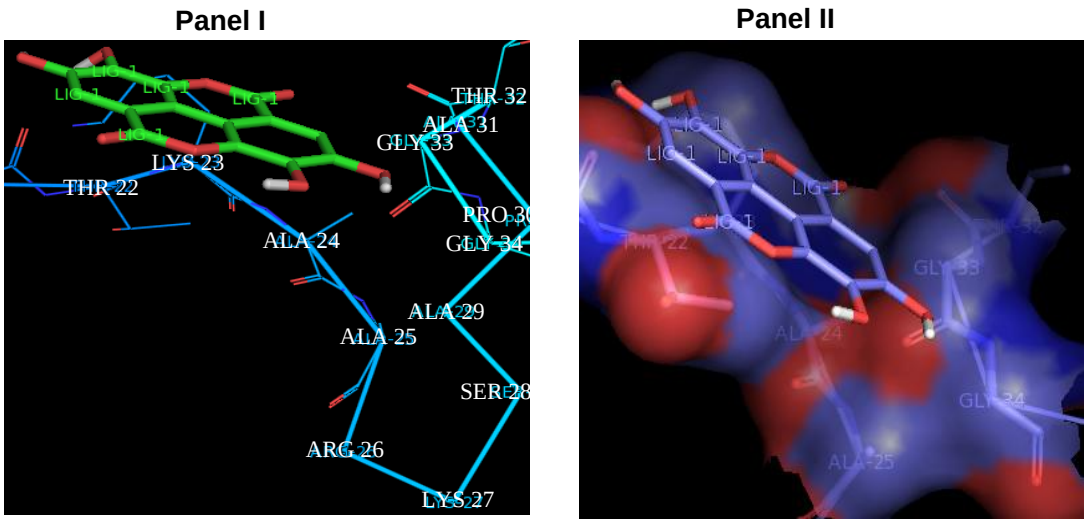


Figure 3.13.Molecular modeling studies with TBBD and the pentapeptide motif (A) *TBBD is modeled into the docked complex of CARM1 and H3 pentapeptide. (Green – CARM1, Magenta – H3 pentapeptide, Red – TBBD)* ***(B)*** *Figure showing the docked conformation of TBBD near the KAPRK motif of histone H3. The free energy of binding for this ligand conformation is ΔG=-5.36 kcal/mol.* ***(C)*** *Figure showing the docked conformation of TBBD near the KAARK motif of histone H3. The free energy of binding for this ligand conformation is ΔG=-3.72 kcal/mol, which is higher than the free energy of binding at the KAPRK motif.*

3.6.2. The proline residue of the histone H3 tail is essential for TBBD interaction with the ES complex:

The only difference between the two motifs, KAPRK and KAARK, is the presence of proline at the third position. This residue, P16, in histone H3 is predicted to form hydrophobic interactions with TBBD, thus giving this conformation the lowest free energy of binding, indicating the possibility of the proline residue playing a critical role in determining the inhibition. Involvement of the P16 residue of histone H3 in bringing about the inhibition of CARM1 methylation at a single site was further validated by using site directed mutagenesis. As described earlier, TBBD interacts with histone H3 at a single site as determined by ITC measurements **(section 3.4.2**). Since, the molecular modeling study indicates a difference in the interaction between the two motifs KAPRK and KAARK, we speculated that the interaction might also be different. The mutants A25P and P16A

described in **section 2.2.9,** were subjected to similar ITC studies to determine any difference in their interacting potentials, i.e. binding affinities. Data for the point mutant A25P, which has two proline residues adjacent to the arginine residue, indicates the presence of two binding sites (n = 2.40 ± 0) for TBBD on histone H3 (**Figure 3.14.A**). The binding of TBBD to this mutant substrate is also enthalpy driven, ΔH = -15.8 kcal/mole (**Table 3**). Significantly, the P16A mutant, which lacks the proline residue preceding arginine, does not show any binding (n = 7.30 ± 1.59E3) with TBBD (**Figure 3.14.B**).

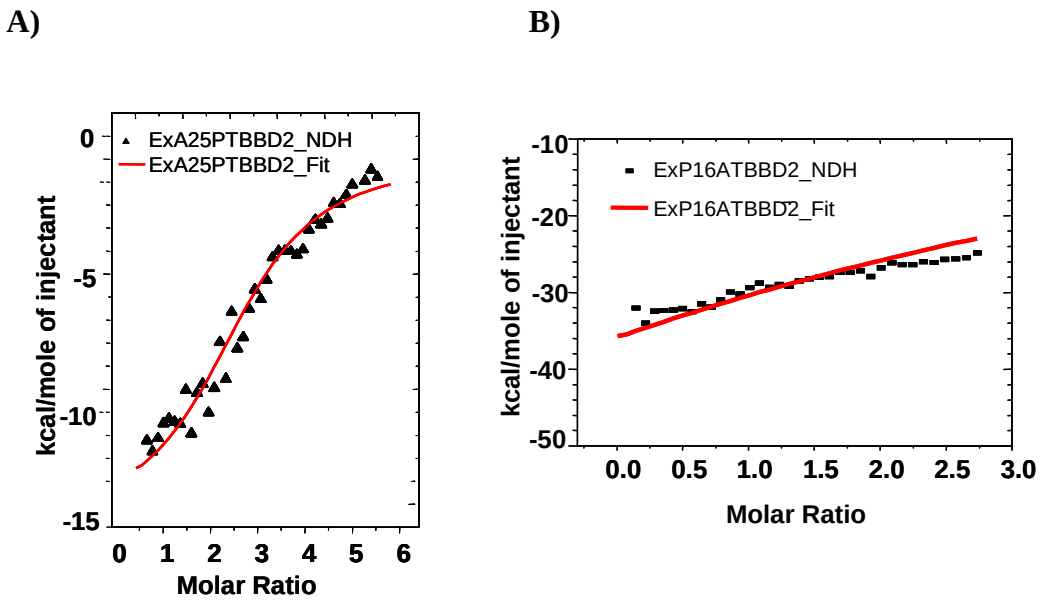


Figure 3.14. (A) *Isothermal calorimetric (ITC) titration was carried out by titrating histone H3 mutant A25P (7 µM) against the ligand TBBD (140 µM) at 25°C. The one-site model with two binding sites is shown.* ***(B)*** *Isothermal calorimetric (ITC) titration was carried out by titrating histone H3 mutant P16A (7 µM) against the ligand TBBD (140 µM) at 25°C. No interaction was observed. The dots represent the heat change associated with the titration. The fit represents the curve fitting done by the software.*

3.6.3. The proline residue of the histone H3 tail is essential for TBBD-mediated inhibition of arginine methyltransferase activity:

As mentioned above, wild-type histone H3 (**Figure 3.15.A**) is methylated by CARM1 at two sites, R17 and R26. As revealed by the docking data, TBBD binds the KAPRK stretch of histone H3, preventing methylation of H3R17 (**Figure 3.15.B, panel I, lanes 2-4**). However, KAARK is not bound by TBBD, thus allowing R26 in this motif to be

methylated (**Figure 3.15.B, panel II, lanes 2-4)**. A mutant histone H3 (**Figure 3.15.A**) in which the alanine residue was mutated to proline (A25P) showed inhibition of H3R17 methylation by TBBD as expected (**Figure 3.15.B, panel I, lanes 6-8**).

A)

B)

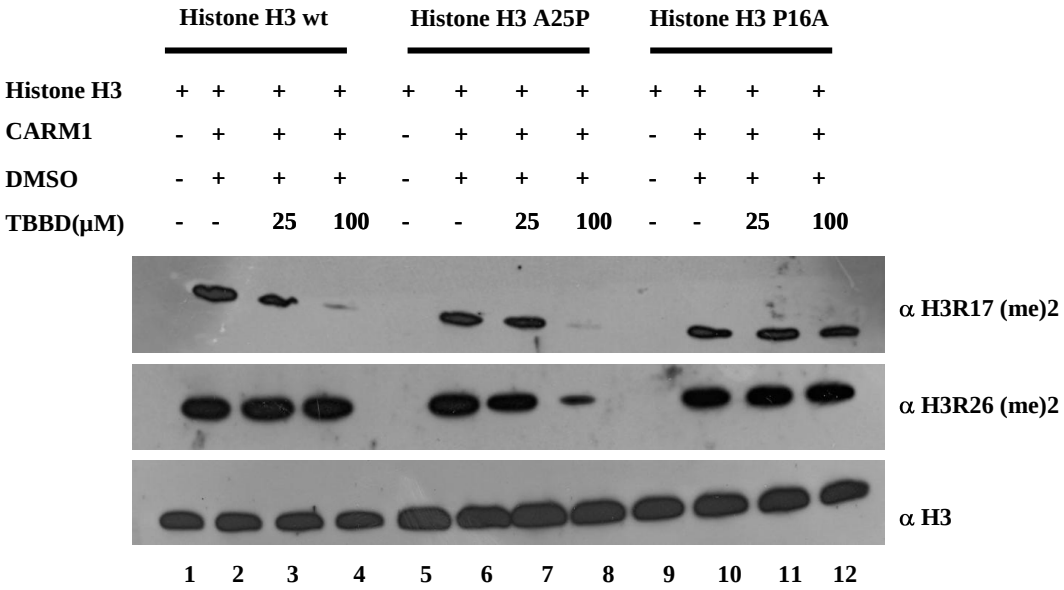


Figure 3.15. (A) *Sequence details of the histone H3 proteins used for the assay.* ***(B)*** *In vitro histone methyltransferase assays were performed with CARM1 in the presence or absence of TBBD by using histone H3 (lanes 1-4), mutant A25P histone H3 (lanes 5-8), and mutant P16A histone H3 (lanes 9-12) as substrate. Histone H3 in the absence of enzyme (lane 1); or in the presence of enzyme and DMSO (lane 2); or in the presence of 25 µM and 100 µM TBBD (lanes 3-4). Histone H3 was probed with antibodies against dimethylated H3R17 (Panel I) or dimethylated H3R26 (Panel II). As a loading control, histones were probed with antibody against histone H3 (Panel III).*

Significantly, it was observed that the presence of proline at position 25 (A25P) led to inhibition of R26 methylation by TBBD (**Figure 3.15.B, panel II, lanes 6-8**). However, a point mutant P16A (**Figure 3.15.A**), in which proline at residue 16 was mutated to alanine, did not show any inhibition of methylation at both sites (**Figure 3.15.B, panel I and panel II, lanes 10-12**), establishing that it is indeed the proline residue which is responsible for TBBD-mediated inhibition of CARM1 methylation. Thus, in agreement with the mechanistic differences in the methylation of H3R17 and R26 as observed by X-ray crystallographic analysis, TBBD-mediated inhibition of R17 methylation is also uniquely specific.

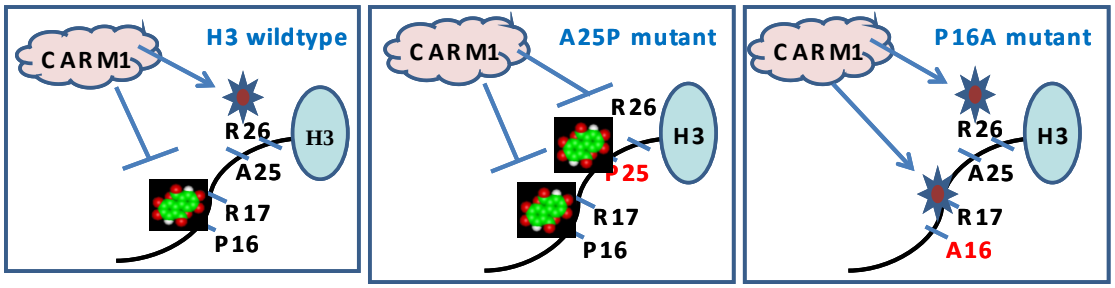
Table 3: **Themodynamic parameters of TBBD interaction with histone H3 wildtype and mutants**

	K_d, μM	n (no.of binding sites)	ΔH (kcal)/mole	ΔS (e.u)
Histone H3 wt	4 ± 0.736	1.43 ± 0.0372	-18.3	-35.4
CARM1	-	-	-	-
Histone H3A25P	7.9 ± 1.24	2.4 ± 0	-15.8	-26
HistoneH3 P16A	-	$7.30 \pm 1.59\ 10^{3}$	-	-

3.7. A novel mechanism of enzyme inhibition

Taken together, these observations suggest that P16 of histone H3 is the docking site for TBBD, and thus this single amino acid determines the inhibitory effect of TBBD on CARM1 activity. The novel CARM1-specific inhibitor TBBD, selectively blocks methylation of H3R17 but not of H3R26. Remarkably, the sequence of the histone H3 tail (specifically the proline residue at position 16) determines the specificity of the inhibition brought about by TBBD (**Figure 3.16 (A and B)**). Most importantly, the data establish a new mechanism of specific enzyme inhibition determined by the amino acid sequence of the substrate.

A)



B)

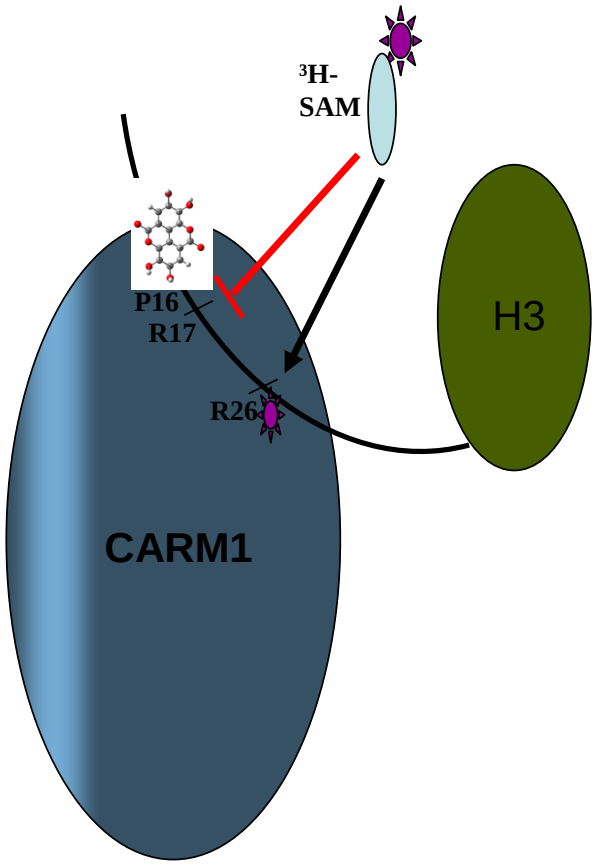


Figure 3.16. ***(A)*** *Cartoon representation of the differential interaction of TBBD with wildtype and mutant histone A25P and P16A thereby influencing the enzyme inhibition.* ***(B)*** *TBBD is a novel inhibitor of arginine methyltransferase CARM1 wherein the substrate sequence directs the specificity of the enzyme inhibition.*

The mechanism of action of TBBD on CARM1 activity is a novel enzyme inhibition effect. Although in classical biochemistry there are examples of the substrate being

sequestered by inhibitor and thereby leading to inhibition (Li et al., 2008), TBBD recognizes the substrate sequence and thus brings about inhibition at a single site of modification without affecting the other residue modification. The significance of the ES complex involving the KAPRK motif for R17 methylation inhibition was further confirmed by verifying the modification status of the flanking residues, K14 and K18 acetylation which were not affected on TBBD treatment. The sequence recognition and the subsequent enzyme inhibition seems to require an active site in the context of the residue being recognized. Although the characterization of the inhibition reported here, is with respect to histone H3, the protein arginine methyltransferase CARM1 also methylates a few nonhistone proteins including the acetyltransferase CBP, HuR, PABP1 and TARPP. In order to have a further understanding of the molecular mechanism of TBBD mediated inhibition of the arginine methyltransferase activity, these substrates also need to be subjected to similar studies.

3.8. The physiological significance of inhibition of arginine methylation

The protein arginine methylation is an event that is not restricted only to the histones, rather it is more prevalent on the nonhistone proteins. However, as compared to PRMT1, the nonhistone substrates of CARM1 are fewer than PRMT1. And importantly, most of the biological activities of CARM1 are either due to its methyltransferase or its transcriptional co-activation property. The histone H3R17 methylation is a mark that is associated with both these functional attributes of CARM1. In such a scenario, the identification of an uncompetitive inhibitor that binds to the ES complex and inhibits the modification of H3 R17 methylation would be highly useful in deciphering the role of this modification in the physiological contribution of CARM1 arginine methyltransferase. This was indeed possible and this would be discussed in chapter 5.

The small molecule inhibitor, TBBD (ellagic acid), reported here is a major component of pomegranate crude extract, which has been used against various ailments such as parasitic diseases, diarrhoea, ulcers and most importantly as an anti-cancer agent (Dorai and Aggarwal, 2004). The anti-tumor activity has been shown for several cancers especially prostate cancer, breast cancer and also for colorectal cancer (Losso et al., 2004). However, the active component from the crude extract and the exact molecular target within the

physiological system has not yet been identified. Possibly, the so far unidentified molecular target for TBBD is CARM1 activity. The crude extract could inhibit both the p300 acetyltransferase activity as well as the arginine methyltransferase activity of CARM1. This could be because of the various tannins and polyphenolic components of the pomegranate crude extract. However, this scaffold can be further derivatized to obtain better and more efficient inhibitors.

Chapter 4

IDENTIFICATION OF A NOVEL SCAFFOLD FOR p300/KAT3B INHIBITION

This chapter highlights the identification of a p300 acetyltransferase inhibitor from the roots of a medicinally important plant Plumbago rosea. The characterization of the mechanism of inhibition has led to the identification of the functional group responsible for HAT inhibition. A series of chemical substitutions revealed the significance of the hydroxyl group for the inhibition. Presumably, the hydroxyl group is involved in hydrogen bonding with a critical lysine residue in the p300 minimal HAT domain. The experimental evidences that establish the significance of this novel scaffold for HAT inhibition is presented in this chapter.

Chapter outline:

4.1. p300/KAT3B acetyltransferase:

p300 acetyltransferase is referred to as the master regulator of gene expression (Ogryzko et al., 1996) due to its different biological activities. p300 and its twin homologue CBP are probably the most widely studied acetyltransferases. These enzymes are important transcriptional co-activators and are components of the transcription machinery (Janknecht and Hunter, 1996). Their role in transcription and other physiological functions is a combination of various biological activities such as the acetyltransferase function, the co-activation property, their ability to act as scaffold or bridges connecting different proteins (reviewed in Giles et al., 1998). Significantly, not all the activities require the enzymatic function rather their interactomes (Manning et al., 2001) also influences the functional outcome.

p300 is a large protein with well documented role in processes such as transcription (Ogryzko et al., 1996), cell cycle (Giordano et al., 1999), DNA repair (Goodman and Smolik, 2000, Chan and La Thangue, 2001), pluripotency maintenance (Miyabayashi et al., 2007), differentiation (Zhong and Jin, 2009) etc. Though, the exact structural information of this protein though is not available, an elegant interdisciplinary approach utilizing the Surface enhanced Raman spectroscopy (Kumar and Arif et al., 2006) has provided significant details of the secondary structural organization of this protein. This was followed by the report of the minimal HAT domain crystal structure complexed with the p300 specific inhibitor Lysyl CoA (Liu et al., 2008). The enzyme p300 is modulated not only by the various interacting proteins, but is also self-regulated through an autocatalytic mechanism (Thompson et al., 2004). Earlier work from Philip Cole's group has revealed the existence of an autoacetylation loop in the enzyme which consists of twelve acetylation residues which undergo very rapid acetylation, essential for the acetylation activity of the enzyme (Thompson et al., 2004). The lysyl CoA complexed p300 HAT domain crystal structure also led to the identification of the mechanism of enzyme action. For a long time, p300 was considered to follow a ping-pong mechanism of action (Thompson et al., 2001), but the crystal structure data and the biochemical validation, suggest a Theorell-Chance mechanism of enzyme action proposing a hit and run mechanism (Liu et al., 2008), thus shedding light on its wide

repertoire of substrates that get modified. This mechanism of enzyme activity precludes the need for a proper enzyme-substrate complex formation. Instead, the enzyme exists in an active autoacetylated form and different substrates transiently interact with the active site of the enzyme to undergo modification.

The substrates that get modified by p300 are indeed many with a proposed number of more than 100 substrates (Marmorstein, 2001). It is probably the only histone acetyltransferase that catalyzes acetylation of all four histone tails (reviewed in Lee and Workman, 2007). It also acetylates several transcription factors such as p53 (Gu and Roeder, 1997), NF-κB (Chen et al., 2001), SP1 (Xiao et al., 2000), c-myc and regulates their activities. The histone chaperone NPM1 has been identified as a p300 acetyltransferase specific substrate wherein acetylation regulates its transcriptional ability as well as its oncogenic potential (Swaminathan et al., 2005, Shandilya et al., 2009). A search for the consensus motif for p300 acetylation has shown that there does not seem to exist any characteristic recognition motif, however the presence of a G/SK sequence relative to the acetylation site is a probable recognition motif of p300 acetyltransferase (Bannister et al., 2000).

Since, the involvement of p300 in physiological functions is immense and it regulates almost all essential cellular processes, it is an obvious corollary that its dysfunction may lead to several abnormal or diseased states. Indeed the hyper-activation or hypo-activation of this enzyme has been linked to a wide range of diseases from cancer, asthma, respiratory disorders to metabolic diseases like diabetes (reviewed in Selvi and Kundu, 2009). One of the major mediator of the inflammatory response is the transcription factor NF-κB, which exhibits a strict requirement of acetylation of its p65 subunit for its transcriptional activity. Thus, NF-κB which is one of the main players in the field of disease manifestation due to its obvious association with the first step of disease, i.e. inflammation is directly regulated by p300 mediated acetylation and thus p300 influences several disease associated pathways. It is important to remember that the acetylation and the deacetylation mechanisms that exist in the cell maintain a homeostatic balance that controls the smooth functioning of the different cellular processes. And it is this balance that is lost in most disease states. The balance can be lost by several mechanisms such as

increased or decreased enzyme activities or mutations in any of the enzyme components or sequestration of the proteins or most importantly altered post translational modifications which might change the interacting proteome. All these changes need not essentially occur on only acetyltransferases and deacetylases, most often the alterations are in both the enzyme machineries. For example, in cancer there is hyperacetylation associated with the gene rich regions whereas there is hypoacetylation at the promoter regions. Hence, it is essential to understand the exact cause and mechanism of these dysfunctions so as to design and execute measures towards therapeutic purposes. The most widely used method has been the knockout strategy wherein the entire protein is absent thus addressing the effect of that protein *in toto* on the cellular system. This strategy although has been extremely useful in providing new insights into the protein function, it is slowly being realized that such an information might not be useful in delineating the role of the enzyme in the cellular processes. The advantage of an enzyme associated activity is the ability to modulate it by varying substrate concentrations, or by using modulators (activators/inhibitors) or by just changing the optimal activity conditions. Thus, if it becomes clear that the enzymatic activity is what is responsible for its functions and thereby its possible role in disease manifestation, an inhibitor would have immense therapeutic potential. Most importantly, a modulator might help in understanding the enzyme and its mechanism of action. Thus, the identification of specific modulators of an enzyme are valuable tools to understand the enzyme function in physiological conditions as well as to modulate the same enzyme function in pathophysiological states. This concept was realized in the early 2000. The increased use of histone deacetylase inhibitors led to the realization that there should also exist modulators of the forward reaction, i.e. histone acetylation inhibitors/activators. The first known p300 specific HAT inhibitor is a synthetic molecule, Lysyl CoA (Lau and Kundu, et al., 2000). Although, it is a decade since the first HAT inhibitor was discovered, the field of HAT inhibitors consists of less than a dozen representatives. The most important fact is that none of these inhibitors, inspite of their proven specificities, have been considered for any clinical trials. The identification of HAT inhibitors from natural source such as anacardic acid from cashewnut shell liquid (Balasubramanyam

et al., 2003) and garcinol from *Garcinia indica* (Balasubramanyam et al., 2004), opened up a newer source for HAT inhibitors. Although, these inhibitors were highly potent, their nonspecific action on other chromatin modifying enzymes necessitated the search for specific inhibitors. The identification of p300 specific inhibitors, curcumin (Balasubramanyam et al., 2004a) and LTK-14 (Mantelingu and Reddy et al., 2007) has immensely contributed towards understanding the role of p300 in transcription (Black et al., 2006) and global gene regulation (Mantelingu and Reddy et al., 2007). But curcumin is a well known antioxidant with multiple pleotropic effects (reviewed in Bar-Sela et al., 2010). The garcinol derivative LTK-14 has anti-HIV property as it inhibits the viral syncytia formation. However detailed clinical studies and investigations are essential to exploit the therapeutic potential of LTK14. Interestingly, the DNA binding intercalator, Sanguinarine which is a putative anti-cancer therapeutic was also found to be a potent inhibitor of both p300 and PCAF with an IC_{50} of 10 μM (Selvi and Pradhan et al., 2009), but it had a broad spectrum of inhibitory activity. Although, the role of enzymes like p300 have been implicated in diseases such as neurodegenerative disorders (Rouaux et al., 2003), several inflammatory disorders (reviewed in Dekker and Haisma, 2009) and it has also been shown that the HAT inhibitors can effectively downregulate NF-κB transcription (Ralhan et al., 2009), it is surprising that none have been taken forward for therapeutic applications. One of the possible reason could be that most of these inhibitors have an IC_{50} of micromolar concentrations (**Table 1**) which is not suitable for clinical investigations. Furthermore, the available information about these inhibitors are all based on cell line experiments with very minimal data on whole animals. Hence, the apparent *in vivo* toxicity and the bioavailability of these inhibitors are not known. All these facts necessitate the identification of other HAT inhibitors especially from the natural sources so that an active scaffold could then be modified for better and efficient inhibition.

4.2. Search for p300 specific inhibitor:

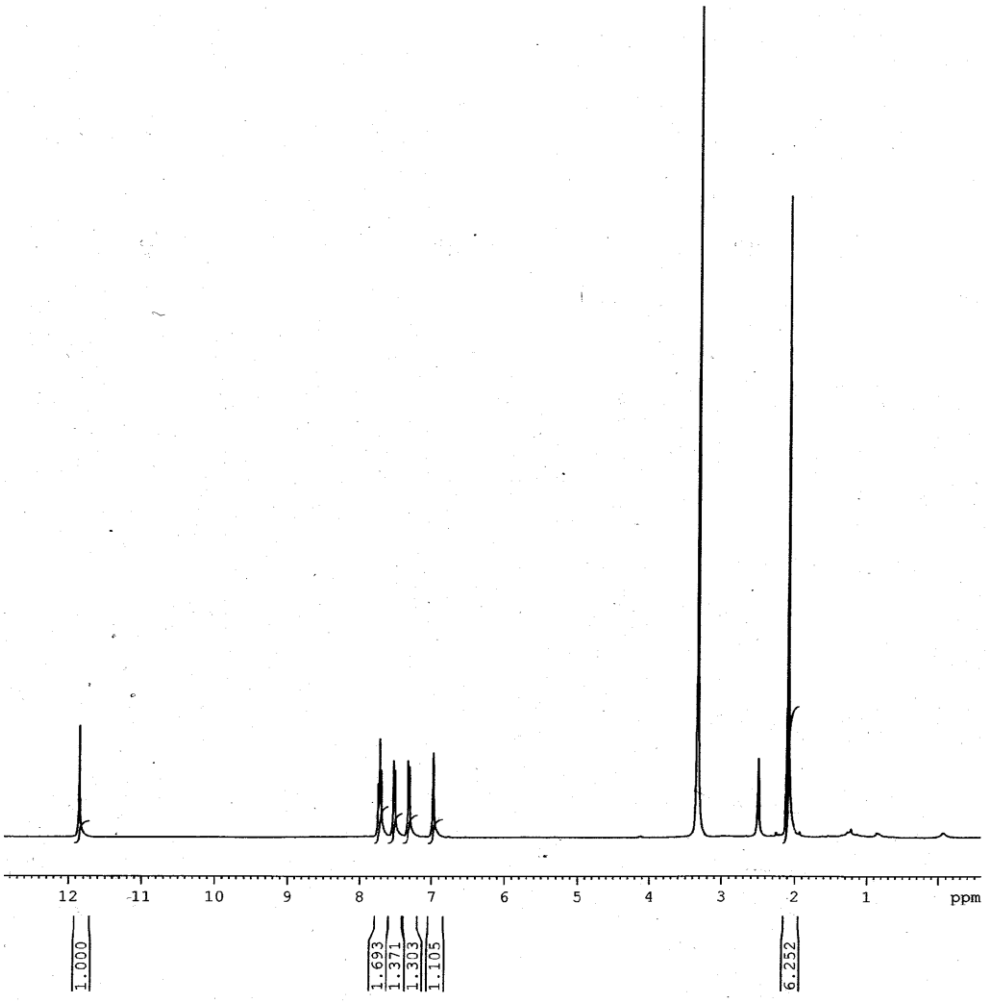
We have established a screening system of medicinal plants (described in the Indian ayurvedic literature by Charaka and Sushruta) with known anticancer properties for HAT modulation activity. A systematic literature survey was carried

out to determine the possible candidate for p300 inhibition. The ayurvedic medicines are generally crude extracts of various plant components in a suitable form. Few are topical and few are taken internally. One way to search for active components is to first determine the diseases in which that particular enzyme (in this case, p300) is involved. The diseases with a more direct involvement are better candidates to consider. The next step is to identify the plant materials that are used for treatment of those diseases that have been screened in the first survey. Such an extensive search led to the identification of the root extract of *Plumbago rosea* as a treatment measure for several inflammatory and microbial diseases (reviewed in Krishnaswamy and Purushothaman, 1980). The more striking observation was that the active component of this root extract, plumbagin was used for the treatment of hepatic ailment (Parimala and Sachdanandam, 1993) and also exhibited anticancer activity against the hepatocellular carcinoma cell line (HCC) HepG2 (Shih et al., 2009) by inhibiting the invasion and migration of the cells. Interestingly, although majority of cancer cases report hypoacetylation citing loss in p300 activity (Muraoka et al., 1996, Koshishi et al., 2004) or mutated p300 (Roelfsema and Peters, 2007), there are few cancers such as oral cancer and hepatocellular carcinoma which exhibit histone hyperacetylation. In the case of oral cancer, the histone hyperacetylation is related to the hyperacetylation of a p300 substrate, NPM1 leading to expression of genes associated with oral cancer manifestation (Shandilya et al., 2009). The histone hyperacetylation in HCC is causally related to the hyperacetylation of p300 regulated MEF2 (Bai et al., 2008) leading to a mechanism contributing towards tumorigenesis. Taking all these facts into consideration, we decided to test the root extract of *Plumbago rosea* for its effect on acetylation.

4.3. Plumbagin isolated from *Plumbago rosea* is a histone acetylation inhibitor

Plumbago rosea roots were obtained and authenticated. The roots were processed as described in the methods **section 2.9.2**. The active component was tested for purity on Thin Layer chromatography plates which showed the presence of a single spot. This was further characterized by mass spectrometric and ^{1}H-NMR spectroscopic methods (**Figure 4.1.A**)

A)



B)

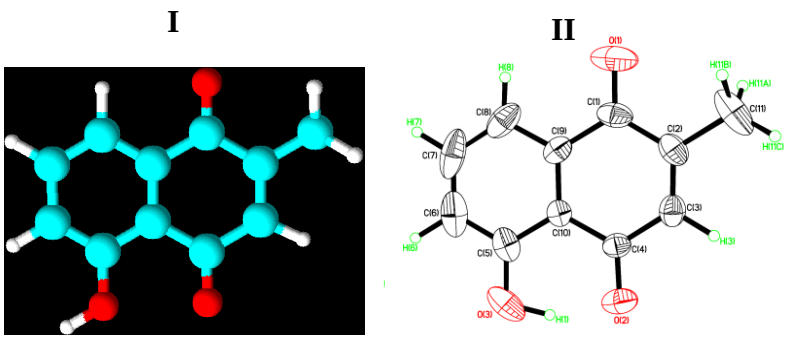
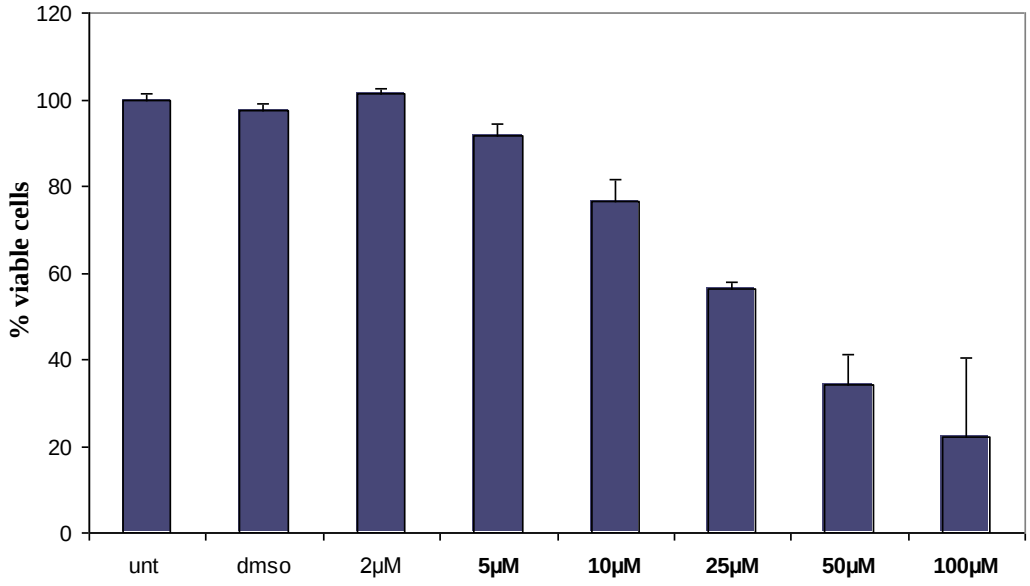


Figure 4.1. Plumbagin/RTK1. A) *Proton NMR spectra of plumbagin (RTK1).* ***B)*** *Ball and stick model of plumbagin where blue colour denotes carbon, white indicates hydrogen and red represents oxygen (panel I), X-ray crystal structure (panel II) of plumbagin*.

The spectral details of the molecule plumbagin was found to be as follows: ^{1}H NMR (400 MHz, DMSO- d_6) δ 11.96 (s, 1H, OH), 7.64 (t, 1H, Ar-H), 7.63 (d, 1H, Ar-H), 7.28 (d, 1H, Ar-H), 6.82 (s, 1H, CH), 2.22 (s, 3H, CH_3); HRMS (m/z): $[M]^+$ Calcd. for $C_{11}H_8O_3$, 189.18; found, 189.36. The molecule was crystallized and the X-ray diffraction data suggested that it is a monoclinic crystal. The solved crystal structure data is represented in (**Figure 4.1.B**).

To investigate the effect of plumbagin on the histone acetylation in cells, an initial screening assay was done to establish the compound concentration that could be used without causing any cytotoxicity. Hence, HeLa cells were treated with different concentrations of plumbagin ranging from 5 µM to 100 µM for 24 hours. A MTT assay was done to determine the level of toxicity and it was evident that the concentrations higher than 10 µM were toxic to the cells, with almost 50% reduction in cell viability at 25 µM concentration (**Figure 4.2. A**), in a 24 hour treatment period as observed by the MTT assay. Furthermore, in an independent experiment, HeLa cells were treated with DMSO, 10, 25 and 100 µM concentration for 12 hours and subjected to FACS analysis. There was no apparent cytotoxicty in cells treated upto 25 µM concentration for 12 hours as evident by the FACS data (**Figure 4.2.B**). However, the higher concentration of 100 µM RTK1 led to an increase in the apoptotic population.

