Elucidating the Relevance of Eukaryotic Acetyltransferases in Early Development and Cellular Homeostasis

A thesis submitted for the degree of

Doctor of Philosophy

By

Moumita Basu

To

Molecular Biology and Genetics Unit

Jawaharlal Nehru Centre for Advanced Scientific Research

(A Deemed University)

Jakkur P.O., Bangalore 560064, INDIA

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जवाहरलाल नेहरु उन्नत वैज्ञानिक अनुसंधान केन्द्र
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CERTIFICATE

This is to certify that the work described in this thesis entitled **'Elucidating the Relevance of Eukaryotic Acetyltransferases in Early Development and Cellular Homeostasis'**, is the result of the investigations carried out by Ms. Moumita Basu at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre 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 for any other diploma, degree or fellowship.

Date: Nov 15, 2021
Place: Bangalore - 64.

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DECLARATION

I hereby declare that this thesis entitled '**Elucidating the Relevance of Eukaryotic Acetyltransferases in Early Development and Cellular Homeostasis**' 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 anywhere else for the award of any other degree.

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

Moumita Basu

Date: 15.12.2021

Place: Bengaluru

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Dedicated to my grandparents

(Dadu, Thamma, Dadubhai and Dimma)

who have meant and continue to mean so much to me

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Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 1

Introduction

This chapter introduces the basic concepts of epigenetics, chromatin dynamics, and histone post-translational modifications. A brief review on lysine acetylation, and the chromatin modifying enzymes that regulate this dynamic covalent modification has been included. The focus of this chapter is on two specific lysine acetyltransferases; one of the acetyltransferases is p300/CBP (KAT3A, KAT3B), which is a well-characterized transcriptional coactivator indispensable for multiple cellular functions, and, the other acetyltransferase being TFIIIC220, a poorly characterized acetyltransferase whose primary role has been elucidated as a transcription factor for RNA polymerase III. A comprehensible account of history, functions, and regulation of these two epigenetic enzymes is presented in this chapter.

1.1. Protein posttranslational modification

Nascent protein does not often become fully-functional immediately after template-based biosynthesis from ribosome, it must undergo certain chemical modifications outside ribosome. After folding the backbone and functional groups in the side chains of proteins serve as chemical synthons for a wide array of enzymatic, non-enzymatic or self-catalysed reactions that range from inclusion of small functional groups or complex biomolecules to side chain or backbone cyclization, proteolytic cleavages or even splicing. While some modifications required for protein processing and protein folding such as n terminal methionine excision, N-α acetylation, N-myristoylation *etc.* happen co-translationally in the RER, a wide array of modifications which are posttranslational in nature may happen in cytoplasm, nucleus and many other cell organelles such as RER, Golgi apparatus, endosomes, lysosomes and secretory vesicles (reviewed in Shandala *et al.,* 2011; Millar *et al.,* 2019). Together these protein modifications are the second route to proteome diversification, the first route being transcriptional level mRNA splicing. Protein covalent modifications occur much more extensively in nucleated cells as compared to that in prokaryotes. ~5% of the genome in higher eukaryotes are dedicated to enzymes related to proteome diversification through protein modifications (Walsh *et al.,* 2005).

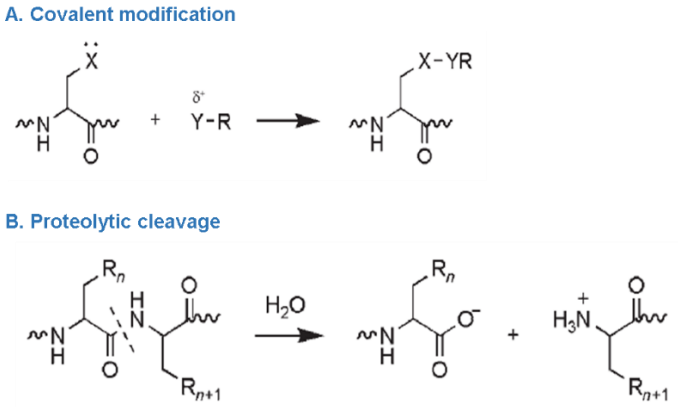


Figure 1.1. Schematic representation of mechanisms for two different types of protein post-translational modifications.

Protein posttranslational modifications (PTMs) can be broadly categorized into two classes; the first category is enzyme-catalyzed covalent addition of electrophilic chemical groups or biomolecules to an electron rich nucleophile sidechain of amino acids in proteins, and, the second category of PTM is protease mediated or autocatalytic cleavage. Proteolysis can regulate the localization, activity and lifespan of a protein. While proteolytic cleavage of a protein is irreversible in nature the covalent posttranslational modifications are reversible (Wang *et al.*, 2014; Blom *et al.*, 2004).

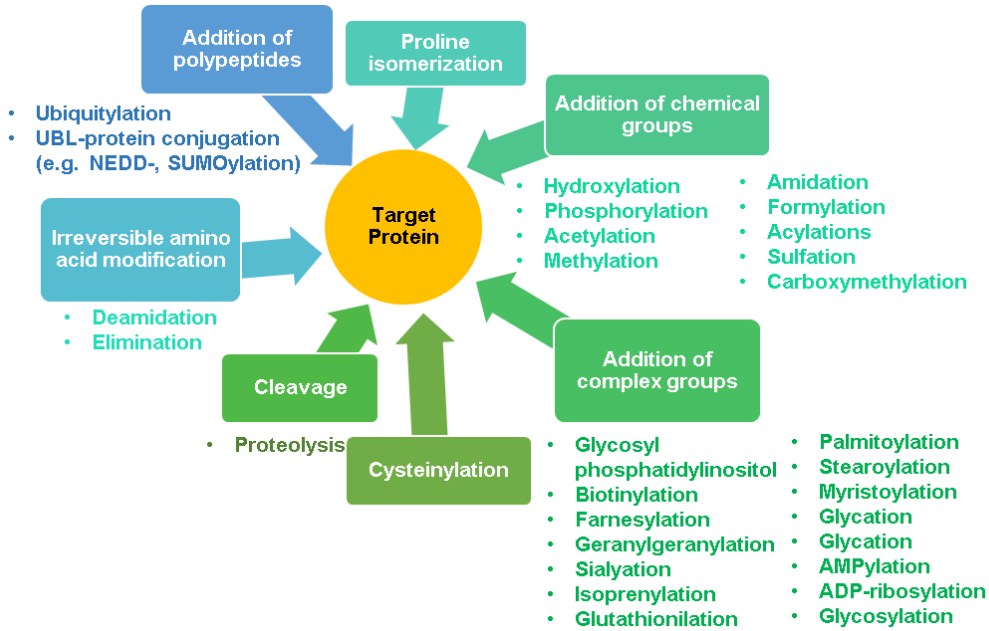


Figure 1.2. Categorization of protein posttranslational modifications based on various chemical modifications.

Table 1.1. Posttranslational modifications of different amino acid residues in protein *(Adapted from Walsh et al., 2005)*

Res.	Modifications	Examples
Asp	Phosphorylation Isomerization to isoAsp	Protein tyrosine phosphatases; response regulators in two-component systems
Glu	Methylation Carboxylation Polyglycination Polyglutamylation	Chemotaxis receptor proteins Gla-residues in blood coagulation Tubulin Tubulin
Ser	Phosphorylation O-glycosylation Phosphopantetheinylation Autocleavages	Protein serine kinases, phosphatases Notch Fatty acid synthase Pyruvamidyl enzyme formation
Thr	Phosphorylation O-glycosylation	Protein threonine kinases/phosphatases
Tyr	Phosphorylation Sulfation Ortho-nitration TOPA quinone	Tyrosine kinases/phosphatases CCR5 receptor mutation Inflammatory responses Amine oxidases reductase
His	Phosphorylation Aminocarboxypropylation N-methylation	Sensor protein kinases in two-component regulatory systems Diphthamide formation Methyl CoM reductase
Lys	N-methylation N-acylation (by acetyl-, biotinyl-, lipoyl-, ubiquityl- groups) C-hydroxylation	Histone methylation Histone modifications, SUMO-, Ubiquitin-tagging proteins Collagen maturaton
Cys	S-hydroxylation (S-OH) Di-sulfide bond formation Phosphorylation S-acylation S-prenylation Protein splicing	Sulfenate intermediates Protein in oxidizing environments PTPases Ras Ras Intein excisions
Arg	N-methylation N-ADP-ribosylation	Histones $G_{S\alpha}$
Met	Oxidation to sulfoxide	Met sulfoxide reductase
Asn	N-glycosylation N-ADP-ribosylation Protein splicing	N-glycoproteins eEF-2 Intein excision step
Gln	Transglutamination	Protein cross-linking
Trp	C-mannosylation	Membrane proteins
Pro	C-hydroxylation	Collagen, HIF1α
Gly	C-hydroxylation	C-terminal amide formation
Leu, Ile, Val, Ala, Phe side chains are not known to undergo PTM		

PTMs can affect a wide range of protein characteristics and function such as protein folding, localization, solubility, lifespan, enzymatic function, assembly, protein-protein

or protein -molecule interaction, cell-cell or cell-matrix adhesion, molecular trafficking, secretion, receptor activation (Rayslava *et al.*, 2013; Marshall, 1993; Haltiwanger and Lowe, 2004; Goulabchand *et al.*, 2014). Therefore, these modifications are associated with various biological processes such as signal transduction, gene expression and its regulation, chromatin organization, DNA repair, cell cycle *etc*. (Wang *et al.*, 2015a; Strumillo and Beltrao, 2015; Wei *et al.*, 2017). Multiple posttranslational modifications can crosstalk with each other guiding coordinated effect as well.

Covalent post-translational modifications can be further categorized based on the amino acid residues that are modified, the functional groups or molecules that are added to the protein backbone or by the new gain of function that is achieved post modification all of which are discussed in table 1.1.

As many as 460 different types of PTMs have been enlisted in UniProt database and out of those modifications protein acetylation is the second most prevalent type of modification; as many as ~14000 sites of only N-ε acetylation of lysine have been identified in 664 different species (The UniProt consortium, 2013).

These modifications can be classified based on several factors. 15 out of 21 amino acids are known to undergo modification, some of the most commonly occurring modifications have been listed in table 1.1. PTMs can be also classified based on the fragment of co-substrate or coenzyme that is enzymatically coupled to the protein and the concomitant chemical nature of the protein modification. This catalogue includes S-adenosylmethionine (SAM)-dependent methylation, ATP-dependent phosphorylation, acetyl-CoA dependent acetylation, NAD-dependent ADP ribosylation, CoASH-dependent phosphopantetheinylation, and phosphoadenosinephosphosulfate (PAPS)-dependent sulfurylation (Walsh *et al.*, 2005). Thirdly, PTMs can also be categorized based on the type of chemical modifications they undergo which has been broadly covered in the Figure 1.2.

1.2. Protein acetylation

Protein acetylation can further be classified into two categories, N-α acetylation and N-ε acetylation. N -terminal acetylation is an irreversible covalent modification that occurs in almost 80% of mammalian proteins and is required for protein stability, interactions,

or subcellular localization (Aksens *et al.,* 2016). N-α acetyltransferases ('Nat's: A-F) catalyze acetyl group transfer from acetyl-CoA to the first N terminal residue of nascent emerging polypeptide to block further modification of positively charged amino group in general; it is generally a co-translational modification but might also occur post-translationally. NATs belong to GNAT superfamily and can differ in their subunit composition and substrate specificity (Drazic *et al.,* 2016). Nat C, E and F catalyzes acetyl-group transfer to initiator methionine (iMet) if it is followed by hydrophobic residues; Nat A and D can acetylate A-, S-, T-, V-, C-, and sometimes G-residues starting at N-termini after proteolytic cleavage of iMet by methionine aminopeptidases (MetAPs) (Bonissone *et al.,* 2013). So far, NAT D is known to acetylate only histones H2A and H4. The frequency of N-α acetylation increases with complexity of the organisms, in eukaryotes >80% proteins undergo N-α acetylation but is rare in prokaryotes and archaea (Aksens *et al.,* 2016; Beltrao *et al.,* 2013).

N-ε acetylation is posttranslational irreversible modification that occur exclusively on lysine residues in proteins. Lysine acetylation has been discussed in detail later.

Apart from lysine, recent works have identified other internal residues such as serine, threonine, histidine to undergo acetylation as well. Acetyltransferases from pathogenic species (e.g., *Yersinia pestis*, *Pseudomonas syringae*, *Ralstonia solanacearum etc.*) acetylate eukaryotic host cell proteins on these unusual residues. YopJ superfamily acetyltransferases (HopZ1, PopP2 *etc.*) can O-acetylate host mammalian kinases, plant proteins on S-, T-, H- residues preventing effective immune signaling and facilitating infection (Lee *et al.,* 2015; Paquette *et al.,* 2012). O-acetylation can be reversed by promiscuous esterases (Munger *et al.,* 1991; Vincent *et al.,* 2003).

1.3. Lysine acetylation

Lysine acetylation is posttranslational modification where an acetyl moiety from acetyl Coenzyme A (acetyl-CoA) is transferred onto the ε-nitrogen on lysine residue of a protein neutralizing the positive electrostatic charge of that position. It was first described as a covalent modification that occur in nucleus and is associated with active transcription (Alfrey *et al.,* 1964; Struhl, 1996). Further studies have revealed that lysine acetylation have much wider implications in the context of cellular functioning. It affects protein function, chromatin architecture, DNA repair, transcription, replication to metabolic

activities and cellular homeostasis (Capell and Berger, 2013; Unnikrishnan *et al.*, 2010; Vo and Goodman, 2001; Chatterjee *et al.*, 2012; Kouzarides, 2007; Shogren-Knaak *et al.*, 2006). Lysine acetylation is ubiquitous and occurs in almost every cellular compartment; around 1750 different proteins in cell were found to carry lysine acetylation mark (Choudhury *et al.*, 2009; Zhao *et al.*, 2010). Currently over 35,000 acetylation sites have been identified in human cells; the abundance of acetylation is comparable to that of phosphorylation (Hornbeck *et al.*, 2012). Interestingly, mitochondria have emerged as organelle in which acetylation is more prevalent than phosphorylation and plays a key role in integrating metabolic cues with bioenergetic equilibrium of the cell (Gnad *et al.*, 2010).

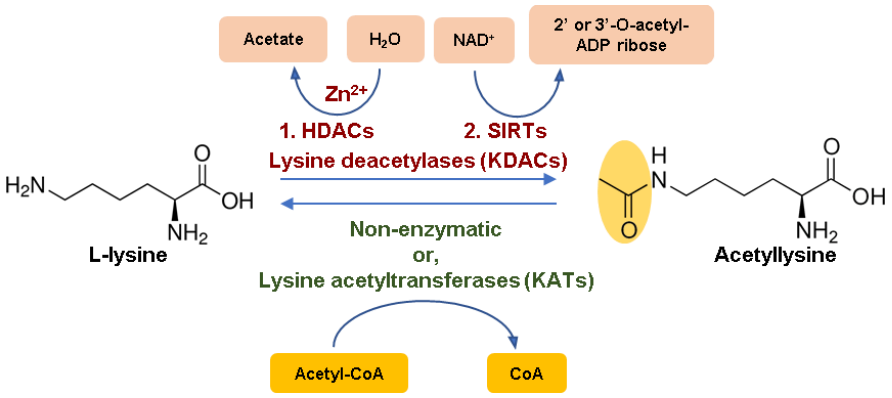


Figure 1.3. Lysine acetylation is a reversible process. *Lysine acetyltransferases (KATs) catalyze the transfer of acetyl group from acetyl-CoA to lysine residues of target protein whereas lysine deacetylases (KDACs, here mentioned as HDACs and SIRTs) removes acetyl groups from lysine residues with the help of different cofactors such as zinc or NAD+.*

Histones are not the only proteins to undergo acetylation, many nonhistone proteins such as nonhistone chromatin proteins (e.g., PC4, HMGs), DNA-binding transcription factors (p53, ELKF, TCF, NF-κB, MyoD, GATA1, E2F1, HNF4 *etc.*), transcriptional coactivators (e.g., ATCR, CIITA, β-catenin, RB, RIP140), general transcription factors (TFIIE, TFIIF, TFIIB *etc.*), chromatin remodeler (Brm), DNA replication factor (MCM3), chromatin cohesion proteins (cohesin subunits), DNA metabolic enzymes (Flap endonuclease 1, thymine DNA glycosylase, Werner DNA helicase *etc.*), cytoskeletal proteins (tubulin), signaling components (Smad-7), viral proteins (e.g., Large T antigen, adenoviral E1A, HIV Tat) are subjected to acetylation; in fact, some of the acetyltransferases (e.g., PCAF, p300/CBP, MOF, Tip60 *etc.*) undergo autoacetylation

that modulates their activities (Roth *et al.*, 2001; Gu and Roeder, 1997; Imhof *et al.*, 1997; Boyes *et al.*, 1998; Munshi *et al.*, 1998; Waltzer and Bienz, 1998; Martínez-Balbás *et al.*, 2000; Marzio *et al.*, 2000; Bannister *et al.*, 2000; Santos-Rosa *et al.*, 2003; Wang and Chen , 2010a; Yang *et al.*, 2012; Yuan *et al.*, 2012).

1.3.1. Readers, writers, erasers of lysine acetylation

Lysine acetylation is a highly regulated process, cellular acetylation is driven by the localization of enzymes, metabolites, and cofactors required to balance acetylation and deacetylation levels. Once lysine on target protein is modified by 'writer' lysine acetyltransferase (KAT), the acetylated lysine 'code' is read by 'reader' proteins which relay downstream signaling, and finally, the acetylation is removed by 'erasers' lysine deacetylases (KDACs) from target proteins.

- **Writers:**

Lysine acetylation can occur nonenzymatically in mitochondria due to high pH and high local concentration of acetyl-CoA; naturally occurring hydroxide ions deprotonate lysine primary amine priming it for nucleophilic attack to acetyl-CoA terminal carbonyl group. A transient tetrahedral intermediate is formed before decomposing into the final products of acetyl-lysine, coenzyme A, and, hydroxide (Santo-Domingo and Demaurex, 2012; Wagner and Payne, 2013). The enzymatic reaction of lysine acetylation is carried out by an army of 21 putative lysine acetyltransferases that has been characterized in mammals so far. The structure, mechanism and function of the acetyltransferases have been discussed later in this chapter.

- **Readers:**

In total three domains are responsible for recognizing acetylated proteins: 1. bromodomains (BrD), 2. tandem plant homeodomain (PHD) and, 3. YEATS domain.

Bromodomain was first discovered in Drosophila brahma (brm) gene, a regulator in SWI/SNF chromatin remodelers (Tamkun *et al.*, 1992). Bromodomain is approximately 110 amino acids long and sequence conservation is observed from yeast to human. A bromodomain containing protein may contain more than one bromodomains (Ali *et al.*, 2019). 61 different bromodomains have been identified in 46 proteins in mammals;

nearly all bromodomain containing protein are nuclear factors that bind to chromatin to regulate its structure and function. Some bromodomain containing protein act as transcriptional coactivators (e.g., p300, GCN5, PCAF, BRDs), repressive functions of certain bromodomain-containing proteins are also known (e.g., BAZ2A, ZYMND11). Bromodomain of GCN5 is involved in chromatin remodeling and sequential acetylation events (Syntichaki *et al.*, 2000). Bromodomain is composed of conserved four left-handed α-helices bundle known as 'BrD fold' connected by two variable loops. A hydrophobic cavity formed by this structure serves as acetyl-lysine binding site. A hydrogen bond is formed between a conserved Asn (asparagine) residue in bromodomain and acetyl-lysine carbonyl group, and, tyrosine residues lining hydrophobic cleft aid in ligand positioning via π–π stacking and hydrogen bond formation with critical water molecules (Dhalluin *et al.*, 1999). Some bromodomains (e.g., BRDT) cooperatively bind multiply acetylated peptides. Often adjacent domains such as helicase, SAND *etc.* modulate specificities and strength of binding of bromodomains *in vivo*.

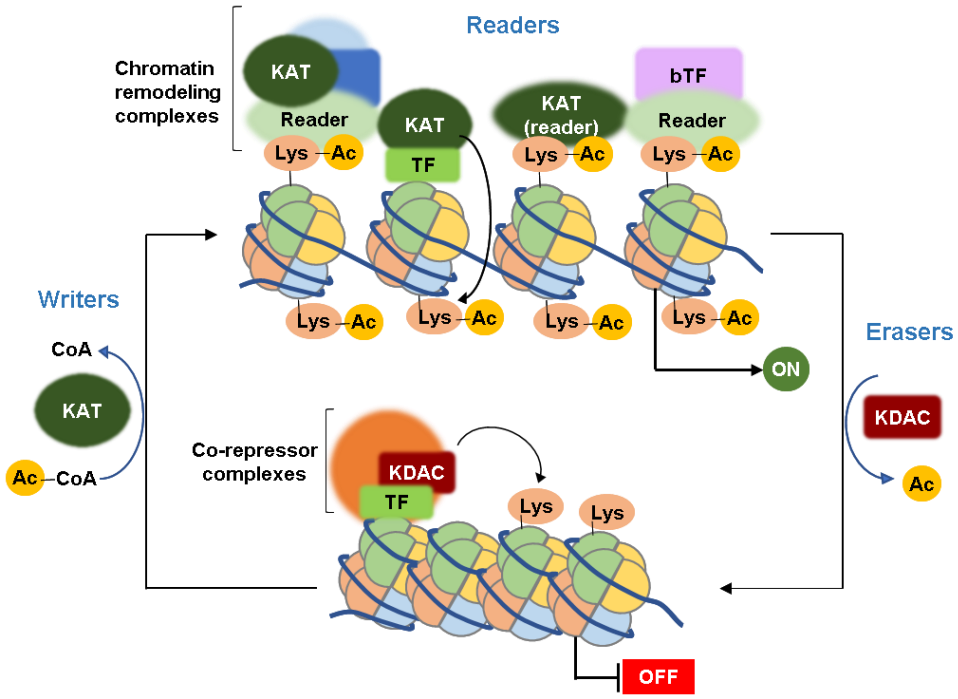


Figure 1.4. The sequential action of 'writer', 'reader', and 'eraser' of nucleosomal lysine acetylation. *Acetyltransferases (KATs)transfer acetyl-group from acetyl-CoA to nucleosomal substrates leading to various effects brought about by readers containing acetyl-lysine binding domains. Readers may recruit chromatin remodeling complexes or basal transcription machinery (bTF) to a now accessible template; or, can also recruit other posttranslational modifiers including other KATs. Deacetylases (KDACs) erase off*

acetyl-group from lysine residues leading to compact chromatin formation. KDACs can further recruit co-repressor complexes or repressive modifiers and proteins.

Tandem plant homeodomain (PHD) has aspartic acid (Asp) that forms hydrogen bond with acetyl-lysine, it also recognizes N-α acetylated proteins. Tandem PHD fold has antiparallel two- strand β-sheet and an α-helix stabilized by two zinc atoms, ands, is present in chromatin remodeler such as CHD4 or transcriptional coactivator such as a few members of MYST family acetyltransferases *e.g.,* MOZ or in proteins such as DPF3b (Zeng *et al.,* 2010; Musselman *et al.,* 2012; Qiu *et al.,* 2012).

YEATS (Yaf9, ENL, AF9, Taf14, Sas5) domain has been identified in five human proteins that it is named after, YEATS2, ENL, AF9, TFIIF, and GAS41. AF9 and ENL are components of super elongation complex (SEC), GAS, YEATS2 belong to chromatin remodeling complexes and TFIIF is a transcription factor. Structurally, YEATS domain adopts an eight-strand immunoglobulin fold with an aromatic cage lined with seine residues (Li *et al.,* 2014a; Zhao *et al.,* 2017; Ali *et al.,* 2019). While ENL YEATS domain recognizes H3K27ac specifically, AF9 YEATS domain binds to H3K9ac specifically. AF9 YEATS domain has an expanded binding repertoire of acetyl-lysine marks, and it can also accommodate modifications, such as crotonylation. Similarly, Gas41 YEATS domain can recognize succinylation besides H3K27 and 14 acetylation (Wan *et al.,* 2017; Li *et al.,* 2016; Hsu *et al.,* 2018).

Apart from acetyl-lysine reader domains, readers that recognize unmodified lysine residues have been identified recently. SET and SET-like domains present in proteins such as VPRBP, DAXX, PELP1 *etc.* bind to p53 C-terminal acidic domain only when it is not acetylated. Additionally, SET acidic domains are also known to recognize non-acetylated lysine-rich domains of H3, KU70 and FOXO1 (Wang *et al.,* 2016).

JQ1, I-BET are two most well characterized bromodomain inhibitors. Almost all BET inhibitors are synthesized based on pharmacophore methyltriazole that function primarily by forming hydrogen bond with a critical asparagine residue that otherwise binds to acetyl-lysine. More than 20 BET inhibitors are currently under clinical trial; non-BET inhibitors of bromodomains are also being developed but are still in primitive stage (Ali *et al.,* 2018).

Erasers:

The indication of the existence about lysine deacetylation mechanism came to the surface when butyrate treatment led to hyperacetylation followed by differentiation of Friend erythroleukemic cells (Riggs *et al.*, 1977). Once trapoxin was also found to be an inducer of hyperacetylation, the first KDAC was isolated from bovine calf thymus lysates using a Trapoxin affinity matrix (Tauton *et al.*, 1996) and followed by this several other deacetylases were identified. deacetylases are structurally and mechanistically distinct; yet, they are grouped into four classes based on their structural homology with yeast transcriptional repressors and unique catalytic mechanisms.

Table 1.2. Classification of mammalian KDACs.

Yeast homolog	Co-factor	Human KDAC	Distribution	Localization	Target substrates	Interacting proteins	Protein complexes
Class I (RPD3)	Zn^{2+}	KDAC1 KDAC2 KDAC3 KDAC8	Ub Ub Ub Ub	N N N/C N	Histones, p53, NF-kB	Rb, NF-κB, MyoD, BRCA1, ATM, MBD2, DNMT1, p53, MeCP2, EST1B, DNMT3A, Hsp70	NuRD, SIN3, N-CoR, SMRT
Class IIa (HDA1)	Zn^{2+}	KDAC4 KDAC5 KDAC7 KDAC9	TS TS TS TS	N/C N/C N/C/Mt N/C	Histones	MEF2	N-CoR, SMRT
Class IIb (HDA1)	Zn^{2+}	KDAC6 KDAC10	TS Ub	C C	Histones, Tubulin, HSP	Rb, Hsp 90	
Class III (SIR2)	NAD^{+}	SIRT1 SIRT2 SIRT3 SIRT4 SIRT5 SIRT6 SIRT7	Unknown	N/C N/C Mt Mt Mt/N/C N N/No	Histones, Tubulin, p53, TAF	p53	
Class IV (RPD3)	Zn^{2+}	KDAC11	Ts	N	Histones	IL10	

(Ub- ubiquitous, TS- tissue specific, N- nucleus, C- cytoplasm, Mt-mitochondria)

Class I, II and IV deacetylases are Zn^{2+} dependent, they share similar mechanism of deacetylation where an active site histidine deprotonates a water molecule to enable nucleophilic attack on acetyl group carbonyl and forms oxyanionic tetrahedral intermediate which decomposes to release acetate and deacetylated lysine. Zn^{2+} positions

and polarizes water molecule and is held by aspartic acid and histidine residues of classical catalytic triad (charge-relay network) (Seto and Yoshida, 2014; Delcuve *et al.,* 2012). This zinc ion is a critical target of inhibitors of class I, II, IV KDACs. Panobinostat, Romidepsin, Vorinostat are non-specific yet potent and widely used inhibitors of these classes of KDACs. Class I deacetylases mainly reside in nucleus whereas class II and IV deacetylases shuttle between nucleus and cytoplasm. KDACs are often found in large, macromolecular complexes that mainly function as repressors. For example, CoREST, NuRD, and Sin3 complexes harbor a catalytic core composed of a KDAC1:KDAC2 dimer, and the NCoR complex contains KDAC3 (Watson *et al.,* 2012).

Class III deacetylase require nicotinamide adenine dinucleotide (NAD^+) as cofactor for its catalytic activity. Of all the SIRTs, SIRT1, 2, and 3 are robust deacetylases and activities of SIRT 5, 6, and 7 are more limited. SIRT4 has no deacetylase activity (Nakagawa *et al.,* 2009; North *et al.,* 2003; Liszt *et al.,* 2005; Ford *et al.,* 2006; Laurent *et al.,* 2013). The reaction mechanism of Sirtuins involve formation of C1′-O-alkylamidate intermediate through nucleophilic addition of acetyl oxygen to anomeric (C1') carbon of the nicotinamide ribose followed by extraction of electron from 2' hydroxyl group of NAD^+ ribose by a histidine residue which then attacks the C1′-O-alkylamidate carbon generating a bicyclic intermediate. Deprotonated water molecule attacks bicyclic intermediate to generate deacetylated lysine and O-acetyl-ADP-ribose (Sauve and Schramm, 2003). Sirtuins SIRT4 and 6 also possess weak ADP ribosyl transferase activity which utilizes similar catalytic sites and mechanism (Haigis *et al.,* 2006). Additionally mitochondrial SIRTs accept wide range of substrates including malonyl- and succinyl-lysines (Du *et al.,* 2011; Park *et al.,* 2013; Rardin *et al.,* 2013a). Unlike other KDACs sirtuins are not known to exist in complexes.

Aberrant expression of HDACs have been associated with many human diseases including cancer, making them important therapeutic targets (Lane and Chabner, 2009). Numerous structurally diverse KDAC inhibitors (KDI) have been isolated from natural resources and have been synthetically produced that can induce apoptosis, differentiation, growth arrest, inhibit angiogenesis in many types of cancers (Hess-Stumpp *et al.,* 2005). KDIs are categorized into five groups (Eckschlager *et al.,* 2017):

(i) hydroxamic acids (vorinostat, belinostat, panobinostat, givinostat, resminostat, abexinostat, quisinostat, rocilinostat, and practinostat)

(ii) short chain fatty acids (valproic acid, butyric acid, and phenylbutyric acid)

(iii) benzamides (entinostat, mocetinostat, tacedinaline, and 4SC202)

(iv) cyclic tetrapeptides (romidepsin)

(v) sirtuin inhibitors (nicotinamide, cambinol, sirtinol, and EX-527)

Some of these KDIs are pan-inhibitors (phenylbutyrate, vorinostat, and belinostat) and some are selective inhibitors (for example, valproic acid and sodium butyrate inhibit both HDAC I and IIa). Bezamides and cyclic peptides exclusively inhibit HDAC I only (Wawruszak *et al.,* 2016). Sirtuin inhibitors are categorized into two groups based on interacting sites. One group bind to NAD^+ interacting site while the other bind to acetyllysine binding sites. Nicotinamide is widely used as inhibitor of Sirtuins (Jiang *et al.,* 2017).

Many reports suggest better efficacy of KDIs when used in combination of other drugs in anticancer treatment (Zhang *et al.,* 2017). Since KDIs are relatively nontoxic to normal cells and show selective anti-proliferative effect against various cancer cells many KDIs are used for treatment of cancer. Vorinostat (suberanilohydroxamic acid or SAHA), Romidepsin, Panobinostat, Belinostat are among few to be approved by FDA, USA as drugs and many are extensively studied in clinical trials for treatments of different hematological cancers and solid cancers such as breast cancer, pancreatic cancer and NSCLC (Richon *et al.*, 1998; Nakajima *et al.*, 1998; George *et al.*, 2005; Plumb *et al.*, 2003; ClinicalTrials.gov).

1.4. Lysine acetyltransferases

Lysine acetyltransferases are grouped into two major subclasses based on their cellular localization. While acetylation mediated by cytoplasmic B type of KATs is linked to transport of newly synthesized histones to nucleus, the nuclear type A KATs catalyze transcription-related events. Besides histones, a pool of 250 proteins in cytoplasm has been found to be acetylated in cytoplasm (Choudhury *et al.,* 2009).

B-type KATs

Type B KATs are known to have housekeeping functions, they acetylate newly synthesized histones in cytoplasm to facilitate their transport to nucleus. Type B acetyltransferases include HAT1, HAT2, HatB3.1, Rtt109, and HAT4. The catalytic core of type B KAT is Hat1, the first ever KAT to be characterized; it acetylates K5 and 12 of

newly synthesized histone H4. Hat2, an ortholog of Hat1and a component of multisubunit complex that includes Hat1, recognizes H3 tails and aids in interaction between Hat1 and newly synthesized H3/H4 tails. Both Hat 1 and 2 have been found to be associated with chromatin remodeling complexes. Yet, mutation in any of these proteins fail to generate any significant defect in cells underlining the fact that KAT activities and covalent marks in histone are highly redundant in eukaryotes. (Chicoine *et al.*, 1996; Sobel *et al.*, 1995; Verreault *et al.*, 1998; Kaufman *et al.*, 1995; Qin and Parthun, 2006). Though categorized as cytosolic acetyltransferases Hat1 and 2 are predominantly found in nucleus active at replication fork and telomeres, involved in mismatch repair (Ruiz-Garcia *et al.*, 1998; Kelly *et al.*, 2000). Cytosolic fraction of Hat1 was found to be increased in colorectal tumor or during early development (Lebel *et al.*, 2010). Hat4, another member of B-type KAT superfamily anchored to Golgi apparatus and is involved in H4 acetylation prior to chromatin assembly (Yang *et al.*, 2011). Type-B HATs share homology with general control non-derepressible 5 (Gcn5) and belong to the GNAT superfamily (Dutnall *et al.*, 1998; Yang *et al.*, 2011).

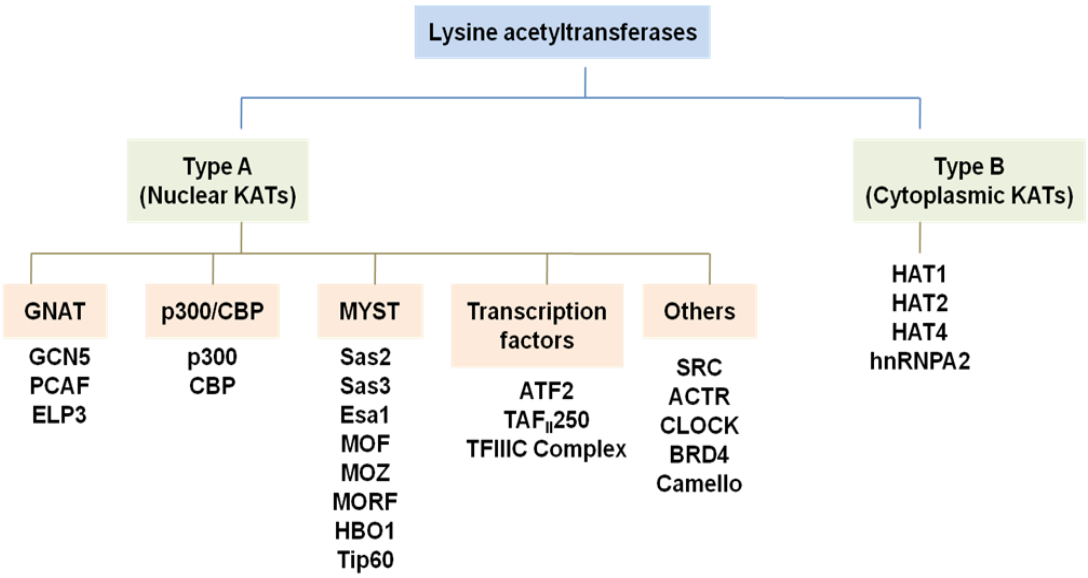


Figure 1.5. Classification of prominent mammalian acetyltransferases *(the list is not exhaustive)*

A-type KATs

Nuclear KATs which acetylate nucleosomal histones, are predominantly located inside nucleus but increasing number of studies put forward evidences of their nucleocytoplasmic transport. For instance, autoacetylation or growth-factor induced

phosphorylation of PCAF, GCN5 induces their nuclear transport and deacetylation in the nuclear localization signal (NLS) in PCAF increases cytoplasmic accumulation (Blanco-Garcia *et al.*, 2009). The localization of p300/CBP can be compared to that of Hat1. During oocyte maturation p300/CBP is found in cytoplasm before being transported into nucleus (Kwok *et al.*, 2006). Intracellular localization of p300/CBP has been found to be affected in cancers (Fermento *et al.*, 2010).

Nuclear acetyltransferases can be categorized into superfamilies based on their sequence homology. Out of all the acetyltransferases found in eukaryotic system GNAT and p300/CBP family of acetyltransferases are the most characterized due to their potency and involvement in many cellular pathways. Other designated acetyltransferases such as SRC1 or Tip60 have very weak and often undetectable activity towards histones (Yamamoto and Horikoshi, 1997); others such as BRCA2 has no detectable intrinsic activity under certain conditions (Siddique *et al.*, 1998; Fuks *et al.*, 1998).

Table 1.3. Lysine acetyltransferases and their substrate specificities.

