Histone acetylation and gene expression in neural cells: Probed by small molecule modulators

A Thesis Submitted for the Degree of

MS (by Research)

By

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To

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DECLARATION

I hereby declare that this thesis entitled "**Histone acetylation and gene expression in neural cells: Probed by small molecule modulators**", 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.

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Certificate

This is to certify that the work described in this thesis entitled, "**Histone acetylation and gene expression in neural cells: Probed by small molecule modulators**", is the result of the investigations carried out by Dr Mohankrishna D V, 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: Prof. Tapas K Kundu

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MohanKrishna D V

Synopsis (MS by Research)

Thesis Title: Histone acetylation and gene expression in Neural cells; probed by small molecule modulators.

Submitted by: Mohan Krishna D V

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The eukaryotic genome is a highly compact and dynamic nucleoprotein complex comprising of DNA, histones, several nonhistone proteins and RNAs. This compact and folded organization is a deterrent to the essential cellular processes like transcription, replication and repair. Hence there are machineries such as ATP-dependent remodeling complexes, nonhistone chromatin associated proteins and most importantly chromatin modifying enzymes, which regulate the dynamic folding and unfolding of the chromatinised genome. These enzymes bring about modification of histone tails as well as other chromatin components like non histone chromatin associated proteins and DNA, thereby fine tune the process of gene expression intricately. Of these chromatin modifications known, the best studied include reversible Lysine acetylation, Lysine/ Arginine methylation and Serine/ Threonine phosphorylation. All these modifications together set up a complex network referred to as 'Epigenetic landscape', which helps in establishing the transcriptionally competent state of chromatin. Modifications, like these, especially acetylation is considered to be the ultimate switches for regulation of gene expression. Acetylation of histone tail generally results in a permissive environment for chromatin templated phenomena like transcription and thus is a

mark of active transcription. The reversible acetylation and deacetylation of chromatin in a cellular context is a highly regulated phenomena.

p300/ CBP is an important class of histone acetyltransferases (HATs), often referred to as a 'master regulator' of acetylation dependent transcription. Besides transcriptional coactivation property p300 possesses different intrinsic enzymatic activities of which acetyltransferase activity is the most important. p300/CBP mediated acetylation homeostasis is vital for neuronal gene expression, with significant outcome in terms of neuron survival and physiology. Small molecule modulators of chromatin modifying enzymes are efficient tools for understanding the complex functional network. Small molecules help in delineating the role of enzymatic activity versus coactivation property in various physiological outcomes. This research work was initiated towards understanding the cellular physiology of histone lysine acetylation and associated epigenetic marks in neurons using small molecule modulators.

RTK1, also known as Plumbagin , is a well known natural compound, with anti-proliferative activity. It is known for its ability to generate Reactive oxidative Species and inhibit microtubule network. Recently, our laboratory has found that it is a potent inhibitor of p300 HAT activity. In the present research project RTK1 has been used to elucidate the epigenetic state of histone modifications in neural cells i.e., SH-SY5Y, a neuroblastoma cell line. SH-SY5Y cells are known to be dopamine beta-hydroxylase active, acetylcholinergic, glutamatergic and adenosinergic, which are characteristics of neurons. SH-SY5Y cell line is an established culture model for neurons and hence has been used in our experiments. SY-SY5Y cells were treated with RTK1 to investigate the epigenetic landscape and its consequences in altering gene expression, cell survival and apoptosis in neural cells. Histones were isolated from RTK1 treated SH-SY5Y cells and

acetylation levels on a global scale was analysed by western blotting. We observed that histone acetylation decreased drastically on histone H3K9, 14; histone H2AK5 and histone H4K5, 8 residues. These are also sites for p300 and PCAF mediated acetylation. Since these modifications are marks of transcriptional activation we also analysed epigenetic marks, other than acetylation. Interestingly, we found that Histone H3S10 phosphorylation and H3K4trimethylation significantly decreased post treatment of RTK1. However, transcription repressive marks like Histone H3K9 di/ tri-methylation did not alter. Collectively, these data suggested that RTK1 could inhibit epigenetic marks specific for transcriptional activation in the cellular context. Analysis also revealed that such drastic alteration of Epigenetic mark preceded neural cell death. RTK1 treatment up regulated pro- apoptotic genes belonging to the p53 pathway. Contrary to what was expected, RTK1 mediated decrease in histone acetylation also activated some neuroprotective genes that are p300 coactivation dependent. Recent reports describe neuroprotective roles for RTK1. This contradictory finding matches with such studies. Presently, we are analyzing the effects of RTK1 treatment on normal neurons using primary cultures.

Histone acetylation being vital for neuronal gene expression, its decrease is reported in various neurodegenerative states. Incidentally, broad spectrum Histone deacetylase inhibitors (HDACi) have been proven to have protective effect in neurodegenerative disorders . Presumably, neuroprotection was achieved mainly due to induction of histone hyperacetylation. However, HDAC inhibitors are generally non specific or possess broad spectrum activity. Considering that these degenerative pathologies are associated with reduced p300/CBP mediated histone acetylation, an alternative way of reversing the same would be to activate p300 ezymatic activity. We had already established CTPB as a small molecule activator

p300.To generate more efficient molecules we further derivatised CTPB to various analogues and screened them for their p300 activation potential. Through this screening we could achieve a potent molecule, TTK21. TTK21, in a dose dependent manner activated both p300 and CBP similar to its parent molecules CTPB and CTB. However, TTK21, like CTPB, was impermeable to cells. Previously, we had employed self-fluorous, cell permeable carbon nanospheres which also entered cell nucleus, to improve permeability of CTPB and thus observed histone hyperacetylation in cells as well as mice brain. TTK21 will be conjugated to these carbon nanospheres in an effort to improve its permeability and thus, ability of TTK21 to induce acetylation of histones in cell culture system and in animal models will be studied.

In conclusion, this research work has led to the elucidation of histone acetylation associated epigenetic network with the help of a HAT inhibitor, RTK1. It has also led to identification of TTK21 as a novel HAT activator. These small molecules have been used as probes to understand cellular physiology effected by histone acetylation and associated epigenetic marks. This work highlights the importance of HAT mediated histone acetylation in neural gene expression and neuron cell survival. This research has also elucidated the role for novel activators of HAT and novel delivery agents that could be used to activate HAT in vivo. This is a novel approach for therapeutics as well.

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Chapter 1

INTRODUCTION TO NEURONAL EPIGENETICS

This chapter describes the significance of the different chromatin modifications with special emphasis on histone acetylation, in the regulation of neuronal gene expression, neuronal physiology and neurodegeneration. It also debates in favor of an inhibitor based approach to understand the roles of the enzymes involved in these modifications especially p300/CBP histone acetyltransferase in the context of neurons.

Chapter outline:

1.1. Chromatin modifications

1.2. Cross talk of chromatin modifications, the epigenetic language

1.3. HAT Enzymes as transcriptional co-activators

1.4. Signaling to the neuronal chromatin

1.5. Towards Epigenetic therapy for Neurodegenerative diseases

1.6. Chromatin acetylation, HAT/ HDAC balance in neurons

1.7. Small Molecule Modulators of Histone Acetyltransferases

1.8. Understanding the enzymatic role of transcriptional co activators in neurons

1.9. Research Focus

1.1. CHROMATIN MODIFICATIONS:

1.1.1. Chromatin

DNA, which is the blueprint of life, is condensed into chromatin and chromosome structures in order to incorporate the long 2m strand into the small confines volume of the nucleus of the eukaryotic cell. The process, involving packaging of DNA, into a discrete cellular organelle, the

Nucleus, is a general phenomenon across eukaryotes. DNA is packaged as a nucleoprotein complex called 'Chromatin'. Chromatin is also the physiological template for all nuclear processes involving genomic DNA, including transcription by RNA polymerase II (Pol II). The structural unit of chromatin, the nucleosome, comprises 147 bp of DNA wrapped around an octamer of core histone proteins (two copies each of H2A, H2B, H3, and H4) in ~1.7 turns (Figure 1.1) (Richmond and Davey, 2003). However the wrapping of DNA into chromatin also limits the accessibility of the DNA to factors involved in these processes, thus implicating regulation at a higher scale, making it more accessible in a spatio-temporal manner, to overcome the hindrances to physiological processes that are DNA templated. It is immediately observed that chromatin exists in multiple, functionally distinct structural states, that are defined by their protein composition and level of compaction that directly influences one pivotal process, Transcription (e.g., transcriptionally active euchromatin and transcriptionally repressed heterochromatin (Horn and Peterson, 2002; Woodcock and Dimitrov, 2001). The dynamic interconversion between these different chromatin states can play an important role in transcriptional regulation (Horn and Peterson, 2002; Woodcock and Dimitrov, 2001). The establishment of distinct chromatin domains can be achieved through (1) ATP dependent chromatin remodeling factors (2) specific covalent modification of histones (e.g., acetylation, methylation), (3) nucleosome assembly with histone variants (e.g., H2A.Z, CENP-A), (4) Involvement of RNAs in gene silencing and heterochromatinization or (5) incorporation of nucleosome binding non-core histone proteins, such as the linker histone H1, the heterochromatin-associated protein HP1, poly(ADP-ribose) polymerase-1 (PARP-1) and PC4 (Brown, 2003; Horn and Peterson, 2002; Kellum, 2003; Kim et al. 2004; Das et al. 2006) (**Figure 1**). The formation and disruption of higher-order chromatin structures is regulated by the histone-modifying enzymes (e.g., acetyltransferases, deacetylases) and also by chromatin remodeling complexes (e.g., SWI/SNF family). These factors act locally to modify individual nucleosomes at specific gene promoters (Horn and Peterson, 2002). Thus, chromatin structure and chromatin templated activity is modulated by its constituent proteins and a diverse group of regulatory enzymes.

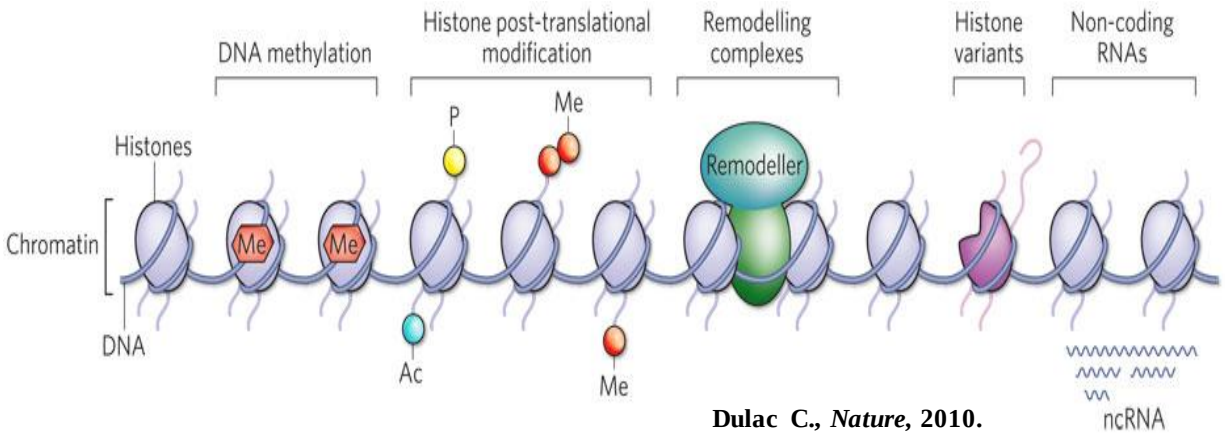


Figure 1.1: Epigenetic players in regulating gene expression (Adapted from Dulac C., 2010)

A. ATP dependent remodeling factors:

The ATP dependent remodeling facilitates either the condensation or de-condensation of chromatin. The remodelers utilize energy in the form of ATP hydrolysis for the remodeling activity and hence the name. Broadly, there are three different groups of ATP-dependent chromatin-remodeling complexes. All these complexes are characterized by an ATPase subunit that belongs to the SNF2 super family of proteins. Such proteins are further classified into (1) SWI2/SNF2 group (2) Imitation SWI (ISWI) group (3) Mi-2 group of complexes (Eisen et al., 1995). The Swi/Snf families of proteins are well conserved across species and have a bromodomain apart from the conserved ATPase subunit. The ISWI family consists 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 (**n**ucleosome-**r**emodeling **f**actor), and CHRAC (**ch**romatin **a**ccessibility **c**omplex). (Ito et al., 1997, Tsukiyama and Wu, 1995, Varga-Weisz, 1997). The third group of remodeling complex coupled to which is the 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 deacetylase activity along with 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 that recognize

acetylated lysines within proteins, in the remodeling complexes. 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 (Vignali et al., 2000). 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 and chromatin associated proteins:

Apart from histones, there are several other nonhistone chromatin associated proteins which also modulate the chromatin dynamicity and thereby the chromatin functions. One of the major components of this nonhistone protein group are the chromatin modifying enzymes. Apart from these there are the chromatin associated proteins like high mobility group proteins (HMG), heterochromatin proteins (HPα, β, γ), H1, PC4. They are called chromatin associated proteins as they are bound to the chromatin (individually to DNA/ histones or to the nucleosome) and they are always isolated upon chromatin fractionation. The transcription factors are other important group of proteins that mediate the structural organization of chromatin (McBryant et al., 2006). There are also other chromatin-associated proteins like the scaffold proteins which comprise of the insulators and the domain boundary elements.

C. Histone chaperones: Histone chaperones are important escort proteins of histones during transcription, replication and DNA repair (reviewed in De Koning et al., 2007). The main role of histone chaperones is to act as a sink and source of histones in various aspects of histone metabolism. They also avoid aggregation of histones during storage. Thus histone chaperones are defined mainly as factors that associate with histones and stimulate a reaction involving histone transfer without being part of the final product. Histone chaperones play important role in histone deposition and eviction (reviewed in Park and Luger, 2008), thus indicating an important role alongside ATP dependent remodeling complexes for remodeling processes and influencing transcriptional outcomes. HIRA and CAF-1 are histone chaperones that play a significant role in

histone H3 and H4 deposition. There are other chaperones like Asf-1 that do not have the property of histone deposition. Rather, they store histones that are used by HIRA and CAF-1 for histone deposition. Considering the different roles played by chaperones in histone metabolism they can broadly be classified into three groups. (A) Chaperones that can bind and transport or transfer histones without necessarily involving additional partners—for example, Asf1; (B) multichaperone complexes that combine several histone chaperone subunits—for example, the CAF-1 complex; and (C) chaperones that provide histone-binding capacity within large enzymatic complexes—for example, actin-related protein-4 (Arp4) in the INO80 chromatin-remodeling complex. Human histone chaperone Nucleophosmin 1 (NPM1) belongs to the first category of histone chaperones. NPM1 interacts with core histones H3, H2B and H4. But this interaction is neither enough for its chaperone activity nor does this interaction facilitate transcriptional activation. However, NPM1 enhances the acetylation-dependent chromatin transcription (Swaminathan et al., 2005). NMP1 is exclusively acetylated by p300 and this acetylated form of NPM1 not only shows an increased affinity toward acetylated histones but also shows enhanced histone transfer ability. NPM1 disrupts the nucleosomal structure in an acetylation-dependent manner, resulting in transcriptional activation. In our laboratory we could elucidate a very interesting link between NPM1, and its role in oral cancer manifestation (Shandilya et al., 2009). It was also observed from our laboratory that the acetylation status of histones in oral cancer is high. A combination of hyper acetylated histone and hyper acetylated NPM1 resulted in activation of many pro-inflammatory genes like TNF-α, presumably due to increased histone eviction at the promoters, as a result of increased chaperone function of NPM1. Targetting this hyperacetylation through a novel small molecule inhibitor of p300 proved to be effective against oral cancer mice model (Arif et al., 2010). Thus these proteins are more than mere histone escorts and are key players in various chromatin functions in health and disease along with other epigenetic players.

D. RNA: 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) led to the speculation 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). Also presence of several non coding RNAs, mainly small non coding RNAs and long non coding RNAs adds to the repertoire of factors that regulate gene expression. The best known are the small non coding RNA like the siRNA, miRNA and piRNA which have vital contributions in epigenetic regulation of gene expression.

1.1.2. Different chromatin modifications:

Among the modifications (**Figure 1.2**), the best studied is the acetylation and deacetylation of histones and nonhistone proteins (Li et al., 2007). This is brought about by a class of enzymes called ' Histone acetyltransferases' (HATs), which however of late have been called as 'Lysine acetyltransferases' (KATs), as they are not restricted to histones alone, rather have a wide array of substrates from transcription factors to enzymes regulating metabolism. In addition to acetylation, chromatin also gets methylated and phosphorylated by 'Histone methyltransferases' and various 'kinases' respectively. Reversal of such modifications is inevitable for cellular functions and is brought about by 'Histone deacetylases'(HDACs), 'Histone demethylases', and 'phosphatases' for removal of acetyl-, methyl- , and phosphoryl- groups respectively. Based on the residue that gets modified, the histone methyltransferases (HMTs) are classified into lysine- and arginine- methyltransferases. Distinct residues on histone tails and nonhistone proteins undergo either serine, threonine or in a few cases tyrosine phosphorylation. The acetylation, methylation and phosphorylation modifications, very intricately regulate chromatin dynamics and gene expression. Other cellular events like signal transduction and cell cycle are also regulated by these modifications. All the above mentioned modifications and others (**Figure 1.2**), 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. However, other modifications like Ubiquitination, SUMOylation, Citrullination, Biotinylation, ADP-Ribosylation etc do play important roles in regulation of transcription and thereby in, gene expression.

1.1.3. Chromatin acetylation:

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) represent two enzyme classes that, respectively, catalyze forward and backward reaction kinetics of lysine residue

acetylation in specific protein substrates. These substrates most importantly include nucleosomal histones and various transcription factors (TFs), which form part of the transcription initiation complex. Accordingly, HATs and HDACs are found embedded in large multiprotein complexes near euchromatic regions of the chromatin. HATs modify core histone tails by post-translational acetylation of specific lysine residues, thereby creating appropriate 'histone code' for chromatin modification and enhanced DNA accessibility of TFs

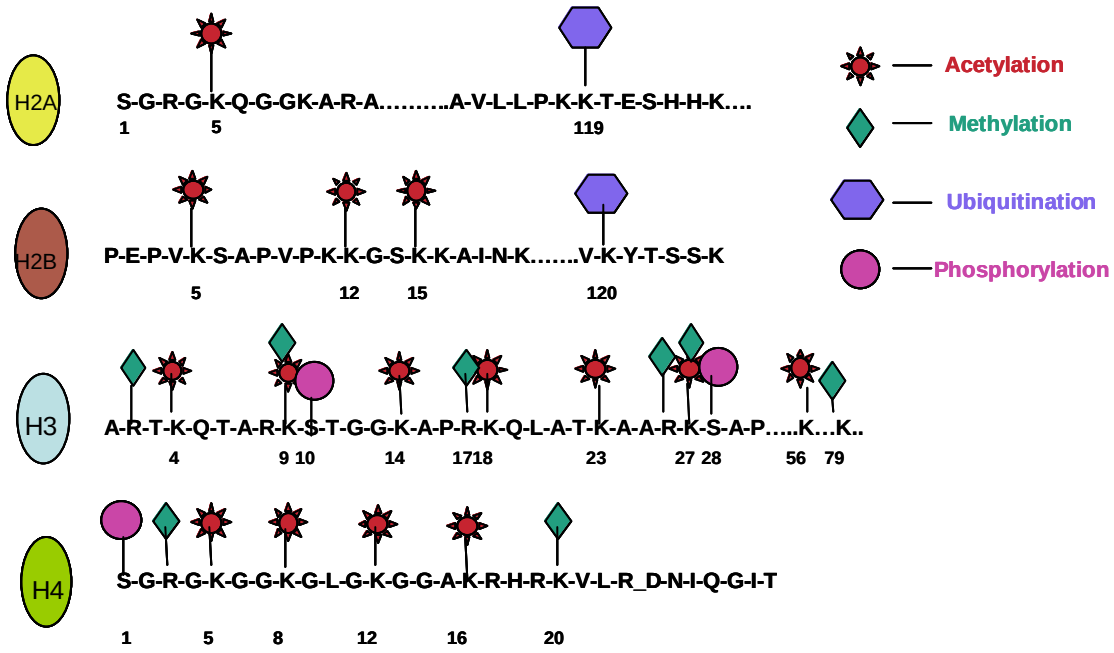


Figure 1.2: Post-translational modifications of human nucleosomal histones. The modifications include acetylation, methylation, phosphorylation and ubiquitination. Most of the known histone modifications occur on the N-terminal tails of histones, with some exceptions including ubiquitination of the C-terminal tails of H2A and H2B and acetylation and methylation of H3 within the globular domain.

Acetylation is a well-characterized modification with vital implications on dynamicity of chromatin and transcription. There are many ways acetylation could alter the pre-existing state of chromatin in terms of transcription. Acetylation of histones results in deposition of a negative charge on the lysine residue which acts as a neutralizer of the strong positive charge of lysine to which DNA (with a negatively charged phosphate back bone) is electrostatically bound and thus loosens the histone - DNA contacts. Acetylation of histones also leads to the recruitment of bromodomain containing ATP dependent chromatin remodeling complexes that read acetylation

mark and act as effectors by loosening the compaction (Li et al., 2007). In fact, acetyltransferases and deacetylases exist usually in a complex consisting of remodeling factors. The yeast SAGA complex consisting, Gcn5 acetyltransferase human STAGA complex containing GCN5 are activator complexes while, drosophila sin3 complex containing rpd3 deacetylase are repressor complexes. Moreover, TFs like RelA, E2F, p53 and GATA1 are also acetylated by HATs (reviewed in Batta et al., 2007). In addition to enhancing protein stability, such acetylation, in most cases, enhance their transactivation potential by facilitating their interactions with DNA and other proteins of the transcription apparatus. Contrarily, HDACs attenuate transcription process at a particular site by deacetylating these targets. Taken together, the HAT–HDAC system, owing to their involvement in turnover of histone and transcription machinery regulation, is one of the ultimate regulatory switches of gene expression. In addition to transcriptional regulation, HAT–HDAC system is also postulated to modulate other chromatin-associated processes like replication, site-specific recombination and DNA repair, thereby playing a major role in modulating overall cellular fate.

1.1.3.1. Histone acetylation:

Histone acetylation and its association with transcriptional activity was first identified by Vincent Allfrey in 1964 (Allfrey et al., 1964). However, recognition of the phenomena and the realization of its importance dawned much later, with the discovery of a tetrahymena HAT through an ingenious 'in-gel assay' by David Allis's group in mid 1990s (Brownell et al., 1995). Following this, there has been a spurt of research in this field, which has led to the identification of acetylated nucleosomes as indispensable 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. Thus, histone acetylation is now considered as a major determinant of the transcriptional competence of chromatin. Several of the acetyltransferases exist in complexes with remodeling factors thus providing the explanation for the conformational changes associated with acetylation (Marmorstein and Roth, 2001, Lee and Workman, 2007). Many HATs also have transcriptional co-activation property and interact with other transcription factors and hence facilitate efficient transcription. 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 divided into two classes on the basis of their cellular 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. The cytoplasmic variant is called as type B HATs. 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. Nuclear HATs are many in number, with further subdivision into five main classes based on their functional characteristics (**Table 1**). Based on homology even cytoplasmic HATs like Hat1 are related to Gcn5 superfamily and hence classified together. HATs are also known to be components of multiprotein complexes. The presence of HAT enzymes in complexes allows for the histone acetyltransferase activity to be targeted to promoters of active genes or to be directed for some other purpose. For example, both the Esa1-containing NuA4 and the Gcn5-containing SAGA HAT complexes bind directly to transcriptional activators and the combination of this activator binding with HAT activity activates transcription from a chromatin template. It is essentially to confer specifity, facilitate recognition and other subsequent activities. HAT catalytic subunits could be essential to direct acetyltransferase activity towards different substrates in a substrate specific manner compared to the HAT enzymes alone. For example, recombinant Esa1 acetylates histone H4 tails preferentially in free histones, but fails to acetylate H4 tails in a nucleosomal substrate. In contrast, the NuA4 complex acetylates H4 tails both in free histones and in nucleosomes. Similarly, recombinant Gcn5 generally acetylates H3 tails in free histones but not in nucleosomes whereas the Gcn5-containing SAGA complex acetylates H3 tails in both free histones and in nucleosomes. This suggests that accessory proteins in NuA4 and SAGA enable Esa1 and Gcn5, respectively, to acetylate nucleosomal substrates (Tan, 2001). However, unless the structures and substrates of all the HATs are known it would be impossible to conclusively prove the case. Nevertheless, it is certain that the presence of other factors along with the HATs themselves have a vital role in targeting HAT activity to a specific promoter or a specific substrate. It could also be possible that the accessory factors relay upstream signals to the acetyltransferases and thus influence their activities.

GNAT (Gcn5) family members were the first to be identified, the founding member being 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

HAT family	HAT members	Known functions	HAT complexes
GNAT	yeast GCN5	Transcriptional co-activator	SAGA, ADA,ATAC
	human GCN5	Transcriptional co-activator	STAGA, TFTC
	PCAF	Transcriptional co-activator	PCAF complex
	Hat1	Acetylation of non-nucleosomal histones	HAT-B
	Elp3	Transcriptional elongation	Elongator for RNA Pol II
MYST	Esa1	Cell cycle progression	NuA4
	Sas2, Sas3	Transcriptional Silencing	Sas2-unknown; Sas3-NuA3
	Tip60	Tat interaction, DNA damage response	TIP60 complex
	MOF	Dosage compensation	MSL complex
	MOZ	Leukemogenesis	Unknown
	HBO1	ORC replication complex interaction	HBO1 complex
CBP/ p300	CBP/ p300	Transcriptional Co-activator	Unknown
TAFII250	human TAFII250 and homologs	Transcriptional co-activator	TFIID
Nuclear Receptor Co-activtors	SRC1	Steroid Receptor Co-activator	**Unknown**
	ACTR	Steroid Receptor Co-activator	
	TIF2	Nuclear Receptor Co-activator, Leukemogenesis	

Table 1: **Lysine acetyltransferase (KAT) families, their function and their complexes**

factor) was also identified which has a similar active site structure as the above member. It has several non-histone substrates apart from acetylating the histone tails. The second family is the p300/CREB-binding protein (p300/CBP) family. These proteins, arguably, are the most studied amongst HATs. With well documented and diverse effects in various cellular processes such as transcription, replication, repair, differentiation, disease, apoptosis, cell survival and cell death, they are aptly referred to as the 'master regulators of gene expression'. The third important class of HATs is the MYST family. The founding members of the MYST family are MOZ, Ypd3, Sas2 and TIP60. While having a preference for H4 acetylation (H4Lys16) in vivo these have been implicated in DNA damage repair and telomere silencing. H4 acetylation dramatically influences nucleosomal structure, results in an open conformation that is essential for active transcription, repair and even apoptosis where an open form of chromatin is essential for DNA cleavage. These enzymes can form fusion proteins resulting in cancers. General Transcription Factors have been

HATs	Histone substrate specificity
GNAT family	
GCN5	H2B; H3-K9, -K14, -K18, -K23, -K27; H4-K8, -K16
PCAF	H3-K14; H4-K8
MYST family	
TIP60	H2A-K5; H3-K14; H4-K5, -K8, -K12
MOZ	H3; H4
p300/CBP family	
p300	H2A-K5; H2B-K12, -K15, -K20; H3-K14, -K18, -K23; H4-K5, -K8, -K12
CBP	H2A-K5; H2B-K12, -K15, -K20; H3-K14, -K18, -K23; H4-K5, -K8
Nuclear receptor coactivators	
AIB1	H3; H4
General transcription factors	
$TAF_{II}250$	H3; H4

Table 2: Classes of HATs/ KATs responsible for histone acetylation

shown to possess acetyltransferase activity, thereby regulating transcription directly, thus bringing them into a separate class of acetyltransferases. ATF2, TAF1, TAFII250, TFIIIC90 are all GTFs with intrinsic HAT activity. 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). Histone H3 and H4 lysines are the most acetylated by p300/CBP, PCAF and GCN5 at specific residues as depicted in **Table 2** and such acetylation mostly results in transcriptional activation. However as mentioned before ,H4 acetylation of LYS16 by TIP60 is a mark associated with DNA repair while p300 mediated H3K56 acetylation is a mark involved in DNA damage, histone deposition and chaperone function. Few residues of histone H2A and H2B also get acetylated, majorly by p300 e.g., H2BK15 acetylation, which is a mark of non apoptotic cells and is decreased in apoptosis (Ajiro et al., 2010). The specific epigenetic language of each of these modifications in conjunction with methylation and phosphorylation will be discussed in the section on the cross talk established by these modifications. It is important to note that all these modifications are reversible. The reverse reaction is carried out by a group of enzymes called histone deacetylases (HDACs). There are four classes of HDACs; Class I (HDAC1, 2, 3, 8), II (HDAC4, 5, 6, 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).

1.1.3.2. Mechanism of histone acetylation:

The histone acetylation reaction is a bi-susbtrate reaction involving the transfer of the acetyl group from the pseudo substrate, acetyl-coenzyme A onto the ε-amino group of the lysine residue on protein substrates. GNAT family follows 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 Coenzyme A (Tanner et al., 1999). For the MYST family two mechanisms have been proposed. The first implicates a cysteine residue, which functions as an acetylated intermediate leading to a ping-pong mechanism of catalysis (Yan et al., 2002). The other mechanism concludes 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 HAT also showed a sequential mechanism similar to the other families of acetyltransferase (Sagar et al., 2004). Though a ping-pong mechanism of action was proposed (Thompson et al, 2001) the final proof for the mechanism of action of p300 was obtained by the crystal structure data and biochemical evidences (Liu et al., 2008) which suggested a Theorell-Chance ('kiss and run') 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).

1.1.3.3. Nonhistone protein acetylation:

It is well known that HATs have nonhistone substrates that play a vital role in major cellular processes. Recent revelations of the cell's acetylome, substantiates this fact and makes acetylation the most prevalent post translational modification rivaled only by phosphorylation (Choudhry et al, 2009). 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. Posttranslational modifications differentially regulate p53 function, depending on the types and sites of modification. For example, phosphorylation of residue S15 in p53 releases MDM2 from p53 and enhances the interaction with acetyltransferases p300/CBP (Shieh et al., 1997). p300/CBP-mediated acetylation of residue K373 in p53 leads to apoptosis, whereas acetylation of residue K320 by PCAF leads to cell cycle arrest (Knights et al., 2006). Acetylation of p53 is also reported to augment its DNA binding ability (Gu et al et al., 1997). Many other nonhistone protein acetylations have since been elucidated. The human positive co-activator 4 (PC4) was initially identified as a general co-activator for activator-dependent transcription. It is also a DNA

binding protein and binds non-specifically to both double and single stranded DNA. The activity of PC4 is influenced significantly by its post translational modifications. PC4 is known to get phosphorylated by Casein Kinase II (CKII). Very interestingly, we demonstrated that, PC4 can be acetylated specifically by p300 (Kumar et al., 2001). The activity of PC4 is negatively regulated by phosphorylation (Ge et al., 1994; Jonker et al., 2006). While phosphorylation negatively regulates PC4's co-activator function and double-stranded DNA (dsDNA) binding ability, acetylation of PC4 enhances its dsDNA binding ability. Phosphorylation of PC4 by CKII in turn inhibits the p300-mediated acetylation in vitro. However, acetylated form of PC4 could still be phosphorylated by CKII. This type of exclusivity in posttranslational modifications where the presence of one modification prevents the occurrence of the other may have a role in many functional outcomes. Incidentally, it was found that PC4 could transactivate p53. PC4 enhances DNA binding ability of p53. Indeed, acetylated PC4 is a better transactivator of p53. On incubation with acetylated PC4, p53 bind to its cognate sites better than with unmodified PC4. PC4 is a DNA bending protein and its ability to bend DNA contributes for p53 activation, and this function is enhanced by acetylation. Thereby, acetylation of PC4 enhances; while phosphorylation abolishes; its ability to bind and bend DNA, activate p53 DNA binding, and, thereby, regulate p53 functions (Batta et al., 2007). Hence, different acetylation sites have different cellular outcomes thus increasing the level of regulation brought about by a single modification on a single protein. Similar to autophorphorylation events of various kinases, the acetyltransferases too undergo autoacetylation, in *cis*- and *trans*- that positively influences their HAT activity by making them more active. Apart from this, acetylation of several signaling associated molecules like STAT1, transcription factors like NF-κB, GATA-4, c-myc, Tat, histone chaperones (NAP1, NPM1, Asf1) etc play important role in transcriptional regulation, which when perturbed lead to disease states (Batta et al., 2007). Several metabolic enzymes and even cytosolic proteins like α-tubulin get acetylated (Westermann and Weber, 2003). Thus, acetylation has emerged as means of regulating gene expression by either promoter specific histone acetylation, that modulates the local chromatin environment so as to facilitate access to cellular machineries or as acetylation of non histone proteins that 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 (Yang and Seto, 2008). However all these functions are not unique to acetylation alone and they are brought about by a repertoire of other

modifications which either synergize or antagonize with acetylation. This cooperation between different posttranslational marks within histones and with DNA methylation, referred to as 'cross talk', is essential for the establishment of the combinatorial 'histone code'. However, unlike a code, the combinations are not a rule. It need not be the case that a set of modifications always code for transcriptional activation. Rather, it is the context in which the modifications are introduced that decides the downstream events.

1.2. CROSS TALK OF CHROMATIN MODIFICATIONS, THE EPIGENETIC LANGUAGE:

Epigenetics has been 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 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 context dependent cassette of modifications.

1.2.1. Cross talk between DNA methylation and Histone modifications:

The cross talk first identified was between DNA methylation of the CpG islands and H3K9 methylation. Several studies indicate the positive regulatory effects of the above modifications on each other. In fact, treatment of the DNA methylation inhibitor 5-aza dc, in addition to decreasing global DNA methylation also reduces the H3K9 methylation (Zhang et al., 2007). Knockout studies of DNA methyl transferases (DNMTs); H3K9 methyltransferases; in MEFs, ES cells and mammalian cells have 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 an absolute prior requisite for H3K9 methylation to occur. 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, that deacetylate 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.

1.2.2. Cross talk between histone acetylation and histone methylation:

Methylation-induced acetylation has been found to occur on histone H4. PRMT1 (Protein arginine methyltransferase 1), an H4-R3 methyltransferase, induces p300-mediated H4 acetylation and transcriptional activation (An et al., 2004). This H4 acetylation in turn inhibits H4 methylation. It is also observed that methylating and acetylating enzymes targeting the same histone residue can be indirectly inhibitory towards one another. For example, acetylated lysine 9 on histone H3 will inhibit Suv39h1 (Suppressor of variegation 3 – 9 homologue1)-mediated methylation of the same residue. Similarly, methylated H3-K9 will prevent acetylation. These two mutually exclusive modifications have this effect on one another because of their opposing transcriptional outcomes as H3K9 methylation results in gene silencing whereas H3-K9 acetylation induces gene activation (Keppler et al., 2008). Furthermore, the methyltransferase disruptor of telomeric silencing-1 (Dot1); is known to cross talk with the heterochromatin protein silent information regulator-3 (Sir3), a member of the SIR complex that also includes Sir4 and the NAD-dependent H4K16 HDAC Sir2 (Altaf et al., 2007). The SIR complex is associated with transcriptional repression; gene silencing; cell cycle progression and chromosome stability (Brachmann et al., 1995). Misregulation of Dot1 is linked with leukemogenesis (Okada et al. 2005). Dot1 is responsible for methylating histone H3 at K79. This methylation is dependent on the ability of Dot1 to interact with a short basic region on the N-terminal tail of histone H4. Sir3, known to associate with unmodified H3 and H4 tails, competes with Dot1 by interacting with the same basic region on H4 and by binding H3 adjacent to K79. GCN5-mediated H4-K16 acetylation displaces Sir3 on H4, thereby allowing Dot1 to interact with H4 and subsequently methylate H3-K79. H3-K79 methylation in turn further blocks Sir3–H3 interactions. This series of events serves to define a heterochromatin boundary and allow for transcriptional elongation. H3K4 methylation by MLL is a signal for MOF mediated acetylation of H4K12 (Dou et al., 2005).

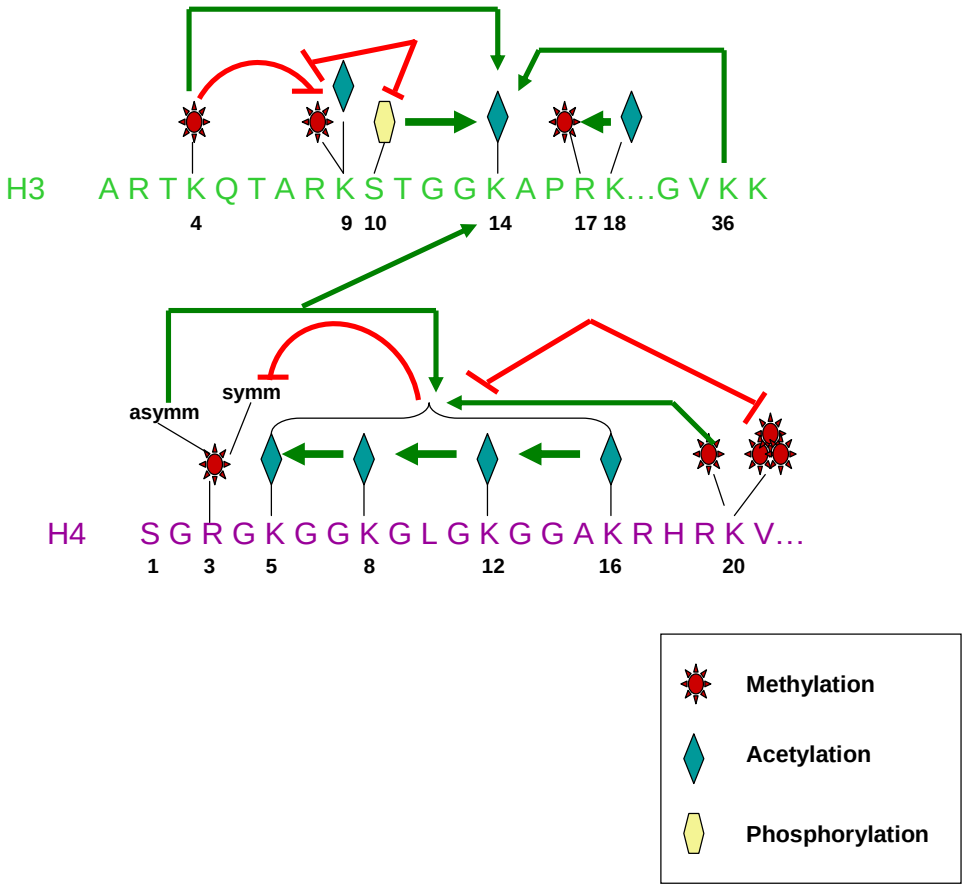


Figure 1.3. Cross talk of histone H3 and H4 modifications: The amino acid residues and their corresponding positions of the histone H3 and H4 N-terminal residues are shown. The positive regulatory modifications are represented by the green arrow, whereas the red lines indicate the negative modulatory 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.