A)



B)

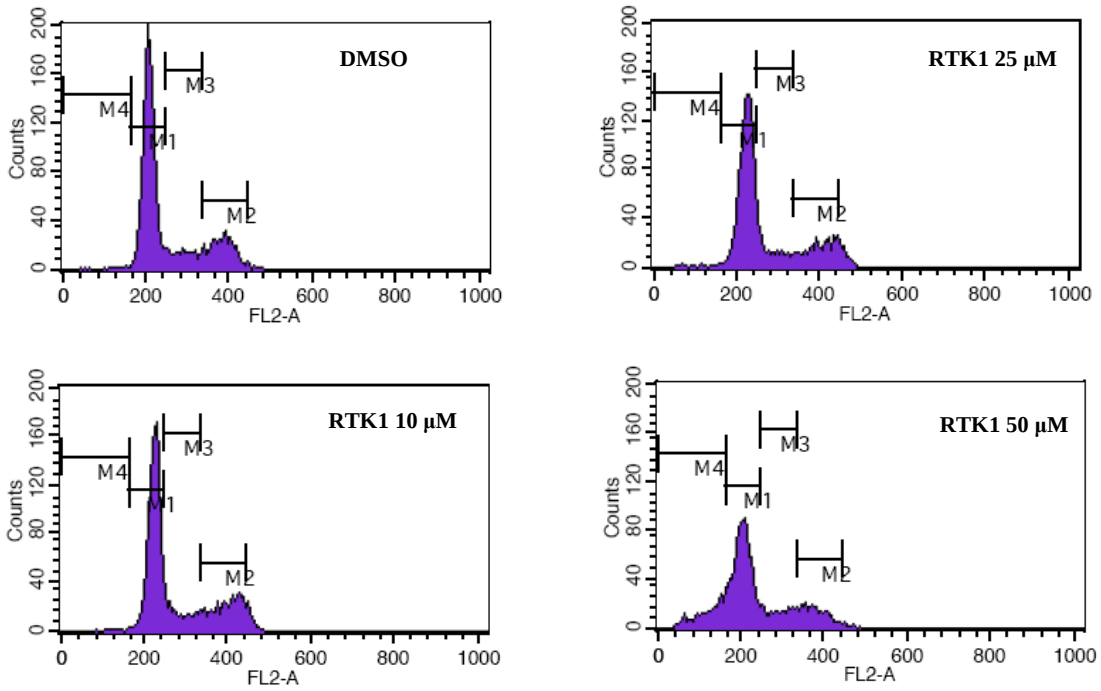


Figure 4.2. Cytotoxicity profiling of plumbagin/RTK1. A) *Cytotoxicity assay: HeLa cells were untreated (lane 1), treated with DMSO (lane 2), treated with 2, 5, 10, 25, 50, 100 µM RTK1 (lanes 3-8) for 24 hours and subjected to MTT assay. The cell viability was calculated as a measure of the ability to reduce the MTT and plotted as percent viable cells. The experiment was performed in replicates and the error bar represents the standard deviation.* ***B)*** *FACS analysis of PI stained HeLa cells treated with DMSO, RTK1 (10, 25, 100 µM) for 12 hours. The cells were gated in M1, M2, M3 and M4 region in the FL2 channel.*

Since, the initial anticancer activity of plumbagin was shown on HepG2 cell line (Bai et al., 2008), the effect of RTK1 on histone acetylation was also tested on the HepG2 cell line. Incidentally, the HCC HepG2 exhibits hyperacetylation of histones. The cells were treated with the compound at low micromolar concentrations from 5 to 50 µM for 12 hours. The histones were isolated from the treated cells. It was observed that the compound exhibited almost 50% reduction in the acetylation levels at 5 µM concentration itself (**Figure 4.3.A, lane 3 versus lane 2**). The subsequent increase in concentration to five fold (i.e. 25 µM) drastically reduced the

acetylation levels with almost complete loss of acetylation at the highest concentration (50 µM) used (Figure 4.3.A, lane 4 and 5 respectively).

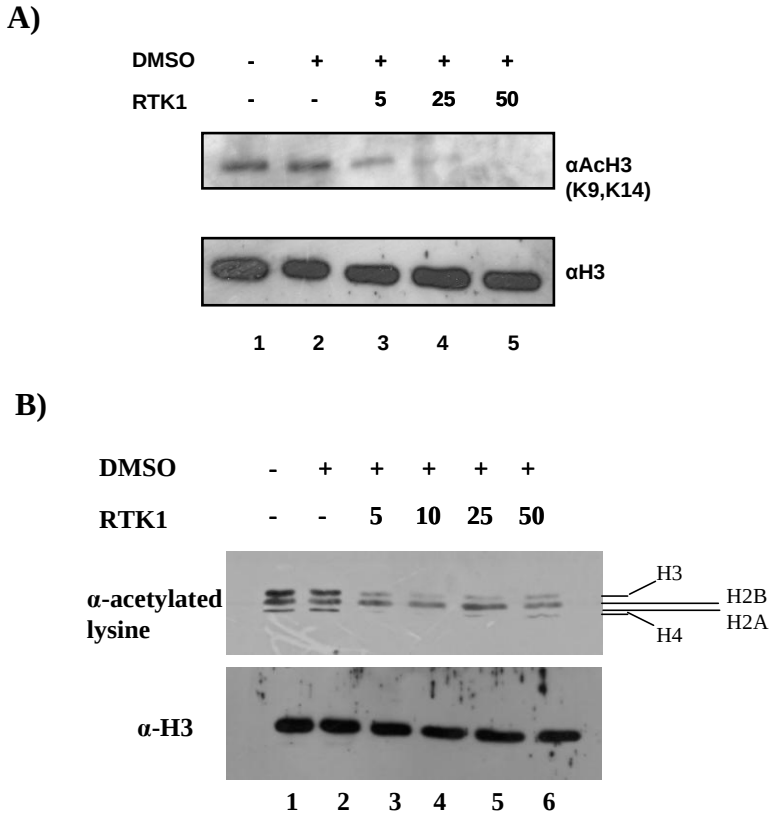


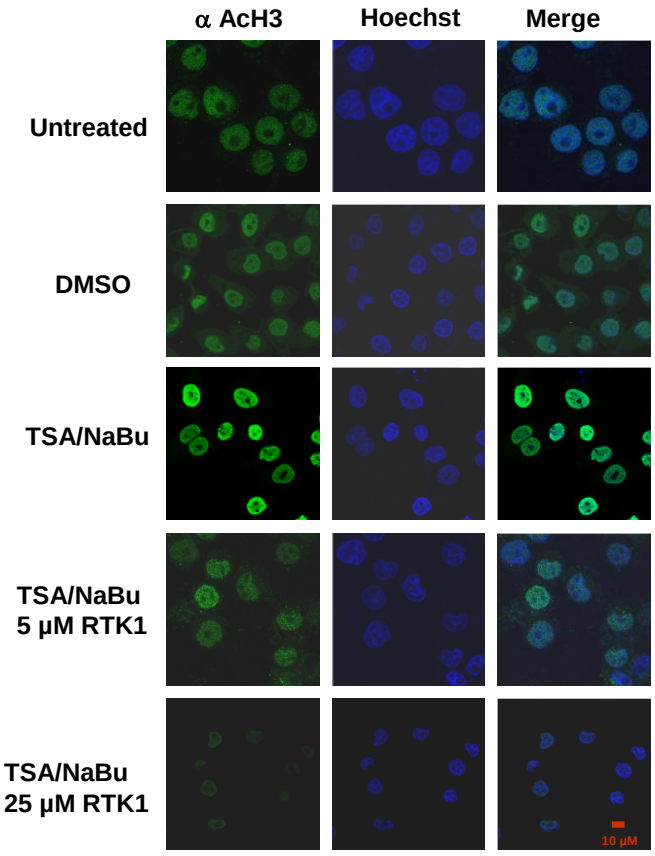
Figure 4.3. Effect of plumbagin/RTK1 on histone acetylation. A) *HepG2 cells were treated as indicated, for 12 hours; histones were isolated from untreated cells (lane 1); DMSO treated cells (lane 2); RTK1 treated cells (lanes 3-5). The histone acetylation was probed by immunoblotting using acetylated H3 antibody. As a loading control the blots were probed with histone H3 antibody.* ***B)*** *HepG2 cells were treated as indicated, for 12 hours; histones were isolated from untreated cells (lane 1); DMSO treated cells (lane 2); RTK1 treated cells (lanes 3-6). The histone acetylation was probed by immunoblotting using acetylated lysine antibody. As a loading control the blots were probed with histone H3 antibody.*

Since, the above treatment showed a decrease in the acetylated histone H3 levels, we also investigated the modulation of the bulk histone acetylation status on plumbagin treatment. HepG2 cells treated with plumbagin at the similar concentrations were immunoblotted using a pan acetylated lysine antibody on isolated histones from these treated cells. It was observed that indeed both H3 and H4

acetylation was drastically reduced in a dose dependent manner (**Figure 4.3.B, lanes 3-6**).

This inhibition was also verified in another cell line, HeLa by immunofluorescence analysis using acetylated histone H3 antibody which recognizes H3K9 and K14 acetylation status. The HeLa cells show low levels of H3 acetylation as compared to the HepG2 cells, hence, the HeLa cells were treated with the HDAC inhibitors (Trichostatin A/Sodium butyrate, TSA/NaBu) to induce hyperacetylation (Figure 4.4.A, panel 3) and were also treated with plumbagin and the acetylation status was examined. Similar to the observation in HepG2 cells by western blotting, the immmunofluorescence results also showed a dose dependent decrease of histone acetylation across 5 to 25 µM concentrations of plumbagin (Figure 4.4.A, panel 4 and 5). The lower concentration image shows a mosaic sort of acetylation pattern wherein there are few cells which show decreased acetylation whereas there are few which are hyperacetylated.

A)



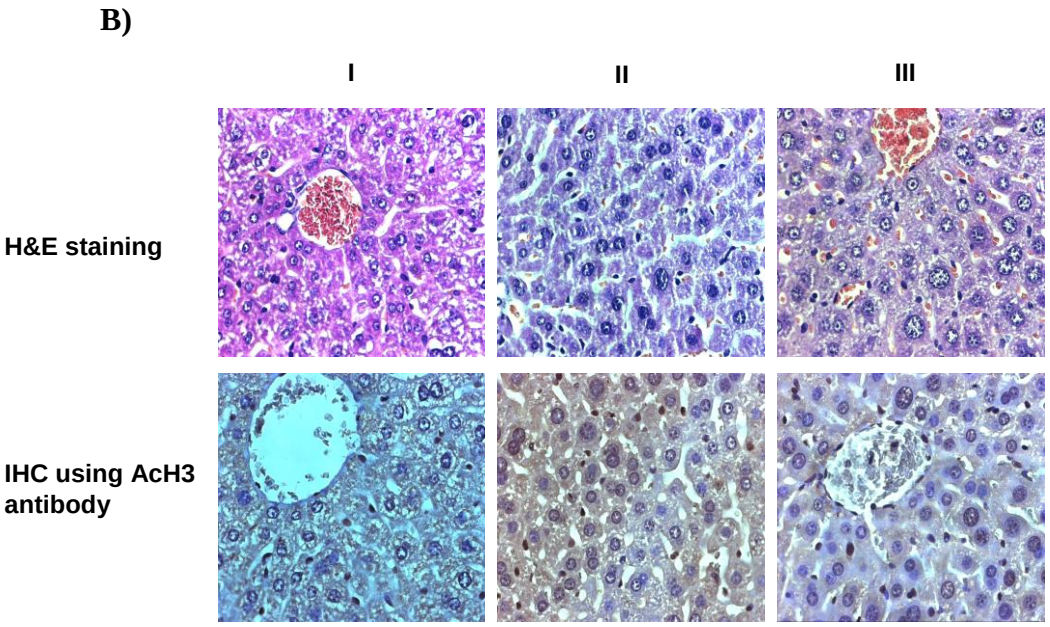


Figure 4.4. Plumbagin/RTK1 inhibits histone acetylation in vivo. ***A)*** *Inhibition of histone acetylation was visualized by immunofluorescence analysis of RTK1 treated HeLa cells probed with antibodies against acetylated histone H3. The Hoechst stained images represent DNA. Cells were either treated with DMSO, or with RTK1 (10 µM and 25 µM) as indicated for 12 hours.* ***B)*** *Liver tissue from mice injected with water (panel I), DMSO (panel II) and RTK1 (panel III) was stained with hematoxylin and eosin or immunostained with antiacetylated histone H3 antibody as indicated.*

In this case, it needs to be remembered that this treatment is a combination treatment of the HDAC inhibitor and the acetylation inhibitor (plumbagin). However, this heterogeneity disappears at higher concentrations wherein there is maximum decrease in acetylation levels (**Figure 4.4.A, panel 5**).

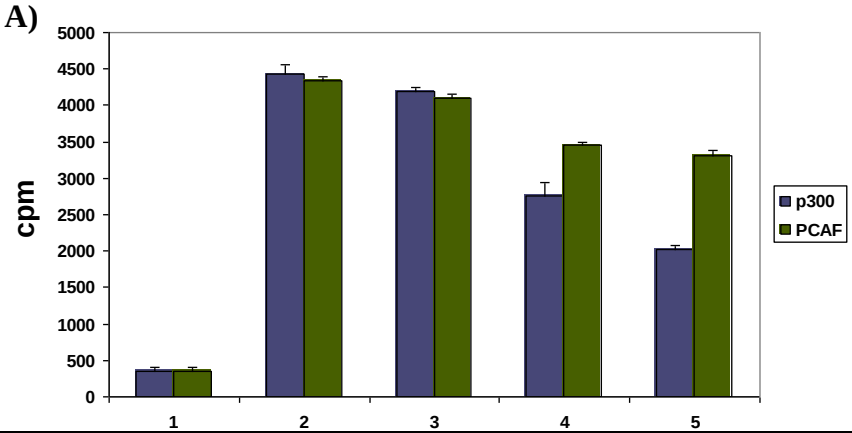
The acetylation inhibition property of plumbagin was also verified in another model system. The compound plumbagin has been used for various medicinal purposes. Hence, the characterization of the acetylation inhibition had to be validated in multiple systems. Mice were injected with plumbagin and following six hours of treatment, the mice was sacrificed and the liver was subjected to immunohistochemistry as described in the methods **section 2.6.3.** As visualized by immunohistochemistry using antibody against acetylated histone H3 (K9, K14), there was a significant decrease in histone acetylation in RTK1 treated mice liver as compared to the normal and DMSO treated controls. The acetylation status of

histones in the untreated mice liver, was taken as the control for comparing the treated sections (**Figure 4.4.B, panel I, H&E staining and AcH3 staining**). There was no gross loss in morphology of cells as indicated by the H&E staining. The solvent control sections show strong to moderate positivity in hepatocytes indicating the solvent's ability for inducing the acetylation in these cells (**Figure 4.4.B, panel II, H&E staining and AcH3 staining**). On the other hand, RTK1 dissolved in the DMSO solvent shows negative staining for Ac-H3 antibody indicating the inhibitory activity of acetylation by RTK1 in this liver sample (**Fig. 4.4.B, panel III of H&E staining and AcH3 staining**).

Taken together, these data suggest that plumbagin isolated from *Plumbago rosea* is an inhibitor of histone acetylation *in vivo.*

4.4. Plumbagin is an inhibitor of p300 acetyltranferase

Having validated the fact that indeed plumbagin is an acetylation inhibitor, the next task was to identify its molecular target of inhibition. We resorted to the *in vitro* screening process using the candidate representative members of acetyltransferases and methyltransferases by filter binding and gel fluorography assay, to identify the target of plumbagin. The *in vitro* assay using recombinant p300 and PCAF acetyltransferase indicated that plumbagin preferentially inhibits the p300 acetyltransferase activity at low micromolar concentrations as observed by both filterbinding and gel flurography assays (**Figure 4.5.A and 4.5.B, lanes 4-5**). On the other hand, there was very minimal effect of plumbagin on the methyltransferases, G9a (lysine methyltransferase) and CARM1 (arginine methyltransferase as observed by a filter binding assay (**Figure 4.6, lanes 4-7**).



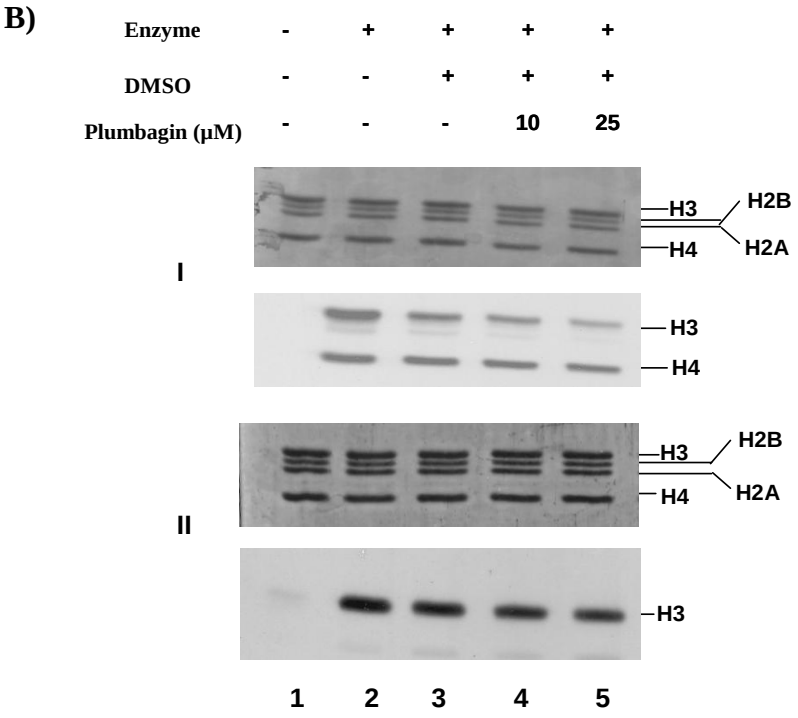


Figure 4.5. Effect of plumbagin/RTK1 on histone acetyltransferases. *Filterbinding* ***(A)*** *and Gel Fluorography assay* ***(B)*** *for inhibition of histone modification. HAT assays were performed either with p300 (I) or PCAF (II) in the presence or absence of RTK1 by using highly purified human (HeLa) core histones and processed for filterbinding or fluorography. Core histones without enzyme (lane 1); histones with enzyme (lane 2); histones with enzyme in the presence of DMSO (lane 3); histones with enzyme in the presence of increasing concentration of RTK1, 10 µM and 25 µM (lanes 4-5). Error bars are standard deviations of mean of duplicate reactions. In each set of gel assay the top panel is coomassie staining and the bottom panel is the autoradiogram.*

Even with a four fold higher concentration of inhibitor than the p300 inhibition concentration, there was very minimal effect on the methyltransferases indicating the specificity towards acetylation. Since, there was a preferential inhibition of p300 by plumbagin at lower concentrations, the inhibitory potential was compared to the other known p300 inhibitors (both specific and non specific). The filter binding assay indicated that the IC_{50} of plumbagin was very similar to the other p300 specific inhibitors curcumin and LTK14 (**Figure 4.7, lanes 6, 7, 8 vs lane 2 and 3**). The almost similar IC_{50} value of plumbagin in comparison with the other known p300 inhibitors encouraged us to look for other systems to prove the preferential inhibition of p300 activity.

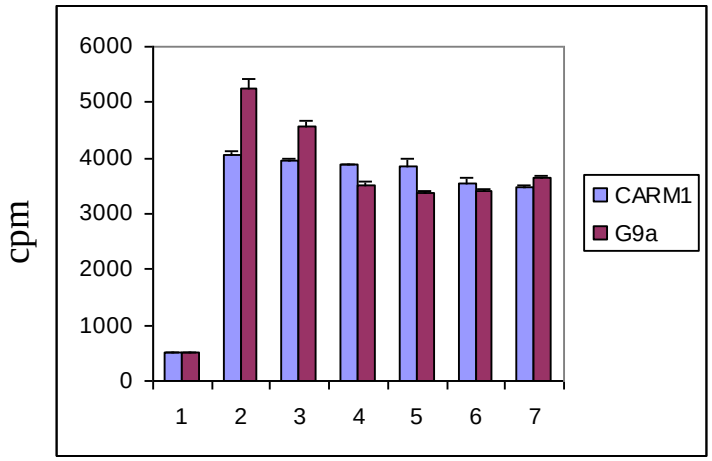


Figure 4.6. Effect of plumbagin/RTK1 on histone methyltransferases. *Filterbinding assay for inhibition of histone (arginine and lysine) methylation. Histone methylation assays were performed either with CARM1 (I) or G9a (II) in the presence or absence of RTK1 by using highly purified HeLa core histones and processed for filterbinding assay. Core histones without enzyme (lane 1); histones with enzyme (lane 2); histones with enzyme in the presence of DMSO (lane 3); histones with enzyme in the presence of increasing concentration of RTK1, 10 µM, 25 µM, 50 µM and 100 µM. Error bars represent standard deviation.*

Table 1: HAT inhibitors

Sl.No.	HATi	IC_{50} p300 (µM)	IC_{50} PCAF (µM)	IC_{50} CBP(µM)	IC_{50} Gcn5 (µM)
1.	Lysyl-CoA	0.5	200	-	-
2.	H3-CoA-20	200	0.3	-	-
3.	Anacardic acid	8.5	8.5	-	-
4.	Garcinol	7.0	5.0	-	-
5.	Curcumin	25	-	-	-
6.	Isothiazolones	1-50	1-50		
7.	γ-butyrolactones	-	-	500 to 2000	100
8.	LTK14	20			
9.	EGCG	100			
10.	Sanguinarine	10	10		

4.5. Plumbagin is a p300 specific inhibitor

The inhibition of histone acetylation by plumbagin was investigated by an antibody raised against the the peptide resembling histone H3 tail, in which the K9 and K14 residues are acetylated. *In vivo*, these residues are acetylated by HATs p300/CBP and PCAF, which are also referred to as Factor Acetyltransferases or Protein acetyltransferases. This is due to their ability to act on several nonhistone substrates leading to distinct functional consequences.

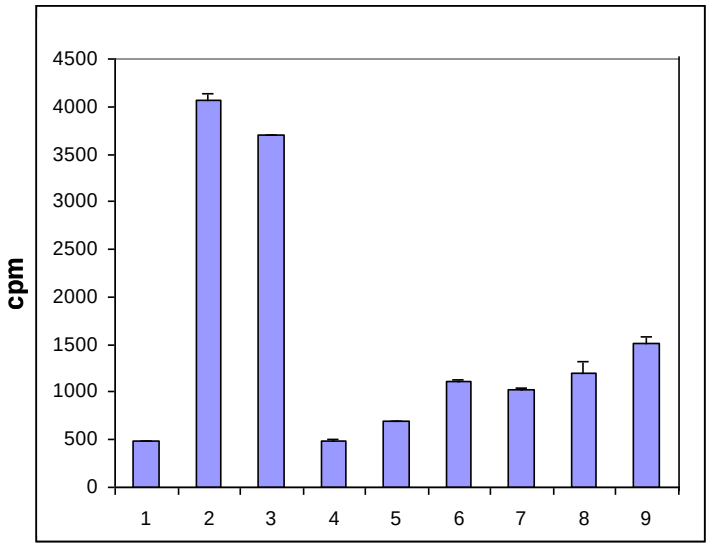
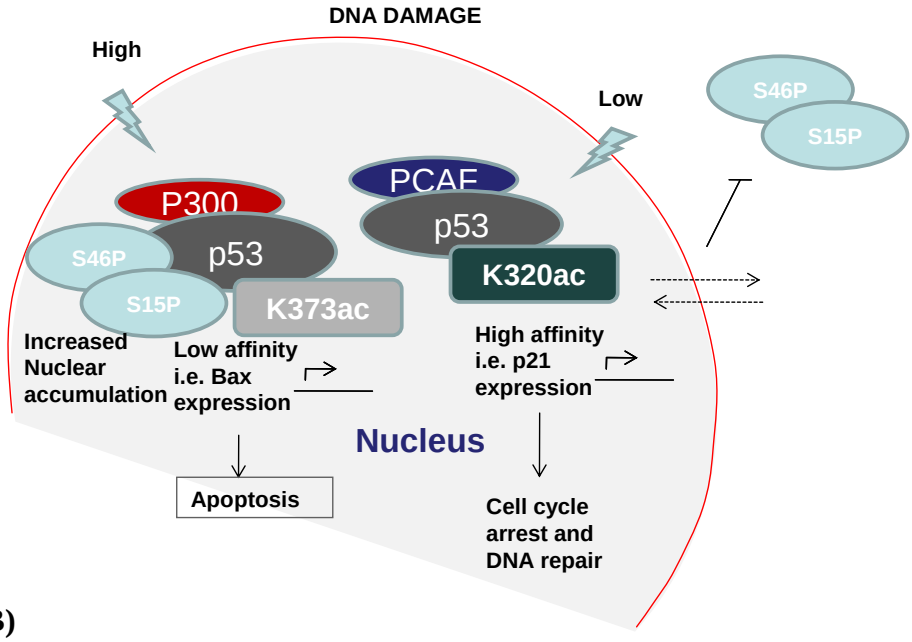


Figure 4.7. Filter binding HAT assay for inhibition of histone acetylation. *Assays were performed with hexa- histidine tagged, baculovirus expressed fulllength p300 in the presence or absence of HAT inhibitors at their reported IC50 concentrations, by using highly purified HeLa core histones. Core histones without p300 (lane 1); with p300 (lane 2); with p300 in the presence of DMSO (lane 3); Anacardic acid (10 µM) (lane 4); Garcinol (10 µM) (lane 5); Isogarcinol (10 µM) (lane 6); Curcumin (25 µM) (lane 7); LTK14 (20 µM) (lane 8); RTK1 (25 µM) (lane 9). Error bars represents standard deviation.*

p53 is an *in vivo* substrate of both these histone acetyltransferases. p300 and PCAF acetylate different lysine (K) residues, (K373 and K320 respectively) of p53 upon DNA damage (**Figure 4.8.A**). The PCAF acetylation site K320 is a site that binds to the high affinity p53 RE on *p21* and other survival associated gene promoters. Hence, this residue acetylation is a mark for cell survival. On the other

hand, the p300 acetylation site K373 binds to the low affinity p53 RE on *p21* and other apoptotic gene promoters hence skews the cell towards the apoptosis pathway.

A)



B)

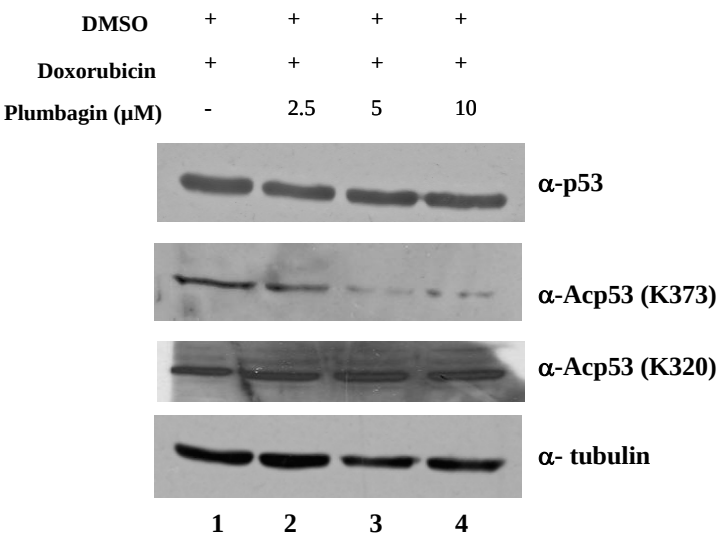


Figure 4.8. Acetylation of p53 at different sites and its functional consequences. A) *The different acetylation sites on p53 with their functional outcomes.* ***B)*** *HEK293 cells were treated with either DMSO (lane 1) or RTK1 with the indicated concentrations (lanes 2, 3, 4) for 3 hours. Following the treatment the cells were treated with doxorubicin for 6 hours. The cell lysates were subjected to immunoblotting analysis using either anti acetyl p53 K373 (panel II) or anti acetyl p53 K320 (panel III), or anti p53 DO-1 (panel I) or anti tubulin (panel IV). Tubulin and p53 antibody were used to verify the equal amount of protein loaded in each lane.*

To investigate any possible specificity of RTK1 to HAT activity in the cellular system, we selected the phenomenon of p53 acetylation. For this purpose, we treated HEK293 cells with different concentrations of RTK1 for 3 hours following which acetylation of p53 was enhanced by treating cells with doxorubicin for different time points. Whole cell lysates prepared from the treated cells (as described above) were subjected to western blotting analysis using antibodies against p53, acetyl p53 (K373), acetyl p53 (K320) and β actin. It was found that the acetylation of p53 at lysine 373 (by p300) was inhibited by treating cells with increasing concentrations of RTK1 (**Figure 4.8.B, panel II, lane 1 vs. lanes 2, 3, 4**). Significantly, a further increase in the concentration of RTK1 did not affect the acetylation of p53 at lysine 320 (by PCAF) (**Figure 4.8.B, panel III, lane 1 vs. lanes 2, 3, 4**). Levels of p53 and actin were used as protein expression controls (**Figure 4.8.B, panels I and IV**).

Yet another nonhistone substrate of p300 is positive cofactor 4, (PC4), whose transcriptional coactivator function on p53 is controlled by its acetylation (Batta et al., 2007). The *in vitro* acetylation of PC4 by p300 was found to be inhibited by RTK1 in a gel fluorography assay (**Figure 4.9**).

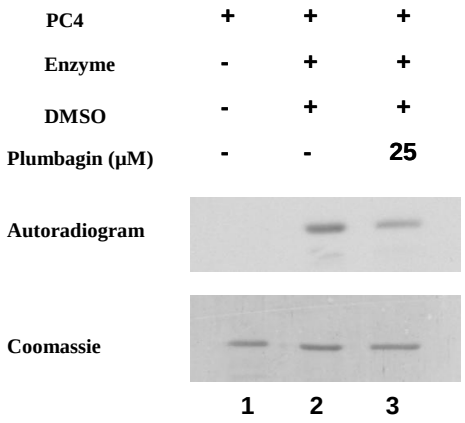


Figure 4.9: Effect of plumbagin/RTK1 on PC4 acetylation. *Gel fluorography assay of PC4 is shown with the coomassie staining as the bottom panel and the autoradiogram as the top panel. PC4 without p300 (lane 1), with p300 in the presence of DMSO (lane 2), with p300 in the presence of 25 µM RTK1 (lane 3).*

These results establish that indeed RTK1 preferentially targets p300 factor acetyltransferase activity in the physiological context. Significantly, the treatment of RTK1 in the HepG2 cells, did not alter the expression of p300 (**Figure 4.10**),

suggesting that the inhibition observed is not due to reduced protein level of p300, rather is an inhibition of the enzymatic activity.

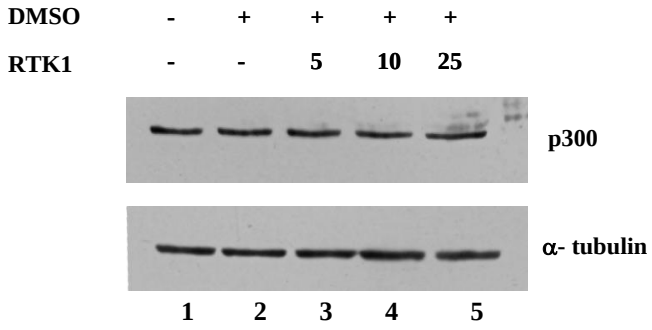


Figure 4.10. Effect of plumbagin/RTK1 on protein levels of p300. *HepG2 cells were treated as indicated, for 12 hours; lysate from untreated cells (lane 1); DMSO treated cells (lane 2); RTK1 treated cells (lanes 3-5). The levels of p300 and α tubulin was probed by immunoblotting using the respective antibodies.*

4.6. Plumbagin is a noncompetitive inhibitor of p300

In order to understand the mechanism of plumbagin mediated inhibition of p300 activity, the enzyme inhibition kinetic analysis was performed. Since, the acetylation is a bi-substrate reaction, using core histone as a main substrate and the acetyl group donor, acetyl CoA as a pseudo-substrate, the kinetic characterization was performed in two sets. In one case, the acetyl CoA concentration was kept constant whereas the histone concentration was varied. In the second experimental set up, the core histone concentration was kept constant and the acetyl CoA concentration was varied. The kinetic analysis of the RTK1 mediated inhibition of full length p300 acetyltransferase, revealed a noncompetitive pattern with both histones and acetyl CoA substrates (**Figure 4.11 A and B**).

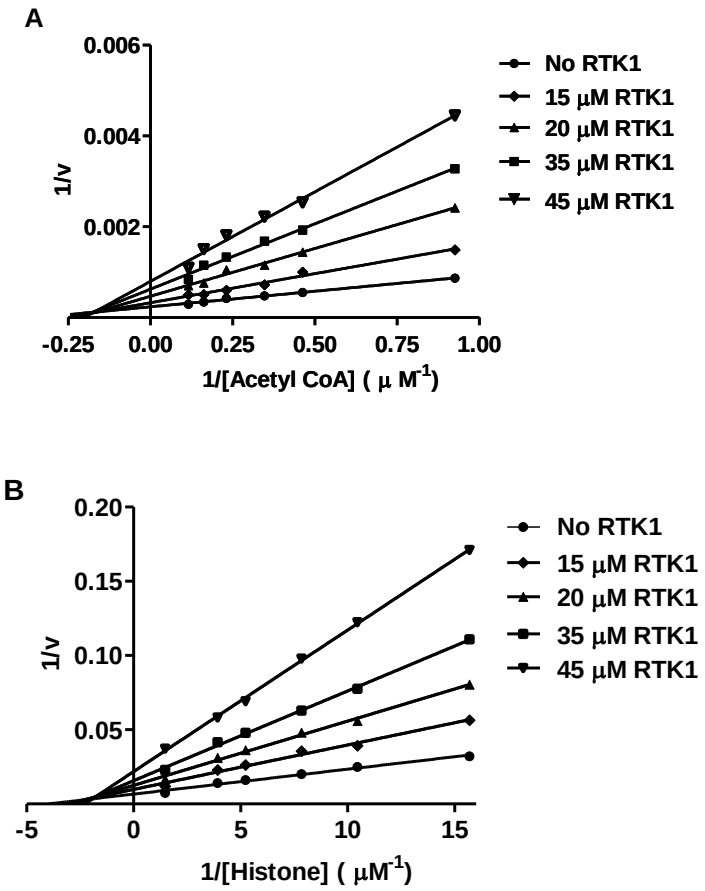


Figure 4.11.A), B) Kinetics of full length p300 HAT inhibition by RTK1*. Lineweaver Burk Plot representation of RTK1 mediated inhibition of p300 HAT activity at a fixed concentration of core histones (1.6 μM) and increasing concentration of [³H-acetyl CoA] in the presence (15, 20, 35, 45 μM) or absence of RTK1. D) Lineweaver Burk plot representation of RTK1 mediated inhibition of p300 HAT activity at a fixed concentration of [³H-acetyl CoA] (2.78μM) and increasing concentration of corehistones in the presence (15, 20, 35, 45 μM) or absence of RTK1. The results were plotted using the Graph Pad Prism software.*

The mechanism of inhibition therefore involves the inhibitor binding to a site other than the active site of the enzyme which was subsequently confirmed by docking studies and also isothermal titration calorimetry. The baculovirus expressed full length p300 often gets degraded and may also contain some interacting proteins even after high quality purification. Since, the biophysical experiments require proteins which are highly pure, the subsequent experiments were carried out with the

bacterially expressed p300 minimal HAT domain (aa 1284-1673) described in **methods section, 2.2.8.** RTK1 inhibits the HAT activity of the p300 catalytically active domain with very low IC_{50} value of 2 µM indicating the possible interaction of RTK1 with this domain (**Figure 4.12**).

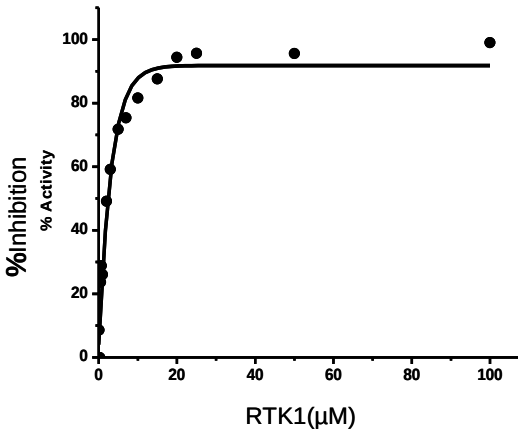
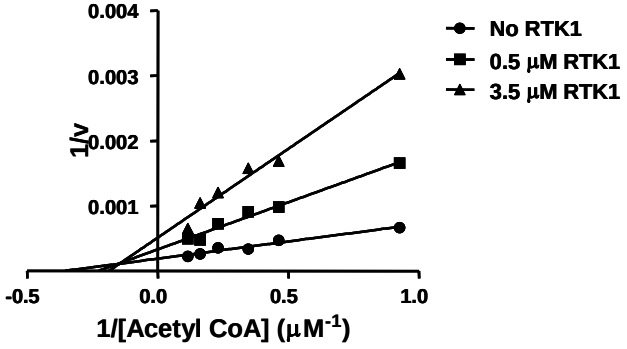


Figure 4.12. Effect of plumbagin/RTK1 on p300 minimal HAT domain. *Percentage of inhibition of HAT activity, performed with p300 minimal HAT domain in the presence or absence of RTK1 (0.5- 100 µM), by using highly purified HeLa core histones and processed for filter binding assay.*

RTK1 could inhibit the minimal HAT domain more potently than the full length p300. There was a reduction in the IC_{50} value (2 µM) for HAT domain which is ten fold lower than the IC_{50} value (20 µM) of full length p300. However, the pattern of HAT inhibition was similar to that of p300 full length i.e. noncompetitive in nature (**Figure 4.13. A and B**). These results further suggest that RTK1 augments its p300 HAT inhibitory activity by directly targeting the minimal HAT domain of p300. The HAT domain is a minimal catalytic domain which consists of the region responsible for acetyltransferase activity. Further, it should be noted that full length p300 has the CH1, CH2, CH3, bromodomain, repressive KIX domain and the unstructured glutamine (Q) rich region (**Figure 4.14**). The site of interaction of RTK1 may not be easily accessible in the full length protein due to its multi domain organization. However, in the minimal HAT domain the interaction site might be easily accessible, which could be the cause of reduction in the IC_{50} value. In agreement with this data, it has been shown that the p300 specific inhibitor LTK14,

also has a ten times lower IC_{50} value with the HAT domain than the full length enzyme.