KAT super-family	KATs	Transcription-related functions/effects	Substrate specificity *in vitro*	Known native KAT complexes and specificities *in vivo*
GNAT	Hat1(KAT1)	- (cytoplasmic KAT)	H4	Sc HAT-B, HAT- A3 complexes (no nucleosomal acetylation)
	Gcn5 (KAT2) hGcn5(KAT2A)	Coactivator (adaptor)	H3/H4	Sc ADA, SAGA (H3/H2B); Hs GCN5 complex, STAGA, TFTC (H3)
	PCAF(KAT2B)	Coactivator	H3/H4	Hs PCAF complex (H3, weak H4)
	Elp3(KAT9)	Transcriptional elongation	H3/H4/H2A/H2B	Elongator, pol II holoenzyme (H3/ weak H4)
	Hpa2(KAT10)	-	H3/H4	Hpa2
	Hpa2	-	H4	
	Nut1	Transcription factor	H3/H4	Mediator
MYST	Sas2 (KAT8)	Silencing	H4	SAS
	Sas3(KAT6)	Silencing	H3/H4/H2A	NuA3 (H3)
	Esa1 (KAT5)	Cell cycle progression	H4/H3/H2A	NuA4 (H4/ H2B), Pic.NuA4
	MOF(KAT8)	Dosage compensation	H4/H3/H2A	MSL complex
	Tip60(KAT5)	HIV-Tat interaction, DNA repair	H4/H3/H2A H4/H3/H2A	Tip60 complex
	MOZ(KAT6A)	Leukomogenesis	-	MOZ/MORF
	MORF(KAT6B)	-	H4/H3/H2A	MOZ/MORF
	HBO1(KAT&)	ORC interaction	-	HBO1 complex (H3/H4)
p300/CBP	p300 (KAT3B) CBP (KAT3A)	Global coactivators	H3/H4/H2A/ H2B	
Nuclear receptor coactivator	SRC1 (KAT13A) ACTR (KAT13B) TIF2 (KAT13C) CLOCK(KAT13D)	transcriptional response to hormone signals	H3/H4 H3/H4 - H3	
Others	TAF1 (KAT4)	RNAPII general transcription factor	H3/H4	TFIID
	TFIIIC220	RNAPIII transcription initiation	-	
	TFIIIC110		-	TFIIIC(H2A/H3/H4)
	TFIIIC90 (KAT12)		H3	
	Rtt109 (KAT11)		H3	
	CMLO3		H4	

In the recent years, three new proteins were added to the growing list of lysine acetyltransferases: Camello, which was identified as an unrelated KAT protein essential for zebrafish development (Karmodiya *et al.*, 2014), the bromodomain protein 4 (BRD4), an epigenetic reader protein which was identified as a lysine acetyltransferase required for histone eviction (Devaiah *et al.*, 2016), and hnRNPA2, a mitochondrial protein, recently discovered as a KAT which bears structural resemblance to p300/CBP family of KATs (Guha *et al.*, 2016).

1.4.1. GNAT family lysine acetyltransferases

The first ever A-type acetyltransferase to be characterized belong to GNAT superfamily, one of the best-understood set of acetyltransferases till date. At least 12 different KATs acetylating both histone and nonhistone substrates have been included in GNAT superfamily. Gcn5, PCAF, Hat1, Elp3 and Hpa2 are the most prominent KATs in this family. Overall GNAT family KATs show very little pairwise sequence similarity (3-23%) but harbor four conserved motifs- C, D, A, and B, in N- to C-terminal order. Motifs C and D are required for protein stability and A and B contain acetyl-CoA and substrate binding motifs respectively. While motif C is exclusive to GNAT family KATs, motif A is shared with MYST family acetyltransferases. A conserved stretch of Arg/Gln-X-X-Gly-X-Gly/Ala known as 'P loop' in A motif recognizes and binds to pyrophosphate moiety of acetyl-CoA (Dutnall *et al.*, 1998; Wolf *et al.*, 1998; Sterner and Berger, 2000). GNAT family members have ~160 residue long KAT domain and a C-terminal bromodomain.

Gcn5 (general control nonderepressible-5) was discovered in *Tetrahymena thermophilia* nuclear extract as a 55 kDa protein (p55) based on its ability to transfer radiolabeled acetyl-CoA to a histone impregnated gel slice (Brownell *et al.*, 1995; Brownell *et al.*, 1996). Subsequently in yeast *Saccharomyces cerevisiae* Gcn5 was characterized as a transcriptional adaptor establishing interaction between certain activators and transcription complexes (Berger *et al.*, 1992; Georgeakopoulos *et al.*, 1992; Marcus *et al.*, 1994; Silverman *et al.*, 1994). Gcn5 has then been identified and characterized in various organisms including human and found to possess a conserved function throughout eukaryotes, Gcn5 KAT domains from different species can substitute each other *in vivo* (Candau *et al.*, 1996; Wang *et al.*, 1997). *In vitro* recombinant Gcn5

acetylates free histones H3 strongly and H4 weakly but are unable to acetylate nucleosomal histones (Grant *et al.,* 1997; Kuo *et al.,* 1995; Ruiz-Garcia *et al.,* 1997). Effective acetylation by Gcn5 in physiological conditions requires aid of multisubunit complexes such as SAGA and ADA or alternatively spliced version of Gcn5 that contains N terminal sequence required for chromosomal substrate recognition (Xu *et al.,* 1998b). C terminal bromodomain in Gcn5 interacts with DNA-dependent protein kinase and phosphorylation inhibits Gcn5's KAT activity *in vivo* (Barley *et al.,* 1998). Acetylation by Gcn5 is observed in promoters and it affects chromatin remodeling, transcription *in vivo* and cell growth (Kuo *et al.,* 1995; Gregory *et al.,* 1998; Wang *et al.,* 1998).

PCAF (p300/ CREB-binding protein associated factor) bears ~80% similarity with Gcn5 and interacts with p300/CBP mediating transcriptional activation of genes (Yang *et al.,* 1996). Similar to Gcn5, PCAF acetylates free or nucleosomal histones H3 strongly and H4 weakly. PCAF acts as a transcriptional coactivator when bound to a promoter proximal site or distal enhancer site by acetylating local nucleosomal histones or chromatin proteins or transcription-related proteins such as HMGs, p53, Tat, MyoD, TFIIIF, TFIIE etc. Therefore, PCAF's role has been implicated in myogenesis and nuclear receptor-mediated, growth factor-signaled activation (Blanco *et al.,* 1998; Krumm *et al.,* 1998; Leo *et al.,* 2000; Puri *et al.,* 1997; Xu *et al.,* 1998a).

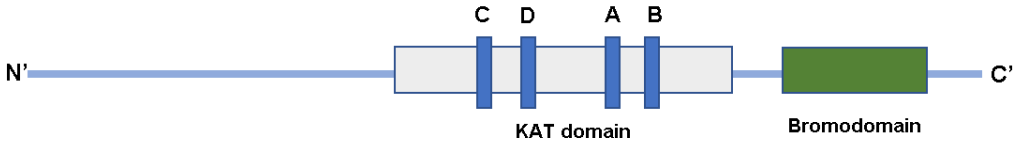


Figure 1.6. Schematic representation of general features of GNAT family acetyltransferases*. GNATs possess KAT domain located in mid-region of the protein comprising of four short stretches of conserved regions identified as C, D, A and B. These KATs also possess a bromodomain located in C terminal.*

Elp3 was discovered in yeast as the smallest elongator subunit of three-subunit elongator complex that tightly binds to hyperphosphorylated CTD of RNA polymerase II (Otero *et al.,* 1999). *In vitro* recombinant Elp3 could acetylate all four core histones (Wittschieben *et al.,* 1999). Elp3 null mutants are viable with slow activation of some genes related to growth adaptation, salt or temperature sensitivity *etc.* (Otero *et al.,* 1999).

Hpa2 is GNAT family KAT that acetylates H3 and H4; Hpa3, a Hpa2 homolog in yeast also possesses weak acetyltransferase activity and both are known to undergo extensive autoacetylation. Hpa2 forms dimer or tetramer *in vitro, in vivo* Hpa2 function is yet to be characterized; as Hpa2 null mutants do not show any apparent growth defect (Angus-Hill *et al.,* 1996).

SSAT (Spermidine/spermine N1-acetyltransferase) is another GNAT family KAT that acetylates polyamines spermidine/spermine promoting their degradation. SSAT is associated with different diseases, including Alzheimer's, cystic fibrosis and cancer (Pegg *et al.,* 2008).

- **Role of GNAT family KATs:**

GNAT family acetyltransferases are important for cell growth and development. Gcn5L null mice are embryonic lethal (Xu *et al.,* 2000). Yeast Gcn5 is required for normal progression through G2-M boundary and mitotic gene expression (Zhang *et al.,* 1998; Krebs *et al.,* 2000). Mammalian cell cycle regulatory proteins such as c-Myc and E2F family of activators actively recruit GNAT complexes through TRRAP subunits (McMahon *et al.,* 2000; Lang *et al.,* 2001). Similarly, SAGA complex also contacts with various transcriptional activators, or, recruit chromatin remodeling complexes such as SWI/SNF complex facilitating further factor binding and induction of transcription (Syntichaki *et al.,* 2000; Hassan *et al.,* 2002). Interestingly, opposing effect of SAGA on transcription has also been recorded, it can also mediate repression through ArgR/Mcm1 complex (Ricci *et al.,* 2002). Mammalian GCN5L containing complexes STAGA and TFTC are involved in DNA repair. STAGA associates with UV-damaged-DNA binding protein (UV-DDB) p127 and the p127-related protein SAP130 on irradiated DNA and TFTC associates with SAP130 leading to increased HAT activity required for DNA repair through XRCC (Martinez *et al.,* 2001; Brand *et al.,* 2001; Feaver *et al.,* 1993). Yeast Hat1 is involved in double-strand break repair (Qin and Parthun, 2002).

1.4.2. p300/CBP family acetyltransferases

p300 is a ubiquitously expressed, essential protein possessing more than one enzymatic function: acylation (acetyl-, butyryl-, propionyl-, crotonyl- transferase) and E3/E4 ligase activities. p300 also acts as a nodal integrating point for several signaling pathways and

is enriched on crucial gene regulatory elements such as promoters, enhancers modulating several gene expression. p300 plays critical role in several developmental and differentiation programs which has been discussed below.

A brief history of p300/CBP:

Ep300 or p300 (E1A-binding protein, 300kDa), also known as KAT3B and RSTS2, was discovered as an adenoviral E1A interacting phosphoprotein which was later found to be indispensable for the transforming activity of E1A (Yee and Branton, 1985; Whyte *et al.*, 1989). p300 paralog, CBP (CREB-binding protein; also known as KAT3A, RSTS1) was discovered as a nuclear protein co-precipitated with phosphorylated CREB (c-AMP response element binding protein) that facilitates cAMP response gene expression (Chrivia *et al.*, 1993). After discovery both p300 and CBP were characterized as transcriptional coactivator facilitating certain gene expression; later in 1996, p300/CBP were found to have intrinsic acetyltransferase activity opening up new avenues for regulatory functions for this family of KATs (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996).

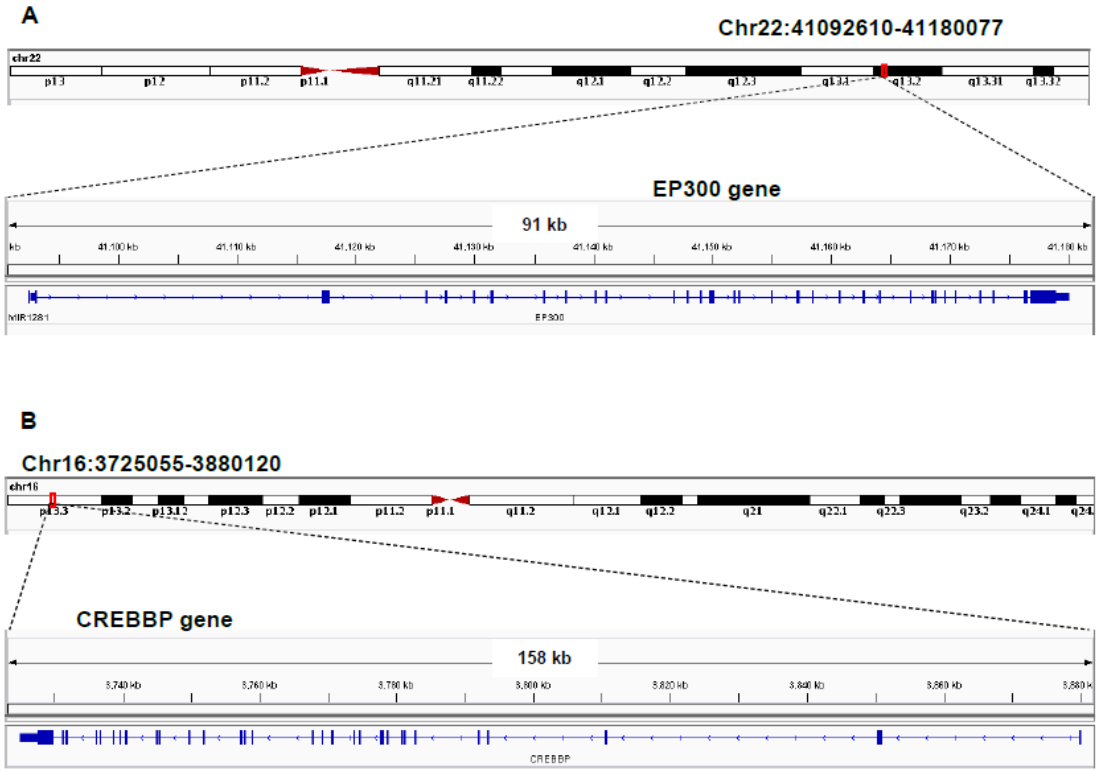


Figure 1.7. Genomic location of the EP300 gene and its homologous CREBBP gene. *EP300 is located on chromosome 22: q13.2 while its homolog CREBBP is located on chromosome 16: p13.3. EP300 and CREBBP are large genes spanning approximately*

90 kb and 150 kb in length respectively. Visualization of gene loci was done using the Integrative Genomics Viewer (Robinson et al., 2011).

p300 and CBP (KAT3 family) were found to be highly homologous proteins and were predicted to be members of a family of coactivators (Arany *et al.*, 1994; Lundblad *et al.*, 1995). Human EP300 (Ensembl ID: ENSG00000100393) occupies ~91 kb genomic region on the long arm of chromosome 22 (Chromosome 22: 41,092,610-41,180,077; + strand) and CBP is located on chromosome 16 (Chromosome 16: 3,725,055-3,880,120; - strand) spanning over 150 kb. Although p300 and CBP originated from a single gene, over the course of evolution these proteins have diverged considerably, acquiring unique functions which render them indispensable for the development of multicellular organisms. Mammalian p300/CBP has evolved to possess multiple functions along with ateast three different types of enzymatic functions which has been listed in Figure 1.8.

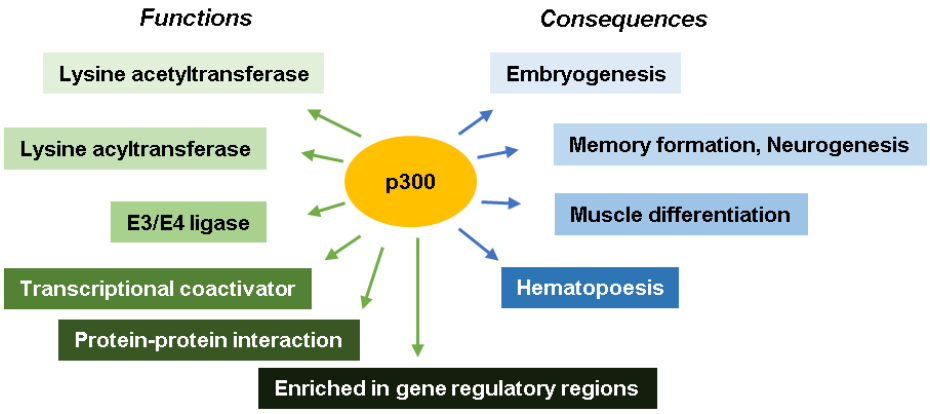


Figure 1.8. The multiple intrinsic enzymatic/non-enzymatic activities of p300. *p300 is a general transcription coactivator possessing several enzymatic functions, of which its lysine acetyltransferase activity is well studied. It is an essential protein with functional consequences in numerous developmental/ differentiation programs.*

Functional Domains of p300/CBP:

p300 is a large protein comprising of 2414 amino acids and multiple functional domains connected with unstructured flexible loops (Dyson and Wright, 2016). 2442 amino acid long CBP shares these conserved functional domains with p300. The N-terminal domains include the Nuclear Receptor Interacting Domain (NRID), the Cysteine/ Histidine-rich domain 1 (CH1) and KIX domain. The central catalytic core domains comprise of the Bromodomain, CH2 domain (consisting of the RING and PHD), and Lysine/histone acetyltransferase (also acyltransferase) domain (KAT/HAT). The C-terminal domains

are the CH3 domain and the Interferon binding domain (IBiD). The CH1 and CH3 domains contain transcriptional zinc-finger adapter motifs, TAZ1 and TAZ2 respectively. The CH3 domain also contains another zinc-finger motif known as ZZ motif. The IBiD domain comprises of Nuclear Coactivator Binding Domain (NCBD), a glutamine rich stretch followed by a proline-rich PxP motif (Bedford *et al.*, 2010; Yang and Seto, 2008). p300 and CBP share a number of similarities between them: apart from being transcriptional coactivators for RNAPII, these proteins share high sequence, structure and functional homology. Acetyltransferase and bromodomains of p300 and CBP share ~90 and 93% homology. Additionally, both the proteins show almost identical substrate specificity at least for histone substrates and both the proteins are important for proper growth and development (Kalkhoven, 2004; Schiltz *et al.*, 1999).

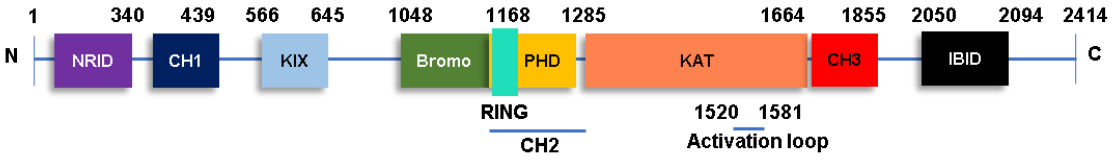


Figure 1.9. Domain Architecture of p300. *p300 consists of (from N-terminal) the Nuclear Receptor Interacting Domain (NRID), Cysteine/ Histidine-rich domain (CH1), KIX, Bromodomain, CH2 (RING, PHD), Lysine/histone acetyltransferase domain KAT, CH3 and Interferon binding domain (IBID).*

The KAT domain of p300 comprises of a central β-sheet containing seven β-strands surrounded by nine α-helices and several loops (Liu *et al.*, 2008) While the central core region of p300 KAT domain bear structural similarity with that of Gcn5/PCAF and MYST family KAT domains (Liu *et al.*, 2008; Trievel *et al.*, 1999; Yan *et al.*, 2002), two main features made p300 KAT domain distinctive. Firstly, p300 KAT domain catalytic tunnel is decorated with an exceptionally long substrate binding loop L1, and secondly, p300 KAT domain, especially the substrate binding site, has an overall negative surface charge contrasting to deeper, polar substrate binding sites of the other KAT domains.

The long L1 loop favors not only orderly and stable binding of adenosine ring of acyl-CoAs but also establishes extensive intramolecular interactions with the substrates. The KAT domain harbors two distinct shallow electronegative patches, of which one (P1) makes contacts with the lysine moiety of the Lys-CoA bisubstrate while the other (P2) located ~10 Å away was shown to be important for substrate binding and catalysis (Liu *et al.*, 2008; Wang *et al.*, 2008a). Typically, p300/CBP substrates have p300/CBP-

acetylated lysine residues flanked by positively charged amino acid residues either three to four residues upstream or downstream (~10 Å) from the acetylation site (Thompson *et al.*, 2001). The flexibility of the shallow substrate binding P2 groove, which governs substrate recognition, could account for the promiscuous substrate specificity of p300 in comparison to the other KATs (Wang *et al.*, 2008a). p300/CBP acetylates a wide variety of proteins, both histone as well as non-histone proteins. p300-mediated histone acetylation is a mark of actively transcribing promoters (eg. H2AK5ac, H3K9ac, H3K18ac, H3K23ac, H3K27ac, H4K5ac, H4K8ac, H4K12ac) and active enhancers (H3K27ac). Furthermore, there is a growing interest in the non-histone substrates of p300/CBP. A brief overview of a few p300/CBP substrates and the functional consequences of p300/CBP-mediated acetylation on their functions have been listed in Table 1.4.

Table 1.4. p300/CBP-mediated non-histone protein acetylation and its consequence

Effect	Substrate	References
Enhanced transcriptional activation or, activation of downstream signaling	p50 (K431, K440, K441) WNT/β-catenin (K354) p53 (K117, K161, K162) ERα (K229, K299, K302 and K303) AR (K630, K632, K633) RFPL3 Ku80 Snail (K146,K187) Smad2 (K19, K20, K39) Smad3 (K378) p73a (K321, K327, K331) HMGB1 (K2, K11)	(Deng and Wu, 2003) (Garcia-Jimenez et al., 2014; Levy et al., 2004) (Li et al., 2012) (Wang et al., 2001) (Fu et al., 2003; Fu et al., 2000) (Qin et al., 2015) (Hsu et al., 2014) (Xiao et al., 2015) (Tu and Luo, 2007) (Inoue et al., 2007) (Costanzo et al., 2002) (Pasheva et al., 2004; Sterner et al., 1979)
Increased DNA binding	RelA (K218, K221, K310) STAT3 (K685) p53 (C-terminal) PC4 (Lysine-rich domain) RUNX1 (K24, K43)	(Chen et al., 2002; Huang et al., 2009) (Wang et al., 2005a; Zhuang, 2013) (Gu and Roeder, 1997) (Kumar et al., 2001) (Wang et al., 2009)
Enhanced protein function or, enhanced cognate interactor binding	p53 (K382) PTEN (K402) pRB (K873, K874) HMGA1 (K65, K71) NPM1(K212, K215,K229, K230, K257, K267 and K292)	(Li et al., 2007; Mujtaba et al., 2004) (Ikenoue et al., 2008) (Chan et al., 2001; Nguyen et al., 2004) (Munshi et al., 1998) (Shandilya et al., 2009; Swaminathan et al., 2005)
Increased protein stability	c-Myc (C-terminal domain) Smad7 (K64, K70) HIF-1α (K709)	(Vervoorts et al., 2003) Grönroos et al., 2002; Simonsson et al., 2005) (Geng et al., 2012)
Decreased DNA binding	Rel A (K122, K123) FoxO1 K242, K245 and K262	(Kiernan et al., 2003) (Calnan and Brunet, 2008)
Repression of protein function	cMyc (K143, K157, K275, K317, K323, and K371) Beclin-1 (K430, K437) HDAC1 (K218, K220, K432, K438, K439, and K441) E1A (K239)	(Wasylishen et al., 2014; Zhang et al., 2005) (Sun et al., 2015) (Qiu et al., 2006; Yang et al., 2015a) (Deng et al., 2005)
Decreased protein stability	MATIIα (K81)	(Yang et al., 2015b)

Biochemical studies revealed that p300 follows the Theorell-Chance (or 'hit and run') catalytic mechanism where p300 forms a stable complex with acetyl-CoA, followed by the transient binding to the protein substrate at the P1 pocket, stable ternary complex is not formed. After acetylation of the lysine residue, the protein substrate immediately dissociates from the enzyme. This mechanism is consistent with the broad spectrum of substrate specificity observed in the case of p300/CBP. Among the catalytic residues Tyr 1467 (functions as a general acid to protonate the CoA leaving group) and Trp 1436 (guides the substrate lysine side chain into attacking acetyl-CoA by vander Waal's contact) are critical (Wang *et al.*, 2008a).

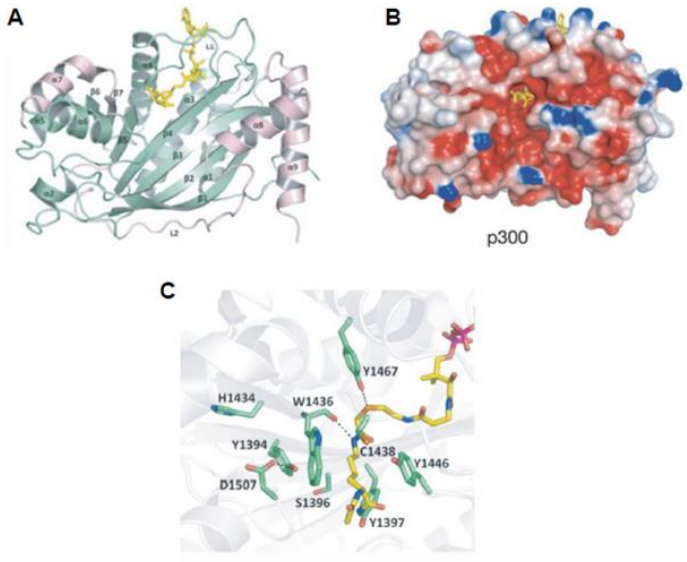


Figure 1.10. Structure of p300 HAT domain. *(A) Structure of the p300 HAT domain with N and C subdomains colored in green and pink, respectively. Lys-CoA is shown in yellow stick figure representation. (B) Electrostatic surface representations of the HAT domains of p300. (C) The active site of p300 showing residues that are in position to play potential catalytic roles (Liu et al., 2008).*

- **Modulation of p300/CBP function:**
- **Autoinhibition of acetyltransferase function by RING domain:**

Further investigation into p300 core catalytic region showed that four central domains (bromodomain, PHD, RING and KAT domain) form a compact conformation in which the RING domain is positioned such that it obstructs the substrate binding of the KAT

domain. Mutation that repositions RING domains enhanced acetyltransferase activity of p300 (Delvecchio *et al.*, 2013).

- **Autoinhibitory loop of p300 KAT domain:**

Besides RING domain, an auto-inhibitory loop resides in the p300 KAT domain that modulates the catalytic activity of p300 (Thompson *et al.*, 2004). This lysine-rich loop interacts with the electronegative patch present in the catalytic site acting as a pseudo-substrate inhibiting efficient substrate binding. Trans-autoacetylation masks the positive charges on the lysine residues in the loop allowing it to be displaced from the catalytic site leading to activation of the enzyme (Karanam *et al.*, 2006; Thompson *et al.*, 2004).

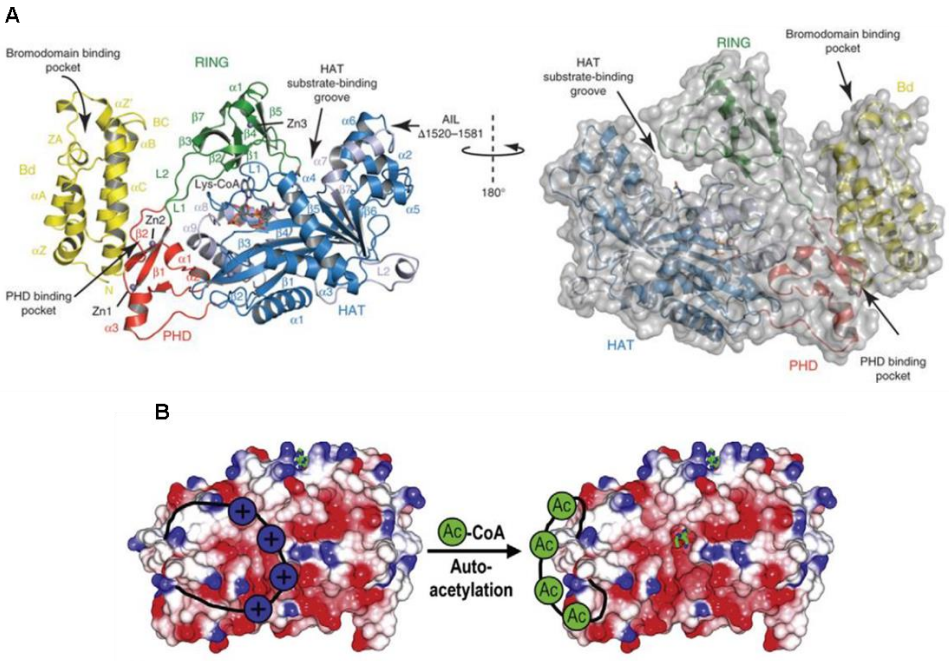


Figure 1.11. A. Structure of p300 core catalytic domain. *Ribbon and surface representations of the p300 core structure with labeling of secondary-structure elements. The bromodomain (Bd), RING and PHD domains are shown in yellow, green and red, respectively. The N and C subdomains of the HAT domain are shown in blue and grey, respectively. The position of the deleted autoinhibitory loop (AIL) is indicated with an arrow. Lys-CoA is shown in stick representation (Delvecchio et al., 2013). B. Modulation of p300 catalytic activity through autoacetylation. A model for the regulation of p300/CBP by autoacetylation is shown where it is proposed that the lysine-rich basic activation loop blocks the substrate-binding site when unacetylated but is displaced upon autoacetylation (Wang et al., 2008a).*

p300 autoacetylation is modulated by various factors. Inflammatory signals such as lipopolysaccharide (LPS) or interferon γ prompts glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to translocate to nucleus where p300 acetylates GAPDH and GAPDH in turn stimulates catalytic activity of p300/CBP activating further downstream targets such as p53 (Hara *et al.*, 2006; Hara and Snyder, 2006; Sen *et al.*, 2008).

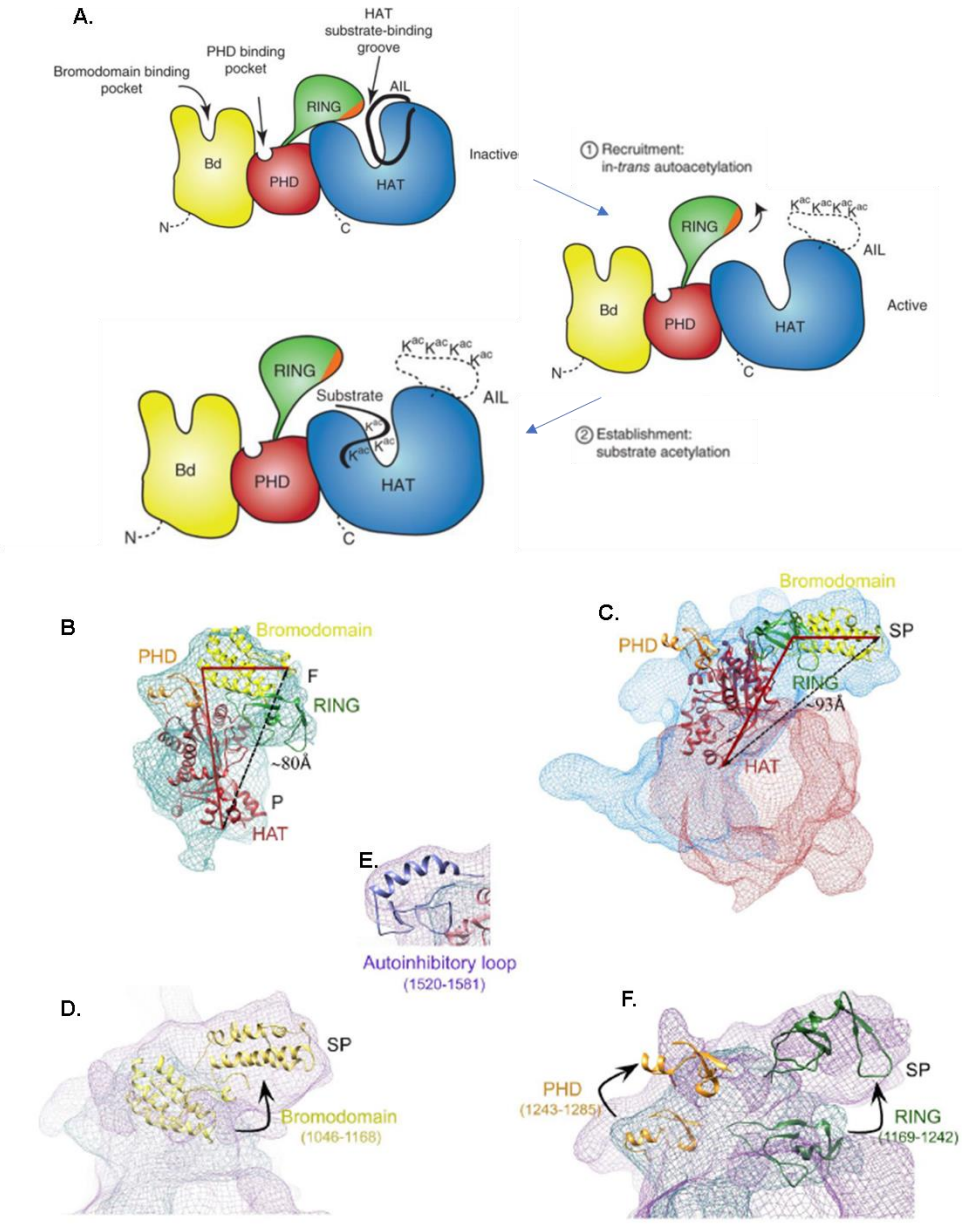


Figure 1.12. Model for p300 autoregulation. *(A) Theoretical model: In the inactive state (top), the RING domain occludes the catalytic domain (substrate-binding groove in the HAT domain). The model proposes that p300 activation will be initiated by the recruitment of at least two copies of p300 resulting in autoacetylation in trans of the autoinhibitory loop (AIL) and displacement of the RING domain. Rearrangement of the*

core catalytic domains exposes the HAT active site, resulting in substrate acetylation. Yellow, bromodomain (Bd); green, RING; red, PHD; blue, HAT; Kac, acetyl-lysine; X, any amino acid (Delvecchio et al., 2013). (B-F) Experimental model: Conformational changes in the central domain of p300 upon complex formation with p53 (Ghosh et al., 2019). Fitting of different subdomains (Bromo (yellow), RING (green), PHD (orange) and HAT (red)) of the central domain of p300 in (B) p300 (dark cyan mesh, contour level 0.02) and (C) p300-p53 complex (p300, blue mesh, p53, red mesh) maps. Subdomains are shown in cartoon representations. Opening up of the kink between the Bromo and HAT domains is clearly detected. Close-up views of the superposed cryo-EM maps of p300 and p300-p53 complex (dark cayan mesh, p300, purple mesh, p300-p53 complex) with the corresponding fitted structures showing (D) reorientation of the Bromo-domain (yellow), (E) loop-like density seen on the back of p300 density in the complex that can accommodate a model of the autoinhibitory loop, and (F) RING (green) and PHD (orange) sub-domains of p300 central domain are displaced following complex formation.

Histone chaperone NPM1 (Nucleophosmin 1) is another factor which translocates to nucleus under chronic inflammatory signals and induces p300 autoacetylation (Shandilya *et al.,* 2009; Arif *et al.,* 2010; Kaypee *et al.,* 2018; Kaypee *et al.,* 2018b). Mastermind-like protein 1 (MAML1), a transcription coactivator of the NOTCH pathway, directly interacts with p300 and enhances its autoacetylation followed by transcription co-activation (Hansson *et al.,* 2009). Recent study has elucidated that tumor suppressor (p53) and its gain-of-function mutants expressed in various types of cancer cells interact with p300 in its tetrameric conformation and induce p300 autoacetylation which is subsequently enriched in the target gene TSS inducing their expression in cancer (Kaypee *et al.,* 2018).

- **Modulation of p300 activity by PTMs:**

Other than factor-mediated autoacetylation p300/CBP undergo a vast array of post-translational modifications and many of these PTMs appear to be conserved in both proteins. The reported PTMs such as phosphorylation, arginine methylation, SUMOylation and acetylation have varying consequences on their enzymatic activity, transactivation potential, protein stability and interactome.

p300 was first discovered as highly phosphorylated nuclear protein (Yee and Branton,1985). Several kinases such as protein kinase A (PKA), protein kinase C (PKC), cyclin dependent kinase 1 (CDK1), mitogen associated protein kinase (MAPK), salt-inducible kinase 2 (SIK2) *etc.* and depending on the site of phosphorylation p300 activity as well as stability are known to be affected. While ERK1/2-mediated phosphorylation of p300 on S2279, S2315 and S2366 (Chen *et al.*, 2007b), and Akt-mediated phosphorylation on S1834 increase the KAT activity of p300 (Huang and Chen, 2005), while phosphorylation on S89 by SIK2 and PKC kinase inhibits its catalytic activity (Bricambert *et al.*, 2010; Yuan *et al.*, 2002). On the other hand, in response to double-strand breaks (DSB) induced by etoposide or ionizing radiation (IR), ataxia-telangiectasia mutated (ATM) kinase phosphorylates p300 on S106 which results in p300 stabilization (Jang *et al.*, 2010; Jang *et al.*, 2011). Contrastingly, p38 MAPK-mediated phosphorylation on S1834 induces p300 degradation following DNA damage response (Poizat *et al.*, 2005; Wang *et al.*, 2013). In lung cancer, p300 is phosphorylated on S1038 and S2039 by CDK1 and ERK1/2 leading to its degradation, resulting in increased cell proliferation and metastasis (Wang *et al.*, 2014).