1.2.3. Cross talk between histone phosphorylation and histone acetylation:

Histone phosphorylation is known to modify nucleosomal structure. H3 phosphorylation is synergistically coupled to histone acetylation, thereby increasing the levels of complexity and control of transcriptional regulation (Cheung et al., 2000). A combination of phosphorylated and acetylated histones is believed to display different recruitment signals for transcription factors and regulatory complexes than histone with single modification. However, the precise mechanism and physiological roles of this multifaceted cross-talk relationship is not fully understood but two models have been proposed: the synergistic model and the parallel-independent model (Biel et al., 2005). A number of HATs are known to preferentially acetylate H3-S10 phosphorylated forms of their substrates as opposed to the unmodified forms, thus giving rise to the synergistic model. For example, Msk1/2-mediated H3-S10 phosphorylation enhances the binding affinity of the HAT, general control of nuclear-5 (GCN5) to H3, which in turn leads to H3-K14 acetylation and transcriptional activation (Lo et al., 2000). In the parallel-independent model, H3-S10 phosphorylation does not necessarily lead to H3-K14 acetylation, and the absence of H3 phosphorylation does not necessarily decrease levels of H3 acetylation. This model is supported by the observation that, at times under certain conditions, Msk1/2 mutations or inhibition resulting in decreased H3-S10 phosphorylation do not affect levels of histone acetylation (Keppler et al., 2008)

1.2.4. Cross talk between histone phosphorylation and histone acetylation/ methylation:

Phosphorylation, acetylation and methylation machinery is also known to function together in a cross-talk tertiary loop (Figure 1.3). For example, histone H3K9 methylation inhibits S10 phosphorylation to repress gene transcription. On the other hand, in the case of gene activation, S10 phosphorylation facilitates K4 methylation, inhibits K9 methylation and enhances K14 acetylation, which in turn helps to further inhibit K9 methylation (Cheung et al., 2000; Rea et al., 2000; Zhang et al., 2001). The absence of a repressive methyl group on K9 then allows for K9 acetylation and further chromatin decondensation. These modifications work in concert to compose distinct histone codes, which are recognized by specific chromatin-interacting proteins in order to precisely drive the necessary physiological processes in the cell (Keppler 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 to each other.

1.2.4. Crosstalk between Biotinylation, Ubiquitination and other modifications:

An intriguing modulation is the H2BK123 monoubiquitination in positively regulating the H3K4 and K79 methylation. This is very interesting since ubiquitination is a common specific signal for degradation or repression (Nakanishi et al., 2009).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.

1.3. HAT ENZYMES AS TRANSCRIPTIONAL COACTIVATORS:

1.3.1. General introduction

In addition to having enzymatic activity that modifies chromatin many of these modifiers are also 'scaffold proteins', that connect different transcription factors; 'mediators', between DNA and the transcriptional factors; and 'co-activators' of transcription. These proteins are generally termed as transcriptional co-activators, because they, unlike 'activators', do not bind to DNA themselves, but streamline and increase the efficiency of transcription.

1.3.2. Transcriptional co-activators, Lysine acetyltransferase p300/KAT3B: Role in development and disease.

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 1.4.** It is characterized by three Zinc finger domains. It has a bromo domain, and most importantly the acetyltransferase domain, which is active without the full-length form, and thus has enabled 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 1.4, 1.5**). p300 HAT domain has a loop, which is made of about 12 lysine residues which is normally inhibitory in nature but when it gets sequentially acetylated gives rise to a hyperactive p300. Crystallization was difficult due to the presence of unstructured loops within the HAT domain. However, deletion of some of these loops and

binding of lysyl coA lead to the formation of a structure stable enough to be crystallized (Thompson et al., 2005). 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

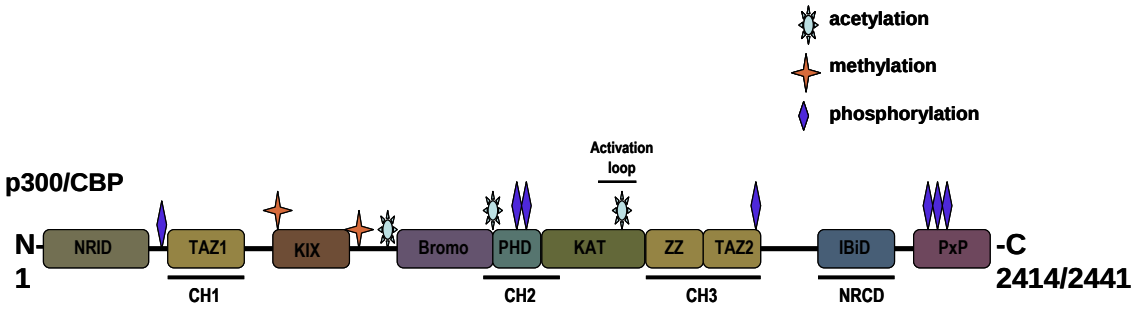


Figure 1.4. p300 domain architecture: Domains of histone acetyltransferase p300. Apart from the minimal HAT domain (1284-1673), three CH domains, a single bromodomain and the KIX domain are preset. The domains are marked for their propensity to get modified post translationally.

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. p300/ CBP mediated histone and non-histone acetylation results in various functional outcomes ranging from transcription, replication, and repair. 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. Although p300 and CBP share extensive homology, genetic and molecular analyses suggest that they perform not only overlapping but also unique functions. The p300-/-, cbp-/- and p300+/- cbp+/- mice show similar, embryonic lethal phenotypes (Yao et al., 1998), together with similar defects in growth and neural tube closure (Yao et al., 1998), which suggests that p300 and CBP have overlapping roles during embryonic development.

Furthermore, some *p300* and *Cbp* heterozygous mice suffer early lethality (Yao et al., 1998), indicating that p300/CBP gene dosage, and therefore the level of p300/CBP proteins, is probably important during development. CBP and p300 are known to contribute in diametrically opposed

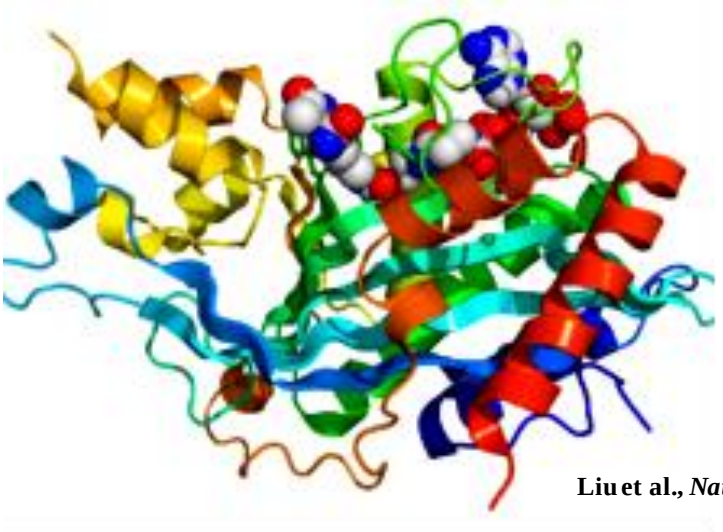


Figure 1.5. p300 crystal structure. Overall structure of the p300 HAT domain with N and C terminus coloured in blue and red, respectively. Lys-CoA is shown as a space filled model. carbon = white, oxygen = red, nitrogen = blue, phosphorous = orange. (Adapted from Liu et al., *Nat Str Mol Biol*, 2008).

cellular processes. It is shown that it participates in various tumor-suppressor pathways. It has been shown that mice engineered to contain a null mutation in one CBP allele developed a variety of hematological abnormalities, including extramedullary myelopoiesis and erythropoiesis, lymph node hyperplasia, and splenomegaly (Goodman et al., 2000) which also supported from the fact that human patients with the Rubinstein-Taybi syndrome (RTS), due to CBP heterozygosity, also have an increased incidence of malignancy (Goodman et al., 2000). Whether CBP and p300 promote apoptosis or cell proliferation appears to be highly context dependent. Cell proliferation and growth is also known to get influenced by p300/CBP activity (Stein et al., 1990; Yao et al., 1998) which is supported by the fact that p300-/- embryos are significantly smaller than their wild-type littermates and show defects in cell proliferation (Yao et al., 1998). Further it is also shown that p300/CBP-PCAF protein complex can arrest cell cycle progression (Yang et al., 1996) and might regulate target genes that are involved in controlling the G1/S transition, such as p21WAF1 (Missero et al., 1995). p300 was first implicated in oncogenic pathways when it was originally identified as an adenovirus E1A oncoprotein binding

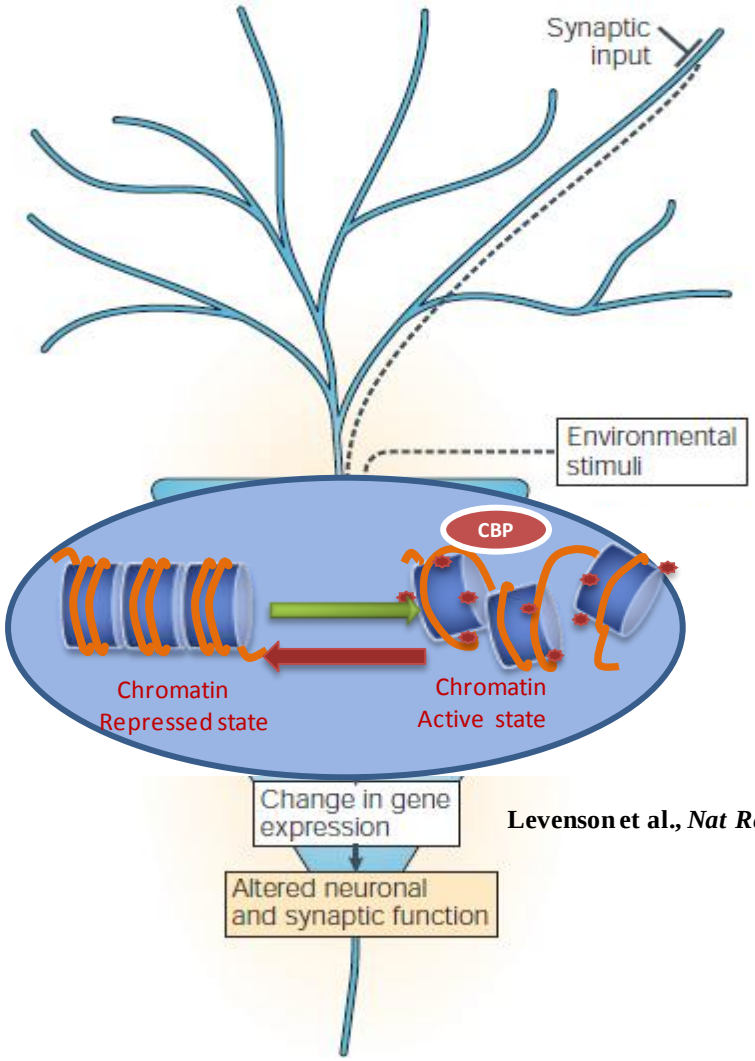
partner (Stein et al., 1990) . Similar observations were made when mutations in the gene encoding p300 are found in breast, colorectal, gastric and epithelial cancers. Glioblastoma and other malignancies can also arise as a result of loss of heterozygosity at the p300 locus (Gayther et al. 2000).

With evidence piling, implicating p300/CBP in various disease states, it was still not certain to what extent altered HAT activity contributed to the pathology in diseases. However, it is now clear and well elucidated with elegant studies that in various kinds of disorders, dysfunction of HAT regulation is the key feature. In Acute Myeloid Leukemia (AML), a fusion protein is created in which CBP is fused with a MYST domain-containing MOZ (Yang. 2004; Borrow et al. 1996). This CBP–MOZ chimera, possessing protein interacting domains from both the p300/CBP and MYST families of HATs, exhibits gain of function characteristics leading to unchecked hyperacetylation and aberrant transcriptional activation. Histone acetylation, controlled by HATs and HDACs plays a critical role in the regulation of inflammatory genes and in mediating the anti-inflammatory effects of corticosteroids in asthma patients (Mroz et al. 2007). It was observed that the HAT activity is higher in asthmatic patients, which leads to increased expression of multiple inflammatory genes that are regulated by pro-inflammatory factors, such as NF-κB. It is proposed that the activities of HATs/histone deacetylases (HDACs) affect the regulation of transcription of genes critical for beta-cell function and metabolic homeostasis, and therefore may play a crucial role in the pathogenesis and/or management of diabetes (Gray et al., 2005). In a mutant mouse model of a HAT, mice heterozygous for the CBP demonstrated an increased insulin sensitivity and glucose tolerance even while demonstrating a marked lipodystrophy of white adipose tissue (Yamauchi et al., 2002). More recently, it was also demonstrated that the regulation of expression of insulin by glucose is under the control of histone hyperacetylation, suggesting an important role for HATs and HDACs in the regulation of this critical gene (Gray et al., 2001). Different studies have shown the involvement of HAT p300 in cardiac hypertrophy and development. It has been shown that Phenylephrine (PE) could activate p300 and CBP through p42/p44 MAPK (Gusterson et al., 2002). The importance of p300 in normal cardiac transcription was further illustrated by p300 knockout mouse embryos that have reduced expression of muscle structural proteins such as myosin heavy chain and α-actin, as a consequence, cardiac structural defects (Yao et al., 1998). In contrast, role of the related protein, CBP was found to be less important in cardiovascular development (Roth et al.,

2003; Tanaka et al., 2000). Over expression of p300 was sufficient to induce hypertrophy, but the HAT domain of p300 was found to be required for this activity (Gusterson et al., 2003). The above study clearly suggested the importance of p300 in regulating the hypertrophic program in response to PE.

With histone acetylation playing such vital roles in transcription and gene expression it was obvious that it would affect life processes right from the stage of cells to the organs but its role was clearer in some tissues while not readily manifested in others. The nervous system has been one such tissue where this phenomenon was not well understood until recently. Though nervous system is much more than just the Brain, our present knowledge is mostly limited to some areas of the brain like the hippocampus and the striatum that are responsible for learning, establishment of memory and retrieval of stored memory, than other constituents like the spinal cord or the peripheral nervous system in the context of histone acetylation. Organic disorders always showed evidence of what went wrong in the brain while inorganic diseases never show any pathological changes. Many psychiatric disorders with no obvious brain pathology still cause significant morbidity, if not mortality. Most neurodegenerative disorders are associated with some degree of leaning and memory defects. In fact, it is so common that in most of the cases, forgetfulness is the only complaint of the patients! The brain functions in a stimulation dependent way, constantly establishing newer circuits and removing older circuits and synapses that depend majorly on environmental stimulation or lack of it, thereby bringing about our daily sense, perception, behavior, learning, memory and even forgetfulness (**Figure 1.6**). Any disturbance in this process leads to neurological and psychiatric disorders. One characteristic feature of the brain is to learn and store the information in the form of memory. In cognitive psychology, memory is usually divided into short term and long term memories. Short term memory is most often stored as sounds, especially in recalling words or as images. It provides a working space for short computations and then transfers it to other parts of the memory system or discards it. Short term memory can be converted to long term depending on the importance and emotional value. Otherwise the memory system discards it. Short Term Memory is vulnerable to interruption or interference. The biological basis for this form of memory is unclear and may involve the pattern of events occurring at the synapse and presynaptic terminals like neurotransmitter release and its endocytosis. This does not require structural alterations at the

synapse. However, short-term memory, which is a temporary potentiation of neural connections, can become long-term memory through the process of



Levenson et al., *Nat Rev Neuro sci*, 2005

Figure 1.6.Epigenetics in the adult nervous system. Regulation of the epigenetic state of the genome in adult neurons occurs in response to synaptic inputs and/or other environmental stimuli. These external stimuli result in changes in the transcriptional profile of the neuron and, ultimately, neural function (Adapted from Levenson et al., 2005).

rehearsal and meaningful association. This results in Long-Term Memory being relatively permanent storage. Information is stored on the basis of meaning and importance with inputs from the emotional centres of the brain like the limbic system. Establishment of long memory needs structural change in the circuits. One of the mechanisms of long term memory formation is the induction of Long term potentiation (LTP) (Bliss et al., 1993; Malenka et al., 2004; Cooke et al., 2006) . Long-term potentiation (LTP) is a persistent increase in synaptic strength following high-frequency stimulation of a chemical synapse. There are two phases to LTP: an early induction phase and a late maintenance phase. On the whole this translates to establishment of a new circuit and to more number of synapses in an established circuit that can propagate the particular signal with greater ease so that the information is never missed. The post synaptic cell harbours receptors that are more active and more in number for the particular signal from the particular presynaptic cell. There are also more number of synaptic proteins like synaptophysin in the synapses. This places demands to the cell's biochemical apparatus that modulate gene expression and translation (Costa-Mattioli et al., 2008) (**Figure 1.6 and 1.7**). This is where important transcription factors like CREB and co-activators like CBP along with their HAT activity play a pivotal role. Indeed, in a learning model called Contextual fear conditioning, which is a hippocampus dependent learning model, by which an animal learns to associate a novel context with an aversive stimulus, acetylation of histone H3, but not H4, is significantly increased after an animal undergoes contextual fear conditioning (Levenson et al., 2004). Also, as mentioned during the description of long term potentiation; formation of long-term contextual fear memories requires NMDA (*N*-methyl-D-aspartate)-receptor-dependent synaptic transmission and the MEK–ERK/MAPK signalling cascade (where MEK referes to MAPK/ERK kinase) in the hippocampus and inhibition of either of these processes blocks the increase in acetylation of H3. To further determine the role of CBP in long-term memory formation, two recent studies have generated CBP-deficient mice that lack the severe developmental problems of the CBPDN+/– animals. The first study linked the dominant-negative allele of CBP to an inducible promoter (CBPI-DN+/–) (Korzus et al., 2004). Activation of the dominant negative allele after animals had developed normally led to impaired learning of the spatial water maze task and novel object recognition. In the second study, mice that lacked one allele of CBP (CBP+/–) had impairments in contextual and cued fear memory, and novel object recognition (Alarcon et al., 2004). In both studies, administration of an HDAC inhibitor, that blocks

deacetylase activity and increases histone acetylation, restored normal long-term memory formation. Also, the induction of late-phase LTP, which requires transcription, was significantly impaired in CBP+/– animals. Treatment of hippocampal slices from CBP+/– animals with the HDAC inhibitor suberoylanilide hydroxamic acid significantly improved late-phase LTP

Protein	Activity	Neuronal Function
MSK1	CREB phosphorylation H3-S10 phosphorylation	Long-term memory formation
Rsk-2	CREB phosphorylation H3-S10 phosphorylation	Long-term memory formation
PP1	H3-S10 dephosphorylation	Long-term memory formation
G9a/GLP	H3-K9 di-methylation	Cognition and adaptative behavior
SMCX	H3-K4 tri-demethylase	Cognitive functions
MeCP2	Methyl-CpG-binding protein	Memory and synaptic plasticity
CBP	Histone acetyltransferase	Long-term memory consolidation
HDAC2	Histone deacetylase	Negative regulator of synaptogenesis and memory formation

Table 3: Chromatin modifying enzymes with distinct effects on neuronal function with their modifications

induction, which indicated that inhibition of HDACs had compensated for HAT haploinsufficiency. Indeed, inhibitors of HDACs, Trichostatin A and Sodium butyrate when added to slice cultures could induce LTP, which was significantly better than untreated slices. In addition, LTP in the Amygdala that was induced by Forskolin, which is an activator of CREB downstream genes, was also enhanced by the HDAC inhibitor Trichostatin A. These studies indicate that the epigenetic state of the genome, more importantly chromatin acetylation, affects the induction of long-term forms of mammalian synaptic plasticity and thus influence memory. There are other modifications that play a role in neuronal function apart from acetylation (**Table 3**). Phosphorylation at H3S10 and di/ tri methylation of H3K4 is known to be necessary for

memory functions. However, H3K9 methylation, which is generally a transcription repressive mark is also known to be necessary for some aspects of cognitive functions in animal models. DNA methylation is also a major determinant of active and inactive chromatin and thus influences neuronal function (Levenson et al., 2005).

1.4. SIGNALS TO THE NEURONAL CHROMATIN:

1.4.1. Signaling to neuronal chromatin:

Gene expression is tightly regulated spatio-temporally and is governed by various signals from the environment and from within the cell. It is the same case for the neurons. When the diverse functions of neurons are taken into consideration it will be understood that environmental cues play a major part in neuronal cell signaling that in turn influences gene expression. The signals either result in immediate and short term changes like neuron depolarization or long term changes that necessitate gene expression changes and structural alterations like dendritogenesis. Most of the signals however seem to converge on one particularly important transcription factor, cyclic AMP Response Element Binding Factor (CREB) (**Figure 1.7 and 1.8**). The cAMP/CREB signaling pathway has been strongly implicated in the regulation of a wide range of biological functions such as growth factor-dependent cell proliferation and survival, glucose homeostasis, spermatogenesis, circadian rhythms and the synaptic plasticity that is associated with a variety of complex forms of memory including spatial and social learning indicating that CREB may be a universal modulator of processes required for memory formation. Deletion of CREB in neurons of the developing CNS (Central Nervous System) results in apoptosis, and postnatal silencing of CREB results in neuronal degeneration in adulthood. Neurons of the adult striatum and hippocampus, which are the centers for learning and memory in the mammalian brain, are particularly vulnerable to CREB deficiency. Most importantly, CREB interacts with its nuclear partner CBP (CREB Binding Protein) and drives the transcription of a large number of genes that is dependent on its intrinsic lysine acetyltransferase activity. The crucial event in the activation of CREB is the phosphorylation of Ser133 in KID (Kinase-Inducible Domain). This domain includes several consensus phosphorylation sites for a variety of kinases like PKA (Protein Kinase-A), PKC (protein Kinase C) CSNK (Casein Kinases), CaMKs (Calmodulin Kinases),

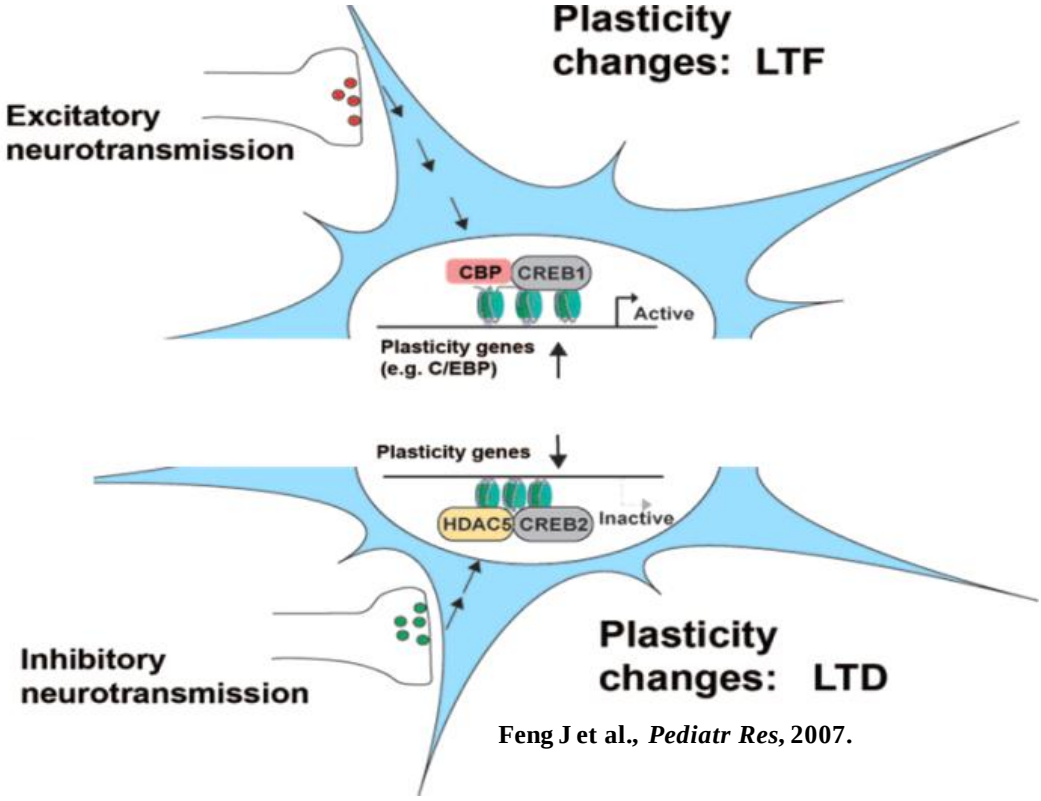


Figure 1.7. Histone modifications mediate alterations in neural gene expression and long-term neural plasticity. A schematic drawing of excitatory (*A*) and inhibitory (*B*) synaptic transmission in a sensory neuron in *Aplysia* that can lead to long-term synaptic plasticity changes such as LTF (long-term facilitation) and LTD (long-term depression). Histone acetylation through HAT proteins (*e.g.* CBP) or deacetylation by HDAC5 is involved in the stable alterations in the expression of the plasticity genes such as C/EBP (Adapted from Feng J et al., 2007)

GSK3 (Glycogen Synthase Kinase-3) and p70S6K that can either increase or decrease the activity of CREB. Ser133 phosphorylation of CREB can be induced by electrical activity, Growth Factors, Neurotransmitter or Hormone action on GPCR (G-Protein-Coupled Receptors), or by Neurotrophin effects on RTKs (Receptor Tyrosine Kinases) (**Figure 1.8**) (Mayr B et al., 2001). Since several different protein kinases possess the capability of driving this phosphorylation, making it a point of potential convergence for multiple intracellular signaling cascades. Upon stimulation of cellular GPCR (G-Protein-Coupled Receptors) and Growth Factor Receptors, AC (Adenylate Cyclase) is activated, by various G-proteins leading to increases in cAMP. This in turn activates PKA-C by dissociating the regulatory (PKAR) from the catalytic (PKAC) subunits. In the basal state, PKA resides in the cytoplasm as an inactive heterotetramer of paired regulatory and catalytic subunits. Induction of cAMP liberates the catalytic subunits.

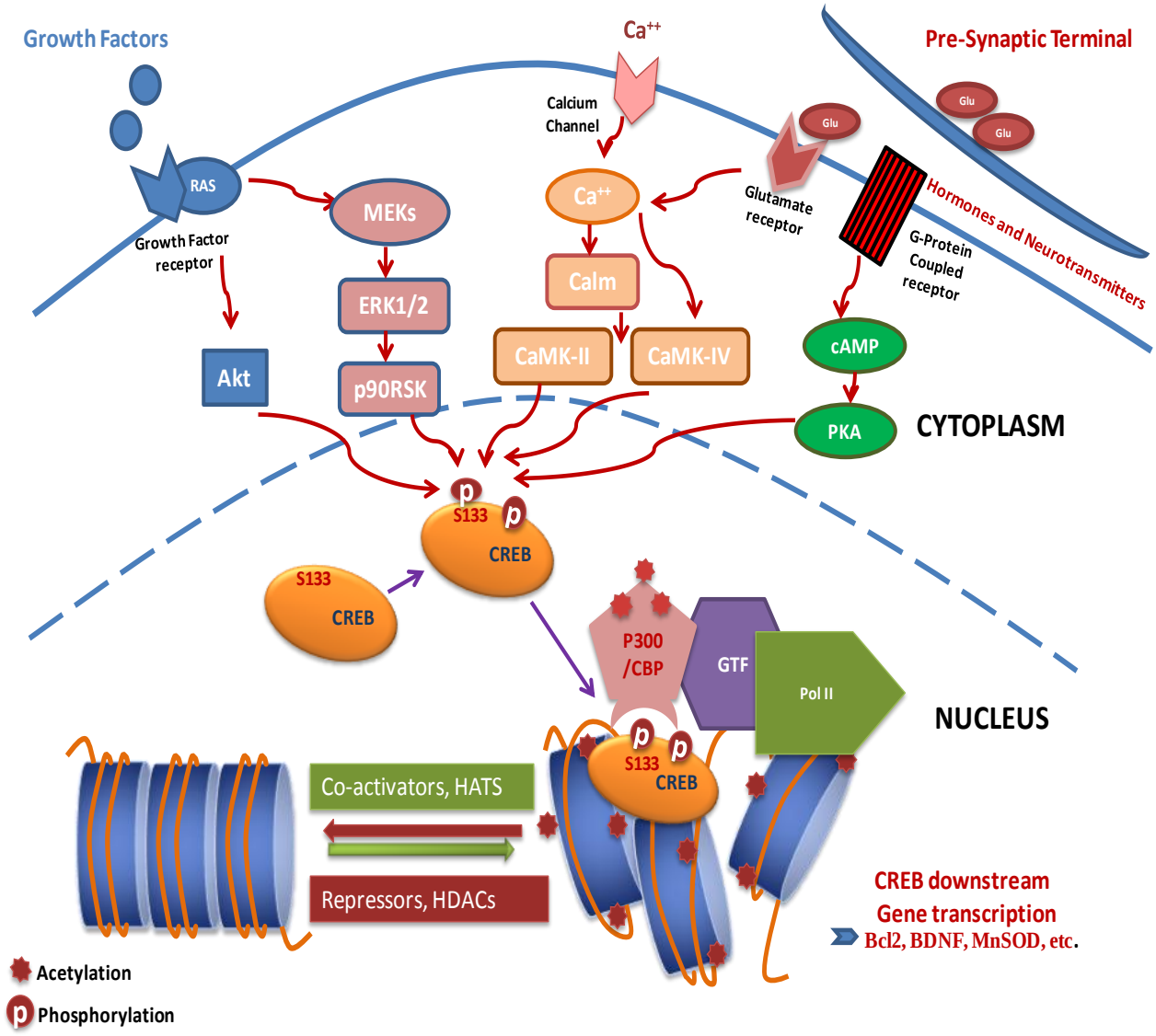


Figure 1.8 : Neuronal excitation drives activity dependent signaling through various kinases. Most of the signals however seem to converge on one particularly important transcription factor, cyclic AMP Response Element Binding Factor (CREB) which gets phosphorylated at Ser133 and makes it active. This recruits CBP and results in promoter proximal histone hyperacetylation.

This activated PKA then recruits the Ca2+/CalmK-IV (Calmodulin (Calm)-dependent Kinases), MEK (MAPK/ERK Kinases)/ ERK1/2 (Extracellular Signal-Regulated Kinases) and together they translocate to the nucleus (Choe et al., 2002; Ahmed et al., 2005). In the nucleus they lead to the recruitment of the transcriptional co activators CBP (CREB Binding Protein) and p300 by phosphorylating Elk1. Elk1 is a part of a TCF (Ternary Complex Factor) that activates RSKs

(Ribosomal S6 Kinases) and binds SRF (Serum Response Factor) to the SRE (Serum Response Element). Phosphorylation of Elk1 increases its transcriptional ability to form ternary complexes with SRF at the SRE in the promoter region of many genes, such as c-Fos (Finkbeiner S., 2001). Further, CBP/p300 stimulates gene expression by interacting with components of the general transcriptional machinery or by promoting the acetylation of specific lysine residues in nucleosomes located near transcriptionally active promoters thus creating access to the gene for the basal transcriptional machinery. The basal transcriptional machinery includes TBP (TATA-binding protein), TFIIB (Transcription Factor-II-B), and RNA Pol-II (RNA Polymerase-II) . The accumulation of cAMP in response to activation of GPCR also induces PLC-Gamma (Phospholipase-C-Gamma) that catalyzes the formation of DAG (Diacylglycerol), a PKC activator through PI (Phosphatidylinositols). PI3K (Phosphoinositide-3kinase) is responsible for activation of Akt/PKB (Protein Kinase-B), which directly or indirectly affects CREB.

In addition to the modification of CREB, these signals also modify p300/CBP itself. These posttranslational modifications of p300/CBP are known to modify its interacting ability and more importantly its HAT activity. A brief description regarding such modifications is presented below.

1.4.2 Signal transduction cascades affecting Posttranslational modifications of p300/CBP and its HAT activity:

Several posttranslational modifications have been reported to regulate CBP activity. Phosphorylation of CBP or p300 at an unidentified site near the carboxyl terminus by MAPK and CaMKIV has been reported to increase their HAT and transcriptional activity during neuronal activity (Ait-Si-Ali et al., 1999; Impey et al., 2002). Neuronal activity-dependent activation of CBP depends on CAMK-IVdependent phosphorylation on Ser 301. CBP is also activated by elevation in cAMP level. cAMP-mediated activation of CBP is dependent on type-I PKA-mediated phosphorylation. Growth factors (like NGF) can trigger CBP phosphorylation via p42/p44 MAPK, which has been shown to interact directly with CBP. NGF can also trigger Ras pathway, to mediate phosphorylation of 90 kDa Ribosomal S6 kinase (RSK), which binds to CBP and leads to expression of genes dependent on Ras pathway. However, this interaction inhibits expression of genes with CRE elements in PC12 cells. In one study it is also found that Akt-dependent p300 phosphorylation at Ser 1384 can increase p300 HAT activity and contribute

to inflammatory gene expression by TNF-α (Huang and Chen, 2005). However, phosphorylation of p300 at Ser 89 by PKCα and PKCδ appears to repress its transcriptional activity, probably leading to cell growth inhibition (Yuan et al., 2002; Yuan and Gambee, 2000). Besides phosphorylation, methylation by Coactivator- Associated Arginine Methyltransferase 1 (CARM1) (Xu et al., 2001) and sumoylation by Ubc9 (Girdwood et al., 2003) have also been reported to regulate the transcriptional activities of CBP and p300. p300/ CBP methylation by (CARM1) causes a transcriptional switch from CREB-regulated to nuclear hormone receptor-regulated gene expression (Xu et al., 2001). A recent work indicated that IKKα-mediated phosphorylation of CBP not only changes its protein binding preference but also is required for its HAT and intrinsic transcriptional activities. However, blockage of IKKα or substitution of CBP Ser- 1382/Ser-1386 with Ala could not completely abolish the HAT activity, suggesting that IKKα mediated phosphorylation has other alternative ways of activating CBP.

1.5. Chromatin acetylation, HAT/ HDAC balance in neurons:

During normal conditions, protein concentration (availability) and enzymatic activity of HATs (like CBP and p300) and HDACs remain in a highly harmonized state of balance where adequate active molecules from either group are present to effectively regulate chromatin and TF acetylation in a controlled manner. Such equilibrium manifests a neuronal homeostasis and is responsible for regulated gene expression leading to normal neurophysiological outputs like long-term potentiation, learning and memory. This equilibrium is maintained very stringently tinkering with the HAT or HDAC dose and/ or activity is not appreciated by cellular homeostasis machinery. Treatment of neurons with HDAC inhibitors like trichostatin A (TSA) in normal conditions induces neuronal apoptosis. Similarly, over expression of CBP in resting CGC neurons under prosurvival conditions also leads to chromatin condensation and cell death. However, overexpression of an HAT domain-deleted CBP mutant did not generate similar results. CBP conveys the same fate in normal neurons as is observed by blocking HDAC activity as both measures alter the precious HAT: HDAC balance towards enhanced histone acetylation, resulting in open chromatin, expressing genes that otherwise remain quiescent.

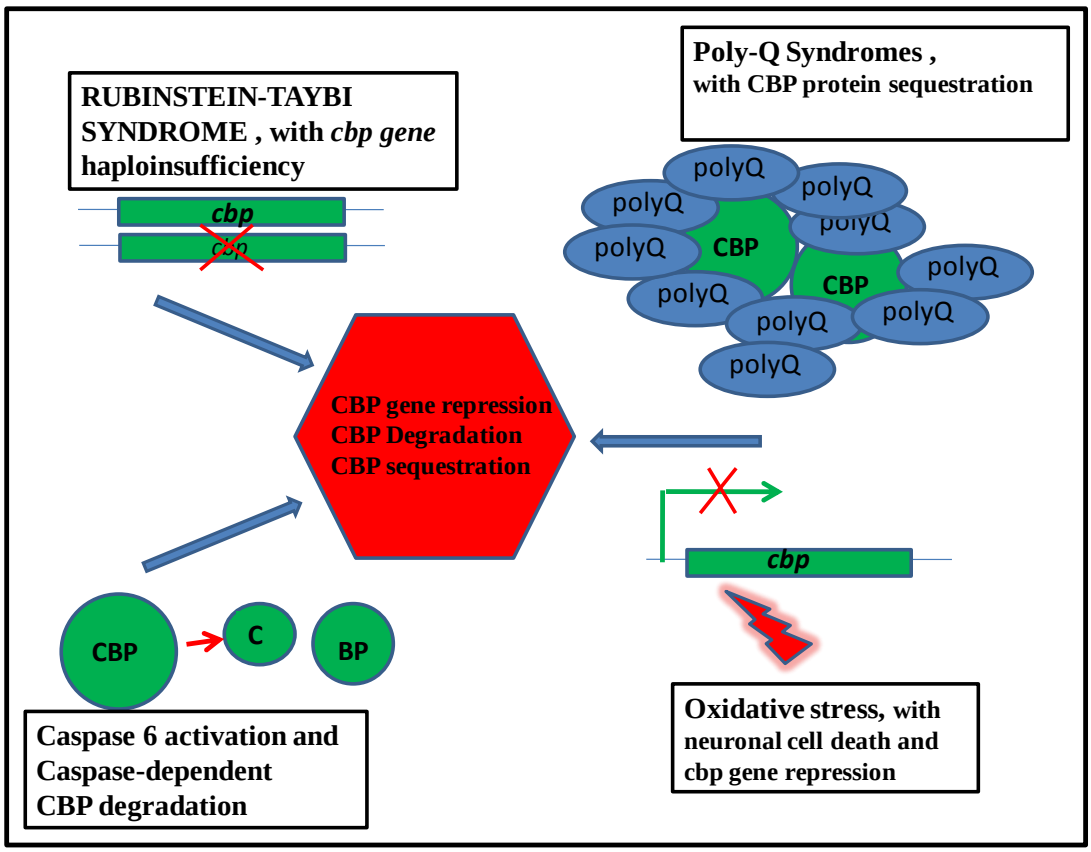


Figure 1.9: Decreased amounts of functional CBP protein and subsequent CBP's loss of function has been observed in different contexts of neurological disorders and neuronal apoptosis. RTS (Rubinstein-Taybi Syndrome) results from a mutation on one *cbp* gene allele. In several cases of polyQ diseases, CBP can be sequestered by the mutated polyQ proteins, forming aggregates in the cytoplasm or the nucleus. CBP proteasomal degradation was also shown to be favoured by polyQ proteins. CBP is a caspase-6 substrate in cerebellar granule neurons (CGN) deprived of potassium modeling caspase-dependent apoptosis. Finally, *cbp* gene repression has been observed in oxidative stress-induced death of a motor neuronal cell line.