A)



B)

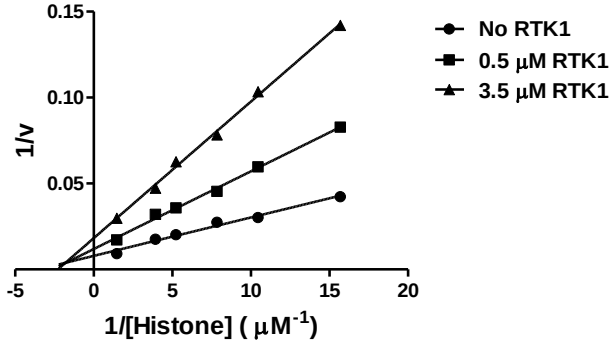


Figure 4.13.A) and B) Kinetics of p300 HAT domain inhibition by RTK1*: C) Lineweaver Burk Plot representation of RTK1 mediated inhibition of p300 HAT activity at a fixed concentration of core histones (1.6 µM) and increasing concentration of [³H-acetyl CoA] in the presence (0.5 µM or 3.5 µM) or absence of RTK1. D) Lineweaver Burk plot representation of RTK1 mediated inhibition of p300 HAT activity at a fixed concentration of [³H-acetyl CoA] (2.78µM) and increasing concentration of corehistones in the presence (0.5 µM or 3.5 µM) or absence of RTK1. The results were plotted using the Graph Pad Prism software.*

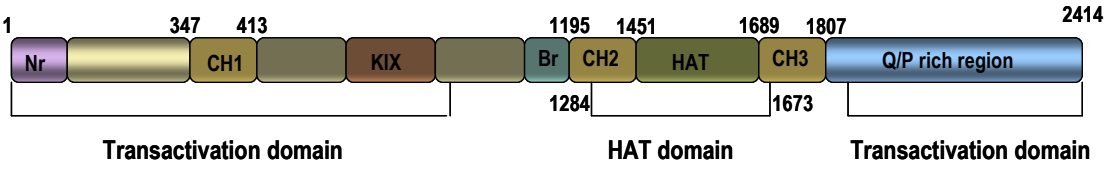


Figure 4.14. Domains of histone acetyltransferase p300. *Apart from the minimal HAT domain (1284-1673), three CH domains, a single bromodomain and the KIX domain are present.*

4.7. Plumbagin binds to p300 HAT domain at a single site

Considering all the above parameters, it was evident that plumbagin is indeed a p300 specific inhibitor with more affinity towards the HAT domain. To further verify this observation and also to obtain a thermodynamic perspective of the inhibitor and p300 HAT domain interaction, the binding of the inhibitor to the enzyme was also confirmed from isothermal titration calorimetry studies (**Figure 4.15**). The interaction was found to be entropy driven with high affinity (K_d = 2 ± 0.25 μM, ΔH = - 2.25 ± 0.23 kcal/mole, ΔS = 17.2 e.u.).

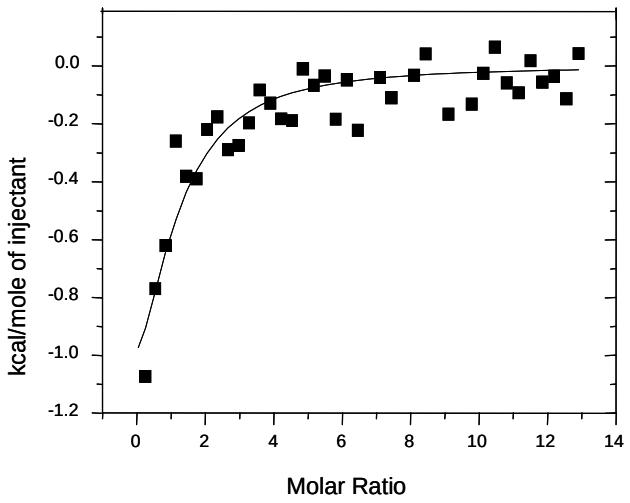


Figure 4.15: Determination of enthalpy of association of RTK1 with p300 HAT domain*. Iosthermal calorimetric titration of 3.4 μM p300 HAT domain into 200μM RTK1 was carried out at 25 ºC in 10 mM Tris pH 7.5. The exothermic heat exchanged per mole of injectant as a function of molar ratio of HAT domain to RTK1 is represented. The data were fitted with "One set of sites" binding model. Solid line represents the fit of the binding isotherm.*

The binding isotherm could be fitted to 1:1 stoichiometry. The magnitude of enthalpy value is consistent with the range of enthalpy values reported for a hydrogen bond such as the one, proposed between RTK1 and p300 HAT domain. The source of positive entropy may be twofold: release of bound water from the surface of the protein upon association with the ligand and conformational entropy change of the protein as a sequel to binding. From the above observations, it can be proposed that RTK1 binds to HAT domain at a single site which is not a part of the active site. The

dissociation constant value agrees well with the IC_{50} value (2μM) as estimated from the **Figure 4.16**, thereby supporting the validity of the thermodynamic parameters generated from ITC. Apart from this a fluorescence titration was also performed to further validate the dissociation constant value (**Figure 4.16**). The K_d value obtained from the fluorescence is 6.38 ± 0.95 μM, which is similar to the value (2 μM) obtained by the isothermal titration study. The Equation used for fitting the values is as follows:

1/(Fo-F) = 1/Fmax + Kd/Fmax(1/[L])

Fo Fluorescence intensity of HAT domain (1.1μM) without plumbagin

F Fluorescence intensity of HAT domain (1.1μM) with plumbagin

Fmax Y-intercept

Kd Dissociation constant of binding of plumbagin with HAT domain

L Concentration of plumbagin in μM.

Equation for inner filter effect: Fcorrected = Fobs. Antilog [(Abs290 nm + Abs 320 nm)/ 2]

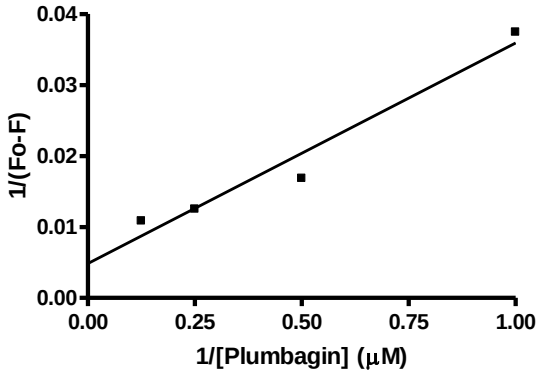
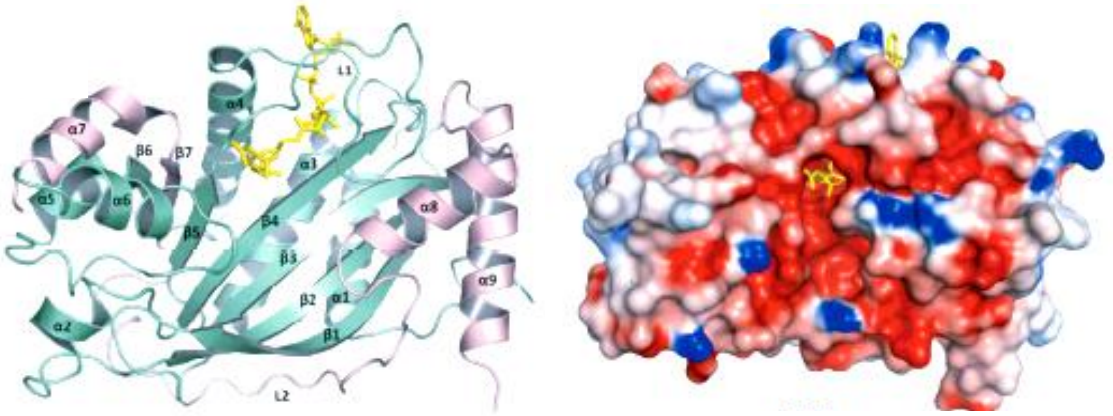


Figure 4.16. Curve fitting analysis to evaluate the dissociation constant for the association of plumbagin (RTK1) with the HAT domain *in buffer containing 10 mM Tris, pH 7.9, 0.2 mM EDTA, pH 8.0 and 100mM KCl at 25°C. The protein concentration used was 1.1μM for each point and the inhibitor concentration is depicted in the X axis.*

4.8. Plumbagin/RTK1 docks on to the p300 HAT domain through hydrogen bonding to K1358 residue with functional consequences

The crystal structure of the acetyltransferase domain of p300 HAT liganded to a synthetic specific inhibitor, Lysyl CoA has been reported recently (**Figure 4.17**).



Adapted from Liu et al., 2008

Figure 4.17. Overall structure of the p300 HAT domain *with N and C subdomains coloured in green and pink, respectively. Lys-CoA is shown in yellow stick figure representation. Electrostatic surface representations of the HAT domains of p300, Lys-CoA in the p300 HAT domain*

Using this structure, the docking studies with RTK1 has been performed. The p300 HAT domain and inhibitor complex showed the interaction of the hydroxyl group on the 5th Carbon in RTK1. The residues involved in the interaction are LYS 1358, GLU 1380, MET 1376 and TYR 1421 (**Figure 4.18**). As per the docking model, the -OH group interacts with LYS 1358 on HAT domain and further facilitates the further interaction with the remaining three residues (**Figure 4.19 Panel II**).

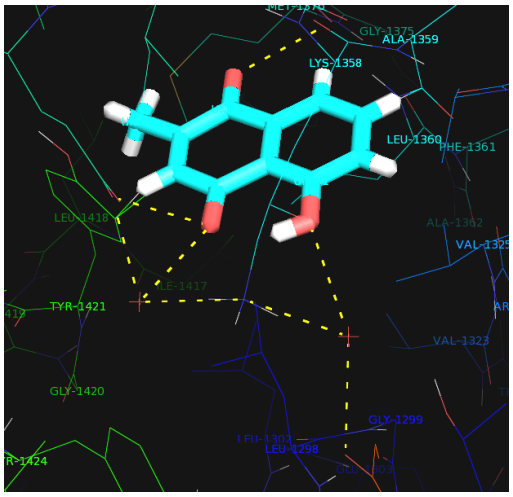


Figure 4.18. *The inhibitor plumbagin forms hydrogen bond with the K1358 residue which facilitates the interaction with the other three residues, E1380, M1376, Y1421.*

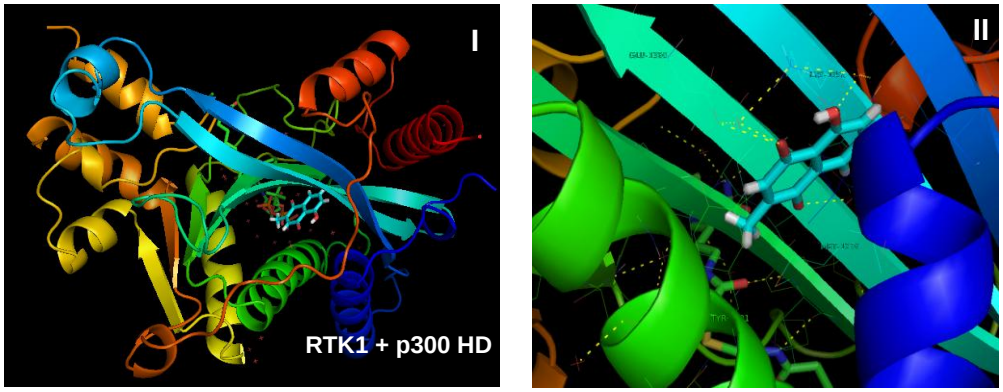


Figure 4.19. *Docking of RTK1 on to the p300 HAT domain (Panel I), Enlarged image of the docking site with the contact residues (Panel II).*

Since the lysine residue was found to be the primary residue mediating the interaction and the ITC data also suggested a single binding site, this residue (K1358) was mutated to alanine. An *in silico* mutant K1358A was generated which did not show any interaction with the inhibitor (**Figure 4.20**).

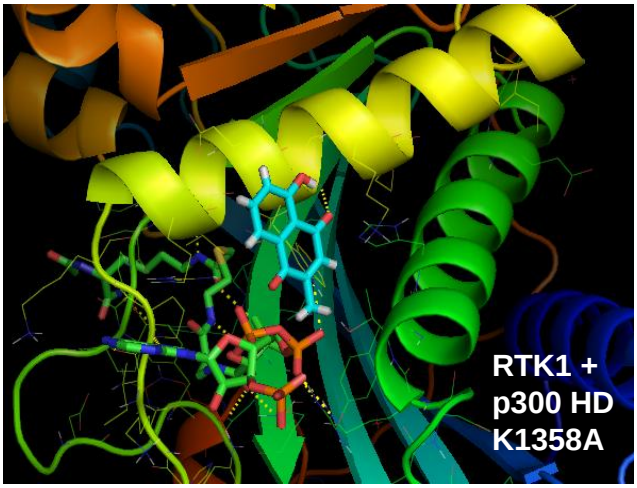


Figure 4.20*. Docking of RTK1 on to the in silico generated mutant K1358A.*

Subsequently, the same mutant was cloned and expressed as described in the methods section. The mutant K1358A showed a similar tryptophan fluorescence spectra as the wild type, suggesting that the overall protein structure did not alter due to the mutation (**Figure 4.21**).

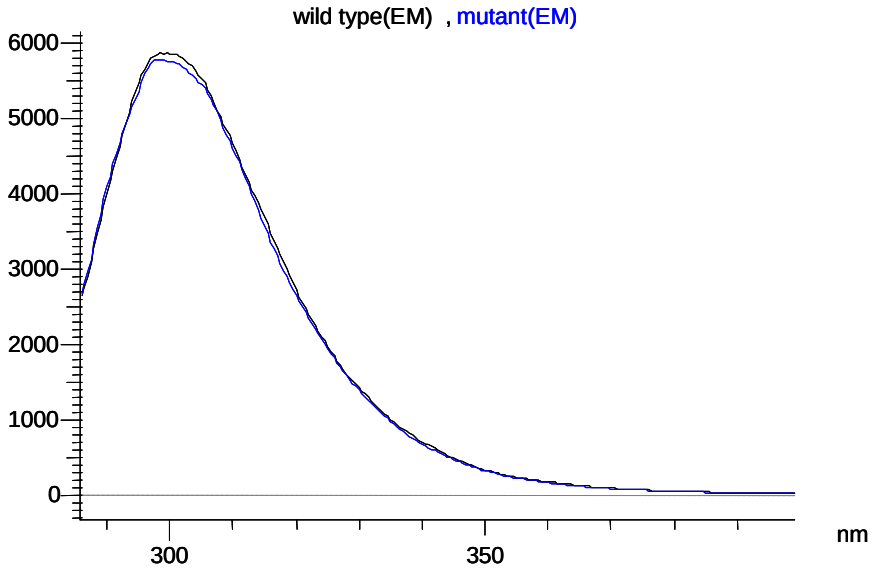


Figure 4.21. ***Fluorescence emission spectrum of p300 minimal HAT domain*** *(Wildtype and mutant K1358A), by excitation at 280 nm. Wildtype (black line), Mutant (blue line).*

Remarkably, this lysine residue was found to be absolutely critical for the HAT activity. It was found that the HAT domain mutant (K1358A), completely lost its acetyltransferase activity in a filterbinding assay (**Figure 4.22**).

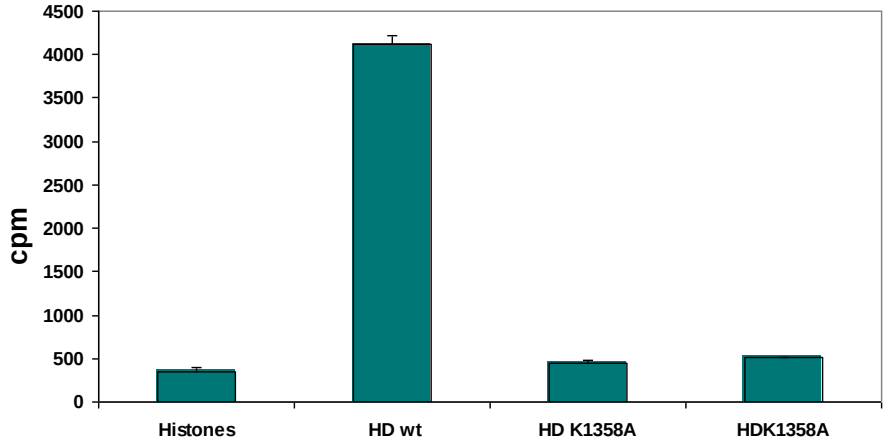
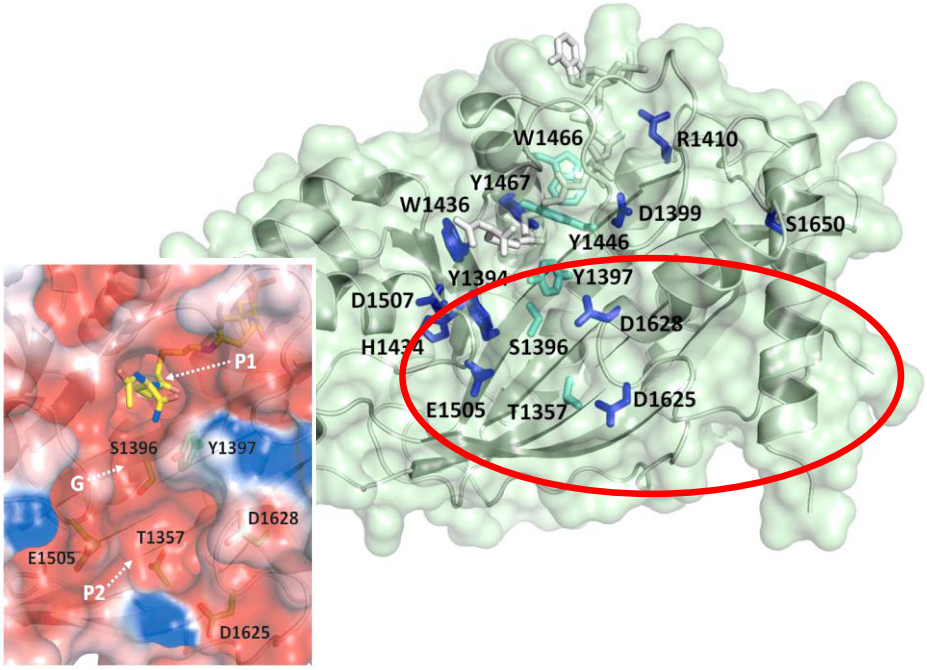


Figure 4.22: ***Filter binding HAT assay for histone acetylation***. *Assays were performed with p300 HAT domain (HD wildtype) or HD K1358A by using highly purified HeLa core histones. Core histones without enzyme (lane 1); with HD wildtype (lane 2); with HD K1358A (lane 3); with increased concentration of HD K1358A (lane 4). Error bars represents standard deviation.*

The small molecule inhibitor RTK1, through its hydroxyl group possibly forms a specific interaction with a single lysine residue (K1358) in the p300 HAT domain. The K1358 residue, is not a part of the activation loop of p300 and has not been mapped as an important residue in the p300 HAT domain structure. However, one of its adjacent residue T1357 has been shown to be essential for the structural maintenance of the HAT domain. The T1357 residue is a component of the negative environment chamber for the activation loop (**Figure 4.23**).



Adapted from Liu et al., 2008

Figure 4.23*. The presence of the K1358 residue in the vicinity of the T1357 residue which is a part of the activation loop.*

Presumably, the K1358 residue also plays an essential role in the activation loop stability directly and/or by affecting the chemical environment around the T1357 residue and hence the acetyltransferase function. The lysine residue K1358 is in the proximity of the following residues in the HAT domain (M1376, H1377, V1378, E1380, H1327, D1614 as represented in **Figure 4.24.** The exact significance of these proximal residues and K1358 needs to be investigated in the light of their essentiality for acetyltransferase activity.

4.9. Single hydroxyl group of Plumbagin/RTK1 is essential for HAT inhibition

The docking study and the point mutation of K1358A, indicated that the single hydroxyl group of RTK1 is possibly involved in interacting with the HAT domain. Therefore, the chemical entity in 'RTK1', which could be involved in the inhibition of p300 HAT activity was investigated. RTK1 was derivatized by using the single hydroxyl group at 5th carbon position with various substituents (**Figure 4.25**).

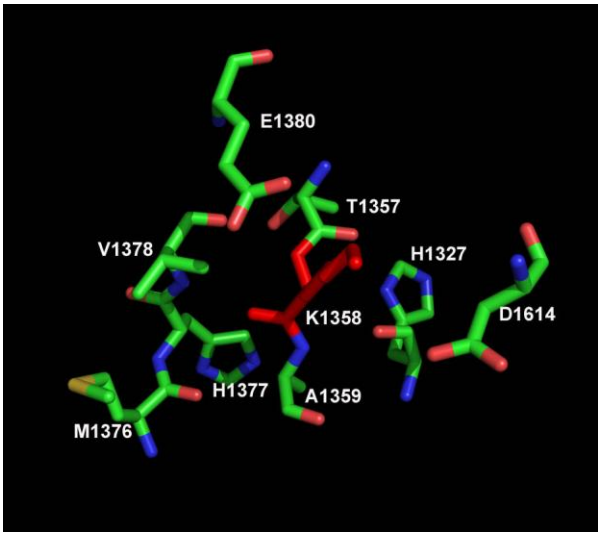


Figure 4.24*. K1358 residue (shown in red) in the p300 HAT domain with its proximal amino acid residues.*

Alkyl substitutions at the –OH position which increased the chain length could not inhibit HAT activity (**Figure 4.26, lanes 5-7**). When groups like acetyl and sulfonyl were substituted for hydroxyl group of RTK1, a mild reduction (<10%) of HAT activity was observed (**Figure 4.26, lanes 8-9**). Heterocyclic substitutions such as piperidine, morpholine and an ester substitution also showed a mild reduction of HAT activity (**Figure 4.26, lanes 10, 11, 12**). However, N,N-dimethylamine substituent did not affect the HAT activity (**Figure 4.26, lane 13**). All the derivatives were checked for their HAT inhibitory potential at the IC_{50} value (20 µM) of RTK1.

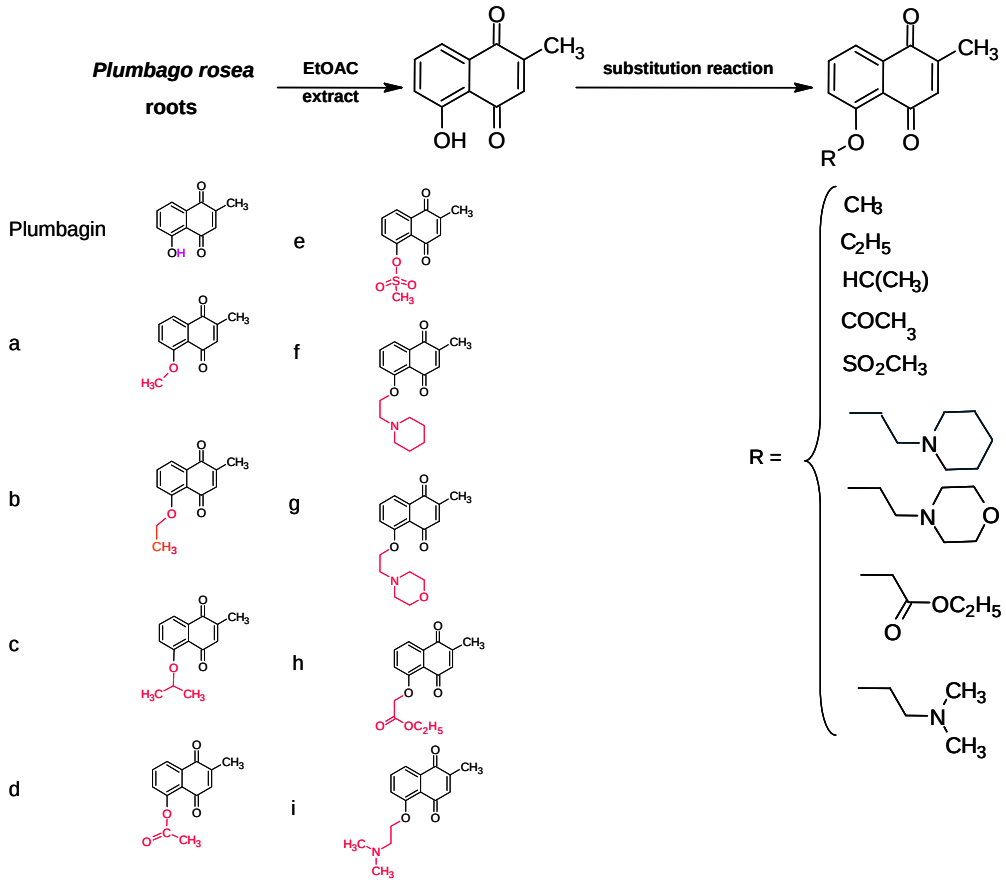


Figure 4.25. *Synthesis scheme and structures of RTK1 and its derivatives (a-i).*

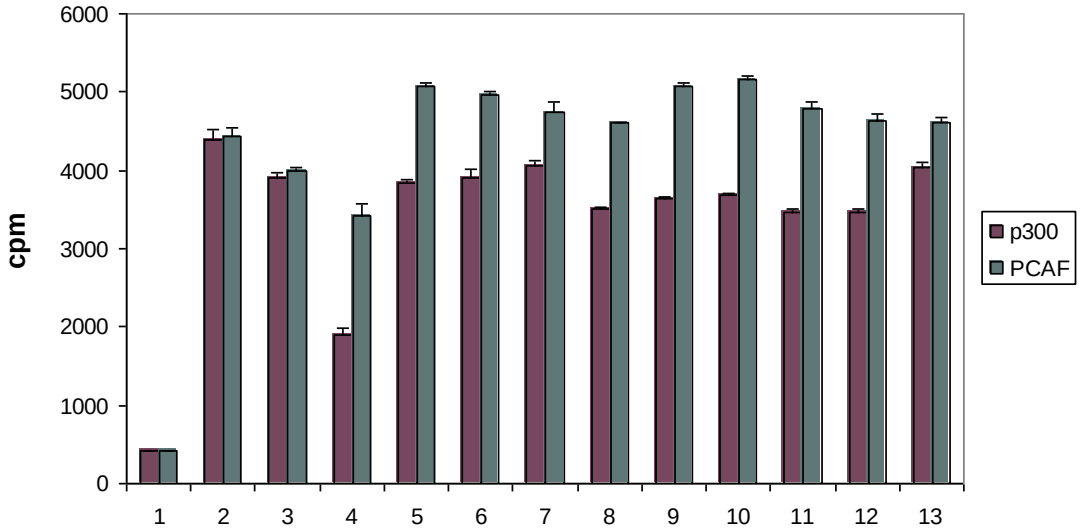


Figure 4.26. *HAT assays were performed either with p300 (I) or PCAF (II) in the presence or absence of RTK1 and its derivatives by using highly purified HeLa core histones. Core histones without enzyme (lane 1); with enzyme (lane 2); with enzyme in the presence of DMSO (lane 3); RTK1 (25µM) (lane 4), RTK2-RTK10 [Scheme I] at 50µM concentration (lanes 5-13 resp.). Error bars represent standard deviation.*

As compared to RTK1, all the derivatives were inactive for HAT inhibition at the tested concentration. Thus, the substitution of the hydroxyl group with any other group almost abolishes the inhibition activity, suggesting that the 'hydroxyl' group in RTK1 is essential to bring about the HAT inhibition (**Figure 4.26, lane 4 vs lanes 5-13**). One of the derivative, RTK2 **(Figure 4.26, panel II**), a methoxy substituted compound was crystallized and selected as a representative of the inactive molecules for the detailed characterization. This molecule was confirmed by NMR spectroscopy (**Figure 4.27)** and used for further characterization.

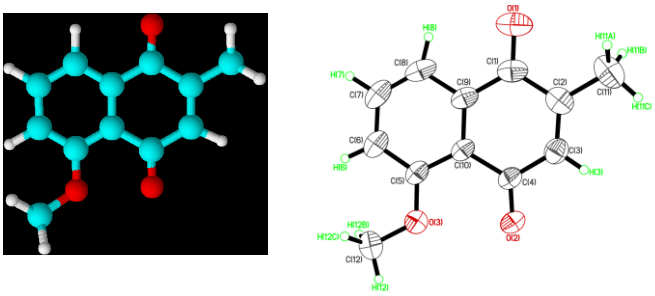


Figure 4.27*. Ball and stick model of methoxy substituted derivative RTK1 (first panel), X ray crystal structure of hydroxyl group substituted RTK1, RTK2 (methoxy derivative), second panel.*

To further validate if there are any differences in the interaction (binding) of the hydroxyl derivative with the HAT domain in comparison with the parent compound, plumbagin, an isothermal titration calorimetric study with RTK2 and p300 HAT domain was carried out. The enthalpy change corresponding to the addition of RTK2 to p300 HAT domain could not be fitted to any binding model as apparent from the improbable large values of standard errors of deviation (binding site N= 1.06 ± 338, ΔH = 946 ±30000 kcal/mole). It implies absence of any physical association of the –OH group substituted derivative, RTK2 with p300 HAT domain (**Figure 4.28**), This is in contrast with the strong interaction of RTK1 with p300 HAT domain.

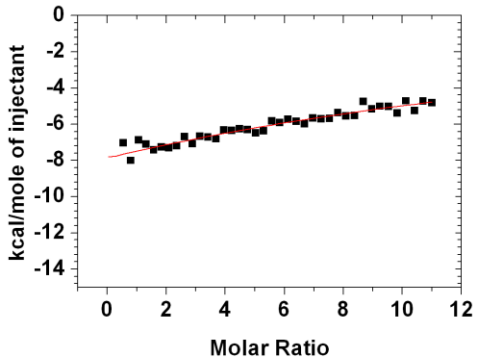


Figure 4.28: Estimation of enthalpy of association of RTK2 with p300 HAT domain*. Isothermal calorimetric titration of 3.4 µM p300 HAT domain into 200 µM RTK2 at 25 ℃ in 10 mM Tris pH 7.5. Shown is the exothermic heat exchanged per mole of injectant as a function of molar ratio of HAT domain to RTK2. The data could not be fitted with any binding model giving rational values for enthalpy change and dissociations constant. It implies absence of association.*

The absence in interaction was also further confirmed from the docking analysis of the HAT domain with RTK2, which does not show the formation of hydrogen bond or any other interaction with RTK2 (**Figure 4.29**). These data argue for the mechanistic and structural requirement of -OH group to facilitate the p300 HAT inhibition.

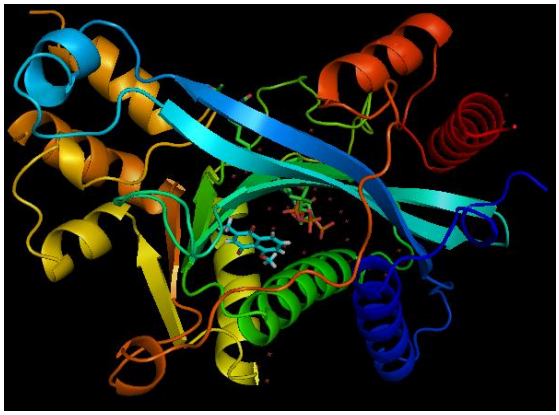


Figure 4.29. *Docking of RTK2 on to the p300 HAT domain*

The earlier work on acetylation inhibitors has shown that several natural compounds, like anacardic acid (Balasubramanyam et al., 2003), curcumin (Balasubramanyam et al., 2004), garcinol (Balasubramanyam et al., 2004) possess

HAT inhibitory activity. Interestingly, all these molecules are hydroxyl compounds (**Figure 4.30**). The recently reported HAT inhibitor from natural source, Epigallocatechin-3-Gallate (EGCG) is also a polyhydroxy compound (Choi et al., 2009). Anacardic acid has a single hydroxyl group and is a potent and nonspecific inhibitor of HATs. The amide derivative of anacardic acid, CTPB was found to be a HAT activator and not inhibitor. A detailed derivatization study of these amides revealed the importance of the pentadecyl hydrocarbon chain and the presence of electronegative groups (-CF_3, -Cl) at the para position for the HAT activation (Mantelingu and Kishore et al., 2007).

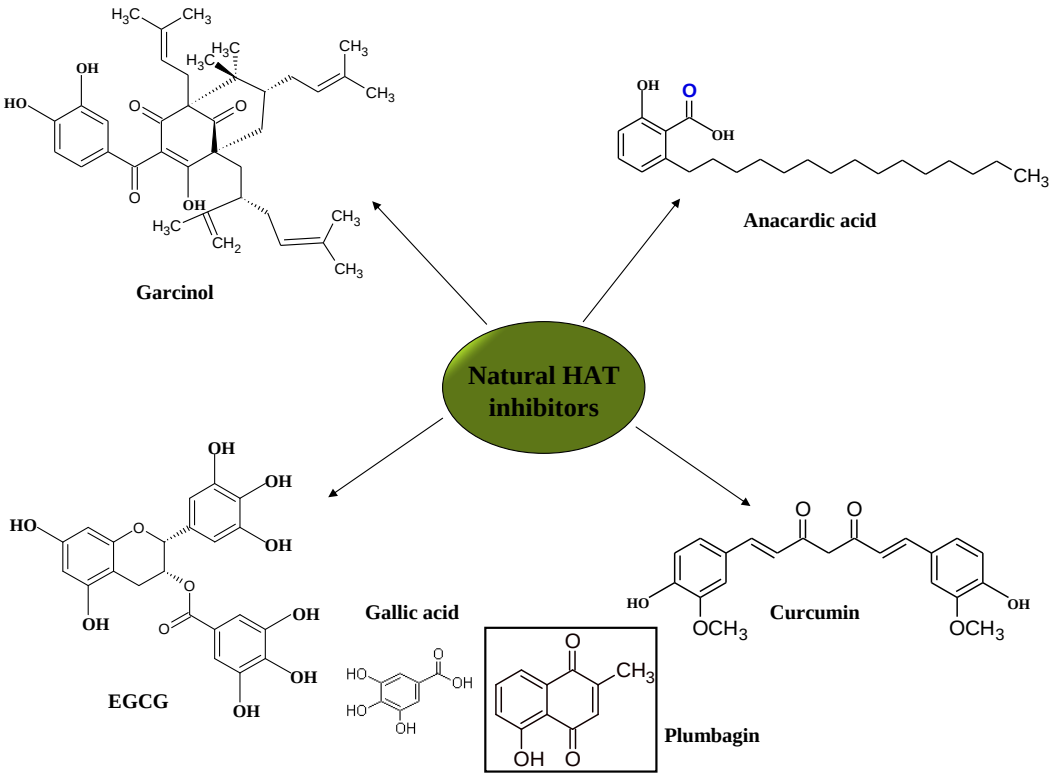


Figure 4.30: Structures of different HAT inhibitors from natural source

Curcumin, a natural p300 HAT specific inhibitor, also has two hydroxyl groups. Garcinol, a polyhydroxyl benzophenone, which has three hydroxyl groups is a potent nonspecific HAT inhibitor with high cellular permeability and cellular toxicity. The cyclization of garcinol, through the enolic hydroxyl group, yielded

isogarcinol. Although, isogarcinol retained the nonspecific, HAT inhibitory activity, it was found to be less potent (with higher IC_{50}) as compared to garcinol. The 14, mono methyl substituted derivative of isogarcinol, LTK14 has a single hydroxyl group. This derivative is a specific inhibitor of p300 HAT and is almost nontoxic in nature (Mantelingu and Reddy et al., 2007). However, LTK14 is a less potent HAT inhibitor in comparison with garcinol. This raises the possibility that multiple hydroxyl groups in garcinol might be responsible for its potent HAT inhibition ability. In agreement with the obtained results, the disubstitution of isogarcinol yielded compounds (devoid of any hydroxyl groups) that could not inhibit the HAT activity. The only exception is the methyl-suphonyl disubstituted isogarcinol (LTK19), which was also found to be a p300 HAT specific inhibitor. However, the similar functional group substitution in RTK1 did not show any inhibitory activity. One of the new acetylation inhibitor gallic acid which is a non specific acetylation inhibitor is also a poly hydroxy functional group containing compound. This indicates that probably the –OH group in the context of the acetyltransferases seems to be an essential chemical entity for HAT inhibition (**Figure 4.30**).

In summary, this chapter explains the characterization of a small molecule inhibitor of the lysine acetyltransferase (KAT), p300 from a natural source which also led to the elucidation of the chemical entity responsible for the inhibition of the KAT activity (Ravindra and Selvi et al., 2009). All the natural KAT inhibitors (specific and nonspecific) discovered so far possess 'hydroxyl' group. The substitution of the single 'hydroxyl' group with any functional group (**Figure 4.25**), present in plumbagin / RTK1 completely ceases its ability to inhibit the KAT activity. These data suggest that possibly, the 'hydroxyl' group-mediated interaction of RTK1 with p300 could be essential for the inhibition of acetyltransferase activity, as hypothesized previously. This presumption could be general and might be one of the mechanisms associated with HAT inhibition by these inhibitors.

Interestingly, in the p300 minimal HAT domain, a novel site K1358 has been identified which is essential for the acetyltransferase activity of p300. This residue is not a part of the catalytic site nor the activation loop, yet regulates the enzyme function. The exact role of this residue needs to be characterized. Furthermore, the

interaction of the other p300 HAT inhibitors with this critical residue also needs to be investigated. The reported mutation studies for p300 in various cancers ranging from glioblastoma (4%), squamous cell carcinoma (67%) to lung cancer (8%), show several frame shift mutations wherein this residue is lost due to an earlier frame shift event (reviewed in Iyer et al., 2004). However, no deletion or point mutation report with respect to lysine 1358 is available. Only a detailed analysis of different patient samples of different disease states might address this concern.

Plumbagin has been reported to suppress NF-κB activation leading to the potentiation of apoptosis (Sandur et al., 2006). Since, the process of NF-κB activation requires p300 mediated acetylation of the p65 subunit of NF-κB, the suppression observed could be due to the p300 specific inhibition in the physiological condition. Although, plumbagin is a putative anticancer agent (Shih et al., 2009), its major limitation to be used as therapeutic molecule could be the cellular toxicity. The recent report of plumbagin interaction with the tubulin and modulating tubulin dynamics (Acharya et al., 2008) signifies its role in cytoskeleton mechanisms which might be the reason for its cytotoxicity. However, the scaffold identified through plumbagin is a small scaffold which needs to be exploited for the synthesis of efficient nontoxic p300 specific KAT inhibitors, with an intact hydroxyl group, could be highly useful, both as a therapeutic as well as a biological tool for modulating acetyltransferase function (reviewed in Swaminathan et al., 2007).