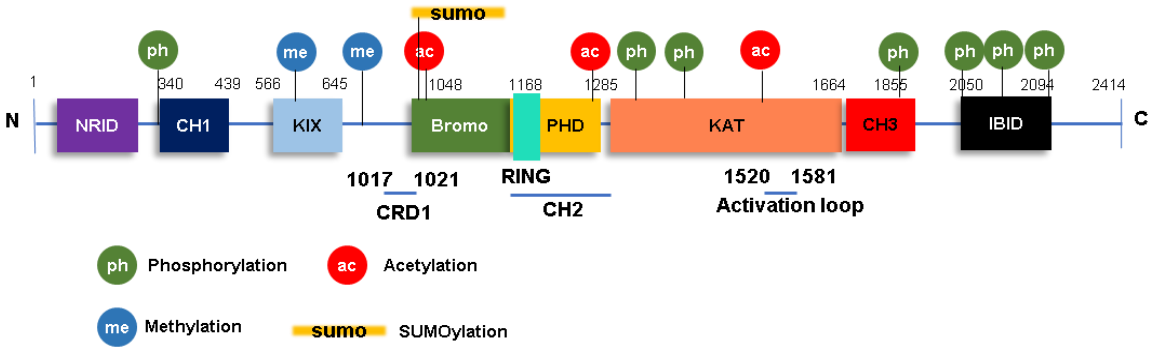


Figure 1.13. Post translational modifications of p300. *Domain organization of human p300 protein. Acetylation, phosphorylation, methylation, and sumoylation sites are denoted by circles containing the letters ac, ph, me, and su, respectively.*

Arginine methylation in p300 is known to enable the protein being involved in differential signaling pathway. p300 is a substrate of arginine methyltransferase coactivator associated arginine methyl transferase 1 (CARM1) (Bedford and Clarke, 2009). Methylation of R580 in KIX domain of p300 abrogates its interaction with CREB interaction shutting off cAMP-induced transcription (Xu *et al.*, 2001) whereas, in C terminal R754 methylation promotes steroid hormone receptor mediated transcription and BRCA1 recruitment to p21 promoter in response to DNA damage (Chevillard-Briet

et al., 2002, Lee *et al.,* 2011). The methylation and demethylimination of p300 by CARM1 and peptidyl deiminase 4 (PAD4), respectively, on the sites R2088 and R 2142 are important for the interaction of p300 with the coactivator GRIP-1 (Lee *et al.*, 2005). Arginine methylation on these sites regulate the assembly and disassembly of p300 coactivator complexes, thus modulating transcriptional outcomes (Lee *et al.*, 2005). CBP methylation by CARM1 is critical for CBP recruitment to ER-responsive target gene promoters (Ceschin *et al.,* 2011).

p300 is SUMOylated on the Cell Cycle Regulatory domain 1 (CRD1) which is located within the bromodomain between the residues 1017 and 1029. SUMOylated p300 is involved in transcriptional repression which is mediated by the specific recruitment of the histone deacetylase, HDAC6 (Girdwood *et al.,* 2003). The Anaphase Promoting Complex/Cyclosomes (APC/C) subunits APC5 and APC7 enhance p300 autoacetylation which is required for proper cell cycle progression (Turnell *et al.,* 2005). p300 autoacetylation is negatively regulated by the Class III lysine deacetylase, SIRT2 and as expected, the suppression of p300 autoacetylation leads to the reduction of its enzymatic activity (Black *et al.*, 2008). A recent study has elucidated a unique mechanism for induction of p300 autoacetylation through the binding of eRNAs (Bose *et al.,* 2017).

The autoacetylation of p300/CBP can be enhanced by certain small molecules as well. The first reported activator of p300 activity is N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (CTPB), an anacardic acid derivative (Balasubramanyam *et al.,* 2003). Since this molecule is cell impermeable, a carbon nanoshere (CSP) was used as a carrier (Selvi *et al.,* 2008). Further derivatization of CTPB molecule led to the synthesis of TTK21 (N-(4-chloro-3-trifluoromethyl-phenyl)-2-N-propoxy-benzamide), another potent KAT3 activator *in vitro* and *in vivo*. TTK21, when conjugated to CSP, could effectively cross the blood brain barrier and enhance neurogenesis and long-term memory formation in adult mice (Chatterjee *et al.*, 2013). These small molecule activators of p300/CBP appear to be promising therapeutics in diseases where CBP or p300 proteins are down-regulated or inactivated.

- **Transcriptional integration by p300/CBP:**

p300/CBP can affect cellular transcription in the following ways (i) p300/CBP can act as adaptor proteins bridging the basal transcription machinery (eg. TATA box-binding protein (TBP), TFIIB, TFIIE, TFIIF) to DNA sequence-specific transcription factors (eg.

p53, NFκB, cMyc) (Bannister and Kouzarides, 1996; Chan and La Thangue, 2001; Ogryzko *et al.,* 1996). (ii) p300/CBP can act as scaffold proteins interacting with multiple transcription factors/co-factors through their multiple domains (Chan and La Thangue, 2001; Dyson and Wright, 2016). By virtue of their structural plasticity, p300/CBP can physically interact with a vast array of transcription factors thereby functioning as nodal integrators of transcription (Bedford *et al.,* 2010; Dyson and Wright, 2016; Lee *et al.,* 1998; Perissi *et al.,* 1999; Ravi *et al.,* 1998). (iii) Finally, p300/CBP can covalently modify histones to facilitate the loosening of chromatin resulting in enhanced transcription (Chen *et al.,* 2007a; Sabari *et al.,* 2015).

p300 and CBP are referred to as molecular integrators owing to their complex network of protein-protein interactions. They integrate multiple transcriptional cues which are highlighted by their ability to synergize the transactivation of multiple transcription factors bound to the same promoter elements in cis. In contrast, the limited intracellular pool of these transcription co-activators leads to constant competition among transcription factor for the binding of p300/CBP. This could also dictate the preference of a transcription signaling pathway over another. Therefore, it is evident that p300/CBP play a pivotal role in the decision of transcriptional outcomes. This function of p300/CBP is endowed by their ability to interact with a multitude of cellular proteins. It is estimated that p300 can interact with over 400 proteins in the cell, most of which are related to transcription regulation.

- **Dissimilarity between p300 and CBP:**

Despite of having high level of homology between sequence, structure and function (in terms of substrate specificity) p300 and CBP have been implicated in diverse cellular pathway. Outside the conserved domains the homology between these two proteins are quite different. A detailed study has highlighted the substrate level selectivity and specificity of these two enzymes (Henry *et al.,* 2015). During development certain organs were shown to more tolerant to p300 or CBP loss as compared to whole organism indicating p300 and CBP possess nonredundant functions *in vivo*. Heterozygous inactivation of p300 or CBP gave rise to cellular and organismal defects that were not compensated by presence of the other homologue. While heterozygous inactivation of p300 led to more severe abnormalities in heart, lung, small intestine than inactivation of CBP (Tanaka *et al.,* 1997; Yao *et al.,* 1998), mutations in CBP have been majorly linked

to Rubinstein-Taybi syndrome, congenial neurodevelopment disorder, and fetal alcohol syndrome (Roelfsema *et al.,* 2005; Lopez-Atalaya *et al.,* 2012; Viosca *et al.,* 2010; Guo *et al.,* 2014). p300 mediated aberrant acetylation plays more prominent role in carcinogenesis (Ozdag *et al.,* 2002; Gayther *et al.,* 2000; Santer *et al.,* 2011).

- **Deregulation of p300/CBP:**

p300/CBP being important coordinator and effector of several signal nodes disruption of these protein functions including their acetyltransferase activity has profound implications. Disruption of p300 activity may lead to cell cycle arrest in G1/S phase, accumulation of senescent markers, or, defects in cell proliferation (Yan *et al.,* 2013; Ait-Si-Ali *et al.,* 2000; Yao *et al.,* 1998). Rubinstein-Taybi syndrome (RTS) caused by germline mutations either in EP300 or CREBBP gene allele (Roelfsema and Peters, 2007; Rubinstein and Taybi, 1963). Interestingly, CBP deficiency is associated with hematological malignancy which is also an associated symptom of RSTS (Ghizzoni *et al.,* 2010). p300/CBP null mutant chimeric mice are susceptible to tumor formation (Rebel *et al.,* 2002).

p300 and CBP are important for maintenance of cellular homeostasis. In cancers, p300 and CBP can function either as oncogenes or tumor suppressors, depending on the context which govern their cellular functions. The loss of heterozygosity (LOH) in cell lines and primary tumors at the EP300 or CREBBP gene loci due to chromosomal loss or inactivating mutations such as missense mutations, frameshift or truncations, have indicated a probable tumor suppressive role of these enzymes. LOH at the EP300 locus (22q13) has been observed in numerous cancers including oral, breast, ovarian, hepatocellular, colorectal, gastric cancers and glioblastomas (Iyer *et al.,* 2004). The biallelic loss and inactivating mutations are relatively rarer at the CREBBP locus (16p13). CREBBP gene mutations have been observed in colon, breast, lung, and ovarian cancers (Kishimoto *et al.,* 2005; Ozdağ *et al.,* 2002; Tillinghast *et al.,* 2003). In hematological malignancies, chromosomal instability at the CREBBP or EP300 loci leads to translocation events. Although, the events of translocations occur at a low frequency, they are often associated with poor prognosis of the disease (Diab *et al.,* 2013). Chromosomal translocations often result in fusion KAT proteins which retain the functional HAT and bromodomains. In-frame translocations confer oncogenic potential to these chimeric proteins, such as in the case of mixed lineage leukemia (MLL)-CBP

t[11;16] (q23;p13) or in MLL-p300 t[11;22](q23;q13) fusions (Krivtsov and Armstrong, 2007). Apart from chromosomal aberration p300/CBP can interact with oncoproteins such as c-Myc, c-Myb, HIF1α, β-catenin, and androgen receptor (AR) (Bannister and Kouzarides, 1995; Bannister *et al.,* 1995; Dai *et al.,* 1996; Hecht *et al.,* 2000; Liu *et al.,* 2008; Sobulo *et al.,* 1997). The expression levels of p300/CBP are dysregulated in malignancies; overexpression of p300 strongly correlates with aggressiveness of hepatocellular carcinoma (HCC) and is a predictive marker for the poor prognosis of the disease (Li *et al.,* 2011; Yokomizo *et al.,* 2011). In prostate cancer, p300 levels correlate with the grade and tumor size and the higher p300 expression increases the risk of recurrence among patients (Debes *et al.,* 2003; Isharwal *et al.,* 2008). p300 expression is also correlates with the aggressiveness of cutaneous squamous cell carcinoma (CSCC) and nasopharyngeal carcinoma (Chen *et al.,* 2015; Liao *et al.,* 2012). In colorectal adenocarcinomas, p300 and CBP appear to have contrasting roles in disease outcome. The higher expression of p300 is predictive of poor prognosis while higher CBP expression correlates with longer survival in patients (Ishihama *et al.,* 2007). Upregulation in p300 expression is also linked to recurrence and poor prognosis in breast cancer and non-small cell lung carcinoma, while in the case of bladder cancer cells the high expression of p300 may confer doxorubicin-resistance (Hou *et al.,* 2012; Takeuchi *et al.,* 2012; Xiao *et al.,* 2011).

As discussed previously, post-translational modifications play a major role in the modulation of p300/CBP function. Phosphorylation of p300 on S1038 and S2039, by CDK1 and ERK1/2, results in its degradation which promotes the progression of lung cancer (Wang *et al.,* 2014). In oral cancer p300 activity is dysregulated due to the enhancement in its autoacetylation by NPM1 and GAPDH (Arif *et al.,* 2010b). Overall, it is apparent that the regulation of p300/CBP expression and activity is important for the normal functioning of the cell. In circumstances where the precise regulation of p300 or CBP is lost may lead to disorders such as cancers, neurodegenerative disorders, and metabolic diseases.

1.4.3. MYST family acetyltransferases

MYST family is named after its founding members: MOZ, Ybf2/Sas3, Sas2, and Tip60 (Borrow et al, 1996). Other proteins such as Esa1, MOF, HBO1, MORF *etc.* are also

included into this family later based on the presence of a homology region (Neuwald and Landsman, 1997).

Tip60 (Tat-interactive protein, 60kDa) was the first human MYST protein to be discovered and demonstrated to interact with the activation domain of HIV-1 transactivation protein (Tat) (Kamine *et al.,* 1996). However, Tat binding inhibited catalytic activity of Tip60 which otherwise acetylates free and nucleosomal H2A, H3 and H4 in general (Westendorp *et al.,* 1995). In cells Tip60 is present in a complex that possesses ATPase, DNA helicase and DNA binding activities and have implications in DNA repair and apoptosis (Ikura *et al.,* 2000). Tip60 is also known to act as a coactivator in ligand dependent manner (Brady *et al.,* 1999).

MOZ (Monocytic leukemia zinc finger protein) is a MYST family protein that is involved in oncogenic transformation leading to leukemia. Due to partial chromosomal translocation in acute myeloid leukemia N terminal of MOZ and C terminal of CBP fuses to form chimeric protein with acetyltransferase and transactivation properties (Borrow *et al.,* 1996). MOZ can also fuse with TIF2, a nuclear receptor coactivator KAT and fused protein exert aberrant acetylation leading to leukomogenesis similar to that of MOZ-CBP (Carapeti *et al.,* 1998, Liang *et al.,* 1998).

MORF (Moz-related protein) is highly homologous to MOZ that can acetylate free as well as nucleosomal histones with preference for H4 and H3. N-terminal repression region of MORF inhibits its catalytic activity and transactivation and C terminal activation domain can work independent of its KAT domain (Champagne *et al.,* 1999).

Sas2 and **Sas3** were initially discovered in yeast as components of transcriptional silencing machinery. Sas (Something about silencing) acetyltransferases can acetylate free histones H3, H4, H2A *in vitro* and Sas3 is a component of NuA3 (John *et al.,* 2000). KAT activity of Sas2 requires the presence of Sas4 and is stimulated by Sas5 (Sutton *et al.,* 2003). Sas2 promoted silencing in HML and telomere but inhibited it at HMR; Sas3 mediated silencing is weaker and only restricted for mating loci (Ehrenhofer-Murray *et al.,* 1997).

Esa1 (Essential Sas family acetyltransferase 1) was identified through its homology with MYST proteins. Esa1 is essential for cell cycle progression and null mutant of its gene is inviable (Smith *et al.,* 1998; Clarke *et al.,* 1999). In vitro recombinant Esa1 acetylated

free histones H3 and H2A weakly and H4 strongly but did not acetylate nucleosomal histones. Esa1 is also a component of NuA4 activator complex.

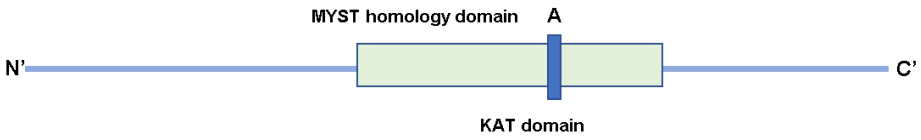


Figure 1.14. Schematic representation of general features of MYST family KATs. *MYST family shares homology region A with GNATs which also corresponds to acetyl-CoA binding motif. MYST homology domain (~90% conserved domain) is shared among this family of KATs which spans their KAT domains.*

MOF (Males absent on the first) mediated H4 acetylation is linked to X chromosome inactivation during dosage compensation (Hilfiker *et al.*, 1997). In vivo MOF is present in MSL complex and actively acetylate nucleosomal histones while recombinant fragment of MOF could acetylate H4 strongly and H3, H2A weakly (Smith *et al.*, 2000).

HBO1 (histone acetyltransferase bound to ORC) was discovered as interactor of ORC1 subunit of the origin recognition complex (ORC). It acetylates histones H3 and H4 robustly as free histones and weakly as nucleosomal histones (Iizuka and Stillman, 1999).

Interestingly, **JADE1**is another acetyltransferase that primarily acetylates H4 is incorporated into HBO1 complex to accentuate its acetyltransferase function; JADE1-HBO1-Tip60 can stabilize each other and often reported to show compensatory function in terms of acetylation (Foy *et al.*, 2008).

Role of MYST family acetyltransferases:

MYST family acetyltransferases are involved in wide range of functions related to transcriptional coactivation, DNA damage repair, cell cycle control, apoptosis to gene silencing. Tip60 forms stable complex with ATM complex and initiates DNA damage repair and apoptosis in appropriate context, in Drosophila Tip60 homolog exchanges histones at damage sites (Sun *et al.*, 2005; Kusch *et al.*, 2004). Tip60 also controls fundamental node for cell cycle progression and apoptosis by interacting and inducing Mdm2, and also, acting as a transcriptional coactivator for p53in a context dependent manner (Legube *et al.*, 2004; Legube *et al.*, 2002). Like Tip60, MOZ also interacts with ATM and regulates cell cycle checkpoint G2/M (Smith *et al.*, 2005). MSL complex containing MOF is involved in dosage compensation in Drosophila male flies (Akhter *et*

al., 2000). MOZ and Querkopf have been implicated in self-renewal of hematopoietic and neural stem cells respectively (Thomas *et al.,* 2006). One of the intriguing sets of function of MYST family acetyltransferases is their involvement in gene silencing. Esa1p is involved in transcriptional silencing (Clarke *et al.,* 2006). In Drosophila HBO1 homolog is involved in maintenance of polycomb repressive group mediated Hox gene silencing (Grienenberger *et al.,* 2002). HBO1 has been demonstrated to associate with ORC (origin recognition complex) and acetylation by HBO1 dictates binding of prereplicative complex (Aggarwal and Calvi, 2004).

1.4.4. Other acetyltransferases

1.4.4.1. Nuclear receptor coactivators

Coactivators such as ACTR, SRC-1 interact with nuclear hormone receptors and bring about transcriptional activation by hormone signals thereby defining a unique functional family of KATs. This family of KATs possess N-terminal basic helix-loop- helix/ PAS region, receptor-coactivator interaction domains and C-terminal KAT domain.

SRC1 (Steroid receptor coactivator 1), also known as p160 and NCoA-1 in mice (Kamei *et al.,* 1996, Torchia *et al.,* 1997) is a human nuclear receptor cofactor originally discovered by way of its interaction with the human progesterone receptor (PR). Later on, SRC1 was found to interact with and activate targets of glucocorticoid receptor (GR), estrogen receptor (ER), thyroid receptor (TR), and retinoid X receptor (RXR) (Onate *et al.,* 1995). SRC1 acetylates free and mononucleosomal H3 and H4 (Spencer *et al.,* 1997). SRC1 is also known to interact with other KATs such as p300/CBP, PCAF (Kamei *et al.,* 1996; Smith *et al.,* 1996; Yao *et al.,* 1996; Spencer *et al.,* 1997).

ACTR (activin receptor), also known as RAC3, AIB1, TRAM-1 in human and p/CIP in mice, was identified as a novel interactor of retinoic acid receptor (RARb) interactor (Chen *et al.,* 1997; Li *et al.,* 1997; Anzick *et al.,* 1997; Takeshita *et al.,* 1997; Torchia *et al.,* 1997). Like SRC1, ACTR interacts with multiple nuclear hormone receptors and stimulate transactivation. ACTR acetylates free and nucleosomal H3 and H4 with its C-terminal KAT domain (Chen *et al.,* 1997). ACTR is also regulatory target of other KATs such as CBP and PCAF (Chen *et al.,* 1999).

TIF2 (transcriptional intermediary factor 2), also known as GRIP1, SRC2, NCoA-2 in mice (Hong *et al.*, 1997; Torchia *et al.*, 1997), binds to a number of nuclear hormone receptors like steroid, thyroid, retinoic acid, vitamin D receptors and stimulates their transactivation (Voegel *et al.*, 1996).

In addition to the KAT families discussed above a number of proteins have acetyltransferase activity which show little sequence and functional homology with each other. TBP-associated factor 250kd (TAFII250 (KAT4)), αTubulin acetyltransferase (αTT1), circadian locomotor output cycles protein kaput CLOCK (KAT13D) etc. are a few examples of these KATs. Here, we have discussed about some acetyltransferases which act as transcription factor as their primary function.

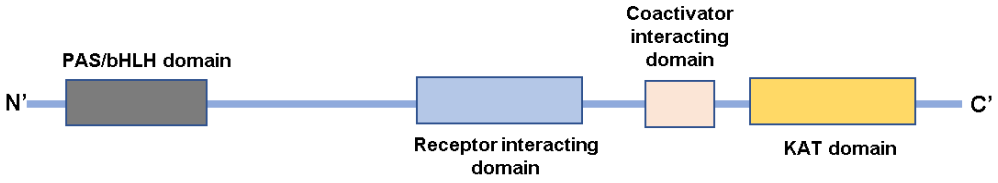


Figure 1.15. Schematic representation of general features of nuclear receptor coactivator family KATs. *The characteristic conserved domains in this family include PAS/basic helix-loop-helix homology (bHLH) domain, nuclear receptor interaction regions, and the general area of interaction for coactivators p300/CBP and PCAF. KAT domain is located near their C terminal.*

1.4.4.2. Transcription factor acetyltransferases

Acetyltransferases with primary function as transcription factors establish direct connection between acetylation and transcriptional stimulation.

$TAF_{II}250$, a subunit of general transcription factor TFIID in human, possesses acetyltransferase activity. Homologs of this protein $TAF_{II}230$ in Drosophila and $TAF_{II}145/130$ in yeast were also shown to have KAT activity. TAF has KAT domain in its central region and it weakly acetylates H3 and H4 from core histones and recombinant H2A. Interestingly, KAT domain of TAF bears little apparent sequence similarity with that of other families of KATs but shares a similar acetyl-CoA binding motif Gly-X-Gly pattern with GNAT family acetyltransferases. Human and *Drosophila* TAF possess two bromodomains that recognize acetylated H4 but yeast TAF does not have bromodomain

suggesting against a major role of bromodomain in acetyltransferase activity of TAF (Jacobson *et al.,* 2000; Mizzen *et al.,* 1996). Apart from being an acetyltransferase TAF also possesses a bipartite kinase and ubiquitin activating/ conjugating function (Dikstein *et al.,* 1996; O'Brien and Tjian, 1998; Crane-Robinson, 1999; Pham and Sauer, 2000).

TFIIIC complex, a general transcription factor for RNAPIII, possesses acetyltransferase activity. Functionality-wise TFIIIC mediated acetylation might be different from all other type-A acetyltransferases which are involved with transcription by RNAPII. The acetyltransferase function of TFIIIC complex has been discussed later.

TFIIB and **TFIIF** are associated with weak autoacetylation functional relevance of which is assumed to be limited for induction of RNAPII-mediated transcription (reviewed in Torchia *et al.,* 1998; Choi *et al.,* 2004)

There are few other acetyltransferases that has been discovered recently and have not been categorized into any group in particular.

Tau proteins, that are the building blocks of neurofibrillary tangles (NFTs) found in a range of neurodegenerative tauopathies, including Alzheimer's disease was found to be an acetyltransferase that autoacetylates. Interestingly, it was found to share catalytic mechanism with MYST family acetyltransferase (Cohen *et al.,* 2013). **BRCA1** has been demonstrated to autoacetylate but its activity is not detectable *in vitro*. (Siddique *et al.,* 1998; Fuks *et al.,* 1998). A recently characterized acetyltransferase is **Brd4**, a BET family protein that acetylates H3 and H4 *in vitro* and is associated with global nucleosome eviction through its acetyltransferase activity (Devaiah *et al.,* 2016). In the past years, the **Camello** family has also been included in the list of type A KATs; Camello is a functional HAT that has perinuclear localization and acetylates H4. It has been identified in zebrafish (Karmodiya *et al.,* 2014).

1.4.5. Inhibitors of lysine acetyltransferases

The general mechanism of acetylation:

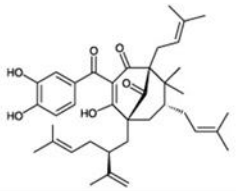
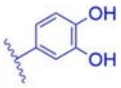
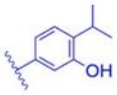
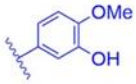
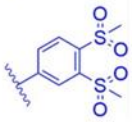
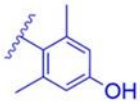
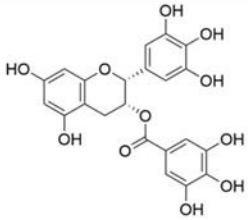
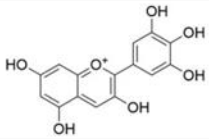
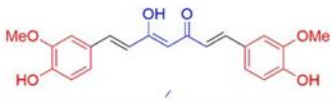
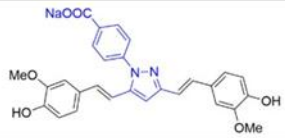
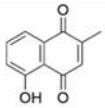
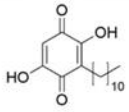
Despite having considerable divergence in sequence KATs from distinct families exhibit structurally homologous acetyl-CoA binding regions, which generally adopt a globular α/β fold. However, the flanking regions are not conserved, these residues guide substrate

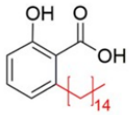
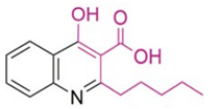
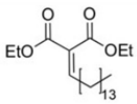
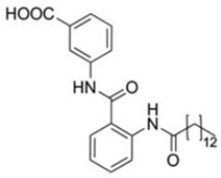
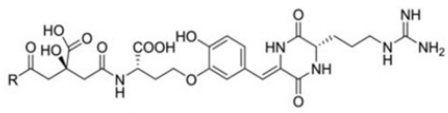
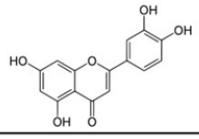
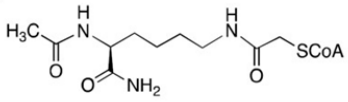
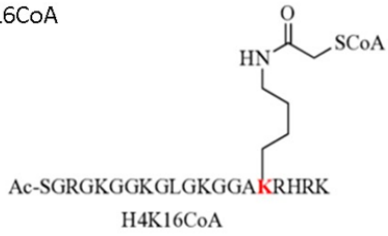
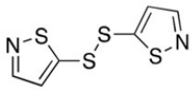
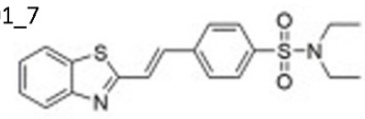
positioning (Berndsen and Denu, 2008). Three different mechanisms of acetylation have been proposed for KAT families. GNAT and MYST families share similar mechanisms, in GNATs an active site glutamate is required to deprotonate the lysine ε-amine, enabling nucleophilic attack of the acetyl-CoA carbonyl, followed by formation of a transient tetrahedral intermediate and its subsequent collapse into acetyl-lysine and coenzyme A (Jiang *et al.,* 2012). MYST family acetyltransferases possess cysteine residue in active site and forms acetyl-cysteine intermediate. However, mutation in cysteine residue did not have any effect on enzymatic activities. But, instead of cysteine, mutagenesis of an active site glutamate ablates activity without reducing levels of autoacetylation (Yuan *et al.,* 2012). As described earlier, p300/CBP family utilize 'hit and run' mechanism which is ordered, rapid and occurs through ternary complex formation. In place of basic residues in active site, aromatic residues lining its shallow catalytic pocket lower pKa to attack nucleophilic attack on acetyl-CoA. A tyrosine residue then acts as an acid to protonate the sulfhydryl of CoA. This mechanism of catalysis allows substrate promiscuity of p300/CBP (Zhang *et al.,* 2014)

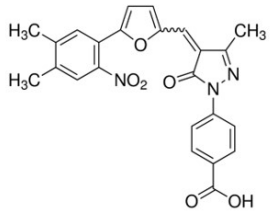
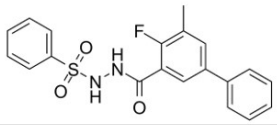
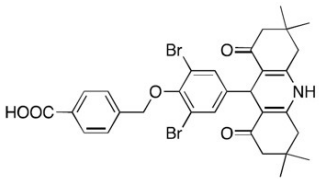
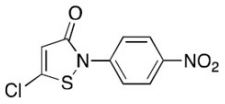
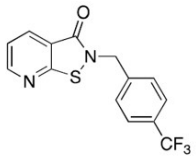
KAT inhibitors:

The connection between anomalous protein acetylation and the onset of different diseases has inspired the research on KAT inhibitors with the final goal of developing therapeutics, as well as chemical probes to further investigate KAT function. Over the years natural products have served as synthon for a large number of drugs and biological tools, and not surprisingly those are also utilized for developing KAT inhibitors (KATi). Natural compounds usually present a preference of oxygen more than sulphur or nitrogen as heteroatoms, as well as a higher number of stereogenic centres and fused rings (Cherblanc *et al.,* 2013). Bioisoteric replacement or structural simplification of natural molecules can modulate its potency and target specificity. Among all KATs, p300/CBP family has emerged as the most prominent therapeutic target as it has been implicated in respiratory diseases, HIV infection, metabolic diseases, and cancer (Dekker *et al.,* 2009). Among the KAT inhibitors of p300/CBP, the most potent and specific inhibitor that has been discovered and characterized till date is A-485. This molecule has been demonstrated to suppress proliferation in 61 cancer cell lines with an EC50 < 2 μM highlighting the potential therapeutic property of the molecule (Lasko *et al.,* 2017).
A few other prominent KAT inhibitors and their targets are listed in table 1.5.

Table 1.5. List of acetyltransferase inhibitors

KATi	Target acetyltransferases (IC_{50})	References
Garcinol 	p300 (5μM) PCAF (7μM)	Balasubramanyam et al., 2004a
Isogarcinol 	p300 (5μM) PCAF (7μM)	Cen et al., 2013
LTK13  LTK14  LTK19 	p300 (5-7 μM)	Mantelingu et al., 2007b
EML425 	p300 (2.9 μM) CBP (1.1 μM)	Milite et al., 2015
Epigallocatechin -3-gallate (EGCG) 	p300 (30μM) CBP (50μM) PCAF (60μM) Tip60 (70μM)	Choi et al., 2009
Delphinidin 	p300 (30μM) CBP (30μM)	Seong et al., 2011
Curcumin 	p300 (25μM)	Balasubramanyam et al., 2004b
CTK7A 	p300 PCAF	Arif et al., 2010
Plumbagin 	p300 (20μM) PCAF (50μM)	Vasudevarao et al., 2014
Embelin 	PCAF (7.2 μM)	Modak et al., 2013

KATi	Target acetyltransferases (IC_{50})	References
Anacardic acid 	p300 (8.5- 1000µM) PCAF (5-667.1µM) Tip60 (64- 347.6 µM)	Balasubramanyam et al., 2003 Wu et al., 2009 Ghizzoni et al., 2012 Wapenaar et al., 2015
MC1823 	p300/CBP (50µM)	Mai et al., 2006
SPV106 	p300/CBP (~50µM) PCAF (~100µM)	Sbardella et al., 2008 Castellano et al., 2015
10e 	PCAF (~100µM)	Park and Ma, 2012
14a (NK13650A)peptide  R = NH-Aspartic acid	p300 (11.5nM)	Tohyama et al., 2012
Luteolin 	p300 (7.5µM)	Selvi et al., 2015
Lysyl-CoA 	p300 (0.5 µM) PCAF (200 µM)	Lau et al., 2000
H4K16CoA 	Tip60 (17.59 µM) Esa1(5.51 µM) p300 (6.62 µM)	Wu et al., 2009
NU9056 	Tip60 (2µM)	Coffey et al., 2012
DC_M01_7 	MOF (6µM)	Zhang et al., 2018

C646 	p300 (1.6 µM)	Bowers et al., 2010
WM-8014 	MOZ (8 nM) MORF (28 nM)	Baell et al., 2018
DC-G16-11 	GCN5 (6.8 µM)	Xiong et al., 2018
CCT077791 	PCAF (2.2 µM)	Stimson et al., 2005
PU141 	p300 (5.92µM) CBP (2.85µM)	Furdas et al., 2014

1.4.6. Functions of acetyltransferases

KATs and KDACs develop a well-balanced and complex interaction in many cells' functions. Acetylation does not only modulate the properties and interaction of proteins that are involved in various signaling pathways, due to consumption of acetyl-CoA during acetylation and NAD^+ during deacetylation by specific KDACs acetylation also interferes with metabolic processes and energy homeostasis. Hyperacetylation is observed in actively diving cells in normal tissues or in tumors, local acetylation sites are observed on proteins related to DNA damage, cell-cycle control, transcription *etc.* (Kim *et al.*, 2006; Choudhary *et al.*, 2009). In mitochondria proteins associated with cellular metabolic processes undergo frequent acetylation and are enriched in highly metabolically active tissues (Rardin *et al.*, 2013; Lundby *et al.*, 2012). Cytoplasmic acetylation is relatively understudied though tubulin was the second protein discovered to be acetylated (L'Hernault and Rosenbaum, 1983; Piperno and Fuller, 1985; Piperno *et al.*, 1987).

Lysine acetylation can be detected using acetylation specific antibodies, mass-spectrometric analysis. Recent use of SILAC (stable isotope labeling of amino acids in

culture) and label-free approach has been used to assess the dynamicity of protein stoichiometry (Zhu *et al.*, 2016; Rardin *et al.*, 2013). Mass spectrometry analyses of acetylation have been conducted in a wide variety of species ranging from bacteria to budding yeast, to plants, to eukaryotic human pathogens, rodents, and humans (Liu *et al.*, 2016b; Kosono *et al.*, 2015; Downey *et al.*, 2015; Konig *et al.*, 2014; Smith-Hammond *et al.*, 2014a; Smith-Hammond *et al.*, 2014b). In mammalian cells individual acetylation sites are conserved across species but are tissue specific (Strahl and Allis, 2000). Unlike phosphorylation, acetylation only occurs in regions with defined secondary structures.

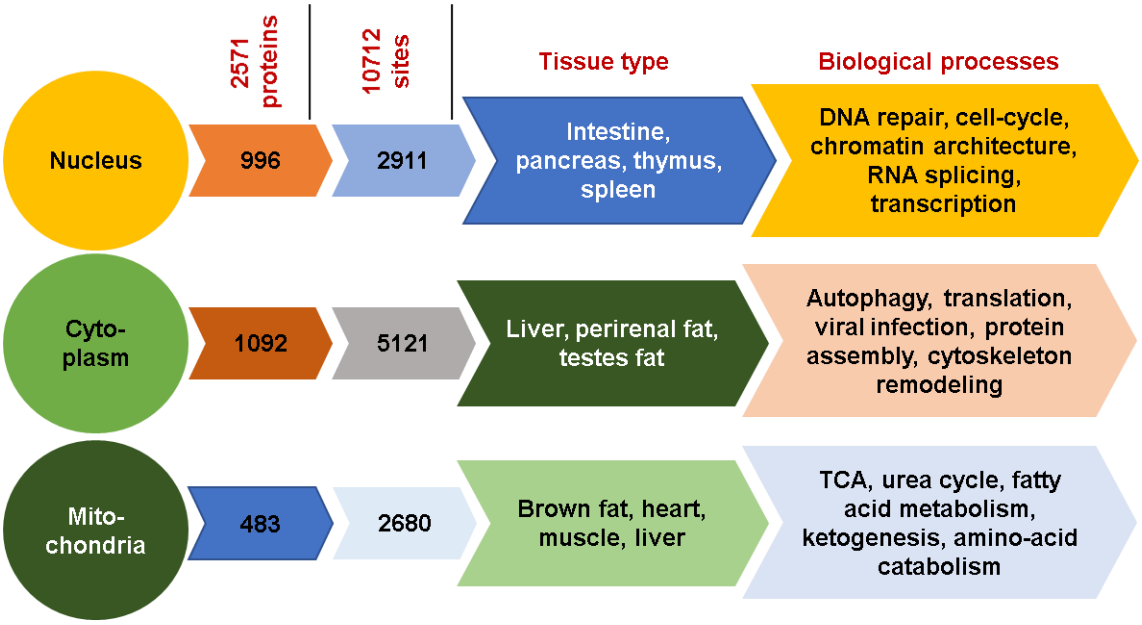


Figure 1.16. Relevance of acetylation in eukaryotes. *Pathways relevant to tissue specific acetylation in human. Tissue specificity could be linked to subcellular localization of acetyltransferases (Adapted from Ali et al., 2018)*

- **Modulation of protein properties:**

Acetylation can modulate protein properties in the following ways:

Function:

Depending on the substrate and site of acetylation protein function can be induced or disrupted. Acetylation of certain transcription factors influence their DNA binding and promoter specificity. For example, acetylation of RAR-related orphan receptor (RORγ) inhibits its interaction with cognate binding sites and deacetylation activate DNA binding and lineage-determining (Wu *et al.*, 2015). Deacetylation of RORγ increases

transcription of IL17 gene but inhibits transcription of IL2 gene (Lim *et al.,* 2015). Acetylation of FoxO proteins by p300 facilitates dissociation from promoters of genes such as p27 and MnSOD (Daitoku *et al.,* 2015). Acetylation of Forkhead protein P3 (FOXP3) enhances its stability and function as a transcription factor that regulates T cell identity; interestingly acetylation of Tip60 by p300 enhances its ability to acetylate FOXP3 establishing multiple layers of KAT cooperation for appropriate signaling (Xiao *et al.,* 2014).

Acetylation of p53, E2F1, EKLF, GATA1 *etc.* proteins also in sites adjacent to DNA binding domain stimulates their DNA binding ability, whereas acetylation in DNA binding domain of HMGI (Y) results in disruption of its DNA binding (Gu and Roeder, 1997; Boyes *et al.,* 1998; Zhang and Bieker, 1998a; Martínez-Balbás *et al.,* 2000).

- **Interaction:**

Acetylation is also known to regulate protein-protein interactions. Acetylated histones can recruit bromo-domain containing proteins; one such class of proteins are acetyltransferases themselves (Dhalluin et al. 1999). Acetylation of co-activator ACTR inhibits its binding to cognate nuclear receptors (Chen *et al.,* 1999). Acetylation at K221 of RelA disrupts its interaction with negative regulator IκBα, allowing for nuclear translocation and increased DNA binding (Chen *et al.,* 2002).