These studies, therefore, strongly support the maintenance of precise balance between HATs and HDACs as a prerequisite of neuronal survival in normal conditions. One of the first evidences implicating loss of HAT activity to neurodegeneration came when CBP mutations rendering the HAT enzymatically inactive were seen in Rubenstein–Taybi syndrome. This syndrome is a developmental disorder associated with mental retardation and childhood cancers of neural crest origin (Petrij et al., 1995). The molecular basis of selective neuronal apoptosis during neurodegenerative diseases has revealed the role of acetylating and deacetylating agents during the process. Several studies have now successfully manipulated neuronal vulnerability by

influencing the dose and enzymatic activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs), enzymes regulating acetylation homeostasis within the nucleus, thus focusing on the importance of balanced acetylation status in neuronal vitality (Saha et al. 2006). It is now increasingly becoming clear that acetylation balance is greatly impaired during neurodegenerative conditions. Such equilibrium manifests neuronal homeostasis which is responsible for regulated gene expression leading to normal neurophysiological outputs like long-term potentiation, learning and memory (Saha et al., 2006). Neurodegenerative conditions (Rouaux et al., 2003) reflect a malfunctioning acetylation apparatus. Such malfunction, as suggested by several observations, is manifested by loss of HATs like CBP and p300 during various neurodegenerative challenges (Rouaux et al., 2003; Jiang et al., 2003). As reflected by the deacetylation of histones in apoptotic conditions where this loss is an early event during apoptosis (Rouaux et al., 2003) and is specific for affected neurons only. In one study, immunocytological detection of CBP with oxidative stress-challenged murine cortical neurons was done which revealed the presence of CBP only in the nucleus of neurons surviving the hypoxic stress, whereas in the condensed or fragmented nuclei, representing neurons undergoing apoptosis, the HAT was not present (Jin et al. 2001). Furthermore, the critical nature of the loss of HAT is illuminated by several overexpression studies of CBP, all of which demonstrate enhanced neuronal viability in response to various challenges (Rouaux et al., 2003; Nucifora et al., 2001; McCampbell et al., 2001; Taylor et al., 2003). It may be mentioned here that, among all HATs, loss of CBP appears to be pivotal in facilitating neurodegenerative cascade of events (**Figure 1.9**). Various mechanisms are now known to reduce CBP HAT availability in several models of neuronal insult. Nuclear translocation of expanded polyglutamine-containing neurotoxins (like mutated huntingtin protein), implicated in at least nine neurodegenerative diseases; selectively enhance ubiquitination and degradation of CBP by proteosomal pathway (Jiang et al., 2003). Additionally, during Alzheimer's disease progression, Presenilin-1-dependent epsilon-cleavage product N-Cad/CTF2 binds to CBP and facilitates its proteosomal degradation (Marambaud et al., 2003). Alternatively, CBP is redistributed from their normal nuclear location into Huntington aggregates, which compromises their availability for normal functions (Nucifora et al., 2001). Furthermore, caspase-6-dependent CBP proteolysis has been reported in low K^+ shock model of neurodegeneration (Rouaux et al., 2003). Active caspase-6 is also reported in neuropil threads, neuritic plaques and neurofibrillary tracts of Alzheimer brain,

suggesting that CBP may be lost in Alzheimer's disease by caspase cleavage. Like CBP, p300 protein level also decreases during neurodegenerative conditions (Rouaux et al., 2003). However, the mechanism behind such loss is not well known. Epigenetics, especially histone acetylation, is also implicated in

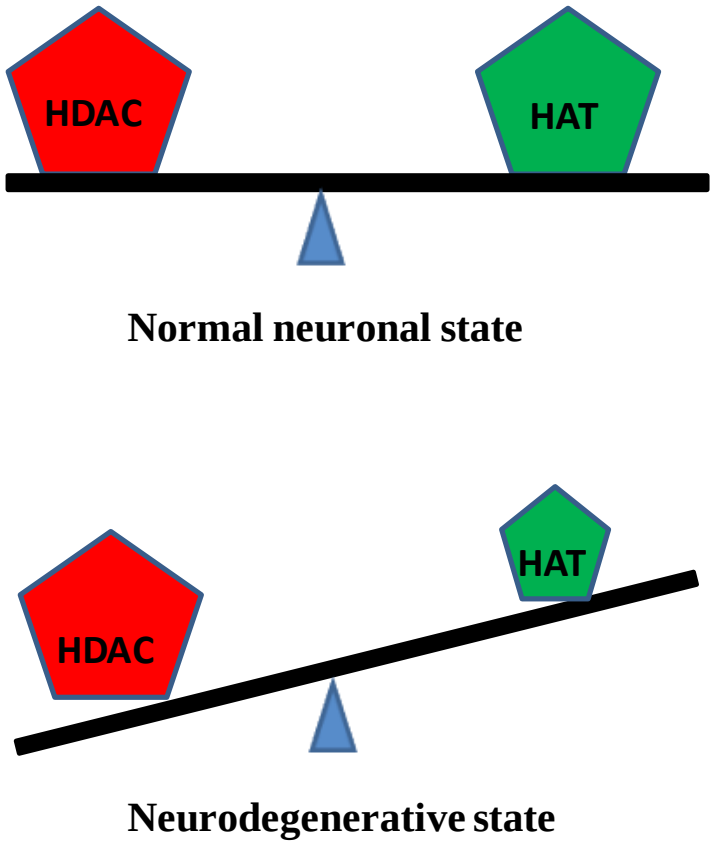


Figure 1.10: Chromatin acetylation status, transcription and survival: a balance between HAT and HDAC activities. A fine tuning of HAT/HDAC activities orchestrates neuronal death and survival. On one hand, acetylation levels can be decreased hypoacetylation because of CBP loss of function, as observed during apoptosis and neurodegeneration.

neuropsychiatric conditions like cocaine addiction. While histone hypoacetylation due to various reasons has been the cause of neurodegeneration, abnormal histone hyperacetylation is also implicated in some disorders. It is hypothesized that cocaine administration could induce global level histone acetylation change within Striatum, which is the the major neural substrate for the addiction (Kumar et al., 2005). Indeed, cocaine administration induced different histone modifications at different gene promoters. At the cFos gene promoter, H4 hyperacetylation was

seen instantly after fast cocaine injection, whereas no histone modifications were seen with chronic cocaine administration, consistent with cocaine's ability to induce cFos acutely (Levine AA et al., 2005). In contrast, at the BDNF and Cdk5 promoters, genes that are induced by chronic cocaine, H3 hyperacetylation was observed with chronic cocaine only. This shows clearly that addiction induces neuroadaptations through epigenetic regulation of gene expression. Furthermore, it also provides evidence that individual gene expressions are associated with different epigenetic regulatory mechanisms.

1.6. Towards Epigenetic therapy for Neurodegenerative diseases:

Despite differences in disease etiology and cellular properties of affected neurons in various forms of neurodegenerative disorders, loss of acetylation homeostasis appears to represent a critical and decisive mechanism commonly underlying neuronal dysfunction and degeneration. Once this was realized, research in this field focused on experimental therapeutics and drug development that would allow proper acetylation levels resetting. The first attempts were performed by Huntington disease researchers, who tried to fine-tune the altered HAT-HDAC balance by using general HDAC inhibitors to re-establish the acetylation threshold in dying neurons. On the opposite, targeting HAT loss reversal has also been proposed. Resetting HAT/CBP functionality is possible by three means: either over expressing the protein, trying to enhance the activity of the remaining protein with HAT activators or blocking its degradation in the first place. Over expression studies of CBP have been conducted by different laboratories and they all demonstrated increased neuronal survival in different context of apoptosis. For example, the androgen receptor with an expanded polyglutamine repeat, which is the cause of Spinal and Bulbar Muscular Atrophy (SBMA), interacts with CBP, leading to cell toxicity. This cell death can be mitigated in part by increased expression of CBP, which retains histone acetyltransferase activity (McCampbell *et al.,* 2001). Overexpression of CBP rescued polyglutamine-induced (huntingtin or atrophin-1 with expanded polyglutamine repeats) neuronal toxicity of N2a cells (Nucifora *et al.,* 2001). Using a *Drosophila* model of polyglutamine toxicity, Taylor et al., demonstrated complete functional and morphological rescue by upregulation of endogenous Drosophila CBP. Rescue of the degenerative phenotype was associated with eradication of polyglutamine aggregates, recovery of histone acetylation, and normalization of the transcription

profile (Taylor *et al.,* 2003). Thus, the success of using CBP over expression to rescue polyQ toxicity in cell models suggests that a gene therapy approach could be of benefit. Attempts to prevent cell death in CGN deprived of potassium by the over expression of CBP also to be proved successful, but only with a CBP protein that still had an intact HAT domain (Rouaux *et al.,* 2003). However, high levels of CBP expressed in neuroprotective conditions ultimately leads to cell death, likely because of unwanted hyperacetylation. This therapeutic strategy thus implies a restricted gene targeting to damaged neurons.

HDAC inhibitors are also effective in animal models of neurodegeneration demonstrating that these *in vitro* findings are translatable *in vivo*. As mentioned above, butyrates are considered to be most effective *in vivo* in terms of their ability in crossing the blood brain barrier. In *Drosophila* models of Huntington's disease, the HDAC inhibitors SAHA and sodium butyrate arrest the progressive neuronal degeneration and lethality (Steffan et al., 2001). SAHA and sodium butyrate have also been demonstrated to extend survival, ameliorate motor deficits and delay characteristic neuropathology in the mouse Huntington's disease model, R6/2 (Ferrante et al., 2003; Hockly et al., 2003). NaBu has also been reported to be effective in treating SBMA (Minamiyama et al., 2004), an inheritable motor neuron disease caused by an expanded polyglutamine-repeat within the androgen receptor. VPA was also shown to induce persistent activation of extracellular signal-regulated kinase (ERK), and its downstream effectors RSK and CREB, thus promoting neurite growth (Yuan *et al.,* 2001). The mode of action for HDAC inhibitors is highly non-specific and not targeted: if the context of neurodegeneration were CBP's loss of function, clearly, HDACi do not specifically target CBP's regulations only, but rather unlock repressive conformations at promoters of essential genes. This implies that more genes 'than needed' will be activated, and this might end up being cytotoxic. In this respect, while HDACi have been proved efficient in several *in vitro* and *in vivo* models of apoptosis, it is worth considering the death signaling pathway involved in each pathological situation. It is noteworthy that the CBP/p300 proteins are present in limited quantities thus creating a competition between transcription factors. CBP sequestration or reduction in CBP level, while inducing specific gene downregulation, could also represent a way to allow specific re-activation of a given gene. HDACi treatment may worsen this problem. Determining the genes that promote survival, especially those under CBP's control, could facilitate the development of novel drugs and specific therapeutic strategies with lower adverse side effects than those

currently available. In this scenario, it needs to be known as to what would be the changes in gene expression and epigenetics, when an inhibitor for HAT activity is used without altering total protein levels of HATs. There is need to understand the extent of hypoacetylation and hyperacetylation of histones that could be tolerated by neurons. There is also a need to elucidate the genes that would be regulated directly by CBP mediated acetylation. This would be possible by using small molecule modulators of HATs that would activate or inhibit the enzymatic activity thereby providing an easy alternative to investigate histone acetylation. Many of these small molecules have already been used in other settings like cancer and have been found to be highly specific and efficacious in cell culture models. There is a brief description of the HAT inhibitors that have been discovered in the last one decade. Though none of them have been used in the background of neuronal physiology, their effects on other cells have been studied to considerable extent

1.7. Small Molecule Modulators of Histone Acetyltransferases:

Several small molecule modulators of p300/CBP and PCAF have been developed (reviewed in Selvi et al, 2009). Though, synthetic bisubstrate analogues, that inhibit p300, PCAF, and TIP60 are known, not much information regarding their in vivo effects are known due to their poor cellular permeability. However, Spermidinyl CoA is one such synthetic and general inhibitor of HATs that has been reported recently to induce cell cycle arrest by sensitizing cells to radiation (Bandyopadhyay K et al., 2009). However, naturally occurring products have shown better in vivo effects. The first naturally occurring HAT inhibitor, anacardic acid, was isolated from cashew nut shell liquid, which inhibits the HAT activity of both p300 and PCAF very effectively (Balasubramanyam *et al.,* 2003). Anacardic acid is not known to be cell permeable. However several reports describe meticulously, its in vivo activity. Anacardic acid potentiates apoptosis induced by cytokine TNF-α and chemotherapeutic agents like cisplatin, doxorubicin etc. This increase in apoptosis correlates with downregulation of various gene products that mediate proliferation (cyclin D1 and cyclooxygenase-2), survival (Bcl-2, Bcl-xL, cFLIP, cIAP-1, and survivin), invasion (matrix metalloproteinase-9 and intercellular adhesion molecule-1), and angiogenesis (vascular endothelial growth factor), all known to be regulated by the NF-$\kappa\beta$. Anacardic acid inhibited both inducible and constitutive NF-$\kappa\beta$ activation (Sung et al., 2008). Anacardic acid also inhibits the activity of TIP60, a MYST family acetyltransferase and

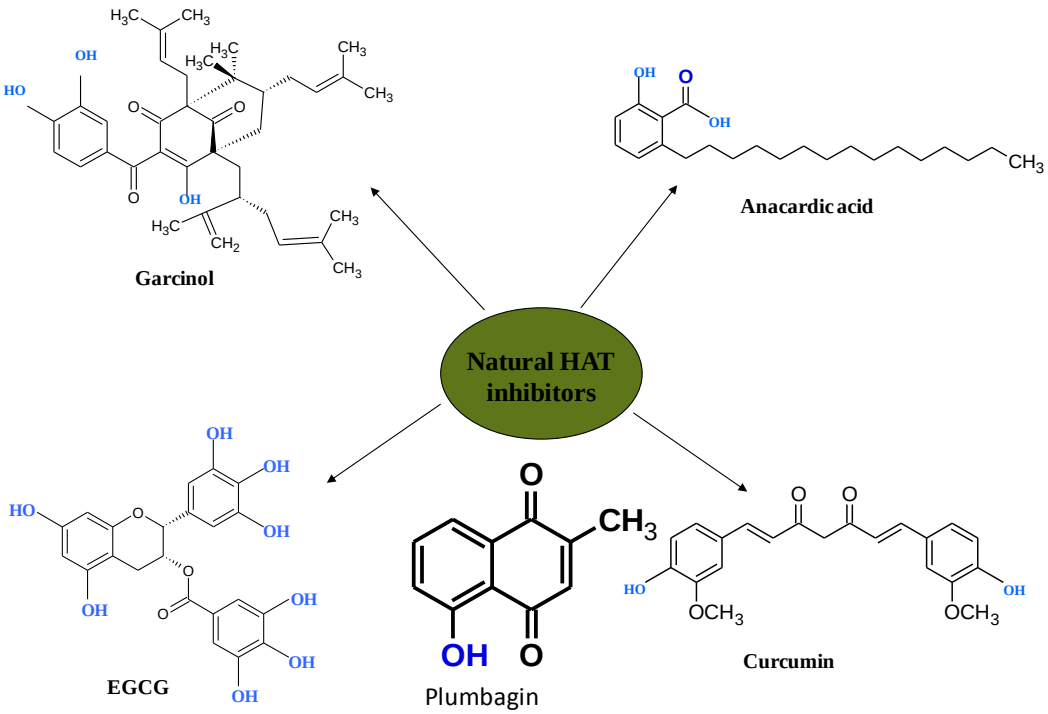
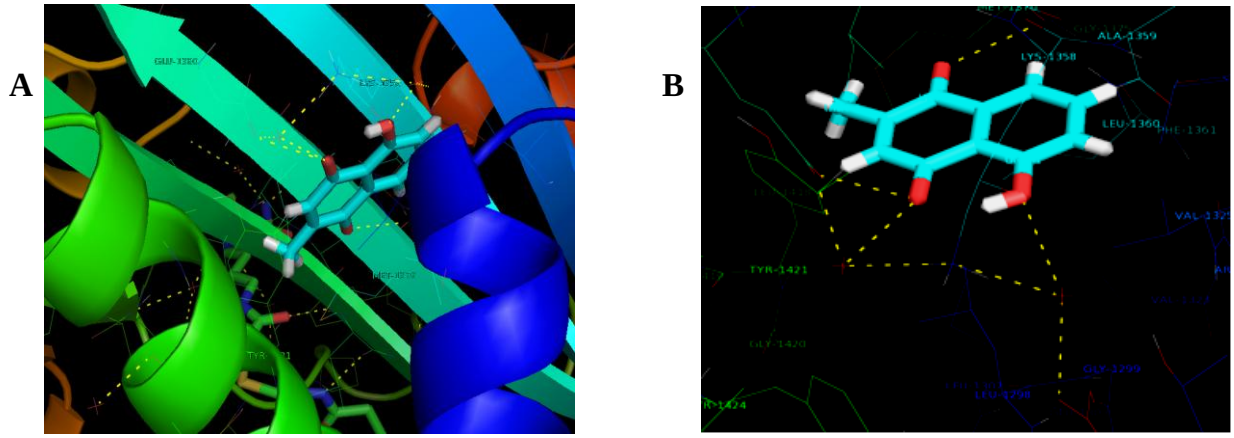


Figure 1.11: Structures of naturally occurring small molecules known to be HAT inhibitors. The presence of hydroxyl- group is highlighted in blue

sensitizes tumor cells to ionizing radiation (Sun et al., 2006). It was also found that Curcumin (diferuloylmethane), a major curcumanoid in the spice turmeric, is a specific inhibitor of the p300/CBP histone acetyltranferase (HAT) activity but not of PCAF both *in vitro* and *in vivo*. Curcumin could also inhibit the p300-mediated acetylation of p53 *in vivo*. p300/ CBP HAT activity dependent transcriptional activation from chromatin but not a DNA template is repressed (Balasubramanyam et al., 2004). Yet another naturally occurring molecule, polyisoprenylated benzophenone Garcinol inhibits non-specifically both PCAF and p300. Garcinol inhibits histone acetylation in cells even when cells are pretreated with the HDAC inhibitors like Trichostatin or sodium butyrate. Garcinol induces apoptosis and predominantly downregulates global gene expression in HeLa cells (Balasubramanyam et al., 2004). Plumbagin (RTK1), isolated from *Plumbago rosea* root extract, has been recently investigated to inhibit histone acetyltransferase activity potently *in vivo*. RTK1 specifically inhibits the p300- mediated acetylation of p53 but not the acetylation by the other HAT p300/CREB-binding protein –associated factor, PCAF, upon induction by doxorubicin treatment. Presence of hydroxyl- group is observed commonly across most of the naturally occurring small molecule inhibitors of HATs, leading a speculation

that these groups may confer activity in terms of HAT inhibition (**Figure 1.11**). In case of Plumbagin, a single hydroxyl group is observed at the 5th position. When we investigated whether this hydroxyl group has any role to play in HAT inhibition, we observed that this single hydroxyl group of RTK1 makes a hydrogen bond with the lysine 1358 residue of p300 HAT domain and almost abolishes its HAT activity (**Figure 1.12**). In agreement with this observation, it has been found that the hydroxyl group substituted plumbagin derivatives lost the acetyltransferase inhibitory activity. This study describes for the first time that a chemical entity (hydroxyl group) is required for p300 HAT inhibition (Ravindra et al., 2009). Plumbagin is a potent inhibitor of NF-κB pathway and its downstream gene products (Sandur et al., 2006). It is expected that one of the mechanisms of NF-κB inhibition would involve Plumbagin mediated p300 HAT inhibition and thereby hypoacetyation of p65, amongst other mechanisms, though it is yet to be proved. EGCG is a novel HAT inhibitor with global specificity for the majority of HAT enzymes. Interestingly it has no activity toward other epigenetic enzymes including HDAC, SIRT1, and Histone methyltransferase activity. EGCG abrogates p300-induced p65 acetylation in vitro and in vivo, increases the level of cytosolic Iκβα, and suppresses TNF- α induced NF-κβ activation. EGCG also prevents TNF-α induced p65 translocation to the nucleus. EGCG treatment inhibited the expression of NF-κβ target genes in response to various stimuli (Choi et al., 2009). Our laboratory has synthesized a water soluble inhibitor, CTK7A, a derivative of



Ravindra KC et al., *J Biol Chem*, 2009.

Figure 1.12: Docking of RTK1 onto the p300 HAT domain (A) and image of the docking site with the contact residues showing the hydrogen bond (B).

curcumin. This molecule is active against both p300 and PCAF acetyltransferase activities and has been found to target the hyperacetylation of histone and non histone proteins by p300 in oral cancer and reduce cancer load effectively in nude mice models (Arif et al., 2010). A new synthetic HAT inhibitor has been shown to be cell permeable and works in nanomolar concentrations. This molecule, C646, slows cancer cell growth by impeding intracellular histone acetylation (Bowers et al., 2010).

With more HAT inhibitors being discovered HAT activators have not been left far behind. By using anacardic acid as a synthon, an amide derivative of anacardic acid, CTPB, has been synthesized (Balasubramanyam et al., 2003). However, cells are impermeable for this particular molecule. This hurdle was crossed when novel drug delivery agents in the form of glucose derived carbon nanospheres were discovered which could ferry small molecules into the nucleus (Selvi et al., 2008). These nanoparticles could even cross the blood brain barrier. This discovery opens up new ways of targeting drugs to the neurons. The fact that CTPB is selective to p300 adds to the value and could help as a tool to study p300/CBP specific activation in the neurons and in the background of neurodegeneration. Incidentally, newer HAT activators have been discovered. Nemorosone has been reported to be a p300/ CBP specific HAT activator. Long chain alkylidenemalonates have been reported to activate PCAF specifically, while inhibiting p300/CBP *in vitro*. In this research project we report a new activator of p300 and CBP that is derived from the CTPB scaffold.

1.8. Understanding the enzymatic roles of these transcriptional coactivators in neuronal chromatin:

It is indeed clear that the acetyltransferase p300 regulates key cellular processes, not only by its enzymatic activity but also by participating in the process of transcription as coactivators. 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 further validations such as examining the important promoters for these proteins or

pulldowns, are helpful one efficient way to understand the enzyme function is through an inhibitor based approach. Small molecule modulators (inhibitors/activators), of enzymes have been used very successfully to unravel the mechanistic details of enzyme function.

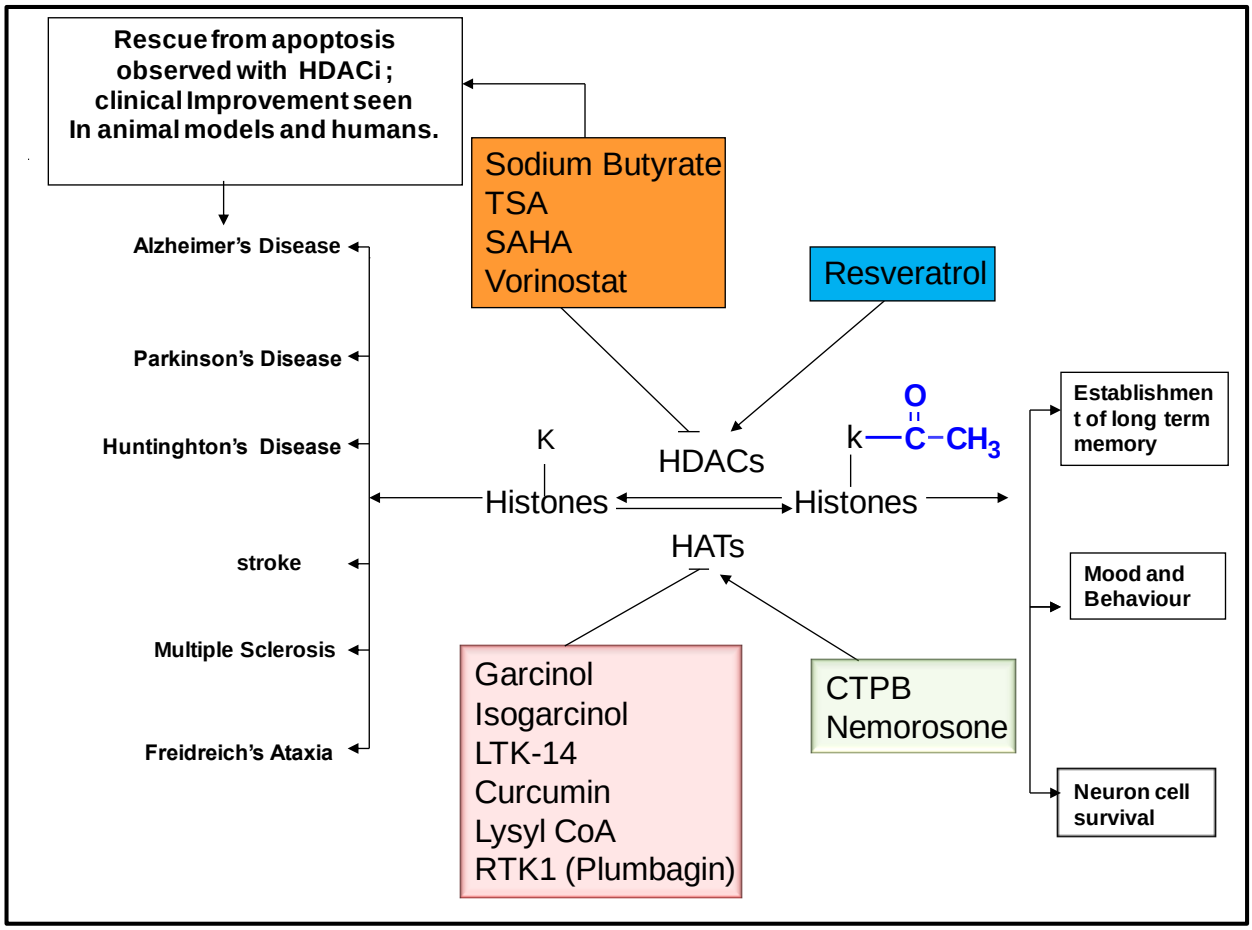


Figure 1.11: Small Molecule Modulators of HATs and HDACs; Implications in Neuronal physiology: Histone acetylation balance is important for neuronal cell survival and neuron physiology. HDAC inhibitors have been known to rescue Neuronal cells from apoptosis in various degenerative states. But the role of HAT inhibitors/activators, in neuronal physiology, is unknown.

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 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. In the context of neurons, though much is known about the role of p300/CBP as has been discussed all through, the role of HAT activity of these enzymes versus their role as co activators and bridging proteins has not been dissected. It is still unclear as to the actual changes in gene expression that would occur with a defective acetylation of histones. Though we know that HDAC inhibitors have been proven efficacious in neurodegenerative settings, it is still not known as to what genes they de-repress to reverse the pathology. Though histone acetylation is important for the neurons, we do not know much about the associated epigenetic marks on histones, though DNA methylation has been addressed to some extent. So, this warrants the use of HAT inhibitors that target the enzymatic activity of p300/CBP and /or other HATs that play a crucial role in neuronal physiology (**Figure 1.11**). This would also serve as an easy alternative and/ or is complementary to gene ablation and silencing studies. Some of these small molecule modulators that activate p300 could be of immense use in the neurodegenerative setting. There is also need for better and efficient activators that has been addressed in this work. These activators could also be the beginning of next generation epigenetic therapeutics as will be discussed later in the future perspectives.

1.9. RESEARCH FOCUS:

The area of chromatin research has advanced tremendously 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 talks is still in debate, since for every modification network, there seems to exist 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). We

decided to investigate the physiological role of histone acetylation and the associated epigenetic marks, in the global perspective of gene regulation by an inhibitor based approach taking the following into consideration.

a. p300/ CBP is an enzyme with multiple cellular functions which are both 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 the use of enzyme specific inhibitors and activators. The cellular physiology of neurons to these HAT inhibitors needed to be studied.
b. Though the role of histone acetylation has been highlighted in most of the reports which have studied neuronal physiology in the chromatin context, there is very less knowledge as to what are the other modifications that would be affected by tampering with acetylation. Indeed, most studies look at specific modifications like H3 and H4 acetylation while actually, in the in-vivo context, a repertoire of modifications constitutes a code for regulation of gene expression. Hence we wanted to understand the epigenetic network associated with histone acetylation in the neurons.
c. Though HAT inhibitors are increasingly being studied for applications in diseases that does not concern with the nervous system like Cancer and Diabetes Mellitus, their toxic effects on the neurons cannot be neglected, considering the excessive sensitivity of neurons for both hypo and hyperacetylation of histones.
d. Even though hypoacetylation of histones have been implicated in neurodegeneration, no efforts have been done in the direction of specifically activating the involved HAT. Since sequestering, degradation or hypoexpression of CBP is involved in most cases, till now there are no drugs that would specifically activate the enzyme in a bid to increase acetylation, through whatever CBP is available for function and thus keep the acetylation specific to CBP downstream genes, thereby reducing toxicity. In this context, it has to be noted that HDAC inhibitors may have been proved to alleviate the progression of neurodegeneration, but due to its non specific nature it also causes neuronal apoptosis. Hence, an effective alternative to overcome the pleotropic effects of indiscriminate activation by HDACs is specific activation of the involved HATs. Hence we have

intitiated search for efficient activators of p300/CBP provided their role in neuroprotective pathways.

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. Protein Expression and Purification

2.3. Histone modifying enzyme assays in vitro

2.4. Histone/Protein modification analysis in cells

2.5. Gene expression studies

2.6. Cell viability assays

2.1. General Methods

2.1.1. Reagent information:

The molecular biology reagents were purchased from either SIGMA, USB or Invitrogen for the different experiments as indicated alongside. Buffers for routine use were prepared from Qualigenes AR grade chemicals. Mammalian cell culture requirements were purchased from SIGMA or Invitrogen. Insect cells requirements were purchased from SIGMA. Tissue culture plasticware used were from NUNC. Primary antibodies used were essentially from Upstate, Abcam, Millipore or Calbiochem, whereas the secondary antibodies were purchased from Bangalore Genei or Invitrogen. Plumbagin (RTK1) and its analogue RTK2 has been synthesized in the laboratory according to previously published protocols (Ravindra et al., 2009). Synthesis of derivatives of CTB like TTK21 and the TTK series has been done in the laboratory (Vedamurthy and Kundu , unpublished).

2.1.2. Poly Acrylamide Gel Electrophoresis (PAGE):

SDS-PAGE: Sodium Do-decyl Sulphate – Polyacrylamide Gel Electrophoresis (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).

2.1.3. Western blot analysis:

The separated proteins on a 12% SDS-PAGE gel were blotted to PVDF/Nitrocellulose membrane using Biorad 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.

2.1.4. Mammalian Cell culture:

SH-SY5Y, C6 and HeLa cells were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (US biological) 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 cryocooler 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.

2.1.5. 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 10 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.

2.1.6. 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. Re-precipitation was done using 3 M sodium acetate, pH 5.2 and equal volume iso-propanol. Pellet was washed with water, allowed to air dry and finally was resuspended in ultra pure water.

2.1.7. 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 1mM 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.

2.1.8. 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.

$$C = A_{260} X\ \theta$$

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.

2.1.9. 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 Ethidium Bromide (10 μg/100 ml H_2O) with gentle rocking. Nucleic acids were visualized on U.V. lamp in gel documentation system (Bio-rad).

2.2. 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.).** Aliquots were flash frozen in liquid nitrogen and stored in -80ºC.

2.2.2. 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). 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.

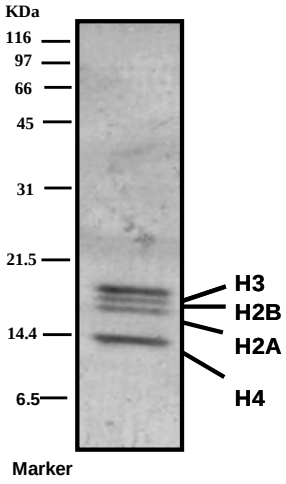


Figure 2.1. The core histone octamer profile on a 15% SDS-PAGE representing all the four histones, H3, H2B, H2A and H4.

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 **c**hecked on 8% SDS PAGE **(Figure 2.2.A).** The activity of the protein was verified by filterbinding assay and fluorography gel assay (**Figure 2.2.B**)

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

Sf21 insect cells (5 million cells per 150 mm plate) were infected with the recombinant CBP 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

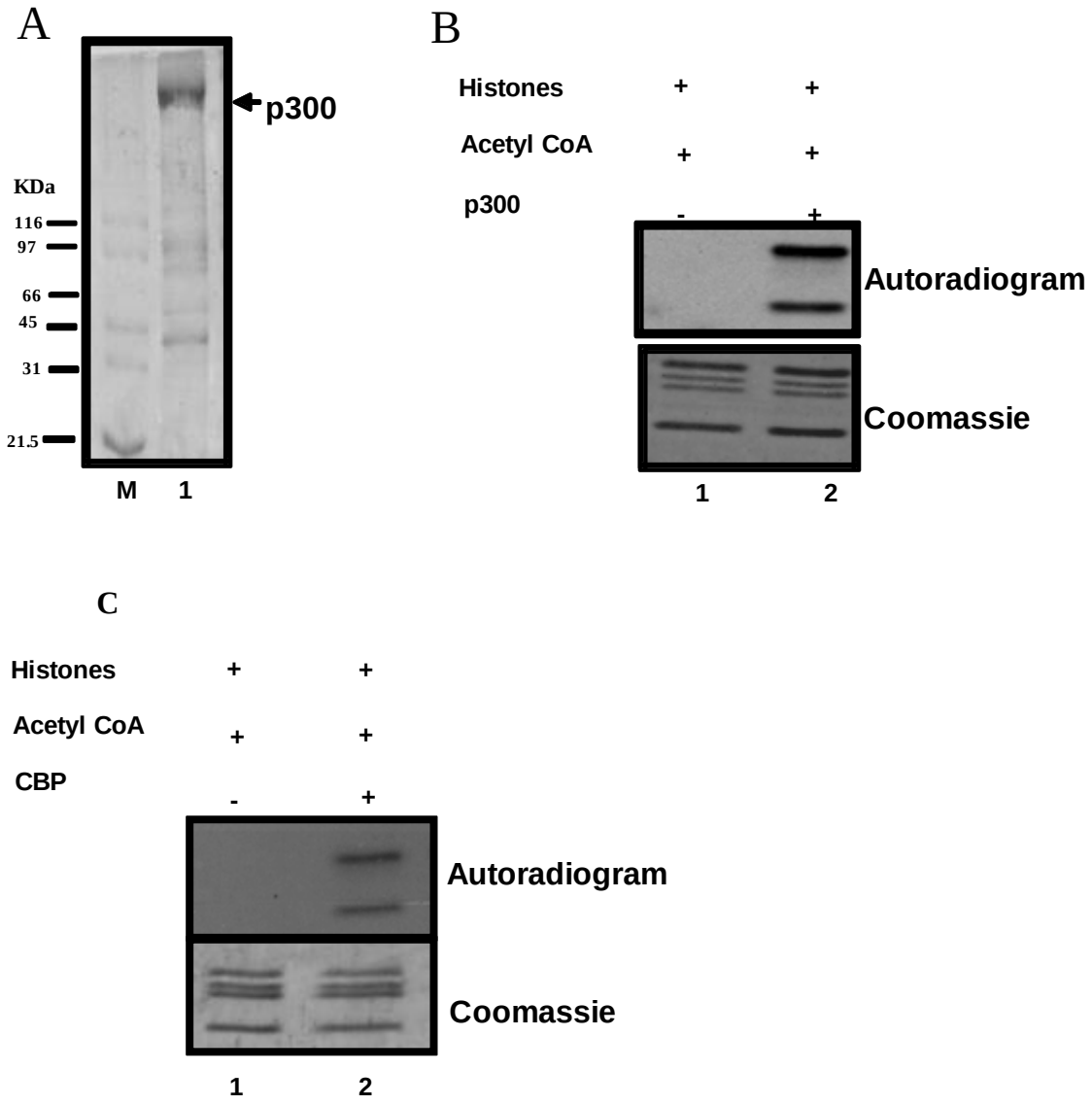


Figure 2.2. Recombinant histone acetyltransferases. Protein profile of **(A)** full length p300, **(B)** The activity of the p300 enzyme was verified by the gel fluorography assay as represented in lane 1 versus lane 2. Lane 1, histones without enzyme: lane 2, histones with enzyme. The upper panel represents the autoradiogram profile whereas the lower panel is the coomassie stained gel.**(c)** Activity of purified Flag-CBP

PMSF. The supernatant was kept for binding with M2-agarose beads (SIGMA) followed by washes with buffer consisting of 20 mM Tris, pH 7.4, and 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 .The activity of the protein was verified by filterbinding assay and fluorography gel assay (**Figure 2.2.C)**

2.3. Histone modifying enzyme assays in vitro:

2.3.1. 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 CBP 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. For gel fluorography assays, the histones were TCA precipated using 25% TCA. Precipitates were washed twice with acetone, dissolved in 2x SDS loading dye,heated for 5 minutes and separated using 15% SDS-PAGE. The gel was stained by coomassie to ascertain the presence of histones in equal amounts in each of the reaction and was later dehydrated in DMSO for 1hour. Later they were incubated in scintillation fluid (PPO solution in DMSO) for 45 minutes and rehydrated again in distilled water for 4 hours. The gel was later dried using a gel drier and exposed in an X-ray cassette using a film for 5 days in -80 degree cooler. The film would be developed later to get intensity profiles for each of the reaction.

2.4. Histone/Protein modification analysis in cells and tissues:

2.4.1. **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 minutess at room temperature. Cells were then permeabilized using 1% Triton X-100 (in PBS) for 10 minutes and subsequently washed with PBS for 10 mins, 3 times. Nonspecific sites were blocked using 5% FBS (in PBS) for 45 minutes 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.

2.4.2. 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.

2.4.3. 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.

2.4.4. Whole cell extract using Laemmli Buffer:

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 Laemmli buffer (SDS 4%, Glycerol 20%, Tris-HCl 0.125 at pH 6.8). Samples were boiled for 5 minutes at 90 degree centigrade and allowed to cool till they attained room temperature. They were then subjected to brief sonication. 5X SDS loading dye was added to samples and boiled for 5 more minutes. These lysates were later used for western blotting.