Chapter 5

Histone Arginine methylation and Lysine acetylation in transcription regulation; probed by the enzyme specific inhibitors.

This chapter highlights the role of arginine methylation and acetylation in the regulation of gene expression, probed by the two specific inhibitors, TBBD and Plumbagin of CARM1 and p300 respectively. Besides investigating the p53 responsive gene expression,a detailed global gene expression analysis has also helped in understanding the role of arginine methylation in several signaling and developmental pathways, few of which were known but many unknown. In an attempt to understand, these events in distinct cellular states with respect to both the modifications, the embryonic stem cell line was used. The inhibitors could repress the pluripotency associated marker gene expression implicating the role of arginine methylation and lysine acetylation in maintenance of pluripotency. Finally, the breast cancer cell line, MCF7 was used to explore the possibility of TBBD as a therapeutic agent and the results obtained will also be discussed.

Chapter outline:

5.6. Histone H3R17 methylation regulates important signaling and ribosome biogenesis associated genes.

5.7. Effect of H3R17 methylation inhibition on breast cancer cell line.

5.1. Transcription regulation by arginine methylation.

The role of arginine methylation in transcriptional regulation is a three-pronged effect as represented in **Figure 5.1.**

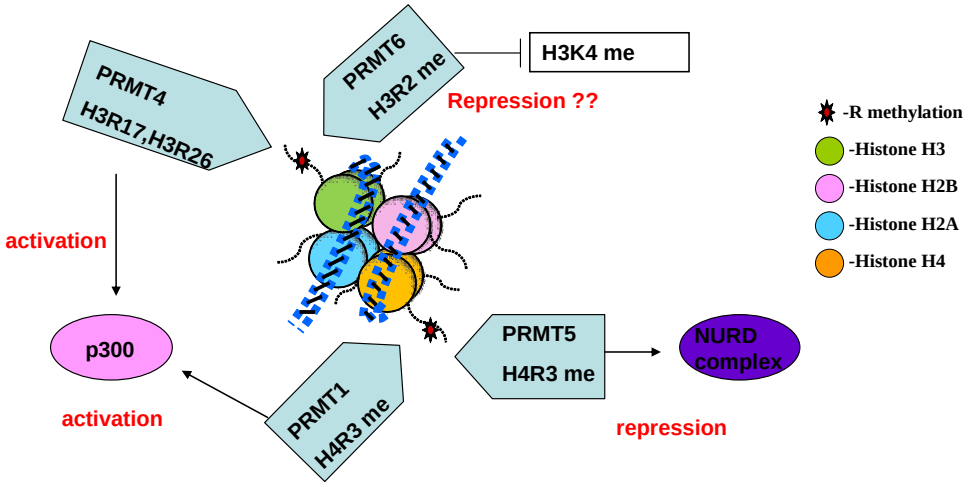
I. Histone arginine methylation of H3R2, H3R17, H3R26 and H4R3, which leads to the recruitment of different transcription associated machineries and thereby regulates the transcriptional output. Histone H3 R17 and R26 methylation by CARM1 are associated with the p300 mediated K18 acetylation (Daujat et al., 2002) and hence are marks of transcriptional activation. Similarly, histone H4R3 methylation by PRMT1 aids in p300 acetylation and hence also forms a transcriptional activation network (An W et al., 2004). The symmetric arginine methylation of histone H4R3 by PRMT5, is associated with the action of the DNA methyltransferase and deacetylase associated with the NURD complex (Le Guezennec et al., 2006) which are components of the PRMT5 complex, hence leads to transcriptional repression. An indirect evidence of negative regulation by H3R2 is also shown wherein PRMT6 mediated H3R2 methylation acts as a deterrent to histone H3K4 methylation by the MLL complex, which is a mark of transcriptional activation (Kirmizis et al., 2007, Guccione et al., 2007). Thus, possibly the histone H3R2 modification is a negative modulatory modification.

II. Arginine methylation of nonhistone proteins modulates the transcriptional outcome by different mechanisms. Apart from the histone modifications, the arginine methylation of CBP by CARM1 is also known which regulates the transcriptional co-activation property of CBP (Chevillard-Briet et al., 2002). Modification of Spt5 facilitates transcriptional elongation (Kwak et al., 2003), arginine methylation of HIV Tat protein regulates the viral gene transcription (Boulanger et al., 2005). Although, this is not a generalized mechanism, it implicates that such individual events can regulate the transcriptional efficiency of any particular system.

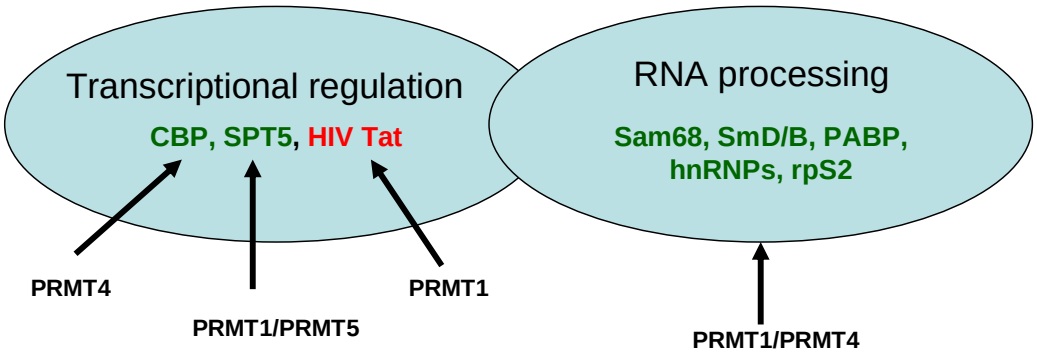
III. Finally, the transcription efficiency can also be modulated by the arginine methyltransferases themselves, since enzymes such as PRMT1 and PRMT4 are transcriptional co-activators (Stallcup et al., 2000, Koh et al., 2001).

Each of the above mechanisms can also be influenced by other epigenetic marks associated with transcriptional activation such as lysine methylation and acetylation. The co-existence of modifications have been shown on several promoters poised for transcriptional activation (Zhang et al., 2009). The histone arginine methylation mark has not yet been tested for its whole genome occupancy, partly due to the debate that still exists on whether this modification is a general transcription associated mark or a specific promoter associated mark.

A) Histone methylation



B) Nonhistone protein methylation



C) Co-activation

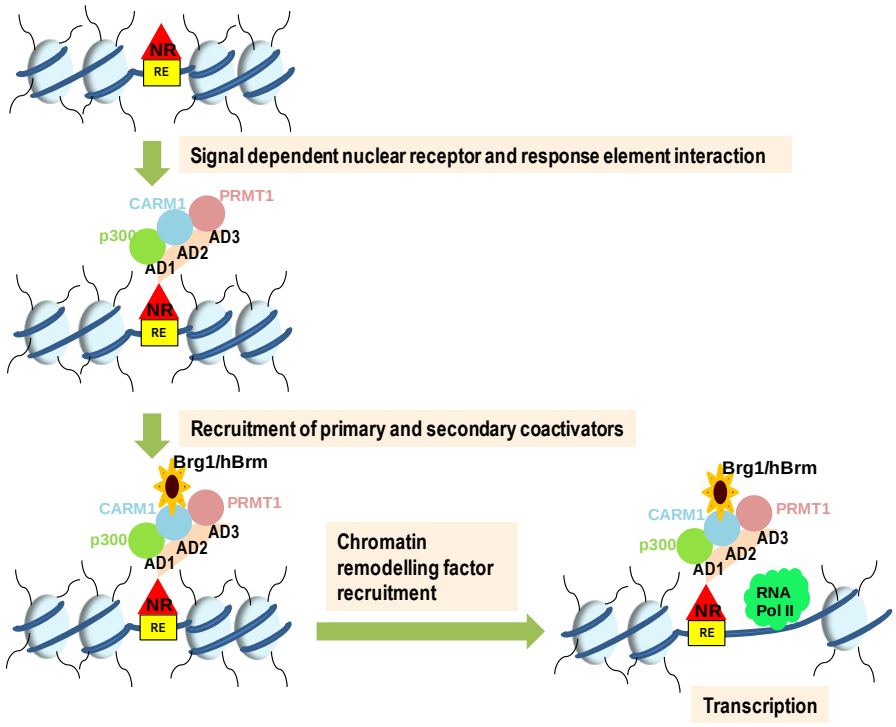


Figure 5.1. Transcriptional regulation by arginine methylation. *A) Histone arginine methylation by different PRMTs and their modification sites on the core nucleosome structure are represented. Based on the enzyme catalyzing the modification, the recruitment of different complex is facilitated resulting in transcriptional activation/repression. H3R2 methylation has been shown to antagonize the activation associated H3K4 methylation, but no direct evidence of H3R2 in transcriptional repression has been shown, hence the question mark. B) Several nonhistone proteins are methylated by the arginine metyltransferases which regulate transcription both at the initiation as well as elongation level. Further, RNA processing has also been shown to be regulated by arginine methylation. The substrates represented in green indicate modifications that have a positive regulatory effect whereas the substrate represented in red has a negative effect. The arrows indicate the enzymes that act on the respective substrates. C) The PRMTs are co-activators which in co-operation with other co-activators like p300 regulate the transcriptional activation property .The co-activators help in recruiting the remodeling machinery and thus facilitate the process of transcription.*

5.2. Arginine methylation and its associated epigenetic mark, acetylation regulates p53 downstream target gene expression:

The DNA damage associated p53 transcriptional activation is one such example wherein this co-operativity has been fine mapped to the ordered recruitment of

PRMT1, p300 and CARM1 in bringing about the histone modifications and thereby regulating the transcription of p53 responsive genes (An et al., 2004). The molecular dissection of the co-operativity can be answered in many ways, and one of the time tested solution is to use inhibitors of the key players in the system. Therefore the search for the inhibitors of the arginine methyltransferase and histone acetyltransferases was initiated, not only to use it as a probe to understand the enzyme mechanism , but more so as a biological tool to fine map the co-operativity and thereby understand their role in global gene regulation and also in disease manifestation.

Tumor suppressor, p53:

The nonhistone protein p53 is a well known tumor suppressor protein (Winchester, 1983), regarded as the guardian of the genome. Majority of cancers show mutations in this gene and thus its role in DNA damage response and other repair pathways are well recognized and acknowledged. All the physiological events are mainly regulated by the role of p53 in transcription, wherein it aids activation/repression (Dony et al., 1985). The p53 dependent DNA damage pathway is one of the properly worked out signal dependent activation system with new facets of molecular recognition being discovered frequently. This regulation is brought about by several factors namely the protein-protein interactions as well as the post translational modifications (reviewed in Yang and Seto, 2008). Incidentally, p53 was the first nonhistone protein acetylation substrate with functional significance to be identified (Gu and Roeder, 1997), alongside the identification of acetyltion of important transcription factors (Imhof et al., 1997). These discoveries brought about a revolutionary change to the field of acetylation which was initially considered to be restricted to histones and thus considered to be involved in the structural organization alone. However, the identification that a nonhistone protein can also be acetylated indicated that this modification might have important regulatory roles within the cellular system. Since then there have been several nonhistone protein substrates identified and the present number is more than hundred. The presence of multiple modifications on p53 also signifies the important regulatory role played by the different posttranslational modifications on its function.

p53 does not undergo arginine methylation. The lysine methylation has also been identified recently and the functional consequences of p53 lysine methylation are currently

being studied (Chuikov et al., 2004, Huang et al., 2010). Although, p53 does not get arginine methylated, the regulation of p53 dependent gene expression has been shown to be influenced by histone arginine methylation as well as citrullination (Li et al., 2008). PRMT1 mediated histone H4R3 methylation and PRMT4 mediated histone H3R17 methylation present on the p53 responsive gene promoter enhances p53 dependent transcription of target genes. It has also been shown that petidyl arginine deiminase (PAD4) which is an arginine demethylase (resulting in the formation of citrulline), regulates the p53 downstream target gene expression by repressing the genes. Thus, there seems to be a role of reversible arginine methylation on p53 downstream target activation.

The acetylation of p53 leads to activation of transcription by multiple mechanisms such as enhancement of specific DNA binding, inhibition of non-specific DNA binding, as well as acting as tags for recognition motifs such as bromodomain present in different recognition modules (reviewed in Yang and Seto, 2008). Apart from the structural intricacies, p53 acetylation also acts as a regulator of its stability. Many of the acetylation sites are also ubiquitination targets which finally lead to the protein degradation. The acetylation precludes any other modification at the same site and hence helps in conferring protein stability. Recent studies revealed that Lys 370, 372 and 382 can also be methylated (Huang et al., 2006, Shi et al., 2007, Kurash et al., 2008), indicating that acetylation would inhibit the susbsequent modification, methylation. p53 acetylation may also crosstalk with neighboring modifications similar to the histone code. For example, p53 acetylation at Lys 370 and 372 might communicate with Ser 371 phosphorylation (Brookas and Gu, 2003). Strikingly, a recent study revealed that Lys 372 methylation is required for p53 acetylation at multiple sites. The methylation likely recruits TIP60 via its chromodomain and promotes Lys 120 acetylation, which might then form docking sites to enlist PCAF and p300 for acetylation.

Thus, it is evident that there are multiple acetylation sites on p53. Interestingly, it has been shown that distinct residues get acetylated by specific acetyltransferases with distinct functional consequences which has been discussed in the previous chapter. The role of acetylation on p53 target gene expression is of dual modality, wherein the promoter histone acetylation regulates the transcriptional outcome as well as p53 acetylation itself regulates the functional outcome.

The cooperativity of these modifications on the p53 dependent promoter genes has been shown with the representative, *GADD45*. The Growth Arrest and DNA damage genes comprise a class of genes, *GADD45A*/gadd45, *GADD45B*/MyD118 and *GADD45G*/CR6 (Fornace et al., 1992). These act as sensors of environmental, physiological stress and modulate the DNA damage response by interacting with other proteins such as PCNA, p21, Cdc2/CyclinB1, MEKK4 and p38 kinase (Liebermann et al., 2002).

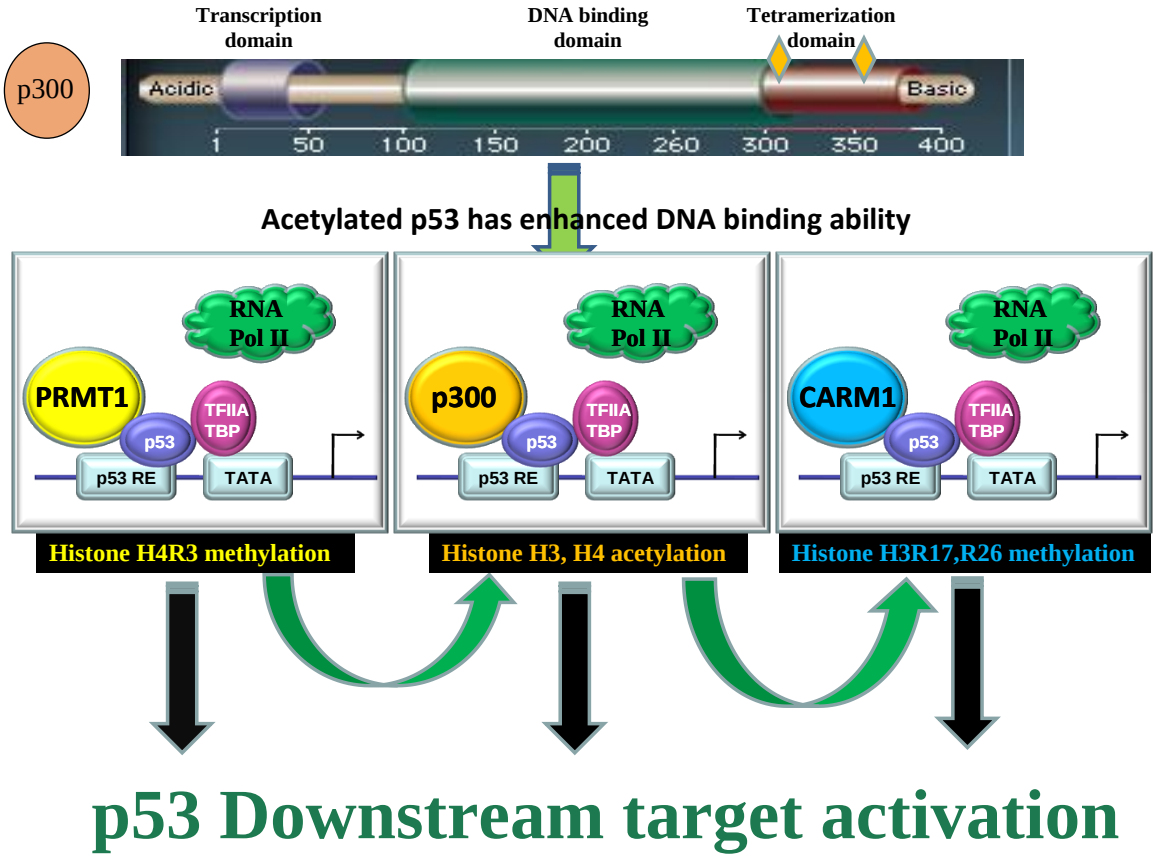


Figure 5.2. Cooperativity of histone modifications on p53 dependent gene expression. *The ordered recruitment of histone modifying enzymes, PRMT1, p300 and PRMT4 lead to a sequential modification reaction marked by histone H4R3 methylation, H4 and H3 acetylation followed by histone H3R17 and R26 methylation. These modifications influence the p53 downstream target gene expression both individually but also in a co-operative manner depicted by the curved green arrow.*

The histone modifications especially histone H3 and H4 arginine methylation as well as acetylation on the *GADD45* promoter was found to increase the p53 dependent gene expression. There was a sequential order of modifications, PRMT1 mediated histone H4R3

methylation led to increased p300 mediated acetylation of histone H3 and H4 which made the histone H3, a more amenable substrate for CARM1 mediated histone H3R17 and H3R26 methylation. All these modifications influences the downstream gene expression, both individually as well as exert a co operative effect (**Figure 5.2**). However, several questions remain unanswered in the above transcription activation model, which include the following.

(a) Is there a universality of these modifications, i.e. the epigenetic language, in all p53 dependent gene expression?

(b) Are other PRMTs also a part of this epigenetic language? One of the modification site, histone H4R3 is a common modification for both PRMT1 and PRMT5 albeit with different functional consequences. PRMT1 mediated asymmetric arginine methylation results in transcriptional activation whereas the symmetric methylation by PRMT5 is associated with transcriptional repression. Could there be a reversible role of the H4R3 methylation, since that is the primary modification that sets the stage for the entire co-operative activation process.

(c) Since, the arginine methylation inhibitor, TBBD (discussed in chapter 3), is a specific inhibitor of H3R17 methylation, could it be used to understand the significance of H3R17 methylation in regulating p53 dependent gene expression?

5.3. Effect of arginine methylation and acetylation on p53 dependent gene expression: probed by enzyme specific inhibitors.

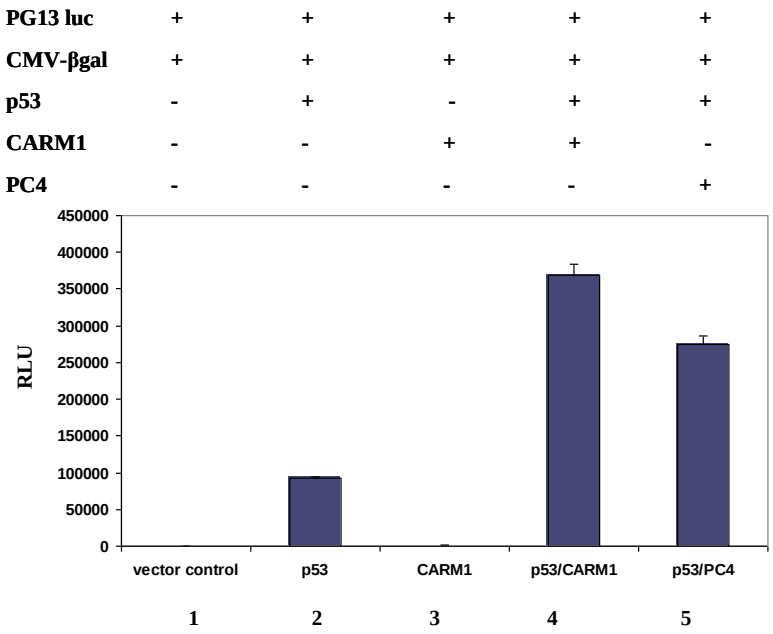
Methylation of R17 and R26 has been linked to transcriptional activation (Bauer et al., 2002). However, the significance of the individual contribution of H3R17 methylation could not be assigned. The functional interactions of p300, CARM1 and PRMT1 in p53-responsive gene expression has been well-established (An et al., 2004). Since, TBBD inhibits CARM1-mediated histone H3R17 methylation in a specific manner, it provided an opportunity to determine the effect of CARM1 methylation (especially of H3R17) on p53-responsive gene expression.

5.3.1. Effect of H3R17 methylation on p53 dependent transcriptional activation:

The p53 null cell line H1299 was selected for testing the effect of the H3R17 methylation on p53 dependent gene expression. Mammalian wild type p53 was transfected alongwith CARM1 and after 36 hours of transfection, the transcriptional activity was tested

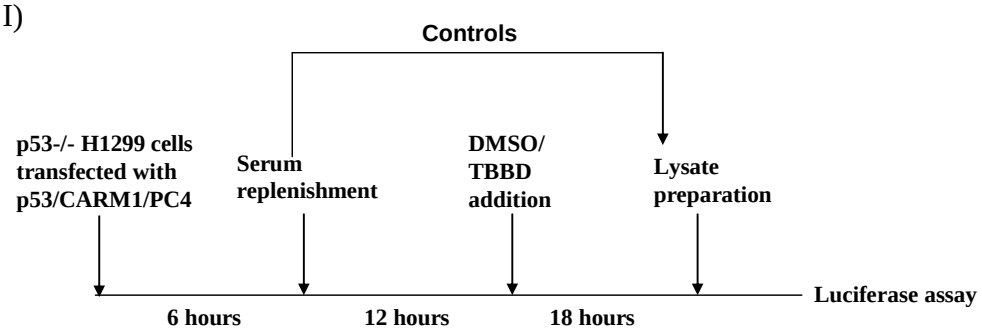
by examining the luciferase activity. Human Positive transcriptional Co-activator 4 (PC4), an activator of p53 (Banerjee et al., 2004) was used as the positive control. As expected, PC4 could activate the transcriptional outcome by several fold above the CMV-β-gal vector control (**Figure 5.3.A, lane 5 versus lane 1**). CARM1 and p53 transfected together could also enhance transcriptional activity (**Figure 5.3. A, lane 4)** as reported earlier.

A)

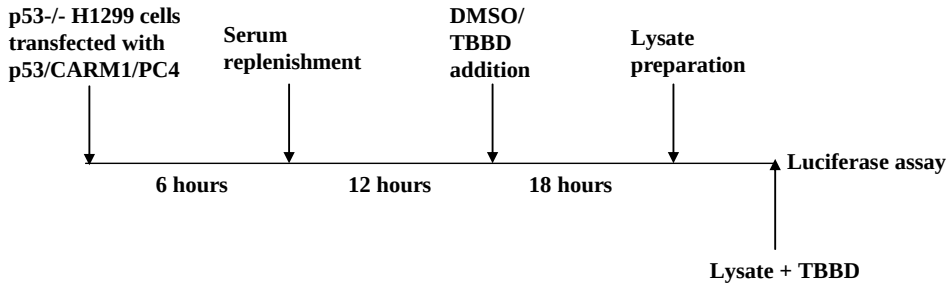


B)

I)



II)



III)

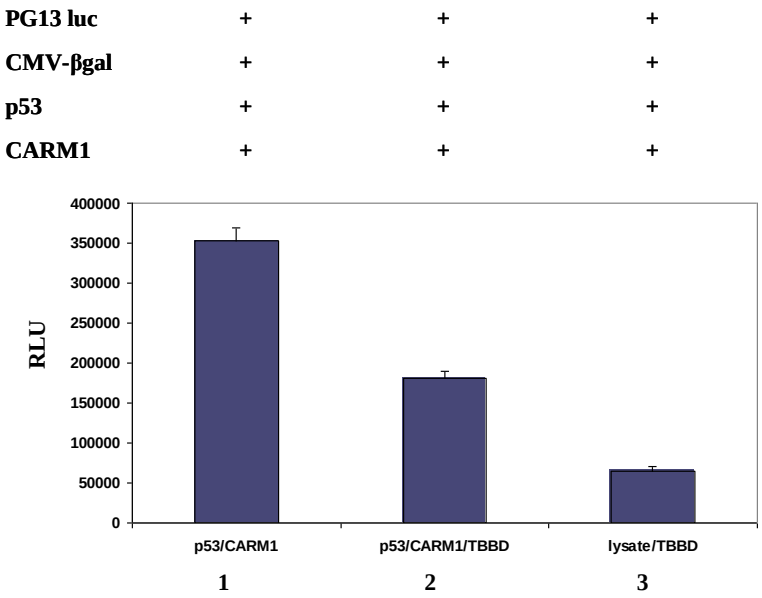


Figure 5.3. Effect of H3R17 methylation on p53 dependent transcriptional activation. *A) H1299 cells were transfected with the respective DNA as indicated using lipofectamine reagent. Following 36 hours of transfection, the lysates were tested for their luciferase activity. The transcriptional activator, p53 enhanced the transcriptional activity (lane 2). The cotransfection of the coactivators, CARM1 (lane 4) and PC4 (lane 5), led to an increase in transcriptional activity measured as relative light units. The experiment was performed in replicates and the error bar represents the standard deviation. B) The transfection reaction as depicted in I) was performed using p53 and CARM1 III), (lane 1) followed by TBBD treatment for 18 hours (lane 2). Lane 3 represents the experiment in scheme II, wherein the lysate of lane 1 was incubated with TBBD for 30 minutes followed by the luciferase assay.*

However, TBBD is an antioxidant (Majid et al., 1991) which could potentially interfere with the luciferase assay system. This was verified by incubating the lysate with TBBD which indicated a drastic reduction in the luciferase counts (**Figure 5.3. B, lane 3**). Although, the TBBD treated cells showed a decrease in the luciferase transcriptional activity (**Figure 5.3.B, lane 2**), to avoid any wrong interpretation of the results, we decided to examine the p53 downstream target gene expression directly by examining the transcript levels which is described in the subsequent section.

5.3.2. p21, a p53 responsive gene:

p21/Waf1 is a cyclin dependent kinase inhibitor and belongs to the family of the Cip/Kip family of kinase inhibitors. It functions as a cell cycle checkpoint by inhibiting the cyclin dependent kinases at the G1/S and G2/M interfaces. This is brought about by the direct interaction of p21 with the cyclin-cdk complexes, preventing Rb phosphorylation and thereby blocking the E2F pathway (Garner and Raj, 2008). The exact functional role of p21 is a much debated concept since it has been shown to be both aniapoptotic as well as proapoptotic. These two opposing functions are possible due to tis different interacting proteins and its ability to respond to different signals. The expression of p21 is generally controlled at the transcriptional level by the p53 depdendent or independent pathway, however, reports suggest a post-transcriptional regualtory mechnaism too. The functional role of p21 varies from promoting apoptosis, protecting cells from undergoing apoptosis, inhibit differentiation, as well as promte differentiation.

However, the well characterized role of p21 is as a mediator of p53-induced growth arrest and a direct regulator of CDK activity (El-Deiry et al., 1993). Although p53 is not required for *p21* transcription, the regulation of *p21* has been shown to be through p53-dependent mechanism following DNA damage (Zeng and El-Deiry, 1996). There are few indirect evidences of histone acetylation and p21 in a positive regulatory mode (Xiao et al., 2000) however, not much information is available about the role of arginine methylation. An earlier report had indicated that arginine methylation is not essential for regualting the p21 gene expression. However, several groups have now shown peptidyl arginine deiminase (PAD4) to be involved in the regulation of *p21* gene expression via citrullination (Li et al., 2008). This modification is a result of deimination reaction of arginine methylation and hence indicates the possibility of arginine methylation to be functional in *p21* gene expression as well.

The dual conflicting role of p21 is partly regulated by the binding of p53 on the *p21* promoter to its cognate sites (**Figure 5.4**). On the *p21* promoter, there exists a high affinity binding site, which when bound by p53, signals to the survival pathway. On the other hand, there are several low affinity binding sites, which when bound by p53 signals for the apoptotic pathway.

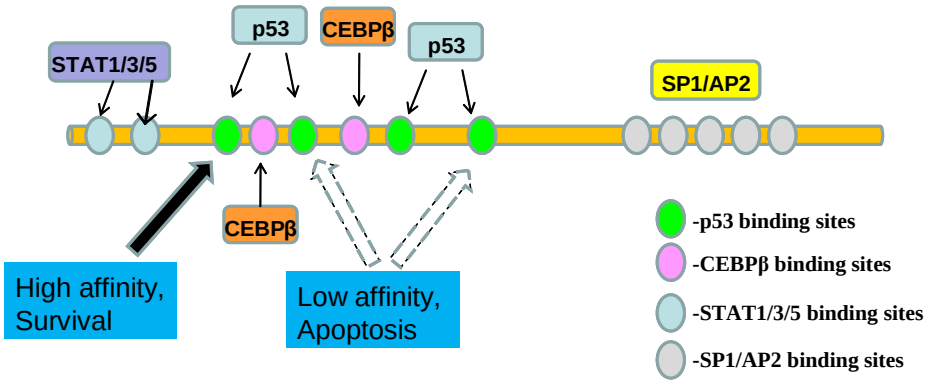


Figure 5.4. p53 RE on the p21 promoter. *Several transcription factor binding sites are present on the p21 promoter. There are two types of binidng sites for p53 on the p21 promoter. The high affinity binding site denoted by the filled arrow, which skews the cells towards the survival pathway whereas the low affinity binding site denoted by the dotted arrow, which signals towards the apoptotic pathway.*

The immediate response to DNA damage is usually the cell survial pathway and hence, it was essential to use this response element (RE) for the susbsequent assays. Yet another regulatory role is exerted by p53 acetylation itself. The PCAF mediated K320 acetylation signals for the cell survival pathway whereas the K373 acetylation by p300 is involved with binding to the low affinity sites (Sakaguchi et al., 1998) and thus finally leading to apoptosis (**described in chapter 4**).

5.3.3. Histone H3R17 methylation regulates the p53 dependent p21 gene expression:

The effect of TBBD inhibition of H3R17 methylation of histones at the promoter of the p53-responsive gene, p21, in HEK293T cells which have endogenous wildtype p53 was investigated. HEK293T cells were subjected to genotoxic insult by doxorubicin to induce p53 followed by TBBD treatment for 24 hours and processed for ChIP or gene expression analysis (**Figure 5.5**, **A**). H3R17 methylation at the p21 promoter was verified by ChIP analysis (**Figure 5.5. B, lane 1 vs lane 2**). However, the H3R26 methylation at this promoter region was minimally affected by the treatment (**Figure 5.5. B, lane 1 vs lane 2).** Strikingly, reduced H3R17 methylation (upon TBBD treatment) at the p21 promoter was significantly correlated with repression of p21 expression (**Figure 5.5. C, lane 1 vs lane 2**)

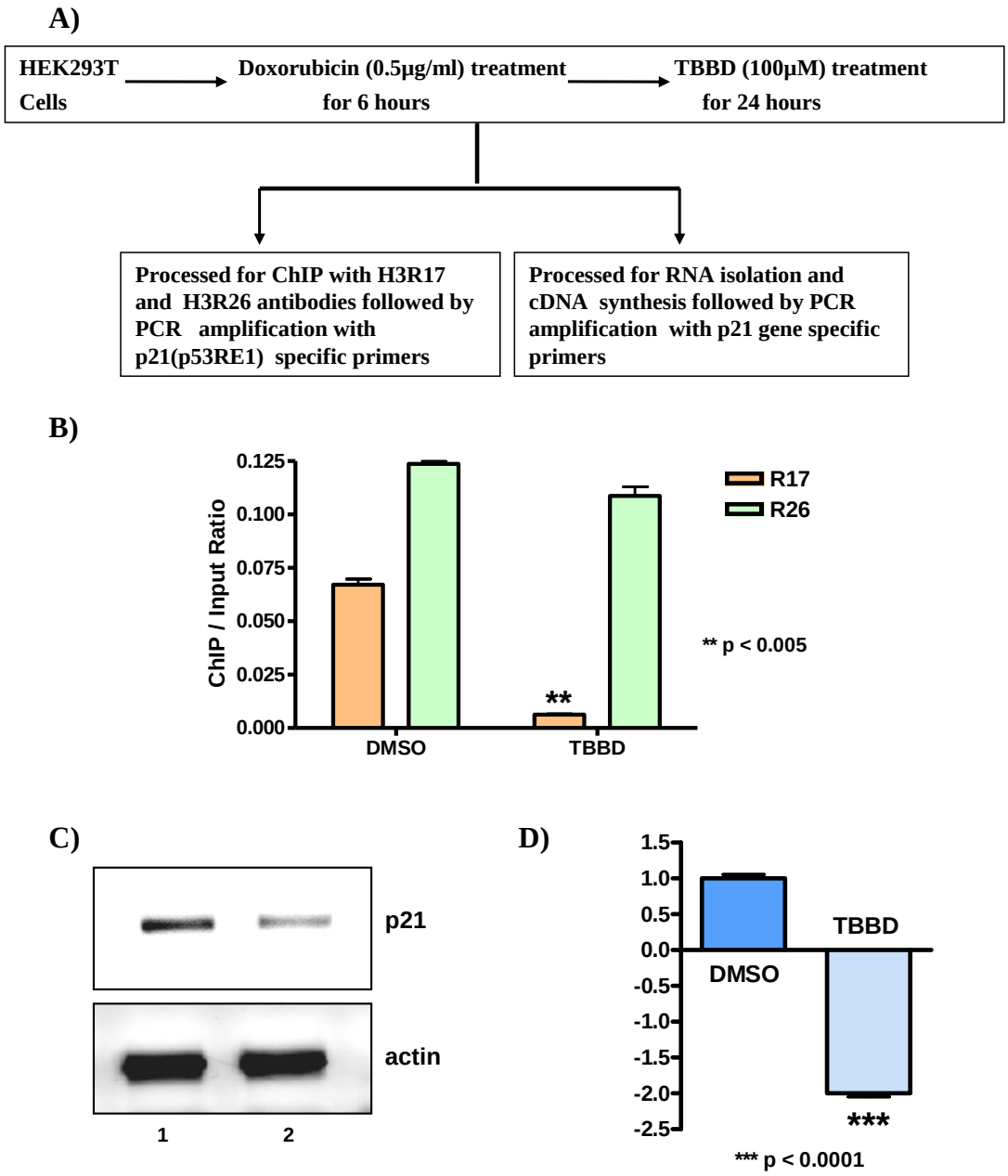


Figure 5.5. Histone H3R17 methylation regulates p53-responsive gene expression*. (A) Scheme representing the experimental procedure. The HEK293T cells were treated as indicated and processed for either ChIP or gene expression analysis. (B) State of histone H3R17/H3R26 methylation in the p53-responsive element (p53 RE) of the p21 promoter analyzed by ChIP. ChIP by input ratio in cells treated with DMSO (lane1) or TBBD (lane 2). (n=3, p<0.005). (C) Repression in p21 expression was observed on TBBD treatment in the HEK293T cell line. Reverse transcriptase PCR amplification of p21 expression in cells treated with DMSO (lane 1) or TBBD (lane 2). (D) The expression of p21 is repressed on TBBD treatment. Realtime PCR quantification of fold change in p21 gene expression in cells treated with DMSO (lane 1) and treated with TBBD (lane 2). (n=3, p<0.001).*

The p21 expression upon TBBD treatment was found to be about two fold reduced as compared to the solvent control (**Figure 5.5. D, lane 1 vs lane 2**). Similar results were obtained with H1299 cells transfected with p53. Taken together, these results suggest that H3R17 methylation by CARM1 is essential for p53-dependent regulation of p21 gene expression. Significantly, TBBD treatment caused a significant reduction of p21 gene expression in H1299 and HEK293T cells (**Figure 5.5**). The latter cells were subjected to DNA damage which leads to activation of p53 downstream targets. Previous work showed that p53-dependent p21 expression is modulated by arginine methylation, and because H3R17 regulates the transcriptional activation of p53-responsive genes, our results also implicate methylation of H3R17 in p53 dependent gene expression. FACS analysis of TBBD-treated HeLa cells (which lack functional p53) showed that there are no cell-cycle defects (**Figure 5.6**).