- **Stability:**

Stability and half-life of a protein may also be dependent on acetylation status of the protein. Acetylation of E2F1 increases its half-life in vivo (Martínez-Balbás *et al.,* 2000). Acetylated α-tubulin and microtubule stability are correlated (Takemura *et al.,* 1992). In case of p53, acetylation can directly compete with ubiquitylation. Upon DNA damage p53 CTD is acetylated by p300 and MOZ preventing MDM2 mediated ubiquitination and degradation (Rodriguez *et al.,* 2000). Acetylation can be promoted by cross-talk with other PTMs such as lysine methylation; lysine methyltransferase 7 (KMT7, SET7/9)-mediated monomethylation at K372 promotes acetylation and stabilization of p53 by disrupting its interaction with the deacetylase SIRT1(Liu *et al.,* 2011). Furthermore, acetylation of p53 is recognized by p300/CBP and TAF1 bromodomains to facilitate acetylation of histone H3 and histone H4 at p53 response genes, which induces cell-cycle

arrest or apoptosis (Barlev *et al.,* 2001). Acetylation of RelA has been reported to stabilize RelA by inhibiting methylation of adjacent lysine residues (Yang *et al.,* 2010).

Localization:

Acetylation regulates cellular localization, mainly, nuclear import and export of various proteins such as PCNA, SRY, p53, Abl, CtBP2, PAP, CIITA, β-catenin, RECQL4 *etc.* p300/CBP, PCAF are the major acetyltransferases known to be involved in these processes (Naryzhny *et al.,* 2004; Thevenet *et al.,* 2004; Kawaguchi *et al.,* 2006; di Bari *et al.,* 2006; Zhao *et al.,* 2006; Spilianakis *et al.,* 2000; Shimazu *et al.,* 2007; Dietschy *et al.,* 2009; Soutoglou *et al.,* 2000). In general, acetylation can modify a protein's interaction either with its binding partner which might sequester the protein in particular subcellular compartment or with nuclear import/ export factors. Acetylation can disrupt interaction of RECQL4, PAP with nuclear import factors leading to their accumulation in cytoplasm whereas SRY acetylation facilitates its nuclear import (Thevenet *et al.,* 2004; Shimazu *et al.,* 2007; Dietschy *et al.,* 2009). In case of p53, acetylation prevents p53 oligomerization facilitating its interaction with nuclear exporter CRM1 (Kawaguchi *et al.,* 2006), contrarily, acetylation of STAT3(K685) by p300/CBP facilitates homodimerization and nuclear translocation (Xu *et al.,* 2016).

Regulation of cellular transcription and DNA-templated processes

Eukaryotic transcription is a tightly regulated process and acetylation plays a major role in that regulation. Acetyltransferases can establish control over cellular transcription on multiple levels, they themselves can act as transcription coactivator, can acetylate chromatin proteins, transcription factors. Acetyltransferases are found to be enriched in gene regulatory regions of active genes; for example, CBP is found to be enriched at the promoters of nearly all expressed genes and regulate promoter-proximal pausing of RNAPII (Boija *et al.,* 2017). Acetyltransferases can influence cellular transcriptions in the following ways:

- **Histone acetylation:**

Eukaryotic genome is confined in the nucleus in a form of nucleoprotein complex, chromatin, which is fundamentally made up of smaller units of 11nmcalled nucleosomes. Nucleosomes are octamer consisting of consisting of H3-H4 tetramer and two H2A-H2B dimers. The core histones and their variants are important in chromatin regulation and

gene expression. Linker histone H1 and its variants compacts nucleosomes into denser 30nm fibers known as chromatosomes (Robinson and Rhodes, 2006). Histones were the first proteins discovered to undergo acetylation (Philips *et al.,* 1963; Allfrey *et al.,* 1964). Almost all the histones undergo acetylation; predominantly N terminal tails undergo acetylation but lysine residues in globular domain are also acetylated (Tweedie-Cullen *et al.,* 2012). Acetylation of lysine residues mask positive charges on histones and negatively affect electrostatic interaction between histones and negatively charged DNA backbone exposing local DNA to transcription machinery. (Lugar *et al.,* 1997). Acetylation of core histones is positively correlated to transcriptional activation (Puerta *et al.,* 1995). H4K16ac is known to disrupt chromatin structure resulting in transcription activation (Shogren-Knaak *et al.,* 2006). Similar observations were made for H3K56, H3K64 and H3K122ac (Tessarz and Kouzarides, 2014). H3K56ac is specially targeted by Sir2 in yeast to maintain heterochromatin state near transcriptionally silent regions like telomeres (Xu *et al.,* 2007). Acetylation can also disrupt histone-histone interaction; H4K91ac affects H2A-H2B dimerization and weakens histone octamer altering heterochromatin structure (Ye *et al.,* 2005). H3K122ac facilitates histone eviction thus inducing transcription (Tropberger *et al.,* 2013). In contrast to methylation marks which are species specific, acetylation sites in core histones are well conserved (Garcia *et al.,* 2007). Together the specific array of histone posttranslational modifications is known as 'histone code' which serves as dynamic regulatory control of gene expression. Additionally, recruitment of various reader proteins on acetylated histone increases the number of regulatory factors. Turnover of histone acetylation is different for different sites. Turnover rate is higher (<30 min) for acetylation on histone tails (except for H3K4ac, H2AK13ac, and H2AK15ac) and lower (>2hrs) for globular domains (Zheng *et al.*, 2013). Linker histone H1 is acetylated on 11 lysine residues at lower frequency (Wisniewski *et al.*, 2007). Conventionally H1 is thought to repress gene expression as it is involved in chromatin compaction, H1 can counteract H3 and H4 acetylation-mediated chromatin decompaction (Sun *et al.,* 2015; Bernier *et al.,* 2015). H1.4K34ac is detected in distal and proximal promoter regions of highly transcribed genes in stem cells and in cancer cells (Kamieniarz *et al.,* 2012). Single residue modifications on H4K16ac as well as K12ac are known to inhibit 30nm chromatosome formation (Allahverdi *et al.,* 2011; Shogren-Knaak *et al.,* 2006; Dhall *et al.,* 2014).

- **Transcription factor acetylation:**

Acetylation of transcription factors can affect their nuclear translocation, stabilization, sterically prevent ubiquitination, modify complex composition, or facilitate chromatin binding specificities as discussed earlier.

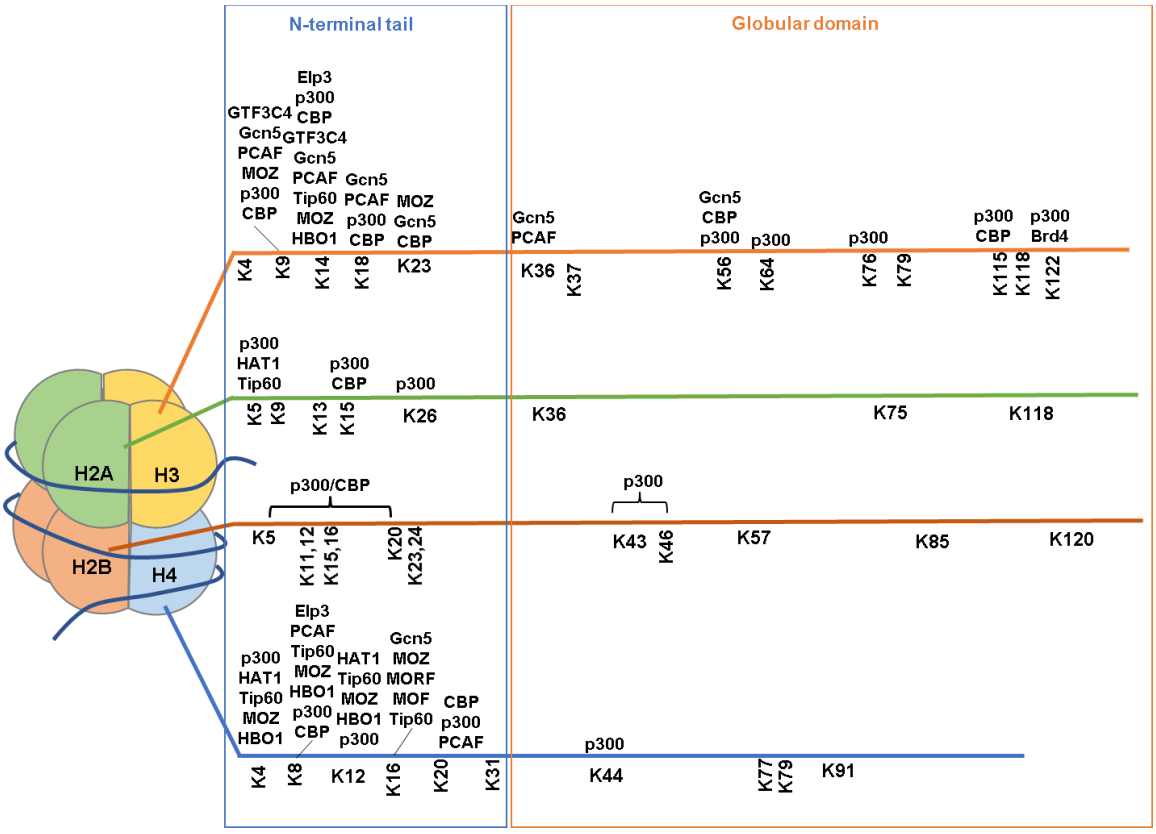


Figure 1.17. Site specific core histone modification by nuclear acetyltransferases in mammals.

- **Acetylation of basal transcription machinery:**

In addition to transcription factors, several other factors associated with the RNAP transcription complexes are known to be acetylated.

Components of TFIID complex in RNAPII transcription machinery, TAFs contribute to transcription initiation by RNAPII; acetylated TAF_I68 in TIF-IB/SL-1complex increases its interaction with rDNA and enhances RNAPI mediated transcription (Choudhary *et al.*, 2009; Muth *et al.*, 2001). Acetylation of cyclin T1 dissociates from 7SKRNP complex and activates CDK9 in p-TEFb (positive transcription elongation factor b) inducing Ser2 phosphorylation in RNAPII CTD thereby activating transcriptional elongation (McNamara *et al.*, 2016). CDK9 is also regulated by acetylation; K44ac

disrupts ATP binding and kinase activity whereas K48ac facilitate p-TEFb activation (Sabo *et al.,* 2008; Fu *et al.,* 2007). p300 is known to directly regulate downstream gene expression by acetylating lysine in mammalian RNAPII CTD stalled in transcription start site (Schroder *et al.,* 2013). RNAPI and III subunits are also known to undergo acetylation (Choudhary *et al.,* 2009). Acetylation of PAF53, a regulatory subunit of RNAPI is regulated by CBP and SIRT7 and deacetylation facilitates rRNA transcription by RNAPI Glucose deprivation suppresses SIRT7 activity leading to hyperacetylation of PAF53 and suppression of rRNA transcription (Chen *et al.,* 2013).

- **Chromatin organization**

Apart from nucleosomal particles, chromatin associated non histone proteins and noncoding RNA; several other factors such as DNA methylation, post-translational modifications of histones, histone chaperones, chromatin remodelers and different histone variants together maintain the dynamicity of chromatin which is essential for important nuclear processes, for example, DNA replication, transcription, repair etc. (Dulac, 2010). Chromosomes are located in nucleus in nonrandom radial manner giving rise to specific chromosome compartments based on gene density; however, chromosome can form spatial loops and permitting large scale functional chromatin interaction and establishing transcription factories, repair centers, optimizing the nuclear resources for efficient responses to external cues (Nora et al, 2017). Nucleosome assembly is brought about by histone chaperones and ATP dependent chromatin spacing and organizing factors in replication dependent or independent manner (Haushalter *et al.,* 2003). Bulk histone synthesis occurs in cytoplasm during S-phase. The newly synthesized histones possess transient acetylation marks (H4K5, K8 and K12) deposited by cytoplasmic KAT Hat1; another cytoplasmic KAT, Hat4, has be shown to acetylate free H4 on the globular lysine sites, H4K79ac and H4K91ac. They are then loaded onto DNA by specific histone chaperones such as chromatin assembly factor 1 (CAF1), anti-silencing factor (Asf1) depositing H3-H4 tetramers, nucleosome assembly protein (NAP1), histone regulator (HIR) preferentially depositing H2A-H2B dimers etc. Also, histone chaperones are responsible for replacing canonical histones with noncanonical histone variants (H3.3, H2Az *etc.*) and induce cellular response under various circumstances. The regular array of nucleosomes is organized through an energy driven process involving ATP dependent chromatin remodelers such as ATP-utilizing chromatin assembly and remodeling factor (ACF) complex in Drosophila, remodeling

and spacing factor (RSF) complex in human *etc.* (Vereault *et al.*, 1996; Yang *et al.*, 2011; Haushalter *et al.*, 2003).

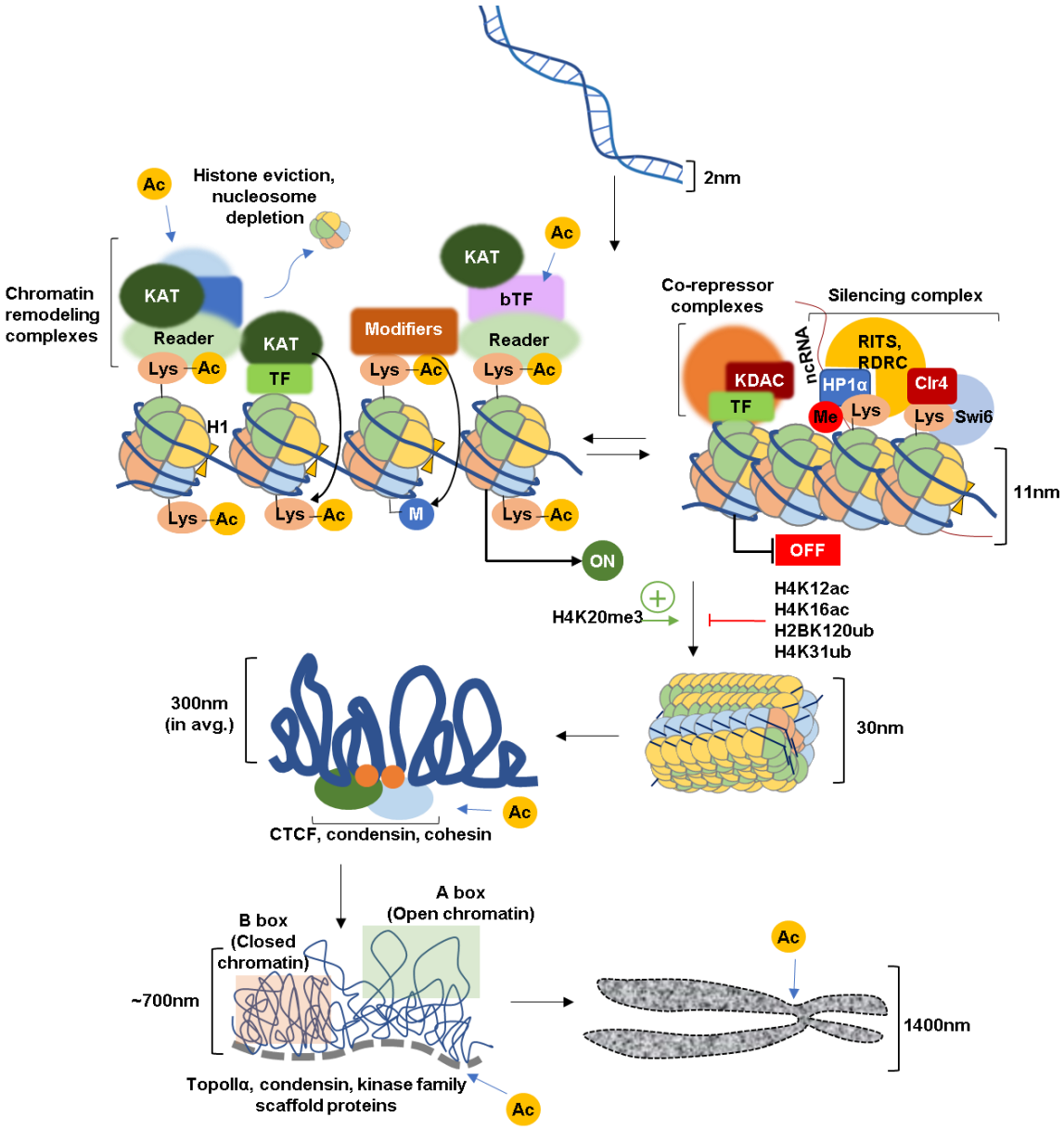


Figure 1.18. Regulation of chromatin dynamics through acetylation. *Schematic representation of involvement of acetylation in different stages of DNA and chromatin compaction. The components modified by acetylation are indicated with blue arrows tagged with 'Ac'. The diameters of DNA to chromatin and finally chromosomes are indicated in terms of nm. Abbreviations: bTF- basal transcription machinery, m- histone PTMs, TF- transcription factor, 'on' and 'off' denotes status of transcription.*

Chromatin exists broadly in two distinct functional forms; viz. heterochromatin and euchromatin. Heterochromatin is highly condensed form, which has hypoacetylated

histones and hypermethylated DNA in general are associated with silent genes. The nucleation of heterochromatin is position specific; such as in telomere it requires site specific binding of Rap1p and then serial recruitments of Sir4, Sir 3p and Sir2p which deacetylates H4K16 and promotes the spread of heterochromatin; but, in centromeres RNAi pathway gets activated which induces the binding of Clr4 methyltransferase resulting in H3K9 methylation and subsequent recruitment of heterochromatin protein 1 (HP1) (Volpe *et al.,* 2002).

Euchromatin is looser decondensed form which undergoes localized, targeted reorganization to allow the progress of cellular processes that requires DNA binding factors such as replication, repair, transcription, recombination. Acetylation is one of the best documented PTM that function *in vivo* regulating chromatin structure and assembly. In general, euchromatin harbors high levels of acetylated histones; in β-globin gene locus DNaseI accessible sites are decorated with hyperacetylated H4 (Kiefer *et al.,* 2008). Additionally, acetylation is prevalent in active promoters, enhancers, regions with increased nuclease sensitivity (Wang *et al.,* 2008b; Roh *et al.,* 2007). *In vivo* studies showed that treatment of cells with HDAC inhibitor, TSA, leads to hyperacetylation that increased pore size of chromatin (Görisch *et al.,* 2005).

- **DNA damage repair**

Surge of acetylation has been detected upon infliction of DNA damage. Acetylation can increase accessibility for repair enzymes to damaged sites. Certain patterns of acetyl-lysine residues in histones (for example, H4 acetylation by Tip60, Esa1p) is essential for initiation of repair (Bird *et al.,* 2002). Several nonhistone proteins involved in repair process are also known to undergo acetylation which modulates their functions. Acetylation of DNApol β impaired its dRP-lyase activity essential for short patch base excision repair (Hasan *et al.,* 2002); acetylation of Werner DNA helicase stimulates it function in long patch base excision repair (Moftuoglu *et al.,* 2008). Acetylation stimulates strand displacement, Okazaki fragment synthesis (Balakrishnan *et al.,* 2010). Lack of p300 mediated acetylation affects mismatch repair in cells (Piekna-Przybylska *et al.,* 2016).

Apart from transcription and repair acetylation regulates properties of proteins involved in various other DNA/chromatin templated pathways such as DNA replication, heterochromatin assembly, sister chromatid cohesion etc. (Choudhary *et al.,* 2009) and influence these pathways.

- **Metabolic regulation:**

At least 1/3rd of mitochondrial proteins undergoes acetylation, mitochondrial acetylome is conserved from Drosophila to human (Anderson and Hirschey, 2012; Weinert *et al.*, 2011). Mitochondrial metabolism results from high concentrations of acetyl-CoA from aerobic catabolism and acetylated proteins in mitochondria play major roles in multiple process, namely, TCA cycle, oxidative phosphorylation, amino acid metabolism, urea cycle, β-oxidation of lipids, nucleotide metabolism, urea cycle *etc.* (Zhao *et al.*, 2010). The mechanism of acetylation in mitochondria is intriguing, though handful of acetyltransferases has been discovered to be functional in mitochondria, a high concentration of acetyl-CoA (in millimolar) raises the possibility of nonenzymatic acetylation of mitochondrial proteins. Indeed, increased mitochondrial protein acetylation is associated with physiological conditions that result in higher level of acetyl-CoA (e.g., fasting, calorie restriction, high fat diet, ethanol intoxication (Schwer *et al.*, 2009; Hirschey *et al.*, 2011; Fritz *et al.*, 2012; Picklo, 2008). Among the mitochondrial deacetylases (SIRT3, 4 and 5), SIRT3 is the most robust deacetylase. Mice lacking SIRT3 accumulates significant hyperacetylated mitochondrial proteins that control shift to a fasting metabolism when the source of energy is switched from glucose to lipids and amino acids (Lombard *et al.*, 2007). Thus, SIRT3 is linked to energy status of the cells and is expressed in highly metabolically active tissues such as liver, kidney and heart (Ahn *et al.*, 2008).

Though p300 mediated acetylation has been earlier shown to get involved in mitochondrial function, siRNA mediated screening of KATs that can increase mitochondrial consumption has identified only one candidate facilitate this syndrome, and the acetyltransferase is GCN5L1 (also known as Bloc1s1). GCN5L1 is a homolog of GCN5 and is involved in biosynthesis of lysosome-related organelles and also in mitophagy (Scott *et al.*, 2012; Webster *et al.*, 2014).

ACAT1is a metabolic enzyme that moonlights as an acetyltransferase for components of mitochondrial pyruvate dehydrogenase complex and negatively affects its function. This in turn limits the conversion of pyruvate to acetyl-CoA necessary for oxidative phosphorylation by TCA cycle, thereby facilitating anabolic 'Warburg' metabolism and cancer cell growth (Fan *et al.*, 2014).

- **Stability of cytoskeletal structures:**

In cytoplasm a non-canonical KAT, α-tubulin acetyltransferase (αTAT1) acetylates α-tubulin at K40 which is a marker for tubulin stability, overexpression of KDAC6 and SIRT2 deacetylases destabilizes microtubules (Kalebic *et al.,* 2013; Hubbert *et al.,* 2002; North *et al.,* 2003). Actin and its regulatory elements such as Arp2/3, cortactin function and stability are modulated by acetylation; cortactin acetylation inhibits its localization in cell periphery perturbing actin dynamics in cell periphery and altering cell motility (Zhang *et al.,* 2007; Zhang *et al.,* 2009). Acetylation inhibits RhoGDI promoting Rho GTPase mediated actin assembly in stress fibre and filopedia formation, γ-actin acetylation of K61 residue stabilizes actin stress fibers (Kim *et al.,* 2006). Other intermediate filaments such as vimentin, cytokeratin 8 are also known to undergo acetylation which destabilizes the polymers (Drake *et al.,* 2009; Leech *et al.,* 2008).

Acetylation of microtubules are also known to improve kinesin-1, dynei*n etc*. mediated cargo transport, and hyperacetylation of microtubules are known to rescue BDNF transportation defect observed in Huntington disease (Dompierre *et al.,* 2007; Reed *et al.,* 2006).

Tau, a microtubule associated protein (MAP), along with MAP2 and 4 are regulated by acetylation. Apart from self-acetylation these proteins get modified by several other acetyltransferases such as p300/CBP, PCAF, several nuclear SIRTs and KDACs. Several acetylation sites on Tau have been identified. Acetylation on K274, 281 led to mislocalization of Tau, K280ac promoted Tau aggregation, K174 ac slowed cellular turnover of Tau, all contributing to cognitive disorders (Cohen *et al.,* 2011; Tracy and Gan, 2017). Several other PTMs, including lysine methylation and ubiquitination, can competitively inhibit Tau acetylation *in vitro* and *in vivo* (Morris *et al.,* 2015).

- **Quality control for translation, cytoplasmic clean-up**

A huge number of proteins associated with protein synthesis has been found to be acetylated, which might have roles to play in translational control mechanisms (Choudhary *et al.,* 2009). As discussed previously, acetylation plays important role in quantity control of cellular proteins by affecting their stability and half-life, a mechanism that works alongside with transcriptional and translational level regulation.

Chaperones are known to assist in protein folding, misfolded proteins are cleared off by proteasomal degradation. In case of massive conformational defects due to prolonged, excessive stress, aging or certain diseases cause accumulation and aggregation of proteins in cytoplasm leading to stress granule, aggresome formation and induction of autophagy pathway. Hsp90, a stress inducible protein and chaperone, is known to undergo acetylation on multiple residues influencing glucocorticoid receptor (GR) or mineralocorticoid receptor (MR) signaling and chaperonic activity (Kovac *et al.,* 2005; Jiménez-Canino *et al.,* 2016). On the other hand, immediate activation of Hsp90 requires an HDAC6-mediated deacetylation which is essential for its ATP binding, Deacetylation of K294 mediates cochaperone binding and Rac1 activation that leads to actin reorganization and cell migration (Bali *et al.,* 2005; Gao *et al.,* 2007). Crystallins, DNAJB8are other chaperones whose activities are also known to be influenced by acetylation, where mostly deacetylation activates chaperones to suppress cytotoxic aggragation (Lin *et al.,* 1998; Lapko *et al.,* Hageman *et al.,* 2010).

There are some functions of acetyltransferases that are termed as noncanonical functions; following are a few examples of those functions.

- **Acylation:**

Apart from acetylation several other acylation modifications such as propionylation, butyrylation, crotonylation, succinylation, malonylation, 2-hydroxyisobutyrylation, glutarylation, β-hydroxybutyrylation , benzoylation have been identified in eukaryotes that are involved in transcriptional regulation, metabolic sensing, maintenance of pluripotent states, DNA repair, spermatogenesis, reversal of HIV latency *etc.* (reviewed in Barnes *et al.,* 2019). p300 is a promiscuous enzyme which is able to accept acyl-CoAs with varying length; p300 is able to process propionyl- and butyryl coA *in vitro* and *in vivo* and this property is also shared by CBP, GCN5 and PCAF (Chen *et al.,* 2007; Leemhuis *et al.,* 2008; Montgomery *et al.,* 2014). A recent study has indicated that p300 is capable of transferring pentonyl-, succinyl-, glutaryl- groups to H4 peptide substrate (Tan *et al.,* 2014). Such cofactor promiscuity of p300 (or other KATs) may enable them to function as metabolic sensors relaying signals about acyl-coA levels to histone or non-histone proteins and mediate their activities (Wellen *et al.,* 2009; Cai *et al.,* 2011). In fact, a recent study has demonstrated the ability of p300 to function as a metabolic sensor

in suppressing autophagy at an elevated nucleo-cytosolic acetyl-CoA levels (Marino *et al.,* 2014).

- **RNA acetylation:**

Elp3, a versatile acetyltransferase, can facilitate acetyl-CoA based modification of a very different class of macromolecule, RNA. Mutational analysis showed that Elp3 KAT domain was required for incorporation of xm^5U modifications into yeast tRNA. Genetic ablation of Elp3 confers resistance to the killer yeast toxin zymocin, an xm^5U-specific RNase (Huang *et al.,* 2008). The unique presence of Fe-S cluster in Elp3, unlike other multidomain acetyltransferases, is essential for radical SAM-based mechanism for Elp3-catalyzed uridine carboxymethylation (Selvadurai *et al.,* 2014). In addition to Elp3, another eukaryotic protein acetyltransferase has been demonstrated to catalyze post-transcriptional acetylation of tRNA and rRNA cytidine residues; for example, Fe (II)-ketoglutarate-dependent dioxygenase family members catalyze removal of methyl groups from protein, RNA, and DNA (Ito *et al.,* 2014; Gerken *et al.,* 2007; Ng *et al.,* 2007; Aas *et al.,* 2003).

- **Regulation of nuclear shape:**

Human Nat10, a nucleolar acetyltransferase, known for its function as regulator of telomeric gene activation, harbors tubulin acetyltransferase function (Aas *et al.,* 2003; Shen *et al.,* 2009). Depletion of Nat10 impaired tubulin acetylation and cytokinesis. Recent study has proposed therapeutic targeting of Nat10 as a treatment for Hutchinson−Gilford progeria syndrome (HGPS), a premature-aging disease result from mutation of the gene LMNA, which encodes the nuclear membrane proteins lamin A and C (Larrieu *et al.,* 2014; Gordon *et al.,* 2014). Functional Nat10 acetyltransferase domain is required for maximal induction of lamin-dependent nuclear shape defects in model systems and HGPS patient-derived cells. A derivative of the initial small molecule hit, 'remodelin', was shown to reduce DNA damage and cell senescence phenotypes normally observed in HGPS cell lines. Nat10-catalyzed acetylation stabilizes microtubule anchorage, which in turn exerts an external force on the nucleus that contributes to irregular morphology in cells where progerin has aberrantly accumulated in nuclear membranes. By reducing these external forces, small molecule inhibitors of Nat10 can restore normal nuclear shape and thus provide a new therapeutic avenue for treatment of the symptoms associated with this phenotype in HGPS (Larrieu *et al.,* 2014).

Due to the crucial functions played by acetyltransferases in organisms, mutations, loss of allele of acetyltransferases that lead to aberrant function are associated with various diseases including cognitive dysfunction, neurodegeneration, cancer, infectious diseases *etc.* (reviewed in Drazic *et al.*, 2016).

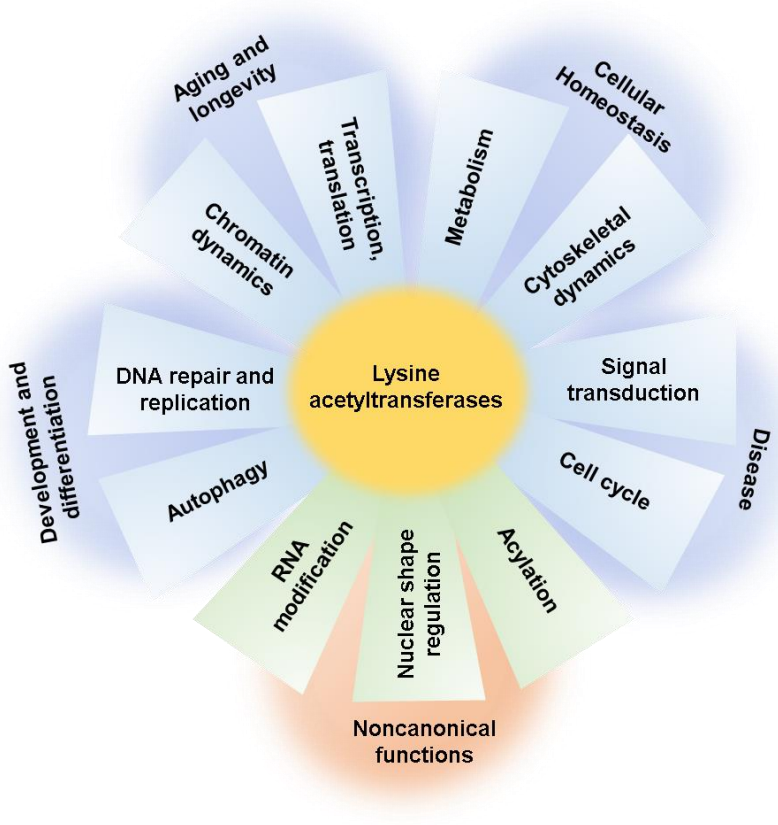


Figure 1.19. Scope of biological functions regulated by eukaryotic acetyltransferases. *The primary shell represents individual pathways regulated by acetyltransferases through histone and nonhistone acetylation (acylation), the secondary shell represents coordinated biological phenomena regulated by acetyltransferases.*

1.5. Transcription by RNAPIII

Eukaryotic transcription of nuclear DNA is carried out by at least three RNA polymerases, namely, RNAPI, II, and III. While RNAPII transcribes all protein-coding mRNAs and some non-coding RNAs (ncRNAs) such as small nuclear- (sn-), small nucleolar- (sno-), micro- (mi-) and long non-coding (lnc-) RNAs; majority of non-coding RNAs are transcribed by RNAPI and RNAPIII. RNAPI is located in the nucleolus and transcribes the precursor rRNA (pre-rRNA), which is processed into 28S, 5.8S and 18S

rRNAs in human. rRNAs account for ~90% (by mass) of all RNAs transcribed in cell at a time. RNAPIII resides in nucleoplasm and transcribes as many as ~200 different types of noncoding RNAs which are involved in various cellular pathways. The canonical targets of RNAPIII are 5SrRNA and tRNAs both of which are required for protein synthesis and account for ~10% of total mass of RNAs synthesized in cells at a time (Palazzo *et al.,* 2015). Among the other types of noncoding-RNAs that are synthesized by RNAPIII are U6 snRNA, 7SL RNA, 7SK RNA, vault RNA, hY RNA, H1 RNA, MRP RNA. SINEs etc. 7SK RNA is directly involved in RNAPII-mediated transcription regulation as it along with HEXIM1/2 sequesters positive Transcriptional Elongation Factor-b (pTEFb) in an inactive complex (Yik *et al.,* 2003). Several other pol III transcripts also play functionally-related roles in pre-rRNA or pre-tRNA processing complexes (RNase MRP and RNase P RNAs), serve as guide RNAs for rRNA methylation (*e.g.,* yeast snR52) or are required for co-translational insertion of nascent polypeptides across the membrane of the endoplasmic reticulum (7SL RNA). U6 snRNAs are involved in pre-mRNA splicing, the RNA component of telomerase (TERC) and the 7SK RNA regulatory complex of the pol II transcription elongation factor P-TEFb. Many other noncoding RNAs whose function are yet to be elucidated are also transcribed by RNAPIII (Dieci *et al.,* 2007 and references therein). RNAPIII is also associated with transcription of some microRNAs, such as hsa-mir565 (a tRNA fragment), hsa-mir-1975 (overlaps with the hy5 gene), and hsa-mir-886 (overlaps with a vault gene) (Canella *et al.,* 2010). Interestingly, the length of the transcripts generated by RNAPIII are not longer than 400bp which is consistent with elongation properties of RNAPIII. Also, all these genes possess long stretch of T residues as termination signal. However, the promoters of these genes are diverse in nature and can be broadly divided into four categories.

The first type of promoter resembles that of 5SrRNA gene which consists of A box, intermediate element (IE) and C box, together known as internal control region (ICR) (Bogenhagen et al. 1980; Sakonju et al. 1980). tRNAs, adenoviral VAI gene contain type II promoters that also has an ICR consisting of A box, IE and, B box (Galli et al. 1981). These promoters' regions are highly conserved as they encode T- and D- loops in tRNA, but the length of IE region can vary. A-box in type II promoters can bind to different transcription factors. U6snRNA, 7SK RNA, H1RNA *etc*. are regulated by type III promoters that bear resemblance with that of RNAPII targets. These genes have external

control region that consists of TATA box, proximal sequence element (PSE) and often distal sequence element (DSE) (Hernandez and Lucito 1988). Intriguingly, the specificity of RNAPIII for some of its target promoters (snRNA) can be switched to RNAPII and vice versa by abrogation or inclusion of TATA box (Lobo and Hernandez 1989).

Yeast U6 snRNA is regulated by a hybrid promoter consisting of A box, B box and, external TATA box. B-box is located downstream of gene body. All three control regions are required for efficient *in vivo* transcription of this gene (Brow and Guthrie 1990). In fact, careful analysis has identified the presence of external TATA box in almost all 5SrRNA and tRNA genes in *Schizosaccharomyces pombe* (Hamada et al. 2001). Also, 5SrRNA genes in yeast contain only C-box as ICR (Challice and Segall, 1989).

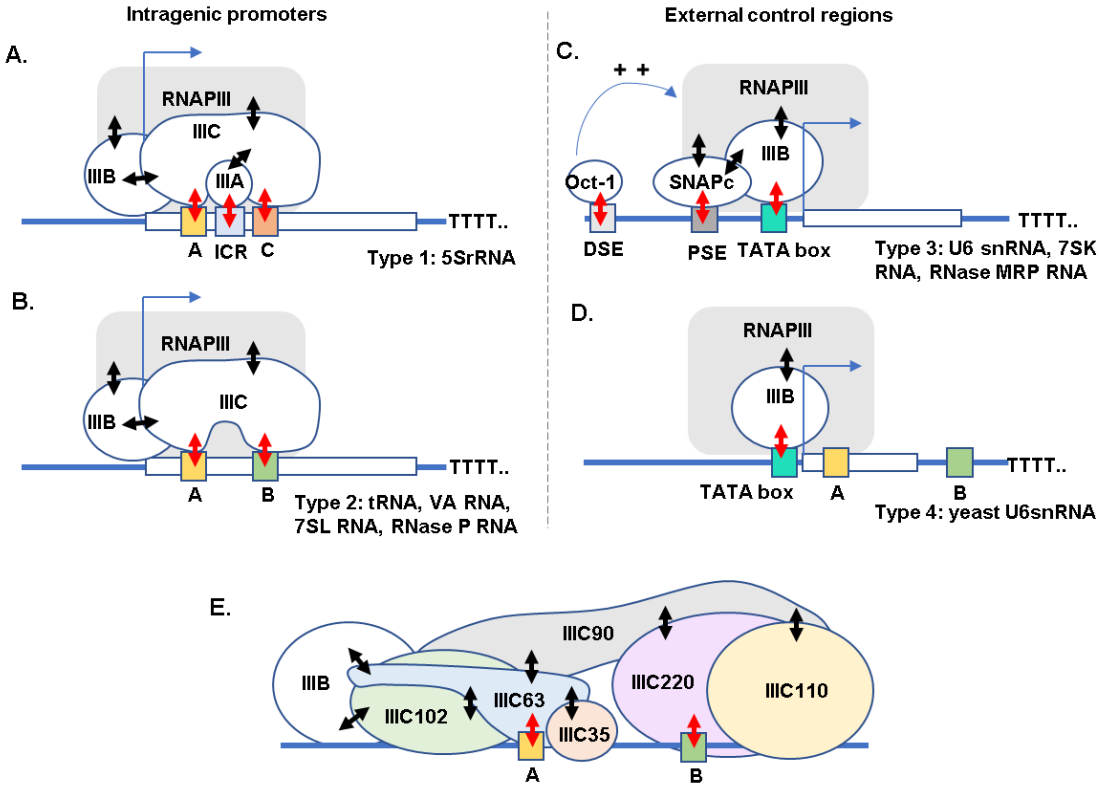


Figure 1.20. Placement of TFIIIC at preinitiation complexes formed at RNAPIII targeted promoters. *(A-D) Interaction of the components of preinitiation complex (IIIC-TFIIIC, IIIB-TFIIIB, RNAPIII and IIIA- TFIIIA) interacting with different regulatory elements of all 4 types of target promoters of RNAPIII. (E)Assembly of human TFIIIC subunits (220- 35) on a type 2 promoter after extrapolating the relative positions of their homologs from solved structure of yeast TFIIIC holo-complex as mentioned in Male et*

al., 2015. The DNA-protein interactions and protein-protein interactions are indicated with red and black arrows respectively.