2.4.5 Immunohistochemistry:

All sample tissues were collected in three separate vials (a).for IHC (fixed in 10% formalin) ; (b). for protein isolation (in protease inhibitor cocktails; Roche) ; (c). for RNA isolation (in RNA latter solution; Ambion). Tissues samples except for IHC were quick frozen in liquid nitrogen and stored at -80°C. Patient samples to be used in IHC were fixed in 10% formalin for 1–2 hr and transferred to 70% ethanol overnight followed by paraffin embedding. Paraffin blocks of fixed oral tissues were sectioned at 5 µm. The sections were mounted on silane coated glass slides. The deparaffinization was done by keeping the slides at 42°C hot.

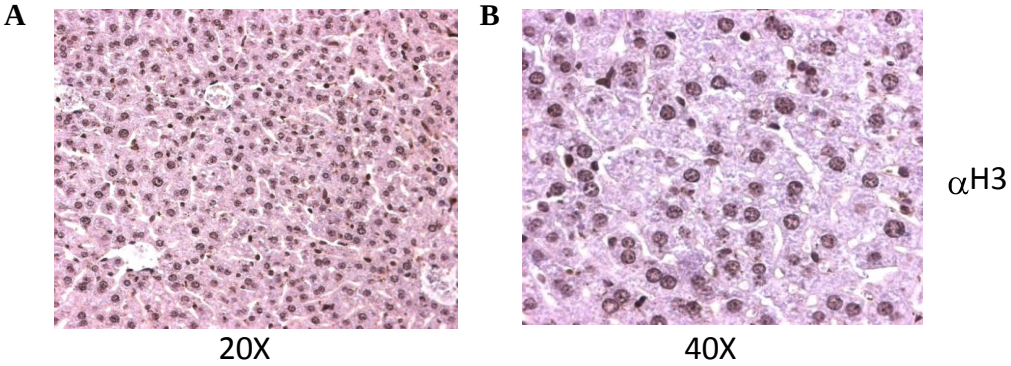


Figure 2.3: Immunohistochemical analysis of mouse liver using anti H3 antibody. Prescence of brown coloured precipitate indicates the localization of protein against which antibody has been used. Prescence of histone H3 in nucleus can be made out by the nuclear localization of brown colour.

platform (overnight), hydrated and heated by microwave for 5 min at 93–98 °C in citric buffer (10mM, pH 6.0).The slide were further incubated in same buffer for 20 min at room temperature (RT). The slides were washed with PBS (3 min, two times). Blocking was done at RT with 5% fat free milk. Slides were again washed with PBS (3 min, four times). The sections were then incubated overnight at RT with different antibodies as mentioned in the text (1:30 dilution). Then, the Envision kit (Dako, Denmark) was used according to the manufacturer's recommendations. Sections were counterstained with Mayer's hematoxylin, mounted in DPX and air dried. Prescence of brown coloured precipitate indicates the localization of protein against which antibody has been used. Prescence of histone H3 in nucleus can be made out by the nuclear localization of brown colour (**Figure 2.3**)

2.5. **Gene expression studies:**

2.5.1. Endogenous gene expression assay

HeLa and SH-Sy5Y cells were treated with RTK1 to induce histone hypoacetylation . 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 SyBR green supermix (BioRad) and gene-specific primers of *Actin, PUMA, NOXA, BDNF,*and *Bcl2*.

Sequences of the primers used are as follows:

Actin: FP, 5'-GTGGGGCGCCCCAGGCACCA-3',
RP, 5'-CTCCTTAATGTCACGCACGATTTC-3'.

PUMA: FP, 5′-GACCTCAACGCACAGTA-3′,
RP, 5′-CTAATTGGGCTCCATCT-3′;

Noxa: FP, 5′-ACTGTTCGTGTTCAGCTC-3′,
RP, 5′-GTAGCACACTCGACTTCC-3′.

p300: FP, 5'-ATGGCCGAGAATGTGGTG-3',
RP, 5'-ATTATCCCTTGTCCATTGCC-3'

PCAF: FP, 5'- GACCTGCAGCAAATAATTC-3',
RP, 5'- GTAAAATAGACTTTCTCAAG-3'.

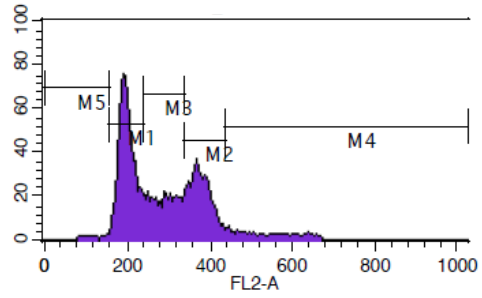
BDNF: FP, 5'-AAACATCCGAGGACAAGGTG-3',
RP, 5'- AGAAGAGGAGGCTCCAAAGG-3'.

2.6. Cell Viability assays:

2.6.1. 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 for 30 mins to ensure only DNA staining. 50 µg/ml Propidium Iodide was added for staining at 37ºC for 30 mins. Cells were sorted and analyzed by flow cytometry for the cell cycle distribution using inbuilt software of BD FACS caliber instrument. Analysis was done in FL2 channel.

A



B

	% Gated	Stage of cell cycle
All	100	All inclusive
M1	43.91	G1 phase
M2	27.5	G2 phase
M3	24.2	S phase
M4	4.49	Ploidy levels greater than 2n
M5	0.74	Sub-G1, apoptotic population

Figure 2.4: FACS analysis of PI stained HeLa cells. A. The cells were gated in M1, M2, M3 and M4 and M5 region in the FL-2 channel. **B.** Tabular representation of percentage of cells gated in relation to their cell cycle stages.

2.6.2. 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.

2.6.3. DNA Fragmentation Assay:

RTK1-induced apoptosis was monitored by the extent of chromatin fragmentation. DNA was extracted from the untreated and RTK1 treated HeLa/ SH-SY5Y cells. The cells ($3x10^6$ per 90-mm dish) were seeded and treated with the compound for 24 h. Harvested cells were washed with PBS and then lysed with lysis buffer containing 0.5% Triton X-100, 20 mM Tris, and 40 mM EDTA at room temperature for 15 min. The lysate was treated with RNase (0.1 mg/ml) and proteinase K (2 mg/ml) for 1 h, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and DNA was precipitated by incubating the upper aqueous phase with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 1 volume of isopropyl alcohol overnight at -20 °C. The pellet obtained on centrifugation was washed with 70% ethanol and dissolved after air drying in 50 µl of TE buffer. The extracted DNA was analyzed on a 1.8% agarose gel and visualized by ethidium bromide staining.

Chapter 3

Plumbagin alters epigenetic landscape and induces apoptosis in neuronal cells.

This chapter discusses the role of p300 acetyltransferase inhibition by RTK1 (Plumbagin), isolated from the roots of a medicinally important plant Plumbago rosea, in neuronal cell survival. It also discusses HAT inhibition mediated alterations in the epigenetic state of neuronal and non neuronal cells. Alterations in gene expression that follow these epigenetic changes has been investigated. The experimental evidences that signify the importance of histone acetylation balance in neuronal cell survival and apoptosis is presented in this chapter through a small molecule inhibitor based approach.

Chapter outline:

3.1. p300/KAT3B acetyltransferase

3.2. RTK1 is a potent anti cancer agent.

3.3. RTK1 inhibits p300 HAT enzyme in vitro.

3.4. RTK1 inhibits histone acetylation in neuroblastoma cells.

3.5. RTK1 alters epigenetic network; inhibits transcriptional activation mark.

3.6. RTK1 induces apoptosis of neuroblastoma cells.

3.7. RTK1 induces expression of both apoptotic and neuroprotective genes

3.8. RTK1 mediated perturbation of epigenetic network is true even in non neuronal cells and animal models.

3.1. 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. Their role in transcription and other physiological functions is a combination of various biological activities such as the acetyltransferase function, the co-activation property and 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 and DNA repair (Goodman and Smolik, 2000, Chan and La Thangue, 2001), differentiation (Zhong and Jin, 2009) etc. Though, the exact structural information of this protein is not available, an interdisciplinary approach utilizing the Surface enhanced Raman spectroscopy (Arif et al., 2007) 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 and 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. 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 (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. 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 HAT inhibitors especially from the natural sources so that an active scaffold could then be modified for better and efficient inhibition. Considering the role of histone acetylation to be so important, in cell survival, it has to be seen how these HAT inhibitors will affect systems in vivo. Each tissue is different in terms of physiology and histone acetylation would be tuned to its needs. Thus, HAT inhibition in different tissues could result in different physiological outcomes. These are the kinds of studies that are the need of the hour. Hence, one aspect of our work is to discover and establish newer HAT inhibitors and to look at their in vivo effects.

3.2. RTK1 is a potent anticancer agent:

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. Incidentally plumbagin inhibits many cancer cell growth. Potential role of plumbagin, as an anti-cancer agent has been recognized (Parimala and Sachdanandam, 1993; Naresh et al., 1996; Sugie et al., 1998; Hazra et al., 2002) and its anti-cancer effects have been reported in diverse cancer models such as prostate (Powolny and Singh, 2008), lung (Hsu et al., 2006; Gomathinayagam et al., 2008), cervical (Srinivas et al., 2004; Nair et al., 2008), ovarian (Srinivas et al.,2004), Breast (Amir Ahmad et al , 2008). Also, majority of cancer cases report hypoacetylation citing loss in p300 activity (Muraoka et al., 1996) 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 had earlier tested the root extract of *Plumbago rosea* for its effect on histone acetylation. Surprisingly, plumbagin turned out to be a potent HAT inhibitor.

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

RTK1 (plumbagin) was used in histone acetyltransferase assays to check its HAT inhibition, As observed in **Figure 3.1,** a drop in acetylation levels is clearly observed at 25μM concentration of plumbagin. Though the decrease in observed in bothp300 and PCAF enzymes, the inhibition is better for p300 compared to PCAF (**Figure 3.1, lane 5**). RTK1 was also tested in cell culture models. RTK1 could potently inhibit histone acetylation in HepG2 cell line. The same inhibition was also observed in HeLa cell line. However, in the cells it was not clear whether the inhibition is because of inhibition of PCAF or p300.However through an ingenious and established assay we could dissect the preferential inhibition of RTK1 of p300 but not PCAF by RTK1. DNA damage results in

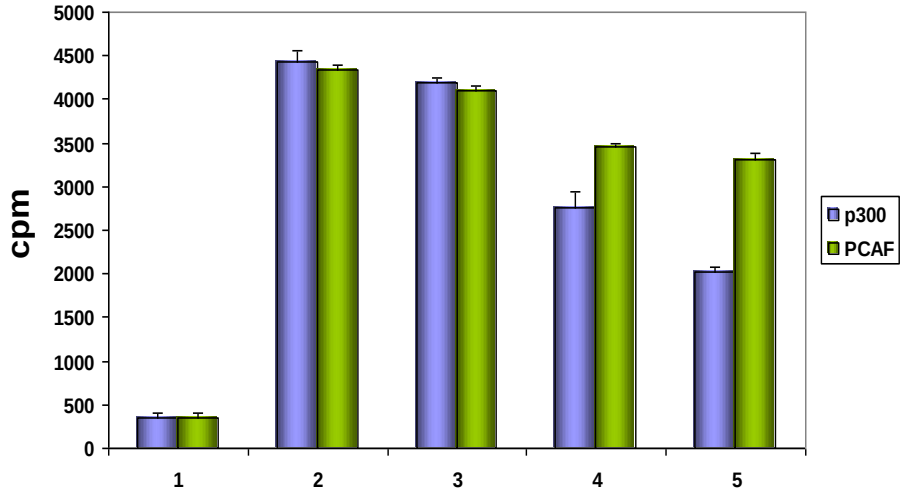


Figure 3.1. Effect of plumbagin/RTK1 on histone acetyltransferases: Filter binding assay for inhibition of histone modification: HAT assays were performed either with p300 (blue bar) or PCAF (green bar) in the presence or absence of RTK1 by using highly purified human (HeLa) core histones and processed for filterbinding. 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 assays.

p53 getting acetylated at various lysines, mediated by both p300 and PCAF, which is important for different functional outcomes. Doxorubicin, an anthracycline and DNA intercalating agent, when treated to cells, increases p53 accumulation and p53 dependent transcription. p300 and PCAF acetylate different lysine (K) residues, (K373and K320 respectively) of p53 upon DNA damage. 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. HEK293 cells were 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 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 (Ravindra KC et al., 2009).The concentration of RTK1 added did not affect the acetylation of p53 at lysine 320 (by PCAF). This suggested that there was preferential inhibition of p300 in the cells and not PCAF in the assay, within the range of concentration tested. It has to be noted that protein levels of p300 did not change after addition of RTK1 to cells. It was also elucidated that RTK1 is a non competitive

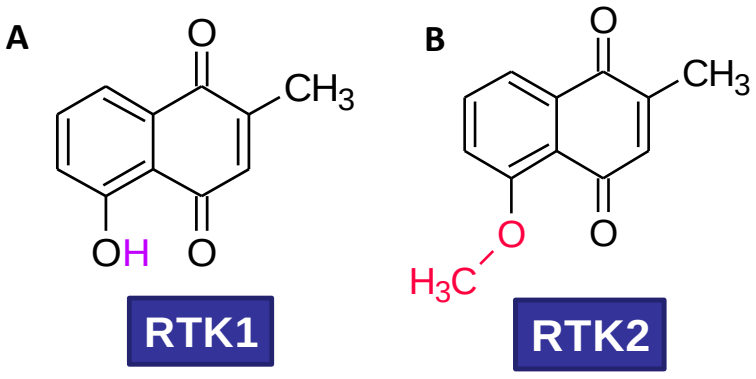


Figure 3.2: Structure of RTK1 with single hydroxyl group at the 10th position and methoxy- substitution at the same position in RTK2

inhibitor of p300 and a single hydroxyl group within the molecule was very important to preserve its HAT inhibitory activity. When the hydroxyl group was changed to other functional groups there was a complete loss of HAT inhibitory activity (**Figure 3.2**). This hydroxyl group also was responsible for interaction of RTK1 with the HAT domain of p300 and removal of the group, as observed in the structure of RTK2, resulted in the molecule losing its HAT inhibitor activity (Ravindra et al., 2009). The presence of a hydrogen bond between this single hydroxyl group and a particularly important Lys 1358 of HAT domain of p300 as observed by docking studies suggested that this binding is important for HAT inhibitory activity. The mutation of lysine 1358 to alanine completely abrogated HAT activity. Hence, this site specific binding was possible because of the presence of a hydroxyl- group and the absence of the same lead to loss of inhibitory activity. With this information in our consideration we set out to understand the effect of RTK1 on neuronal cell acetylation and its functional consequences.

3.4.1 RTK1 inhibits histone acetylation in neuroblastoma cells:

Our goal has been to elucidate the effects of HAT inhibitor in neuronal cells. SH-SY5Y cells were grown as mentioned in materials and methods section. The cell line SH-SY5Y is a third generation Neuroblastoma cell line, cloned from SH-SY5. This cell line has been used as a model for human neuronal cells. SH-SY5Y cells are known to be dopamine beta hydroxylase active, acetylcholinergic, glutamatergic and adenosinergic. SHSY5Y cells are well established in vitro models for studying neuronal cell physiology especially neurotransmitter, neuronal differentiation and neuropharmacologic studies. RTK1 was treated at two concentrations, 10μM and 20μM to SH-SY5Y cells. Treatment of plumbagin at 20μM induces drastic changes in cell shape. This morphological change could be due to the fact that plumbagin is known to inhibit microtubule polymerization (Acharya et al., 2008). This has been investigated in many cell types like A549 which are lung cancer cells. Since microtubule dynamics is very important in maintenance of cell shape, motility and intracellular transport, the same could be true even in neuronal cells like SH-SY5Y (**Figure 3.3 A**). Also, Western blotting was done using specific Acetylated H3 (H3K14 acetylation) antibody to ascertain HAT inhibition. This site on

histone H3 is acetylated by p300. The same blot was stripped of antibody binding and re-probing was done with H3 antibody to confirm equal loading of histones. It was seen that RTK1 inhibited acetylation at 20μM concentration (**Figure 3.3 B**). In Figure 3.3 B, when compared to lane 1 in which cells were treated with DMSO solvent, lane 2 and 3 show decreased intensity as a result of decreased acetylation. In Lane 4, histones of cells

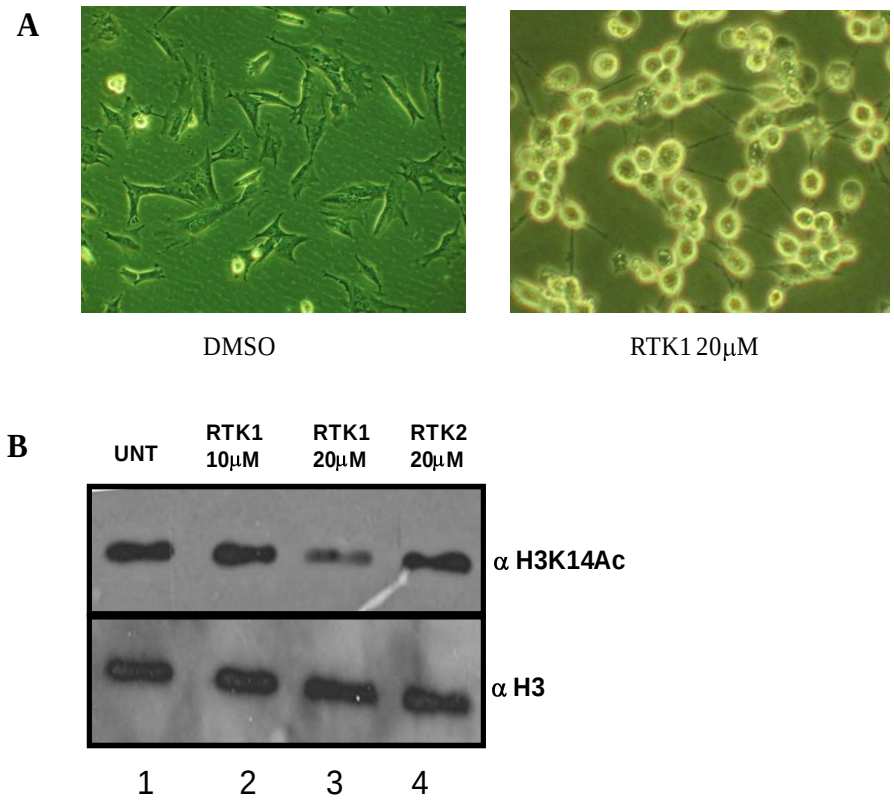


Figure 3.3: RTK1 alters cell morphology and inhibits p300 mediated histone acetylation in SH-SY5Ycells; A. Representative image of SH-SY5Y cells treated with DMSO or RTK1 20μM; **B.** Western blot analysis of RTK1 untreated and treated cells. Lane 1- Untreated cells with DMSO solvent added to culture, Lane 2 -10μM RTK1 treatment for 6 hours, Lane 3 – 20μM RTK1 treatment for 6 hours, and Lane 4- 20μM RTK2 treatment for 6 hours. Immunoblotting was done using Anti acetylated H3 K14 antibody. As loading control re-probing of the same blot was done by anti H3 antibody.

treated with RTK2 was loaded and acted as negative control. As expected from our filter binding assays, RTK1 inhibited histone acetylation in neuronal cells in a dose dependent

manner, its effect being maximum at 20μM. RTK2, a derivative of RTK1, does not inhibit p300 in vitro. The same is reflected when RTK2 was treated to cells. No change was seen in histone acetylation levels upon RTK2 treatment. Hence RTK1 inhibited histone acetylation.

3.4.2. RTK1 inhibits p300 autoacetylation in neuroblastoma cells:

Within the cell, the activity of p300 is controlled by various mechanisms. Interacting partners, posttranslational modifications etc, modify the histone acetyltransferase activity of p300/CBP. One such vital posttranslational modification is autoacetylation of p300 (Thompson et al., 2004). In the normal state there is a stretch of lysine rich, inhibitory loop of amino acids that cover the acetyl-coA binding site in the HAT domain of p300 (**Figure 3.4A**).Under appropriate conditions; there is acetylation of this loop. This modification will pave way for a more active p300. This phenomenon of p300 autoacetylation has been observed in cell free systems and is expected to regulate the activity of p300. Hence gauging the extent of autoacetylation is one way of estimating the activity status of p300 in both in vitro and in vivo. As reported earlier, RTK1 could inhibit p300 mediated acetylation in different cell lines. We found a similar pattern of inhibition in neuronal cell line. To confirm whether this inhibition is also an effect of RTK1 on p300 activity, we tested the autoacetylation status of p300 upon RTK1 treatment in cells. We hypothesized that it may affect autoacetylation levels of p300. In order to prove that RTK1 could affect the autoacetylation of p300, it was treated at two concentrations, 10μM and 20μM to HeLa cells. Western blotting was done using specific Acetylated p300 (K1499 acetylation) antibody. The same nitrocellulose blot was stripped of antibody binding and re-probing was done with tubulin antibody to ascertain loading of cell lysates (**Figure 3.4, B**). In Figure 3.4B, when compared to lane 1 in which cells were treated with DMSO solvent, lane 3 shows decreased intensity as a result of decreased acetylation. In Lane 4, lysate of cells treated with RTK2 was loaded and acted as negative control. It was seen that RTK1 inhibited autoacetylation in a dose dependent manner, which was not observed when RTK2 was treated to cells. The panel adjacent to the western blot data quantifies the inhibition observed in the immunoblot It is observed

that there is nearly 60 percent inhibition in the autoacetylated status of p300 as observed by the drop in intensity to 40% of the DMSO treated lane. Thus the decrease in histone

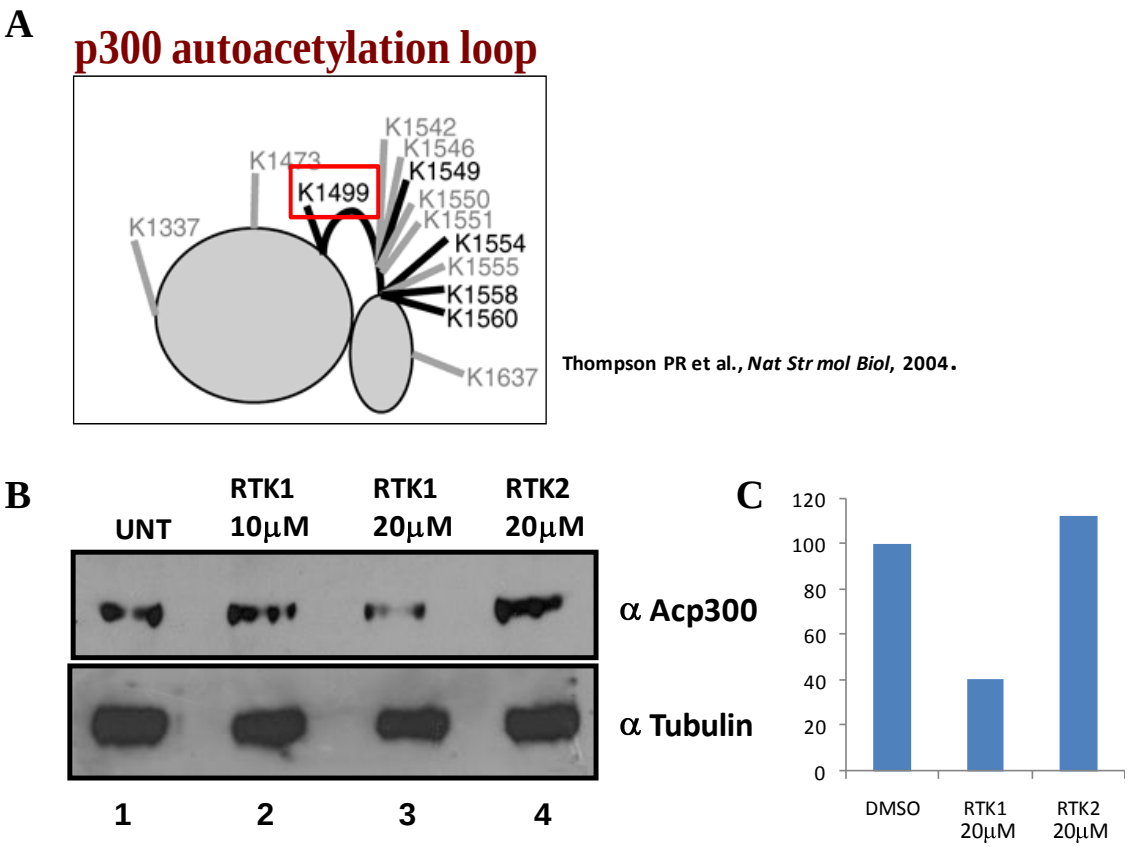


Figure 3.4: A. Cartoon depicting lysine rich inhibitory loop of p300 that gets autoacetylated. The last and the most critical lysine in the series is lysine 1499. Measure of this acetylated lysine of p300 is a measure of its activity (Adapted from Thompson et al., 2004). **B.** RTK1 inhibits p300 autoacetylation in HeLa cells in a dose dependent manner. Immunoblot analysis of cell lysates treated with solvent DMSO (lane 1), 10 μM RTK1 (lane 2), 20 μM RTK1 (lane 3) and 20 μM RTK2 (lane 4); Probed with antibody against acetylated p300. As loading control re-probing of the same blot was done by tubulin antibody; C. Quantification of western blot showing the percentage of inhibition.

acetylation observed in cells after treatment of RTK1 is due to inhibition of p300 enzyme, which is also evident from decrease in autoacetylation status of p300.

3.5.1.1. RTK1 alters epigenetic network; inhibits transcriptional activation mark.

It has been established that there is a cross talk between histone modifications in the cells that influences transcription and thus gene expression. Acetylation of histone is an

important mark that is generally associated with transcriptional activation. However, it is the presence of combinatorial modification of histone on gene promoters that finally decides the transcriptional state of the gene. Among these modifications, methylation and phosphorylation of histones play a very important role. More specifically the presence of acetylation on N-terminal histone H3 (H3K9, 14, 18 etc) and H4 tail (H4K5, 8, 12, 16)

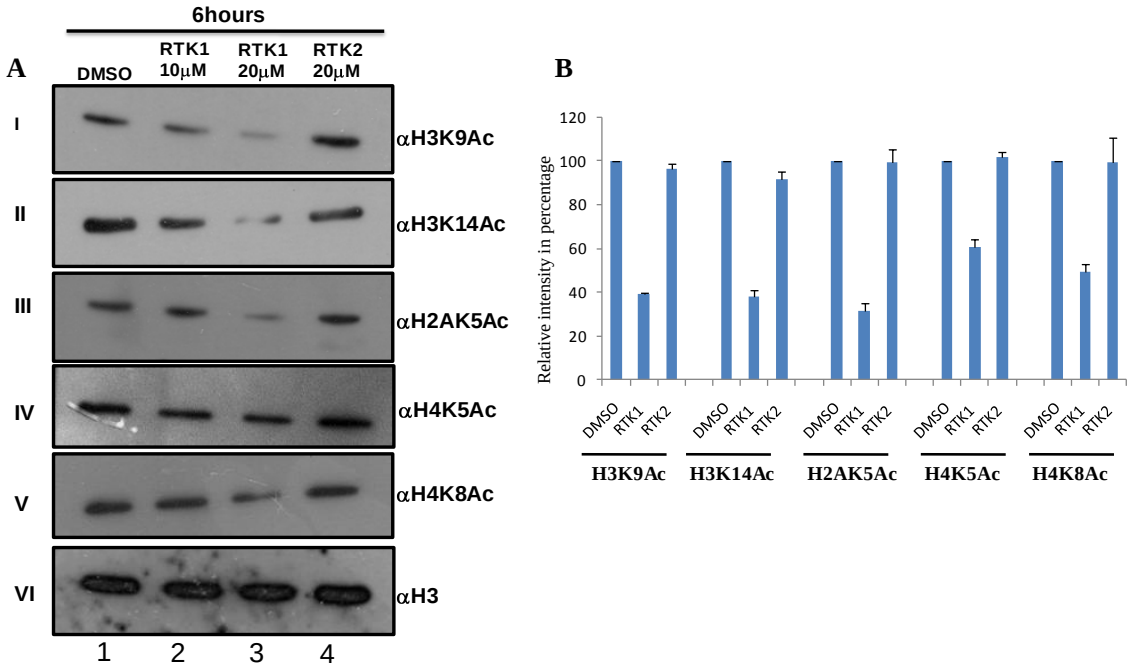


Figure 3.5: RTK1 inhibits histone acetylation marks responsible for transcriptional activation in SH-SY5Ycells; A. Western blot analysis of solvent treated and RTK1and treated cells. Lane 1- cells treated with DMSO solvent, Lane 2-10μM RTK1 treatment for 6 hours, Lane 3 – 20μM RTK1reatment for 6 hours, Lane 4- 20μM RTK2 treatment for 6 hours. Probing was done using anti acetylated H3K9 antibody (panel I), anti acetylated H3K14 antibody (panel II), anti acetylated H2AK5 antibody (panel III), anti acetylated H4K5 antibody (panel IV), anti acetylated H4K8 antibody (panel V). As loading control, re-probing of the same blot was done by anti H3 antibody (panel VI); **B.** The experiments were performed in duplicates, the levels of histone acetylation marks obtained by western blotting were quantified using ImageJ software and graph was plotted for relative intensity (in percentage) considering the intensity of DMSO treated lanes as 100%. Only the solvent treated, 20μM RTK1 and 20μM RTK2 lanes are quantified.

are transcriptional activation marks. But these modifications do not act alone and there are intricate cross- talks between H3K4 tri-methylation and H3S10 phosphorylation. In

order to investigate the epigenetic changes associated with HAT inhibition we analysed the changes using immunoblotting using various antibodies against the above mentioned activation marks after treatment with RTK1. RTK1 was treated at two concentrations, 10μM and 20μM to SH-SY5Y cells with RTK2 20μM as negative control. Western blotting was done using specific antibodies to histone modifications like acetylated H3 (H3K9, H3K14, H2AK5, H4K5 and H4K8 acetylation). Most of these sites are acetylated by p300. H2AK5 is reported to be a p300 specific site. The same nitrocellulose blot was stripped of antibody binding and re-probing was done with H3 antibody to ascertain equal loading of histones. It was seen that RTK1 could inhibit acetylation at all these sites in a dose dependent manner. As depicted in **Figure 3.5 A**, we observe that acetylation at H3K9 (panel I), H3K14 (panel II), H2AK5 (panel III), H4K5 (panel IV), H4K8 (panel V) decreases at the same concentration of RTK1 suggesting that all the events are because of the inhibitor treatment and not other indirect events. Also, RTK2 that cannot inhibit p300 acetyltransferase does not cause any of these epigenetic changes similar to RTK1 treatment. After treatment with RTK1 or RTK2 at 20μM it

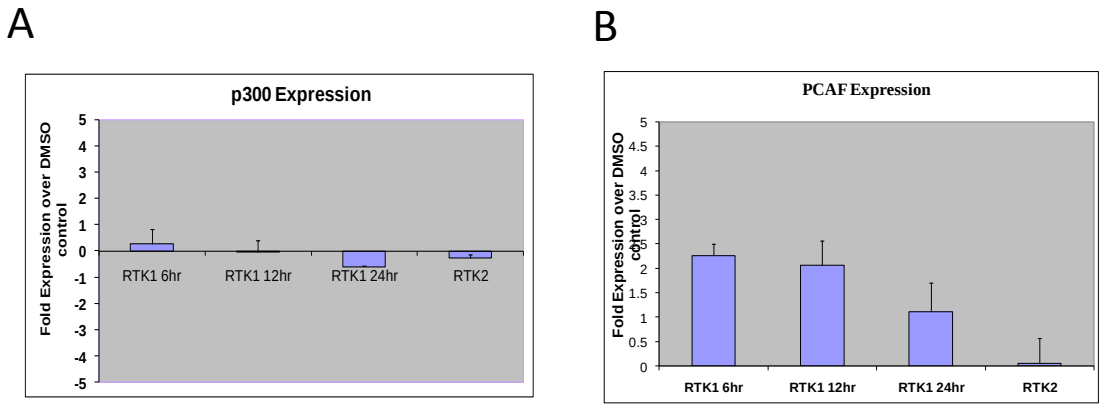


Figure 3.6: RTK1 treatment does not alter p300 expression: Cells were treated with DMSO or 20μM plumbagin for 6, 12, and 24 h. Cells were harvested total RNA was extracted, and reverse transcription was performed as described in the methods section. RT-PCR was performed as described elsewhere using gene specific primers for p300 (**A**) or PCAF (**B**) Results are mean fold expression change over DMSO treatment.

was found that RTK1 inhibits all the tested acetylation marks to nearly 60% as indicated by the drop in intensity to 40% of DMSO treated lane (**Figure 3.5, B**). Thus RTK1

inhibits major transcriptional activation marks, on all histones analyzed, on a global scale, which are meant for transcriptional activation. We speculated that this reduction in histone acetylation upon inhibitor treatment could also be a result of a downregulation on the acetyltransferase expression itself. Hence, either p300 or PCAF, which acetylate the above mentioned sites could be reduced due to their reduced expression and thus acetylation could come down. To ascertain whether major cellular HATs like p300 or PCAF show any change in expression patterns pos t treatment we performed a real time PCR assay. After treatment with RTK1 at 20μM or solvent DMSO (control) for various time points like 6, 12 and 24 hours we harvested cells, isolated RNA and made cDNA using the RNA. Using primers specific for p300 (**Figure 3.6A**) and PCAF (**Figure 3.6B**) we performed SyBrGreen based RT-PCR assay. We plotted graphs as fold expression change over solvent DMSO treatment. We did not see any change in expression patterns of p300 compared to the solvent treated cells. Very interestingly there was a significant two fold up regulation of PCAF expression at 6 hours. The same trend continued till 12 hours. However, by 24 hours the expression decreased though it was still 1.5 fold more than DMSO treated cells. However, it has to be noted that RTK2 did not cause any change in the expression of either of the enzymes at 24 hours of treatment. This suggests that, the decrease in acetylation in histones after treatment of RTK1 is because of inhibition of enzyme activity and not because of hypoexpression of the enzymes.