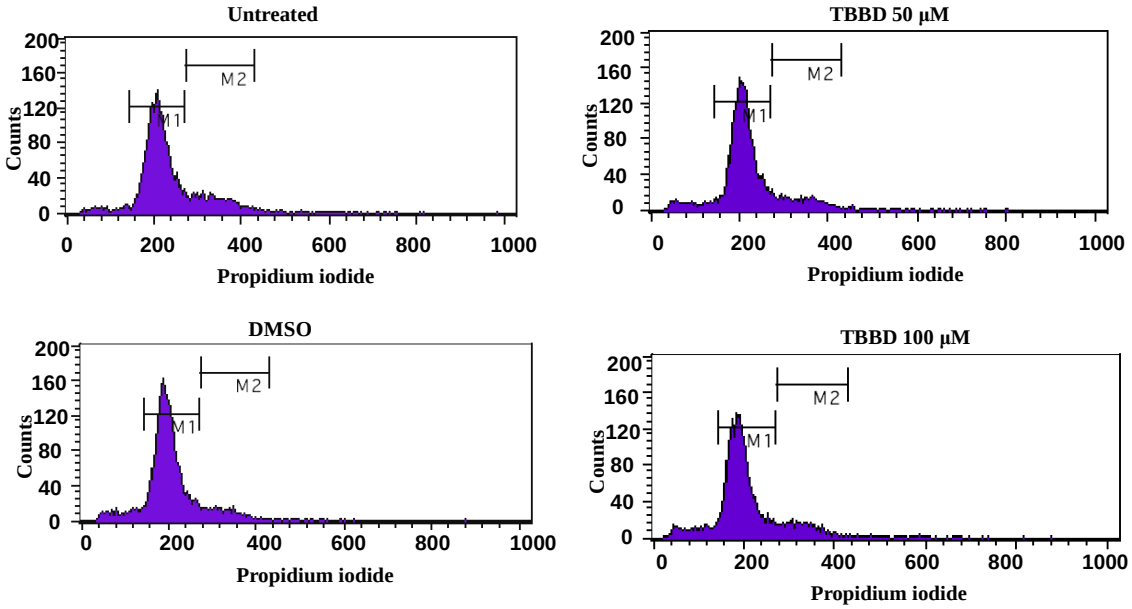


Figure 5.6. FACS analysis of TBBD treated cells*. The propidium iodide staining fluorescence was captured and the cells were gated in M1, M2, M3 regions. The cell populations in different stages do not show any change across the inhibitor treatment.*

Possibly, H3R17 methylation is an important modulator of tumor suppressor function. However, a more intensive analysis of CARM1-mediated H3R17 methylation in different cell lines under different conditions should be undertaken to elucidate the spatiotemporal importance of H3R17 methylation.

5.3.4. Histone H3R17 methylation and acetylation regulates the p53 dependent p21 gene expression:

To understand the role of p300 mediated acetylation on p53 dependent p21 expression, similar experiments were performed with HEK293T cells subjected to genotoxic insult followed by treatment with RTK1. The inhibition of acetylation was verified by immunofluorescence analysis using antibody against H3K9, K14 acetylation and the TBBD treated cells on genotoxic insult were verified by immunofluorescence using H3R17 dimethylation antibody.

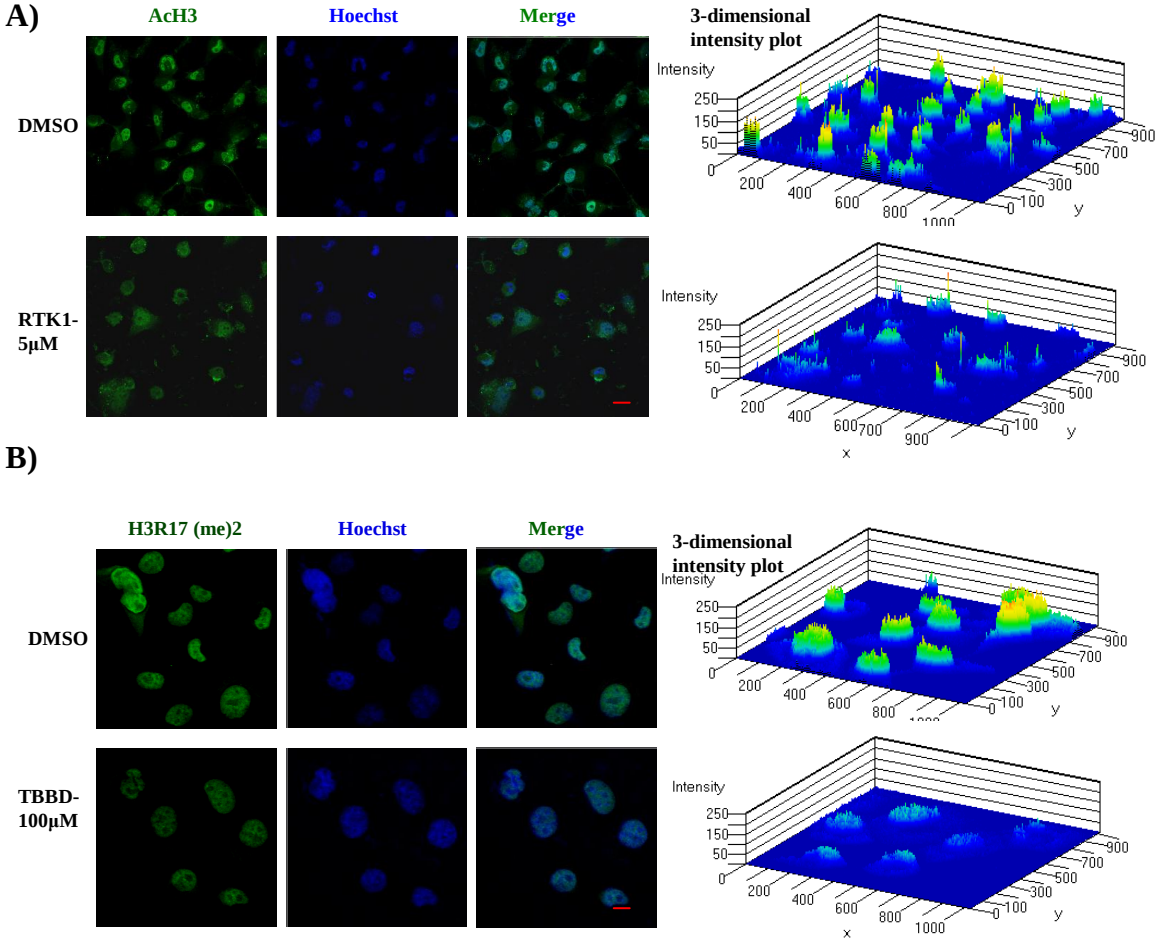
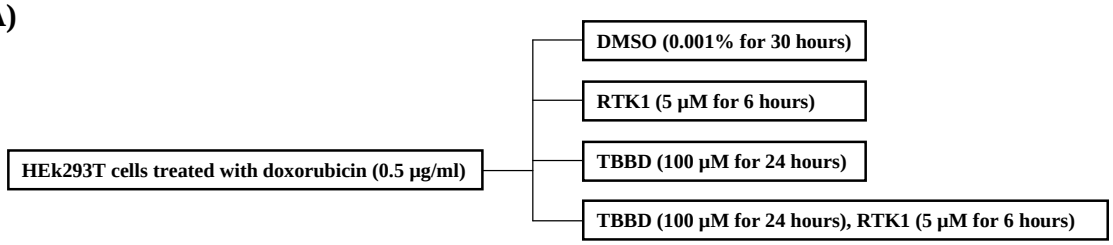


Figure 5.7. Effect of TBBD and RTK1 on the histone modification status of HEK293T cells subjected to genotoxic insult*. Immunofluorescence analysis of the treated cells A) probed with acetylated histone H3 antibody and B) H3R17 methylated antibody. The different panels indicate the different staining as represented. The green fluorescence is from the antibodies tested (AcH3 K9,K14 or H3R17me2), the blue staining arises from Hoechst staining of DNA and the last panel represents the merged of the antibody and DNA staining. DMSO treated cells were used as solvent control.*

To elucidate the combinatorial role of H3R17 methylation and acetylation, cells were treated with TBBD for 24 hours first followed by RTK1 treatment in the last 6 hours after the genotoxic insult. However, the reverse treatment wherein cells that were treated first with RTK1 followed by TBBD, cytotoxic effects was observed. Acetylation of H3K18 is considered a pre-requisite for arginine methylation and hence the prior acetylation inhibition as well as inhibition of arginine methylation might have been toxic to the cells.

As expected, the acetylation inhibition by RTK1 led to a repression of almost four-fold in both *p21* as well as *bax* expression (**Figure 5.8. maroon bar, lane 1 vs lane 2**). The TBBD treatment decreased *p21* (two-fold) and *bax* expression by about four-fold (**Figure 5.8. yellow bar, lane 1vs lane 2**) as observed earlier (**Figure 5.5**).

A)



B)

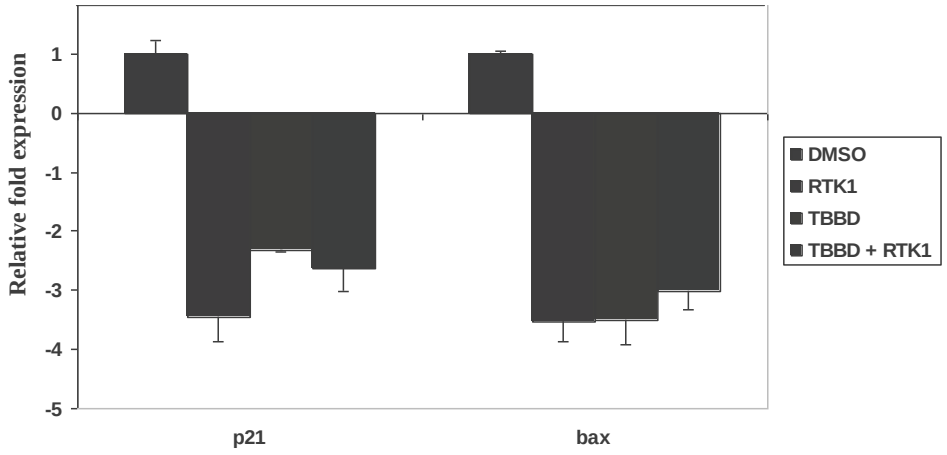
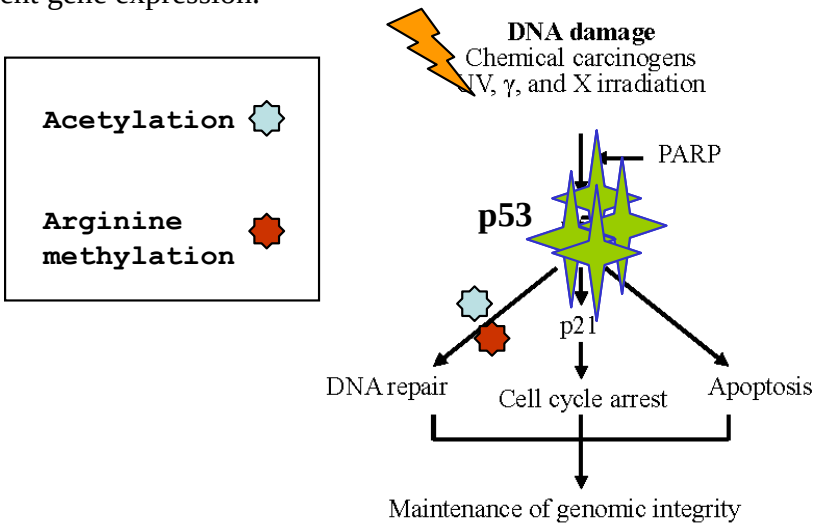


Figure 5.8. Role of histone arginine methylation and acetylation in p53 dependent p21 and bax gene expression. *A) Schematic of the experimental plan, B) The expression of p21 and bax is repressed on TBBD treatment. Realtime PCR quantification of fold change in p21 (lane 1) and bax (lane 2) gene expression in cells treated with DMSO (blue bar), RTK1 (maroon bar), TBBD (yellow bar) and TBBD followed by RTK1 treatment (green bar).*

Acetylation inhibition seems to repress the gene expression to a greater extent as compared to the arginine methylation inhibition. This could be due to the dual regulatory role of acetylation on p53, wherein, p53 acetylation itself regulates its functionality as well as the acetylation of histones on p53 responsive gene promoters. Hence, this could have a more drastic effect on the gene expression. However, the combined treatment of TBBD followed by RTK1 also decreased the gene expression to comparable levels. The expression of *bax* was equally affected by both acetylation as well as arginine methylation inhibition indicating that the probably, these modifications regulate the different p53 dependent genes to differential extent.

The interplay of these modifications, i.e. acetylation and arginine methylation has been earlier shown to be essential for the DNA damage response by modulating the expression of *GADD45* gene expression. The use of the specific inhibitors RTK1 (p300 specific) and TBBD (CARM1 specific), indicate that arginine methylation and acetylation are essential for both *p21* and *Bax* expression as well. However, there was no synergistic effect on using both the inhibitors together, rather the fold repression was similar to the TBBD treatment alone. The cells that were first treated with RTK1 followed by TBBD underwent apoptosis at very early time points, implicating that these modifications are essential for the survival pathway. Thus, H3R17 methylation and histone acetylation form an epigenetic language for the establishment of p53 dependent gene expression both in the cell cycle pathway as well as the apoptosis pathway (**Figure 5.9**). However, it remains to be concluded whether this language is universal across all cell types and across all p53 dependent gene expression.

A)



B)

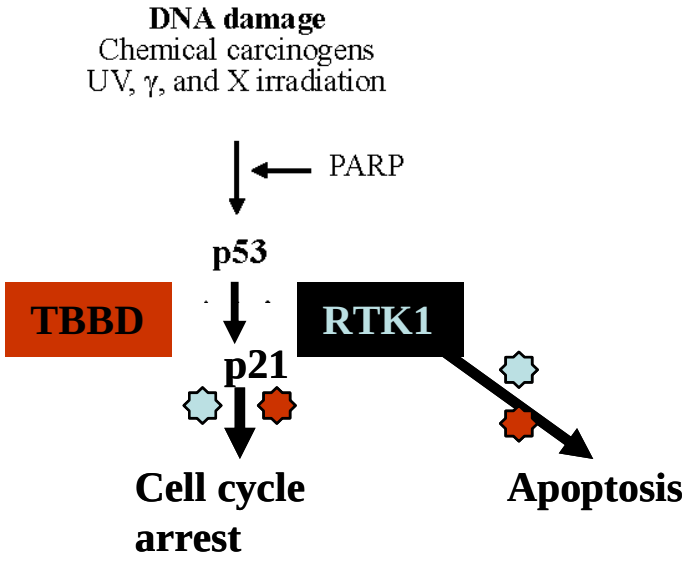


Figure 5.9. Role of histone arginine methylation and acetylation in p53 dependent gene expression.
A) Upon DNA damage, p53 is activated and based upon the extent of damage, the pathway towards DNA repair (through GADD45) or Apoptosis (through Bax) are upregulated. During this process, cell cycle arrest is induced because of the action of p21. Earlier reports implicate arginine methylation and acetylation in GADD45 expression in a p53 dependent manner. B) The results presented here extend the role of arginine methylation and acetylation in p53 dependent p21 and bax expression, thus implicating a regulatory role of these modifications p53 dependent downstream gene activation.

5.4. The role of histone H3R17 methylation in global gene expression:

It is indeed evident that arginine methylation is not just involved in nuclear receptor associated transcriptional activation, but also plays an important role in other transcription events such as the p53 dependent gene expression. In addition to its role in p53-responsive gene expression, CARM1-mediated H3R17 methylation has been implicated in breast cancer development in an estrogen receptor (ER)-dependent manner, as well as androgen receptor (AR) dependent manner. However, these apparently contradictory results (tumor suppression versus tumor promotion) were obtained using different cell lines. Hence, to understand the role of H3R17 methylation in cancer development in the absence of both p53 function and ER-dependent regulation, we performed an analysis of global gene expression

in HeLa cells treated with 100 μM TBBD. The cells were treated in replicates and a part of the cells were processed for immunoblotting analysis using the modification specific antibodies to verify the inhibition. As observed earlier, TBBD treatment led to almost 95% reduction in histone H3R17 methylation (**Figure 5.10 A, panel I, lane 1 versus 2**) whereas H3R26 levels were unaffected (**Figure 5.10 A, panel II, lane 1 versus 2)**. Histone H3 was used as the loading control **Figure 5.10 A, panel III, lane 1 versus 2)**. From the same samples, RNA was isolated (**Figure 5.10 B**) and a quality check was performed with Bioanalyzer (**Figure 5.10 C**) to verify the RNA profile. Integrity of RNA samples was checked as a measure of the RNA Integration Number (RIN) by the RNA nano 6000 eukaryotic assay (**Table 1**).

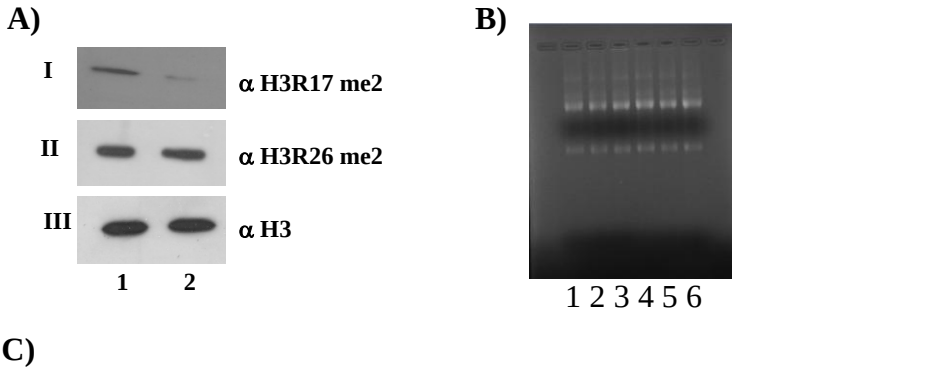


Figure 5.10. Quality check of RNA used for microarray hybridization. A*) HeLa cells were treated with DMSO (lane 1), TBBD 100 μM (lane 2), for 24 hours and the histones were probed with the indicated antibodies.* ***B)*** *RNA samples used for the hybridization. DMSO treated (lanes 1, 3,5) and TBBD treated (lanes 2, 4, 6).* ***C)*** *Bioanalyzer profile of RNA samples as indicated.*

The microarray hybridization data indicate that about 28 genes were upregulated by TBBD treatment whereas 40 genes were downregulated (**Figure 5.11 and Table 2**) compared to expression in HeLa cells treated with DMSO (solvent). Although the gene expression analysis was done on a 44,000 whole genome array, only about 70 genes were differentially regulated upon inhibition of H3R17 methylation. TBBD treatment led to a 65% down regulation of the differentially regulated gene expression, which is expected since it is a mark associated with transcriptional activation.

However, its specific inhibition could also upregulate the expression of several genes, suggesting that the role of methylated H3R17 residue in gene regulation is much more complex. Recent reports have indicated that the presence of CARM1 and thereby the H3R17 methylation is not a mark associated with transcriptional activation alone, rather the role of CARM1 in the coactivator complex assembly and dissassembly indicate that it might also have a repressive role.

Table 1: RNA Integration Number (RIN) of RNA samples used for microarray analysis.

SAMPLE	RNA INTEGRATION NUMBER (RIN)
DMSO 1	9.0
TBBD 1	8.4
DMSO 2	8.6
TBBD 2	8.8
DMSO 3	8.4
TBBD 3	8.9

RIN (RNA integrity number) > 7 is good
RIN (RNA integrity number) 6-7 is optimal
RIN (RNA integrity number) 5-6 is Admissible
RIN (RNA integrity number) < 5 is Degraded

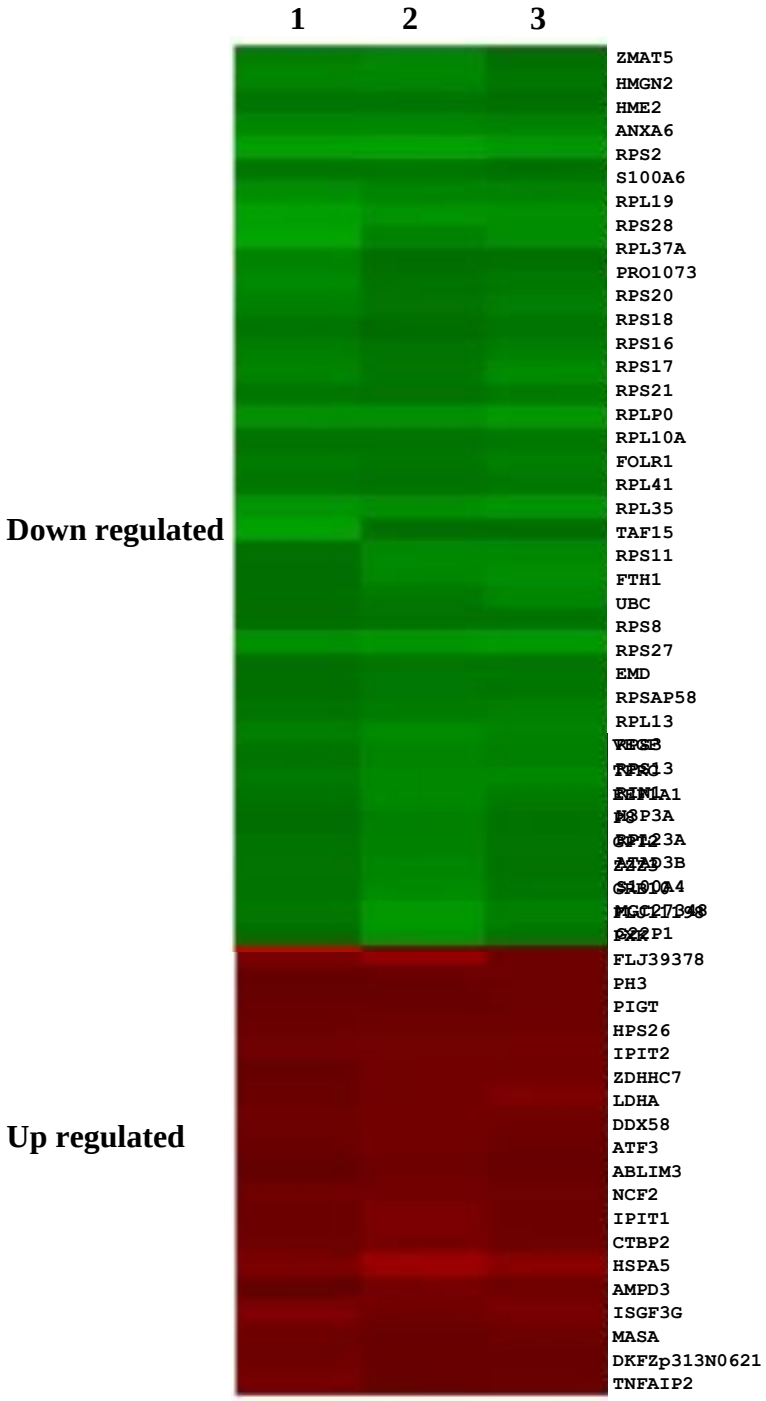


Figure 5.11. Histone H3R17 methylation modulates global gene expression. *A combined heatmap representing the upregulated and downregulated genes. The three samples are biological replicates of treated samples.*

Table 2: List of common up regulated and down regulated genes across all samples

UP REGULATED GENES	*ABLIM1, AMPD3, ATF3, CTBP2, DDX58, DKFZp313N0621, EEF1A1, FLJ11196, FLJ39370, GPT2, GRB10, HSPA5, IFIT1, IFIT2, ISGF3G, LDHA, MASA, NCF2, P8, PIGT, PIM3, PXK, RPS26, TFRC, TNFAIP2, VEGF, ZDHHC7, ZZZ3*
DOWN REGULATED GENES	*ANXA6, ATAD3B, EMD, FGFBP1, FOLR1, FTH1, G22P1, H3F3A, HMGN2, RPSAP58, ZMAT5, MGC27348, NME2, PRO1073, RIN1, RPL10A, RPL13, RPL19, RPL23A, RPL3, RPL35, RPL37A, RPL41, RPLP0, RPS11, RPS13, RPS16, RPS17, RPS18, RPS20, RPS21, RPS27, RPS28, RPS3, RPS8, S100A4, S100A6, TAF15, UBC*

The recent estrogen receptor cistrome (set of *cis*-acting or *trans*-acting factors on the genome), also indicates that H3R17 methylation is present on the promoters which are repressed but get activated in response to estrogen stimulation. All these validate our observations that the role of CARM1 and H3R17 methylation is more complex and needs to be addressed in different cell lines with differential signals. This is further strengthened by the data obtained from the silencing of CARM1 in the human embryonic stem cells which shows a greater percentage of genes with up regulated expression than down regulation.

5.5. Effect of H3R17 methylation on differentiation:

The global gene expression modulation brought about by TBBD treatment showed a distinct pattern of several pathways being differentially altered. The significance of these pathways in the light of their involvement in cancer manifestation will be discussed subsequently. An interesting observation was the up regulation of few differentiation and

development associated genes in individual samples across the microarray analysis. The few important differentiation associated genes that showed upregulated expression are enlisted in **Table 3.**

Table 3: Differentiation associated genes that were upregulated on TBBD treatment

Neural cell, nerve development

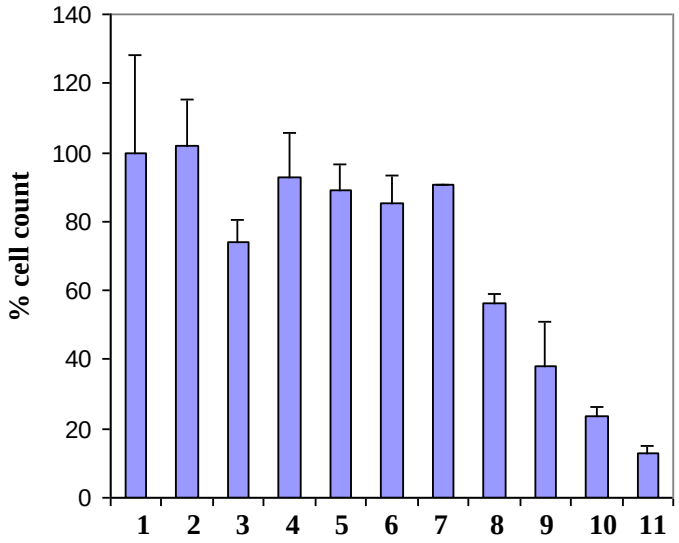
- **SCNN1D – Sodium channel nonvoltage gated delta (expressed in cerebral cortex)**
- **DLGAP4- Discs, large homolog associated protein (guanylate kinase in neuronal cells)**
- **VLDLR- very low density lipoprotein receptor (cerebellar function, CNS development)**
- **TIAM2- T cell lymphoma invasion and metastasis, (neural cell development)**
- **SLCIA3, SLC16A2- Solute carrier protein, (cranial nerve development)**
- **NKX2-5, involved in axon guidance**

- **EYA4- Eyes absent 4 (eye develpoment)**
- **CILP2- cartilage intermediate layer protein (heart development)**
- **BGLAP- osteocalcin (skeletal system development)**
- **Klf9- Kruppel like factor (differentiation and development)**

These data indicate that H3R17 methylation might have a repressive role on the differentiation process. But, these genes were differentially expressed in the HeLa cells which is an already differentiated cell type. Earlier reports have conflicting indications on the role of CARM1 and histone H3R17 methylation in differentiation. CARM1 by virtue of its co-activation property of MEF-2 and PPAR-γ enhances the muscle (Chen et al., 2002) and adipocyte differentiation (Yadav et al., 2008) respectively. On the other hand, the methyltransferase property of CARM1, by methylating TARPP (Kim et al., 2004) and Sox-9 (Ito et al., 2009) regulates thymocyte and chondrocyte differentiation. The most recent evidences link CARM1 with neural crest formation (Bhattacherjee et al., 2009) and most importantly with pluripotency maintenance (Torres-Padilla et al., 2007, Wu et al., 2009). Incidentally, p300 has also been implicated in neural crest formation (Bhattacherjee et al., 2009) as well as pluripotency maintenance (Miyabayashi et al., 2007, Zhong and Jin, 2009). Hence, to understand these differences, TBBD was treated to human ES cell lines. Initially, HUES7 cells were treated with the arginine methylation inhibitor TBBD and the p300 specific acetylation inhibitor, LTK14 (Mantelingu and Reddy et al., 2007) to verify the toxicity profile since the ES cells are highly sensitive to any stress and cytotoxic agents. The histone acetylation inhibitor, RTK1 was found to be toxic to mammalian cells at very low

concentrations and hence, the initial cytotoxicity assays were initially performed with the relatively nontoxic p300 specific inhibitor, LTK14. TBBD exhibited toxicity at concentrations higher than 25 µM within 48 hours of treatment, as observed in **Figure 5.12 A, B.** Hence, a sub-lethal concentration of 10 µM concentration was used for the subsequent assays.

A)



B)

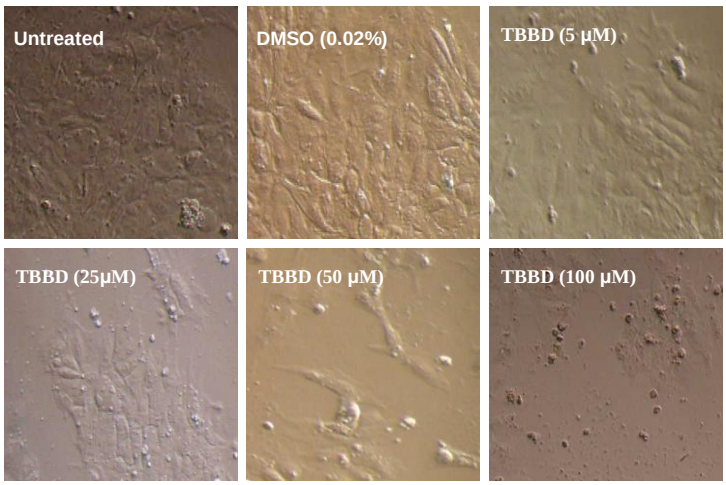


Figure 5.12. Cytotoxicity profiling of TBBD on human ES cell line, HUES7. *A) Toxicity profiling of TBBD treatment on HUES7 cells, untreated(lane 1), treated with0.02% DMSO (lane 2), 0.1% DMSO (lane 3), with TBBD (lanes 4-11), 1, 2, 5, 10, 25, 50, 75, 100 µM respectively, and cell count was performed after 48 hours. B) Representative images of the inhibitor treatment*

Similar experiments with the p300 inhibitor, LTK14 showed the cytotoxicity to be high with low micromolar concentrations itself (**Figure 5.13, A and B**). Hence, 0.2 µM concentration was used for the subsequent experimentation. Since, RTK1 was more toxic as compared to the LTK14, the initial toxicity profiling was determined using LTK14, but subsequently, RTK1 was also included in the experiments at similar concentrations.

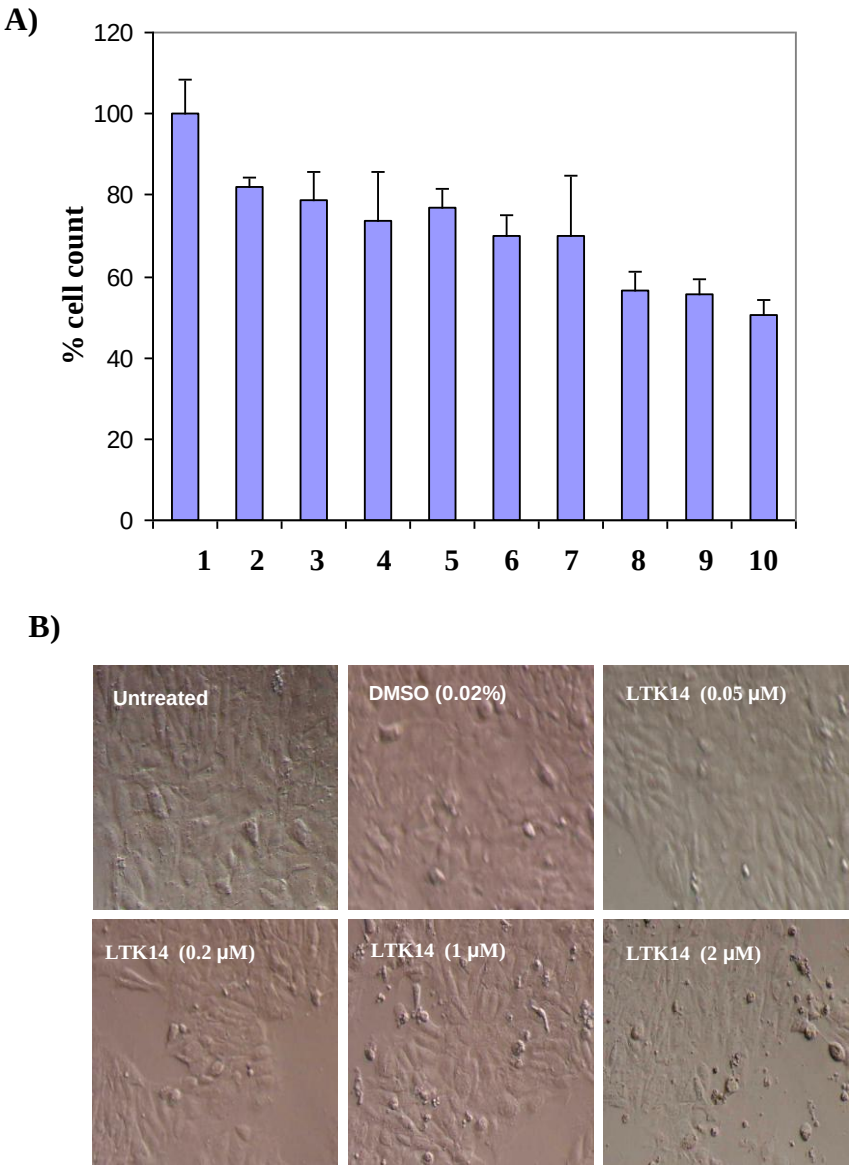


Figure 5.13. Cytotoxicity profiling of LTK14 on human ES cell line, HUES7. *A) Toxicity profiling of LTK14 treatment on HUES7 cells, untreated(lane 1), treated with 0.02% DMSO (lane 2), with LTK14 (lanes 3-10), 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2 µM respectively, and cell count was performed after 48 hours. B) Representative images of the inhibitor treatment*

Subsequently, BG01V cells were treated with the different inhibitors and the inhibition was verified by immunofluorescence analysis using modification specific antibodies. It was observed that 10 µM TBBD treatment itself could inhibit the histone H3R17 methylation as observed by the immunofluorescene analysis (**Figure 5.14,** compare 3-dimensional intensity profile of DMSO versus TBBD). DMSO seems to induce a slight increase in the methylation which is decreased on the inhibitor treatment.

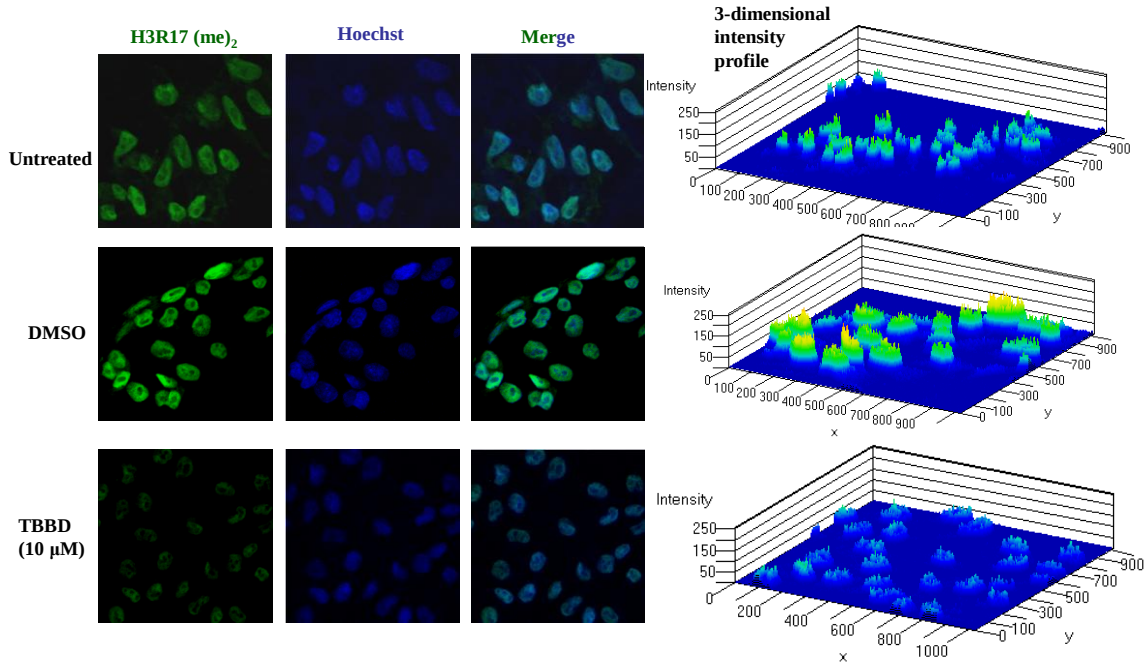


Figure 5.14. TBBD inhibits H3R17 methylation in human ES cell line, BGO1V. *Immunofluorescence analysis of cells untreated, treated with DMSO, TBBD (10 µM), with H3R17 methylation antibody (green), counter stained with hoechst for DNA staining (blue) and the merged image of the two. The 3-dimensional profile represents the intensity profile of the green fluorescence of the antibody used for staining. Each circle represents a single cell and the peaks represent the intensity of the modification.*

The use of the p300 specific acetylation inhibitor, RTK1 at the pre-determined dose of 0.2 µM did not cause any apparent cytotoxicity. The inhibition of histone acetylation was verified by performing an immunofluorescence analysis using the dual acetylation, K9, K14 specific acetylated histone H3 antibody. There was a slight increase in the level of histone acetylation on treatment with DMSO which is expected since DMSO is a mild

HDAC inhibitor. However, there was a drastic decrease in acetylation with RTK1 treatment as observed in **Figure 5.15**, compare 3-dimensional intensity profile of DMSO versus RTK1.