Fractionation of HeLa cell extract over phosphocellulose column identified three fractions that were important for RNAPIII mediated transcription. Fraction A (in 100mM KCl flowthrough) corresponded to a polypeptide identified as TFIIIA and was found to be required for only type I promoter. Fraction B (100-350mM KCl step up solution) contained TFIIIB, a complex of TBP (TATA box binding protein), Bdp (B double prime 1), Brf1 or 2 (TFIIB related factor). TFIIIB was found to be essential for transcription initiation from all types of RNAPIII target promoters. Brf1 containing TFIIIB complex drives transcription form type I and II promoters in human whereas type III promoters require binding of Brf2 containing TFIIIB that form a weak complex. Fraction C (350-600mM KCl step up solution) contained another complex TFIIIC which was found to be required for type I and II promoters only. An additional fraction D (in 600-1000mM KCl step up solution) was found to be involved in transcription from type III promoters (Segall *et al.*, 1980; Lobo *et al.*, 1991; Schramm *et al.*, 2000; Willis, 2002).

In case of all RNAPIII promoters the final step of PIC formation involves RNAPIII recruitment by promoter bound TFIIIB complex; and, TFIIIB in promoter is in turn recruited by a series of other factors. ICR and C-box in type I promoter is bound by zinc finger containing TFIIIA which then guides TFIIIC bind to A and B box. TFIIIC then recruits TFIIIB through SANT domain protein Bdp1 (Lasser *et al.*, 1983). In case of type II promoter B-box is readily recognized by TFIIIC complex which then recruits TFIIIB (Lasser *et al.*, 1983). In type III promoters, PSE is recognized by a five-subunit complex SNAPc (snRNA activating protein complex) containing PBP (PSE-binding protein), PTF (PSE- transcription factor). TFIIIB is then recruited to TATA box through SNAPc, TBP interaction (Schramm *et al.*, 2000). In all the cases, TFIIIB has been shown to be sufficient for driving several rounds of successful transcription once other components of PIC has been stripped off after transcription initiation indicating that the other components mentioned above acts as recruitment factors only (Kassavetis et al. 1995; Rüth et al. 1996).

Chromatin modifications identified near active RNAPIII-occupied sites partially match with that of active RNAPII targets, H3K4me3 and H3K9ac are enriched at both RNAPII and RNAPIII target active promoters (Barski *et al.*, 2010; Moqtaderi *et al.*, 2010; Oler *et*

al., 2010). However, H3K36me3 or H3K79me2 were not found to be associated with transcribed RNAPIII genes, and H327me3 enrichment was found to be weakly correlated (Barski *et al.,* 2010). TFIIIC-mediated recruitment of p300 in the RNAPIII promoters facilitates downstream transcription from chromatinized template (Mertens and Roeder, 2000). c-Myc, Fos, Jun, Stat1, Cyclin T1- a subunit of pTEFb, even some basal RNAPII transcription factors were found to be in close proximity of RNAPIII target promoters Interestingly, RNAPII was found to enriched with 2kb of RNAPIII transcribed gene TSS and mostly RNAPII mediated transcription occurred in the opposite direction of RNAPIII transcription 153. A subset of tDNAs with highest enrichment of RNAPIII is decorated with CCCTC- binding factor (CTCF) (Barski *et al.,* 2010; Oler *et al.,* 2010; Raha *et al.,* 2010). RNAPIII is also known to be involved in formation of subcellular structures such as perinucleolar (PNC) domain in tumor cells, these represent favorable sites for transcription. tRNA induced and condensin mediated organization of chromatin domains can result in recruitment of tRNA genes to nucleolus or co-recruitment of RNAPII genes to these sites favoring repression or expression in context-dependent manner (Haeusler and Engelke, 2004; Haeusler *et al.,* 2008).

1.5.1. Multifunctional TFIIIC complex

TFIIIC complex recruits TFIIIB and RNAPIII onto type I and III promoters. Chromatographically TFIIIC complex can be separated into three fractions: TFIIIC1-like (TFIIIC0), TFIIIC1 and TFIIIC2. Among these TFIIIC2 is a stable complex of six subunits and binds to B-box tightly. TFIIIC1 stimulates binding of TFIIIC2 complex to type II promoters, TFIIIB and PBP. TFIIIC1 is required for 7SK transcription (Yoon *et al.,* 1995; Schramm and Hernandez, 2002). The details about exact components of TFIIIC1 and TFIIIC0 still remain obscure. Both the complexes are functionally equivalent and can be replaced by Bdp1(Weser *et al.,* 2004). The components of TFIIIC1 have molecular mass corresponding to 70, 50, 45 and 40 kDa (reviewed in Schramm and Hernandez, 2002).

TFIIIC2 is a large complex with a combined molecular mass of about 600kDa in human. Electron microscopic studies have shown that yeast TFIIIC complex has a dumbbell shape with two globular regions separated by a stretchable linker region that allows the protein to span a surprisingly long distance (both A and B box binding). This complex

is also quite flexible; when the distance between boxes A and B was zero, TFIIIC appeared as a large blob on the DNA and with increasing distance between boxes A and B, TFIIIC appeared as two globular domains separated by a linker of increasing length between them (Deprez *et al.*, 1999). Out of six sunubits in human TFIIIC2 complex only two subunits, TFIIIC102 and 63 are homologs of yeast Tfc4 and 1; and rest of the complex are strikingly distinct from yeast TFIIIC. Interestingly, acetyltransferase activity has been attributed to more than one subunit of human TFIIIC2 complex (Kundu *et al.*, 1999) whereas yeast TFIIIC complex is enzymatically inactive. Both in yeast and human TFIIIC has been found to occupy many sites without co-occupancy of RNAPIII or TFIIIB. Thses sites are termed as 'extra TFIIIC (ETC) loci' and are known to function as barrier (Moqtaderi and Struhl, 2004; Moqtaderi *et al.*, 2010). In murine cells more than 80% TFIIIC binding sites are known to be ETC (Carrière *et al.*, 2012).

The noncanonical functions of TFIIIC in presence or absence of RNAPIII are summarized below:

- **Regulation of RNAPII activity**

Apart from generating transcript 7SKRNA that regulates pTEFb function and interferes with RNAPII function directly, in the chromosomal tDNA-RNAPII gene loci assembled RNAPIII complex function as insulator preventing UAS from promoter activation (Simms *et al.*, 2008). In this case, inhibition of TFIIIC binding by introducing mutation to tDNA promoter increased transcription from adjacent RNAPII promoter and this RNAPII activation was dampened by deletion of UAS of a divergently transcribed upstream gene (Simms *et al.*, 2004; Simms *et al.*, 2008). This insulator like effect of t-DNA is known as tRNA gene mediated silencing (tgm silencing) or tRNA position effect and is not altered by disruption of sub-nuclear localization (Simms *et al.*, 2008; Hiraga *et al.*, 2012; Hull *et al.*, 1994; Bolton and Boeke, 2003).

RNAPIII along with TFIIIC can transcribe SINE RNAs that can influence RNAPII activity as described recently, RNAPIII-mediated transcription of enhancer RNA (FosRSINE1) upon stimulation of neuronal activation regulates RNAPII mediated transcription of Fos gene (Policarpi *et al.*, 2017).

- **Barrier and insulator function of tDNA**

Aforementioned tRNA genes (e.g., $tRNA^{Thr}$) utilize specific chromatin remodelers and histone modifiers to counteract the propagation of heterochromatin and act as barrier element (Oki and Kamakaka, 2005; Dhillon *et al.*, 2009). Insulation by tDNA cluster decreases upon overexpression of Sir deacetylases in yeast (Valenzuela *et al.*, 2009). Here on/off rates of TFIIIB/C occupancy at tDNA determines its efficiency as insulator. Cluster of tDNAs are shown to perform better as insulators as compared to single tDNA copy and RNAPIII occupancy is strictly not necessary (Donze and Kamakaka, 2001; Noma *et al.*, 2006). TFIIIC can bind to other B-box sequences and some tDNAs (e.g., $tRNA^{Phe}$) without co-occupancy of TFIIIB or RNAPIII. These ETC sites are recognized as COC (chromosome organizing clumps) in yeast. ~90% of COC sites were within a few hundred base pairs of promoters of RNAPII transcribed genes including divergent promoters and IRs (Noma *et al.*, 2006). In human most of the ETC sites localize adjacent to RNAPII transcription start sites (Oler *et al.*, 2010), many sites are found in perinuclear and pericentric heterochromatin regions. In mammals, about 500,000 copies of Alu repeats that account for 5% of the genome content contain B-box sequences (Okada *et al.*, 1991), representing a large fraction of potential TFIIIC binding sites in spatial genome organization. However, it is observed that tRNA genes and ETC located near pericentromere with modest TFIIIC enrichment only act as weak boundary element and require the presence of other factors to establish strong boundary activities. These insulators and barriers are often found to be bound by CCCTC-binding factor (CTCF) that recruits cohesin complex (Wendt *et al.*, 2008). Mutation in conserved domains of cohesin severely disrupted tDNA-mediated insulator activity (Donze *et al.*, 1999). ETC sites additionally function as loading sites for condensin (D'Ambosio *et al.*, 2008). In yeast, condensin and TFIIIC play opposing roles in localizing RNAPIII genes within nucleus which is important for proper transcription; binding of condensin reduces transcription of tDNAs, reduced condensin binding induces TFIIIC binding and transcription; depending on TFIIIC condensin binding the 3D location of the gene changes (Iwasaki *et al.*, 2010). In human, TFIIIC serves as a sequence specific anchoring site that recruits condensin II to condensin II–TFIIIC sites (CTS) that are marked with active H3K4me3 marks and that condensin binding facilitates transcription from adjacent promoters (Yuen *et al.*, 2017).

Apart from tDNAs, previously described SINE (short interspersed nuclear elements), such as, 7SLDNA derived SINE B1 and pre-tDNA derived SINE B2 elements can also function as barriers and enhancer-blocking insulators (Lunyak *et al.,* 2007; Roman *et al.,* 2011). Inhibition of various acetylating complexes such as NuA4, SAGA, SAS-I etc. and chromatin remodelers such as RSC and Isw2 have shown to abolish insulation by tDNAs.

- **Regulation of RNAPII function independent of RNAPIII**

TFIIIC bound ETC sites were found upstream of Tfc6 gene in yeast, mutation in the ETC site increase Tfc6 transcription and overexpression of Tfc6p downregulates its own expression indicating the possibility of TFIIIC complex regulating its own expression (Moqtaderi and Struhl, 2004; Kleinschmidt *et al.,* 2011). TFIIIC when bound to B-box sequence near inverted repeats (IRs) occupied by RNAPII in yeast facilitate downstream transcription (Noma *et al.,* 2006).

- **Targeting of retroelement insertion by tDNAs**

TFIIIC is involved in retrotransposon integration directed by tDNA that protects the host from deleterious effect as tDNAs contain intragenic promoter and are not inactivated by flanking site alteration (Boeke and Devine, 1998). In yeast, Ty3 transposon in yeast is inserted immediate upstream of tDNA TSS, Ty1transposon insertion occurs with ~700bp window near tDNA TSS in an approximately 80bp periodic fashion (Chalker and Sandmeyer, 1990; Ji *et al.,* 1993; Devine and Boeke, 1996; Bachman *et al.,* 2004). TFIIIC, TFIIIB, ISW2 chromatin remodeling complex, SET3C deacetylase complex facilitate and RNAPIII interfere insertion of these transposons (Kirchner *et al.,* 1995; Connolly and Sandmeyer, 1997; Bachman *et al.,* 2005; Mou *et al.,* 2006).

- **Nucleosome displacement by tDNAs**

tDNA placement adjacent to a strong nucleosome positioning sequence can override the positioning, in fact it was observed that in vivo tDNAs are nucleosome free regions. Interestingly, it was also observed that ETC sites were nucleosome free as well giving rise to the idea that TFIIIC binding is dominant to nucleosome formation at B-box sequences *in vivo* and might be a consequence of unusually strong binding of TFIIIC complex to DNA (Morse *et al.,* 1992; Jansen and Verstrepen, 2011; Mavrich *et al.,* 2008; Xu *et al.,* 2009; Donze *et al.,* 2012).

- **Chromatin boundaries and direct RNAPII regulation by RNAPIII complexes**

tDNA element can function as a functional barrier for yeast HMR mating locus, can limit gene silencing from ribosomal DNA locus. mutation or deletion of tDNA or B-box element results in partial repression of downstream genes in these cases (Donze *et al.*, 1999; Donze and Kamakaka, 2001; Biswas *et al.*, 2009; Simms *et al.*, 2004). Ectopically placed ETC site, binding only TFIIIC without the rest of RNAPIII apparatus was found to be sufficient for mediating barrier function; in fact, both tDNAs and ETC sites that bind to only TFIIIC individually can act as effective barriers that limit spreading of heterochromatin mediated repression to adjacent euchromatic domains (Simms *et al.*, 2008; Valenuela *et al.*, 2009; Partridge *et al.*, 2000; Noma *et al.*, 2006; Scott *et al.*, 2006).

Contrarily, it was also observed that tDNA can maintain complete silencing at HMR under conditions of reduced Sir2 activity indicating that it may also serve as a barrier to the propagation of histone acetylation from adjacent euchromatic domains to heterochromatic region (Lynch and Rusche, 2010).

- **Replication fork pause at tDNAs**

A genome-wide chromatin immunoprecipitation (ChIP) study with replication fork associated protein revealed that practically all tDNAs colocalized with replication fork pause (RFP) sites (Sekedat *et al.*, 2010). Paused forks at tDNAs are dependent on TFIIIC binding and induce attenuation of RNAPIII transcription to release and modulate recombination upon activation of replication stress checkpoint pathways. Misregulation of this process may result in increased genetic instability in tumor formation (Clelland and Schultz, 2010).

- **Cohesin, condensin, clustering and genome-wide organization**

Cohesin and condensin are SMC (Structural Maintenance of Chromosomes) category proteins that are involved in sister chromatid cohesion, chromatin organization, and chromosome segregation. recruitment of cohesin is crucial for establishment of silencing at yeast HMR locus; mutation in cohesin subunits led to loss of barrier activity of tDNA at HMR (Donze *et al.*, 1999; Lazarus and Holmes, 2011). TFIIIC shows strong co-localization with cohesin loading complex and condensin subunits at barrier region;

condensin is also recruited to ETC sites and ectopically engineered B-box and TFIIIC acts as a loading factor (D'Ambrosio *et al.*, 2008).

In short, TFIIIC may act as a stably bound, global "bookmark" within chromatin to establish, maintain, or demarcate chromatin states as cells divide or change gene expression patterns (Policarpi *et al.*, 2017; reviewed in Donze, 2011 and Van *et al.*, 2012).

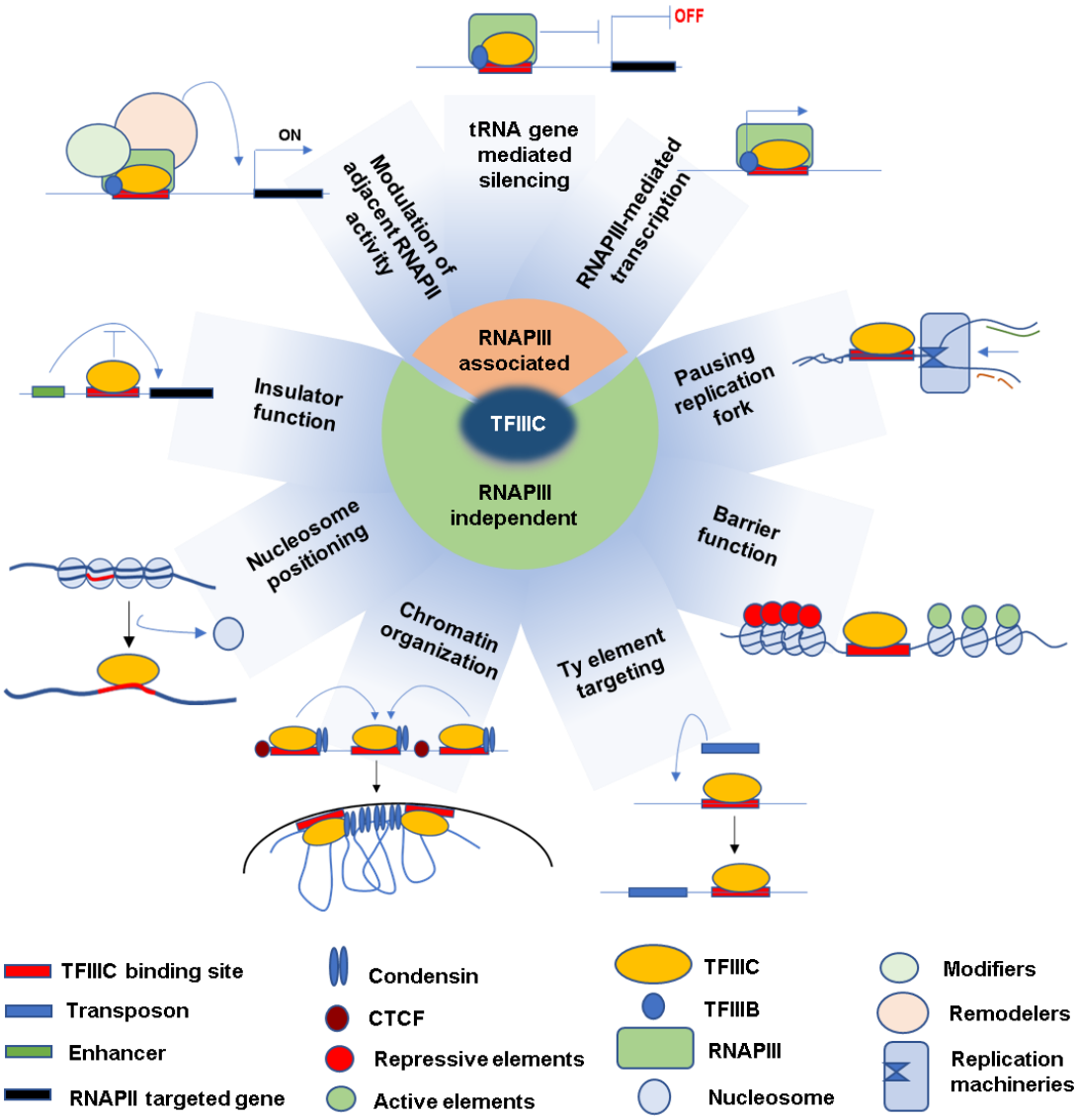


Figure 1.21. Schematic representation of canonical and noncanonical functions of TFIIIC.

Evolution of TFIIIC complex

Human TFIIIC2b is reported to have six subunits, viz. α (220 kDa) binds to the box B promoter element, β (110 kDa) stabilizes interactions of TFIIIC2 with TFIIIC1, it also possesses acetyltransferase activity, γ (102 kDa) directly binds tRNA and virus-associated RNA promoters, δ (90 kDa) transfer acetyl group to free nucleosomal H3, ε (63 kDa) tightly associated component of TFIIIC2 and recently discovered 35 kDa subunit. TFIIIC in yeast also has been reported to have six subunits of which TFC7 (homolog of TFIIIC35), TFC1 (homolog of TFIIIC102), TFC8 (homolog of TFIIIC63) bears sequence homology with their human counterpart. The evolutionary conservation of TFIIIC complex across species is summarized in table 1.6.

Table 1.6. Homologs of human TFIIIC complex subunits present in various organisms

Characteristics of TFIIIC complex	Yeast *(S. cerevisiae)*	Drosophila *(D. melanogaster)*	Zebrafish *(D. rerio)*	Human *(H. sapiens)*
Characterized subunits of TFIIIC	TFC1 (95), TFC3 (138), TFC4 (131)TFC6 (91), TFC7 (55) and TFC8 (60)	Only 2 subunits are characterized (TFIIIC220 and TFIIIC35)	Predicted orthologs are reported but not annoted, TFIIIC complex is not characterized	TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90, TFIIIC 63, TFIIIC 35
DNA binding activity	Binds to A and B box sequences of RNAPIII target promoters	Binds to A and B box sequences of RNAPIII target promoters	Not known	Binds to A and B box sequences of RNAPIII target promoters
B box binding subunits	TFC3 (138)	dTFIIIC220	Not characterized	hTFIIIC220
'ExtraTFIIIC' or ETC function	Yes	Yes	Not known	Yes
Acetyltransferase activity	No	Maybe (Recently Bombyx mori insect TFIIIC complex was shown to possess acetyltransferase activity)	Not known	Yes

1.5.2. Context dependent regulation of RNAPIII transcription

Multiple factors are known to regulate RNAPIII mediated transcription and most of those were identified studying yeast or similar unicellular eukaryotes that adjust their gene expression pattern on environmental ques. Favorable growth conditions promote transcription and unfavorable conditions limit rate of transcript generation; as RNAPI and III transcripts that contribute to translational apparatus in a cell consuming ~80%

nucleotides in a cell are restricted during nutrient deprivation. 53, 54, 55. Multiple pathways such as secretory signaling pathway, TOR pathway, Ras-PKA pathway, DNA damage pathway etc. are known to affect transcription by RNAPIII. 54,56-60. Secretory pathway defect represses 5SrRNA and tRNA expression through MAF1, a downstream effector of protein kinase C (PKC). Maf1 is the global negative regulator of RNAPIII. Maf1 was first discovered in yeast mutant which accumulated high level of tRNA despite of having a growth defect (Boguta *et al.*, 1997; Pluta *et al.*, 2001).

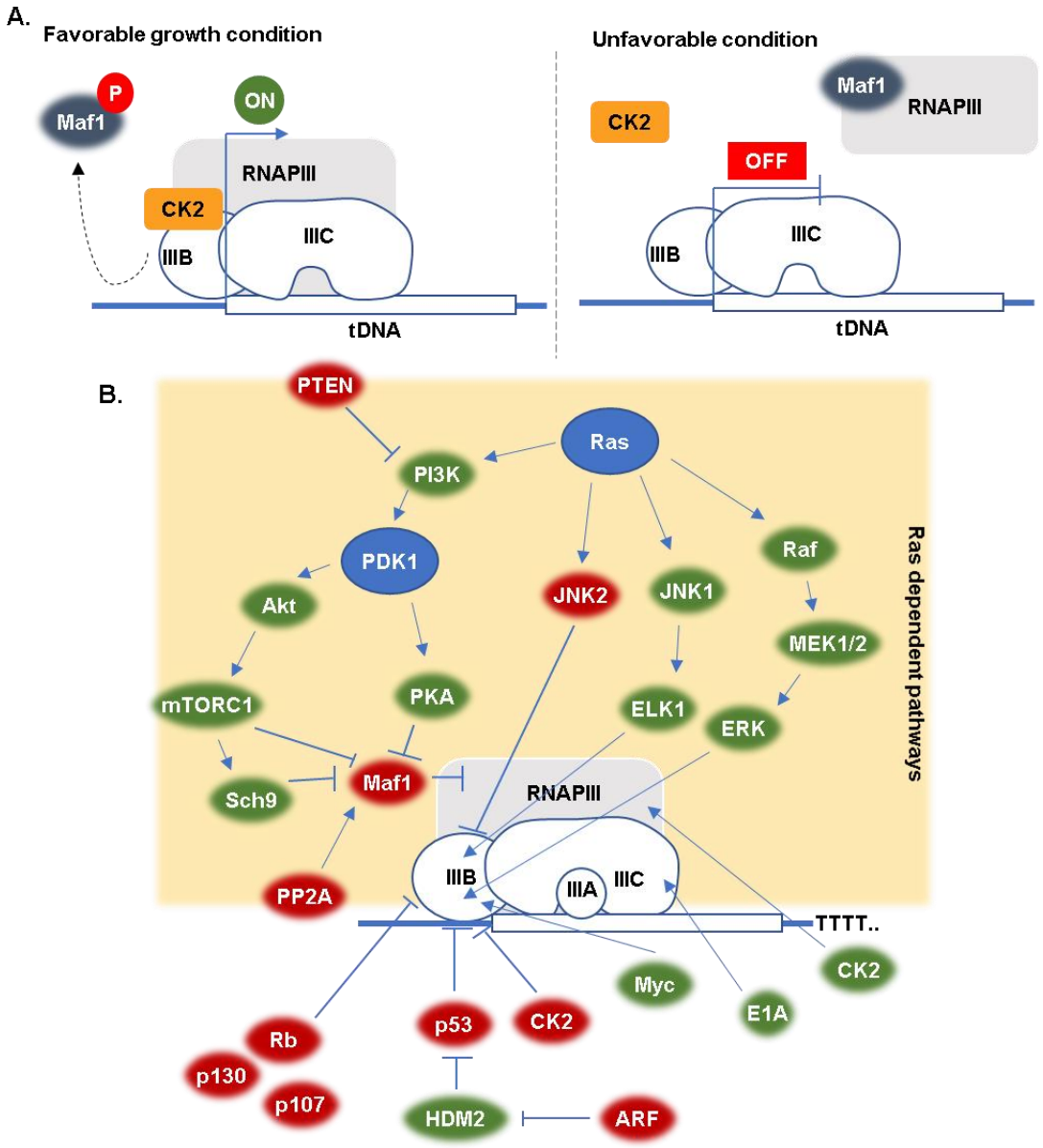


Figure 1.22. Regulation of Pol III transcription. *(A) Under favorable growth conditions CK2 mediated phosphorylation of Maf1fails to inhibit RNAPIII PIC complex, in unfavorable growth condition Maf1perturbs RNAPIII recruitment inhibiting transcription. (B) Compilation of different signaling pathways regulating RNAPIII-*

mediated transcription. Blue nodes represent nodal points, green and red nodules represent activators and repressors respectively. Arrow ends represent targets of the regulators (Adapted from Dumay-Odelot et al., 2010).

Maf1 orthologs that function as RNAPIII repressor have been identified in mammals, flies, worms, plants, and parasites (Johnson *et al.,* 2007; Rideout *et al.,* 2012; Cai *et al.,* 2016; Soprano *et al.,* 2013; Romero-Meza *et al.,* 2016). Under favorable growth conditions, Maf1 is phosphorylated and is sequestered in cytoplasm but under repressive conditions, Maf1 is dephosphorylated by protein phosphatase 2A (PP2A) and translocates to nucleus where it interacts with RNAPIII complex preventing RNAPIII transcription (Towpik *et al.,* 2008; Oficjalska-Pham *et al.,* 2006). Maf1 binds to RNAPIII clamp and also weakens its interaction of RNAPIII with Brf1 subunit of TFIIIB thus impairing RNAPIII recruitment to promoters; however, Maf1 does not bind to preassembled RNAPIII-Brf1-TBP-DNA initiation complex and the interaction (Cabart *et al.,* 2008; Vannini *et al.,* 2010; Desai *et al.,* 2005). Apart from nutrient deprivation Maf1 is also known to repress transcription by RNAPIII during Rapamycin treatment, oxidative or endoplasmic reticulum stress (Moir *et al.,* 2006).

Adenoviral protein E1A which activates transcription of immediate early viral genes and stimulates cellular RNAPIII transcripts VA1 and tRNA genes by interacting with TFIIIC subunits (Berger and Folk, 1985; Hoeffler and Roeder, 1985; Gaynor *et al.,* 1985). SV40 small T-antigen stimulates RNAPIII transcription (Loeken *et al.,* 1988). Hepatitis B virus X protein (HepBX) also activates RNAPIII transcription by increased TATA-binding protein (TBP) production (Aufiero and Schneider, 1990; Wang *et al.,* 1995).

Viral proteins such as adenoviral E1A, E1B, papillomaviral E6, E7, SV40 large T antigen also functions through inactivating p53, pRb that also regulate cell cycle. Subsequently, a large number of tumor suppressors such as p53, pRb, ARF, PTEN, BRCA1, ARF were found to be regulators of RNAPIII transcription (White *et al.,* 1996; Chu *et al.,* 1997; Cairns and White, 1998; Chesnokov *et al.,* 1996; Morton *et al.,* 2007; Woiwode *et al.,* 2008; Verus *et al.,* 2009). Hypophosphorylated Rb represses RNAPIII transcription either through direct interaction with TFIIIB and PTF/SNAPc/ PBP subunits or by activation of signal cascade that led to phosphorylation and inactivation of TFIIIB subunits (White *et al.,* 1996; Cairns and White, 1998; Larminie *et al.,* 1997; Hirsch *et al.,* 2004). Chronic overexpression pf p53 has been linked to proteosomal degradation of

BRF1 and repression of RNAPIII transcription 108. BRF1 can also be phosphorylated and inactivated by cyclin dependent kinase 1 (p34 or CDK1) (Gottesfeld *et al.,* 1994; Leresche *et al.,* 1996; Fairley *et al.,* 2003). BDP1, a subunit of TFIIIBα is phosphorylated by CK2 during mitosis inhibiting RNAPIII transcription (Hu *et al.,* 2004). Interestingly, CK2 is also known to stimulate another RNAPIII target U6snRNA transcript production via direct phosphorylation of RNAPIII subunits (Hu *et al.,* 2003). RNAPIII transcription machinery (RNAPIII subunits POLR3G, 3E, 3G, TFIIIC subunits TFIIIC220, TFIIIC110, PTF) also undergoes phosphorylation during mitosis that could contribute to mitotic repression of RNAPIII transcription (Dephoure *et al.,* 2008).

In contrast to tumor suppressor proteins, protooncogene c-Myc and components of MAPK signaling pathway (ERK, JNK1) activated RNAPIII transcription (Gomez-Roman *et al.,* 2003; Felton-Edkins *et al.,* 2003; Zhong *et al.,* 2009). c-Myc promotes recruitment of GCN5-TRRAP coactivator complex with RNAPIII target promoters. JNK1 phosphorylated BRF1 to activate RNAPIII transcription and all proto-oncogene products function through recruitment of TFIIIB to cognate RNAPIII target promoters (Kenneth *et al.,* 2007). During serum starvation or actinomycin induced nucleolar stress c-Myc dependent activation of RNAPIII is perturbed by ribosomal protein L11 (Dai *et al.,* 2010).

RNAPIII is also deregulated during cellar transformation and differentiation. TFIIIC subunits were found to be overexpressed in ovarian cancer patient samples (Winter *et al.,* 2000). Overexpression of BRF1 or $tRNA^{i}_{Met}$ was found to be suffiecient for transforming mouse fibroblasts (Marshall *et al.,* 2008). During cardiomyocyte differentiation induction of BRF1, c-Myc expression and Rb, ERK activation by serum, endothelin 1 or phenylepinephrin induced RNAPIII transcription resulting in improved differentiation (Goodfellow *et al.,* 2006). However, during differentiation of mouse F9 and human NTERA2 embryonic carcinoma cells RNAPIII transcription was reduced because of altered TFIIIB and TFIIIC1 activities (White *et al.,* 1989; Meissner *et al.,* 2007). During the process of differentiation or transformation a subunit of RNAPIII, RPC32α is itself subjected to regulation, higher expression of this protein is linked to increased transformation and it was also found to be required for anchorage dependent growth of HeLa S3 carcinoma cell line. RPC32α downregulation led to increased differentiation of human H1 embryonic stem cells (Haurie *et al.,* 2010).

The deregulation of various components of RNAPIII transcription machinery in different pathophysiological conditions are summarized in table 1.6.

Apart from diseases, RNAPIII is a potential target for aging and longevity as inhibition of RNAPIII in *Drosophila*, *C. elegans*, yeast has been shown to increase lifespan. Contrarily, in mice activation of RNAPIII by impairing Maf1 function has been demonstrated to promote longevity (reviewed in Kulaberoglu *et al.*, 2021).

Table 1.6. Dysregulation of RNAPIII transcription machinery components in various pathophysiological conditions *(Adapted from Park et al., 2017)*

RNAPIII related components	Disease	Status
TFIIIC2	Cancer	Overexpressed
BRF1	Cancer	Overexpressed
	Cerebellar-dental-skeletal syndrome	Mutated
BRF2	Cancer	Overexpressed
BDP1	Non-syndromic hereditary hearing loss	Mutated
POLR3A/B	Syndromic hyperrmyelinating leukodystrophy	Mutated
POLR1C	Leukodystrophy	Mutated
nTr20 (pdt)	Neurological defects	Mutated
BC200 RNA (pdt)	Cancer	Overexpressed
TSEN (pdt)	PCH	Mutated
RNase MRP (pdt)	Cartilage-hair hypoplasia	Mutated
Nc886 (pdt)	Cancer	Silenced by CpG DNA hypermethylation

1.6. Rationale behind this study

As discussed earlier, p300 is a multidomain protein and multiple functions are associated with its different domains; acetyltransferase activity and transcriptional coactivation are two such functions of p300. Conventionally, the two aforementioned functions are positively correlated. p300 KAT domain mediated nucleosomal histone acetylation induces local transcription and factor acetylation induces downstream target gene expression in general. In vitro studies have indicated that p300 C-terminal transactivation

domain (TAD-C terminal) itself is a substrate of p300 KAT domain and acetylation of TAD improves p53 factor binding and activation of downstream genes (Stiehl *et al.,* 2007). p300 KAT domain and TAD can function in co-operative manner as well to induce transcription; for example, p300 recruitment by Myc as a coactivator in the promoter of human telomerase reverse transcriptase gene stimulates its transcription and p300 mediated acetylation of Myc-TAD further stimulated Myc-targeted gene expression (Faiola *et al.,* 2005). Contrarily, in many instances, p300 mediated acetylation negatively affected transcription. p300 mediated acetylation of AFF1, a component of super elongation complex, shuts off early transcription during genotoxic stress (Kumari *et al.,* 2019). p300 mediated acetylation of CH1 domain in N-terminal TAD of p300 dislodges it from HIF1-α and switches off target gene expression (Fath *et al.,* 2006). In fact, multiple biochemical assays overexpressing catalytically dead mutant of p300/CBP suggested that their KAT activity is either not required or only partially required for transcriptional coactivator function (Song *et al.,* 2002; Harton *et al.,* 2001; Hecht *et al.,* 2000; Wang *et al.,* 2000). Hence, the multitude of effector functions brought about by p300 through its various functional domains in different cellular and organismal context may act in synergy or discord. Embryonic development is one such process that is tightly regulated by different posttranslational modifiers, p300 being one of them. Different KATs are required depending on the stage of gene activation. A high functional specificity is crucial for activation of early genes whereas later maintenance stage involve less specific functionality (Kurdistani and Grunstein, 2003; Anamika *et al.,* 2010). While the role of p300 as a transcriptional coactivator during mammalian embryogenesis is quite well studied and has been discussed in latter chapter, the relevance of p300 mediated acetylation in early development remain unclear. Based on the involvement of acetyltransferase activity of p300 (if any) in early differentiation, lineage commitment or survivability of the embryo or embryonic stem cells can be manipulated using different small molecule modulators of p300 acetyltransferase.

The acetyltransferase activity of TFIIIC complex, a general transcription factor for RNAPIII, has been discovered long back (Kundu *et al.,* 1999), yet, its substrate specificity and physiological relevance remain poorly characterized. TFIIIC-holocomplex was found to acetylate multiple core histones (H3, H4 and H2A) but its specificity in vivo or for nucleosomal substrates is not characterized. Inhibition of TFIIIC mediated acetylation in cell-free system using p-hydroxymercuribenzoic acid (PMA)

significantly reduced transcription from chromatinized template but not from DNA template leading to the speculation that TFIIIC-mediated acetylation of nucleosomal histones might be involved in relieving chromatin mediated repression of transcription (Kundu *et al.,* 1999). Interestingly, at least three different forms of TFIIIC complexes (TFIIIC0, 1 and 2) were found to be present in mammalian system; these complexes had different mass, DNA binding properties and dubitably were comprised of different subunits (Yoshinaga *et al.,* 1987; Oettel *et al.,* 1997). Among the subunits identified and characterized in TFIIIC complexes, the largest subunit, TFIIIC220 is the only known subunit that binds to DNA and incidentally was found to possess intrinsic catalytic activity (Kundu *et al.,* 1999). The unique property of TFIIIC220 as a DNA binding acetyltransferase opens up a gamut of possibilities for its effector functions. Apart from facilitating RNAPIII mediated transcription TFIIIC complex is known to be involved in RNAPII transcription, chromatin organization etc. as discussed earlier, having said that, if acetyltransferase activity of TFIIIC220 individually or as an integral part of TFIIIC complex establishes as a mode of regulation remains to be answered.

1.7. Objectives

The overall objective of this study is to understand the role of two acetyltransferases in different cellular contexts and the study has been divided accordingly into two comprehensible parts.