3.5.1.2. Effect of RTK1 on the epigenetic network associated with transcriptional activation:

Since acetylation of histones is not the sole modification for transcriptional activation and is always associated with H3K4 tri-methylation and H3S10 phosphorylation (Berger., 2007), we decided to investigate the effect of RTK1 treatment on the latter two. RTK1 was treated at two concentrations, 10 and 20μM to SH-SY5Y cells for 6 hours (**Figure 3.7 A**). Western blotting was done using specific antibodies against anti phosphorylated H3S10 antibody (panel I), anti tri-methyl H3K4 antibody (panel II), anti di-methyl H3R17 antibody (panel III).As loading control, re-probing of the same blot was done by anti H3 antibody (panel IV). We observe that phosphorylation of H3S10

(panel I) and H3K4 trimethylation (panel II), both decrease drastically after RTK1 treatment , which is also indicated in the graph quantifying the inhibition (**Figure 3.7 B**), whereas no such effect was observed when RTK2 was treated at the same 20μM concentration. However, H3R17 dimethylation, which is also a transcriptional activation mark, known to have a cross talk with CBP mediated histone acetylation at H3K18 (Daujat S et al., 2002), remains unaltered. This was surprising, since being a transcriptional activation mark, it remained unaltered. It could be possible that observing global changes does not reflect sensitive rearrangements. It could also be possible that H3R17 dimethylation is related to a distinct but minor set of genes, regulated by discrete signals. A clue, to be

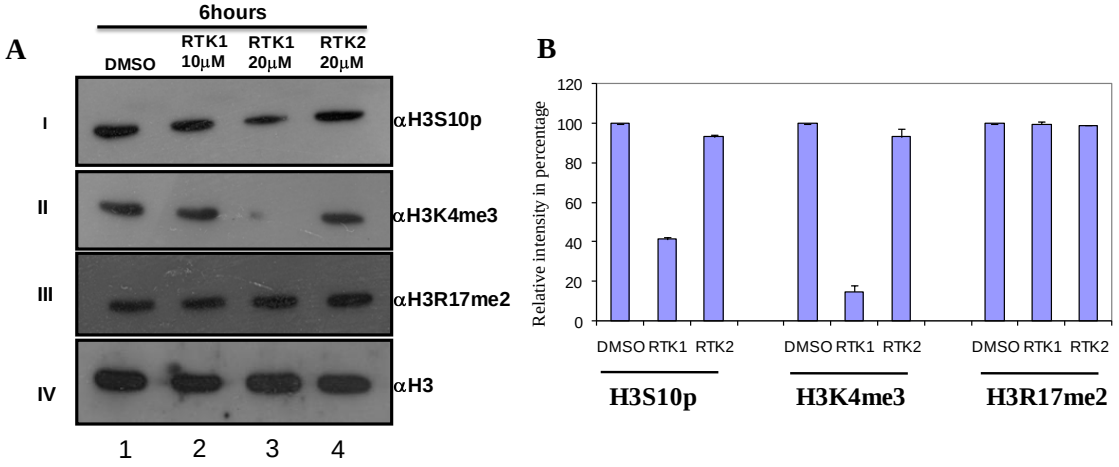


Figure 3.7: RTK1 inhibits transcriptional activation marks associated with histone acetylation in SH-SY5Ycells. A. Immunoblot analysis of solvent treated and RTK1 treated cells. Lane 1, Untreated cells with just DMSO solvent; Lane 2, 10μM RTK1 treatment for 6 hours; Lane 3, 20μM RTK1reatment for 6 hours; Lane 4, 20μM RTK2 treatment for 6 hours. Probing was done using anti phosphorylated H3S10 antibody (panel I), anti tri-methyl H3K4 antibody (panel II), anti di-methyl H3R17 antibody (panel III).As loading control, re-probing of the same blot was done by anti H3 antibody (panel IV); **B.** The experiments were performed in duplicates, the intensities obtained by western blotting were quantified using ImageJ software and graph was plotted for relative intensity (in percentage) considering the intensity of DMSO treated lanes as 100%.

noted here is that CARM1 methylates H3R17, and is responsible for hormone regulated gene expression. This observation however needs to be investigated. The results seemed

to suggest that most of the histone epigenetic marks meant for transcriptional activation, decrease on a global scale after HAT inhibitor treatment. However this would be clear

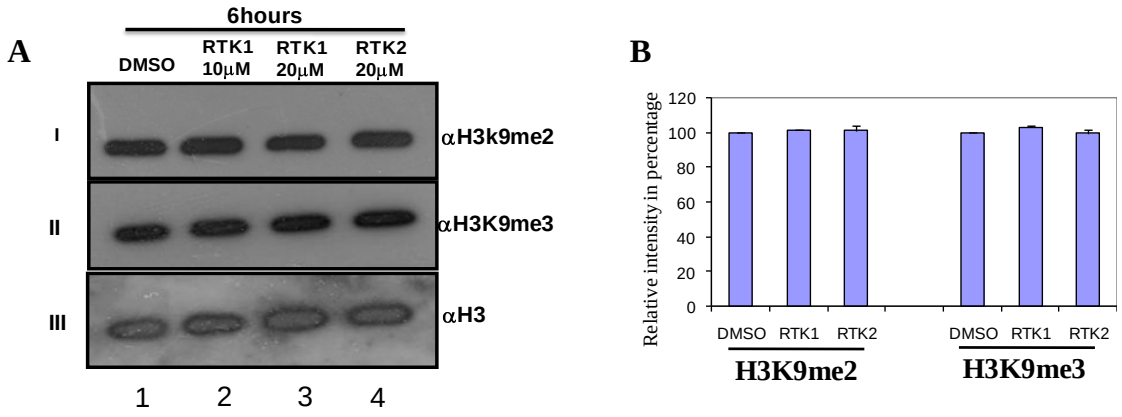


Figure 3.8: RTK1 does not alter marks associated with gene repression in SH-SY5Ycells; A. Western blot analysis of RTK1 untreated and treated cells. Lane 1,Untreated cells with just DMSO solvent; Lane 2, 10μM RTK1 treatment for 6 hours; Lane 3, 20μM RTK1reatment for 6 hours; Lane 4, 20μM RTK2 treatment for 6 hours. Probing was done using anti dimethylated H3K9 antibody (panel I) and anti tri-methyl H3K9 antibody (panel II).As loading control, re-probing of the same blot was done by anti H3 antibody (panel III). **B.** The experiments were performed in duplicates, the levels of histone methyation obtained by western blotting were quantified using ImageJ software and graph was plotted for relative intensity (in percentage) considering the intensity of DMSO treated lanes as 100%.

only if effect on repressive marks on histones can be analyzed. H3K9 di-methylation at promoter proximal histones is indicative of a repressed gene. H3K9 trimethylation is representative of permanently silenced chromatin that occurs in the pericentric heterochromatin (Snowden et al., 2002; Stewart et al., 2002; Rice et al., 2003). We checked whether these two repressive marks would change with treatment of HAT inhibitor RTK1. Hence, we performed western blot for histones isolated from SHSY-5Y cells treated with RTK1 at 10uM and 20uM to SH-SY5Y cells for 6 hours (**Figure 3.8 A**). Western blotting was done using specific antibodies against anti dimethyl H3K9 antibody (panel I) and anti tri-methyl H3K9 antibody (panel II). As loading control, re-probing of the same blot was done by anti H3 antibody (panel III). We

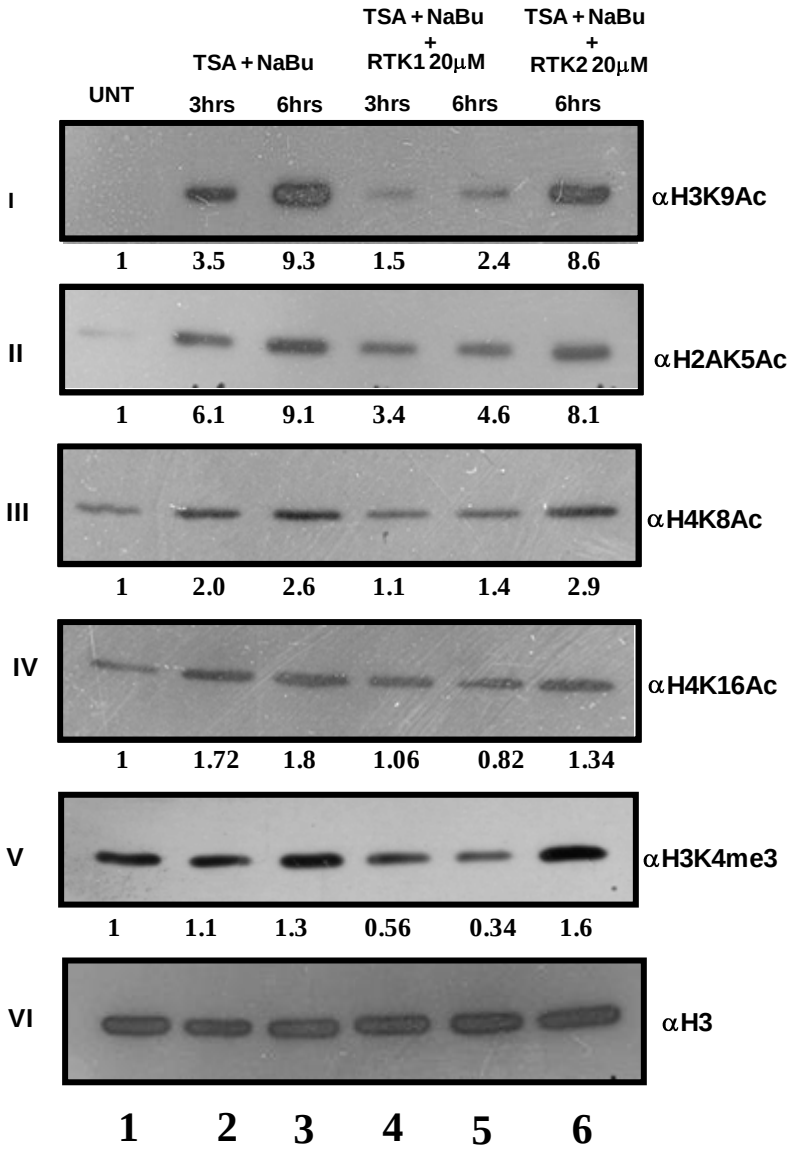


Figure 3.9: RTK1 inhibits HDAC inhibitor treatment induced hyperacetylation in SH-SY5Ycells.Western blot analysis of RTK1 untreated and treated cells in the background of sodium butyrate (NaBu,1mM) and trichostatin (TSA,1μM) treatment . Lane 1, Untreated cells with just DMSO solvent; Lane 2, NaBu and TSA treatment for 3 hours; Lane 3, NaBu and TSA treatment for 6 hours; Lane 4, 20μM RTK1 treatment for 3 hours in presence of NaBu and TSA; Lane 5, 20μM RTK1 treatment for 6 hours in presence of NaBu and TSA; Lane 6, 20μM RTK2 treatment for 6 hours in presence of NaBu and TSA. Probing was done using anti acetylated H3K9 antibody (panel I) and anti acetylated H2AK5 antibody (panel II), anti acetylated H4K8 antibody (panel III), anti-acetylated H4K16 antibody (panel IV) anti trimethylated H3K4 antibody (panel V). As loading control, re-probing of the same blot was done by anti H3 antibody (panel VI). Indicated below each band is the change in intensity with respect to DMSO control.

observe that none of the marks meant for gene repression are altered. Though H3K9 trimethylation is a mark of permanently silenced chromatin where alteration need not occur, H3K9 dimethylation is a mark of repression of erstwhile active genes. Even here no change was observed. The lack of any change in repressive marks is also indicated in the graph quantifying the inhibition (**Figure 3.8 B**). Again, we are only observing the changes in the global histone modification context. Minor rearrangement need not be essentially identified since this cannot be proved by western blot experiments and needs more sensitive chromatin immunoprecipitation of specific genes followed by RT-PCR of their promoter to get information at a higher resolution. Over all, this data suggests that the major change in epigenetic marks after RTK1 treatment to neuronal cells is on transcriptional activation marks and affects all three modifications i.e., acetylation, phosphorylation and methylation. Since RTK1 inhibits histone acetyltransferase it was obvious that it could inhibit acetylation marks on histones. But the alteration of acetylation associated phosphorylation and methylation was interesting. We decided to rigorously establish the fact that RTK1 was first and foremost an inhibitor of HATs and by virtue of the same it causes epigenetic changes. There could be two ways of proving the same. The first is to check what would happen to histone acetylation when RTK1 was added to cell culture media along with broad spectrum HDAC inhibitor like sodium butyrate and trichostastin. If indeed RTK1 is a HAT inhibitor in vivo, it would drastically affect histone acetylation turnover, eventually leading to a hypo-acetylated state. The second is a more indirect and corroborative evidence. The same epigenetic alterations caused by RTK1 should be effected by another known HAT inhibitor. Hence we performed western blot analysis of RTK1 untreated and treated neuroblastoma cells in the background of sodium butyrate (NaBu, 1mM) and trichostatin (TSA,1mM) treatment (**Figure 3.9**). We observe that in untreated cells (lane I) there is not much of acetylation of histones at the sites tested i.e., H3K9ac, H2AK5ac, H4K8ac and H4K16ac (panel I to IV). However upon treatment of Sodium butyrate (NaBu) and Trichostatin (TSA) for 3 hours, there is hyperacetylation observed at all the acetylation sites tested. The quantification of the band intensity showed a drastic 3 fold increase in H3K9 acetylation, 6 times increase in H2AK5 acetylation and a 2 times increase in H4K8

acetylation compared to DMSO treated lane at 3 hours itself which increases further by 6 hours. Acetylation is maximum by 6 hours of treatment (Lane 3, panel I through IV). However this hyperacetylation does not occur in RTK1 treated cells. By 3 hours itself (Lane 4, panel I through IV) the presence of RTK1 prevents hyperacetylation as observed by drop in intensities of the bands. This continues till 6 hours. When lane 3 is compared with lane 5, in all the panels, we see a drastic difference between hyperacetylated histones in lane 3 and hypoacetylated histones in lane 5, at all the tested sites. However, RTK2 which is not a HAT inihibitor does not induce such a hypoacetylation state. Indeed, HDAC inhibitor mediated hyperacetylation of histone is clear in lane 6, at all residues sites (**panel I through IV**) even in the presence of RTK2. To confirm equal loading of histones, the blots were stripped of antibodies and reprobed by H3 antibody. Panel V shows all the lanes to contain equal amount of histones. Thus, RTK1 by virtue of its HAT inhibitory potential prevents hyperacetylation at all the tested modification sites It is important to note that we chose sites on each of the histones except H2B, thus making this a global phenomenon and not a residue specific event. Also, it is important to note that the inhibition is seen as early as 3 hours (lane 2 versus lane 4, panel I through IV). This is suggestive of the rapid permeability of RTK1 to cells and an equally quick and potent inhibition of HATs. This also proves that inhibition of HATs is a direct effect of RTK1 treatment and not a downstream event. Sodium butyrate and Trichostatin, both are HDAC inhibitors known to hyperacetylate histones resulting in enhanced gene expression. They are also known to affect chromatin structure, wherein, their treatment results in a more open form of chromatin. This open form of chromatin requires acetylation of critical residues like H4K16 (Shogren-knaak et al., 2006). When we analyzed the changes by western blotting, HDAC inhibitors caused a significant increase in acetylation of H4K16 (**Figure 3.9; Lane2, 3; panel IV**). However, treatment of RTK1 could abolish this increase at three hour time point itself (**Figure 3.9; lane 4, 5; panel IV**). However, H4K16 site is acetylated by MYST family of HATs and not by p300/CBP family or GCN5/ PCAF family. Hence a direct effect of RTK1 is not very clear. It is possible that RTK1 could inhibit any of the MYST family of HATs directly. There are small molecules HAT inhibitors known, like EGCG, Spermidinyl CoA, where no

specificity is known for any particular HAT. But, they inhibit histone acetylation on a global scale. RTK1 may also inhibit other HATs in the cellular context, which has not been conclusively shown as yet. Hence the decrease in H4K16 acetylation could either be because of a direct effect through inhibition of enzyme acetylating the site, like the MYST family, or this could mean that H4K16 acetylation has cross talks with other acetylation sites. It is known that cross talk between acetylation sites within H4 exists. H4K16 acetylation is absolutely essential for acetylation of H4K12, H4K8 and H4K5. Crosstalk of H4K16 with acetylation sites on H3 is also well known. H3S10 phosphorylation influences H4K16 acetylation as well as H3K14 acetylation (Berger ., 2007; Zippo et al., 2009). Hence inhibition of H3 acetylation could affect H4K16 acetylation as well. Thus, RTK1 inhibits global histone acetylation by virtue of its HAT inhibition, in SH-SY5Ycells. Additionally, the experiments on the elucidation of the epigenetic network perturbation has led to the identification of the inhibition of H3K4 trimethylation as a consequence of HAT inhibition (**Figure 3.9, panel V**). The inhibition of this mark has been observed previously on treatment of RTK1 without sodium butyrate treatment. This mark will however be discussed in the ensuing section that discusses methylation and its crosstalk with acetylation. As discussed above, the changes in epigenetic alteration could be attributed to HAT inhibition if the same set of changes could be observed with a different HAT inhibitor having a different chemical scaffold.

3.5.2. Isogarcinol inhibits HATs and causes effects similar epigenetic alterations, similar to RTK1:

Recently many naturally occurring HAT inhibitors have been reported. We had discovered that Garcinol, a component of fruit rind, is an inhibitor of p300 and PCAF enzymes (Balasubramanyam et al., 2004). Garcinol was a very toxic molecule. In order to reduce its toxicity and make it more specific to p300 we had resorted to derivatization of chemical groups of Garcinol. One such derivative obtained by an internal cyclization was Isogarcinol (IG). IG was subjected to controlled modification and monosubstitution at “14” position to synthesize 14-isopropoxy IG (LTK-13) and 14-methoxy IG (LTK-14) (Mantelingu et al., 2007). These derivatives of isogarcinol were specific to p300.

However, Isogarcinol, by itself is not specific to p300. It could inhibit both p300 and PCAF (**Figure 3.10**). Considering that RTK1 is an inhibitor of p300 and also PCAF to some extent we went ahead to compare epigenetic changes that was caused by RTK1 with those that could be caused by IG. Also, the time point of treatment, and permeability of RTK1 and IG are similar. Hence we chose IG to compare with RTK1. Before we could continue with our assays in neuroblastoma cells we wanted to characterize histone acetylation inhibition in other cell lines. In order to understand whether Garcinol

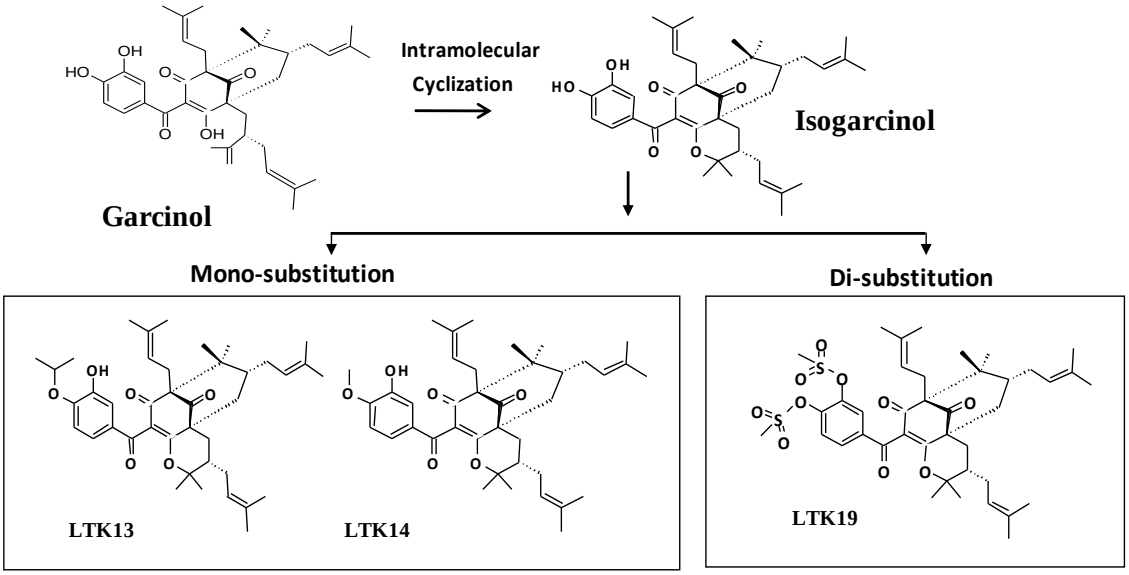


Figure 3.10: Garcinol derivatives are potent inhibitors of p300. Garcinol, a naturally occurring non specific, HAT inhibitory small molecule, was derivatised to isogarcinol (IG) through a process of intramolecular cyclization. IG was subjected to controlled modification and monosubstitution at "14" position to synthesize 14-isopropoxy IG (LTK-13) and 14-methoxy IG (LTK-14). The disubstitution of IG generated 3, 14 disulfoxy IG (LTK-19).

derivatives could inhibit histone acetylation and thus reverse the effects of histone deacetylase inhibitors induced hyperacetylation, LTK14 and Isogarcinol were treated after inducing hyperacetylation in C6 cells by using sodium.

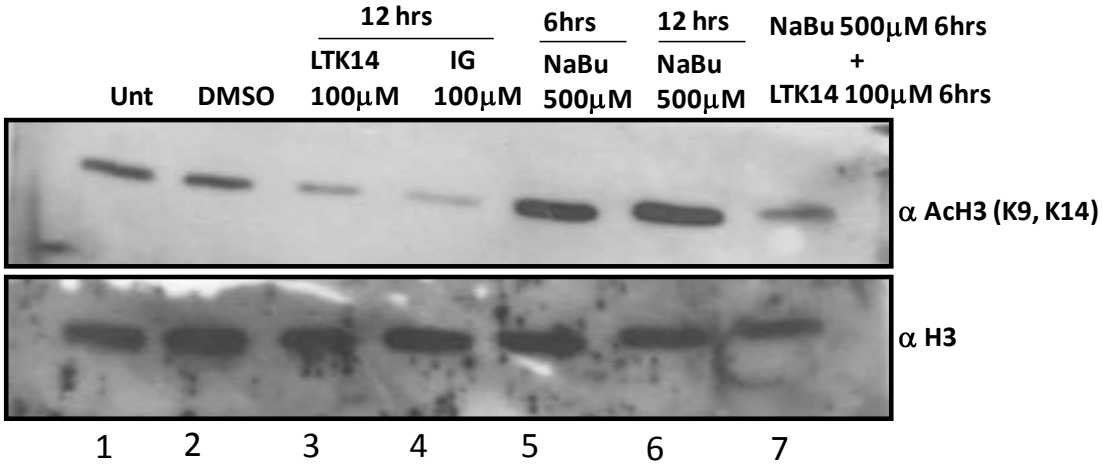


Figure 3.11: Garcinol derivatives, LTK14 and Isogarcinol inhibit histone acetylation in C6 cells and LTK14 reverses hyperacetylation induced by NaBu. Western blot analysis of LTK14 untreated and treated cells. Lane 1- Untreated cells; Lane 2- DMSO treated cells; Lane 3 –100μM LTK14 treatment for 12 hours; Lane 4 - 100uM Isogarcinol treatment for 3 hours; Lane 5 – 0.5mM NaBu treatment for 6 hours; Lane 6 - 0.5mM NaBu treatment for 12 hours; Lane 7 – 0.5mM NaBu treatment for 6 hours followed by a media change with 100mM LTK14 for 6 hours. Probing was done using Anti acetylated H3 K9,14 (bivalent) antibody. As loading control re-probing of the same blot was done by anti H3 antibody.

butyrate, a broad spectrum HDAC inhibitor (**Figure 3.11).** Western blotting was performed to assess the status of histone acetylation in treated and untreated C6 cells. In Figure 3.11, when compared to lane 2, in which cells were treated with DMSO solvent, lane 3 (LTK14, 100μM) and lane 4 (IG, 100μM) show decreased intensity as a result of decreased acetylation. In Lanes 5 and 6, histone hyperacetylation is seen as a result of sodium butyrate (0.5mM) treatment for 6 and 12 hours respectively. In lane 7, cells treated with sodium butyrate for 6 hours were subsequently washed and treated with 100μM of LTK14. As expected, LTK14 could reverse the hyperacetylation induced by HDAC inhibitors in C6 cell line. To observe the effects of these derivatives in SHSY5Y cells, LTK14 was treated at two concentrations, 50μM and 100μM to these cells. IG was treated at a single concentration of 50μM to avoid toxicity. DMSO treated cells were

taken as negative control. Western blotting was done using specific Acetylated H3 (K14 acetylation) antibody. The same nitrocellulose blot was stripped of antibody

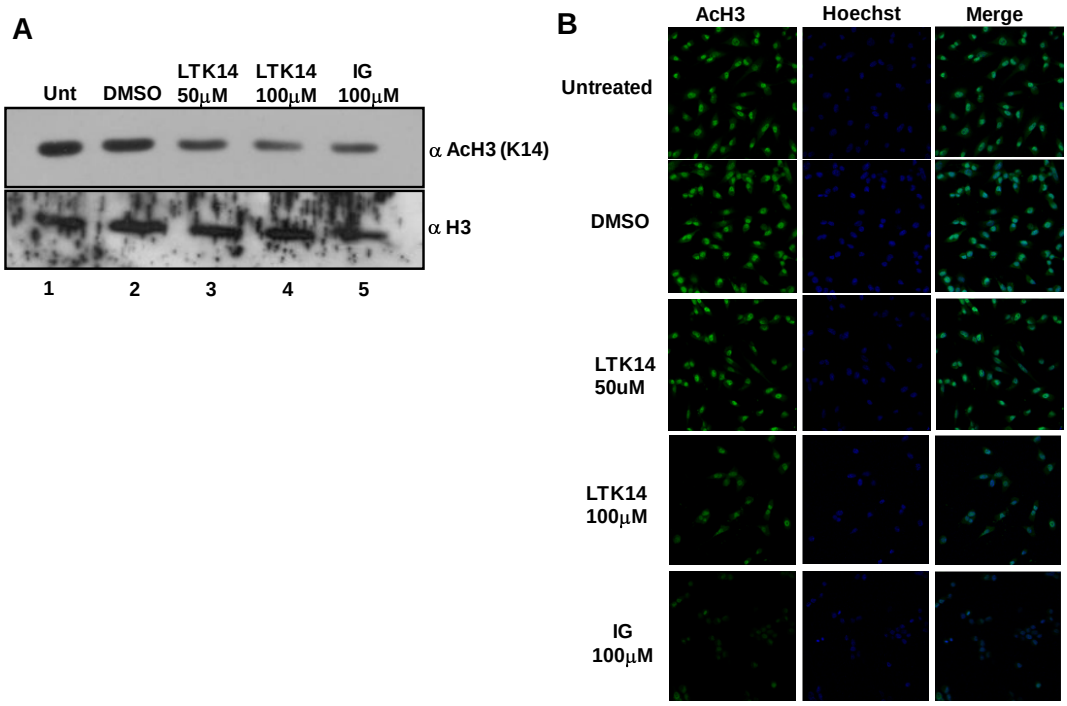


Figure 3.12: Garcinol derivatives LTK14 (p300 specific inhibitor) and IsoGarcinol, inhibit p300 mediated histone acetylation in SH-SY5Y cells in dose and time dependent manner. (A) Immunoblot analysis of solvent treated and LTK14 treated cells. Lane 1- untreated cells, Lane 2- DMSO treated cells, Lane 3 – 50μM LTK14 treatment for 12 hours, Lane 4 – 100μM LTK14 treatment for 12 hours, Lane 5- 100μM Isogarcinol treatment for 3 hours. Probing was done using Anti acetylated H3 for K9 and K14 antibody. As loading control reprobing of the same blot was done by anti H3 antibody. **(B)** Inhibition of acetylation as visualized by immunofluorescence of SY5Y cells with anti AcH3 antibody. Left panel shows immunofluorescence with anti AcH3 antibody; middle panel shows DNA staining by Hoechst and merge of antibody and Hoechst shown in the right panel.

binding and reprobing was done with H3 antibody to ascertain loading of histones. In **figure 3.12 A**, when compared to lane 2 in which cells were treated with DMSO solvent, lane 3 and 4 show decreased intensity as a result of decreased acetylation. In Lane 5,

histones of cells treated with Isogarcinol was loaded which also showed inhibition.Thus

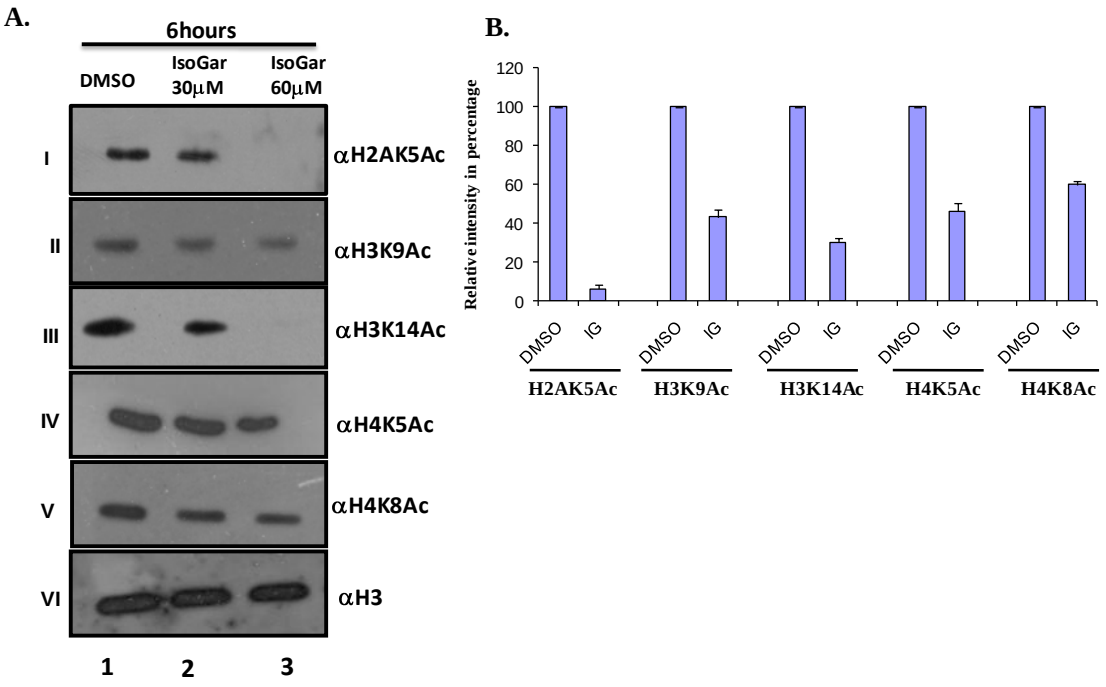


Figure 3.13: Isogarcinol inhibits histone acetylation marks essential for transcriptional activation in SH-SY5Ycells in a dose dependent manner; A. Western blot analysis of solvent DMSO treated and IG treated cells. Lane 1- Untreated cells with just DMSO solvent added to culture, Lane 2-30μM IG treatment for 6 hours, Lane 3 – 60μM IG treatment for 6 hours. Probing was done using anti acetylated H3K2AK5 antibody (panel I), anti acetylated H3K9 antibody (panel II), anti acetylated H3K14 antibody (panel III), anti acetylated H4K5 antibody (panel IV), anti acetylated H4K8 antibody (panel V). As loading control, re-probing of the same blot was done by anti H3 antibody (panel VI); **B.** The experiments were performed in duplicates, the levels of histone acetylation marks obtained by western blotting were quantified using ImageJ software and graph was plotted for relative intensity (in percentage) considering the intensity of DMSO treated lanes as 100%.

LTK14 inhibited p300 mediated acetylation in a dose dependent manner along with IG (**Figure 3.12A**). An immunofluorescence assay was done of SH-SY5Y cells using 100μM LTK14 (**Figure 3.12B**). Two time points 6 hours and 12 hours were chosen for treatment after which cells on coverslips were fixed and processed for immunofluorescence using anti-acetylated H3 antibody. The confocal images clearly show a decrease in intensity at 6 hours of treatment compared to DMSO control. The decrease in inhibition is more after 12 hour treatment implicating a time dependent

inhibition of histone acetylation. Isogarcinol treated cells acted as positive control. Isogarcinol treatment necessitated early processing at 3 hours as the compound is toxic to cells for longer time point treatment. Having characterized the inhibition of Garcinol derivatives in neuronal cell lines we sought to analyze the epigenetic changes that Igogarcinol would have in neuronal cells. The aim was to match the inhibition profile of both RTK1 and IG and thus incriminate HAT inhibition as the common factor for the same set of epigenetic changes. IG was treated at two concentrations, 30μM and 60μM to SH-SY5Y cells for 6hours (**Figure 3.13**). DMSO treated cells were taken as controls. Western blotting was done using specific antibodies to histone modifications like acetylated H3, H2A and H4 (H2AK5, H3K9, H4K8 acetylation); phosphorylated H3S10, tri-methylated H3K4; all of which are marks of transcriptional activation. Western blot has also been done for repressive marks (H3K9me2 and H3K9me3). The same nitrocellulose blot was stripped of antibody and re-probing was done with H3 antibody to ascertain loading of histones. It is observed that in a dose dependent manner, IG inhibits transcriptional activation marks H2AK5Ac, H3k9ac, H3K14Ac, H4K5Ac and H4K8Ac. (**Figure 3.13 A, panel I to V; lane 1 versus 2 and 3**), and H3S10p and H3K4 trimethylation (**Figure 3.14 A, panel I and II, lane 1 versus 2 and 3**) in the same way RTK1 inhibits transcriptional activation marks. Also, transcriptional repressive mark H3K9 tri-methylation is not altered by IG (**Figure 3.14, panel III and IV; lane 1 versus 2 and 3**). It is clearer from the quantification of the western blots that all transcriptional activation marks that have been probed are repressed to nearly 50% from their control levels (**Figure 3.13 B and Figure 3.14 B, upper panel**). It is also clear that IG does not alter transcriptional repressive marks either (**Figure 3.14 B, lower panel**). This is similar to the earlier data on RTK1. The only common factor between these two molecules belonging to different chemical scaffolds is the fact that both are potent HAT inhibitors. This incriminates the role of HAT inhibition in repressing transcriptional activation marks, which could either be acetylation itself or acetylation associated marks like H3S10 phosphorylation. Isogarcinol inhibits HATs and affects similar epigenetic alterations, as RTK1 through its HAT inhibitory activity. It could be argued that the inhibition of marks other than acetylation could be because of a pleotropic effect.

However, two different molecules, bearing two different scaffolds, both of them proven to inhibit p300 HAT, having the same pleotropic effect is distant. However, that possibility cannot be ruled out. At this juncture, it is clear that inhibition of HATs,

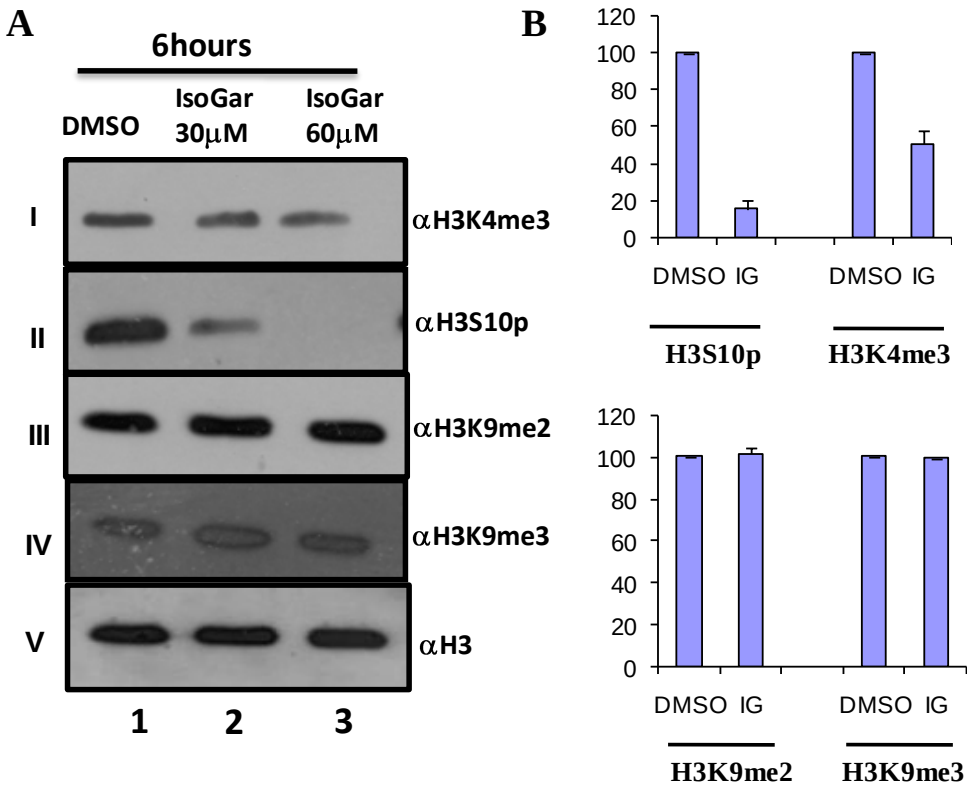


Figure 3.14: Isogarcinol inhibits transcriptional activation marks but not repressive marks; A. Western blot analysis of IG untreated and solvent treated cells. Lane 1- cells with just DMSO solvent added to culture, Lane 2-30μM IG treatment for 6 hours, Lane 3 – 60μM IG treatment for 6 hours. Probing was done using anti phosphorylated H3S10 antibody (panel I), anti trimethylated H3K4 antibody (panel II), anti dimethylated H3K9 antibody (panel III), anti trimethylated H3K9 antibody (panel IV). As loading control, re-probing of the same blot was done by anti H3 antibody (panel V); **B.**The experiments were performed in duplicates, the levels of histone acetylation marks obtained by western blotting were quantified using ImageJ software and graph was plotted for relative intensity (in percentage) considering the intensity of DMSO treated lanes as 100%.

especially p300, has major repercussions in the epigenetic landscape of the cells. Support for this work comes from other studies which have looked into the cross talks between these epigenetic marks in the background of HDAC inhibition. The report

elucidates an increase in acetylation with concomitant increase in other marks like H3K4 trimethylation (Nightingale et al., 2007). Our study concludes just the contrary in the case of HAT inhibition. It has been elucidated that the functional roles of histone H3 acetylation and H3K4 methylation broadly correlate. Enhanced levels of both H3 acetylation and H3K4me3 are detected at the transcriptional start sites of active

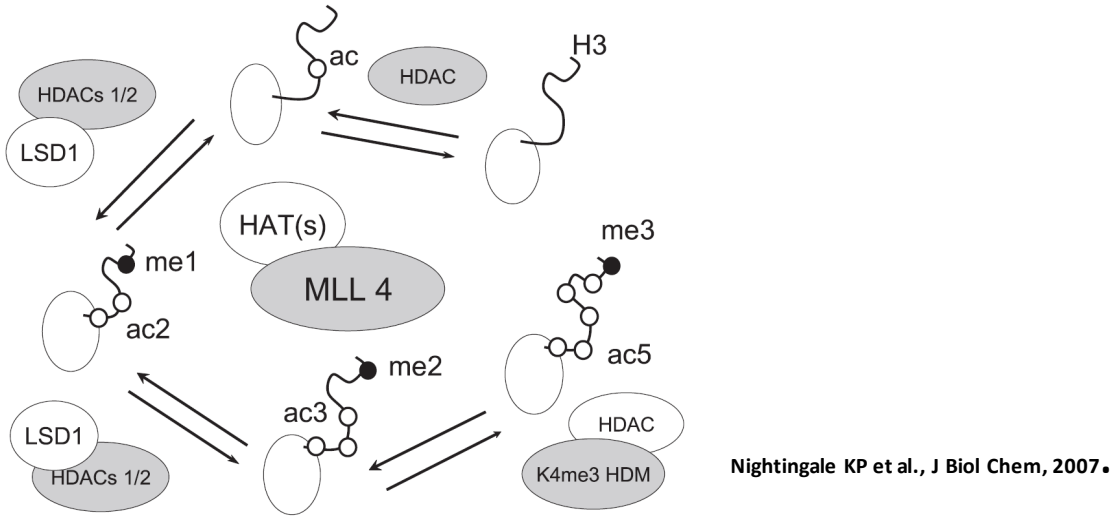


Figure 3.15: Proposed enzymology of histone H3 lysine 4 methylation. The abundance of specific methyl isoforms at H3 lysine 4 (me1, me2, or me3) is linked to the level of H3 acetylation and this is under the regulation of several opposing multienzyme complexes; a MLL4-histone acetyltransferase complex is responsible for depositing these marks. Importantly, the H3 tail must be acetylated for subsequent methyltransferase activity (**Adapted from Nightingale et al., 2007**).

promoters, and both marks exert functional effects by recruiting activating chromatin enzymes through interactions with bromo- and chromo-domain-containing proteins. This correlation is also observed biochemically in the global pool of histones. Mass spectrometry and Western blot analysis demonstrate that the level of histone H3 acetylation is linked to the degree of methylation at H3 lysine 4. It is known now that conditions that increase histone H3 acetylation (*i.e.* treatment with deacetylase inhibitors) are associated with increased levels of H3K4 methylation, primarily the di- and tri-methylated forms, whereas conditions that decrease H3 acetylation (i.e. removal of deacetylase inhibitors) also reduce the abundance of these methyl marks. HDAC inhibition also affects acetylation turnover at promoters of immediate early genes and

results in hyperacetylation at these promoters (Hazzalin CA et al., 2005). In cells that were not treated with HDACi, H3K4 methylation is seen almost exclusively on acetylated (primarily mono-acetylated) H3 isoforms and was virtually absent from the more common non-acetylated form. Further, it was observed that the degree of methylation is linked to the extent of acetylation, with H3 isoforms mono-, di-, and trimethylated at Lys-4 being associated with progressively more highly acetylated H3 isoforms. Infact, it has been reported that the number of acetylated lysines on histone H3 influences the methylation of H3K4, which is due to a direct influence of the acetylation on enzyme activity, in this case MLL4 that tri methylates H3K4. The more the acetylation of H3, the better substrate it becomes for MLL4 resulting in increased H3K4 trimethylation (**Figure 3.15**). Indeed, phosphorylation of H3S10 in conjunction with acetylation, results in a phospho-acetylated histone H3 isoform, which is an even better substrate for trimethylation at H3K4. Kinetics of the modifications reveal that post HDACi treatment, the changes in histone acetylation precede changes in histone methylation. In our observation we observe that after inhibition of histone acetylation, both H3K4 trimethylation and H3S10 phosphorylation levels drop on a global scale. There could be a cross talk operating between these modifications post inhibition of histone acetyltransferase, which needs to be addressed. No study has yet been conducted where changes in epigenetic network is analyzed either post HAT inhibition (p300 and PCAF in this case) or after gene silencing of these major HATs. This area needs to be addressed keeping into consideration the fact that HATs, especially p300, is a master regulator of gene expression. It also has important fallouts into neuronal cell physiology, where hypoacetylation is known to be detrimental to the cells. But, what has not been addressed is the involvement of other modifications, which could be either upstream or downstream of changes in acetylation that could contribute to the pathology and ways to rescue the cells from these harmful perturbations. Our study suggests that post inhibition of HATs, on a global scale, there are major repercussions on other modifications that have been described above. These epigenetic modifications also affect cell survival and physiology. It has already been mentioned that balanced acetylation of histones is vital for neuron cell survival. A loss of acetylation is reported in apoptotic cells. To check the

effect of HAT inhibition of neuronal cells, we performed cell viability assays using neuroblastoma cells post treatment of RTK1. The nature of toxicity would also help elucidating the fact that the epigenetic changes that we have been observing is not a result of apoptosis, but the ensuing epigenetic alterations driven by HAT inhibition would indeed lead to neuronal apoptosis.