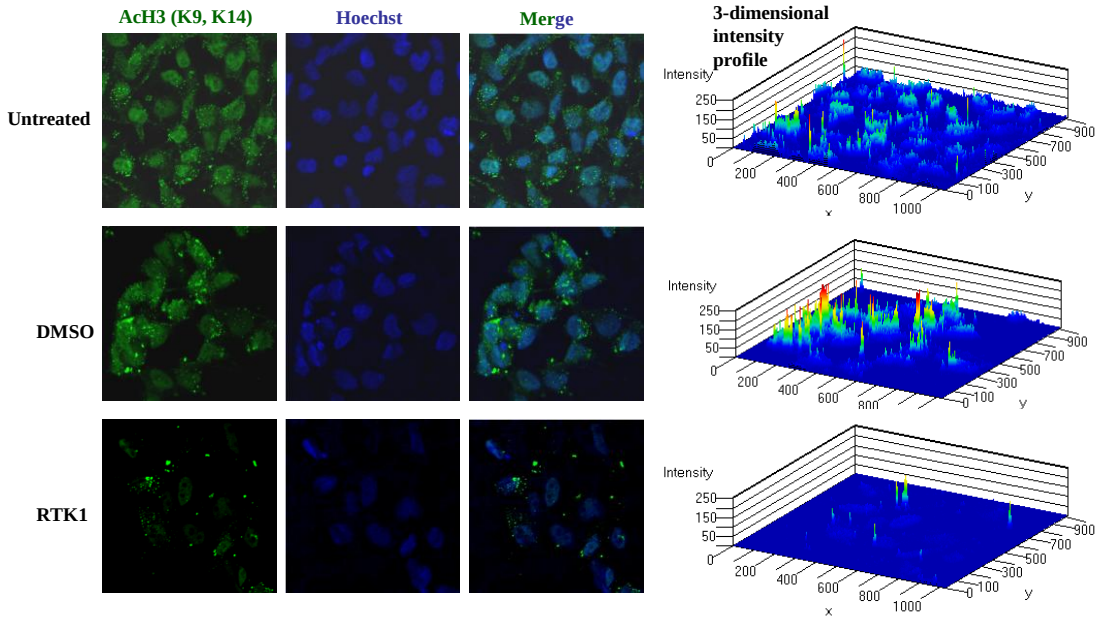


Figure 5.15. RTK1 inhibits histone acetylation in human ES cell line, BGO1V. *Immunofluorescence analysis of cells untreated, treated with DMSO, RTK1 (0.2 μM), with acetylated H3 antibody specific for H3K9/K14 acetylation (green), counter stained with Hoechst for DNA staining (blue) and the merged image of the two. The 3-dimensional profile represents the intensity profile of the green fluorescence of the antibody used for staining. Each circle represents a single cell and the peaks represent the intensity of the modification.*

CARM1 has been shown to be essential for the self-renewal and pluripotency of embryonic stem (ES) cells. When CARM1 is silenced in the ES cells, there is a downregulation of pluripotency associated genes thus promoting their differentiation. Mechanistically, this has been linked to the association of CARM1 with the *Oct4/Pou5f1* and *Sox2* promoters with a concomitant increase in the H3R17 and H3R26 methylation levels (Wu et al., 2009). Since, the inhibition of H3R17 methylation led to an upregulation of several differentiation associated genes, it was possible that TBBD could downregulate pluripotency associated genes.

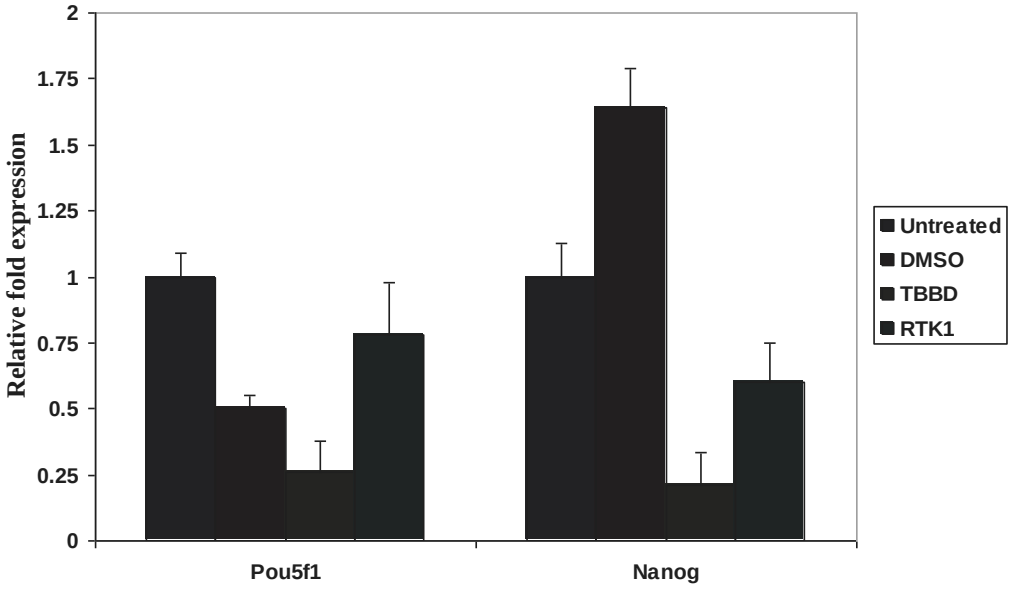


Figure 5.16. Effect of histone arginine methylation and acetylation inhibition on pluripotency associated marker gene expression. *The expression of Pou5f1/Oct4 and Nanog is repressed on TBBD RTK1 treatment. Realtime PCR quantification of fold change in Pou5f1/Oct4 (lane 1) and Nanog (lane 2) gene expression in cells untreated (blue bar) treated with DMSO (maroon bar), TBBD (yellow bar), RTK1 treatment (green bar).*

The pluripotency associated markers, *Oct4/Pou5f1 and Nanog*, were checked by realtime PCR analysis to quantify the gene expression upon inhibitor treatment. Simiilarly, p300 has been shown to be directly involved in modulating Nanog expression. Importantly, epigenetic modification of histone acetylation at the distal regulatory region of *Nanog* was found to be dependent on the presence of p300, which could contribute to the mechanism of p300 regulating *Nanog* expression during the stages of differentiation (Zhong and Jin, 2009). Hence, it was necessary to check the effect of RTK1 on the pluripotency marker gene expression. The experiment was performed by treating the hES cells with the inhibitors for 18 hours, followed by RNA isolation and realtime PCR quantification as represented in **Figure 5.16.**

There were differences in the expression on the different inhibitor treatments. DMSO had differential effects too. However, TBBD which inhibits H3R17 methylation led to a repression of both *Oct4* and *Nanog* levels by four-fold (**Figure 5.16, yellow bar**). It was expected since the earlier report indicates that CARM1 is essential for *Oct4* and *Nanog*

expression, however, this experiment proves that H3R17 methylation has a significant role in this expression. Surprisingly, the treatment with the acetylation inhibitor, RTK1 led to a greater level of *Nanog* down regulation than *Oct4* levels (Figure **5.14, green bar**). The earlier work does suggest that p300 has an important role on *Nanog* expression. Probably, Oct4 levels are not greatly influenced by p300. Since, there was a reduction in the pluripotency associated marker gene expression, the cells were examined for any morphological changes associated with differentiation followed by alkaline phosphatase staining. Alkaline phosphatase expression is observed only in undifferentiated cells and is lost during the process of differentiation.

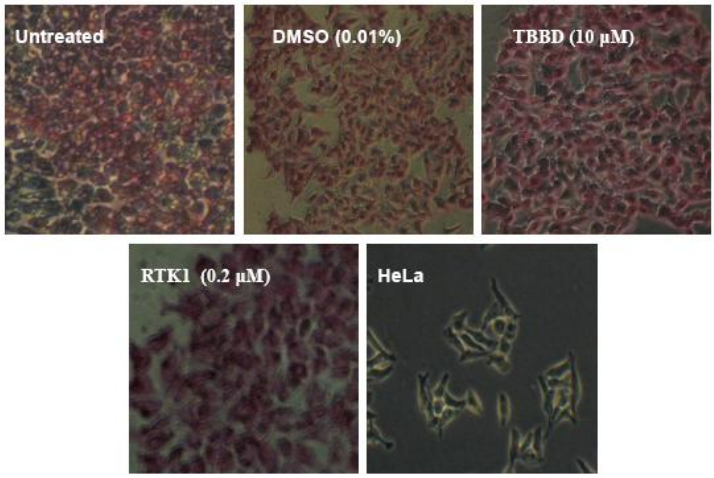


Figure 5.17. Alkaline phosphatase staining of BGO1V cells upon different treatment as represented. HeLa cells were used as a negative control

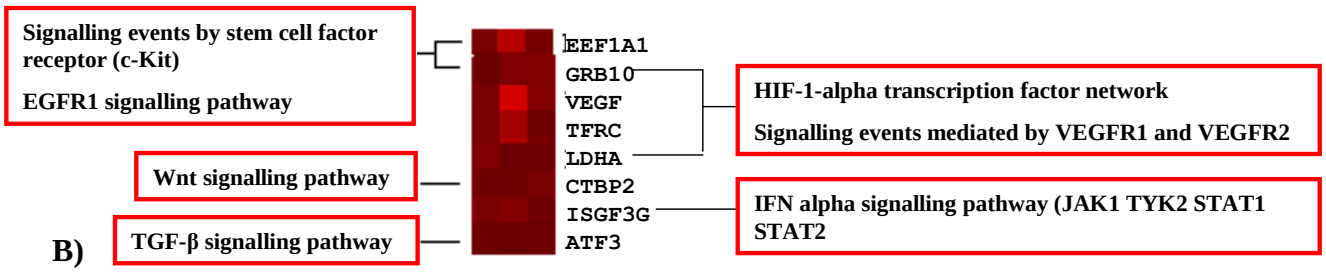
The effect of H3R17 methylation and acetylation inhibition by the specific inhibitors of CARM1 and p300, although led to a decrease in the pluripotency associated marker gene repression, it did not translate directly into an induction of differentiation as observed by the alkaline phosphatase staining (**Figure 5.17**). Alkaline phosphatase is expressed by the undifferentiated cells and the staining is lost on differentiation. Although, there was no apparent change in the staining, upon inhibitor treatment, RTK1 treatment led to a slight decrease in the intensity of the staining. This possibly indicates, that this could either be due to inhibition of acetylation or due to due to some cytotoxic effects. The

absence of any effect on differentiation upon inhibitor treatment could also be due to the time point of the experiment. Possibly, a sustained treatment of the inhibitor might lead to a change in the differentiation status of the human embryonic stem cells. HeLa cells which are differentiated cells did not show any staining (**Figure 5.17**)

5.6. Histone H3R17 methylation regulates important signaling and ribosome biogenesis associated genes

The inhibition of H3R17 methylation, led to an upregulation of several genes in different signalling pathways associated with cancer manifestation (**Figure 5.18. A**). These include the EGFR1 signalling pathway, VEGF signalling pathway, HIF1 alpha transcription network.

A)



B)

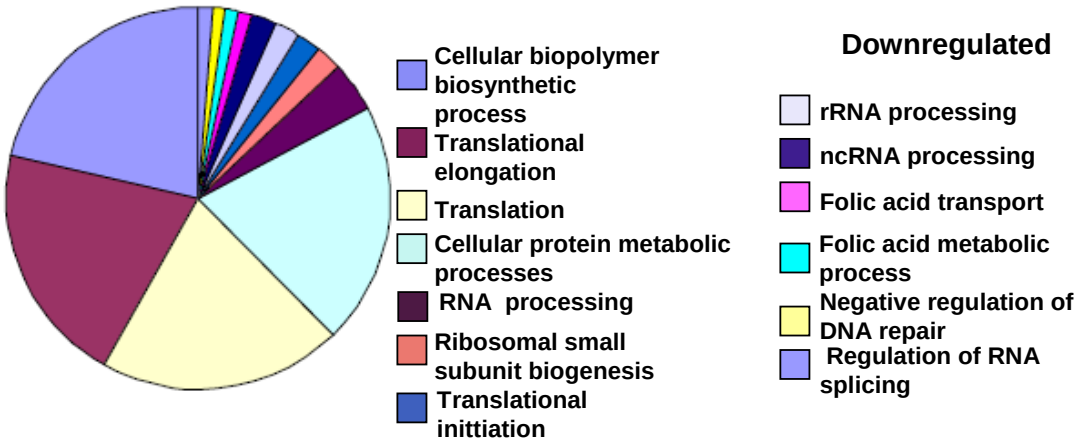


Figure 5.18. H3R17 methylation modulates global gene expression. *A) Important genes and their pathways associated with cancer manifestation that are up regulated in the microarray analysis. B) Percentage distribution of down regulated genes on TBBD treatment.*

Notable among these is the HIF1 alpha network, which is a pathway regulated by p300 acetylation (Semenza et al., 2007). The up regulation of this transcriptional network indicates a possible feedback loop existing between the acetylation and arginine methylation. As expected, the inhibition of H3R17 methylation (a transcriptional activation mark), led to the downregulated expression of quite a few genes. Most of these belonged to the RNA processing pathways (**Figure 5.18. B**).

Out of the differentially regulated genes (**Table 2**), few of the candidate genes were validated (**Figure 5.19**).

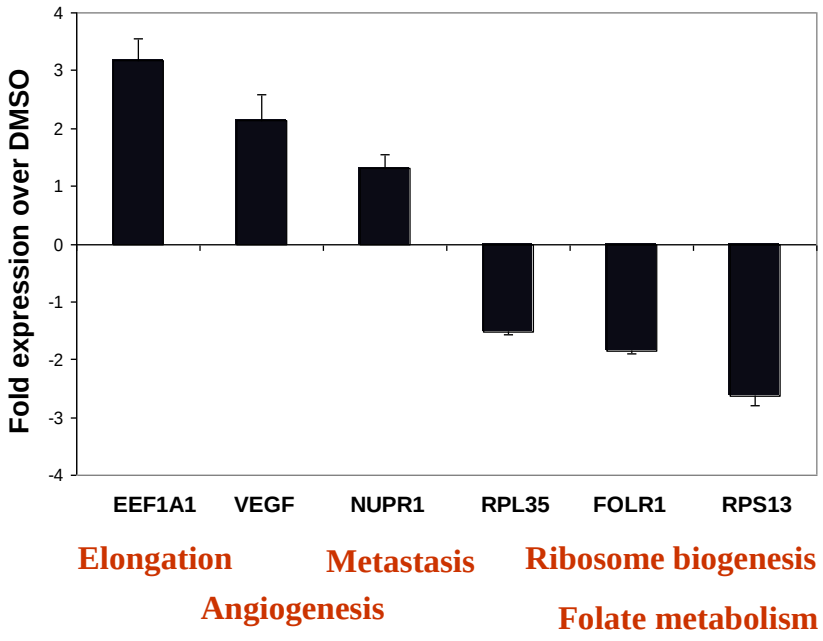


Figure 5.19*. Validation of representative genes that are differentially modulated upon TBBD treatment in HeLa cells.*

Among the up regulated genes, many are known to be involved in disease manifestation by various signalling pathways as depicted in **Figure 5.18. A.** For example, *vascular endothelial growth factor* (*VEGF*) was significantly upregulated. This gene is important in angiogenesis, which is a process necessary for tumor growth. *EEF1A1* (eukaryotic translation elongation factor 1) and *P8/NUPR1* (similar to nuclear protein 1) both have roles in tumorigenesis and differentiation.

Among the genes that were downregulated by TBBD treatment, several are involved in ribosome biogenesis (**Figure 5.18, B**). CARM1-mediated methylation of components of the splicing machinery has been shown to play an important role in mRNA processing. It is quite possible that R17 methylation also plays a role in rRNA processing. However, TBBD treatment did not appear to affect protein synthesis, possibly because rRNA genes are multicopy genes. *FolR1*, encoding a folate receptor, is another example of a gene that exhibited downregulation of gene expression by TBBD treatment **(Figure 5.19).** An interesting observation is the up regulation as well as down regulation of several genes of signalling pathways (**Table 4**) that could potentially lead to cancer manifestation.

Table 4: List of common Up and Down regulated cancer related genes and corresponding pathways across all three data sets

S.No.	Up regulated genes	Corresponding pathways	Down regulated genes	Corresponding pathways
1.	*LDHA, TFRC, VEGFA*	HIF-1 α transcription factor network	*RPS11, RPS13*	TNFα signalling pathway
2.	*GRB10, VEGFA*	VEGFR1, VEGFR2 regulated pathway	*UBC*	CDK mediated phosphorylation and Cdc6 removal
3.	*EEF1A1, GRB10*	EGFR1 signalling pathway	*XRCC6*	Co-regulation of androgen receptor activity
4.	*TFRC*	FOXA2, FOXA3 transcription factor network	*FOLR1*	Folic acid transport
5.	*CTBP2*	Wnt signalling pathway		
6.	*ATF3*	TGFBR signalling pathway		
7.	*IRF9*	IFNα signalling pathway		
8.	*GRB10*	Stem cell factor receptor (c-kit) associated signalling		

Possibly, H3R17 methylation is an important modulator of tumor suppressor function since the inhibition of R17 methylation leads to an increased gene expression in cancer promoting genes. However, there are both pro-angiogenic and anti-angiogenic signals which were differentially regulated, indicating the significance of arginine methylation in maintaining a balance of these signals. An intensive analysis of CARM1-mediated H3R17 methylation in different cell lines under different conditions should be undertaken to elucidate the spatiotemporal importance of H3R17 methylation. An initial attempt towards understanding this difference was attempted which will be discussed in the subsequent section.

Thus, the treatment of TBBD and the subsequent inhibition of H3R17 methylation led to differential modulation of pathways promoting cancer as well as preventing cancer. However, the down regulation of folate receptor gene indicated that the folate metabolism could be compromised. And most interestingly, the extensive down regulation of ribosome biogenesis genes and various other RNA processing machinery were again indicative of a possible anti-cancer activity as these are the events that are markedly up regulated during the process of cancer manifestation. Since, the entire search for the inhibitor for CARM1 was initiated with the literature known about breast cancer, it was decided that the effect of TBBD should be investigated in a breast cancer cell line.

5.7. Effect of H3R17 methylation inhibition on breast cancer cell line:

The earlier data on TBBD (ellagic acid) suggests an anti-cancer activity of this molecule. The microarray experiment suggests that there might be a tumor suppressor promoting role of H3R17 methylation. The results obtained with the p53 target gene expression also signifies an important role of R17 methylation in tumor suppressor function. One way to address this anamoly is the use of a cancer cell line where the anti-cancer effect of TBBD has been shown.

Hence, we selected the breast cancer cell line MCF7, for performing these experiments. The inhibition of H3R17 methylation was verified by performing an immunofluorescence analysis using the dimethylated H3R17 antibody. At very low

micromolar concentrations of 5 μM for 12 hours, there was a drastic decrease in the levels of methylation (**Figure 5.20, 3-dimensional profile**). On further increase of TBBD, there was a dose dependent decrease in the H3R17 methylation levels.

Apart from the ER positive cell line, MCF7, the similar analysis was also carried out on the ER negative cell line, HBL100, shown in **Figure 5.21**. Even in this cell line, a drastic reduction in the methylation of the H3R17 levels by very low concentrations of TBBD (5 μM), was observed as monitored by the immunofluorescence analysis.

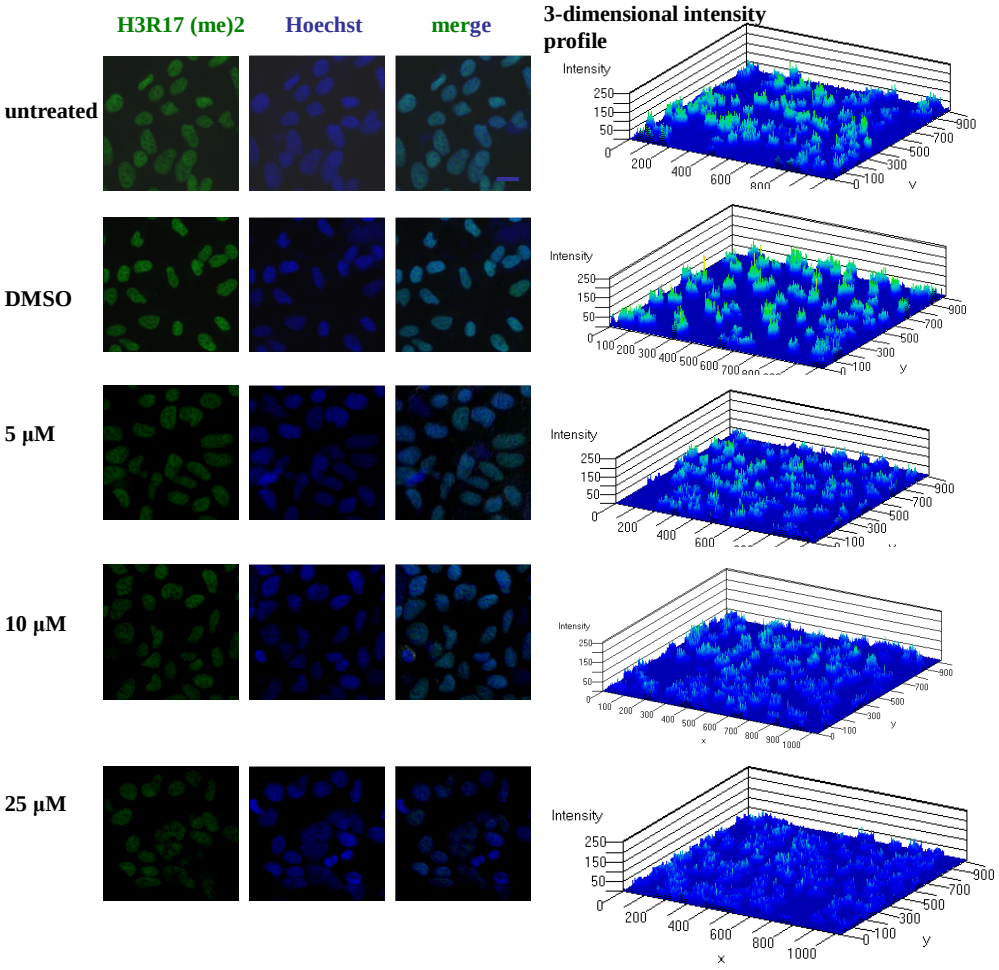


Figure 5.20. TBBD inhibits H3R17 methylation at low micromolar concentrations in breast cancer cell line. *Immunofluorescence analysis of MCF7 cells untreated, treated with DMSO, TBBD (5, 10, 25 μM) with H3R17 dimethylation antibody (green), counter- stained with Hoechst for DNA staining (blue) and the merged image of the two. The 3-dimensional profile represents the intensity profile of the green fluorescence of the antibody used for staining. Each circle represents a single cell and the peaks represent the intensity of the modification.*

A further increase in the concentration upto, five fold, led to a complete loss in the H3R17 methylation levels. These observations indicate that TBBD mediated H3R17 methylation inhibition is indeed true across different cell lines. But, in the breast cancer cell line, MCF7 that has been shown to overexpress CARM1, the H3R17 methylation inhibition occurs at low concentrations and at lesser time point of treatment.

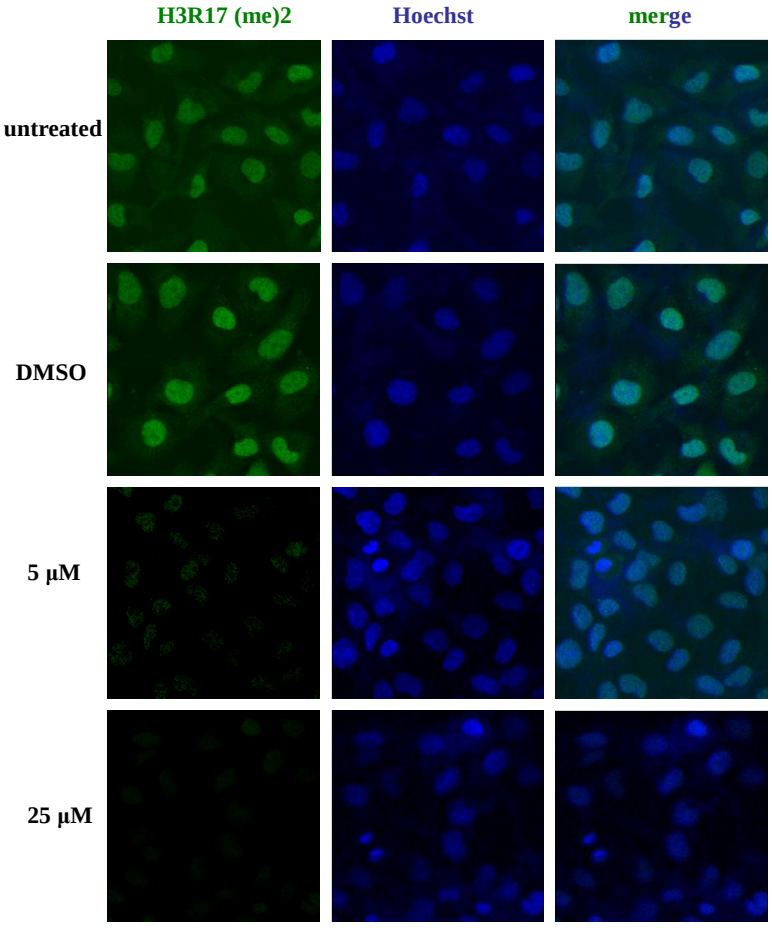


Figure 5.21. TBBD inhibits H3R17 methylation at low micromolar concentrations in breast cancer cell line. *Immunofluorescence analysis of HBL100 cells untreated, treated with DMSO, TBBD (5, 10, 25 µM) with H3R17 dimethylation antibody (green), counter stained with Hoechst for DNA staining (blue) and the merged image of the two.*

The similar level of inhibition on both ER positive and ER negative cell line indicates that the estrogen receptor status is not involved in mediating the effect of TBBD. The treatment although given in different cell line was not subjected to any estrogen induction. Hence, the role of an ER induction cannot be commented upon.

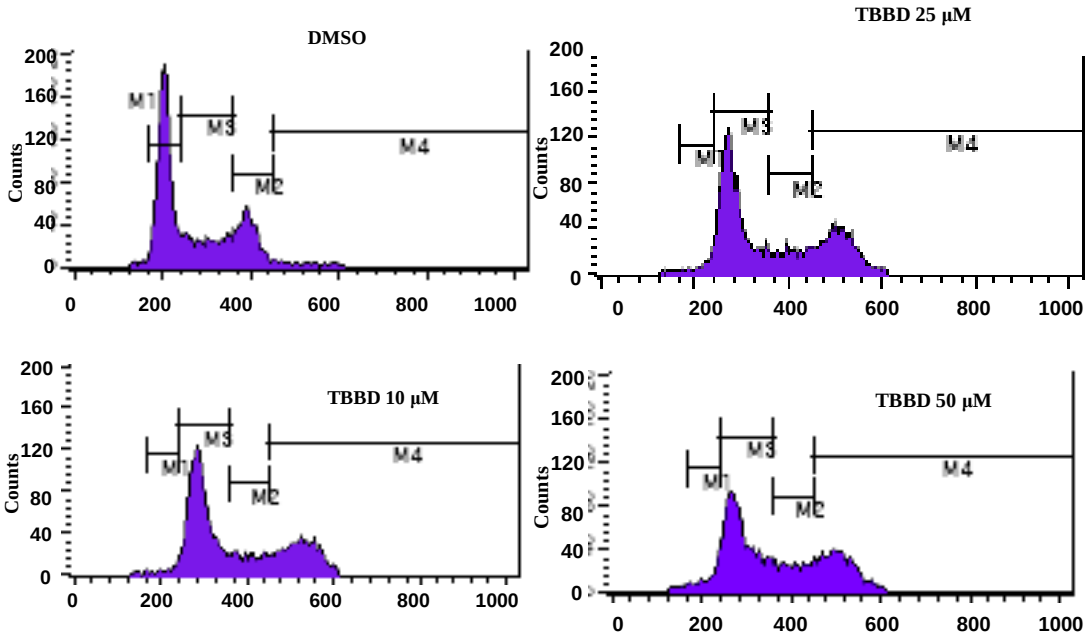


Figure 5.22. FACS analysis of TBBD treated MCF7 cells. *Cells were treated with the indicated concentration of TBBD for 24 hours. PI stained nuclei were gated in M1, M2, M3 and M4 regions in the FL2 channel*

To further verify this apparent cytotoxicity on the MCF-7 cells, a FACS analysis was performed to check the effect on cell cycle. Surprisingly, the 10 µM concentration itself induced a significant accumulation of cells in the M3 region indicating a S-phase arrest **(Figure 5.22).**

In the case of MCF-7 cells, a report indicates the role of H3R17 methylation in the regulation of E2F activation. E2F is a critical regulator of the G1-S transition (Sala et al., 1994). Since, TBBD inhibits this modification, it is possible that there could be a cell-cycle arrest, which is observed in the FACS analysis. It is indeed clear that TBBD mediated

H3R17 methylation inhibition can indeed be an anti-cancer mechanism of action in specific cell types. As can be observed in **Figure 5.21 and 5.22.** the TBBD treatment was highly toxic at concentrations beyond 25 μM and the H3R17 methylation inhibition was also found to occur at low micromolar concentration such as 10 μM. These results clearly indicate a differential effect of TBBD on different cell lines and thereby indicating the role of H3R17 methylation to be also different in different cell lines. This cell line specific effect is similar to the report on CARM1 phosphorylation (Higashimoto et al., 2007; Feng et al., 2009), which occurs in a specific cell type. All these results put together necessitate a detailed analysis in different cell lines for translation into the physiological system.

Thus, the results presented in this chapter using the enzyme specific inhibitors as biological probes has helped in understanding the physiological role of arginine methylation and associated epigenetic mark, acetylation in the regulation of p53 dependent *p21* gene expression. The use of TBBD has also led to the observation that H3R17 methylation is essential for the pluripotency marker gene expression. Thus, out of the different cellular functions, the p53 transcriptional activation and pluripotency maintenance have been delineated to be regulated majorly by the enzymatic activity of CARM1. Apart from the role of these inhibitors in understanding the enzyme function, these could also be used to understand different epigenetic pathways involved in the maintenance of pluripotency and differentiation. Since the enzymatic activities of p300 and CARM1 have been linked to several diseases including cancer, these inhibitors may serve as lead molecules to design new generation therapeutics targeting chromatin (epigenetic) modifications which will be discussed in the next chapter on future perspectives.

Chapter 6

Summary and Future Perspectives

The chapter provides a summary of the research work presented in the thesis, highlighting the significant findings, as well as providing an insight into the future implications.

6.1. Significant findings

The present thesis was focused towards understanding the role of histone arginine methylation and lysine acetylation in the regulation of gene expression by employing an inhibitor based approach. Although, it is well established that CARM1 and p300 are bonafide transcriptional co-activators, their contributions as arginine methyltransferase and lysine acetyltransferase is largely ambiguous. Amongst CARM1 and p300, the arginine methyltransferase CARM1 has been less understood both in terms of the enzyme mechanism as well as its physiological significance. Hence, we decided to elucidate the role of CARM1 in gene regulation by a small molecule modulator and address important pathways alongwith the arginine methylation associated epigenetic mark, acetylation (with the help of an acetyltransferase specific modulator).

In summary, this research work has led to the identification of an arginine methyltransferase, CARM1/PRMT4 specific inhibitor, TBBD (ellagic acid) from pomegranate crude extract (**described in Chapter 3**). TBBD inhibits CARM1 in an uncompetitive manner, hence requiring the enzyme-substrate (ES) complex for inhibition. TBBD was found to interact with the substrate histone H3 with a single binding site. The specificity of the inhibition was retained even in the cellular context and interestingly, TBBD inhibited the CARM1 mediated H3R17 methylation alone without affecting the methylation of another physiological site of the enzyme, H3R26 methylation. Molecular modelling studies using the reported CARM1-histone H3 crystal structure and TBBD

implicated a differential interaction of TBBD with histone H3 at the flanking residues of H3R17 and H3R26 methylation. Proline residue at the 16th position was identified as a critical determinant of the enzyme substrate-inhibitor interaction (ESI) interaction and subsequent inhibition which was also verified by point mutants. Thus, inhibition of CARM1 H3R17 methylation by TBBD established a novel mechanism of enzyme inhibition wherein the substrate sequence determines the specificity of the inhibition (Selvi et al., 2010).

The work highlighted in **chapter 4** describes the identification and characterization of a p300 acetyltransferase specific inhibitor, RTK1 (plumbagin) from the roots of a medicinal plant, *Plumbago rosea*. RTK1 was found to be a potent inhibitor of histone acetylation in vivo, and retained its p300 specific inhibitory activity even in the physiological condition on p53 acetylation. RTK1 inhibits the p300 acetyltransferase in a noncompetitive manner. It shows a very low micromolar IC_{50} against the minimal HAT domain and also had a strong binding affinity. Molecular modelling of RTK1 with the p300 minimal HAT domain crystal structure led to the identification of a lysine residue, K1358 as an important residue in mediating the enzyme-inhibitor (EI) interaction. Subsequent mutation analysis led to the observation that the K1358 residue was essential for the acetyltransferase activity of p300 and thus RTK1 by virtue of interacting specifically with an important residue could potently inhibit p300 acetyltransferase activity. Most importantly, this work also led to the identification of the chemical entity essential for the inhibition of p300 acetyltransferase, since the hydroxy substituted derivatives of the hydroxyl naphthoquinone, plumbagin did not affect HAT activity. All the naturally occuring HAT inhibitors discovered so far possess one or more 'OH' groups. It could be possible that the hydroxyl-group is an important determinant for p300 acetyltransferase inhibition (Selvi et al., 2009).

The results in **chapter 5** signifies the importance of H3R17 methylation and p300 mediated histone acetylation in the regulation of gene expression. The DNA damage dependent p53 transcriptional activation was selected as one of the pathways to study the role of the above modifications. By using TBBD, we conclusively show that H3R17 methylation regulates p53 downstream target *p21* expression. As expected, p300 specific inhibitor also abrogated *p21* expression since acetylation is an important regulator of p53 function. Additionally, another p53 responsive gene, *bax* expression was also repressed

upon the inhibitor treatment implicating the role of H3R17 methylation and histone acetylation in the p53 dependent gene expression. The earlier report (An et al., 2004) implicated the significance of these modifications in the DNA damage pathway, the results described in **Chapter 5** indicate that the modifications also regulate the cell cycle and apoptosis pathway.

A detailed global gene expression analysis has led to the identification of several pathways modulated by H3R17 methylation. Several signalling pathways associated with cancer manifestation showed an up regulated expression on H3R17 methylation inhibition, indicating its importance in promoting tumor suppressor function which was also evident from the effect on p53 dependent gene expression. A few development associated genes were also up regulated and hence the effect of TBBD was tested on human ES cells. Very low micromolar concentration of the inhibitor could downregulate the expression of pluripotency marker genes, *Oct4/Pou5f1* and *Nanog* levels. The histone acetylation had stronger repression on *Nanog* than *Oct4/Pou5f1,* implicating the differential regulatory role of these modifications on pluripotency maintenance.

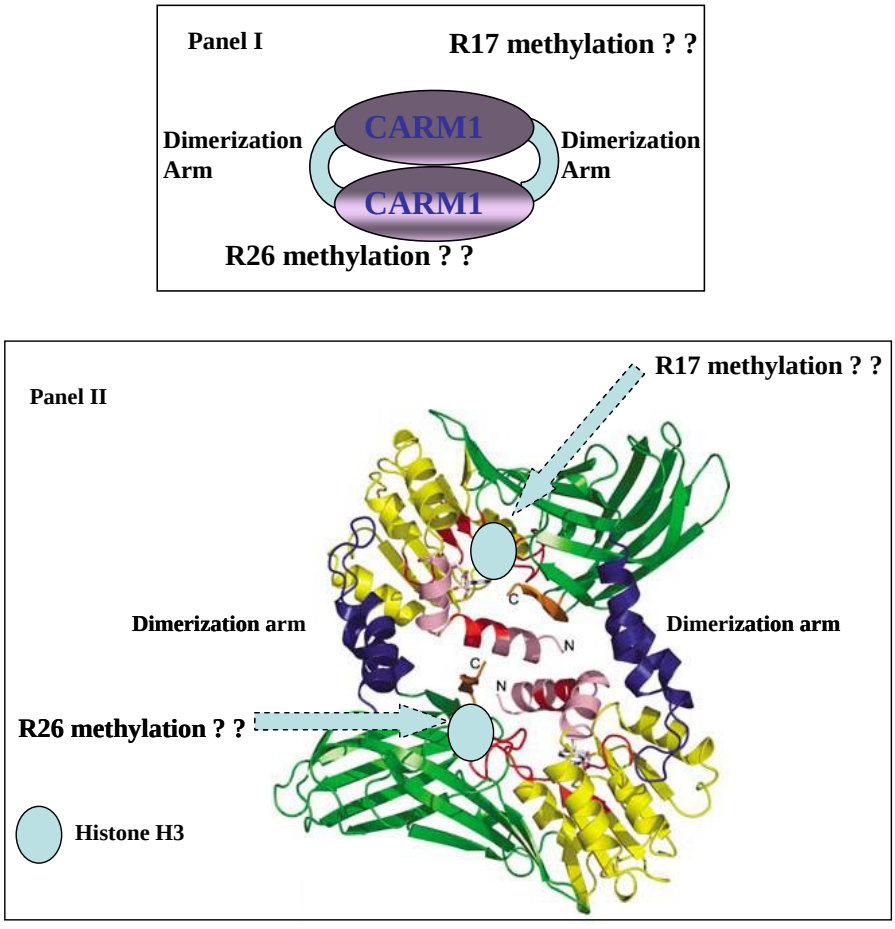
An important observation was the inhibition of H3R17 methylation leading to downregulation of several genes in the ribosome biogenesis pathway. Additionally, folate metabolism associated genes also showed a decreased expression on inhibitor treatment. These set of genes are critical determinants of an anti cancer effect. Hence, the effect of TBBD was tested on a breast cancer cell line, MCF7 which showed a S-phase arrest on treatment with low micromolar concentration (25μM) of TBBD. Although, TBBD was found to have very minimal toxicity on HeLa cells, the similar treatment conditions showed profound cytotoxicity in MCF7 cells indicating a cell type specific response. Thus, TBBD mediated H3R17 methylation inhibition has led to the identification of important signalling pathways regulated by this modification which needs to be investigated in the light of different cell type and signal responses.