1. To understand the role of p300 acetyltransferase activity in early embryonic development.

p300 is a well characterized versatile protein as discussed earlier. Out of the two most important functions of p300, its role as a transcriptional coactivator in maintenance of pluripotency and differentiation thereafter has been well characterized, but, the relevance of acetyltransferase activity of p300 in the process remains elusive. This study aims to elucidate the importance of p300 acetyltransferase activity in the process of embryonic cell differentiation using a specific small molecule modulator of p300 KAT activity.

2. To characterize the acetyltransferase activity of human TFIIIC220

As discussed earlier, TFIIIC220 is a component of TFIIIC complex that belongs to the cohort of poorly characterized acetyltransferases. The objective of the study is to characterize the acetyltransferase activity of the protein *in vitro* and in cells.

Chapter 2

Materials and Methods

This chapter presents a brief overview of the materials and experimental procedures used in this study.

2.1. General Methods

2.1.1. Preparation of bacterial competent cells

E. coli strains DH5α, BL21(DE3), XL10 Gold cells were streaked on Luria broth (10 gm/L tryptone, 5 gm/L yeast extract, and 10 gm/L NaCl) containing 1.5% (w/v) agar culture plates from respective frozen glycerol stocks. The streaked plates were incubated ~12 hours at 37°C. A single colony was inoculated into 10 mL Luria Broth (LB) containing 10 mM $MgCl_2$ and 10 mM $MgSO_4$. The culture was grown at 37 °C in an incubator shaker for 12-16 hours. From this primary culture, 1% inoculum was then inoculated into 100 mL of LB. The secondary culture was grown 37 °C in an incubator shaker till the OD_{600} reached 0.35-0.4 (mid log phase). The culture was chilled to 4 °C and pelleted at 3000g for 10 minutes at 4 °C. The pellet was then resuspended in 12 ml TFB1 (30 mM potassium acetate pH 7.5, 100 mM KCl, 10 mM $CaCl_2$, 15% glycerol) and incubated for 60-90 min at 4 °C. After the incubation, the cells were pelleted at 3000g for 10 minutes at 4 °C. The pellet was resuspended in TFB2 (10 mM Na-MOPS, pH 6.8, 10 mM KCl, 75 mM $CaCl_2$, 15% glycerol), made aliquots and flash-frozen in liquid nitrogen.

2.1.2. Transformation

Frozen aliquot (~50µL) of competent cells were thawed on ice for 5 minutes and ~50 ng of purified plasmid DNA was added. The competent cells in the presence of DNA were incubated for 25 minutes on ice, following which a heat shock at 42 °C was given for 90 seconds. The cells were immediately chilled on ice for around 5 minutes. LB was added to the cells and the cells were allowed to recover at 37 °C in and incubator shaker for around 45 minutes. The transformed cells were subsequently spread over an LB-agar plate containing the appropriate selection antibiotic. The plated cells were then incubated at 37 °C for 12-16 hours.

2.1.3. DNA isolation and estimation

Plasmid was isolated from overnight grown 5mL culture containing appropriate antibiotic for selection by alkaline lysis method. Single colony was inoculated in 5mL fresh LB containing appropriate antibiotic for selection and grown overnight at 37ºC, 180rpm. Cells were pelleted down at 10000g, 4ºC, 2 minutes and resuspended in 100µL of chilled solution I (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA pH 8). 200µL of freshly prepared solution II (0.2N NaOH, 1% SDS) was added and mixed by inverting the tube 3-4 times. 150µL of chilled solution III (5M CH_3COOK, 10M glacial acetic acid in 4:1 v/v ratio) was added immediately and mixed by inverting the tube 6-8 times followed by a spin at 16000g, 4ºC, 10 minutes. Clear supernatant was collected and equal volume of phenol-chloroform-isoamyl alcohol in 25:24:1 v/v ratio (Himedia) was added and vortexed briefly. The mixture was spun at 16000g, 4ºC, 10 minutes and upper aqueous phase was collected. Equal volume of isopropanol was added and incubated at room temperature for 10 minutes followed by a spin at 16000g, 4ºC, 10 minutes. Pellet was washed with 1mL of 75% ethanol and then air dried. Finally, pellet was dissolved in 40µL of TE buffer (10mM Tris-HCl pH 7.5, 0.1mM EDTA pH 8.0) containing 20µg/mL RNaseA (Sigma). Transfection grade plasmids were isolated using NucleoSpin Plasmid kit (Macherey-Nagel, Cat. 740588.50).

Plasmid concentration was estimated using Nanodrop 1000 (Thermo Scientific) where A260 1unit corresponds to 50ng/µL of DNA.

2.1.4. RNA isolation

Mammalian cells were harvested in TRIzol (Ambion) at a ratio of 1mL per 5 million cells and stored overnight at -80ºC followed by centrifugation at 16000g, 4ºC, 20 minutes. Supernatant was mixed with chloroform in 5:1 volumetric ratio and vortexed for 15 seconds followed by 5 minutes of incubation at room temperature. The mixture was spun at 16000g, 4ºC, 10 minutes. The upper phase was collected and mixed with equal volume of isopropanol and kept at room temperature for 15 minutes. After a spin at 16000g, 4ºC, 20 minutes supernatant was discarded and pellet was washed with 75% (v/v) ethanol. The pellet was air dried and dissolved in 30µL of autoclaved RNase free water. ~10µg of RNA was taken for DNaseI (NEB, Cat. M0303) digestion at 37ºC for 10 minutes. Reaction was stopped with 5mM EDTA and by heating at 75ºC for 10 minutes. RNA was precipitated by overnight incubation at -80ºC with 2.5 volume of 100% ethanol and 0.1 volume of 3M sodium acetate pH 5.2 followed by a spin at 16000g,

4ºC, 20 minutes. RNA pellet was air dried and dissolved in 30µL of autoclaved RNase free water.

RNA concentration was estimated using Nanodrop 1000 (Thermo Scientific) where A260 1unit corresponds to 40ng/µL of DNA.

2.1.5. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to visualize DNA/RNA samples. Indicated percentage of agarose was added to 1X TBE buffer (90mM Tris, 90mM Boric acid, 2mM EDTA; final pH 8.3) and dissolved by melting and poured in the pre-sealed gel cast. Samples were prepared in loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol in 5% glycerol) and electrophoresed at different voltages for varying time periods as indicated in 1X TBE buffer. Gels were stained with 50ng/mL ethidium bromide and visualized in gel documentation system (Bio-Rad).

2.1.6. Polyacrylamide gel electrophoresis

Indicated percentage of SDS-PAGE was prepared to quantitate and analyze the purity of protein samples. 8-15% of resolving and 4% stacking gel was prepared from a stock of 30% polyacrylamide containing acrylamide and bisacrylamide in 1:1 v/v ratio (Sigma) in a buffer of 375mM Tris-HCl (pH 8.8) and 375mM Tris-HCl (pH 6.8) respectively containing 0.1% SDS, 0.1% ammonium persulfate (Sigma) and 8% v/v TEMED (Sigma). Gels were cast and run in Bio-Rad mini-PROTEAN system. Samples were prepared in sample buffer (50mM Tris-HCl pH 6.8, 100mM DTT, 0.1% bromophenol blue, 10% glycerol) and heated at 90ºC for 5 minutes and subjected to electrophoresis at 150V in running buffer (25mM Tris-HCl pH 8.3, 250mM glycines, 0.1% SDS). Gels were stained with 0.25% w/v Coomassie brilliant blue in 45% methanol, 10% acetic acid in water followed by destaining with 45% methanol, 10% acetic acid in water.

2.1.7. Western blot analysis

Proteins were run in indicated percentage of SDS PAGE and transferred onto PVDF membrane (Immobilon, Merck Life Science) using semidry western apparatus (Bio-Rad). For protein larger than 75kDa wet transfer (Bio-Rad) was done. PVDF membrane was activated in absolute methanol for 30 seconds and then equilibrated in transfer buffer (25mM Tris, 192mM glycines, 0.038% w/v SDS, 20% v/v methanol). Proteins were transferred onto PVDF membrane at 25V (semi dry) and 100V (wet transfer) for varying

time period according to the size of the protein. Blots after transfer were kept in 5% w/v skim milk in phosphate buffer saline (PBS: 137mM NaCl, 2.7mM KCl, 1.8mM KH_2PO_4, 10mM Na_2HPO_4) for blocking for 30 minutes at room temperature and then incubated with appropriate primary antibody in 1% w/v skim milk in PBS at 4ºC overnight on gel rocker. The blot was washed with 5mL of PBS twice for 5 minutes and then incubated in appropriate HRP conjugated secondary antibodies (abcam) in 1% w/v skim milk in PBS at room temperature for 2 hours. The blot was washed again in 5mL of PBS twice and developed using Supersignal West Pico chemiluminiscence kit (Thermo Scientific) using Versadoc (Bio-Rad) or Omega Lum G (Aplegen) or Bio-Rad ChemiDoc or using GBX developer fixer solution and TMS (Kodak/VMS) films.

Antibodies used:

TFIIIC220: sc-398780 (Santa Cruz Biotechnology)

p53: sc-126 (Santa Cruz Biotechnology)

Actin: ab49900 (abcam)

Tubulin: 62204 (Thermo Fisher Scientific)

Histone and site-specific acetylation antibodies: lab-raised

LC3II: PA1-46286 (Thermo Fisher Scientific)

p62: ab91526 (abcam)

Poly histidine tag: H1029 (Sigma)

γH2AX: 80312 (Cell Signaling Technology)

GAPDH: lab raised

2.1.8. Silver Staining

To detect proteins on a polyacrylamide gel with high sensitivity (in the nanogram range), silver staining of gels is preferred over CBB staining. The proteins are electrophoresed on a polyacrylamide gel followed by fixing in 40% methanol and 10% glacial acetic acid in water. The gel was allowed to fix for 2 hours to overnight. The gel was then rinsed thrice in 50% ethanol in water. The proteins were sensitized with 0.02% sodium thiosulfate for 2 minutes then rinsed in deionized water for a minute. The gel was then incubated with 0.1% silver nitrate for 20 minutes at room temperature. The gels were then rinsed thrice with deionized water. The gel was developed in freshly prepared 0.04% formalin/2% sodium carbonate (~4-20 minutes). The reaction was stopped in 1% glacial acetic acid. The gel was imaged and then stored in 1% glacial acetic acid.

2.1.9. cDNA synthesis

10µL of cDNA was synthesized from 1µg of total RNA. 1µg of RNA were mixed with 1.5µM of OligodT (Sigma) and 1mM dNTPs (NEB) in total of 5µL reaction volume and was heated to 70ºC for 10 minutes followed by 5 minutes incubation at 4ºC. Total volume of the reaction was made up to 10µL by adding 200U MMLV reverse transcriptase (Sigma), MMLV buffer and 20U RNase OUT (Invitrogen). Reaction was incubated at 37ºC for 1 hour and then terminated by heating at 90ºC for 10 minutes and stored at -20ºC.

2.1.10. Nucleosome reconstitution

Nucleosome reconstitutions were performed by salt gradient dialysis as described previously (Hayes and Lee, 1997) 601 DNA (kind gift from Prof. Jonathan Widom) amplified from pGEM3z clone using appropriate primers (listed below) and HeLa core histones and free DNA in 1:1 molar ratio was incubated in Buffer A (10mM Tris-HCl pH 7.9, 1mM EDTA, 1mM β-Me) containing 2M NaCl. Reaction was step dialysed in Buffer A containing 1, 0.8, 0.6, 0.1 M NaCl. Finally, reaction was dialyzed against Buffer B (10mM Tris-HCl pH 7.9, 0.25mM EDTA, 10mM NaCl) for overnight at 4ºC.

601 FP: 5' GCTCGGAATTCTATCCGACTGGCACCGGCAAG 3'

601 RP: 5' GCATGATTCTTAAGACCGAGTTCATCCCTTATGTG 3'

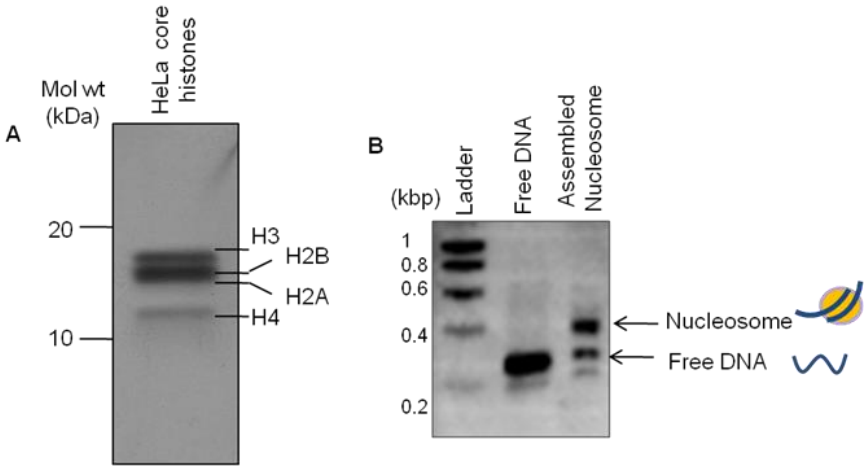


Figure 2.1. Reconstitution of nucleosome. *(A) Core histones isolated from HeLa cells were run on 15% SDS PAGE and stained with Coomassie (CBB). (B) Free 601 DNA (~255bp) and reconstituted nucleosomes (~450 bp) were run on 0.8% agarose gel and stained with ethidium bromide.*

2.1.11. MTT assay

Equal number of E14Tg2a cells (~ $3x10^4$ cells/well) or embryoid bodies were seeded into a 96-well plate and treated with 15µM compound/DMSO for 24 hours in 100µL of respective supplemented media. Post-treatment, 10µL of 5mg/mL MTT reagent (Sigma, M5655, dissolved in 1X PBS) was added and incubated at 37°C for 3 hours in 90µL serum free media. MTT-formazan was dissolved in 100µL DMSO by incubating at 37°C for another 2 hours after discarding the media; absorbance at 540nm was measured using VERSA max microplate reader (Molecular Devices).

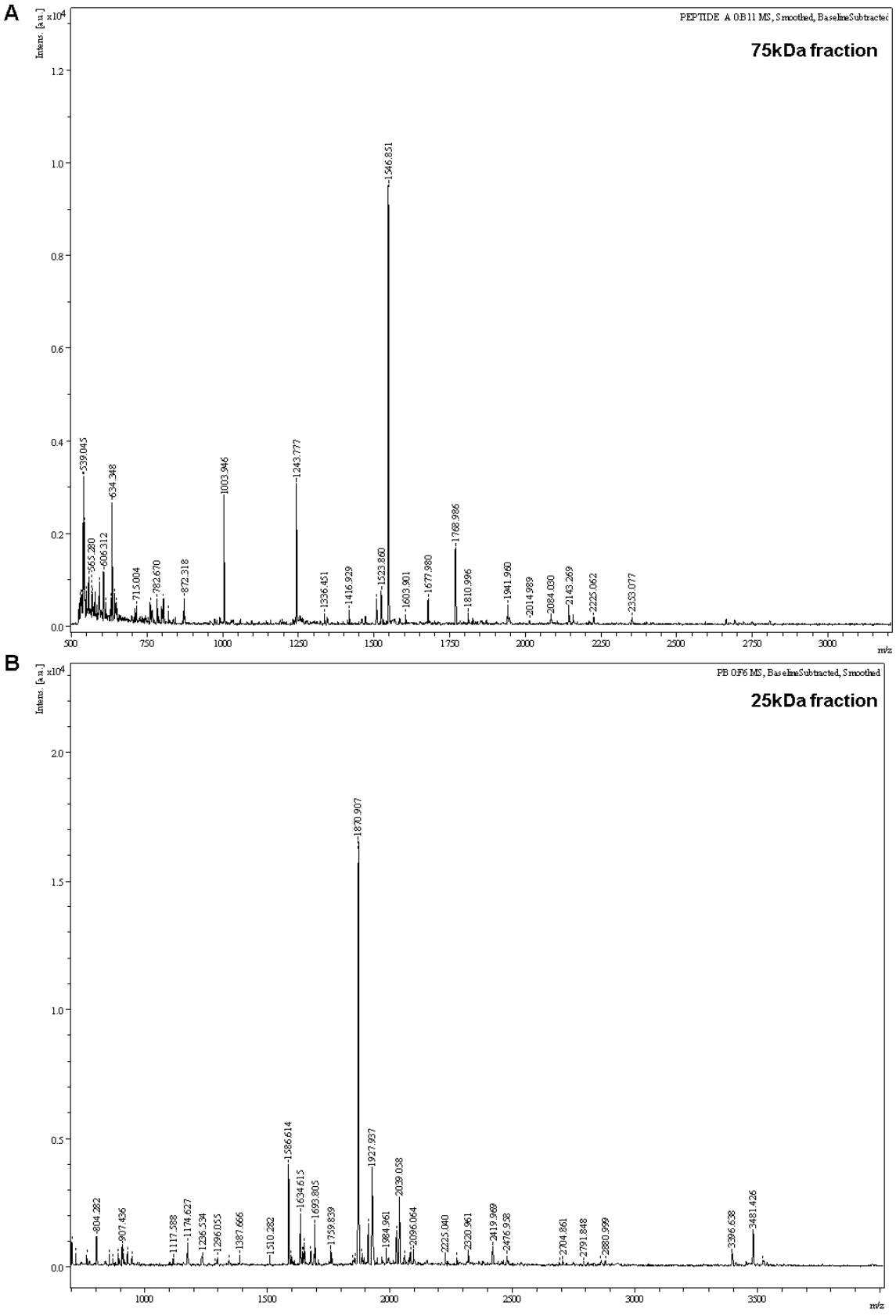
2.1.12. Flow cytometry analysis

For FACS, mES cells were grown to ~80-90% confluency and treated with 15uM Luteolin and equal vol (5uL) of DMSO for 24 hours. Cells were harvested by trypsinization and collected by centrifugation. Cells were resuspended in 1X annexin binding buffer (BD Biosciences kit 556547) by maintaining total population of 1-2 X 10^6 cells/mL. 100 µL cell suspension was incubated with 5 µL Annexin V FITC and 5 µL PI (BD Biosciences- kit cat # 556547) for 10 minutes in dark at RT. Cells were flushed with 400 µL of 1X annexin V binding buffer to stop the reaction. Unstained, single (FITC or PI) stained, heat treated cells were used as controls. Cells were sorted in BD FACS Aria III and analyzed using FCS Express 7 software.

For sorting of TFIIIC220 KD cells based on RFP expression Doxycycline treated cells (~5x 10^6) were trypsinized and resuspended in 3mL media containing 1%v/v FBS and sorted based on high RFP expression in BD Aria-III flow cytometer then seeded into 30mm dish and maintained in 1µg/mL puromycin supplemented media.

For viability assay for TFIIIC220 KD cells Doxycycline treated cells (~1x 10^6) were trypsinized and washed with PBS before resuspending in 1mL PBS containing 0.5µg/mL DAPI and incubating at 37ºC for 5 minutes. Cells were analyzed based on RFP expression and DAPI uptake in BD Aria III flow cytometer.

For cell cycle analysis Doxycycline treated cells (~1x 10^6) were harvested in lysis buffer (1mg/mL sodium citrate, 0.04mg/mL RNase A, 0.03% Tween 20) through pipette. After 10 minutes incubation and brief vortexing nuclei were harvested as pellet by spinning down at 100rcf for 10 minutes at 4ºC. Nuclei was resuspended in 1mL lysis buffer containing 0.1mg/mL Hoechst dye and analyzed in BD Aria III flow cytometer.


A
PEPTIDE A 0B11 MS, Smoothed, BaselineSubtracted
75kDa fraction
Intens. [a.u.]
x10^4
m/z
539.045
565.280
606.312
634.348
715.004
782.670
872.318
1003.946
1243.777
1336.451
1416.929
1523.860
1546.851
1603.901
1677.980
1768.986
1810.996
1941.960
2014.989
2084.030
2143.269
2225.062
2353.077
B
PB 0F6 MS, BaselineSubtracted, Smoothed
25kDa fraction
804.282
907.436
1117.588
1174.627
1236.534
1296.055
1387.666
1510.282
1586.614
1634.615
1693.805
1759.839
1870.907
1927.957
1984.961
2039.058
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3481.426

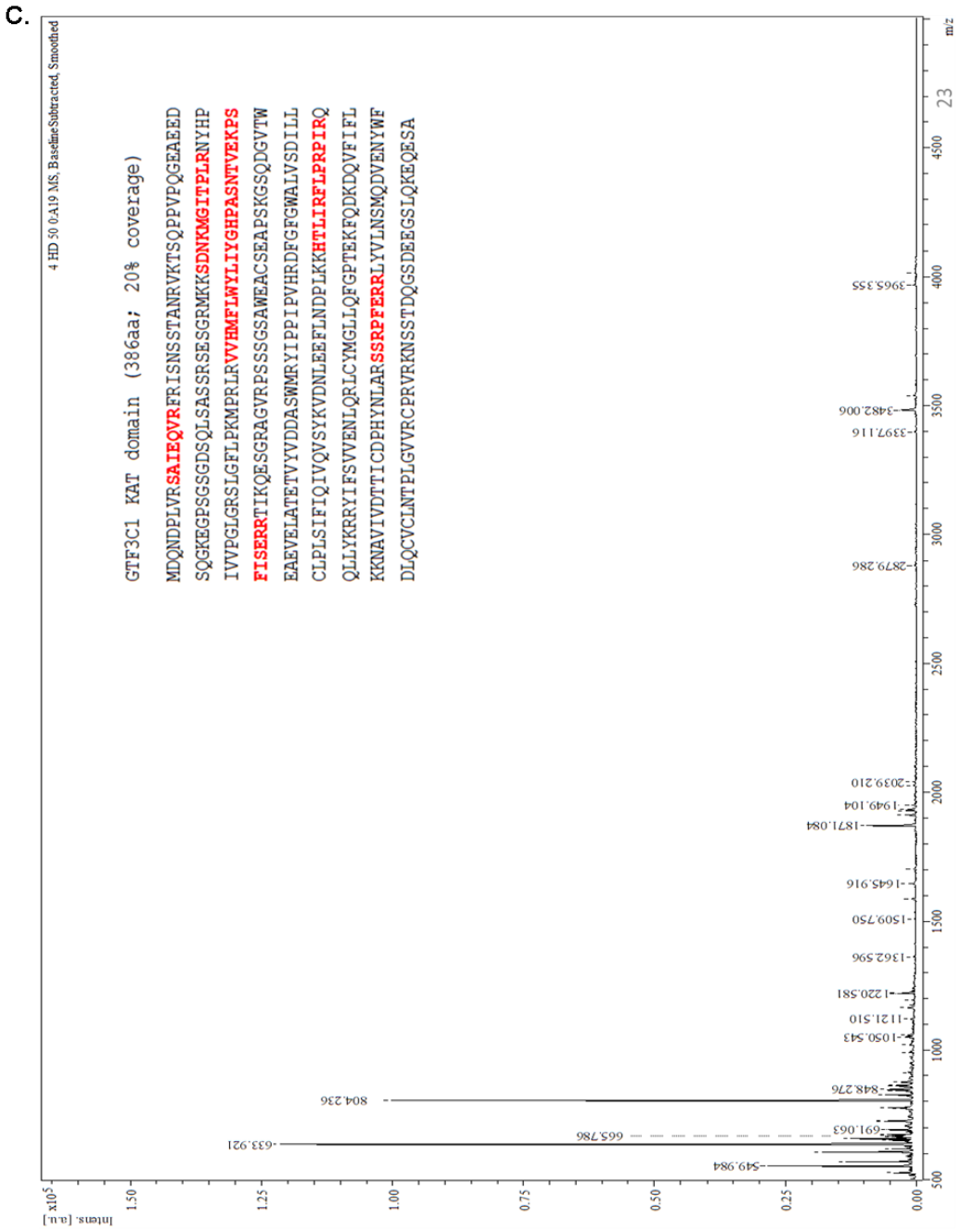

Figure 2.2. m/z values obtained for TFIIIC220 KAT domain (C) and proteins co-eluted with it (A and B). *For TFIIIC220 KAT domain, the peptide fractions corresponding to m/z values obtained from LC/MS result were represented in red letters in the subset.*

2.1.13. Mass-spectrometric analysis

Eluted fraction from Ni-NTA affinity purified TFIIIC220 KAT domain was run on 10% SDSPAGE, stained with Coomassie brilliant blue (dissolved in water- HCl) followed by thorough washing with distilled water. Bands corresponding to molecular weights of 25,

50, 75kDa were excised and proteins were eluted from excised bands following trypsin digestion. Digested proteins were subjected to LC-MS (IISc, Dionex Ultimate 3000). Obtained m/z values were analyzed using Mascot and OMSSA.

2.1.14. Molecular Docking studies

Luteolin (ZINC18185774) and Apigenin (ZINC3871576) structured were downloaded from ZINC database and converted to pdb format using Openbabel. Structures of human p300 and CBP KAT domains were downloaded from RCSB-PDB. The proteins and molecules were processed using Discovery Studio 2021 and docking studies were performed using AutoDock Vina. Docking results were viewed on PyMOL.

2.2. Cell culture

2.2.1. Mammalian cell culture

HeLa S3 (ATCC CCL-2), UMSCC-1(SCC070), and HEK293T (ATCC CRL-3216) cells were maintained at 37ºC in 5% CO_2 incubator in Dulbecco's Modified Eagle's medium (DMEM; Sigma, Cat. D5648) supplemented with 10% fetal bovine serum (FBS; Biological Industries) as recommended by American Type Culture Collection (ATCC). HepG2 (ATCC HB-8065) cells were cultured in Minimum Essential Media (MEM; Gibco, Cat. 11095-080) supplemented with 10% FBS. For cell storage ~2 million cells were resuspended in 1mL freezing mixture (40% incomplete media, 50% FBS, 10% DMSO) and gradually cooled to -80ºC and finally stored in liquid nitrogen. Cells were revived at 37ºC for 3 minutes followed by washing with 10mL of complete media (FBS supplemented media) to remove DMSO. Cells were further seeded in 25mm flask (Eppendorf, Cat. 0030710118) for maintenance. Confluent cells were trypsinized using 0.25% Trypsin-EDTA solution (Himedia) for 2-8 minutes depending on the cell type at 37ºC and then immediately neutralized with complete media. The cells were centrifuged at 200g for 3 minutes and seeded in flasks or dishes as per the experimental requirements.

2.2.2. Generation of stable cell lines

HEK293T cells were transfected with 5µg psPAX2 (Addgene #12260), 0.75µg pRSVRev (Addgene #12253), 1.75µg pCMV-VSV-G (Addgene #8454) and 5µg shRNA (TFIIIC220 shRNA Dharmacon, #RHS4696-200696026 and corresponding nonspecific control) through calcium phosphate co-precipitation. Virus were collected from spent media 48 hours post transfection and filtered through 0.22µm. HeLa S3 and HepG2 cells

were infected with virus for 10 hours in presence of 25ng/mL DEAE and subsequently maintained in 2µg/mL Puromycin (Sigma) selection media. shRNA expression was induced with 2µg/mL Doxycycline (Sigma) for 4 days; Doxycycline was replenished every 24 hours.

For Bafilomycin treatment cells were maintained in 2µg/mL Doxycycline-supplemented culture media for 94 hours. Cells were treated with 100nM Doxycycline (in DMSO) or vector control for 2 hours before harvesting.

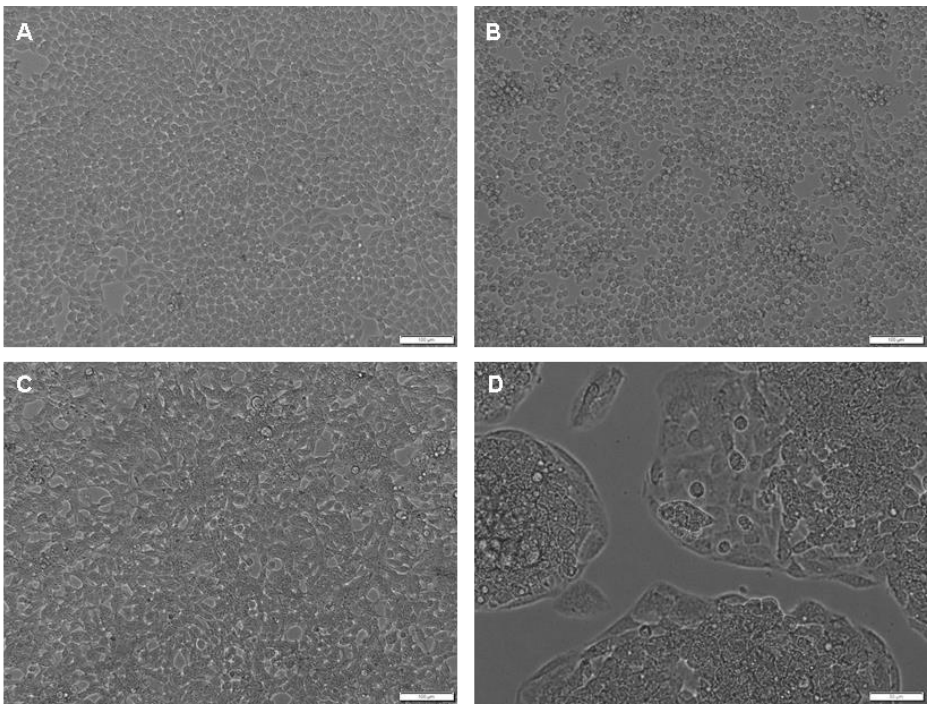

Figure 2.3: Morphological characteristics of mammalian cells grown in a monolayer. *(A)UMSCC-1, (B) HeLaS3, (C) HEK293T, (D) HepG2. Scale bar represents 100 µm for A-C and 50µm for D.*

2.2.3. Transfection of stable cells

2days after Doxycycline treatment stably transfected HepG2 cells were transfected with 500ng of Flag-tagged TFIIIC220 KAT domain with C terminal Myc-tag (NLS) (hereafter mentioned as Flag HD) or similar amount of empty vector (pCMV10 backbone) with Lipofectamine 2000 (Invitrogen). Prior to transfection 1µL Lipofectamine 2000 was incubated with 500ng plasmid in a total of 200µL of OptiMEM (HiMedia) media at room temperature for 20 minutes and then the mixture was added dropwise to 30mm dish containing doxycycline treated cells in 1.8mL OptiMEM supplemented with 0.5%FBS.

6 hours post-transfection cells were maintained in MEM supplemented with 5% FBS and 2µg/mL Doxycycline and incubated for 48 hours before harvesting.

2.2.4. Preparation of mammalian cell lysate

Cells were harvested in complete media after trypsinization as described in section 2.2.1. Cells were washed with 1mL of sterile PBS twice and pelleted down at 200g for 3 minutes at 4ºC. Cell pellet was resuspended in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, Protease inhibitor cocktail (Sigma, Cat.S8830) in 1:10 v/v ratio and incubated at 4ºC for 1 hour. Supernatant was collected after a spin at 16000g, 4ºC for 10 minutes and frozen at -80ºC.

2.2.5. Sf21 insect cell culture

Spodoptera frugiperda ovarian epithelial cells (Sf21; Invitrogen, P/N 51-4000) was cultured in Grace's insect medium (Gibco, Cat. 11605094) supplemented with 10% FBS, antibiotic antimycotic solution (Sigma, Cat. A5955) at 28ºC. Cells were subcultured at 1:4 ratio after gently dislodging the cells with cell scraper. For cell storage ~2 million cells were resuspended in 1mL freezing mixture (40% incomplete media, 50% FBS, 10% DMSO) and gradually cooled to -80ºC and finally stored in liquid nitrogen. Cells were revived at 37ºC for 3 minutes followed by seeding in 10mL of complete media (FBS supplemented media) in 75mm flask for 45 minutes and then media was changed to remove DMSO. For viral infection 10million cells were seeded in each of ten 150mm dishes and grown till 95% confluency. Infected cells were detected under microscope by change in morphology (elongated cells or floating cells or large nucleus etc.)

The sf21 cells were infected with high titer recombinant protein baculovirus stock (~ 2 x 108pfu/ml). Around 6-9 x 10^6 cells were used per flask for the amplification of recombinant virus. The flask was wrapped in Aluminum foil to protect the virus from direct light. The cells were observed after 48 hours for traces of virus infection. Deformed nuclei, elongated and floating cells are hallmarks of a successful infection (Figure 2.6B). The baculovirus was harvested once the cells had burst or exhibited severe features of infection (around 5-7 days post infection). The cell debris was pelleted at 4000 rpm for 5 minutes. The supernatant containing the amplified virus was collected and filter sterilized through a 0.22 µ filter. For regular usage the virus was stored at 4 °C, for storage the virus was frozen at -80 °C.

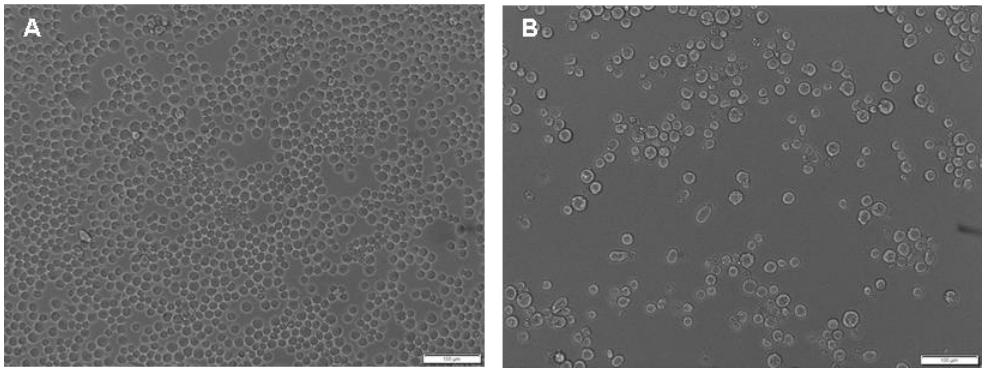

Figure 2.4: Morphological characteristics of the insect cell line Sf21. *(A) uninfected sf21 insect cells, (B) sf21 cells infected with recombinant baculovirus. Scale bar represents 100µm.*

2.2.6. mES cells culture

The mESC line, E14Tg2a was procured from ATCC. Cells were cultured on tissue culture grade plasticware coated with 0.1% gelatin following manufacturer's instructions. Briefly, cells were grown in DMEM media containing 10% fetal bovine serum, 1X non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM beta-mercaptoethanol, and 1000 U/ml leukaemia inhibitory factor (LIF) at 37°C. Medium was changed every 24 h. Confluent colonies were split following trypsinization. EBs were set up using 1×10^6 cells seeded into a low-attachment petri plate and incubated for 48 h before observing the EBs. When effect of compounds on early differentiation was studied, EBs were treated from the time of initiation. EBs were observed and imaged every day using the same imaging conditions. Quantification of EB area was performed using ImageJ.

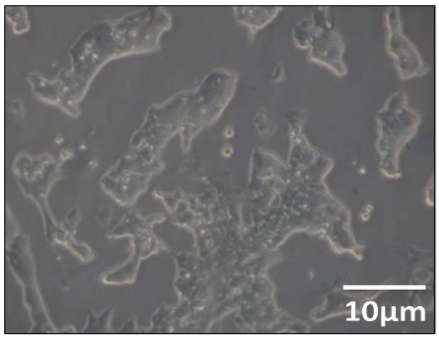

Figure 2.5: Morphological characteristics of E14Tg2A cells *when cultured in presence of LIF in high adherence substratum. Scale bar represents 10µm.*

2.3. Cloning

2.3.1. Generation of KAT domain constructs

KAT domain was amplified from His_6-TFIIIC220 full length construct by PCR using the following primers.

TFIIIC220 KAT domain FP: 5' CATGCCATGGCAATGTTTCTGTGGTAC 3'

TFIIIC220 KAT domain RP: 5' CCGCTCGAGAACCAGTAGTTTTCCAC 3'

The amplified products and pET28b(+) vector were digested with NcoI HF (NEB) and XhoI HF (NEB), and were ligated with T4 DNA ligase (NEB) at 24ºC for 5 hours. Ligation mixture was transformed in DH5α competent cells. Colonies were screened by overnight digestion 37ºC by NcoI HF (NEB) and XhoI HF (NEB). Positive clones were confirmed by sequencing (JNCASR sequencing facility, Eurofin).

Several point mutations were generated in putative acetyl-coA binding motif of TFIIIC220 KAT domain. Primers were generated using QuikChange primer design software (Agilent Genomics). TFIIIC220 KAT domain in pET28b was used as template for generating SDM1 and TFIIIC220 KAT domain SDM1 construct was used as template for generating subsequent point mutations SDM2 and SDM3 using QuikChange II Site-Directed Mutagenesis Kit (Agilent genomics). PCR products were subjected to 1 hours DpnI (NEB) digestion at 37ºC to remove templates and transformed in DH5α. Transformants were confirmed by sequencing and restriction digestion with NotI and XhoI.