3.6. RTK1 induces apoptosis of neuroblastoma cells:

3.6.1. RTK1 inhibits proliferation of SH-SY5Y cells:

RTK1 is toxic to cells and induces apoptosis, which is not surprising, considering the wide array of activities attributed to RTK1. Through unknown mechanisms RTK1 generates Reactive Oxygen Species (ROS); Inhibits tubulin polymerization; Inhibits NF-κB, AP-1 activity; Induces autophagy etc. We recently discovered that RTK1 inhibits HAT and through this work we have elucidated its effects on the epigenetic landscape. It could be argued that all the epigenetic perturbations we observe is due to these activities and unrelated to HAT inhibition. However, HAT inhibition could be central to many of these activities of RTK1. We investigated the role of RTK1 in cellular proliferation. We treated Neuroblastoma cells with increasing concentrations of RTK1 (5, 10, 15, 20μM) while RTK2 (20μM) treated cells were treated as negative control. The treatment time was 6 hours for one set and 24 hours for another set (**Figure 3.16, A** and **B** respectively). We observe that after 6 hours of treatment there is a gradual drop of viability in a concentration dependent manner. Though the drop is not drastic, at the maximum concentration of 20μM, there is a significant drop to 75% from 100% in DMSO treated

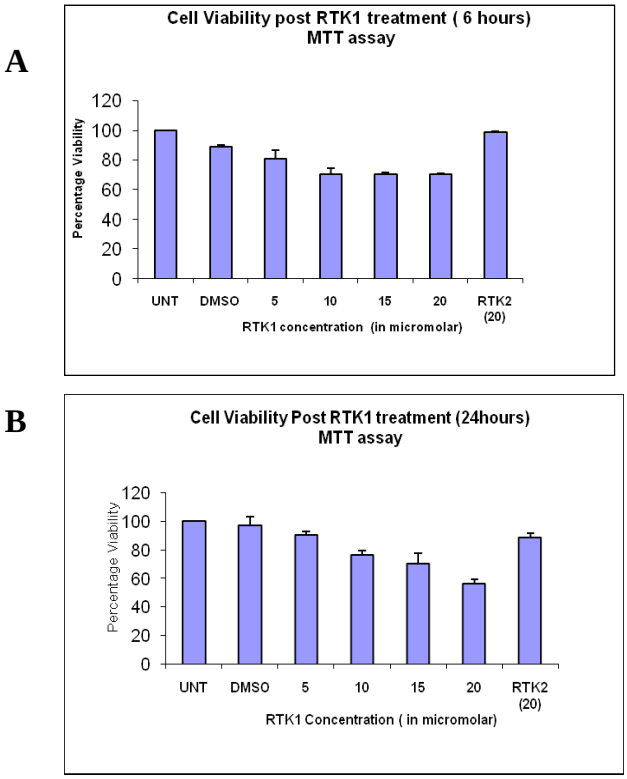


Figure 3.16: Concentration dependent inhibition of cell proliferation by RTK1 measured by MTT assay **A.** 6 hours treatment; **B.** 24 hours treatment.

cells. The drop in viability was drastic by 24 hours. Though dose dependence was observed here too, there was 60% viability at 20μM concentration by the end of 24 hours. Very surprisingly, when RTK2 was treated at the same maximum concentration, no change in cell viability was observed, even after for 24 hours, implicating the HAT inhibitory potential of RTK1 in apoptosis. However, the drop at 6 hours was interesting in another way. RTK1 inhibits tubulin polymerization (Acharya et al., 2008). Treatment of RTK1 results in immediate loss of cell shape. Since we were experimenting with neuronal cells, where cytoskeleton is more than other cells, the loss of cell shape and adherence was imminent. We hypothesized that the drop in viability is actually due to this change in morphology. Since MTT assay involves a step where the culture medium with the dye is syringed out, it was possible that even viable cells would be syringed out as they have lost adherence and are floating in the medium. The assay however did give information that after 24 hours of treatment there is a drastic loss in cell viability which is

not the case when HAT inhibition does not occur.It is yet to be ascertained whether the morphology of the cel is related to HAT inhibition or whether it is due to a different pathway. It is important to note that RTK2 does not cause any morphological change.To have a better estimate as to when apoptosis sets in, we performed DNA fragmentation assay.

3.6.2. RTK1 induces apoptosis of SH-SY5Y cells:

This is a sensitive assay that is designed on the principle that apoptotic cells have fragmented DNA, nearly 200kb in size at the onset, which escapes to the cytoplasm. At later stages, fragments of all sizes are formed giving rise to a ladder when run on agarose gel. At the end of apoptosis, however, DNA is mainly the smaller fragments with hardly any large molecular weight DNA left uncleaved by endonucleases. As observed in

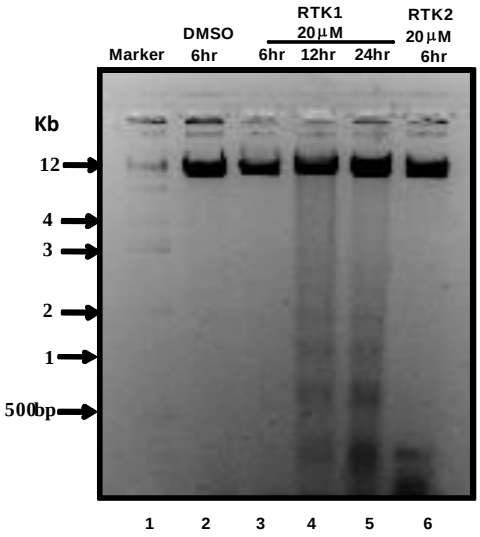
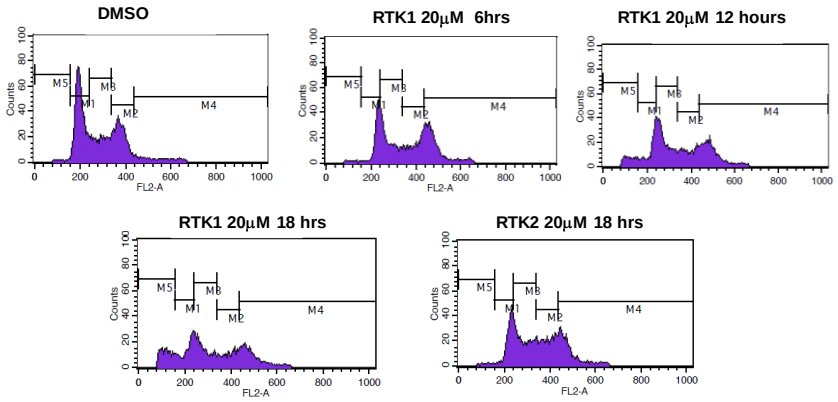


Figure 3.17:RTK1 induces apoptosis (as monitored by DNA fragmentation). Apoptosis sets in at 12 hours as observed by DNA fragmentation. Lane 1, Marker in kilo base; lane 2, DMSO treated cells; lane 3, RTK1 20μM for 6 hours; lane 4, RTK1 20μM for 12 hours; lane 5, RTK1 20μM for 24 hours; lane 6, RTK2 20μM for 24 hours.

Figure 3.17, no DNA fragmentation is observed in either DMSO treated cells (**lane 2**) or RTK1 treated cells (**lane 4**) till 6 hours. The same is the case with RTK2 treated cells (**lane 6**). However, DNA fragmentation is observed by 12 hours of RTK1 treatment (**lane**

4). The appearance of DNA ladders can be seen in lane 4. There is increase in fragmentation of DNA fragmentation by 24 hours as seen by the increase in DNA ladder in **lane 5**.This proves that apoptosis sets in at 12 hours while there is no apoptosis at 6 hours. In our earlier experiments we observe epigenetic changes as early as 3 hours. This observation further strengthens the fact that the epigenetic perturbations are not because of apoptotic cell death or any other downstream event, but due to HAT inhibition by RTK1. As observed in the MTT assay, RTK2, which is not a HAT inhibitor, does not

A



B

	DMSO	RTK1 20μM 6hrs	RTK1 20μM 12hrs	RTK1 20μM 18hrs	RTK2 20μM 6hrs
M1	43.91	19.39	12.25	19.77	19.58
M2	27.51	23.89	19.77	18.18	29.08
M3	24.02	27.77	30.21	25.54	30.20
M4	4.49	29.66	32.37	22.14	21.56
M5	0.74	0.61	6.44	15.43	0.78

Figure 3.18: **A.** FACS analysis of PI stained HeLa cells treated with DMSO, RTK1 20μM (for 6, 12 and 18 hours). RTK2 (20μM) treated cells are considered as negative control. The cells were gated in M1, M2, M3 and M4 and M5 region in the FL-2 channel. **B.** Percentage of cells in various stages of cell cycle post RTK1 treatment.

cause apoptosis. This also proves beyond doubt that HAT inhibition causes apoptosis in neuronal cells. However, DNA fragmentation is a qualitative assay. To be quantitative and more accurate we also performed fluorescence activated cell sorting (FACS) analysis (**Figure 3.18 A and B**). SH-SY5Y cells were treated with DMSO or 20μM RTK1 or 20μM RTK2 for various time points. Later, cells were stained with propidium iodide as

mentioned in materials and methods section and subjected to FACS analysis. Of interest, in FACS analysis, is the appearance of Sub-G1 peaks in the M5 region, which corresponds to the apoptotic population. There is no appearance of Sub-G1 peaks in DMSO treated cells or RTK2 treated cells as expected. RTK1 (20μM) when treated for 6 hours does not yield any apoptotic population of cells. However, by 12 hours of treatment of same concentration of RTK1, there is appearance of a peak in M5 region. This peak increases by 18 hours. The percentage of cells that are in M5 region is mentioned in the tabulation beside each FACS profile. By 12 hours of RTK1 treatment 6% of cells are apoptotic and by 18 hours 15% cells are apoptotic. This analysis confirms two issues. First, the earlier epigenetic perturbations that targeted transcriptional activation marks are not due to apoptosis of cells. The second, is inhibition of HATs, results in apoptosis of neuroblastoma cells. This information adds to the available information that hypoacetylation of histones in neurons is seen predominantly in neurodegenerative conditions, and most of those neurons are in apoptotic state.

3.7. RTK1 induces expression of both apoptotic and neuroprotective genes:

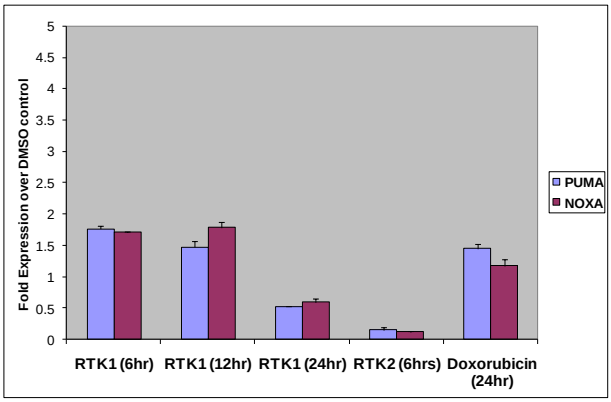


Figure 3.19: RTK1 induces p53 dependent pro apoptotic gene expression: Cells were treated with DMSO or 20μM plumbagin for 6, 12, 24h. Cells were collected, total RNA was extracted, and reverse transcription was performed as described in the methods section. RT-PCR was performed as described elsewhere using gene specific primers for PUMA or NOXA. Results are mean fold expression change over DMSO treatment.

Having ascertained the toxicity profile caused by RTK1, we were interested to know what changes would it cause to gene expression in neuronal cells. More compelling reason was the fact that it inhibited major transcriptional activation marks, mostly those of acetylation. Hence, changes in gene expression especially repression of genes was expected. SH-SY5Y is a p53 positive cell line. However the expression of p53 is considered to be negligible. We checked for expression of PUMA and NOXA genes by RT-PCR analysis as these genes are p53 responsive and get upregulated during p53 mediated apoptosis (Villunger et al., 2003)(**Figure 3.19**). The fold change in expression of treated cells over solvent treated control (DMSO) cells was calculated and data tabulated. These genes are pro apoptotic and are p53 downstream genes. As expected, their expression increased by 1.5 to 2 fold by 6 hours of treatment of RTK1 at 20μM. This high level of expression was maintained till 12 hours. However by 24 hours, the expression decreased, but still was higher than DMSO control. The decrease could be because of the increase in apoptotic population as observed in our earlier data. The increase by 6 and 12 hours was comparable to the induction observed after doxorubicin (DNA damaging agent, induces p53) treatment for 24 hours (10μg/mL). However we were more interested in understanding the gene expression of neuroprotective genes. We chose Brain Derived Neurotrophic Factor (BDNF) and BCL2 genes (**Figure 3.20 A** and **B** respectively). These are genes that protect neurons from death. These two genes are Cyclic AMP Responsive Element Factor (CREB) downstream and they require CBP mediated HAT activity at their promoters for efficient expression (Finkbeiner S et al., 1997). Again, we performed RT-PCR for these two genes after treating neuroblastoma cells with RTK1 at 20μM for 6, 12 and 24 hours. Surprisingly, there is no inhibition in the expression of these genes, notwithstanding the fact that they are CREB downstream. This, despite the fact that histone acetylation is decreasing globally. Recent literature reports that RTK1 at lower concentrations than what has been used here is a neuroprotective agent, and this has been established in the background of cerebral ischemia (Son et al., 2010). This occurs through a different pathway involving the Nrf2/ARE pathway. RTK1 reportedly activates this pathway. In vitro, plumbagin increases expression of neuroprotective genes in primary neuronal cultures upon

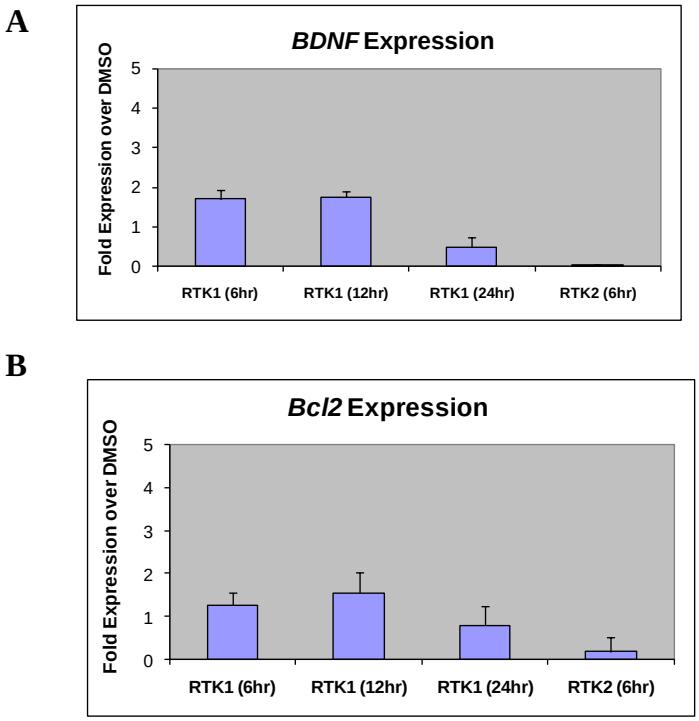


Figure 3.20: RTK1 induces expression of neuroprotective genes: Cells were treated with DMSO or 20μM plumbagin for 6, 12, 24h. Cells were collected, total RNA was extracted, and reverse transcription was performed as described in the methods section. RT-PCR was performed as described elsewhere using gene specific primers for BDNF (**A**) or Noxa (**B**). Results are mean fold expression change over DMSO treatment.

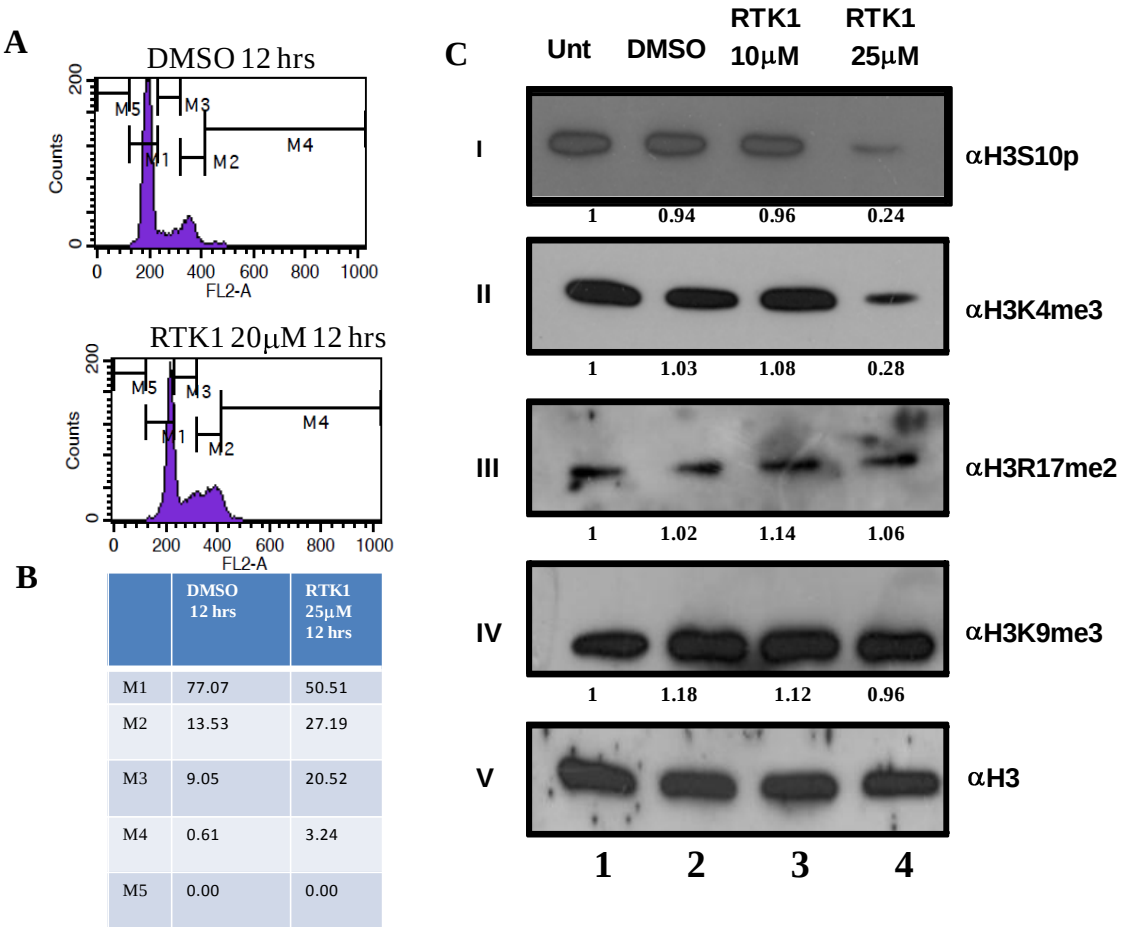
ischemic stress. But, whether the effect is a direct action of plumbagin on the Nrf-ARE pathway or whether this activation is due to the generation of ROS upon RTK1 treatment is unknown. However, it is to be dissected as to what extent the role of RTK1 as a HAT inhibitor would affect the other actions that also influence gene expression. The availability of HATs like p300 in the cell is very limited and thus one way to use them would be to hijack p300 from one pathway to the other. Considering that Nrf2 needs p300 co-activation property it could be possible that Nrf2 responsive genes could be activated even though p300 is inhibited by RTK1. It is not known to what extent p300's HAT activity influences Nrf2 mediated activation. Thus it could be possible that on one hand p300 is inhibited by HAT activity thus repressing a subset of genes. However, the ability of RTK1 that is plumbagin, in inducing the production of ROS could in turn

activate a lot of genes that would be necessary to counter the stress and repair DNA damage which is an inseparable component of ROS mediated stress. Since the role of HATs and the presence or absences of redundancy in these pathways have not been worked out the present up regulation of neuroprotective genes could be a response to ROS production.

3.8. RTK1 mediated perturbation of epigenetic network occurs in non neuronal cells and animal models:

3.8.1. Effect of RTK1 in HeLa cell epigenetic marks.

Having proved that the treatment of RTK1 to neuronal cells alters epigenetic landscape and leads to apoptosis of cells, we wanted to establish the prevalence of this phenomenon across all cell types. This is because, though the expression may vary between tissue and cell types, HATs are present in all cell types. If RTK1 is a HAT inhibitor, then essentially, it should induce similar changes across all cell types since the working principles of epigenetics and transcription are similar across mammalian cells. Also, we wan type specific event. Hence, we treated RTK1 to HeLa cells, which are non neuronal cells and very different in nature from SH-SY5Y cells. We ensured the toxicity levels by initially performing FACS analysis of propidium iodide stained HeLa cells after treatment of 25μM RTK1 for a period of 12 hours (**Figure 3.21 A and B**). We observed no apoptosis at this concentration at 12 hours, as observed by lack of Sub G1 (M5) population of cells, contrary to what occurred in SH-SY5Y cells, which began to die by apoptosis by 12 hours itself. This could be because of the excessive sensitivity of ted to ensure that the observations that we had made is not a onetime event or a cell neuronal cells for hypoacetylation. However, there is an accumulation of cells in S phase and G2 phases of the cell cycle as indicated by increase in M2 and M3 populations. It is known that plumbagin inhibits cell cycle progression in S phase. This feature of plumbagin is attributed to its ability to induce DNA damage. But, it is certain that at the concentration and time point that has been used in the present study there is no cell death by apoptosis which is supported by the FACS data. After ensuring the lack of toxicity, RTK1 was treated at two concentrations, 10μM and 20μM to HeLa cells (**Figure 3.21 C**). DMSO



	DMSO 12 hrs	RTK1 25µM 12 hrs
M1	77.07	50.51
M2	13.53	27.19
M3	9.05	20.52
M4	0.61	3.24
M5	0.00	0.00

Figure 3.21: RTK1 inhibits transcriptional activation marks associated with histone acetylation in HeLa cells in a dose dependent manner. Western blot analysis of RTK1 untreated and treated cells. Lane 1,Untreated cells; lane 2, DMSO treated; Lane 3, 10µM RTK1 treatment for 6 hours; Lane 4, 25µM RTK1reatment for 6 hours. Probing was done using anti phosphorylated H3S10 antibody (panel I), anti tri-methyl H3K4 antibody (panel II), anti di-methyl H3R17 antibody (panel III), anti trimethyl H3K9 (panel IV). As loading control, re-probing of the same blot was done by anti H3 antibody (panel IV).

treated cells acted as negative control. Western blotting was done using specific antibodies like anti phosphorylated H3S10 antibody (panel I), anti tri-methyl H3K4 antibody (panel II), anti di-methyl H3R17 antibody (panel III) and anti tri-methyl H3K9antibody (panel IV). As loading control, re-probing of the same blot was done by anti H3 antibody (panel V). We observe that phosphorylation of H3S10 (panel I) and

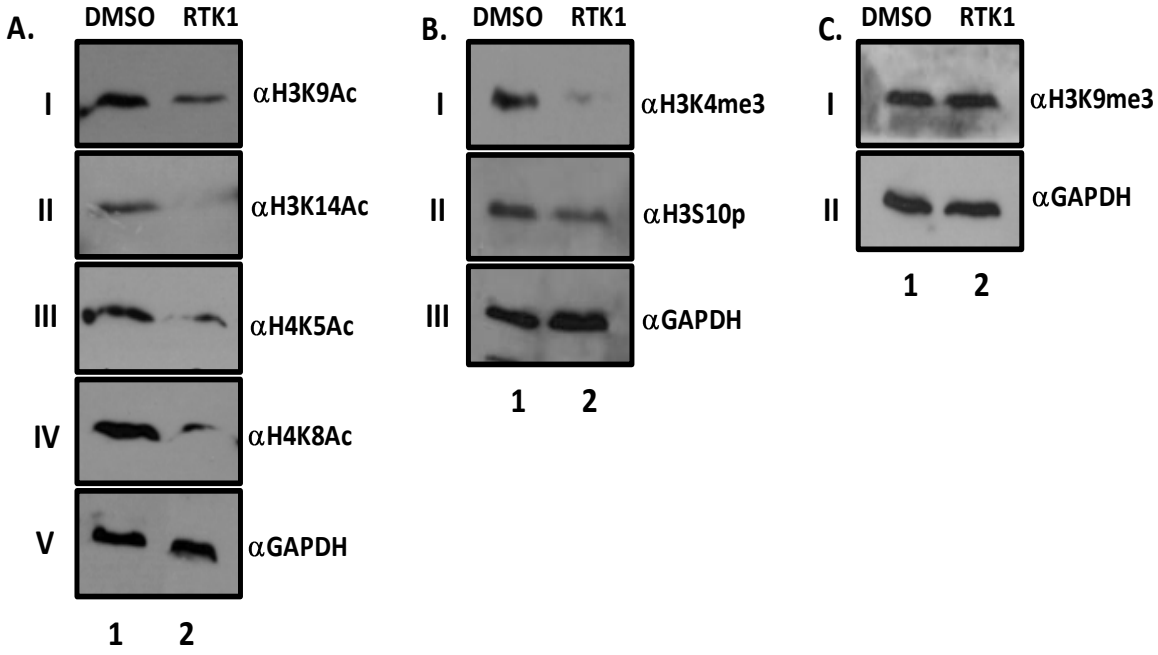


Figure 3.22: RTK1 inhibits epigenetic marks meant for transcriptional activation in mouse brain tissue. Western blot analysis of DMSO and RTK1 (9mg/kg, intra-peritoneal route) injected mouse brain. Lane 1- brain lysate from DMSO injected mouse , Lane 2- brain lysate from RTK1 injected mouse (in **A**,**B** and **C**). **A)** Probing was done to check changes in acetylation status of histones using anti acetylated H3K9 antibody (panel I); anti acetylated H3K14 antibody (panel II); anti acetylated H4K5 antibody (panel III); anti acetylated H4K8 antibody (panel IV) and anti-GAPDH (loading control). **B)** Probing was done to check changes in acetylation associated transcriptional activation marks using anti-phosphorylated H3S10 antibody (panel I); anti-trimethylated H3K4 antibody (panel II); anti GAPDH (panel III) as loading control. **C)** probing was done to check transcriptional repressive mark using anti-trimethylated H3K9 antibody (panel I); anti GAPDH (As loading control).

H3K4 trimethylation (panel II), both decrease post treatment of RTK1 which correlate to the doses where histone acetylation inhibition has been reported. However, H3R17 dimethylation, which is also a transcriptional activation mark remains unaltered at same concentration, as has been observed with SH-SY5Y cells (Figure 3.7). H3K9 tri-methylation too showed not alteration. This result is in line with the earlier data on the same modification in SH-SY5Y cells (**Figure 3.8**).Through these rigorous analysis of

histone post translational modifications we could elucidate the role of HAT inhibition in influencing epigenetic marks and we established RTK1 as a potent HAT inhibitor both in neuronal and non-neuronal cell lines.

3.8.1. Effect of RTK1 in epigenetic alterations in a mouse model:

The ultimate aim of this research is to check for physiological changes ensuing HAT inhibition. This will help to understand the role of histone acetylation in normal physiology better. However, we chose the small molecule inhibitor based approach for the same. Since we had established RTK1 as a HAT inhibitor is detrimental to neuronal cell survival and since acetylation of histones is such a vital physiological process in the mammalian brain, we sought to analyse the epigenetic changes and the physiopathology associated with HAT inhibition in brain, in a mouse model. We observed that most of the acetylation sites, H3K9, H3K14, H4K5, H4K8 acetylation are decreased post RTK1 treatment (**Figure 3.22**, **A**, **panel I-IV respectively**) in mice. To our surprise, there is also a drastic decrease in other transcriptional activation marks, associated with acetylation like H3K4 tri- methylation and H3S10 phosphorylation (**Figure 3.23**, B, panel I and II respectively). As we observed in our cell line based experiments, we do not see any changes in the transcriptional repressive marks like H3K9 tri-methylation even though most of the transcriptional activation marks are repressed. In order to visualise the inhibition of acetylation and inhibition of p300 within the brain tissue we resorted to immunohistochemical analysis. After 6 hours of treatment with either DMSO or RTK1 mouse were decapitated and brain was quickly dissected out. The tissue was then fixed with formalin and embedded with paraffin. Sections were made from the paraffin blocks and stained using anti H2AK5 acetylation and anti acetylated p300 antibodies. Upon completion of the procedure the development of a brown stain is due to the formation of an insoluble brown precipitate from a chromogenic substrate di-amino benzidine (DAB). The sections were counterstained with hematoxylin after immunostaining. We observe a drastic decrease in the H2AK5 acetylation in the RTK1 treated mice brain when compared to DMSO treated brain as indicated by the intense brown nuclear stain taken by tissue in the DMSO injected mice which is absent in the

RTK1 treated mice. There is also a drastic decrease in acetylated levels of p300. The inhibition of autoacetylated forms of p300 with concomitant inhibtion of histone acetylation as observed by western blotting and IHC, proves beyond doubt that RTK1, by virtue of its HAT inhibitory potential inhibits transcriptional marks both in vitro and in vivo. Whether the inhibition of transcriptional activation marks actually translate to

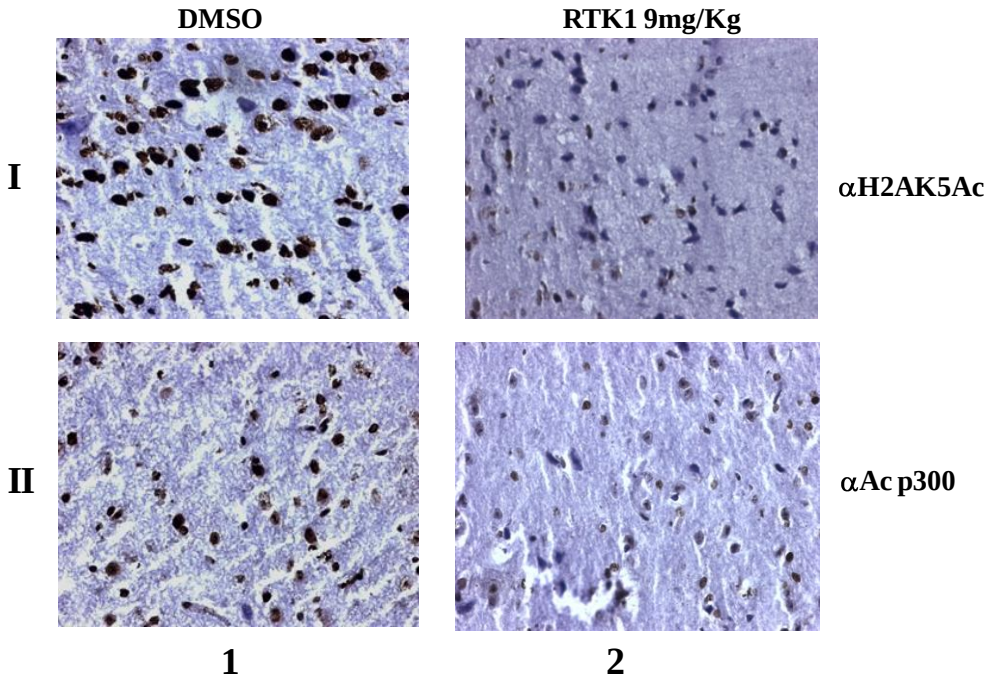


Figure 3.23: Immunohistochemistry was performed using brain tissue from mice which were intra peritoneal injected with DMSO (lane 1) and RTK1 at 9mg/Kg (lane 2) immunostained with antiacetylated histone H2AK5 antibody (panel I) and anti acetylated p300 antibody (panel II).

repression of transcription itself is a subject of further study that is now being conducted. Also, what meaning this form of inhibition of HATs would have in terms of functioning of the brain, especially in the context of learning and memory, as well as neurodegeneration is currently being studied.

Chapter 4

TTK21, a novel p300/CBP histone acetyltransferase activator

This chapter discusses the activators of p300/CBP acetyltransferases. It also includes the screening of various derivatives of CTPB, a HAT activator, to identify and characterize better activators of p300/CBP.

Chapter outline:

4.1. CTPB, a p300/CBP specific activator of acetyltransferase activity.

4.2. Nanoparticles, new tools for drug delivery.

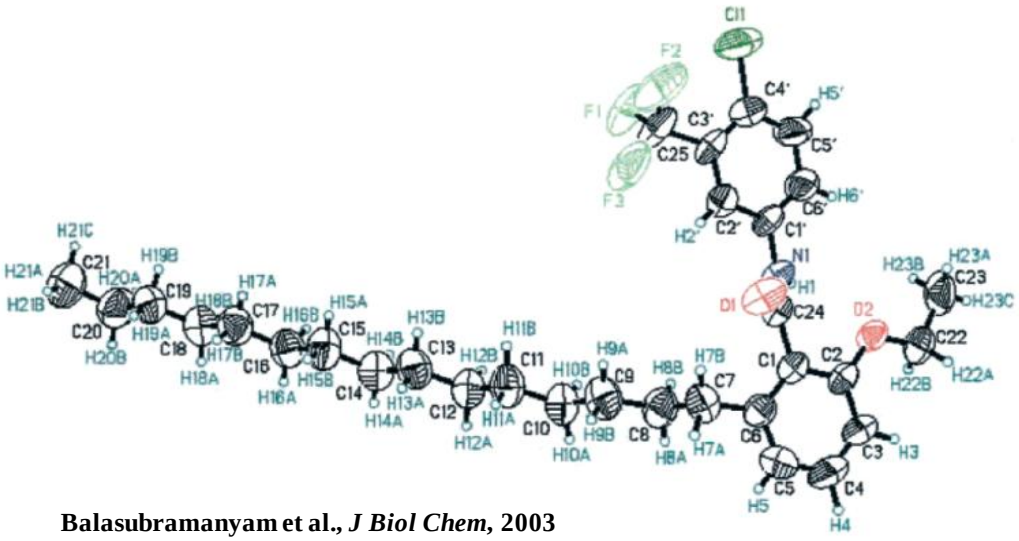
4.3. Identification of a novel, small molecule activator of CBP/p300 and its characterization

4.4. Activation of p300 mediated histone acetylation in vivo using carbon nanospheres as drug delivery vehicle:

4.1. CTPB, a p300/CBP specific activator of acetyltransferase activity.

There are many naturally occurring small molecules that have been isolated and screened from medicinal plants for their anticancer properties. However, not many of these molecules have been tested for their ability to inhibit acetyltransferase activity. In an effort in the same direction, we had isolated cashew nut shell liquid (CNSL) extracts (both polar and non-polar) and tested for their HAT inhibitory activity. Surprisingly, CNSL inhibited HAT activity of both p300 and PCAF (Balasubramanyam et al., 2003). We could also narrow down our search to anacardic acid, which was a very potent but non specific inhibitor of both p300 and PCAF. It was also observed that anacardic acid did not show any changes in cellular histone acetylation which could be due to the fact that these group of molecules were cell impermeable. Due to the apparent lack of specificity toward HATs, we altered the various functional groups of anacardic acid, keeping the parent structure intact, to end up with a molecule with a better inhibitory effect or even selectivity. One such modification involved the acidic group on the anacardic acid which was modified to different amide derivatives using substituted anilides. This resulted in a molecule, CTPB, which surprisingly when tested in an *in vitro* HAT assay, showed an

enhancement in the p300 HAT activity while keeping the PCAF HAT activity mostly unperturbed. Concentration-dependent HAT activity profile revealed a maximum activation for p300 HAT activity at 275 μM CTPB. Thus, CTPB specifically enhanced the HAT activity of



Balasubramanyam et al.*, J Biol Chem*, 2003

Figure 4.1: x-ray crystal structure of CTPB showing the Oak Ridge Thermal Ellipsoid Plot (ORTEP) view of the compound. The color coding of the atoms is as follows: black, carbon; light blue, hydrogen; dark blue, nitrogen; red, oxygen; light green, fluorine; dark green, chlorine.(**Adapted from Balasubramanyam et al.*, J Biol Chem*, 2003**)

p300, a function that is reflected even at the transcriptional level. CTPB could activate in-vitro chromatin templated transcription significantly. However, it did not affect DNA transcription, thus highlighting the fact that CTPB enhances transcription through its ability to activate p300. The resultant increase in chromatin templated transcription is due to hyperacetylation of nucleosomes by p300 in vitro. Molecules which can activate p300 in vivo could be of immense use not only as tools to study the phenomena of acetylation in the living cells and organisms but also as new generation therapeutics in those diseases where decrease in p300/CBP mediated histone acetylation is known. The major drawback with this activator is that it does not permeate into the cells. We treated HeLa cells with various doses of CTPB for a period of 24 hours and analyzed histone acetylation status by western blotting probing acetylated histone H3 antibody against acid extracted histones. Across lanes we do not observe any change in the acetylation status of histones. This is suggestive that the molecule could be impermeable to the cells. We were constantly in the look out for any system that could ferry this drug to the cell nucleus. Such

a system would immensely help investigate the effect of activation of p300 HAT in cells. We hit upon one such drug delivery system in the form of glucose derived carbon nanospheres.