6.2. Implications of the research findings:

6.2.1. Partial elucidation of the CARM1 enzyme mechanism:

One of the important questions in the field of CARM1 enzyme biology is whether there exists a sequential modification of the CARM1 methylation sites on histone H3. Since,

TBBD inhibits H3R17 methylation specifically, it is clear that R17 methylation is not essential for H3R26 methylation to occur, however the reverse may not be true and needs other evidences. CARM1 functions as a dimer and the substrate binding site has been modelled with the PRK residues of histone H3. It was also hypothesized that the H3R26 methylation site might be different. The results obtained suggest that possibly the H3R17 methylation and H3R26 methylation might occur on the different monomers of the functional dimer (**Figure 6.1**). Since, the ITC data suggests that there is a single binding site of TBBD on histone H3, it suggests the presence of the KAPRK motif only once in the CARM1-histone H3 ES complex.



-adapted from Troffer-Charlier et al., 2007

Figure 6.1. Proposed mechanism of CARM1 mediated histone modification. *Panel I represents a cartoon of the possible mode of H3R17 and H3R26 methylation on different monomers of the CARM1 functional dimer. Panel II depicts the same on the reported crystal structure representation. The SAH-bound monomer has been*

modelled as a dimer in which the blue ribbon depicts the dimerization arm. The histone H3 is depicted by the blue filled circle. The dotted arrow indicates the probable modification.

A co-crytallization approach might help in the identification of the exact positioning of TBBD on histone H3 in the CARM1 substrate binding site.

6.2.2. Significance of the p300 K1358 residue :

The lysine residue K1358 seems to be a critical residue for the p300 acetyltransferase activity. It is not one of the residues involved in the autoacetylation loop formation, but it is very close to the negative regulatory region which holds the activation loop. The exact role of K1358 in the p300 enzyme needs to be identified. The significance of this residue in the light of other HAT inhibitors needs to be investigated. The druggability of K1358 is also another aspect that needs urgent attention. There are no disease mutations reported for p300 K1358 as yet, however p300 mutation studies are lesser than the deletion mapping. Hence, apart from cancer, the role of K1358 needs to be investigated in other diseases too.

6.2.3. H3R17 methylation and regulation of gene expression:

The global gene expression analysis showed an up regulation of 35% genes and down regulation of 65% genes amongst the differentially regulated genes. There has been a lot of speculation about the role of H3R17 methylation in transcription. The observation that about 35% genes show an up regulated expression upon inhibitor treatment is an indication towards the transcriptional repression role of CARM1. This has been further strengthened by the gene expression analysis of CARM1 RNAi in ES cells wherein the up regulated gene expression was higher than the down regulated gene expression (Wu et al., 2009). The silencing of CARM1 leads to abrogation of both its methyltransferase as well as co-activation ability. On the other hand, the use of TBBD specifically targets the H3R17 methylation alone, hence a comparison across the gene sets of these two experimental data was attempted. Interestingly, more than 20 genes were differentially modulated across different data sets in the above mentioned experimental conditions. **Table 1** represents the common genes that were differentially regulated in TBBD treated HeLa cells and CARM1 RNAi ES cells.

Table 1: Common gene set between H3R17 methylation inhibition and CARM1 RNAi

UP REGULATED GENES	DOWN REGULATED GENES
CRYAB, CEBPB, EFNA, TNFRS, GADD45 , SERPIN, DUSP, TGFBR IGFBP, KLF, GATA, CLIC, SEMA, IRF, TPBG, MDM, PARP, ARHGE, TES, ETS2, IFI, PRR7, DSG2 , CAMK2, MTPN, WSB, STX3, YWHAB,DDIT3, MBNL2 , RTN, VGLL4, UBE2, COPA, DNAJ	*PLA2G2 , AURK, PFKL , CENP, PRPS1, PIF1, POLD2, HK2, Eno1, Bub1, ADAM, PKM2, PDXP, POLR2E, SNRP, LMN B, MRPL, MRPS, FKBP4, FANC, E2F, XRCC6, SULT4A1, PGK1, MYO1, HIST , SLC , PLEKHA1,ANP, POLD2, NCAPH, ANP32 , PKM2, GALK1 , PRR6, PDXP, SNRP, TMEM , TK1, COX, GPR, KIF, TFB, NME4 , CCDC , WASF1, APOEC3G TUB, RPL13A*

It is pertinent to point out that the cell lines used for both these experiments are different.. However, the up regulation of signalling pathway associated genes such as Insulin growth factor binding protein (IGFBP), Transforming growth factor receptor (TGFBR), Interleukin (IL) etc. indicate the role of H3R17 methylation in these pathways. A detailed analysis of these pathways might reveal novel interactions between the epigenetic mark and signalling events. Several kinases, ribosome biogenesis pathway associated genes were also common across the two data sets implicating the significance of H3R17 methylation in these events.

6.2.4. Role of H3R17 methylation in tumor suppressor function

The results obtained with the p53 transcriptional activation system and the microarray analysis suggest a role of H3R17 methylation in tumor suppressor function. However, the effect of H3R17 methylation on folate metabolism and ribosome biogenesis suggest a role in cancer manifestation. Indeed there are conflicting observations regarding these processes. It is also essential to consider the fact that CARM1 mediated histone H3R17 methylation mark is a modification associated with promoters, hence its role on

different promoters would lead to different effects. Additionally, CARM1 is also a transcriptional co-activator recruited to different promoters by its interacting partners. Apparently, H3R17 methylation mark is associated with tumor suppressor function and its specific recruitment to promoters such as AR, ER, E2F and SRC3 by the interacting proteins skews it towards a cancer promoting role (**Figure 6.2**).

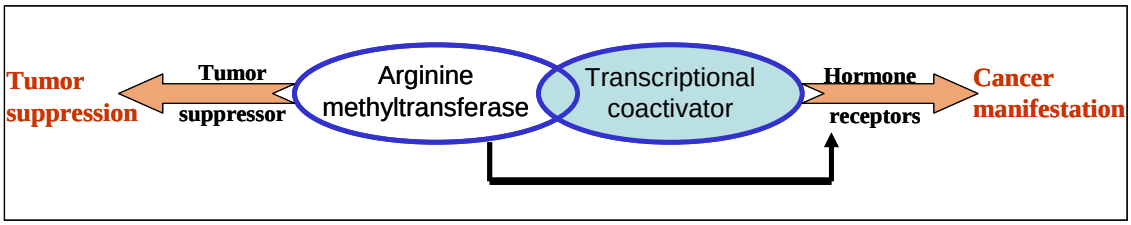


Figure 6.2. Role of CARM1 in tumor suppression/tumor progression. *CARM1 is an arginine methyltransferase as well as a transcriptional co-activator. It facilitates tumor suppressor function, however its specific recruitment to hormone receptors to function as a transcription co-activator influences cancer manifestation. Additionally, the histone methyltransferase activity also supports this process.*

Hence, the balance between these differential promoter occupancy might be the key to H3R17 methylation mediated regulation of cellular homeostasis.

6.3. Future Perspectives

TBBD mediated H3R17 methylation inhibition has led to the identification of important facets of CARM1 associated gene expression. The differential modulation of several signalling pathways implicated an intricate regulation of gene expression which possibly involves other players too. The recent understanding of gene expression suggests a significant regulatory role of micro RNAs (miRNAs) in gene expression (reviewed in Chuang and Jones, 2007). miRNAs bind to their target mRNAs and down-regulate their stabilities and/or translation. When binding to its target mRNA with complete complementarity, the miRNA can lead to degradation of the target. miRNAs can also bind to their targets with incomplete complementarity, often in the 3' UTR regions, and this leads to the translational suppression of their target genes (Esquela-Kerscher and Slack, 2006; Kim and Nam, 2006; Meltzer, 2005). Each miRNA is predicted to have many targets, and each

mRNA may be regulated by more than one miRNA (Rejewsky, 2006; Lewis, 2003; Lim, 2005).

The link between histone modifications and miRNA became evident by an elegant work by Saito et al., 2006 wherein the treatment of DNA methyltransferase inhibitor (DNMTi), 5-Aza-CdR and histone deacetylase inhibitor (HDACi), 4-phenylbutyric acid (PBA) was shown to up regulate the expression of miR-127 leading to reactivation of genes that were silenced. The HDACi, LAQ824 treatment to SKBr3 breast cancer cell line, induced a rapid change in the miRNA expression profile (Scott et al., 2006). Hence, we attempted to understand the role of H3R17 methylation on miRNA expression. A representative member, miR145 is a putative tumor suppressor (Calin and Croce, 2006) and has been shown to be induced by p53 and repress c-myc (Sachdevaa et al., 2009) was selected. Additionally, miR145 has been implicated in smooth muscle development (Cordes et al., 2009). Most importantly, it has been shown to suppress cell growth, invasion and metastasis (Sachdeva and Mo, 2010). Hence, HeLa cells were treated with TBBD and the miR145 profile was checked which indicated an up regulation of miR145 processing (**Figure 6.3**), which might also account for the anti cancer property of TBBD which will be detailed in the next section.

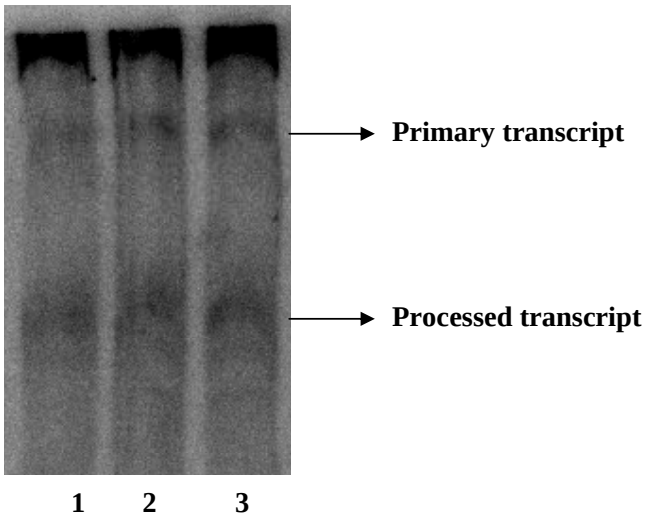


Figure 6.3. Effect of TBBD treatment on miR145 . *HeLa cells were treated with DMSO (lane 1) 25 μM (lane 2) and 100 μM (lane 3) TBBD, and RNA from treated cells was subjected to northern blotting with miR145 as the probe. An induction of miR145 at the primary and processed transcript level was observed with TBBD treatment.*

Thus, there seems to be a modulatory effect of H3R17 methylation on miRNA expression which needs to be further investigated to establish the link between arginine methylation and the miRNA network.

The overall summary of the significant findings is depicted in **Figure 6.4.** The use of the small molecule modulators, TBBD and RTK1 has led to the elucidation of the role of histone H3R17 methylation and acetylation in the regulation of gene expression. It has also led to the identification of important signalling pathways regulated by histone H3R17 methylation.

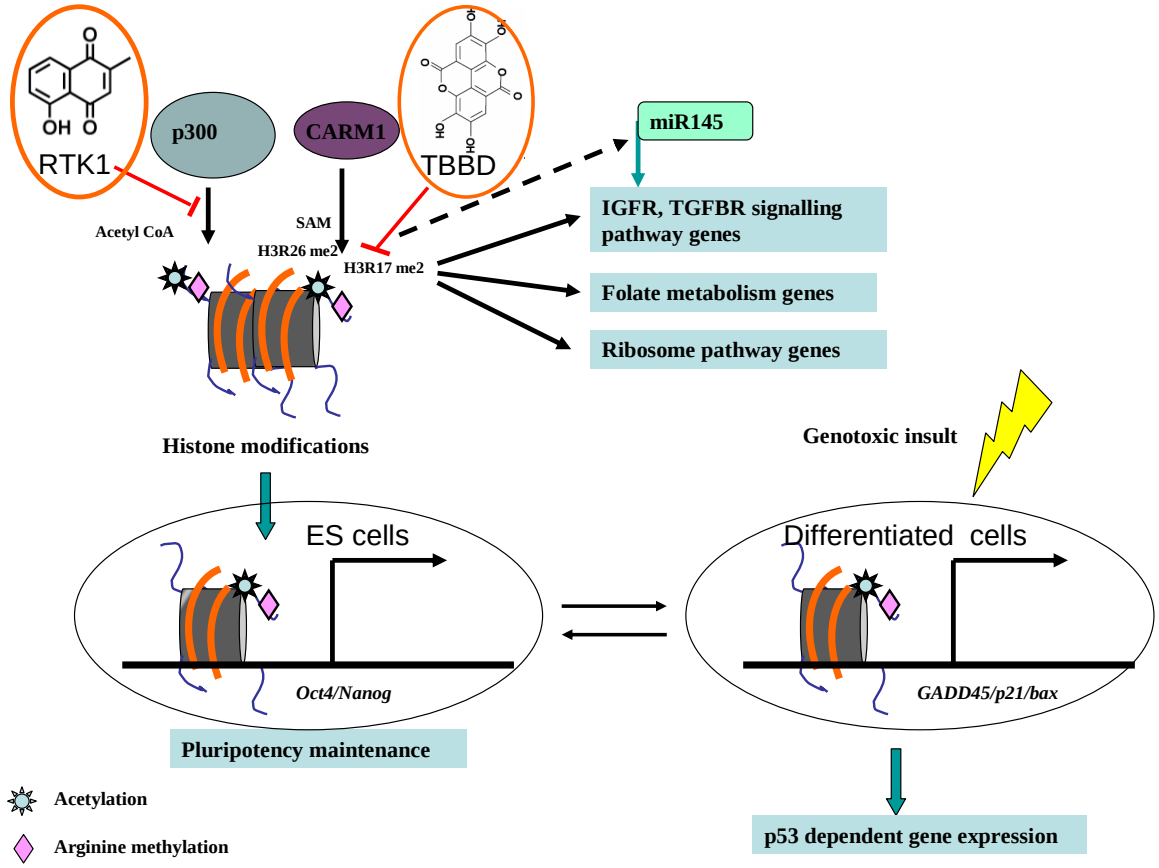


Figure 6.4. Histone H3R17 methylation by CARM1 and histone acetylation by p300 in the regulation of gene expression. *CARM1 methylates histone H3R17 and R26. p300 acetylates histones. In the undifferentiated cells, the Oct4/Nanog promoters show the presence of histones modified by p300 and CARM1, which is essential for pluripotency associated marker gene expression. Different mechanisms lead to the differentiation. When differentiated cells are subjected to genotoxic insult, there is an induction of p53 dependent gene expression which requires H3R17 methylation and acetylation in the p53 responsive promoters. Additionally, H3R17 methylation also regulates the ribosome biogenesis pathway genes, folate metabolism pathway genes and the IGFR and TGFβ pathway genes. IGFR signalling is regulated by miR145, and TBBD mediated H3R17 methylation inhibition probably induces miR145 denoted by the dotted arrow.*

6.4. Therapeutic potential of TBBD (ellagic acid) and RTK1 (plumbagin):

Ellagic acid is a well known anti-cancer molecule whose primary target was unknown. CARM1 arginine methyltransferase is possibly, one of its targets within the cells. The anti cancer activity was exhibited on prostate cancer and breast cancer cell line (Losso et al., 2004) which incidentally shows an up regulation of CARM1 and H3R17 methylation. The S-phase arrest in breast cancer cell line implicates a therapeutic effect. The down regulation of folate metabolism associated genes and ribosome biogenesis pathway associated genes is another aspect in favor of its anti cancer property. A detailed investigation in different cell lines might shed light on its therapeutic abilities. The p300 specific inhibitor, RTK1 has already been shown to have anti cancer effects on hepatocellular carcinoma cell line (Bai et al., 2008). However, the apparent cellular toxicity precludes its use as a therapeutic agent. The derivatization of both these inhibitory scaffolds and subsequent pre-clinical studies might help these molecules in gaining therapeutic efficacy. Since, arginine methylation and acetylation exhibit a cross talk and presumably they form an epigenetic network, the combinatorial targeting of both these modifications by the small molecules could prove to be more beneficial in diseases such as cancer, neurodegenerative disorders, cardiac hypertrophy, which are currently the targets of 'Epigenetic Therapy'.

PUBLICATIONS

Research Papers:

1) **Selvi B R**, Kiran Batta, Kishore HA, Mantelingu K, Radhika A Varier, Balasubramanyam K, Pradhan S K, Dasgupta D, Sriram S, Agrawal S, Kundu T K. (2010) Identification of a novel inhibitor of CARM1 mediated methylation of histone H3R17. *J Biol Chem*. 285, 7143-7152

2) Ravindra KC*, **Selvi B R***, Arif M, Reddy BAA., Thanuja G.R., Agrawal S, Pradhan SK., Dasgupta D, Natesh N, Kundu TK. (2009). Inhibition of lysine acetyltransferase p300/KAT3B by a naturally occurring hydroxynaphtoquinone, plumbagin *J Biol Chem*. 284 (36), 24453-24464.

3) **Selvi, B R***, S Pradhan*, J Shandilya, C Das, B S Sailaja, G Nagashankar, S Gadad, A Reddy, D Dasgupta and Kundu TK. (2009), Putative anticancer therapeutic, Sanguinarine interacts with chromatin; modulates epigenetic modifications and chromatin transcription. *Chem Biol*.,16(2), 203-216.

4) Kumar GV, **Selvi B R**, Kishore AH, Kundu TK, Narayana C. (2008) Surface enhanced Raman spectroscopic studies of coactivator-associated arginine methyltransferase 1. J *Phys Chem B.* 112(21):6703-6707.

5) **Selvi B R***, Ostwal Y B*, Kundu T K. H3R17 methylation, p53 and miRNA network in regulation of gene expression (manuscript under preparation).

* These authors contributed equally.

Scientific Reviews:

1) Selvi B R and Kundu TK. (2009) Reversible acetylation of Chromatin: Implication in regulation of gene expression, disease and therapeutics. *Biotechnol J*. 4(3):375 – 390

2) Selvi B R, Mohan Krishna D V, Ostwal Y B and Kundu T K (2010). Modulators of Chromatin modifying enzymes in transcription and disease – A Connotation to Epigenetic Therapy. (*Communicated*)

3) Selvi B R, Cassel J C, Kundu T K and Boutillier A L. (2010) Tuning acetylation levels with HAT activators: therapeutic strategy in neurodegenerative diseases. (*Communicated*)

Book Chapters:

Swaminathan V, Ashok Reddy BA, **Selvi B R**, Sukanya MS and Kundu TK. (2007): Small Molecule Modulators in Epigenetics: Implication in gene expression and therapeutics. Subcell Biochem. 41:397-428. (*Chromatin and Disease* Ed. Kundu TK and Dasgupta D (Springer Press)

ABBREVIATIONS

µg	microgram
µl	microlitre
µM	micro molar
CARM1	Coactivator Associated Arginine Methyltransferase 1
cDNA	complementary Deoxyribonucleic acid
CPM	counts per minute
CTD	Carboxy terminal Domain
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
HAT	Histone acetyltransferase
HMT	Histone methyltransferase
ITC	Isothermal Titration Calorimetry
FACS	Fluorecence Activated Cell Sorting
kDa	kilo Dalton
min	minutes
mM	millimolar
ng	nano gram
PAGE	polyacrylamide gel electrophoresis
PCAF	p300/CBP associated factor
PMSF	Phenyl Methyl Sulfonyl Fluoride
PRMT	Protein Arginine Methyltransferase
RNA	Ribonucleic acid
RPM	Revolutions Per Minute
RT	Room Temperature
SAM	S-Adenosyl Methionine
SDS	Sodium Dodecyl Sulphate
Swi/Snf	Switch/Sucrose Non Fermenting
TCA	Trichloro Acetic Acid
TE	Tris-EDTA
TEMED	N,N,N'N'-Tetramethyl-ethylene diamine
UV	ultraviolet

REFERENCES:

Acharya, B. R., Bhattacharyya, B., Chakrabarti, G. The natural naphthoquinone plumbagin exhibits antiproliferative activity and disrupts the microtubule network through tubulin binding (2008) *Biochemistry*. **47**:7838-45.

Allfrey, V.G., Faulkner, R., Mirsky, A.E. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis (1964) *Proc. Natl. Acad. Sci. U. S. A.* **51** : 786–794.

Allis, C.D., Berger, S.L., Cote, J., Dent, S., Jenuwein, T., Kouzarides, T., Pillus, L., Reinberg, D., Shi, Y., Shiekhattar, R., Shilatifard, A., Workman, J., Zhang, Y. New nomenclature for chromatin-modifying enzymes. (2007) *Cell*. **131**:633-636

Amur, S. G., Shanker, G., Cochran, J. M., Ved, H. S., Pieringer, R. A. Correlation between inhibition of myelin basic protein (arginine) methyltransferase by sinefungin and lack of compact myelin formation in cultures of cerebral cells from embryonic mice (1986) *J. Neurosci. Res*. **16**:367-376.

An, W., Kim, J., Roeder, R. G. Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. (2004) *Cell*. **117**:735-748

Arif, M., Kumar, G.V., Narayana, C., Kundu, T.K. Autoacetylation induced specific structural changes in histone acetyltransferase domain of p300: probed by surface enhanced Raman spectroscopy. (2007) *J Phys Chem B.* **111**:11877-11879.

Bai, X., Wu, L., Liang, T., Liu, Z., Li, J., Li, D., Xie, H., Yin, S., Yu, J., Lin, Q., Zheng, S. J. Overexpression of myocyte enhancer factor 2 and histone hyperacetylation in hepatocellular carcinoma. (2008). *Cancer Res. Clin. Oncol.* **134**:83-91.

Balasubramanyam, K., Altaf, M., Varier, R. A., Swaminathan, V., Ravindran, A., Sadhale, P. P., Kundu, T. K. Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. (2004). *J. Biol. Chem.* **279**:33716-26.

Balasubramanyam, K., Swaminathan, V., Ranganathan, A., Kundu, T. K. Small molecule modulators of histone acetyltransferase p300. (2003). *J. Biol. Chem.* **278**: 19134-40.

Balasubramanyam, K., Varier, R. A., Altaf, M., Swaminathan, V., Siddappa, N. B., Ranga, U., Kundu, T. K. Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferase-dependent chromatin transcription. (2004) *J. Biol. Chem.* **279**:51163-71.

Ball, L. J., Jarchau, T., Oschkinat, H., Walter, U. EVH1 domains: structure, function and interactions. (2002) *FEBS Lett*. **513**:45–52.

Banerjee, S., Kumar, B. R., Kundu, T. K. General transcriptional coactivator PC4 activates p53 function. (2004) *Mol. Cell Biol*. **24**:2052-2062.

Bannister, A. J., Miska, E. A., Görlich, D., Kouzarides, T. Acetylation of importin-alpha nuclear import factors by CBP/p300. (2000). *Curr. Biol.* **10**:467-70.

Bar-Sela, G., Epelbaum, R., Schaffer, M. Curcumin as an anti-cancer agent: review of the gap between basic and clinical applications. (2010) *Curr. Med. Chem.* **17**:190-7.

Batta, K., Kundu, T.K. Activation of p53 function by human transcriptional coactivator PC4: role of protein-protein interaction, DNA bending, and posttranslational modifications. (2007) *Mol Cell Biol*. **27**:7603-7614.

Batta, K., Das, C., Gadad, S., Shandilya, J., Kundu, T.K. Reversible acetylation of non histone proteins: role in cellular function and disease (2007). *Chromatin and Disease*, Ed. Kundu TK and Dasgupta D, **41**:193-212. Review.

Bauer, U.M., Daujat, S., Nielsen, S. J., Nightingale, K., Kouzarides, T. Methylation at arginine 17 of histone H3 is linked to gene activation. (2002). *EMBO Rep.* **3**:39-44.

Bedford, M.T., Richard, S. Arginine methylation an emerging regulator of protein function. (2005) Mol Cell. 18:263-272. Review.

Bedford, M. T., Clarke, S. G. Protein arginine methylation in mammals: who, what, and why. (2009). *Mol*. *Cell.* **33**:1-13.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. Cell. 2006 Apr 21;125(2):315-26. A bivalent chromatin structure marks key developmental genes in embryonic stem cells.

Bertrand E, Erard M, Gómez-Lira M, Bode J. Influence of histone hyperacetylation on nucleosomal particles as visualized by electron microscopy Arch Biochem Biophys. 1984 Feb 15;229(1):395-8.

Black, J. C., Choi, J. E., Lombardo, S. R., Carey, M. A mechanism for coordinating chromatin modification and preinitiation complex assembly. (2006) *Mol. Cell.* **23**:809-18.
Bode J, Henco K, Wingender E. Modulation of the nucleosome structure by histone acetylation. Eur J Biochem. 1980 Sep;110(1):143-52.

Blomberg, N., Baraldi, E., Nilges, M., Saraste, M. The PH superfold: a structural scaffold for multiple functions. (1999). *Trends Biochem*. *Sci*. **24**:441–445.

Boulanger, M. C., Liang, C., Russell, R. S., Lin, R., Bedford, M. T., Wainberg, M. A., Richard, S. Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression. (2005). *J. Virol*. **79**:124-131.

Brooks, C.L., Gu, W. (2003). Ubiquitination, phosphorylation and acetylation:
the molecular basis for p53 regulation. *Curr. Opin. Cell Biol.* **15**:164–171.

Calin, G. A., Croce, C. M. MicroRNA signatures in human cancers. (2006). *Nat. Rev. Cancer.* **6**:857–866.

Rodríguez-Campos, A., Azorín, F. RNA is an integral component of chromatin that contributes to its structural organization. (2007) PLoS One. 2:e1182.

Chan, H.M., LaThangue, N.B. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. (2001). *J. Cell Sci.* **114**:2363-2373.

Chen, L.F., Fischle, W., Verdin, E., Greene, W. C. Duration of nuclear NF-kappaB action regulated by reversible acetylation. (2001). *Science.* **293**:1653-7.

Chen, S. L., Loffler, K. A., Chen, D., Stallcup, M. R., Muscat, G. E. The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2. (2002) *J. Biol. Chem.* **277**:4324-4333

Chen Y, Sprung R, Tang Y, Ball H, Sangras B, Kim SC, Falck JR, Peng J, Gu W, Zhao Y. Lysine propionylation and butyrylation are novel post-translational modifications in histones. Mol Cell Proteomics. 2007 May;6(5):812-9.

Cheng, D., Côté, J., Shaaban, S., Bedford, M. T. The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. (2007). *Mol. Cell* **25**:71-83.

Cheng, D., Yadav, N., King, R. W., Swanson, M. S., Weinstein, E. J., Bedford, M. T. Small molecule regulators of protein arginine methyltransferases. (2004). *J. Biol. Chem*. **279**:23892-9.

Chesire, D. R., Ewing, C. M., Gage, W. R., Isaacs, W. B. In vitro evidence for complex modes of nuclear beta-catenin signaling during prostate growth and tumorigenesis. *Oncogene.* (2002) **21**:2679-94.

Chevillard-Briet, M., Trouche, D., Vandel, L. Control of CBP co-activating activity by arginine methylation. (2002) *EMBO J.* **21**:5457-66.

Chin, H. G., Patnaik, D., Esteve, P. O., Jacobsen, S. E., Pradhan, S. Catalytic properties and kinetic mechanism of human recombinant Lys-9 histone H3 methyltransferase SUV39H1: participation of the chromodomain in enzymatic catalysis. (2006). *Biochemistry* **45**:3272–3584.

Choi, K. C., Jung, M. G., Lee, Y. H., Yoon, J. C., Kwon, S. H., Kang, H. B., Kim, M. J., Cha, J. H., Kim, Y. J., Jun, W. J., Lee, J. M., Yoon, H. G. Epigallocatechin-3-gallate, a histone acetyltransferase inhibitor, inhibits EBV-induced B lymphocyte transformation via suppression of RelA acetylation. (2009). *Cancer Res.* **69**:583-92.

Chuang, J.C., Jones, P.A. Epigenetics and MicroRNAs (2007) *Pediatr Res*. **61**:24-29

Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gamblin, S. J., Barlev, N. A., Reinberg, D. Regulation of p53 activity through lysine methylation. (2004) *Nature* **432**:353-360.

Cordes, K. R., Sheehy, N. T., White, M. P., Berry, E. C., Morton, S. U., Muth, A. N., Lee, T. H., Miano, J. M., Ivey, K. N., Srivastava, D. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. (2009). *Nature.* **460**:705-10.

Couture, J. F., Hauk, G. Thompson MJ, Blackburn GM, Trievel RC. Catalytic roles for carbon-oxygen hydrogen bonding in SET domain lysine methyltransferases. (2006) *J. Biol. Chem.* **281**:19280–19287

Covic, M., Hassa, P. O., Saccani, S., Buerki, C., Meier, N. I., Lombardi, C., Imhof, R., Bedford, M. T., Natoli, G., Hottiger, M. O. Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression. (2005). *EMBO J.* **24**:85–96.

Cozza, G., Bonvini, P., Zorzi, E., Poletto, G., Pagano, M. A., Sarno, S., Donella-Deana, A., Zagotto, G., Rosolen, A., Pinna, L. A., Meggio, F., Moro, S. Identification of ellagic acid as potent inhibitor of protein kinase CK2: a successful example of a virtual screening application. (2006). *J. Med. Chem.* **49**:2363-2366.

Daujat, S., Bauer, U. M., Shah, V., Turner, B., Berger, S., Kouzarides, T. Crosstalk between CARM1 methylation and CBP acetylation on histone H3. (2002). *Curr. Biol.* **12**:2090-2097.

Dekker, F.J., Hasima, H.J. Histone acetyltransferases as emerging drug targets. (2009) *Drug Discov. Today* **14**:942-948.

De Koning, L., Corpet, A., Haber, J. E., Almouzni, G. Histone chaperones: an escort network regulating histone traffic. (2007) *Nat. Struct. Mol. Biol.* **14**:997-1007.

Dellaire, G., Makarov, E. M., Cowger, J. J., Longman, D., Sutherland, H. G., Lührmann, R., Torchia, J., Bickmore, W. A. Mammalian PRP4 kinase copurifies and interacts with components of both the U5 snRNP and the N-CoR deacetylase complexes. *Mol. Cell. Biol.* (2002) **22**:5141-56.

Denuc A, Marfany G. SUMO and ubiquitin paths converge. Biochem Soc Trans. 2010 Feb;38(Pt 1):34-9. Review.

Dieppois, G., Iglesias, N., Stutz, F. Cotranscriptional recruitment to the mRNA export receptor Mex67p contributes to nuclear pore anchoring of activated genes. (2006) *Mol. Cell. Biol.* **26**:7858–7870.

Dony, C., Kessel, M., Gruss, P. Post-transcriptional control of myc and p53 expression during differentiation of the embryonal carcinoma cell line F9. (1985) *Nature*. **317**:636-639.

Dorai, T., Aggarwal, B. B. Role of chemopreventive agents in cancer therapy. (2004). *Cancer Lett.* **215:**129-140.

Dou, Y., Milne, T.A., Tackett, A.J., Smith, E.R., Fukuda, A., Wysocka, J., Allis, C.D., Chait, B.T., Hess, J.L., Roeder, R.G. Physical association and coordinate function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase MOF. (2005) *Cell* **121**:873-885

Duan, Q., Chen, H., Costa, M., Dai, W. Phosphorylation of H3S10 blocks the access of H3K9 by specific antibodies and histone methyltransferase. Implication in regulating chromatin dynamics and epigenetic inheritance during mitosis. (2008) *J Biol Chem.* 2008 **283**:33585-33590

Egger, G., Liang, G., Aparicio, A., Jones, P.A. Epigenetics in human disease and prospects for epigenetic therapy (2004) *Nature* **429**:457–463

Eisen, J. A., Sweder, K. S., Hanawalt, P. C. Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. (1995). *Nucleic Acids Res*. **23**:2715–2723.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. (1993) *Cell* **75**:817–825.

Esquela-Kerscher, A., Slack, F. J. Oncomirs—microRNAs with a role in cancer (2006) *Nat. Rev.Cancer* **6**:259–269.

Feng, Q., Wang, H., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Struhl, K., Zhang, Y. Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain (2002) *Curr Biol.* **12**:1052-1058.

Feng, Q., He, B., Jung, S. Y., Song, Y., Qin, J., Tsai, S. Y., Tsai, M. J., O'Malley, B. W. Biochemical control of CARM1 enzymatic activity by phosphorylation. (2009) *J. Biol. Chem.* **284**:36167-74.

.

Feng, Q., Yi, P., Wong, J., O'Malley, B. W. Signaling within a coactivator complex: methylation of SRC-3/AIB1 is a molecular switch for complex disassembly. (2006) *Mol. Cell Biol.* **26**:7846-7857.

Fornace, A. J., Jackman, J., Hollander, M. C., Hoffman-Liebermann, B., Liebermann, D. A. Genotoxic-stress-response genes and growth-arrest genes: gadd, MyD, and other genes induced by treatments eliciting growth arrest. (1992). *Annals of the New York Academy of Sciences* **663**:139–153.

Frietze, S., Lupien, M., Silver, P. A., Brown, M. CARM1 regulates estrogen-stimulated breast cancer growth through up-regulation of E2F1. (2008). *Cancer Res*. **68**:301-306.

Frye, R. A. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. (2000). *Biochem. Biophys. Res. Commun.* **273**:793–798.

Garner, E., Raj, K. Protective mechanisms of p53-p21-pRb proteins against DNA damage-induced cell death. (2008). *Cell Cycle*. **7**:277-282.

Gervais, V., Lamour, V., Jawhari, A., Frindel, F., Wasielewski, E., Dubaele, S., Egly, J. M., Thierry, J. C., Kieffer, B., Poterszman, A. TFIIH contains a PH domain involved in DNA nucleotide excision repair. (2004). *Nat. Struct. Mol. Biol.* **11**:616–622.

Giles, R. H., Peters, D. J., Breuning, M. H. Conjunction dysfunction: CBP/p300 in human disease. (1998). *Trends Genet*. **14**:178-183.

Gill, G. Regulation of the initiation of eukaryotic transcription. (2001). *Essays Biochem.* **37**:33–43.

Girdwood, D., Bumpass, D., Vaughan, O.A., Thain, A., Anderson, L.A., Snowden, A.W., Garcia-Wilson, E., Perkins, N.D., Hay, R.T. P300 transcriptional repression is mediated by

SUMO modification (2003) Mol Cell. 11:1043-1054.

Giordano, A., Avantaggiati, M. L. p300 and CBP: partners for life and death.(1999). *J. Cell Physiol*. **181**:218-230.

Goodman, R. H., Smolik, S. CBP/p300 in cell growth, transformation, and development. (2000). *Genes Dev*. **14:**1553-1577.

Gregoretti, I. V., Lee, Y. M., Goodson, H. V. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. (2004) *J. Mol. Biol*. **338**:17–31.

Grossman, S.R., Deato, M.E., Brignone, C., Chan, H.M., Kung, A.L., Tagami, H., Nakatani, Y., Livingston, D.M. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. (2003) *Science* **300**:342-344.

Gu, W., Roeder, R. G. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. (1997). *Cell*. **90**:595-606.

Guccione, E., Bassi, C., Casadio, F., Martinato, F., Cesaroni, M., Schuchlautz, H., Lüscher, B., Amati, B. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. (2007) *Nature* **449**:933-937.

Guo, H. B., Guo, H. Mechanism of histone methylation catalyzed by protein lysine methyltransferase SET7/9 and origin of product specificity. (2007) *Proc. Natl. Acad. Sci. U S A* **104**:8797–8802.

Hassan, Y.I., Zempleni, J. A novel, enigmatic histone modification: biotinylation of histones by holocarboxylase synthetase. (2008) *Nutr Rev.* **66**:721-725. Review.

Havas, K., Flaus, A., Phelan, M., Kingston, R., Wade, P.A., Lilley, D.M., Owen-Hughes T. Generation of superhelical torsion by ATP-dependent chromatin remodeling activities. (2000) *Cell* **103**:1133-1142.

Ho E, Clarke JD, Dashwood RH. Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. J Nutr. 2009 Dec;139(12):2393-6.

Higashimoto, K., Kuhn, P., Desai, D., Cheng, X., Xu, W. Phosphorylation-mediated inactivation of coactivator-associated arginine methyltransferase 1. (2007) *Proc. Natl. Acad. Sci. U S A.* **104**:12318-23.

Hong, H., Kao, C., Jeng, M. H., Eble, J. N., Koch, M. O., Gardner, T. A., Zhang, S., Li, L., Pan, C. X., Hu, Z., MacLennan, G. T., Cheng, L. Aberrant expression of CARM1, a transcriptional coactivator of androgen receptor, in the development of prostate carcinoma and androgen-independent status. (2004). *Cancer*. **101**:83-89.