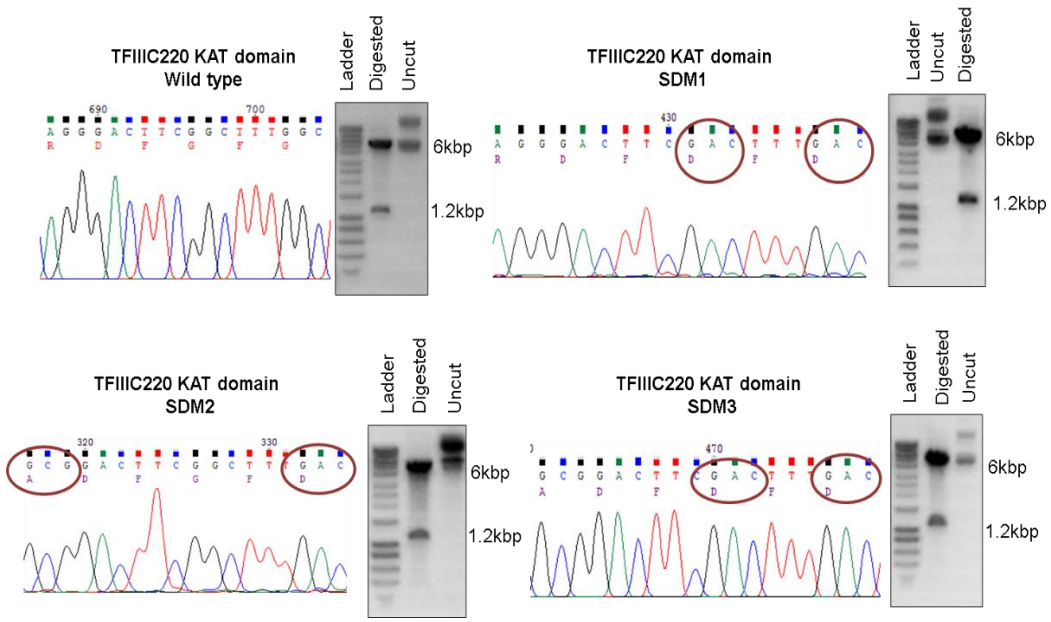

Figure 2.6. Cloning of TFIIIC220 wildtype and mutant KAT domains. *Cloned constructs digested with NcoI and XhoI. Wild type and mutant KAT domain of TFIIIC220*

were cloned in pET28b vectors. Sequences of the mutated residues were highlighted in respective chromatograms obtained by Sanger sequencing and NcoI/XhoI digestion profile of the transformants.

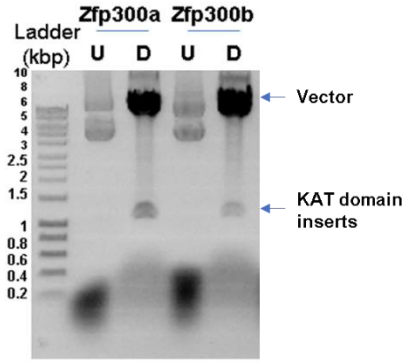

Figure 2.7. Cloning of Zebrafish p300a and b KAT domains*. Cloned constructs digested with NcoI and XhoI. Digested (D) and undigested (U) transformants were run on 0.8% agarose gel.*

Primers used were as follows:

SDM1 FP: 5' GTGTCCCTGAAGCTGAAACTGACCCGAGAGCAG 3'

SDM1 RP: 5' CACAGGGACTTCGACTTTGACTGGGCTCTCGTC 3'

SDM2 FP: 5' GAGCCCAGTCAAAGTCGAAGTCCGCGTGG 3'

SDM2 RP: 5' CCACGCGGACTTCGACTTTGACTGGGCTC 3'

SDM3 FP: 5' GAGCCCAGTCAAAGTCGAAGTCCGCGTGG 3'

SDM3 RP: 5' CCACGCGGACTTCGACTTTGACTGGGCTC 3'

Zebrafish p300a and p300b KAT domain was PCR amplified from total cDNA isolated from 24hpf zebrafish embryo using the following primers and cloned into pET28b vector with C terminal (His)6-tag.

Zfp300a KAT domain FP: 5' CATGCCATGGGCAAAGAGAATAAATATGCTGC 3'.

Zfp300a KAT domain RP: 5' CCGCTCGAGGCACTCATTACAGGTATAG3'.

Zfp300b KAT domain FP: 5' CATGCCATGGGCCGAGTGAACGATTACCT 3'

Zfp300b KAT domain: 5' CCGCTCGAGCCGAAATGTTAGCGTGTCAGG 3'

Catalytic mutant of zebrafish p300a KAT domain was generated by site directed mutagenesis using the following primers and Agilent Quik change II mutagenesis kit as described by the manufacturer.

SDM FP: 5'CCATGAAATCCTTATAGGATATCTTGCTGCTGCCAAAAGACAAG GGTTTACCACA3'

SDM RP: 5'CTGTGGTAAACCCTTGTCTTTTGGCAGCAGCAAGATATCCTATA AGGATTTCATG3'

Clones were confirmed by restriction digestion and sequencing.

2.3.2. Cloning of full length TFIIIC220 in baculovirus

Full-length TFIIIC220 construct in PTRF vector (PTRF-IIICa) clone was a kind gift from Dr. Zhengxin Wang, MD Anderson Cancer Research Centre, Texas. TFIIIC220 full length construct was subcloned into pENTR-DTOPO vector using Topo TA cloning kit (Invitogen) and primers used are listed below. Clone was confirmed by sequencing.

TFIIICFP topo 5' CACCATGGACGCGCTGGAGTC 3'

Topo 1st RP 5' CTCGAGCGGAATTCCGCTCTAGAGGAAGCACTCAGCT 3'

Topo 2nd FP 5' CTAGTCTAGATCAGAAAGTGGACGGATGAAAAAAAG 3'

Topo 2nd RP: 5'CGGAATTCCTTAGGGCTGAACTGAACTTTTC 3'

Topo 3rd FP: 5' CGGAATTCTAACCTTGAAATCCCAGACACAC 3'

Topo 3rd RP: 5' CCGCTCGAGGAGGTGGATCCACTTG 3'

TFIIICRP topo 5' GAGGTGGATCCACTTGTTCCAGTTGACC 3'

Full length TFIIIC220 in Topo vector was cloned into Gateway Entry vector (BaculoDirect Linear DNA) using Baculo-Direct C termExpression Kit (ThermoFisher Scientific, Cat. 12562013). Recombined vector was transfected into Sf21 cells and P1 virus stock was obtained after 7 days and stored in dark at -80°C.

2.4. Protein expression and purification

2.4.1. Purification of His_6 tagged proteins expressed in *E. coli*

All recombinant His_6-tagged proteins were co-expressed with Sirt2 in BL21(DE3) cells to reduce toxic effect of KAT overexpression and were induced with 0.4mM IPTG at 25ºC for 6 hours. Cells were lysed in BC300 (20mM Tris-Cl pH 7.4, 20% Glycerol, 0.2mM EDTA, 300mM KCl, 0.1% NP40, 2mM PMSF, 2mM β-mercaptothenol, Protease Inhibitor Cocktail (Sigma)) containing 10mM imidazole and sonicated until lysate was clear. After a 16000g spin for 30 minutes at 4ºC supernatant fractions were incubated with Ni-NTA resin beads (Novagen) for 3 hours and washed with BC300 containing 30mM imidazole. Proteins were eluted in BC100 (20mM Tris-Cl pH 7.4, 20% Glycerol, 0.2mM EDTA, 100mM KCl, 0.1% NP40, 2mM PMSF, 2mM β-mercaptothenol, Protease Inhibitor Cocktail (Sigma)) containing 250mM imidazole. Purified proteins were run on 12% SDS PAGE and stained with CBB to check the purity.

2.4.2. Purification of His_6 tagged proteins expressed in Sf cells

Sf21 cells were infected with His_6-tagged human TFIIIC220 full length containing baculovirus. 72 hours post infection cells were collected by spinning down at 1500g for 10 minutes at 4ºC. Cell pellet was resuspended in buffer A (10mM Tris-HCl pH 7.5, 0.5M NaCl, 10% glycerol, 0.1% NP40, 15mM β-mercaptoethanol, 2mM phenylmethylsulfonyl fluoride, PIC cocktail (Sigma) and 15mM imidazole) and lysed using Dounce homogenizer (6 strokes, 5 times in 5 minutes interval). Supernatant fraction was collected after centrifuging at 16000g for 30 minutes at 4ºC and incubated with equilibrated Ni-NTA beads for 2 hours at 4ºC. Beads were spun down at 700g and washed with 100mL of buffer B (10mM Tris-HCl pH 7.5, 0.3M NaCl, 10% glycerol, 0.2% NP40, 15mM β-mercaptoethanol, 2mM phenylmethylsulfonyl fluoride, PIC cocktail (Sigma) and 15mM imidazole). Elutions were collected in buffer C (10mM Tris-HCl pH 7.5, 0.2M NaCl, 10% glycerol, 0.2% NP40, 15mM β-mercaptoethanol, 2mM phenylmethylsulfonyl fluoride, PIC cocktail (Sigma) and 250mM imidazole).

Purified TFIIIC220 full length protein was run on 8% SDS PAGE and stained with CBB to check the purity of the sample. Also, elutions were probed with His polyclonal antibody and TFIIIC220 antibody.

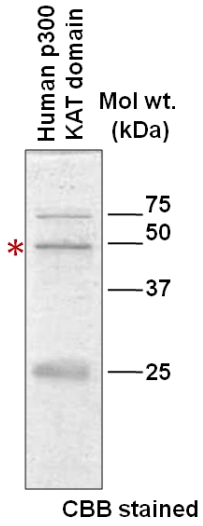

Figure 2.8. Purification profile of human p300 KAT domain. *His_6-tagged p300 KAT domain was affinity purified and eluted fraction was loaded onto 12% SDS PAGE and stained with Coomassie. Protein of expected mass is highlighted with red asterisk.*

2.5. *In vitro* enzymatic reactions

2.5.1. *In vitro* KAT assay followed by filter binding assay

Filter binding KAT assay was performed according to protocol mentioned in Berndsen and Denu, 2005. ~100ng proteins (enzymes) were incubated with 1µg recombinant *Xenopus* H3 (substrate) and 50nCi tritiated acetyl-coA ([3H] Ac-CoA; Perkin Elmer) in KAT buffer (50mM Tris-HCl pH 7.5, 10% glycerol, 1mM DTT, 0.5mM PMSF, 50µM EDTA in water) and 10mM sodium butyrate (Sigma) at 30ºC for 30 minutes in final reaction volume of 30µL. Reaction was stopped by 10 minutes incubation in ice and was spotted on Whatman P81 phosphocellulose paper (Thermo Fisher Scientific, 05-717-2B). 3 washes were given in ~150mL wash buffer (5mM Na_2CO_3 and 50mM $NaHCO_3$ in water) for 10 minutes each to remove residual free acetyl-coA. Dried filter papers were put into 2mL of scintillation fluid (0.5% w/v PPO or 2,5-diphenyloxazole (Sigma) and 0.05% w/v POPOP 1,4-(di-2-(5-phenyloxazolyl))-benzol (Sigma) in toluene) and radioactivity was quantified using Wallac 1409 scintillation counter in counts per minute (cpm) unit.

2.5.2. KAT assay followed by gel assay

Protein activity of TFIIIC220full length, p300 KAT domain and TFIIIC220 KAT domain was normalized by in vitro filter binding assay and the amount of protein having equal enzymatic activities were used for gel assay. ~1µg of recombinant histones or ~280ng of reconstituted nucleosome was incubated with enzymes in KAT buffer containing 10mM sodium butyrate and 0.4mM acetyl-CoA (Sigma) for 1 hour at 30ºC. Reaction was stopped by boiling in sample buffer at 90ºC for 5 minutes and samples were loaded onto 12% SDS PAGE. Histone site specific acetylation antibodies were used to probe the histones after western blotting.

2.5.3. In gel KAT assay

Filter binding KAT assay was performed according to protocol mentioned in Berndsen and Denu, 2005. ~750ng proteins (enzymes) were loaded onto 10% SDS PAGE gel where resolving gel was polymerized along with 1mg/mL calf thymus core histone (Type II, Sigma). Samples were prepared in SDS loading dye without boiling. Gel was run at 4ºC and upper buffer reservoir was supplemented with 0.1mg/mL core histones. SDS was removed by 4 times wash with 100mL buffer I (50mM Tris HCl pH 8.0, 20% isopropanol, 1mM DTT, 0.1mM EDTA in water), 15minutes each. Proteins were

denatured by washing 4 times in 100mL of buffer II (50mM Tris HCl pH 8.0, 8M urea, 1mM DTT, 0.1mM EDTA in water), 15 minutes each. Then the gel was washed twice in 100mL of buffer III (50mM Tris HCl pH 8.0, 0.01% Tween 20, 1mM DTT, 0.1mM EDTA in water) at 4ºC without agitation for 2 hours each and incubated in 100mL buffer III overnight at 4ºC without agitation. After a 30 minutes wash in buffer III at room temperature gel was equilibrated in 100mL of buffer IV (50mM Tris HCl pH 8.0, 10% glycerol, 1mM DTT, 0.1mM EDTA in water) at room temperature for 15 minutes. Acetylation reaction was initiated by placing the gel piece in 3mL of buffer IV containing 5µCi of [3H] AcCoA for 2 hours at 30ºC. Reaction was terminated by adding 50mL of fresh CBB stain for 30 minutes. The gel was destained and washed with water several times before incubation in DMSO for 1 hour. Finally, the gel was incubated in DMSO containing 22.5% w/v PPO at room temperature for 2 hours and washed 4 times with fresh water for 15 minutes each. The gel was dried and exposure was taken on TMS (Kodak/VMS) films for 5-15 days and developed in GBX developer fixer solution.

2.6. Gene expression analysis

2.6.1. RTPCR analysis

200ng of cDNA was mixed with 5µM of appropriate primers, KAPA SYBR-FAST qPCR Master Mix (Sigma, Cat. KK4605) and KSF ROX High (Sigma, Cat. KD4600) in total reaction volume of 10µL. RTPCR was done in Step One Plus real time PCR system (Applied Biosciences). Relative fold change in expression was expressed in terms of $2^{-\Delta\Delta CT}$. Actin, tubulin or GAPDH were used as endogenous controls.

Primers used:

TFIIIC220 RT FP: 5’ CATTCCAGACTCTAGCCATCTC 3’

TFIIIC220 RT RP: 5’ TCCCTATTCTGCAAGGTGTATC 3’

Tubulin RT FP: 5’ CTTCGGCCAGATCTTCAGAC 3’

Tubulin RT RP: 5’ AGAGAGTGGGTCAGCTGGAA 3’

5S rRNA RT FP: 5’ GGCCATACCACCCTGAACGC 3’

5S rRNA RT RP: 5’ CAGCACCCGGTATTCCCAGG 3’

tRNALeu RT FP: 5’ GTCAGGATGGCCGAGTGGTCTAAG 3’

tRNALeu RT RP: 5’ CCACGCCTCCATACGGAGACCAGAAGACCC 3’

tRNATyr RT FP: 5’ CCTTCGATAGCTCAGCTGGTAGAGCGGAGG 3’

tRNATyr RT RP: 5’ CGGAATTGAACCAGCGACCTAAGGATGTCC 3’

7SL RNA RT FP: 5’ GTGTCCGCACTAAGTTCGG 3’

7SL RNA RT RP: 5’ TATTCACAGGCGCGATCC 3’

U1 snRNA RT FP: 5’ ATACTTACCTGGCAGGGGAG3’

U1 snRNA RT RP: 5’ CAGGGGAAAGCGCGAACGCA 3’

U6 snRNA RT FP: 5’ CTTCGGCAGCACATATACTAAAATTGGAAC 3’

U6 snRNA RT RP: 5’ GCTTCACGAATTTGCGTGTCATCCTTGCGC 3’

Actin RT FP: 5’ AGATGTGGATCAGCAAGCAGGAG 3’

Actin RT RP: 5’ TCCTCGGCCACATTGTGAACTTTG 3’

PGDH RT FP: 5’ GCTGGAGTGAATAATGAGA 3’

PGDH RT RP: 5’ GCTGAGCGTGTGAATCCAACT 3’

Survivin RT FP: 5’ GGCATGGGTGCCCCGAGGTT 3’

Survivin RT RP: 5’ AGAGGCCTCAATCCATGGCA 3’

BBC3 RT FP: 5’ CTGCTGCCCGCTGCC TACCT 3’

BBC3 RT RP: 5’ CCGCT CGTACTGTGCG TTGAG 3’

Bax RT FP: 5’ CTCACTCACCATCTGGAAGAAG 3’

Bax RT RP: 5’ GTGTCCCGAAGGAGGTTTATT 3’

JADE1 RT FP: 5’ CCTAGCTTGGTGTCCATTCA 3’

JADE1 RT RP: 5’ GGGTAGAAATAAATACAGGATTGGG3’

p21A RT FP: 5’ GAACTTCGACTTTCTCAGCG 3’

p21A RT RP: 5’ TGGAGTGGTAGAAATCTGTC 3’

p21B RT FP: 5’ GTGGGGTTCAATACTACAGCACAG 3’

p21B RT RP: 5’ TGGTCCTAGCTCTGCCAGTTACTA C 3’

Foxo4 RT FP: 5’ CTTTCTGAAGACTGGCAGGAATGTG 3’

Foxo4 RT RP: 5’ GATCTAGGTCTATGATCGCGGCAG 3’

DRAM1 RT FP: 5’ CGATGGAGTTTCATGCTTCATTT 3’

DRAM1 RT RP: 5’ CACTCTGTTCATTGTGGCATTAC 3’

Ulk1 RT FP: 5’ GTCACACGCCACATAACAGA 3’

Ulk1 RT RP: 5’ CCATCAAGGTGATGAGGAAGAA 3’

AMPKα1 RT FP: 5’ GTCAAAGCCGACCCAATGATA 3’

AMPK α1RT RP: 5’ CGTACACGCAAATAATAGGGGTT 3’

p62 RT FP: 5’ AGGGAACACAGCAAGCT 3’

p62 RT RP: 5’ GCCAAAGTGTCCATGTTTCA 3’

TFEB RT FP: 5’ CAGCAGGTGGTGAAGCAAGAGT 3’

TFEB RT RP: 5’ TCCAGGTGATGGAACGGAGACT 3’

p300 RT FP: 5’ AGGCCTATCAGCAGCGACTCCTTCA 3’

p300 RT RP: 5' TGGGGAGAGCGCACTTGATTGGA 3'
mOct4 RT FP: 5' GGTGAGATGGCTCAGTGGAT 3'
mOct4 RT RP: 5' GCTTAGCCAGGTTCGAGGAT 3'
mNanog RT FP: 5' CCAGGTTCCTTCCTTCTTCC 3'
mNanog RT RP: 5' GGTGAGATGGCTCAGTGGAT 3'
mDppa3 RT FP: 5' CGGGGTTTAGGGTTAGCTTT 3'
mDppa3 RT RP: 5' GGACCCTGAAACTCCTCAGA 3'
mSox1 RT FP: 5' TCAAACGGCCCATGAACGCCTTC 3'
mSox1 RT RP: 5' TCCGGGTGCTCCTTCATGTGC 3'
mSox17 RT FP: 5' CCGATGAACGCTTTCATGGTGTG 3'
mSox17 RT RP: 5' CTCCACCCGCTTCAGCCGCTTC 3'
mTbx2 RT FP: 5' CTGCACGTCTCGGCACTGGGCC 3'
mTbx2 RT RP: 5' CCGCTGACTCGCACCTTGAAGG 3'
mGAPDH RT FP: 5' TCGTCCCGTAGACAAAATGG 3'
mGAPDH RT RP: 5' TTGAGGTCAATGAAGGGGTC 3'
mPax6 RT FP: 5' AACGATAACATACCAAGCGTGT 3'
mPax6 RT RP: 5' GGTCTGCCCGTTCAACATC 3'
mActin RT FP: 5' CTGTCCCTGTATGCCTCTG 3'
mActin RT RP: 5' ATGTCACGCACGATTTCC 3'
mp300 RT FP: 5' GGAGCAAGCTAATGGGGAAGTGAG 3'
mp300 RT RP: 5' CCCCAGCATTTTTGAGAGGAAGAC 3'
('m' stands for mouse)

2.6.2. Immunofluorescence

Mammalian cells were grown on glass coverslips coated with poly L-lysine (Sigma) at 37ºC in 5% CO_2 incubator. Coverslips were collected from culture and washed with PBS to remove media and fixed with 4% paraformaldehyde in PBS for 12 minutes at room temperature. Coverslips were washed thrice with PBS and permeabilized by incubating in 0.5% Triton X-100 in PBS. Triton was removed by washing thrice in PBS and coverslips were incubated at 37ºC for 45 minutes in blocking solution (5% v/v FBS in PBS). Coverslips were incubated in primary antibody solution containing 1% FBS for 1 hour at room temperature on rocker followed by washes with wash buffer (1% FBS in PBS). Alexa Flour secondary antibody (Invitrogen) was diluted in PBS containing 1% FBS and added onto the coverslips and incubated in dark for 1 hour on rocker. Following

two washes in wash buffer cells were incubated in 2µg/mL Hoechst 33258 (Sigma) in PBS for 15 minutes in dark followed by two more washes in PBS. The coverslips were mounted onto glass slides over 70% glycerol and imaging were done using LSM 880 Airy Scan microscope (Carl Zeiss).

Embryoid bodies differentiated using retinoic acid on poly-lysine coated coverslips were treated for 5 days with DMSO or compound. After the treatment, cells were washed with 1X PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were permeabilized using 0.5% Triton X-100 for 10 min. Following washes with 1X PBS thrice for 10 min, blocking was performed using 5% FBS in 1X PBS for 45 min at 37°C. Immunostaining was performed using β-III-tubulin for 1hr at room temperature. Following three washes with wash buffer (1% FBS in 1X PBS), the cells were incubated with secondary antibody (Alexa Fluor- Invitrogen) tagged with a fluorescent dye for 1hr at room temperature. After three washes, nuclei were stained with Hoechst (1 µg/ml) for 20 min. Following washes, coverslips were mounted in 70% glycerol and staining of the EBs was observed using Carl Zeiss confocal microscope LSM510 META.

Antibodies used:

TFIIIC220: sc-398780 (Santa Cruz Biotechnology)

H3K18ac: lab raised

p53: sc-126 (Santa Cruz Biotechnology)

mGFAP: PA1-10004 (Thermo Fisher Scientific)

mβIII tubulin: 4466 (Cell Signaling Technology)

mOct4: 611202 (BD Biosciences)

2.7. Drosophila maintenance

All genotypes were reared on standard cornmeal medium under LD (12hr Light: 12hr Dark) cycles and 25°C. The transgenic lines used in this study are listed below (BDSC-Bloomington Drosophila Stock Center):

UAS.eGFP: BDSC6874

yp-gal4: BDSC58814

UAS.RNAiTF3C1: BDSC57542

Chapter 3

Functional relevance of p300 mediated acetylation in vertebrate embryogenesis

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This chapter presents the evidences that elucidates the role of p300 acetyltransferase activity in vertebrate embryogenesis. This chapter begins with observation on inhibition of p300 acetyltransferase activity using a non-toxic small molecule inhibitor in mouse embryonic stem cells. Inhibition of p300 mediated acetylation negatively affects differentiation of embryonic stem cells. Further analysis shows that absence of p300 mediated acetylation perturbs ectodermal lineage-specific differentiation and induced neurogenesis from mouse embryonic stem cells. In the second part of the study, the acetyltransferase activity of the functional homolog(s) of mammalian p300 in zebrafish is characterized. Zebrafish p300 shows similar substrate specificity to that of human p300. Then a comparative study is performed tallying the deficiency syndromes arising in absence of p300 in zebrafish embryos with the abnormalities rescued by overexpression of active p300 acetyltransferase domain in null background to understand the relevance of p300 mediated acetylation in zebrafish embryonic development. Together these studies conjectured that p300 mediated acetylation is crucial for proper embryonic development and may be particularly essential for ectodermal-specific development; and, the functional relevance of p300 acetyltransferase activity in embryonic development may be evolutionarily conserved in vertebrates.

3.1. Background

Acetylation is one of the epigenetic modifications whose levels are tightly regulated during vertebrate embryogenesis. Acetylation can induce specific transcription and protein expression profile by modulating chromatin compaction in global level or through combinatorial action with other histone modifications in gene regulatory regions. In early embryos, immediately after fertilization, zygotic genome remains silent but transcriptionally competent. In *C. elegans*, *Drosophila* and lower vertebrates like

Xenopus, zebrafish during mid-blastula transition (9-12 mitosis, a few hours post fertilization) and in mammals as early as second cleavage (1-2 days) maternal to zygotic transition (MZT) occurs and prepares the embryonic genome for gastrulation phase where cells migrate and differentiate towards different germ layers (Kane and Kimmel, 1993; O'Farrell *et al.,* 2004, Harvey *et al.,* 2013, Wiekowski *et al.,* 1993). Competing effects of repressive chromatin and transcription machinery relieves global gene repression controlled by maternally deposited factors in early embryos (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b; Prioleau *et al.,* 1994; Almouzni *et al.,* 1995; Veentra et al, 1999). During MZT the embryonic cells undergo several changes ranging from morphological change of nucleocytoplasmic ratio to several changes at molecular level. Zygotic gene activation (ZGA) is preceded by alteration in DNA methylation pattern, establishment of chromatin topologically associated domains (TADs), acquisition of H2AZ in nucleosomes of promoter of zygotic genes. (Guo *et al.,* 2014; Lee *et al.,* 2015; Potok *et al.,* 2013; Ke *et al.,* 2017; Leichsenring *et al.,* 2013). Lastly, active promoters in zygotic genome are bound by specific transcription factor such as Pou5f3, Sox19b, and Nanog in zebrafish (Lee *et al.,* 2013); and NF-Ya (Lu *et al.,* 2016) and DUX TFs in mammals (De Iaco *et al.,* 2017); yet, binding of these TFs alone are not sufficient to trigger transcriptional competency prior to ZGA. Prepatterning of histone modification marks in gene regulatory regions precede ZGA (Lindeman *et al.,* 2011; Vastenhouw *et al.,* 2010). In early embryos maternally defined promoter-permissive H3K4me3 mark and the Polycomb-repressive H3K27me3 mark are constantly modified independent of zygotic transcription. In zebrafish, *Xenopus* and *Drosophila* H3K4me3 is dramatically increased prior to ZGA but the pattern is reversed in mice, where early embryos possess unusual, broad (5-10kb) domains of H3K4me3 and gradually these domains constrict and get restricted to conventional TSSs as the embryogenesis progresses. H3K27me3 can occur with H3K4me3 to demarcate bivalent promoters which are observed in Zebrafish and pluripotent cells but not seen in mice, Drosophila and Xenopus (Liu *et al.,* 2016; Mikkelsen *et al.,* 2007; Li *et al.,* 2014, Akkers *et al.,* 2009, Dahl *et al.,* 2016, Zheng *et al.,* 2016). On the other hand, histone acetylation increases significantly during MZT; in *Drosophila* H4K8ac, H3K18ac, H3K27ac are enriched in active TSS, in mice and zebrafish H3K27ac increases during ZGA (Li *et al.,* 2014; Bernstein *et al.,* 2012; Chan *et al.,* 2019). The pattern of histone acetylation deposition in TSS during ZGA appear to be conserved across many species unlike DNA methylation or histone methylation. In zebrafish embryos acetyltransferases p300 and

Brd4 mediated H3K27ac mark deposition has been found to be sufficient to prepare genome for transcriptional activation and specific inhibition of bromo- and acetyltransferase domains lead to decrease in zygotic transcription followed by restricted gastrulation (Chan *et al.,* 2019). In mice, zygotically defined enhancers that are marked with p300 and DNA-hypermethylation, and, p300 deposition associates with active RNAPII enrichment in target TSS which are mostly differentiation related genes during ZGA and p300 bound clusters increases during development (Hontelez *et al.,* 2015).

p300/CBP are absolutely essential for vertebrate embryogenesis. p300 transcripts are observed as early as E7.5 and expressed ubiquitously with elevated expression in neural tissues; p300 nullizygous mice embryos die during mid-gestation before E11.5 despite the presence of normal amount of CBP; p300 ablation leads to gross developmental arrest at E8.5-9 and shows open neutral tube-defect (NTD) and severe pericardial effusion or defect in embryonic heart development. p300 hemizygous or compound heterozygous (p300+/-; cbp +/-) mice embryos show significant lethality with partial penetrance of exencephaly. p300 deficient mouse embryonic fibroblasts (mEFs) were found to be less proliferative and cell generation stopped after 3-4 cycles of division (Yao *et al.,* 1998). CBP null mouse embryos die within E10.5-12.5 with defective neural tube formation, massive hemorrhage caused by delayed hematopoiesis and defects in mesenchymal cells (Tanaka *et al.,* 2000).

On the contrary, in inner-cell mass (ICM) derived self-renewing and pluripotent mouse embryonic fibroblast deletion of p300 ($p300^{-/-}$) did not affect cellular viability and self-renewal capacity in undifferentiated condition. p300 seemed to be dispensable for the cellular viability of the differentiating embryoid bodies (EBs) but it affected the differentiation process significantly which led to abnormal expression of several germ layer markers (Zhong and Jin, 2009). It was observed that Nanog expression in EBs are dependent on the presence of p300 and in mouse embryonic stem (ES) cells Nanog recruits p300 (via KIX domain) and Brd4 (Boo *et al.,* 2015; Fang *et al.,* 2014).

Further study elucidated the role of p300 as a transcriptional coactivator in embryonic stem cells. p300/CBP is recruited in Nanog binding loci and mediates long range chromatin looping to associate distal enhancer regions to Oct4-Sox2-Nanog cluster and activates the transcription of pluri-genes by enriching TF (including Nanog recruitment) concentration locally. This mechanism of maintaining self-renewal in undifferentiated

cells are shared by p300 and its homologue CBP. p300/CBP KIX and KAT domains were found to be indispensable for maintaining pluripotent identity of mouse ES cells (Fang *et al.,* 2014). Though p300 and CBP are homologous proteins that exhibit high sequence identity in structural domains including bromodomains and acetyltransferase domains and participate in many redundant cellular pathways, in differentiating mouse ES cells p300 and CBP were found to function non-redundantly. p300 deficient mEFs were compromised on retinoic acid receptor activities that interfere with their capabilities in directed differentiation (Yao *et al.,* 1998). Both p300 and CBP deficiency results in manifestation of ectodermal specific and hematopoietic defects but, CBP deficiency did not manifest in cardiac abnormalities which is associated with p300 deficiency (Tanaka *et al.,* 2000). It was also observed that H3K27ac mediated by p300 but not CBP correlated strongly with promoter acetylation and transcription initiation (Martire *et al.,* 2020).

Aforementioned studies elucidated the role of p300 in embryonic development focusing on their transcriptional coactivation abilities. H3K18 and 27 acetylation by p300 and CBP have been found to be relevant for development of vertebrate embryos, the recruitment of the proteins and deposition of the associated acetylation marks does not correlate with a requirement of the gene expression (discussed in Bedford and Brindle, 2012). Conventionally, p300 and CBP mediated acetylation is positively correlated with its transcriptional coactivation function, yet, on several occasions, both the functions have been demonstrated to be dissociative in nature. Transcriptional coactivation by p300/CBP can be supplemented by non-KAT CREB coactivator CRTC (previously known as TORC) (Kasper *et al.,* 2010). The structured domains of p300/CBP are connected through unstructured flexible regions and can function independently; such as KIX domain of CBP is sufficient for its binding to CREB and Myb, and is essential for cognitive function, bZIP domain aids in transcriptional coactivator function which is independent of KAT function *etc* (Montminy *et al.,* 1996; Vecsey *et al.,* 2007; Oliveira *et al.,* 2006). Hence, p300mediated acetylation might regulate vertebrate embryogenesis in a manner that is nonredundant with its transcriptional coactivation function. While loss of acetylation activity of CBP in mice and human patient samples has been linked to cognitive dysfunction attributed to mature circuit function and manifestation of Rubinstein-Taybi syndrome (Wang *et al.,* 2010; Petrij *et al.,* 1995; Murata *et al.,* 2001;

Josselyn *et al.,* 2005; Roelfsema and Peters, 2007), the implication of p300 mediated acetylation in embryonic development remain poorly understood.

3.2.1. Luteolin inhibits p300 mediated acetylation in mouse embryonic stem cells

During early embryo development, ES cells aggregate into a spherical cluster, in which the cells then gradually migrate and differentiate into different germ layers. This feature could be recapitulated (partially) *in vitro* by assembling ES cells into embryoid bodies (EBs) prior to the actual fate determination. In this study the implication of p300 mediated acetylation is investigated in blastocyst derived mouse ES cells (E14Tg2a). These ES cells retain pluripotency and self-renewing capacity when cultured on high adherence substratum and in presence of Leukemia Inhibitory Factor (LIF). In absence of LIF when cultured in low adherence dishes these ES cells form tissue-like spheroid EBs and gradually differentiate into all three germ-layers: ecto-, endo-, and, mesodermal layers (Xu *et al.,* 2001; Pelton *et al.,* 1998; Brickman and Serup, 2017). To inhibit p300 a non-toxic (to non-cancerous cells), cell-permeable, selective small molecule inhibitor, Luteolin (Selvi *et al.,* 2015) was chosen. Luteolin, 3′,4′,5,7-tetrahydroxyflavone, is a dietary flavonoid known for its anti-oxidative, non-mutagenic, anti-tumorigenic and anti-inflammatory properties (Attoub *et al.,* 2011; Manju *et al.,* 2005; Taliou *et al.,* 2013; Zhao *et al.,* 2011; Rao *et al.,* 2012; Lin *et al.,* 2008; Lin *et al.,* 2017; Tsai *et al.,* 2016). Though Luteolin inhibits p300 potently *in vitro* (IC_{50} 7µM) and *in vivo* (inhibitory concentrations 5-25µM), mostly at higher concentrations (>20µM) it exhibits pleotropic effects on cells which seem to be common properties of almost all flavonoids (reviewed in Tuorkey, 2016). Accordingly, to exclude the possibilities of observing flavonoid-like effects of Luteolin, a suboptimal concentration of 15µM was used to treat the embryonic stem cells; additionally, a structurally similar flavonoid, Apigenin, 4′, 5, 7-trihydroxyflavone, was used as a negative control in our study. Structural homolog of Luteolin, Apigenin is known to exert similar beneficial flavonoid-like pleotropic effects as Luteolin when used at a similar dilution (Seo *et al.,* 2012; Mafuvadze *et al.,* 2012; Anter *et al.,* 2011; Funakoshi-Tago *et al.,* 2011; Lindenmeyer *et al.,* 2001; Qin *et al.,* 2016; Zhao *et al.,* 2017; Che *et al.,* 2020; Hong *et al.,* 2018; Wu *et al.,* 2015; Huang et al, 2013; Ma *et al.,* 2008) but unlike Luteolin, Apigenin cannot inhibit p300. Docking studies reveal that both Luteolin and Apigenin bind to catalytic cleft of p300 and establish

interaction with multiple residues, but, only Luteolin binding is stabilized leading to inhibition of p300 mediated acetylation *in vitro* and *in vivo* (Selvi *et al.*, 2015). It was observed Luteolin treatment affects H3K9, H3K14 and H4K8ac level strongly *in vivo* but did not affect H3K9me2 and H3K4me3 level indicating inhibitory effect of Luteolin is specific towards acetyltransferases. Moreover, apart from p300/CBP H3K9residue in vivo is predominantly acetylated by GNAT family acetyltransferases in vivo and the fact that Luteolin did not affect PCAF *in vitro* it further indicated the inhibitory properties of Luteolin to be specific towards p300/CBP family (Selvi *et al.*, 2015)

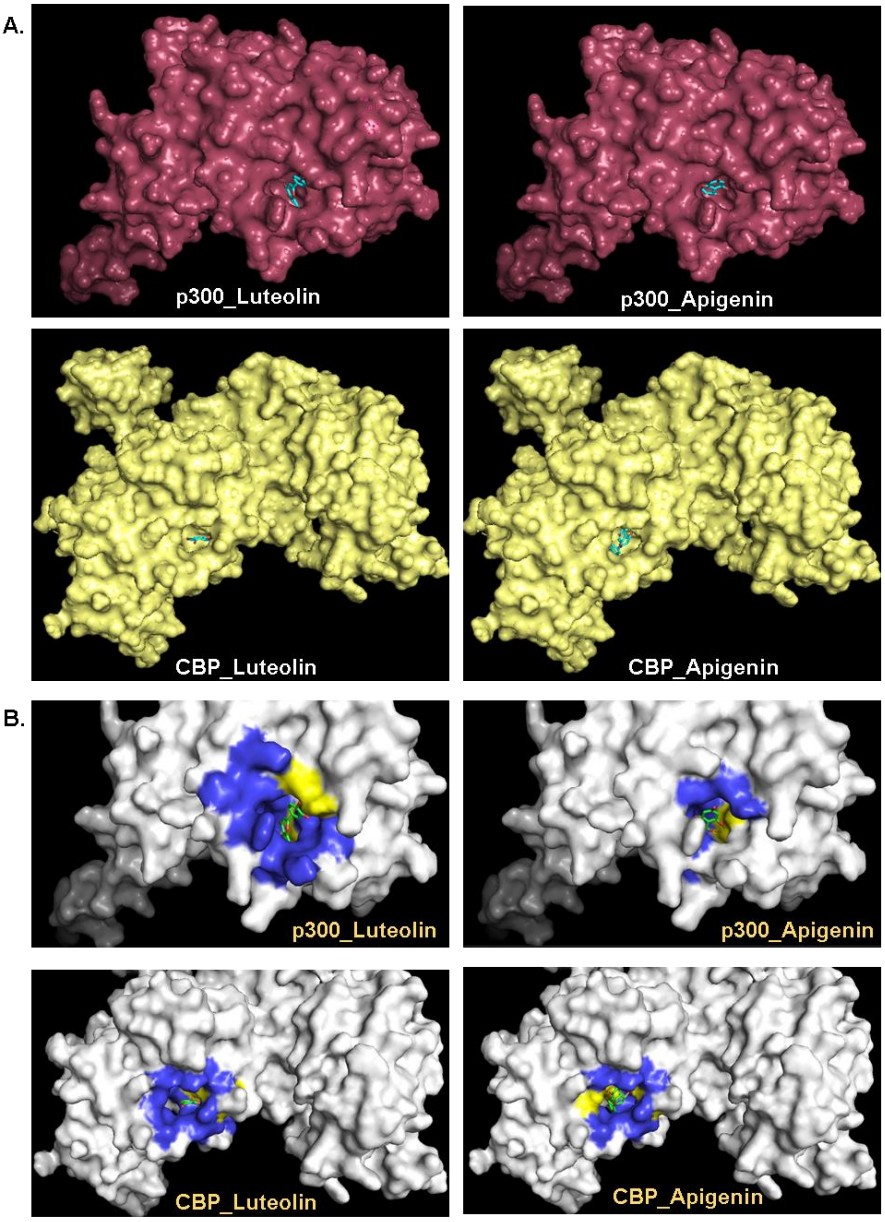


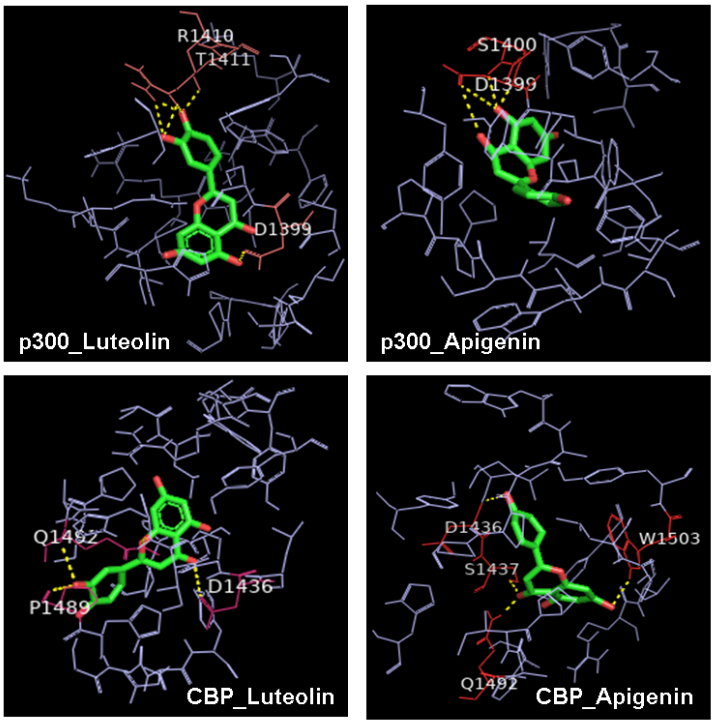
Figure 3.1. Docking of Luteolin and Apigenin to human p300 and CBP catalytic domains. *(A) Surface views of Luteolin and Apigenin (in green color, cartoon format)*

binding to catalytic cleft of p300 KAT domain (PDB ID: 4PZR represented in magenta color) and CBP KAT domain (PDB ID: 57UG, represented in yellow color) (B) Interacting surface of the two catalytic domains with Luteolin and Apigenin is represented in color; blue region corresponds to nonbonded surface and yellow depicts hydrogen bonded polar interacting surface. H-bonding is represented in red.