4.2. Nanoparticles, new tools for drug delivery:

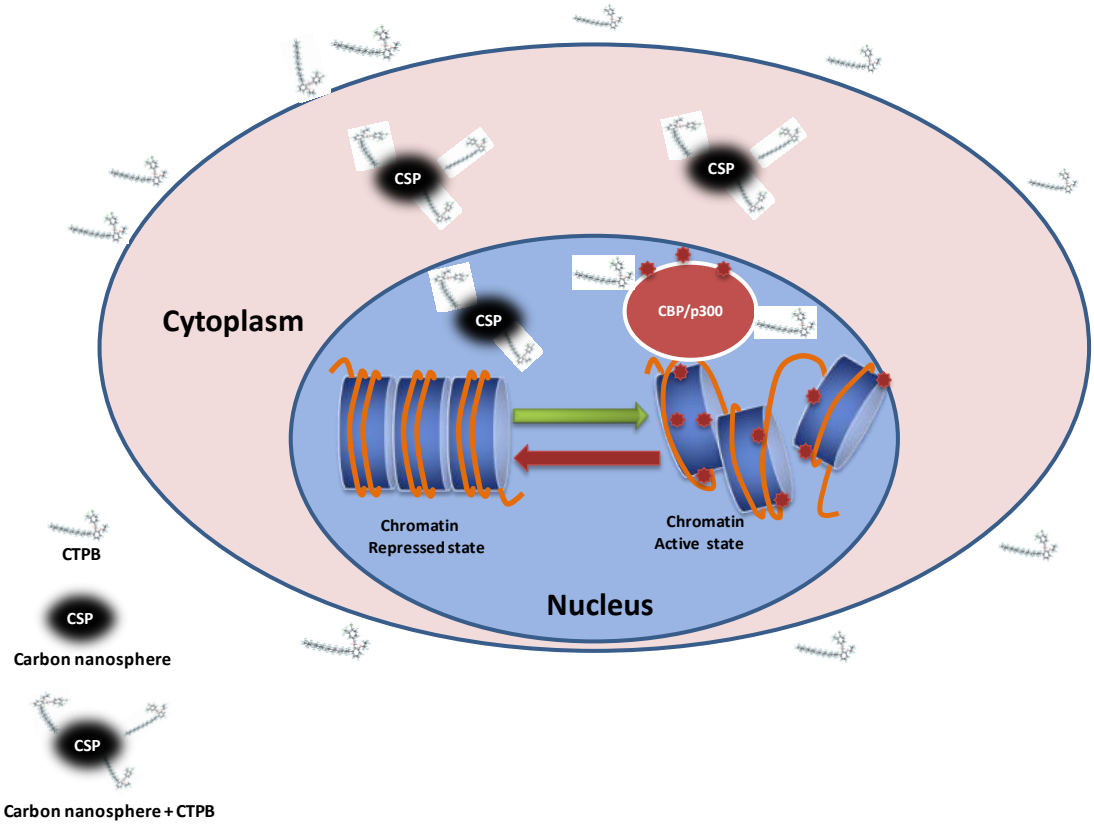


Figure 4.2. Carbon nanospheres bind to CTPB by physical adsorption and ferry them into the nucleus where CTPB activates p300/CBP and results in active state of chromatin.

Nanomaterials in biology has opened the door to a better understanding of cellular processes through imaging and improved therapeutic procedures, especially in drug delivery. Nanoparticles such as silica, LDH clay, micelles, polymer nanoparticles, and carbon nanotubes are being actively explored for the purpose of intracellular drug delivery. Most often, these nanoparticles require several surface chemical modifications to attach the drug molecules and additional fluorescent tags. This inevitably affects the cellular uptake and metabolism of the nanomaterial. However nanoparticles have been useful in transporting nucleic acids, proteins, and drug molecules across the cell membrane. The drawback of these nanoparticles is the inefficiency to

breach the nuclear membrane. This limits their use in many applications. However, nuclear targeting carriers such as peptides and polyethyleneimines do exist. These are positively charged moieties and face intense serum inhibition, since most of the serum is made up of negatively charged proteins. But the carbon nanospheres (CSPs) derived from the hydrothermal treatment of glucose could overcome all of the above shortcomings and entered the cell nuclei **(Figure 4.2).** Importantly, these CSPs are intrinsically fluorescent and do not require any additional fluorescent tags to track them inside the cells (Selvi et al., 2009). Intrinsic fluorescence makes it easy to track them in cells. Once we could track these carbon nanospheres into the cell nucleus we ensured that we could observe the same in animal models. We injected the nanospheres intraperitoneally into rats and then harvested tissues after 72 hours. These tissues were formaldehyde fixed and paraffin embedded. When sections were taken we could observe the intrinsic fluorescence emanating from the nanospheres in almost all of the tissues. Strikingly, these nanospheres could also enter the brain tissue by crossing the blood brain barrier. It was important since any carrier molecule will have to be able to penetrate this barrier in order to deliver small molecules to the brain. It was also observed that there was accumulation of nanospheres more in the brain than in other tissues at 72 hours. The percentage of cells that had nanospheres within them was higher in brain than in either liver or spleen even though it was the liver and the spleen that would be expected to harbor most of these particles considering their roles in removal of particulate foreign particles in the body. Having ensured that the nanosphere could permeate into the nucleus in both cell culture and animal models we also verified whether these could act as efficient carriers. We checked for the ability of these nanospheres to conjugate CTPB. The conjugation of the nanospheres and CTPB was mediated by physical adsorption without requiring any covalent bonding between the carrier and the small molecule. An extended Energy-dispersive X-ray spectroscopy confirmed that the molecule CTPB was indeed conjugated to the glucose derived carbon nanosphere albeit in a physical manner. The surface of carbon nanosphere has hydrophobic pockets and crevices that could account this physical adsorption. Since we had a delivery system in place we decided to look for better and efficient activators of p300. Some of these small molecules were of immense help in understanding the nature of the enzyme activation when probed by Surface Enhanced Raman Spectroscopy (SERS) (Mantelingu et al., 2007; Arif et al., 2007). The earlier works also led to elucidation of the autoacetylation based structural changes in p300 as probed by SERS analysis. Here we had found that the small

molecule activators bind to a region near the HAT domain but not to the HAT domain itself. It was also found that derivatives that lacked the penta-decyl side chain of CTPB could access the binding pocket better than CTPB thus making them better activators.

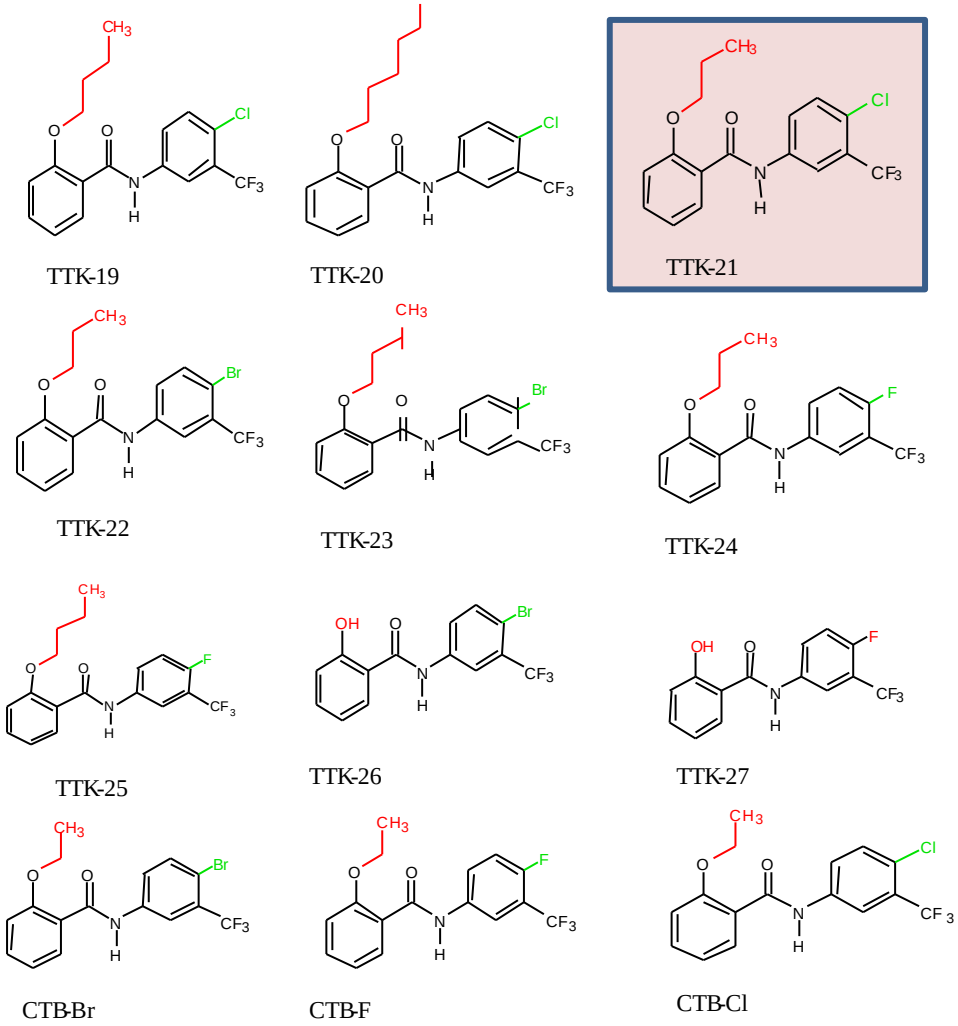


Figure 4.3: Structures of TTK derivatives: Analogues of CTB that have been selected for screening for their HAT activation potential.

4.3. Identification of a novel, small molecule activator of CBP/p300 and its characterization *in vitro*:

In an effort to understand the mechanistic aspects of p300 activation and the chemical entities within these small molecules that are important to activate p300, we derivatized various small

molecules from CTPB. The initial, tail less derivatives, of CTPB were nomenclatured as CTB. CTB was further derivatised to TTK series of molecules (**Figure 4.3**). These molecules were subjected to histone acetyltransferase assays (Filter binding assay) to check for their ability to activate p300/CBP HAT. Core histones purified from HeLa nuclear pellet was used as the substrate and p300 purified from baculovirus infected Sf21 cells was used to determine the HAT

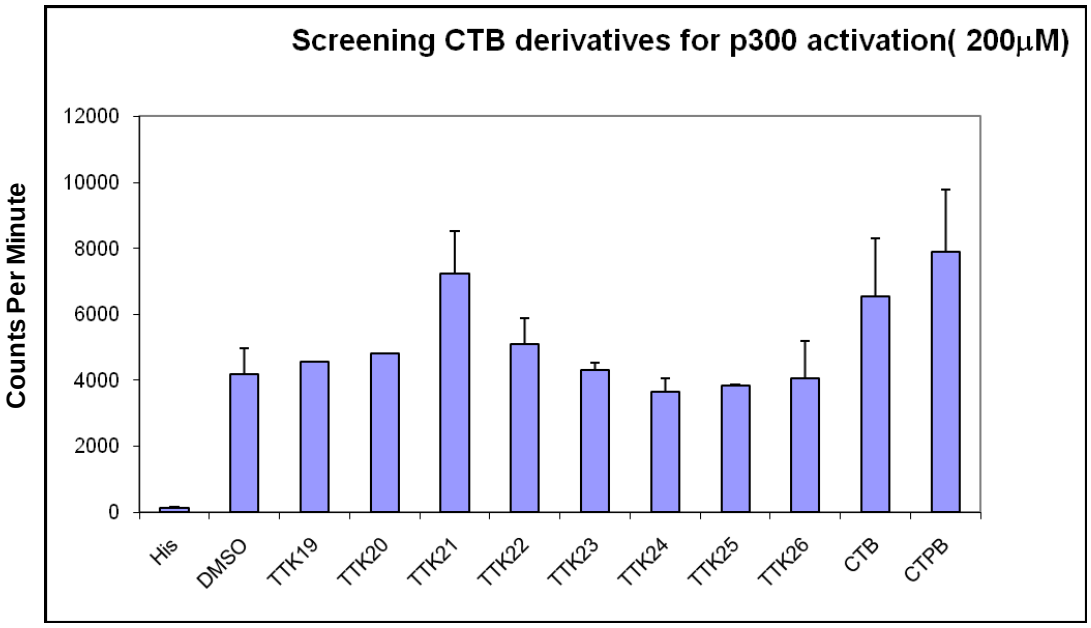


Figure 4.4: Screening TTK series for their HAT modulatory activity: Screening of TTK series (TTK19, TTK20, TTK21, TTK22, TTK23, TTK24, TTK25, TTK26), all derivatives of CTB for their p300 activation activity was done by filter binding assay at 200μM concentration and data tabulated.

modulation activity of these derivatives. All molecules were used at a concentration of 200μM in the HAT assays in the initial screen. TTK21 showed significant increase in radioactivity counts compared to other derivatives (**Figure 4.4**). The activation by TTK21 was comparable to both CTPB and CTB. In fact TTK21 consistently showed better activation than its parent molecule CTB with p300. To test for dose dependent activation by TTK21 of p300, histone acetyltransferase assay were performed in the same way using

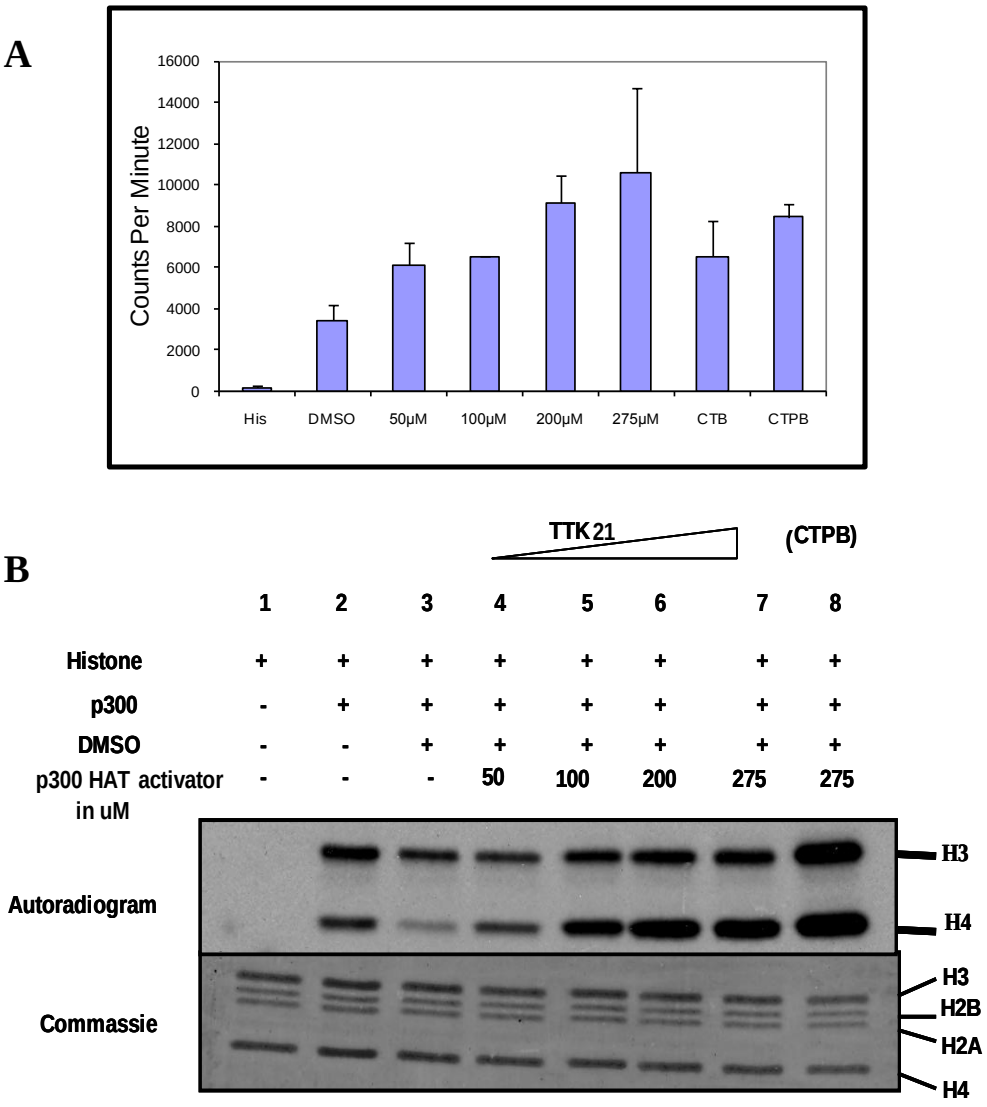


Figure 4.5: TTK21 activates p300 in a dose dependent manner: A. Filter binding assay to show activation of p300 by TTK21. Dose dependent activation of p300 by TTK21 as seen by Filter binding assay using TTK21 at 50µM, 100µM, 200µM, and 275µM. CTPB at 275µM in the last lane serves as positive control. **B.** Gel fluorography assay to show dose dependent activation of p300 by TTK21.TTK21 dissolved in DMSO was added to reaction mixture to the desired concentration. Lane 1- Histone only. Lane 2- p300 enzyme added. Lane 3- DMSO added. Lane 4- 50µM TTK21, Lane 5- 100µM TTK21, Lane 6 – 200µM TTK21, Lane 7 – 275µM TTK21, Lane 8 – 275µM CTPB.

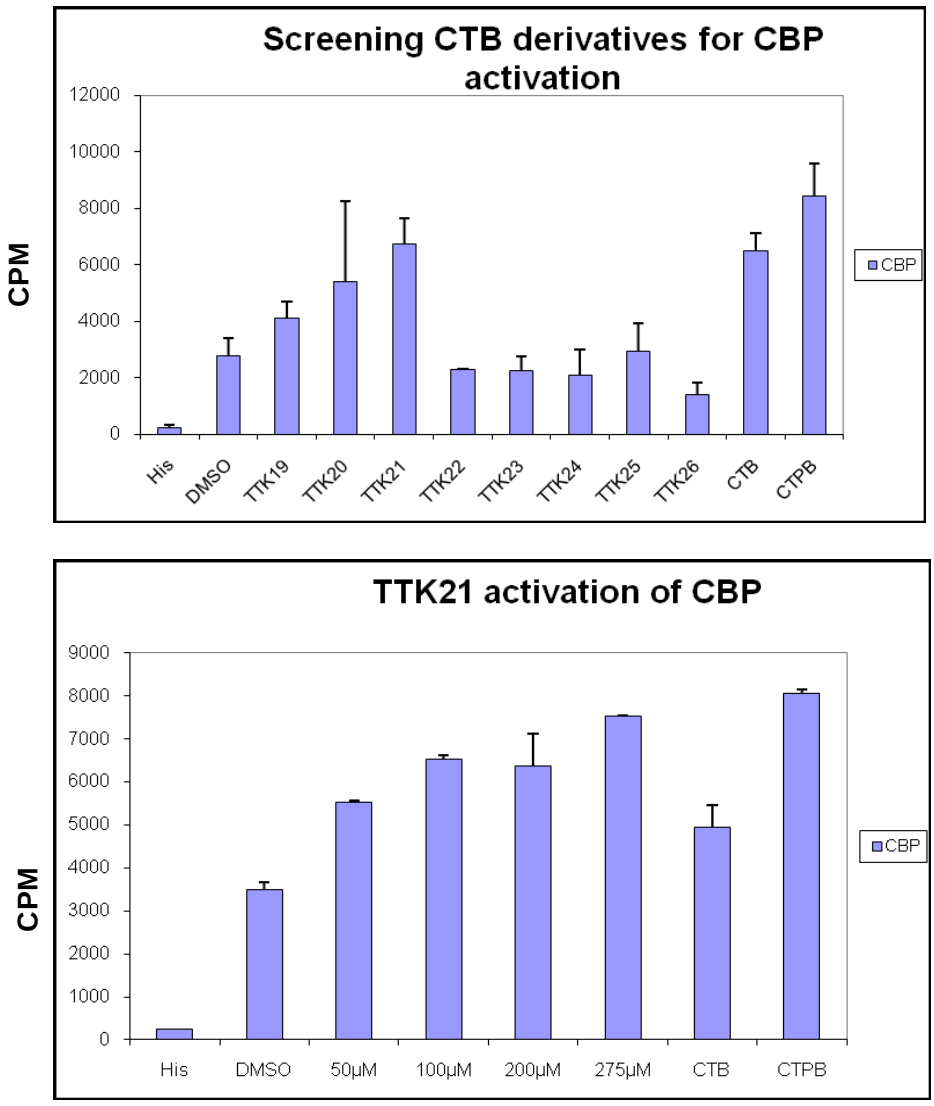


Figure 4.6: TTK21 activates CBP in a dose dependent manner. A. Screening of TTK series (TTK19, TTK20, TTK21, TTK22, TTK23, TTK24, TTK25, TTK26), all derivatives of CTB for their CBP activation activity was done by filter binding assay at 200μM concentration and data tabulated in CPM (Scintillation Counts per Minute) **B**. Dose dependent activation of CBP by TTK21 as seen by Filter binding assay using TTK21 at 50μM, 100μM, 200μM, and 275μM. CTB and CTPB at 275μM in the last lane serves as positive control.

increasing concentrations of TTK21. We tested 50μM, 100μM, 200μM and 275μM concentration of TTK21. TTK21 activated p300 in a dose dependent manner starting from 50μM itself (**Figure 4.5 A**). Maximum activation of TTK21 is seen at 250-275μM range which is similar to its parent molecule CTB. The same is also reported for CTPB (Balasubramanyam et al., 2003). TTK21 mediated activation, was comparable to CTPB. We also performed Gel

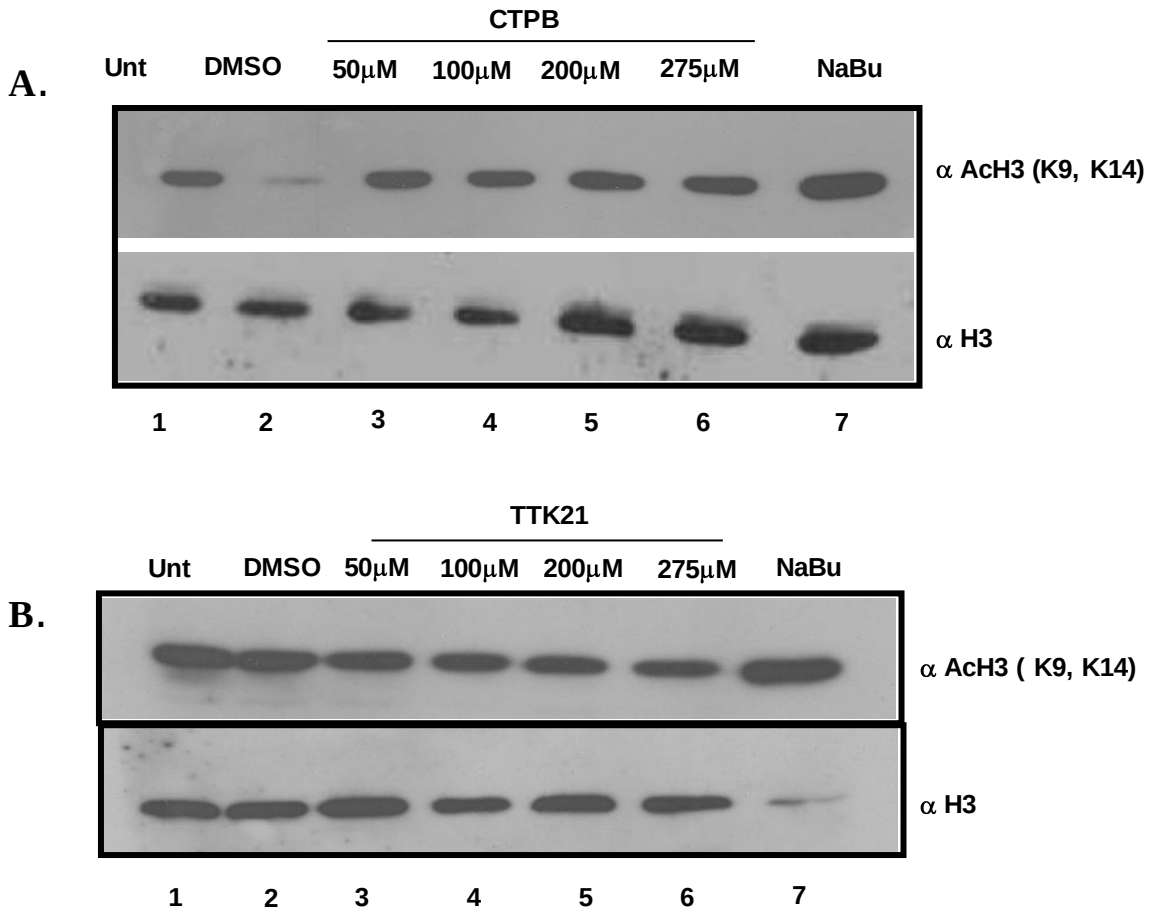


Figure 4.7: CTPB and TTK21 are probably impermeable to cells. **A**. Western blot analysis of CTPB untreated and treated HeLa cells for 24 hours. Lane 1- Untreated cells; Lane 2- DMSO treated cells; Lane 3 – 50μM CTPB treatment; Lane 4 – 100μM CTPB. Lane 5 – 200μM CTPB. Lane 6- 275μM CTPB. Lane 7- 500μM Sodium butyrate (NaBu) treated cells. Probing was done using Anti acetylated H3 for K9 and K14 antibody. As loading control, re-probing of the same blot was done by anti H3 antibody. **B**. Western blot analysis of TTK21 untreated and treated HeLa cells for 24 hours. Lane 1- Untreated cells, Lane 2- DMSO treated cells, Lane 3 – 50μM TTK21 treatment, Lane 4 – 100μM TTK21. Lane 5 – 200μM TTK21. Lane 6- 275μM TTK21. Lane 7- 500μM Sodium butyrate (NaBu) treated cells. Primary probing of the blot was done with AcH3 and re-probing was done by H3 antibody.

fluorography studies. TTK21 activated p300 in a dose dependent manner as observed from the intensity of the bands (**Figure 4.5 B**). Lane 3 contained DMSO and served as negative control. Compared to lane 3, lanes 4-7 show increased intensity of bands. At 275μM activation was

comparable to CTPB in lane 8 of same concentration. The same screening experiment was repeated with full length Flag-tagged CBP and similar result was found. TTK21 significantly activated CBP better than other derivatives and activation was again comparable to CTPB and CTB (**Figure 4.6 A**). Similar results were also obtained in a filter binding assay using TTK21 which activated CBP better than CTB. Filter binding assays were repeated using increasing concentrations of TTK21 with CBP HAT (**Figure 4.6 B**). With p300 and CBP bearing striking sequence homology similar activation for CBP was expected. Consistent with this, TTK21 activated CBP HAT as much as CTPB and CTB as shown. TTK21 activated CBP at 50μM itself very similar to its activation of p300. With increasing concentration of TTK21, at 100μM, 200μM, 275μM, increase in acetylation was observed. This was similar to activation of p300. The activation at 275μM was comparable to that of CTB and CTPB at the same concentration and even better than CTB. Thus we observed that TTK21 is a potent HAT activator of both p300 and CBP. TTK21 is a molecule that is tail less, thus smaller than CTPB but equally potent in activating the HATs. CTPB, as mentioned before, is a p300/CBP specific. Considering the large scale functional significance of acetylation and deacetylation in a cell, we were interested in knowing what effect such an activator would have on chromatin templated phenomena in the cell. Having verified the in-vitro efficacy of TTK21 as a potent HAT activator we wanted to check whether it would activate p300/CBP in cells and thus increase the cellular histone acetylation. Unfortunately, cell membrane is impermeable to CTPB, as well as its derivative TTK21 (**Figure 4.7 A** and **B**). HeLa cells were treated with CTPB or TTK21 in petri dishes for a period of 24 hours. Histones were isolated and Western blot analysis was done using anti-acetylated H3 antibody. In Fig 4.7 A and B; Lane 2; contained histones from DMSO treated cells. Lanes 3-6 contained Histones from cells treated with 50μM, 100μM, 200μM and 275μM of CTPB (A) or TTK21 (B). Lane 2 in A; with histones of DMSO treated cells, shows very less intensity that is lesser than the intensity of the band of untreated cells and thus could be an experimental artifact. Across lanes 3-5 in both panel 1 and panel 2 there is no increase in intensity of the bands suggesting that there is no hyperacetylation of histones. This is an indication that CTPB and TTK21 both are probably impermeable to the cell membrane. So a vehicle that would serve to carry the drug molecule inside would help in vivo activation of histone acetyltransferase. The only other ways of doing this is microinjection of the small molecule. However this is a single cell technique and relevance of the data in actual in vivo

scenario cannot be claimed. Since we had reported for the first time, the delivery of CTPB into the cell by conjugating it with 500nm carbon nanospheres, we wanted to know what effect would TTK21 have on global activation of p300/CBP and global histone acetylation and thus transcriptional regulation and gene expression.

4.4. Activation of p300 mediated histone acetylation in vivo using carbon nanospheres as drug delivery vehicle:

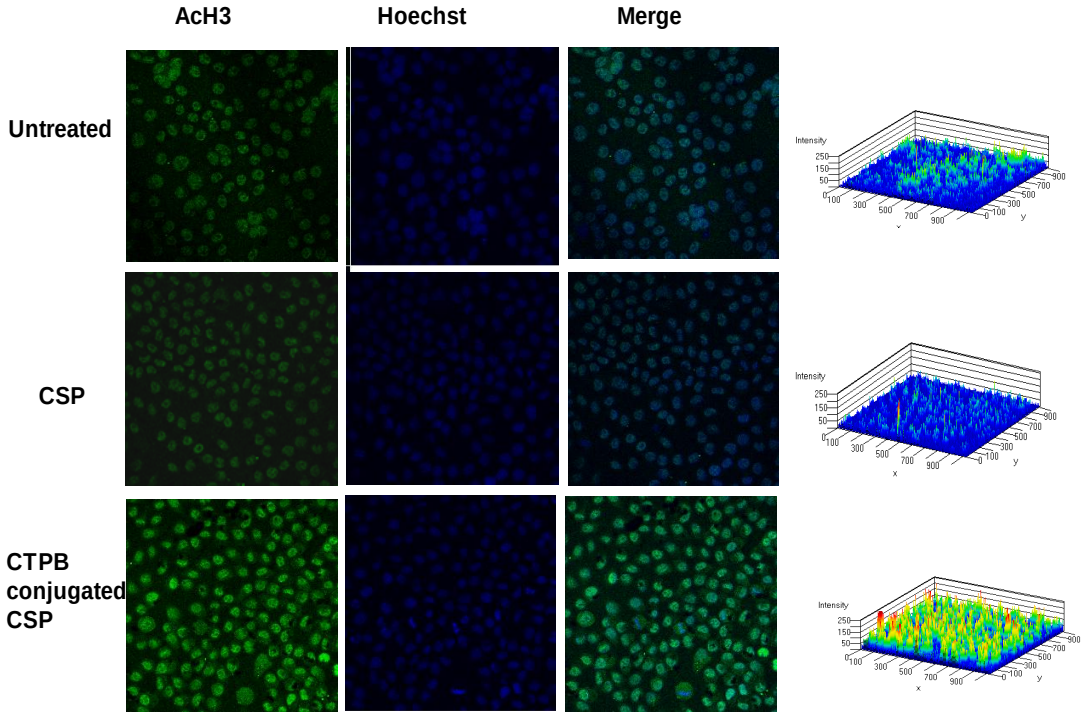


Figure 4.8: CTPB can activate p300 in vivo when conjugated to Carbon nanospheres. (A) Activation of p300 mediated histone acetylation as visualized by immunofluorescence of HeLa cells with anti AcH3 antibody. Panel 1 shows immunofluorescence with anti-AcH3 antibody. Panel 2 shows DNA staining by Hoechst and merge of antibody and Hoechst shown in Panel 3.Intensity plots for each is shown beside Merge images. Intensity plot is shown beside each merge.

In this direction, HeLa monolayer culture was treated with nanosphere conjugated CTPB and its effect on histone acetylation was observed by immunofluorescence using anti-acetylated H3

antibody (**Figure 4.8**). While untreated cells and cells treated with nanospheres without conjugation served as negative control, cells treated with Sodium butyrate, a broad spectrum Histone deacetylase inhibitor, served as positive control. Only cells treated with CTPB conjugated nanospheres (1mg nanosphere to 3 mg CTPB) treated cells showed increased global histone acetylation levels that were reflective of p300/CBP activation by CTPB (Figure 7). Untreated cells and non conjugated nanospheres do not show hyperacetylation. It has already been reported that these nanospheres get accumulated in the animal brain by 3 days after intraperitoneal injection. It is also known that they can cross the blood brain barrier and thus cause hyperacetylation of neurons and glial cells. It was also reported that decreased global acetylation is a feature of apoptotic neurons in neurodegenerative disorders, primarily due to degradation of p300/CBP. It would be interesting to explore the effect of HAT activation as a therapeutic option. In an effort in this direction activation of histone acetylation has been achieved in rat dorsal hippocampi using CTPB conjugated carbon nanospheres (reviewed in Selvi BR et al., *Biochim Biophys acta*, 2010). Since, TTK21 is impermeable to cells a similar approach is being followed. This approach involves conjugation of TTK21 to glucose derived carbon nanospheres. This would overcome the problem of this molecule being impermeable to cells. The conjugation of TTK21 to glucose derived carbon nanospheres in an attempt to deliver it inside the cells is currently being attempted. TTK21 thus adds to the list of very few HAT activators known so far.

Chapter 5

Summary

The chapter provides a summary of the research work presented in the thesis, highlighting the significant findings.

Although, it is well established that p300 is a bonafide transcriptional co-activator and a HAT, its contributions towards the epigenetic phenomena is yet to be clearly understood in terms of cellular physiology. Small molecule modulators of chromatin modifying enzymes are efficient tools for understanding the complex functional network. Small molecules help in delineating the role of enzymatic activity versus coactivation property in various physiological outcomes. This research work was initiated towards understanding the cellular physiology of histone lysine acetylation and associated epigenetic marks in neurons using small molecule modulators. Hence, we decided to elucidate the role of p300 in maintenance of epigenetic marks by using a small molecule inhibitor, RTK1.

RTK1, also known as Plumbagin, has been found to have a potent p300 HAT inhibitory activity. In the present research project RTK1 has been used to elucidate the epigenetic state of histone modifications in neural cells i.e., SH-SY5Y, a neuroblastoma cell line as well as non neuronal cells and animal models. On treatment of RTK1 to cells, histone acetylation decreased drastically on histone H3K9, 14; histone H2AK5 and histone H4K5, 8 residues. These are also sites for p300 and PCAF mediated acetylation. Interestingly, in addition to acetylation we found that histone H3S10 phosphorylation and H3K4 trimethylation significantly decreased post treatment of RTK1. Thus RTK1 decreased transcriptional activation marks in neuronal cells. However, transcription repressive marks like histone H3K9 di/ tri-methylation did not alter. Collectively, these data suggested that RTK1 could inhibit epigenetic marks specific for transcriptional activation in the cellular context. Analysis also revealed that such drastic alteration of Epigenetic mark preceded neural cell death as observed by MTT, FACS and DNA fragmentation analysis. RTK1 treatment up regulated pro- apoptotic genes belonging to the p53 pathway. Contrary to what was expected, RTK1 mediated decrease in histone acetylation also activated some

neuroprotective genes that are p300 coactivation dependent. Recent reports describe neuroprotective roles for RTK1. This contradictory finding matches with such studies. The results in chapter 3 also proves that the same pattern of inhibition of transcriptional activation marks, could happen with the use of IsoGarcinol a different molecule which is also a HAT inhibitor. The same pattern of inhibition with RTK1 was also observed in non neuronal cells like HeLa and in the mouse brain, making these general phenomena downstream of HAT inhibition, and not a cell line or a small molecule specific effect.

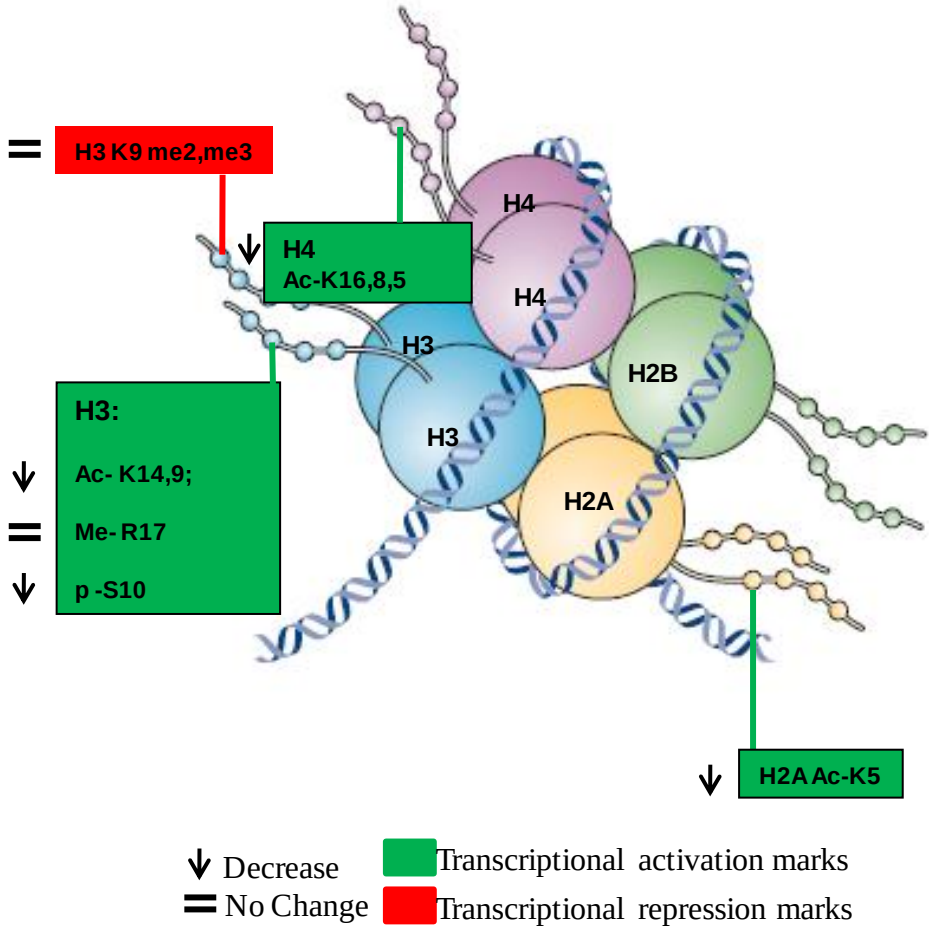


Figure 5.1: Summary of alterations in histone post translational marks post HAT inhibition by plumbagin (RTK1).