Huang, J., Perez-Burgos, L., Placek, B. J., Sengupta, R., Richter, M., Dorsey, J.A., Kubicek, S., Opravil, S., Jenuwein, T., and Berger, S. L. Repression of p53 activity by Smyd2-mediated methylation. (2006) *Nature* **444**:629–632.

Huang, J., Dorsey, J., Chuikov, S., Zhang, X., Jenuwein, T., Reinberg, D., Berger, S. L. G9a and Glp methylate lysine 373 in the tumor suppressor p53. (2010) *J. Biol. Chem*. **285**:9636-9641.

Huang, J., Sengupta, R., Espejo, A.B., Lee, M.G., Dorsey, J.A., Richter, M., Opravil, S., Shiekhattar, R., Bedford, M.T., Jenuwein, T., Berger, S.L. p53 is regulated by the lysine demethylase LSD1. (2007) *Nature* **449**:105-108.

Huynh, T., Chen, Z., Pang, S., Geng, J., Bandiera, T., Bindi, S., Vianello, P., Roletto, F., Thieffine, S., Galvani, A., Vaccaro, W., Poss, M. A., Trainor, G. L., Lorenzi, M. V., Gottardis, M., Jayaraman, L., Purandare, A. V. Optimization of pyrazole inhibitors of Coactivator Associated Arginine Methyltransferase 1 (CARM1). (2009). *Bioorg. Med. Chem. Lett.* **19**:2924-2927.

Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolffe, A. P., Ge, H. Acetylation of general transcription factors by histone acetyltransferases. (1997). *Curr. Biol.* **7**:689-692.

Ito, T., Yadav, N., Lee, J., Furumatsu, T., Yamashita, S., Yoshida, K., Taniguchi, N., Hashimoto, M., Tsuchiya, M., Ozaki, T., Lotz, M., Bedford, M. T., Asahara, H. Arginine methyltransferase CARM1/PRMT4 regulates endochondral ossification. (2009) *BMC Dev. Biol*. **9**:47- 56.

Ito, T., Bulger, M., Pazin, M. J., Kobayashi, R., Kadonaga, J. T. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. (1997). *Cell* **90**:145–155.

Iyer, N. G., Ozdag, H., Caldas, C. p300/CBP and cancer. (2004) *Oncogene* **23**:4225-4231.

Janknecht, R., Hunter, T. Transcription. A growing coactivator network. (1996) *Nature.* **383**:22-23.

Jayne, S., Rothgiesser, K. M., Hottiger, M. O. CARM1 but not its enzymatic activity is required for transcriptional coactivation of NF-kappaB-dependent gene expression. (2009). *J. Mol. Biol*. **394**:485-495.

Jiang, T., Zhou, X., Taghizadeh, K., Dong, M., Dedon, P.C. N-formylation of lysine in histone proteins as a secondary modification arising from oxidative DNA damage. (2007) *Proc Natl Acad Sci U S A.* **104**:60-65.

Kelley, R.L., Kuroda, M.I. Noncoding RNA genes in dosage compensation and imprinting. (2000) *Cell* **103**: 9–12.

Kim, D., Lee, J., Cheng, D., Li, J., Carter, C., Richie, E., Bedford, M. T. Enzymatic activity is required for the in vivo functions of CARM1. (2010). *J. Biol. Chem*. **285**:1147-1152.

Kim, J., Lee, J., Yadav, N., Wu, Q., Carter, C., Richard, S., Richie, E., Bedford, M. T. Loss of CARM1 results in hypomethylation of thymocyte cyclic AMP-regulated phosphoprotein and deregulated early T cell development. (2004). *J. Biol. Chem.* **279**: 25339–25344.

Kim, M.Y., Zhang, T., Kraus, W.L. Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. (2005) *Genes Dev*. **19**:1951-1967. Review.

Kim, S., Park, G.H., Paik, W.K. Recent advances in protein methylation: enzymatic methylation of nucleic acid binding proteins. (1998) *Amino Acids* 15:291-306. Review.

Kim,V. N., Nam, J. W. Genomics of microRNA. (2006) *Trends Genet.* **22**:165–173.

Kirmizis, A., Santos-Rosa, H., Penkett, C. J., Singer, M. A., Vermeulen, M., Mann, M., Bähler, J., Green, R. D., Kouzarides, T. Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation. (2007) *Nature*. **449**:928-932.

Kleinschmidt, M.A, Streubel, G., Samans, B., Krause, M., Bauer, U.M. The protein arginine methyltransferases CARM1 and PRMT1 cooperate in gene regulation. (2008) Nucleic Acids Res. 36:3202-3213

Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. (2006) *Trends Biochem Sci*. **31**:89-97.

Kneipp, K., Kneipp, H., Itzkan, I., Dasari, R.R., Feld, M.S. Ultrasensitive chemical analysis by Raman spectroscopy. (1999) *Chem Rev*. **99**:2957-2976

Koh, S. S., Chen, D., Lee, Y. H., Stallcup, M. R. Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities. (2001) *J. Biol. Chem*. **276**:1089-1098.

Koh, S. S., Li, H. , Lee, Y. H., Widelitz, R. B., Chuong, C. M., Stallcup, M. R. Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators. (2002). *J. Biol. Chem.* **277**: 26031-26035.

Koshiishi, N., Chong, J. M., Fukasawa, T., Ikeno, R., Hayashi, Y., Funata, N., Nagai, H., Miyaki, M., Matsumoto, Y., Fukayama, M. p300 gene alterations in intestinal and diffuse types of gastric carcinoma. (2004). *Gastric Cancer*. **7**:85-90.

Kouzarides, T. Chromatin modifications and their function (2007). *Cell*. **128**:693–705.

Kramer, O. H., Baus, D., Knauer, S. K., Stein, S., Jager, E., Stauber, R. H., Grez, M., Pfitzner, E., Heinzel, T. Acetylation of Stat1 modulates NF-kappaB activity. (2006) *Genes Dev.* **20**:473–485.

Krishnaswamy, M., Purushothaman, K. K. Plumbagin: A study of its anticancer, antibacterial & antifungal properties. (1980) *Indian J. Exp. Biol.* **18**:876-877.

Krivtsov, A.V., Armstrong, S.A. MLL translocations, histone modifications and leukaemia stem-cell development. (2007) *Nat Rev Cancer.* **7**:823-833. Review.

Kuhn, P., Xu, Q., Cline, E., Zhang, D., Ge, Y., Xu, W. Delineating Anopheles gambiae coactivator associated arginine methyltransferase 1 automethylation using top-down high resolution tandem mass spectrometry. (2009). *Protein Sci.* **18**:1272-1280.

Kumar, G.V., Selvi, R., Kishore, A.H., Kundu, T.K., Narayana, C. Surface-enhanced Raman spectroscopic studies of coactivator-associated arginine methyltransferase 1. (2008) *J Phys Chem B.* **112**:6703-6707.

Kurash, J. K., Lei, H., Shen, Q., Marston, W. L., Granda, B.W., Fan, H., Wall, D., Li, E., Gaudet, F. Methylation of p53 by Set7/9 mediates p53 acetylation and activity in vivo. (2008). *Mol. Cell* **29**:392–400.

Kunizaki, M., Hamamoto, R., Silva, F.P., Yamaguchi, K., Nagayasu, T., Shibuya, M., Nakamura, Y., Furukawa, Y. The lysine 831 of vascular endothelial growth factor receptor 1 is a novel target of methylation by SMYD3. (2007) *Cancer Res.* **67**:10759-10765.

Kwak, Y. T., Guo, J., Prajapati, S., Park, K. J., Surabhi, R. M., Miller, B., Gehrig, P., Gaynor, R. B. Methylation of SPT5 regulates its interaction with RNA polymerase II and transcriptional elongation properties.(2003) *Mol. Cell*. **11**:1055-1066.

Lai, M. C., Teh, B. H., Tarn, W. Y. A human papillomavirus E2 transcriptional activator. The interactions with cellular splicing factors and potential function in pre-mRNA processing. (1999). *J. Biol. Chem.* **274**:11832-41.

Langst, G., Becker, P.B. ISWI induces nucleosome sliding on nicked DNA (*2001*). *Mol Cell* **8***:*1085–1092.

Lau, O. D., Kundu, T. K., Soccio, R. E., Ait-Si-Ali, S., Khalil, E. M., Vassilev, A., Wolffe, A. P., Nakatani, Y., Roeder, R. G., Cole, P. A. HATs off: selective synthetic inhibitors of the histone acetyltransferases p300 and PCAF. (2000) *Mol. Cell.* **5**:589-595.

Le Guezennec, X., Vermeulen, M., Brinkman, A. B., Hoeijmakers, W. A., Cohen, A., Lasonder, E., Stunnenberg, H. G. MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties. (2006) *Mol. Cell Biol*. **26**:843-851.

Lee, D.Y., Hayes, J.J., Pruss, D., Wolffe, A.P. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. (1993) *Cell* **72**:73-84

Lee, D.Y., Teyssier, C., Strahl, B.D., and Stallcup, M.R. Role of protein methylation in regulation of transcription. (2004). *Endocr. Rev.* **26**: 147-170.

Lee, J., Bedford, M. T. PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. (2002). *EMBO Rep*. **3**:268-273.

Lee, K. K., Workman, J. L. Histone acetyltransferase complexes: one size doesn't fit all. (2007). *Nat. Rev. Mol. Cell Biol.* **8**:284-295.

Lemmon, M. A., Ferguson, K. M., Abrams, C. S. Pleckstrin homology domains and the cytoskeleton. (2002). *FEBS Lett.* **513**:71–76

Lemmon, M. A. Pleckstrin homology (PH) domains and phosphoinositides. (2007) *Biochem. Soc. Symp.* **74**: 81–93.

Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., Burge, C. B. Prediction of mammalian microRNA targets. (2003) *Cell* **115**:787–798.

Li, B., Gogol, M., Carey, M., Lee, D., Seidel, C., Workman, J.L. Combined action of PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin. (2007) *Science* **316**:1050-1054

Li, B., Carey, M., Workman, J. L. The role of chromatin during transcription. (2007) *Cell.* **128**:707-719.

Li, H., Park, S., Kilburn, B., Jelinek, M. A., Henschen-Edman, A., Aswad, D. W., Stallcup, M. R., Laird-Offringa, I. A. Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. Coactivator-associated arginine methyltransferase. (2002). *J. Biol. Chem.* **277**:44623-30.

Li, P., Yao, H., Zhang, Z., Li, M., Luo, Y., Thompson, P. R., Gilmour, D. S., Wang, Y. Regulation of p53 target gene expression by peptidylarginine deiminase 4. (2008). *Mol. Cell. Biol.* **28**:4745-4758.

Lia, G., Praly, E., Ferreira, H., Stockdale, C., Tse-Dinh, Y.C., Dunlap, D., Croquette, V., Bensimon, D., Owen-Hughes, T. Direct observation of DNA distortion by the RSC complex. (2006) *Mol Cell.* **21**:417-425.

Liebermann, D. A., Hoffman, B. Myeloid differentiation (MyD)/growth arrest DNA damage (GADD) genes in tumor suppression, immunity and inflammation. (2002). *Leukemia* **16**:527–541.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S., Johnson, J. M. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. (2005) *Nature* **433**:769–773.

Linares, L.K., Kiernan, R., Triboulet, R., Chable-Bessia, C., Latreille, D., Cuvier, O., Lacroix, M., Le Cam, L., Coux, O., Benkirane, M. Intrinsic ubiquitination activity of PCAF controls the stability of the oncoprotein Hdm2. (2007) *Nat Cell Biol.* **9**:331-338.

Lippman, Z., Martienssen, R. The role of RNA interference in heterochromatic silencing. (2004) *Nature* **431**: 364–370

Liu, Q., Dreyfuss, G. In vivo and in vitro arginine methylation of RNA-binding proteins. (1995) *Mol Cell Biol.* **15**:2800-2808.

Liu, X., Wang, L., Zhao, K., Thompson, P. R., Hwang, Y., Marmorstein, R., Cole, P. A. The structural basis of protein acetylation by the p300/CBP transcriptional coactivator. (2008) *Nature.* **451**:846-50.

Losso, J. N., Bansode, R. R., Trappey, A2nd., Bawadi, H.A., Truax, R. In vitro anti-proliferative activities of ellagic acid. (2004). *J. Nutr. Biochem.* **15**:672-678.

Ma, K., Chan, J.K., Zhu, G., Wu, Z. Myocyte enhancer factor 2 acetylation by p300 enhances its DNA binding activity, transcriptional activity, and myogenic differentiation. (2005) *Mol Cell Biol.* **25**:3575-3382.

Maison, C., Bailly, D., Peters, A. H., Quivy, J. P., Roche, D., Taddei, A., Lachner, M., Jenuwein, T., Almouzni, G. Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. (2002) *Nat. Genet*. **30**:329-334.

Majid, S., Khanduja, K. L., Gandhi, R. K., Kapur, S., Sharma, R. R. Influence of ellagic acid on antioxidant defense system and lipid peroxidation in mice. (1991) *Biochem. Pharmacol.* **42**:1441-1445.

Manning, E.T., Ikehara, T., Ito, T., Kadonaga, J.T., Kraus, W.L. p300 forms a stable, template-committed complex with chromatin: role for the bromodomain. (2001) *Mol Cell Biol*. **21**:3876-3887.

Mantelingu, K., Kishore, A. H., Balasubramanyam, K., Kumar, G. V., Altaf, M., Swamy, S. N., Selvi, R., Das, C., Narayana, C., Rangappa, K. S., Kundu, T. K. Activation of p300 histone acetyltransferase by small molecules altering enzyme structure: probed by surface-enhanced Raman spectroscopy. (2007) *J. Phys. Chem. B.* **111**:4527-34.

Mantelingu, K., Reddy, B. A., Swaminathan, V., Kishore, A. H., Siddappa, N. B., Kumar, G.V., Nagashankar, G., Natesh, N., Roy, S., Sadhale, P. P., Ranga, U., Narayana, C., Kundu, T. K. Specific inhibition of p300-HAT alters global gene expression and represses HIV replication. (2007). *Chem. Biol.* **14**:645-57.

Marmorstein, R. Structure of histone acetyltransferases. (2001) *J. Mol. Biol.* **311**:433-444.

Marmorstein, R., Roth, S.Y., Histone acetyltransferases: function, structure, and catalysis. (2001). *Curr. Opin. Genet. Develop*. **11**:155–161.

Marshall, W. F., Dernburg, A. F., Harmon, B., Agard, D. A., Sedat, J. W. Specific interactions of chromatin with the nuclear envelope: positional determination within the nucleus in Drosophila melanogaster. (1996) *Mol. Biol. Cell.* **7**:825–842.

Marhold, J., Brehm, A., Kramer, K. The Drosophila methyl-DNA binding protein MBD2/3 interacts with the NuRD complex via p55 and MI-2 (2004) *BMC Mol Biol* **5**:20.

Martin, C., Zhang, Y. The diverse functions of histone lysine methylation. (2005). *Nat. Rev. Mol. Cell Biol*. **6**:838-849.

Mayo, D. W., Miller, F. A., Hannah, R. W. Course Notes on the Interpretation of Infrared and Raman Spectra; Wiley: Hoboken, NJ, (2004) Chapter 8, p 205.

McBryant, S. J., Adams, V. H., Hansen, J. C. Chromatin architectural proteins. (2006). *Chromosome Res.* **14**:39-51.

McGovern, S. L., Helfand, B. T., Feng, B., Shoichet, B. K. A specific mechanism of nonspecific inhibition. (2003). *J. Med. Chem*. **46**: 4265-4272.

Meaburn, K. J., Misteli, T. Cell biology: chromosome territories. (2007) *Nature*. **445**: 379-381.

Melcher, M., Schmid, M., Aagaard, L., Selenko, P., Laible, G., Jenuwein, T. Structure-function analysis of SUV39H1 reveals a dominant role in heterochromatin organization, chromosome segregation, and mitotic progression. (2000) *Mol Cell Biol.* **20**:3728-3741.

Meltzer, P. S. Cancer genomics: small RNAs with big impacts. (2005) *Nature* **435**:745–746.

Miao, F., Li, S., Chavez, V., Lanting, L., Natarajan, R. Coactivator-associated arginine methyltransferase-1 enhances nuclear factor-kappaB-mediated gene transcription through methylation of histone H3 at arginine 17. (2006) *Mol Endocrinol*. **20**:1562-1573.

Miranda, T.B., Jones, P.A. DNA methylation: the nuts and bolts of repression. (2007) *J Cell Physiol*. **213**:384-390. Review.

Miyabayashi, T., Teo, J. L., Yamamoto, M., McMillan, M., Nguyen, C., Kahn, M. Wnt/beta-catenin/CBP signaling maintains long-term murine embryonic stem cell pluripotency. (2007) *Proc. Natl. Acad. Sci. U S A.* **104**:5668-5673.

Monroy, M. A., Schott, N. M., Cox, L., Chen, J. D., Ruh, M., Chrivia, J. C. SNF2-related CBP activator protein (SRCAP) functions as a coactivator of steroid receptor-mediated transcription through synergistic interactions with CARM-1 and GRIP-1. (2003). *Mol. Endocrinol.* **17**:2519-2528.

Monsalve, M., Wu, Z., Adelmant, G., Puigserver, P., Fan, M., Spiegelman, B. M. Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. (2000) *Mol. Cell*. **6**:307-16.

Muraoka, M., Konishi, M., Kikuchi-Yanoshita, R., Tanaka, K., Shitara, N., Chong, J.M., Iwama, T., Miyaki, M. p300 gene alterations in colorectal and gastric carcinomas. (1996) *Oncogene*. **12**:1565-1569.

Muscari, C., Bonafé, F., Carboni, M, Govoni, M., Stanic, I., Gamberini, C., Ricci, F., Tazzari, P.L., Caldarera, C.M., Guarnieri, C. Difluoromethylornithine stimulates early cardiac commitment of mesenchymal stem cells in a model of mixed culture with cardiomyocytes. (2008) *J Cell Biochem*. **103**:1046-1052.

Nakanishi, S., Lee, J.S., Gardner, K.E., Gardner, J.M., Takahashi, Y.H., Chandrasekharan, M.B., Sun, Z.W., Osley, M.A., Strahl, B.D., Jaspersen, S.L., Shilatifard, A. Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1. (2009) *J Cell Biol*. **186**:371-377.

Narlikar, G.J., Fan, H.Y., Kingston, R.E. Cooperation between complexes that regulate chromatin structure and transcription (2002) *Cell* **108**:475–487. .

Nottke, A., Colaiácovo, M.P., Shi, Y. Developmental roles of the histone lysine demethylases. (2009) *Development*. **136**:879-889. Review

Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H., Nakatani, Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. (1996) *Cell.* **87**:953-959.

Oliveira, A.M., Wood, M.A., McDonough, C.B., Abel, T. Transgenic mice expressing an inhibitory truncated form of p300 exhibit long-term memory deficits. (2007) *Learn Mem.* **14**:564-572.

Pal, S., Yun, R., Datta, A., Lacomis, L., Erdjument-Bromage, H., Kumar, J., Tempst, P., Sif, S. mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in transcriptional repression of the Myc target gene cad. (2003) *Mol Cell Biol.* **23**:7475-7487.

Parimala, R., Sachdanandam, P. Effect of Plumbagin on some glucose metabolising enzymes studied in rats in experimental hepatoma. (1993) *Mol Cell Biochem*. **125**:59-63.

Park, Y.J., Luger, K. Histone chaperones in nucleosome eviction and histone exchange. (2008) *Curr Opin Struct Biol.* **18**:282-289. Review

Parthun, M.R. Hat1: the emerging cellular roles of a type B histone acetyltransferase. (2007) *Oncogene* **26**:5319-5328. Review.

Patnaik D, Chin HG, Esteve PO, Benner J, Jacobsen SE, Pradhan S. Substrate specificity and kinetic mechanism of mammalian G9a histone H3 methyltransferase. (2004) *J Biol Chem* **279**:53248–53258.

Pavan Kumar, G.V., Ashok Reddy, B.A., Arif, M., Kundu, T.K., Narayana, C. Surface-enhanced Raman scattering studies of human transcriptional coactivator p300. (2006) *J Phys Chem B.* **110**: 16787-16792.

Perkins, N.D. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. (2006) *Oncogene* **25**: 6717–6730

Podstawka, E., Ozaki, Y., Proniewicz, L.M. Part I: surface-enhanced Raman spectroscopy investigation of amino acids and their homodipeptides adsorbed on colloidal silver. (2004) *Appl Spectrosc*. **58**:570-580.

Pokholok, D.K., Harbison, C.T., Levine, S., Cole, M., Hannett, N.M., Lee, T.I., Bell, G.W., Walker, K., Rolfe, P.A., Herbolsheimer, E., Zeitlinger, J., Lewitter, F., Gifford, D.K., and Young, R.A. (2005) *Cell.* **122**: 517-527.

Poleshko, A., Einarson, M.B., Shalginskikh, N., Zhang, R., Adams, P.D., Skalka, A.M., Katz, R.A.
Identification of a functional network of human epigenetic silencing factors (2010) *J Biol Chem.* **285**:422-433.

Qin, S., Parthun, M.R. Histone H3 and the histone acetyltransferase Hat1p contribute to DNA double-strand break repair. (2002) *Mol Cell Biol*. **22**:8353-8365.

Rajewsky, N. microRNA target predictions in animals (2006). *Nat Genet* **38**:S8–S13

Ralhan, R., Pandey, M.K., Aggarwal, B.B. Nuclear factor-kappa B links carcinogenic and chemopreventive agents. (2009) *Front Biosci (Schol Ed).* **1**:45-60.

Ravindra, K.C., Selvi, B.R., Arif, M., Reddy, B.A., Thanuja, G.R., Agrawal, S., Pradhan, S.K., Nagashayana, N., Dasgupta, D., Kundu, T.K. Inhibition of lysine acetyltransferase KAT3B/p300 activity by a naturally occurring hydroxynaphthoquinone, plumbagin. (2009) *J Biol Chem*. **284**:24453-24464.

Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M., and Puigserver, P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. (2005) *Nature* **434**: 113–118.

Rodríguez-Campos, A., Azorín, F. RNA is an integral component of chromatin that contributes to its structural organization. (2007) *PLoS One.* **2**:e1182.

Roelfsema, J.H., Peters, D.J. Rubinstein-Taybi syndrome: clinical and molecular overview. (2007) *Expert Rev Mol Med.* **9**:1-16.

Rouaux, C., Jokic, N., Mbebi, C., Boutillier, S., Loeffler, J.P., Boutillier, A.L. Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration. (2003) *EMBO J.* **22**:6537-6549

Sachdeva, M., Zhu, S., Wu, F., Wu, H., Walia, V., Kumar, S., Elble, R., Watabe, K., Mo, Y.Y. p53 represses c-Myc through induction of the tumor suppressor miR-145. (2009) *Proc Natl Acad Sci U S A.* **106**:3207-3212

Sachdeva, M., Mo, Y.Y. miR-145-mediated suppression of cell growth, invasion and metastasis (2010) *Am J Transl Res.* **2**:170-180.

Sagar, V., Zheng, W., Thompson, P.R., Cole, P.A. Bisubstrate analogue structure-activity relationships for p300 histone acetyltransferase inhibitors. (2004) *Bioorg Med Chem* **12**:3383–3390

Saito, Y., Liang, G., Egger, G., Friedman, J.M., Chuang, J.C., Coetzee, G.A., Jones, P.A. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. (2006) *Cancer Cell* **9**:435–443

Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. DNA damage activates p53 through a phosphorylation-acetylation cascade. (1998) *Genes Dev*. **12**: 2831–2841

Sandur, S.K., Ichikawa, H., Sethi, G., Ahn, K.S., Aggarwal, B.B. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and IkappaBalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. (2006) *J Biol Chem.* 281:17023-17033.

Scharf, A.N., Meier, K., Seitz, V., Kremmer, E., Brehm, A., Imhof, A. Monomethylation of lysine 20 on histone H4 facilitates chromatin maturation. (2009) *Mol Cell Biol*. **29**:57-67

Schurter, B.T., Koh, S.S., Chen, D., Bunick, G.J., Harp, J.M., Hanson, B.L., Henschen-Edman, A., Mackay, D.R., Stallcup, M.R., and Aswad, D.W. Methylation of histone H3 by coactivator-associated arginine methyltransferase 1. (2001) *Biochemistry.* **40**: 5747–5756.

Scott, G.K., Mattie, M.D., Berger, C.E., Benz, S.C., Benz, C.C. Rapid alteration of microRNA levels by histone deacetylase inhibition. (2006) *Cancer Res* **66**:1277–1281

Selvi, B.R., Pradhan, S.K., Shandilya, J., Das, C., Sailaja, B.S., Shankar, G. N., Gadad, S.S., Reddy, A., Dasgupta, D., Kundu, T.K. Sanguinarine interacts with chromatin, modulates epigenetic modifications, and transcription in the context of chromatin. (2009) *Chem Biol.* **16**:203-216.

Selvi, B.R., Kundu, T.K. Reversible acetylation of chromatin: implication in regulation of gene expression, disease and therapeutics. (2009) *Biotechnol J.* **4**:375-390. Review.

Selvi, B.R., Batta, K., Kishore, A.H., Mantelingu, K., Varier, R.A., Balasubramanyam, K., Pradhan, S.K., Dasgupta, D., Sriram, S., Agrawal, S., Kundu, T.K. Identification of a novel inhibitor of coactivator-associated arginine methyltransferase 1 (CARM1)-mediated methylation of histone H3 Arg-17. (2010) *J Biol Chem.* **285**:7143-7152.

Semenza, G.L. Hypoxia-inducible factor 1 (HIF-1) pathway. (2007) *Sci STKE.* **2007**:cm8

Sexton, T., Schober, H., Fraser, P., Gasser, S.M. Gene regulation through nuclear organization. (2007) *Nat Struct Mol Biol*. **14**:1049-1055.

Shi, X., Kachirskaia, I., Yamaguchi, H., West, L.E., Wen, H., Wang, E.W., Dutta, S., Appella, E., and Gozani, O. Modulation of p53 function by SET8-mediated methylation at lysine 382. (2007). *Mol. Cell* **27**:636–646.

Shih, Y.W., Lee, Y.C., Wu, P.F., Lee, Y.B., Chiang, T.A. Plumbagin inhibits invasion and migration of liver cancer HepG2 cells by decreasing productions of matrix metalloproteinase-2 and urokinase- plasminogen activator. (2009) *Hepatol Res.* **39**:998-1009.

Sims, R.J. 3rd, Nishioka, K., Reinberg, D. Histone lysine methylation: a signature for chromatin function. (2003). *Trends Genet.* **19**:629-639. Review

Smith, B.C., Denu, J.M. Chemical mechanisms of histone lysine and arginine modifications (2009) *Biochim Biophys Acta* **1789** :45-57

Stallcup, M.R., Chen, D., Koh, S.S., Ma, H., Lee, Y.H., Li, H., Schurter, B.T., Aswad, D.W. Co-operation between protein-acetylating and protein-methylating co-activators in transcriptional activation. (2000) *Biochem Soc Trans*. **28**:415-418.

Strahl, B.D., Briggs, S.D., Brame, C.J., Caldwell, J.A., Koh, S.S., Ma, H., Cook, R.G., Shabanowitz, J., Hunt, D.F., Stallcup, M.R., Allis, C.D. Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1.
(2001) *Curr Biol*. **11**: 996-1000

Swaminathan, V., Kishore, A.H., Febitha, K.K., Kundu, T.K. Human histone chaperone nucleophosmin enhances acetylation-dependent chromatin transcription. (2005) *Mol Cell Biol.* **25**:7534-7545.

Swaminathan, V., Reddy, B.A., Ruthrotha Selvi, B., Sukanya, M.S., Kundu, T.K. Small molecule modulators in epigenetics: implications in gene expression and therapeutics. (2007) *Chromatin and Disease* Ed. Kundu TK and Dasgupta D, **41**:397-428. Review.

Tadesse, H., Deschênes-Furry, J., Boisvenue, S., Côté, J. KH-type splicing regulatory protein interacts with survival motor neuron protein and is misregulated in spinal muscular atrophy. (2008).. (2008) *Hum Mol Genet* **17**:506-524.

Tanner, K.G., Langer, M.R., Kim, Y., Denu, J.M. Kinetic mechanism of the histone acetyltransferase GCN5 from yeast. (2000) *J Biol Chem* **275**:22048–22055.

Tanner, K.G., Trievel, R.C., Kuo, M.H., Howard, R.M., Berger, S.L., Allis, C.D., Marmorstein, R., Denu, J.M. Catalytic mechanism and function of invariant glutamic acid 173 from the histone acetyltransferase GCN5 transcriptional coactivator. (1999) *J Biol Chem* **274**:18157–18160.

Taverna, S.D., Li, H., Ruthenburg, A.J., Allis, C.D., Patel, D.J. How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. (2007) *Nat Struct Mol Biol.* **14**:1025-1040. Review.

Teyssier, C., Chen, D., Stallcup, M.R. Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. (2002). *J Biol Chem.* **277**: 46066-46072.

Therrien, E., Larouche, G., Manku, S., Allan, M., Nguyen, N., Styhler, S., Robert, M.F., Goulet, A.C., Besterman, J.M., Nguyen, H., Wahhab, A. 1,2-Diamines as inhibitors of co-activator associated arginine methyltransferase 1 (CARM1). (2009) *Bioorg Med Chem Lett*. **19:** 6725-6732.

Thompson, P. R., Kurooka, H., Nakatani, Y., Cole, P. A. Transcriptional coactivator protein p300. Kinetic characterization of its histone acetyltransferase activity. (2001) *J. Biol. Chem.* **276**:33721–33729

Thompson, P.R., Wang, D., Wang, L., Fulco, M., Pediconi, N., Zhang, D., An, W., Ge, Q., Roeder, R.G., Wong, J., Levrero, M., Sartorelli, V., Cotter, R.J., Cole, P.A. Regulation of the p300 HAT domain via a novel activation loop. (2004) *Nat Struct Mol Biol.* **11**:308-15.

Torres-Padilla, M.E., Parfitt, D.E., Kouzarides, T., Zernicka-Goetz M. Histone arginine methylation regulates pluripotency in the early mouse embryo.(2007). *Nature.* **445**: 214-218

Troffer-Charlier, N., Cura, V., Hassenboehler, P., Moras, D., Cavarelli, J. Functional insights from structures of coactivator-associated arginine methyltransferase 1 domains. (2007). *EMBO J.* **26**: 4391-4401.

Troffer-Charlier, N., Cura, V., Hassenboehler, P., Moras, D., Cavarelli, J. Expression, purification, crystallization and preliminary crystallographic study of isolated modules of the mouse coactivator-associated arginine methyltransferase 1. (2007). *Acta Crystallogr Sect F Struct Biol Cryst Commun*. **63**:330-333.

Tsukiyama, T., and C. Wu. Purification and properties of an ATPdependent nucleosome remodeling factor. (1995) *Cell* **83**:1011–1020

Varga-Weisz, P. D., M. Wilm, E. Bonte, K. Dumas, M. Mann, and P. B. Becker. Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II. (1997) *Nature* **388**:598–602.

Vignali, M., Hassan, A.H., Neely, K.E., Workman, J.L. ATP-dependent chromatin-remodeling complexes. (2000) *Mol Cell Biol.* **20**:1899-1910

Vo, N., Fjeld, C., and Goodman, R.H. Acetylation of nuclear hormone receptor-interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP (2001). *Mol. Cell. Biol*. **21**: 6181–6188.

Wade, P. A., P. L. Jones, D. Vermaak, and A. P. Wolffe. A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. (1998) *Curr. Biol*. **8**:843–846.

Wan, H., Huynh, T., Pang, S., Geng, J., Vaccaro, W., Poss, M.A., Trainor, G.L., Lorenzi, M.V., Gottardis, M., Jayaraman, L., Purandare, A.V. Benzo[d]imidazole inhibitors of Coactivator Associated Arginine Methyltransferase 1 (CARM1)--Hit to Lead studies. (2009) *Bioorg Med Chem Lett.* **19**:5063-5066

Weake, V.M., Workman, J.L. Histone ubiquitination: triggering gene activity. (2008) *Mol Cell.* **29**:653-663. Review

Westermann, S., and Weber, K. Post-translational modifications regulate microtubule function (2003) *Nat. Rev. Mol. Cell Biol*. **4**: 938–947

Wijeratne, S.S,, Camporeale, G., Zempleni, J. K12-biotinylated histone H4 is enriched in telomeric repeats from human lung IMR-90 fibroblasts. (2010) *J Nutr Biochem*. **21**:310-316.

Winchester, G. p53 protein and control of growth. (1983) *Nature*. **303**:660-661.

Xiao, H., Hasegawa, T., Isobe, K. p300 collaborates with Sp1 and Sp3 in p21(waf1/cip1) promoter activation induced by histone deacetylase inhibitor. (2000) *J Biol Chem.* **275**:1371-1376.

Xu, W., Cho, H., Kadam, S., Banayo, E.M., Anderson, S., Yates, J.R. 3rd, Emerson, B.M., Evans, R.M. A methylation-mediator complex in hormone signaling. (2004). *Genes Dev*.**18** :144-156

Xu, L., Glass, C.K., Rosenfeld, M.G . Coactivator and corepressor complexes in nuclear receptor function (1999) *Curr. Opin. Genet. Dev.* **9**:140–147

Yadav, N., Cheng, D., Richard, S., Morel, M., Iyer, V.R., Aldaz, C.M., Bedford, M.T. CARM1 promotes adipocyte differentiation by coactivating PPARgamma. (2008) *EMBO Rep.* **9**:193-198.

Yan, Y., Barlev, N.A., Haley, R.H., Berger, S.L., Marmorstein, R. Crystal structure of yeast Esa1 suggests a unified mechanism for catalysis and substrate binding by histone acetyltransferases. (2000) *Mol Cell* **6**:1195–1205.

Yan, Y., Harper, S., Speicher, D.W., Marmorstein, R. The catalytic mechanism of the ESA1 histone acetyltransferase involves a self-acetylated intermediate. (2002) *Nat Struct Mol Biol* **9**:862–869.

Yang, X.J., Seto, E. Lysine acetylation: codified crosstalk with other posttranslational modifications. (2008) *Mol Cell.* **31**:449-461.

Yao, T.P., Oh, S.P,, Fuchs, M., Zhou, N.D., Ch'ng, L.E., Newsome, D., Bronson, R.T., Li, E., Livingston, D.M., Eckner, R. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. (1998) *Cell* **93**:361-372.

Yu, X., Guo, Z.S., Marcu, M.G., Neckers, L., Nguyen, D.M., Chen, G.A., and
Schrump, D.S. Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. *J. Natl. Cancer Inst*. (2002) **94**: 504–513.

Yue, W.W., Hassler, M., Roe, S.M., Thompson-Vale, V., Pearl, L.H. Insights into histone code syntax from structural and biochemical studies of CARM1 methyltransferase. (2007) *EMBO J* **26**:4402–4412.

Zhang, C., Li, H., Zhou, G., Zhang, Q., Zhang, T., Li, J., Zhang, J., Hou, J., Liew, C.T., Yin, D. Transcriptional silencing of the TMS1/ASC tumour suppressor gene by an epigenetic mechanism in hepatocellular carcinoma cells. (2007) *J Pathol.* **212**:134-142.

Zhang, X., Zhou, L., Cheng, X. Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. (2000) *EMBO J* **19**:3509–3519.

Zhang, X., Yuan, Z., Zhang, Y., Yong, S., Salas-Burgos, A., Koomen, J., Olashaw, N., Parsons, J.T., Yang, X.J., Dent, S.R., et al. HDAC6 modulates cell motility by altering the acetylation level of cortactin. (2007) *Mol. Cell* **27**: 197–213.

Zhang, Y., Lv, J., Liu, H., Zhu, J., Su, J., Wu, Q., Qi, Y., Wang, F., Li, X. HHMD: the human histone modification database. (2010) *Nucleic Acids Res.* **38**(Database issue):D149-154

Zhang, Y., Smith, C.L., Saha, A., Grill, S.W., Mihardja, S., Smith, S.B., Cairns, B.R., Peterson, C.L., Bustamante, C. DNA translocation and loop formation mechanism of chromatin remodeling by SWI/SNF and RSC. (2006) *Mol Cell*. **24**:559-568.

Zhang, Y., Reinberg, D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. (2001). *Genes Dev*. **15**:2343-2360. Review

Zhao, Q., Rank, G., Tan, Y.T., Li, H., Moritz, R.L., Simpson, R.J., Cerruti, L., Curtis, D.J., Patel, D.J., Allis, C.D., Cunningham, J.M., Jane, S.M. PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. (2009) *Nat Struct Mol Biol.* **16**:304-311.

Zhong, X., Jin, Y. Critical roles of coactivator p300 in mouse embryonic stem cell differentiation and Nanog expression. (2009) *J Biol Chem.* **284**: 9168-9175.

Zeng, Y.X., WS, El-Deiry. Regulation of p21WAF1/CIP1 expression by p53-independent pathways. (1996) *Oncogene* **12**: 1557-1564

Zika, E., Fauquier, L., Vandel, L., Ting, J.P. Interplay among coactivator-associated arginine methyltransferase 1, CBP, and CIITA in IFN-gamma-inducible MHC-II gene expression (2005). *Proc Natl Acad Sci U S A.* **102**:16321-16326.

Zofall, M., Persinger, J., Kassabov, S.R., Bartholomew, B. Chromatin remodeling by ISW2 and SWI/SNF requires DNA translocation inside the nucleosome. (2006) *Nat Struct Mol Biol* **13**:339–346.

.