Histone acetylation is vital for neuronal gene expression and its decrease is reported in various neurodegenerative states. Incidentally, broad spectrum Histone deacetylase inhibitors (HDACi) have been proven to have protective effect in neurodegenerative disorders. Presumably, neuroprotection was achieved mainly due to induction of histone hyperacetylation. However, HDAC inhibitors are generally non specific or possess broad spectrum activity. Considering that these degenerative pathologies are associated with reduced p300/CBP mediated histone acetylation, an alternative way of reversing the same would be to activate p300 enzymatic activity. We have established CTPB as a small molecule activator p300. To generate more efficient molecules we further derivatised CTPB to various analogues and screened them for their p300 activation potential. Through this screening we could narrow our search to a new molecule, TTK21. TTK21, in a dose dependent manner activated both p300 and CBP similar to its parent molecules CTPB and CTB. However, TTK21, like CTPB, was impermeable to cells. Previously, we had employed self-fluorescent, cell permeable carbon nanospheres which also entered cell nucleus, to improve permeability of CTPB and thus observed histone hyperacetylation in cells as well as mice brain. By using these carbon nanospheres, an attempt to target TTK21 to the cell nuclei and its effect on activation of histone acetylation will be made, as has been shown for nanosphere - CTPB conjugates which could be delivered to brain resulting in hyperacetylation of histones in brain. The ability of TTK21 to induce acetylation of histones in cell culture system and in animal models is currently being studied.

In conclusion, this research work has led to the elucidation of histone acetylation associated epigenetic network with the help of a HAT inhibitor, RTK1. It has also led to identification of TTK21 as a novel HAT activator. These small molecules have been used as probes to understand cellular physiology effected by histone acetylation and associated epigenetic marks. This work highlights the importance of HAT mediated histone acetylation in neural gene expression and neuron cell survival. This research has also elucidated the role for novel activators of HAT and novel delivery agents that could be used to activate HAT in vivo. This is a novel approach for therapeutics as well.

References

Acharya BR, Bhattacharyya B, Chakrabarti G. 2008. The natural naphthoquinone plumbagin exhibits antiproliferative activity and disrupts the microtubule network through tubulin binding. *Biochemistry*. **29**: 7838-7845.

Ahmad A, Banerjee S, Wang Z, Kong D, Sarkar FH. 2008. Plumbagin-induced apoptosis of human breast cancer cells is mediated by inactivation of NF-kappaB and Bcl-2. *J Cell Biochem*. **105**:1461-71.

Ahmed T,Frey JU. 2005. Plasticity-specific phosphorylation of CaMKII,MAP-kinases and CREB during late-LTP in rat hippocampal slices in vitro. *Neuropharmacology*. **49**:477-492.

Ait-Si-Ali S, Carlisi D, Ramirez S, Upegui-Gonzalez LC, Duquet A, Robin P, Rudkin B, Harel-Bellan A, Trouche D. 1999. Phosphorylation by p44 MAP Kinase/ERK1 stimulates CBP histone acetyl transferase activity in vitro. *Biochem Biophys Res Commun***. 262**:157-162.

Alarcón JM, Malleret G, Touzani K, Vronskaya S, Ishii S, Kandel ER, Barco A. 2004. Chromatin acetylation, memory, and LTP are impaired in CBP+/- mice: a model for the cognitive deficit in Rubinstein-Taybi syndrome and its amelioration. *Neuron.* **42**:947-59.

Altaf M, Utley RT, Lacoste N, Tan S, Briggs SD, Côté J. 2007. Interplay of chromatin modifiers on a short basic patch of histone H4 tail defines the boundary of telomeric heterochromatin. *Mol Cell*. **28**:1002-1014.

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

Arif M, Kumar GV, Narayana C, Kundu TK.. 2007. Autoacetylation induced specific structural changes in histone acetyltransferase domain of p300: probed by surface enhanced Raman spectroscopy. *J Phys Chem B*. **111**:11877-9.

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

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

Balasubramanyam K, Swaminathan V, Ranganathan A, Kundu TK. 2003. Small molecule modulators of histone acetyltransferase p300. *J Biol Chem*. **278**:19134-19140.

Balasubramanyam K, Varier RA, Altaf M, Swaminathan V, Siddappa NB, Ranga U, Kundu TK. 2004. Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferase-dependent chromatin transcription. *J Biol Chem*. **279**:51163-561171.

Bandyopadhyay K, Banères JL, Martin A, Blonski C, Parello J, Gjerset RA. 2009. Spermidinyl-CoA-based HAT inhibitors block DNA repair and provide cancer-specific chemo- and radiosensitization. *Cell Cycle*. **8**:2779-88.

References

Bannister, A. J., Miska, E. A., Görlich, D., Kouzarides, T. 2000. Acetylation of importin-alpha nuclear import factors by CBP/p300. *Curr. Biol*. **10**:467-470.

Bar-Sela, G., Epelbaum, R., Schaffer, M. 2010. Curcumin as an anti-cancer agent: review of the gap between basic and clinical applications. *Curr. Med. Chem*. **17**:190-197.

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.

Berger SL. 2007. The complex language of chromatin regulation during transcription. *Nature*. **447**: 407-412.

Bertrand E, Erard M, Gómez-Lira M, Bode J. 1984. Influence of histone hyperacetylation on nucleosomal particles as visualized by electron microscopy. *Arch Biochem Biophys*. **229:** 395-398.

Biel M, Kretsovali A, Karatzali E, Papamatheakis J, Giannis A. 2004. Design, synthesis, and biological evaluation of a small-molecule inhibitor of the histone acetyltransferase Gcn5. *Angew Chem Int Ed Engl*. **43**: 3974-6.

Black JC, Choi JE, Lombardo SR, Carey MA. 2006. Mechanism for coordinating chromatin modification and preinitiation complex assembly. *Mol. Cell*. **23**:809-818.

Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*.**361**: 31-39.

Bode J, Henco K, Wingender E. 1980. Modulation of the nucleosome structure by histone acetylation. *Eur J Biochem.* **110**:143-152.

Borrow J, Stanton VP Jr, Andresen JM, Becher R, Behm FG, Chaganti RS, Civin CI, Disteche C, Dubé I, Frischauf AM, Horsman D, Mitelman F, Volinia S, Watmore AE, Housman DE. 1996. The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. *Nat Genet*. **14**:33-41.

Boutillier AL, Trinh E, Loeffler JP. 2003. Selective E2F-dependent gene transcription is controlled by histone deacetylase activity during neuronal apoptosis. *J Neurochem*. **84**:814–828.

Bowers EM, Yan G, Mukherjee C, Orry A, Wang L, Holbert MA, Crump NT, Hazzalin CA, Liszczak G, Yuan H, Larocca C, Saldanha SA, Abagyan R, Sun Y, Meyers DJ, Marmorstein R, Mahadevan LC, Alani RM, Cole PA.2010. Virtual ligand screening of the p300/CBP histone acetyltransferase: identification of a selective small molecule inhibitor. *Chem Biol*. **17**:471-482.

Brachmann CB, Sherman JM, Devine SE, Cameron EE, Pillus L, Boeke JD. 1995. The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes Dev*. **9**:2888-28902.

Brownell JE, Allis CD. 1995. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in Tetrahymena macronuclei. *Proc Natl Acad Sci U S A.* **3**:6364-6368.

C. Barbé, J. Bartlett, L. Kong, K. Finnie, H.Q. Lin, M. Larkin, S. Calleja, A. Bush, G. Calleja. 2004. Silica Particles: A Novel Drug-Delivery System. **16**: 1959–1966.

Carlezon WA Jr, Duman RS, Nestler EJ. 2005. The many faces of CREB. *Trends Neurosci*. **28**:436-45.

References

Chan HM, LaThangue NB. 2001. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J. Cell Sci.* **114**: 2363-2373.

Chawla S,Bading H. 2001. CREB/CBP and SRE-interacting transcriptional regulators are fast on-off switches: duration of calcium transients specifies the magnitude of transcriptional responses. *J Neurochem*. **79**:849-858.

Cheung P, Tanner KG, Cheung WL, Sassone-Corsi P, Denu JM, Allis CD. 2000. Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation. *Mol Cell.* **5**: 905-915.

Choe ES,Wang JQ. 2002. Regulation of transcription factor phosphorylation by metabotropic glutamate receptor-associated signaling pathways in rat striatal neurons. *Neuroscience*. **114**: 557-65.

Choi KC, Jung MG, Lee YH, Yoon JC, Kwon SH, Kang HB, Kim MJ, Cha JH, Kim YJ, Jun WJ, Lee JM, Yoon HG. 2009. Epigallocatechin-3-gallate, a histone acetyltransferase inhibitor, inhibits EBV-induced B lymphocyte transformation via suppression of RelA acetylation. *Cancer Res*. **69**:583-92.

Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. 2009. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*. **325**:834-40.

Choy, J. H., Kwak, S. Y., Jeong, Y. J., Park, J. S. 2000. Inorganic Layered Double Hydroxides as Nonviral Vectors. *Angew. Chem.Int. Ed*. **39**: 4041-4045.

Cooke SF, Bliss TV. 2006. Plasticity in the human central nervous system. *Brain*. **129**:1659-73.

Costa-Mattioli M, Sonenberg N. 2008. Translational control of gene expression: a molecular switch for memory storage. *Prog Brain Res*. **169**:81-95.

Dal Piaz F, Tosco A, Eletto D, Piccinelli AL, Moltedo O, Franceschelli S, Sbardella G, Remondelli P, Rastrelli L, Vesci L, Pisano C, De Tommasi N. 2010. The identification of a novel natural activator of p300 histone acetyltranferase provides new insights into the modulation mechanism of this enzyme. *Chembiochem.* **11**:818-827.

Das C, Hizume K, Batta K, Kumar BR, Gadad SS, Ganguly S, Lorain S, Verreault A, Sadhale PP, Takeyasu K, Kundu TK. 2006. Transcriptional coactivator PC4, a chromatin-associated protein, induces chromatin condensation. *Mol Cell Biol*.**26**:8303-15.

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.

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

Dekker FJ, Ghizzoni M, van der Meer N, Wisastra R, Haisma HJ. 2009. Inhibition of the PCAF histone acetyl transferase and cell proliferation by isothiazolones. *Bioorg Med Chem*. **17**:460-466.

Dekker FJ, Hasima HJ. 2009. Histone acetyltransferases as emerging drug targets. *Drug Discov Today.* **14**:942-948.

Denslow SA, Wade PA. 2007. The human Mi-2/NuRD complex and gene regulation. *Oncogene*. **26**:5433-5438.

Dou Y, Milne TA, Tackett AJ, Smith ER, Fukuda A, Wysocka J, Allis CD, Chait BT, Hess JL, Roeder RG. 2005. Physical association and coordinate function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase MOF. *Cell.* **121**:873-885.

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

Dulac C. 2010. Brain function and chromatin plasticity. *Nature*. **465**:728-35.

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

Eisen A, Lucchesi JC. 1998. Unraveling the role of helicases in transcription. *Bioessays*. **20**:634-41.

Feng J, Fouse S, Fan G. 2007. Epigenetic regulation of neural gene expression and neuronal function. *Pediatr Res.* **61**:58-63.

Ferrante RJ, Kubilus JK, Lee J, Ryu H, Beesen A, Zucker B, Smith K, Kowall NW, Ratan RR, Luthi-Carter R, Hersch SM. 2003. Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *J Neurosci*. **23**:9418–9427.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME. 1997. CREB: a major mediator of neuronal neurotrophin responses. *Neuron*. **19**:1031-1047.

Frye RA. 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. *Biophys. Res. Commun*. **273**:793–798.

Gayther SA, Batley SJ, Linger L, et al. (2000). Mutations truncating the EP300 acetylase in human cancers. *Nat Genet*. **24**: 300-3.

Giles RH, Peters DJ, Breuning MH. 1998. Conjunction dysfunction: CBP/p300 in human disease. *Trends Genet.* **14**:178-183.

Girdwood D, Bumpass D, Vaughan OA, Thain A, Anderson LA, Snowden AW, Garcia-Wilson E, Perkins ND, Hay RT. 2003. p300 transcriptional repression is mediated by SUMO modification. *Mol Cell*. **11**:1043-1054.

Gomathinayagam R, Sowmyalakshmi S, Mardhatillah F, Kumar R, Akbarsha MA, Damodaran C. 2008. Anticancer mechanism of plumbagin, a natural compound, on non-small cell lung cancer cells. *Anticancer Res*. **28**:785-92.

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

Gray SG, De Meyts P. 2005. Role of histone and transcription factor acetylation in diabetes pathogenesis. *Diabetes Metab Res Rev*. **21**:416-33.

Gregoretti IV, Lee YM, Goodson HV. 2004. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J. Mol. Biol*. **338**:17–31.

Grossman SR, Deato ME, Brignone C, Chan HM, Kung AL, Tagami H, Nakatani Y, Livingston DM. 2003. Polyubiquitination of p53 by a ubiquitin ligase activity of p300. *Science*. **300**:342-344.

References

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

Gusterson RJ, Jazrawi E, Adcock IM, Latchman DS. 2003. The transcriptional co-activators CREB-binding protein (CBP) and p300 play a critical role in cardiac hypertrophy that is dependent on their histone acetyltransferase activity. *J Biol Chem*. **278**:6838-47.

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

Hazra B, Sarkar R, Bhattacharyya S, Ghosh PK, Chel G, Dinda B. 2002. Synthesis of plumbagin derivatives and their inhibitory activities against Ehrlich ascites carcinoma in vivo and Leishmania donovani Promastigotes in vitro. *Phytother Res*. **16**:133-137.

Hazzalin CA, Mahadevan LC. 2005. Dynamic acetylation of all lysine 4-methylated histone H3 in the mouse nucleus: analysis at c-fos and c-jun**. PLoS Biol**. 3:e393.

Hockly E, Richon VM, Woodman B, Smith DL, Zhou X, Rosa E, Sathasivam K, Ghazi-Noori S, Mahal A, Lowden PA, Steffan JS, Marsh JL, Thompson LM, Lewis CM, Marks PA, Bates GP. 2003. Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. *Proc Natl Acad Sci USA*. **100**: 2041–2046.

Horn PJ, Peterson CL. 2006. Heterochromatin assembly: a new twist on an old model. *Chromosome Res*. **14**:83-94.

Hsu YL, Cho CY, Kuo PL, Huang YT, Lin CC. 2006. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo. *J Pharmacol Exp Ther*. **318**:484-94.

Huang WC, Chen CC. 2005. Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. *Mol Cell Biol*. **25**:6592- 6602.

Impey S, Goodman RH. 2001. CREB signaling--timing is everything. *Sci STKE*. 2001:pe1.

Ito T, Bulger M, Pazin MJ, Kobayashi R, Kadonaga JT. 1997. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. *Cell.* **90**:145-55.

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

Jiang H, Nucifora FC Jr, Ross CA, DeFranco DB. 2003. Cell death triggered by polyglutamine-expanded huntingtin in a neuronal cell line is associated with degradation of CREB-binding protein. *Hum Mol Genet*. **12**:1–12.

Jin K, Mao XO, Simon RP, Greenberg DA. 2001. Cyclic AMP response element binding protein (CREB) and CREB binding protein (CBP) in global cerebral ischemia. *J Mol Neurosci*. **16**:49–56.

Josselyn SA, Nguyen PV. 2005. CREB,synapses and memory disorders: past progress and future challenges. *Curr Drug Targets CNS Neurol Disord*. **4**:481-97.

Kellum R. 2003. Is HP1 an RNA detector that functions both in repression and activation? *J Cell Biol*. **26**:671-672.

References

Keppler BR, Archer TK. 2008. Chromatin-modifying enzymes as therapeutic targets--Part 2. *Expert Opin Ther Targets***. 12**:1457-1467.

Kim MY, Mauro S, Gévry N, Lis JT, Kraus WL. 2004. NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. *Cell*. **17**:803-814.

Korzus E, Rosenfeld MG, Mayford M. 2004. CBP histone acetyltransferase activity is a critical component of memory consolidation. *Neuron***. 42**:961-972.

Kramer OH, Baus D, Knauer SK, Stein S, Jager E, Stauber RH, Grez M, Pfitzner E, Heinzel T. 2006. Acetylation of Stat1 modulates NF-kappaB activity. *Genes Dev*. **20**:473–485.

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

Kumar A, Choi KH, Renthal W, Tsankova NM, Theobald DE, Truong HT, Russo SJ, Laplant Q, Sasaki TS, Whistler KN et al. 2005. Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron*. **48**:303-314.

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

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

Lau OD, Kundu TK, Soccio RE, Ait-Si-Ali S, Khalil EM, Vassilev A, Wolffe AP, Nakatani Y, Roeder RG, Cole PA. 2000. HATs off: selective synthetic inhibitors of the histone acetyltransferases p300 and PCAF. *Mol Cell*. **5**:589-595.

Lee DY, Hayes JJ, Pruss D, Wolffe AP. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell*. **72**:73-84.

Lee KK., Workman JL. 2007. Histone acetyltransferase complexes: one size doesn't fit all. *Nat. Rev. Mol. Cell Biol*. **8**:284-295.

Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD. 2004. Regulation of histone acetylation during memory formation in the hippocampus. *J Biol Chem*. **279**:40545-30559.

Levenson JM, Sweatt JD. 2005. Epigenetic mechanisms in memory formation. *Nat Rev Neurosci*. **6**:108-118.

Levine AA, Guan Z, Barco A, Xu S, Kandel ER, Schwartz JH. 2005. CREB-binding protein controls response to cocaine by acetylating histones at the fosB promoter in the mouse striatum. *Proc Natl Acad Sci*. **102**:19186-19191.

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

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

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

Liu Z, Cai W, He L, Nakayama N, Chen K, Sun X, Chen X, Dai H. 2007. In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat Nanotechnol*. **2**:47-52.

Liu X., Wang L, Zhao K, Thompson PR, Hwang Y, Marmorstein R, Cole PA. 2008. The structural basis of protein acetylation by the p300/CBP transcriptional coactivator. *Nature*. **451**:846-850.

Lo WS, Gamache ER, Henry KW, Yang D, Pillus L, Berger SL. 2005. Histone H3 phosphorylation can promote TBP recruitment through distinct promoter-specific mechanisms. *EMBO J*. **24**:997-1008.

Luo Y, Mughal MR, Ouyang TG, Jiang H, Luo W, Yu QS, Greig NH, Mattson MP. 2010. Plumbagin promotes the generation of astrocytes from rat spinal cord neural progenitors via activation of the transcription factor Stat3. *J Neurochem*. [Epub ahead of print].

Maison C, Bailly D, Peters AH, Quivy JP, Roche D, Taddei A, Lachner M, Jenuwein T, Almouzni G. 2002. Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. *Nat. Genet.* **30**:329-334.

Malenka RC, Bear MF. 2004. LTP and LTD: an embarrassment of riches. *Neuron*. **30**:5-21.

Manning ET, Ikehara T, Ito T, Kadonaga JT, Kraus WL. 2001. p300 forms a stable, template-committed complex with chromatin: role for the bromodomain. *Mol Cell Biol*. **21**:3876-3887.

Mantelingu K, Kishore AH, Balasubramanyam K, Kumar GV, Altaf M, Swamy SN, Selvi R, Das C, Narayana C, Rangappa KS, Kundu TK. 2007. Activation of p300 histone acetyltransferase by small molecules altering enzyme structure: probed by surface-enhanced Raman spectroscopy. *J Phys Chem B.* **111**:4527-34.

Mantelingu K, Reddy BA, Swaminathan V, Kishore AH, Siddappa NB, Kumar GV, Nagashankar G, Natesh N, Roy S, Sadhale PP, Ranga U, Narayana C, Kundu TK. 2007. Specific inhibition of p300-HAT alters global gene expression and represses HIV replication. *Chem Biol*. **14**:645-57.

Marambaud P, Wen PH, Dutt A, Shioi J, Takashima A, Siman R, Robakis NK. 2003. A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell*. **114**:635–645.

Marmorstein, R. 2001. Structure of histone acetyltransferases. *J. Mol. Biol*. **311**:433-444.

Marmorstein R, Roth SY. 2001. Histone acetyltransferases: function, structure, and catalysis. *Curr. Opin. Genet. Develop.* **11**:155–161.

Mayr B. Montminy M. 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol.* **2**: 599-609.

McBryant SJ, Adams VH, Hansen JC. 2006. Chromatin architectural proteins. *Chromosome Res*. **14**:39-51.

McCampbell A, Taye AA, Whitty L, Penney E, Steffan JS, Fischbeck KH. 2001. Histone deacetylase inhibitors reduce polyglutamine toxicity. *Proc Natl Acad Sci USA.* **98**:15179–15184.

Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. 2005. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science*. **307**:538-44.

Minamiyama M, Katsuno M, Adachi H, Waza M, Sang C, Kobayashi Y, Tanaka F, Doyu M, Inukai A, Sobue G. 2004. Sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Hum Mol Genet*. **13**:1183–1192.

References

Missero C, Calautti E, Eckner R, Chin J, Tsai LH, David M. 1995. Livingston and G. Paolo Dotto. Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A-associated p300 protein in terminal differentiation. *Proc Natl Acad Sci U S A.* **92**: 5451–5455.

Mroz RM, Noparlik J, Chyczewska E, Braszko JJ, Holownia A. 2007. Molecular basis of chronic inflammation in lung diseases: new therapeutic approach. *J Physiol Pharmacol*. **58**: 453-60.

Muraoka M, Konishi M, Kikuchi-Yanoshita R, Tanaka K, Shitara N, Chong JM, Iwama T, Miyaki M. 1996. p300 gene alterations in colorectal and gastric carcinomas. *Oncogene.* **12**:1565-1569.

Nair S, Nair RR, Srinivas P, Srinivas G, Pillai MR. 2008. Radiosensitizing effects of plumbagin in cervical cancer cells is through modulation of apoptotic pathway. *Mol Carcinog*. **47**:22-33.

Nakanishi S, Lee JS, Gardner KE, Gardner JM, Takahashi YH, Chandrasekharan MB, Sun ZW, Osley MA, Strahl BD, Jaspersen SL, Shilatifard A. 2009. Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1. *J Cell Biol*. **186**:371-377.

Naresh RA, Udupa N, Devi PU. 1996. Niosomal plumbagin with reduced toxicity and improved anticancer activity in BALB/C mice. *J Pharm Pharmacol*. **48**:1128-32.

Nasongkla N, Bey E, Ren J, Ai H, Khemtong C, Guthi JS, Chin SF, Sherry AD, Boothman DA, Gao J. 2006. Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems. *Nano Lett.* **6**:2427-2430.

Nightingale KP, Gendreizig S, White DA, Bradbury C, Hollfelder F, Turner BM. 2007. Cross-talk between histone modifications in response to histone deacetylase inhibitors: MLL4 links histone H3 acetylation and histone H3K4 methylation. *J Biol Chem*. **282**:4408-4416.

Nucifora FC Jr, Sasaki M, Peters MF, Huang H, Cooper JK, Yamada M, Takahashi H, Tsuji S, Troncoso J, Dawson VL, Dawson TM, Ross CA. 2001. Interference by huntingtin and atrophin-1 with cbpmediated transcription leading to cellular toxicity. *Science*. **291**:2423–2428.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell.* **87**:953-959.

Okada Y, Feng Q, Lin Y, Jiang Q, Li Y, Coffield VM, Su L, Xu G, Zhang Y. 2005. hDOT1L links histone methylation to leukemogenesis. *Cell*. **121**:167-78.

Oliveira AM, Abel T, Brindle PK, Wood MA. 2006. Differential role for CBP and p300 CREB-binding domain in motor skill learning. *Behav Neurosci*. **120**:724-9.

Oliveira AM, Wood MA, McDonough CB, Abel T. 2007. Transgenic mice expressing an inhibitory truncated form of p300 exhibit long-term memory deficits. *Learn Mem*. **14**:564-572.

Parimala, R., Sachdanandam, P. 1993. Effect of Plumbagin on some glucose metabolising enzymes studied in rats in experimental hepatoma. *Mol Cell Biochem***. 125**:59-63.

Park, Y.J., Luger, K. 2008. Histone chaperones in nucleosome eviction and histone exchange. *Curr Opin Struct Biol.* **18**:282-289.

Parthun MR. 2007. Hat1: the emerging cellular roles of a type B histone acetyltransferase. *Oncogene* **26**:5319-5328. Review.

Perkins ND. 2006. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene*. **25**: 6717–6730.

Petrij F, Giles RH, Dauwerse HG, Saris JJ, Hennekam RC, Masuno M, Tommerup N, van Ommen GJ, Goodman RH, Peters DJ, et al. 1995. Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature*. **376**:348-51.

Poleshko A, Einarson MB, Shalginskikh N, Zhang R, Adams PD, Skalka AM, Katz RA. 2010. Identification of a functional network of human epigenetic silencing factors. *J Biol Chem*. **285**:422-433.

Powolny AA, Singh SV. 2008. Plumbagin-induced apoptosis in human prostate cancer cells is associated with modulation of cellular redox status and generation of reactive oxygen species. *Pharm Res.* **25**:2171-2180.

Qin S, Parthun MR. 2002. Histone H3 and the histone acetyltransferase Hat1p contribute to DNA double-strand break repair. *Mol Cell Biol*. **22**:8353-8365.

Ralhan R, Pandey MK, Aggarwal BB. 2009. Nuclear factor-kappa B links carcinogenic and chemopreventive agents. *Front Biosci (Schol Ed).* **1**:45-60.

Ravindra KC, Selvi BR, Arif M, Reddy BA, Thanuja GR, Agrawal S, Pradhan SK, Nagashayana N, Dasgupta D, Kundu TK. 2009. Inhibition of lysine acetyltransferase KAT3B/p300 activity by a naturally occurring hydroxynaphthoquinone, plumbagin. *J Biol Chem*. **284**:24453-64.

Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, Shinkai Y, Allis CD. 2003. Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell.* **12**:1591-8.

Richmond TJ, Davey CA. 2003. The structure of DNA in the nucleosome core. *Nature*. **8**:6936.

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

Roelfsema JH, Peters DJ. 2007. Rubinstein-Taybi syndrome: clinical and molecular overview. *Expert Rev Mol Med.* **9**:1-16.

Roth JF, Shikama N, Henzen C, Desbaillets I, Lutz W, Marino S, Wittwer J, Schorle H, Gassmann M, Eckner R. 2003. Differential role of p300 and CBP acetyltransferase during myogenesis: p300 acts upstream of MyoD and Myf5. *EMBO J***. 22**:5186-5196.

Rouaux C, Jokic N, Mbebi C, Boutillier S, Loeffler JP, Boutillier AL. 2003. Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration. *EMBO J.* **22**:6537–6549.

Sagar V, Zheng W, Thompson PR, Cole PA. 2004. Bisubstrate analogue structure-activity relationships for p300 histone acetyltransferase inhibitors. *Bioorg Med Chem***. 12**:3383–3390.

Saha RN, Pahan K. 2006. HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis. *Cell Death Differ.* **13**:539-550.

Sandur SK, Ichikawa H, Sethi G, Ahn KS, Aggarwal BB. 2006. 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. *J Biol Chem*. **281**:17023-33.

Sbardella G, Castellano S, Vicidomini C, Rotili D, Nebbioso A, Miceli M, Altucci L, Mai A. 2008. Identification of long chain alkylidenemalonates as novel small molecule modulators of histone acetyltransferases. *Bioorg Med Chem Lett.* **18**:2788-2792.

Scharf AN, Meier K, Seitz V, Kremmer E, Brehm A, Imhof A. 2009. Monomethylation of lysine 20 on histone H4 facilitates chromatin maturation. *Mol Cell Biol.* **29**:57-67.

Selvi BR, Cassel JC, Kundu TK, Boutillier AL. 2010. Tuning acetylation levels with HAT activators: therapeutic strategy in neurodegenerative diseases. *Biochim Biophys Acta.* [Epub ahead of print]

Selvi BR, Jagadeesan D, Suma BS, Nagashankar G, Arif M, Balasubramanyam K, Eswaramoorthy M, Kundu TK. 2008. Intrinsically fluorescent carbon nanospheres as a nuclear targeting vector: delivery of membrane-impermeable molecule to modulate gene expression in vivo. *Nano Lett*. **8**:3182-3188.

Selvi BR, Kundu TK. 2009. Reversible acetylation of chromatin: implication in regulation of gene expression, disease and therapeutics. *Biotechnol J*. **4**:375-390.

Selvi BR, Pradhan SK, Shandilya J, Das C, Sailaja BS, Shankar GN, Gadad SS, Reddy A, Dasgupta D, Kundu TK. 2009. Sanguinarine interacts with chromatin, modulates epigenetic modifications, and transcription in the context of chromatin. *Chem Biol*. **16**:203-216.

Shandilya J, Swaminathan V, Gadad SS, Choudhari R, Kodaganur GS, Kundu TK. 2009. Acetylated NPM1 localizes in the nucleoplasm and regulates transcriptional activation of genes implicated in oral cancer manifestation. *Mol Cell Biol*. **29**:5115-5127.

Shi Kam NW, Jessop TC, Wender PA, Dai H. 2004. Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into Mammalian cells. *J Am Chem Soc*. **126**:6850-6851.

Shih YW, Lee YC, Wu PF, Lee YB, Chiang TA. 2009. Plumbagin inhibits invasion and migration of liver cancer HepG2 cells by decreasing productions of matrix metalloproteinase-2 and urokinase- plasminogen activator. *Hepatol Res***. 39**:998-1009.

Shikama N, Lutz W, Kretzschmar R, Sauter N, Roth JF, Marino S, Wittwer J, Scheidweiler A, Eckner R. 2003. Essential function of p300 acetyltransferase activity in heart, lung and small intestine formation. *EMBO J*. **22**:5175-85.

Shogren-Knaak M, Ishii H, Sun JM, Pazin MJ, Davie JR, Peterson CL. 2006. Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science***. 311**:844-847.

Smith BC, Denu JM. 2009. Chemical mechanisms of histone lysine and arginine modifications. *Biochim Biophys Acta*. **1789**:45-57.

Snowden AW, Gregory PD, Case CC, Pabo CO. 2002. Gene-specific targeting of H3K9 methylation is sufficient for initiating repression in vivo. *Curr Biol.* **12**:2159-2166.

Son TG, Camandola S, Arumugam TV, Cutler RG, Telljohann RS, Mughal MR, Moore TA, Luo W, Yu QS, Johnson DA, Johnson JA, Greig NH, Mattson MP. 2010. Plumbagin, a novel Nrf2/ARE activator, protects against cerebral ischemia. *J Neurochem*. **112**:1316-26.

Srinivas G, Annab LA, Gopinath G, Banerji A, Srinivas P. 2004. Antisense blocking of BRCA1 enhances sensitivity to plumbagin but not tamoxifen in BG-1 ovarian cancer cells. *Mol Carcinog.* **39**:15-25.

Srinivas P, Gopinath G, Banerji A, Dinakar A, Srinivas G. 2004. Plumbagin induces reactive oxygen species, which mediate apoptosis in human cervical cancer cells. *Mol Carcinog*. **40**:201-211.

Steffan JS, Bodai L, Pallos J, Poelman M, McCampbell A, Apostol BL, Kazantsev A, Schmidt E, Zhu YZ, Greenwald M, Kurokawa R, Housman DE, Jackson GR, Marsh JL, Thompson LM. 2001. Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. *Nature*. **413**:739–743.

Stewart MD, Li J, Wong J. 2005. Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol Cell Biol*. **25**:2525-2538.

Stimson L, Rowlands MG, Newbatt YM, Smith NF, Raynaud FI, Rogers P, Bavetsias V, Gorsuch S, Jarman M, Bannister A, Kouzarides T, McDonald E, Workman P, Aherne GW. 2005. Isothiazolones as inhibitors of PCAF and p300 histone acetyltransferase activity. *Mol Cancer Ther*. **2005**. 4:1521-1532.

Sugie S, Okamoto K, Rahman KM, Tanaka T, Kawai K, Yamahara J, Mori H. 1998. Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats. *Cancer Lett.* **127**:177-83

Sun Y, Jiang X, Chen S, Price BD. 2006. Inhibition of histone acetyltransferase activity by anacardic acid sensitizes tumor cells to ionizing radiation. *FEBS Lett.* **580**:4353-6.

Sung B, Pandey MK, Ahn KS, Yi T, Chaturvedi MM, Liu M, Aggarwal BB. 2008. Anacardic acid (6-nonadecyl salicylic acid), an inhibitor of histone acetyltransferase, suppresses expression of nuclear factor-kappaB-regulated gene products involved in cell survival, proliferation, invasion, and inflammation through inhibition of the inhibitory subunit of nuclear factor-kappaBalpha kinase, leading to potentiation of apoptosis. *Blood.* **111**:4880-91.

Swaminathan V, Kishore AH, Febitha KK, Kundu TK. 2005. Human histone chaperone nucleophosmin enhances acetylation-dependent chromatin transcription. *Mol Cell Biol.* **25**:7534-7545.

Tanaka Y, Naruse I, Hongo T, Xu M, Nakahata T, Maekawa T, Ishii S. 2000. Extensive brain hemorrhage and embryonic lethality in a mouse null mutant of CREB-binding protein. *Mech Dev*. **95**:133-45.

Tanner KG, Langer MR, Kim Y, Denu JM. 2000. Kinetic mechanism of the histone acetyltransferase GCN5 from yeast. *J Biol Chem*. **275**:22048–22055.

Tanner KG, Trievel RC, Kuo MH, Howard RM, Berger SL, Allis CD, Marmorstein R, Denu JM. 1999. Catalytic mechanism and function of invariant glutamic acid 173 from the histone acetyltransferase GCN5 transcriptional coactivator. *J Biol Chem* . **274**:18157–18160.

Taylor JP, Taye AA, Campbell C, Kazemi-Esfarjani P, Fischbeck KH, Min KT. 2003. Aberrant histone acetylation, altered transcription, and retinal degeneration in a Drosophila model of polyglutamine disease are rescued by CREB-binding protein. *Genes Dev*. **17**:1463–1468.

Thompson PR, Kurooka H, Nakatani Y, Cole PA. 2001. Transcriptional coactivator protein p300. Kinetic characterization of its histone acetyltransferase activity. *J. Biol. Chem*. **276**:33721–33729.

Thompson PR, Wang D, Wang L, Fulco M, Pediconi N, Zhang D, An W, Ge Q, Roeder RG, Wong J, Levrero M, Sartorelli V, Cotter RJ, Cole PA. 2004. Regulation of the p300 HAT domain via a novel activation loop. *Nat Struct Mol Biol*. **11**:308-15.

Tsankova N, Renthal W, Kumar A, Nestler EJ: Epigeneti regulation in psychiatric disorders. 2007. *Nat Rev Neurosci*. **8**:355-367.

Tsukiyama T, Wu C. 1995. Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell.* **83**:1011-20.

Varga-Weisz PD, M Wilm, E Bonte, K. Dumas, M Mann, and PB Becker.1997. Chromatin-remodelling factor CHRAC contains the ATPases ISWI and topoisomerase II. *Nature.* **388**:598–602.

Vignali M, Hassan AH, Neely KE, Workman JL. 2000. ATP-dependent chromatin- remodeling complexes. *Mol Cell Biol.* **20**:1899-1910.

Villunger A, Michalak EM, Coultas L, Müllauer F, Böck G, Ausserlechner MJ, Adams JM, Strasser A. 2003. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science.* **302**:1036-8.

Wade PA, PL Jones, D Vermaak, and AP Wolffe. 1998. A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. *Curr. Biol.* **8**:843–846.

Wijeratne SS, Camporeale G, Zempleni J. 2010. K12-biotinylated histone H4 is enriched in telomeric repeats from human lung IMR-90 fibroblasts. *J Nutr Biochem*. **21**:310-316.

Woodcock CL, Dimitrov S. 2001. Higher-order structure of chromatin and chromosomes. *Curr Opin Genet Dev.* **11**:130-5.

Xiao H, Hasegawa T, Isobe K. 2000. p300 collaborates with Sp1 and Sp3 in p21(waf1/cip1) promoter activation induced by histone deacetylase inhibitor. *J Biol Chem*. **275**:1371-1376.

Xu W, Chen H, Du K, Asahara H, Tini M, Emerson BM, Montminy M, Evans RM. 2001. A transcriptional switch mediated by cofactor methylation. *Science*. **294**:2507-11.

Yamauchi T, Oike Y, Kamon J, Waki H, Komeda K, Tsuchida A, Date Y, Li MX, Miki H, Akanuma Y, Nagai R, Kimura S, Saheki T, Nakazato M, Naitoh T, Yamamura K, Kadowaki T. 2007. Increased insulin sensitivity despite lipodystrophy in Crebbp heterozygous mice. *Nat Genet*. **30**:221-6.

Yan Y, Harper S, Speicher DW, Marmorstein R. 2002. The catalytic mechanism of the ESA1 histone acetyltransferase involves a self-acetylated intermediate. *Nat Struct Mol Biol*. **9**:862–869.

Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature.* **382**:319-24.

Yang XJ, Seto E. 2008. Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol Cell*. **31**:449-461.

Yao TP, Oh SP, Fuchs M, Zhou ND, Ch'ng LE, Newsome D, Bronson RT, Li E, Livingston DM, Eckner R. 1998. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. *Cell*. **93**:361-372.

Yuan LW, Gambee JE. 2000. Phosphorylation of p300 at serine 89 by protein kinase C. *J Biol Chem.* **275**:40946-51.

References

Zhang C, Li H, Zhou G, Zhang Q, Zhang T, Li J, Zhang J, Hou J, Liew CT, Yin D. 2007. Transcriptional silencing of the TMS1/ASC tumour suppressor gene by an epigenetic mechanism in hepatocellular carcinoma cells. *J Pathol*. **212**:134-142.

Zhang Y, Smith CL, Saha A, Grill SW, Mihardja S, Smith SB, Cairns BR, Peterson CL, Bustamante C. 2006. DNA translocation and loop formation mechanism of chromatin remodeling by SWI/SNF and RSC. *Mol Cell.* **24**:559-568.

Zhong X, Jin Y. 2009. Critical roles of coactivator p300 in mouse embryonic stem cell differentiation and Nanog expression. *J Biol Chem*. **284**: 9168-9175.

Zhu H, McShane MJ. 2005. Loading of hydrophobic materials into polymer particles: implications for fluorescent nanosensors and drug delivery. *J Am Chem Soc*. **127**:13448-9.

Zippo A, Serafini R, Rocchigiani M, Pennacchini S, Krepelova A, Oliviero S. 2009. Histone crosstalk between H3S10ph and H4K16ac generates a histone code that mediates transcription elongation. *Cell*. **138**:1122-36.

Zofall M, Persinger J, Kassabov SR, Bartholomew B. 2006. Chromatin remodeling by ISW2 and SWI/SNF requires DNA translocation inside the nucleosome. *Nat Struct Mol Biol.* **13**:339–346.