Based on the similarity between catalytic mechanism and high structural homology between p300 and CBP acetyltransferase domain (discussed in a previous chapter) it is highly likely that Luteolin also have the ability to bind to and inhibit CBP. To calculate binding energy and understand residual level interaction molecular docking was performed between the small molecules and the KAT domains. Due to unavailability of mouse p300 and CBP crystal structure, parallels were drawn from docking of these small molecules with KAT domains of human p300 and CBP. It was observed that both Luteolin and Apigenin bind to respective catalytic pockets of p300 and CBP (Figure 3.1.A). Except for the orientation of the molecules and surface exposure of respective binding sites there is not much apparent difference among the binding patterns; Luteolin when bound to p300 had the highest surface exposure, bonded and nonbonded interacting residues of p300 to Luteolin were mostly present on its surface. In rest of the cases, the binding sites of the small molecules were more or less deep inside the catalytic clefts giving rise to the possibility that due to lesser accessibility of respective binding sites, Luteolin in case of CBP and Apigenin in case of p300 and CBP, might not bind and inhibit catalytic activities (Figure 3.1. B).

Residue level interaction showed that Luteolin can establish 3 hydrogen bonds (H-bonds) with p300 (D1399, R1410, T1411) and CBP (D 1436, P1489, Q1492) each, and Apigenin forms 2 H-bonds with p300 (S1400, D1399) and 4 H-bonds with CBP (D1436, S1437, Q1492, W1503) (Figure 3.2.A). In all of the cases both the molecules also interact with multiple residues without forming polar bonds (listed in Figure. 3.2.B) and many residues were found to be common interacting residues between Luteolin and Apigenin. Among these residues, D1399 in p300 and D1436 in CBP are conserved and both interact with Luteolin and Apigenin through H-bond formation. Luteolin bound R1410, T1411 in p300 are identical with R1447, T1448 in CBP which interact with both Luteolin and Apigenin without forming polar bonds.

A.



B.

Feature	p300		CBP	
Interacting residues	Common	Unique	Common	Unique
Luteolin	L1398, Y1414, C1438, P1439, P1440, Y1446, L1463, W1466, Y1467	S1400, H1402, I1449, Q1455, I1457, P1458, R1462, D1468, K1469	H1439, R1447, T1447, Y1450, C1475, P1476, H1488, K1493, I1494, P1495, R1499, L1500, Y1504	S1437, I1438, G1480, D1481, D1482, W1503
Apigenin		I1395, S1396, Y1397, R1410, T1411, I1435, W1436, A1437, P1458, D1507		L 1435, W1473, A1474, P1477

C.

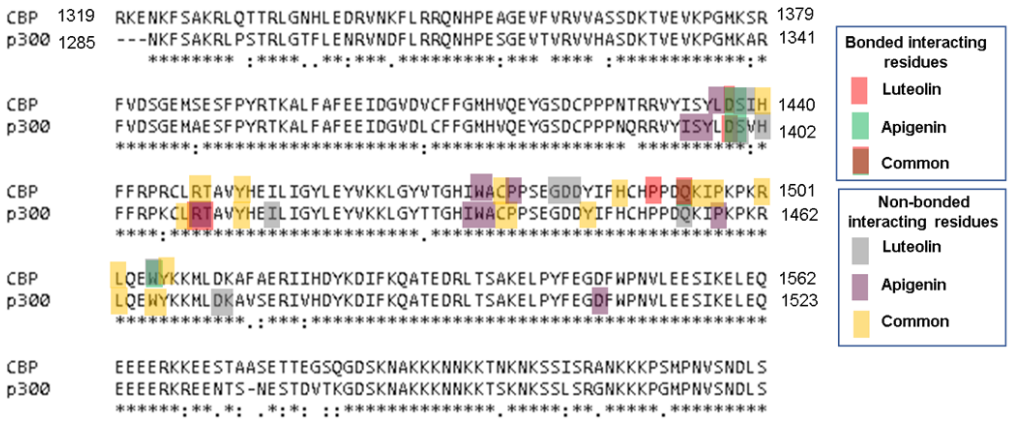


Figure 3.2. Residue level details of interaction of KAT domains with Luteolin and Apigenin. *(A) Interacting residues of p300 (in upper panel) and CBP (in lower panel) with Luteolin and Apigenin (as mentioned, represented in green cartoon). Hydrogen bonds are represented in yellow, polar bonded residues are represented in red, nonbonded interacting residues are represented in blue. (B) All nonbonded interacting*

residues are listed in tabular format. (C) Relative position and conservation of all (bonded and nonbonded) interacting residues between p300 and CBP.

Likewise, there are multiple conserved res residues in p300 and CBP that interact with both Luteolin and Apigenin (represented in Figure 3.2.C) and many of these residues are already implicated in catalytic activities of both the enzymes. In essence, no significant difference in terms of selection of interacting residues was observed between Luteolin and Apigenin that could confer advantage concerning inhibitory properties of either Luteolin or Apigenin to p300 and CBP.

p300_Luteolin

mode	affinity (kcal/mol)	dist from best mode rmsd l.b.	rmsd u.b.
1	-9.6	0.000	0.000
2	-8.8	2.566	6.660
3	-8.5	1.554	6.406
4	-8.3	2.061	2.889
5	-8.2	13.780	16.276
6	-8.1	2.398	7.057
7	-8.1	2.619	7.097
8	-8.0	4.053	8.704
9	-7.8	16.172	19.165

p300_Apigenin

mode	affinity (kcal/mol)	dist from best mode rmsd l.b.	rmsd u.b.
1	-9.3	0.000	0.000
2	-9.2	2.252	3.230
3	-9.0	2.291	6.792
4	-8.5	3.172	5.273
5	-8.4	3.389	5.862
6	-8.0	4.250	8.433
7	-7.9	4.602	7.358
8	-7.9	3.695	6.475
9	-7.9	11.562	14.373

CBP_Luteolin

mode	affinity (kcal/mol)	dist from best mode rmsd l.b.	rmsd u.b.
1	-9.3	0.000	0.000
2	-9.2	2.252	3.230
3	-9.0	2.291	6.792
4	-8.5	3.172	5.273
5	-8.4	3.389	5.862
6	-8.0	4.250	8.433
7	-7.9	4.602	7.358
8	-7.9	3.695	6.475
9	-7.9	11.562	14.373

CBP_Apigenin

mode	affinity (kcal/mol)	dist from best mode rmsd l.b.	rmsd u.b.
1	-9.0	0.000	0.000
2	-8.9	5.095	6.987
3	-8.9	4.291	6.601
4	-8.8	1.718	3.124
5	-8.6	4.531	7.482
6	-8.6	26.456	28.449
7	-8.6	4.927	7.621
8	-8.4	3.183	7.435
9	-8.3	3.891	7.567

Figure 3.3. Affinity of Luteolin and Apigenin for p300 and CBP KAT domains. *Binding energies (in terms of affinity kcal/mol) of Luteolin and Apigenin are listed for their docking with CBP and p300 KAT domain with corresponding rmsd values.*

Next, the binding energies of these molecules to p300 and CBP KAT domains were calculated through docking studies. Both p300 and CBP bind to acetyl-CoA *in vitro* with an unusually high affinity (Km~0.3µm) (Balasubramanyam *et al.*, 2003), the binding energy of acetyl-CoA to p300 KAT domain was found to be approximately -11.6kcal/mol (rmsd u.b.=rmsd l.b.= 0). Since Luteolin and Apigenin binding sites in p300 and CBP partially overlap with respective acetyl-CoA binding sites, it is crucial for them to exhibit similar or higher binding energies in order to displace acetyl-CoA to bind and

inhibit p300 and CBP. The affinity of Luteolin for p300 was found to be the highest (Figure 3.3).

The affinity of Apigenin for p300 was similar to that of Luteolin for CBP and the affinity of Apigenin for CBP was found to be even lower (summarized in Figure 3.3). Since it is already established that Luteolin inhibits p300 and Apigenin does not, together with this set of observation on affinity values of these small molecules to CBP and p300 suggests that neither Luteolin nor Apigenin should inhibit CBP. Collectively from docking studies based on surface accessibility of cognate binding sites and affinity values of these small molecules to KAT domains it can be conjectured that Luteolin inhibits p300 but not its homolog CBP. However, further experiments are required to establish certainty in this regard.

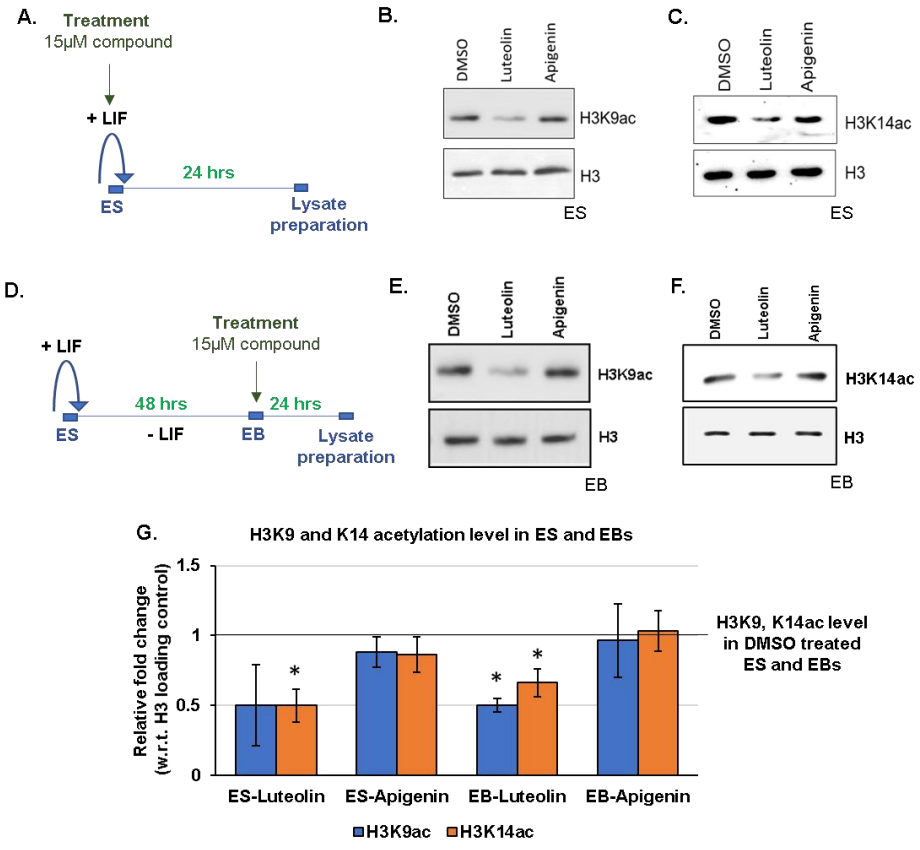


Figure 3.4. Luteolin affects acetylation level in mouse ES cells and EBs formed thereafter. *(A) and (D). Schematic representations for timelines for compound treatment (Luteolin or Apigenin or DMSO solvent control) in ES cells and EBs respectively. Lysates of compound treated ES cells (B and C) and EBs (E and F) were probed with site-specific histone acetylation antibodies (as indicated) by immunoblotting. (G) Quantitation of*

relative intensities of H3K9 and 14ac (with respect to individual H3 loading control of DMSO treated samples) obtained from immunoblots in B, C, E and F. N=3 for ES and N=2 for EBs. Statistical analysis: Students' t test P<0.05, P**<0.01, P***<0.005.*

It is noteworthy that though p300 and CBP share similar substrate specificity for core histones, careful analysis has indicated that the order of preference for these sites are quite different for these two enzymes. p300 has ~10^{10} times higher specificity than CBP for K14 site even with ~3 fold higher k_{cat} value of CBP; in limited acetyl-CoA condition the difference can be upto ~10^{15} fold. The highest specificity for H3K14 site is shown by GCN5irrespective of acetyl-CoA level but GCN5 is not a known target of Luteolin or Apigenin (Henry *et al.*, 2013; Kuo and Andrews, 2013).

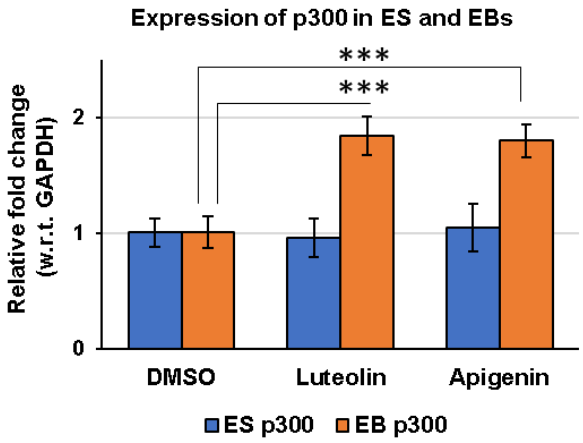


Figure 3.5. Expression of p300 in Luteolin and Apigenin treated ES and EBs. *qPCR analysis of p300 mRNA expression in ES and EBs upon 24 hours of compound treatment; N=3, Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005.*

In presence of LIF, mES cells when treated with 15µM Luteolin for 24 hours (schematic in Figure 3.4. A) acetylation of H3K9 and K14 residues were decreased significantly; and acetylation of H3 was not affected in presence of similar concentration of Apigenin (Figure 3.4. B and C). No significant change in morphology of ES cells was observed upon Luteolin treatment (data not shown). Differentiating EBs were formed from ES cells maintained in absence of LIF in low adherence dish for 2 days; EBs were treated with 15µM Luteolin or Apigenin for 24 hours (schematic in Figure 3.4. D). Similar pattern of inhibition of acetylated H3K9 and K14 was observed in EBs when treated with Luteolin, and no change in acetylation level was observed in Apigenin treated EBs (Figure 3.4.E, F). The reduction in acetylation on both H3K9 and K14 residues in

Luteolin treated ES and EBs were found to be similar (Figure 3.4.E) which indicated that Luteolin permeated ES and EBs equally.

Reduction of acetylation in Luteolin treated ES and EBs might also be an effect of reduced expression of p300 itself. To understand if Luteolin treatment led to reduction on expression of p300 or not expression of p300 mRNA was quantified and compared with DMSO treated ES or EBs. In case of ES cells p300 expression was not changed upon Luteolin or Apigenin treatment; interestingly, in EBs the expression of p300 was almost doubled upon Luteolin and Apigenin treatment (Figure 3.5). However, if p300 is stabilized at protein level in EBs upon Luteolin and Apigenin treatment is yet to be examined. A discrepancy between level of expression of p300 mRNA and protein during EB formation has already been demonstrated. While p300 mRNA accumulation progressively increases till day 4 of EB formation, p300 protein level drastically decreases after day 2 (Zhong and Jin, 2008). Similarly, another study reported increased proteasomal degradation of p300 during differentiation of F9 embryonic carcinoma cell-line; yet, KAT activity of p300 remained remarkably constant throughout differentiation process and might be an effect of increased PKA-mediated phosphorylation of p300 (Brouillard and Cremisi, 2003). In the current study, since H3K14acetylation in Luteolin treated EB was not normalized to that of DMSO treated EBs, and, level of acetylation in Apigenin treated EBs were found to be equivalent to that of DMSO treated EBs it might be presumed that irrespective of mRNA level, p300 might not be stabilized at protein level in Luteolin and Apigenin treated EBs. Interestingly, dietary flavonoids including Luteolin and Apigenin are known to be potent inhibitors of 26S proteasomal pathway in various cancer cells, these inhibitory effect of Luteolin and Apigenin were not observed in nontransformed cells (Chen *et al.,* 2007). If in EBs luteolin and Apigenin aggravates proteasomal degradation of p300 remains to be investigated. On other hand, there might not be any apparent correlation between p300 expression and its acetyltransferase activity that results in comparable level of acetylation in DMSO and Apigenin treated EBs.

3.2.2. Luteolin affects embryoid body formation

2 days old EBs when cultured in differentiation permissive media supplemented with 15µM Luteolin the size (calculated by total surface area observed) of the EBs

progressively decreased over time and EBs were completely disintegrated on day 6 (Figure 3.6. B and C). The morphology of the disintegrated embryoid bodies did not appear similar to that of the ES cells. Whereas, similar affects were not observed in EBs cultured in the presence of Apigenin or DMSO control, the EBs grew in volume progressively as observed till day 12 (data shown till day 6 in figure 3.6.B and C). EBs are known to disintegrate during cavitation process when cell death in the core forms cystic fluid filled cavity (Karbanova and Mokry, 2002); disintegration of once formed EBs may also stem from low growth repression accompanied by progressive differentiation or simply due to high cell death (Sakai *et al.*, 2010). The morphology of Luteolin treated EBs appeared different from that of cystic EBs, the EBs after 2 days of Luteolin treatment had slightly diffused border, nevertheless, the EBs retained their original shapes (as compared to in D0 of Luteolin treatment) and no disintegration was observed from the centre of the EBs; Likewise, cell toxicity might not be the reason behind EB disintegration, as it was observed that in presence of Luteolin, disintegration of EBs occurred gradually over a period of 4 days.

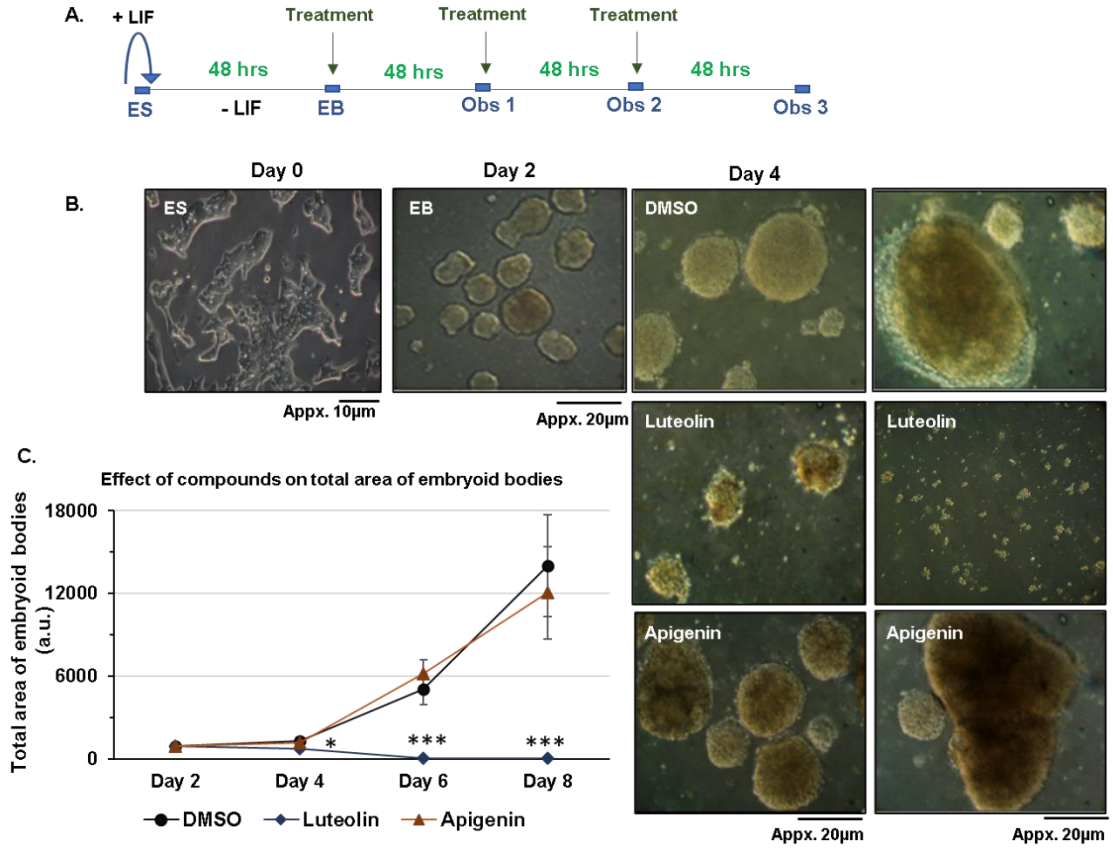


Figure 3.6. Luteolin affects the size of the EBs. *(A) Schematic representation for timelines for compound treatment in EBs. (B) Morphology of ES cells (untreated) and*

EBs upon compound treatment on different days as observed under microscope. (Scale bar: apprx. 10µm for ES cells, 20µm for EBs) (C) Quantitation of relative areas of the EBs upon compound treatment on different days.

3.2.3. Luteolin does not affect cellular viability

Both Luteolin and Apigenin are known to suppress proliferation and induce apoptosis in cancer cells (Attoub *et al.*, 2011; Selvi *et al.*, 2015; Lin *et al.*, 2008; Rao 2012; Zhao 2011; Mafuvadze *et al.*, 2012; Seo *et al.*, 2012; Zhao e al., 2017; Chin and Lin; 2008; Ko *et al.*, 2002; Ding *et al.*, 2014; Han *et al.*, 2016; Wang *et al.*, 2010). However, in case of noncancerous (epithelial cells and stem cells) Luteolin is reported to exert beneficial effects and often promotes proliferation (Lin *et al.*, 2016; Chen *et al.*, 2020; Lin *et al.*, 2015; Wan *et al.*, 2019; Taupin, 2009).

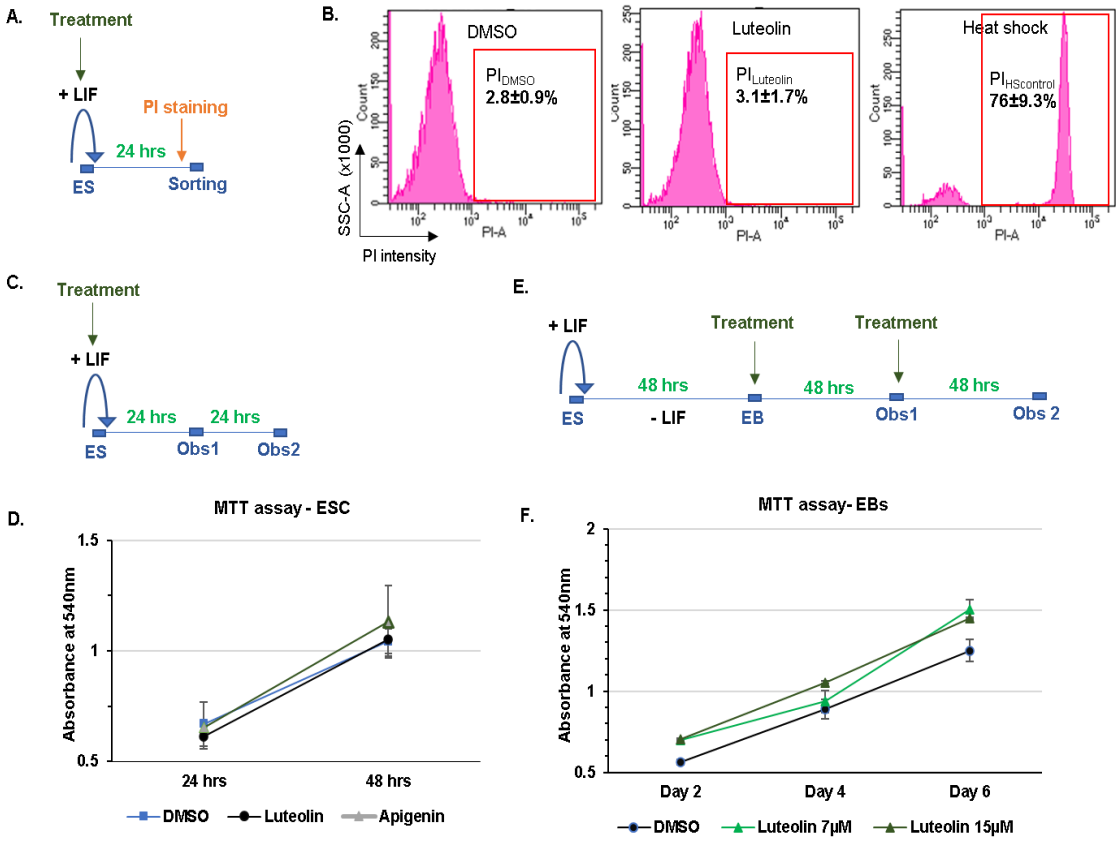


Figure 3.7. Luteolin treatment does not affect cell viability. *(A), (C) and (E). Schematic representations of experimental designs and timelines for compound treatment in ES cells and EBs. (B). Sorting of ES cells based on propidium iodide (PI) staining. PI-stained population is indicated in red box and PI-stained percentage population is*

indicated (N=3). (D). MTT assay plot of compound treated ES cells as observed for 48 hours (N=3; One-way ANOVA, P=0.921). (F). MTT assay plot of compound treated EBs as observed till day 6 (N=3; One-way ANOVA, P=0.35).

To understand if Luteolin treatment led to cell toxicity resulting in smaller embryoid body formation cellular viability assays were performed in differentiating and undifferentiated ES cells. As mentioned earlier, p300 was found to be dispensable for cellular viability of ES or EBs (Zhong and Jin, 2009). Hence, any lethality observed in current study can be directly linked to the small molecules.

ES cells supplemented with LIF when cultured in presence of Luteolin proliferate normally; Luteolin treated ES cells had growth rate (measured over a period of 48 hours) similar to that of ES cells maintained in Apigenin or DMSO control (Figure 3.7. D). To confirm that Luteolin did not induce cell death or compromised membrane integrity ESs treated Luteolin were incubated with propidium iodide (PI), a DNA-binding dye permeable to membrane-damaged or dying cells); no significant difference in PI uptake was observed in case of Luteolin treated ES cells as compared to ES cells treated with DMSO (Figure 3.7. B). In differentiation permissive media in presence of Luteolin EBs could proliferate and actively metabolize MTT till Day 6. The rate of proliferation of EBs in Luteolin and in DMSO were found to be similar (Figure 3.7.F). Due to large size, EBs could not be sorted based on propidium iodide staining. Together these set of observations indicated that Luteolin negatively affect embryoid body formation without exerting toxicity.

3.2.4. Luteolin affects Oct4 level in EBs

The varying sizes of embryoid bodies upon inhibition of p300 can also be associated with improper differentiation (Yao *et al.*, 1998; Zhong and Jin, 2009). In stem cells, Oct4, Sox2, Nanog regulates pluripotency and Oct4 acts as a master regulator and gatekeeper for triggering early differentiation. Oct4 in turn is regulated by factor (e.g., PAF1 complex, Sox2 etc.) binding and epigenetic modification on its gene regulatory proximal and distal sequence elements. Acetylation of H3K9 and 14 in Oct4 promoter in undifferentiated ESs is correlated to its expression (reviewed in Shi and Jin, 2010). An earlier study has shown that in dental pulp (stem) cells Luteolin treatment (1-10µM)

significantly increased Oct4 protein level but not Sox2, whereas, similar dose of Apigenin treatment did not stabilize Oct4 (Liu *et al.*, 2015).

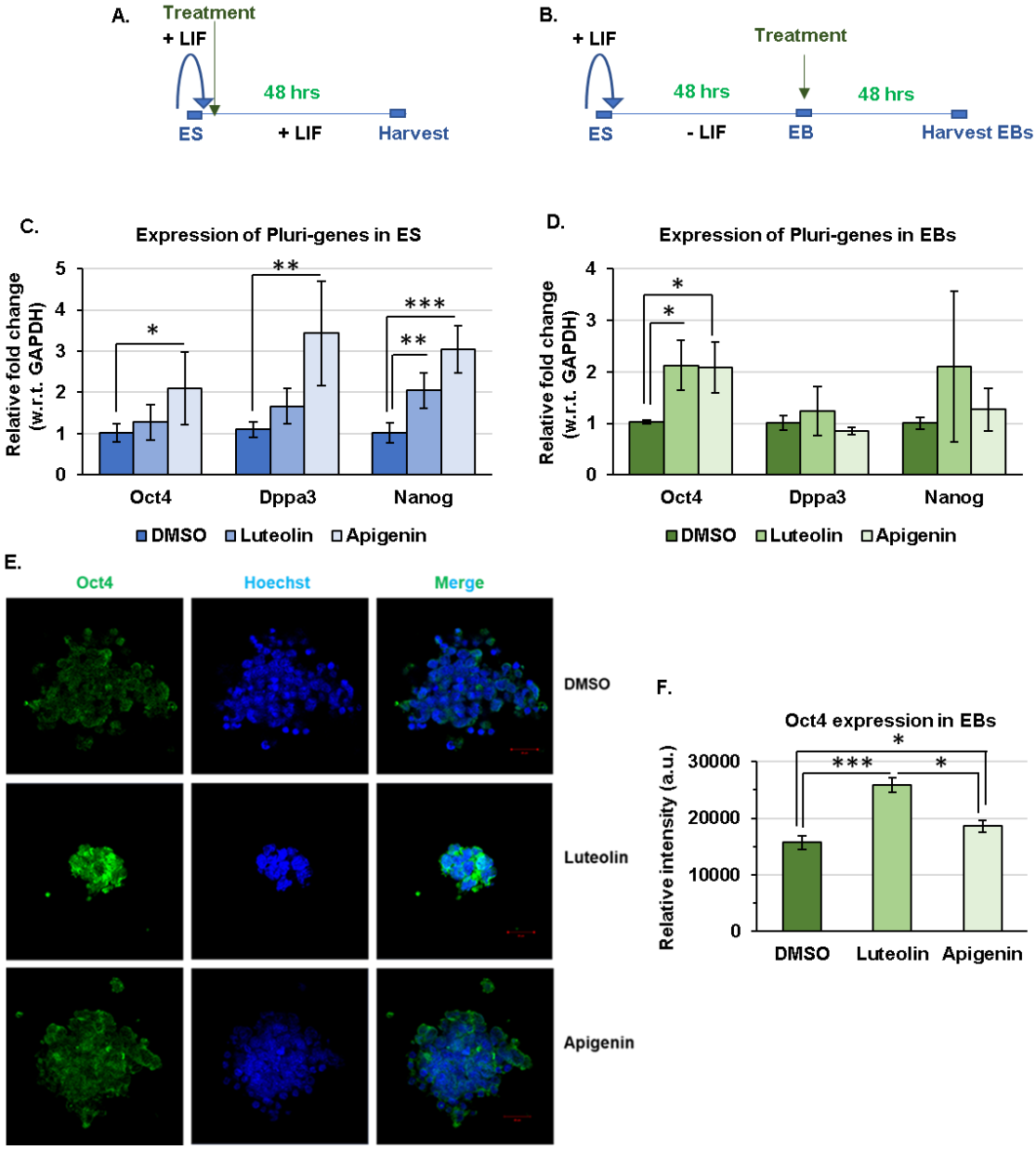


Figure 3.8. Luteolin treatment increases Oct4 protein level in EBs. *(A) and (B) Schematic representations of experimental design (C) and (D) qPCR analysis of Oct4, Dppa3 and Nanog mRNA expression in ES (in C) and EBs (in D) upon compound treatment; N=3, Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005; (E) Immunofluorescence images of compound treated EBs stained with Oct4 antibody. Hoechst stains nucleus. Scale bar: 10µm. (F) Quantitation of relative intensity of Oct4 staining obtained from immunofluorescence study of compound treated EBs. Average of*

total intensity per unit area across the EBs plotted along Y axis. Statistical analysis: Students' t test P<0.05, P**<0.01, P***<0.005.*

In the current study, both Luteolin and Apigenin treatment increased Oct4 mRNA level significantly in differentiating embryoid bodies (Figure 3.8.D). In protein level, however, Apigenin treatment failed to reflect the dramatic increase observed in Oct4 mRNA level as opposed to Luteolin treatment which retained approximately 2-fold higher Oct4 protein as compared to DMSO -treated EBs (Figure 3.8.E and F). In undifferentiating ES cells only Apigenin treatment increased Oct4 mRNA level significantly indicating that the effect of stabilization of Oct4 in protein level (and probably mRNA level as well) is linked to the status of p300-mediated acetylation in EBs. Also, synonymous retention of expression of other pluripotency related genes such as Dppa3, Nanog in ES cells indicate neither Luteolin nor Apigenin treatment affected transactivation property of p300 (Figure 3.8.C).

Oct4 protein stability is regulated by posttranslational modifications such as SUMOylation, ubiquitination, phosphorylation and O-linked β-N-acetylglucosamination (reviewed in Shi and Jin, 2010). Interestingly, Oct4 has also been found to be a substrate of p300, and, p300-mediated acetylation of Oct4 led to its dissociation from Oct-Sox complex (Dai *et al.,* 2014). If p300-mediated acetylation also contributes to degradation of Oct4 remains to be investigated.

3.2.5. Luteolin treatment affects differentiation of EBs

Luteolin treatment affected the EB sizes and upregulated Oct4 protein level significantly (Figure 3.6 and 3.8); both of which reportedly affect the differentiation process. The size of the EBs can influence cell-cell interactions, ECM deposition, diffusion of growth factors. For example, larger EBs (<450µm diameter) showed enhanced cardiogenesis, whereas smaller EBs (<150µm diameter) favored endothelial specific differentiation (Bauwens *et al.,* 2011; Choi *et al.,* 2010; Karp *et al.,* 2007; Khademhosseini *et al.,* 2005; Peerani *et al.,* 2007; Cameron *et al.,* 2006; Zhang *et al.,* 2012; Zandstra and Nagy, 2001). Oct4 is highly expressed in undifferentiated self-renewing mouse ES cells, in differentiation-permissive media (e.g., upon LIF withdrawal or in presence of retinoic acid) Oct4 levels are downregulated with different kinetic (Cauffman *et al.,* 2005; Trouillas *et al.,* 2009a; Trouillas *et al.,* 2009b). Apart from its role in maintaining

pluripotency Oct4 is also known to function as a 'rheostat' during differentiation; low or no Oct4 expression favors trophoblast formation and high Oct4 level promotes primitive endodermal and mesodermal specific differentiation (Niwa *et al.*, 2000; Zeineddine *et al.*, 2006; Stefanovic and Puceat, 2007). To understand the course of differentiation in Luteolin treated EBs expression of three different germ line markers were analyzed. Luteolin treatment 48 hours led to decrease of ectodermal marker Sox1 expression, the expression of mesodermal marker Brachyury (T) and endodermal marker Sox17 were not significantly different from those expressed in Apigenin and DMSO treated EBs. (Figure 3.9.B). Interestingly, a previous report suggested involvement of p300/CBP mediated acetylation in regulation of expression of various regulators of mesodermal differentiation; C646 treatment inhibited Brachyury expression in ES cells (Wu *et al.*, 2018). In current study though Luteolin treatment led to decrease in T expression, the reduction was not found to be significant (Figure 3.9.B). This might be a result of compensation by CBP-mediated acetylation in Luteolin treated EBs.

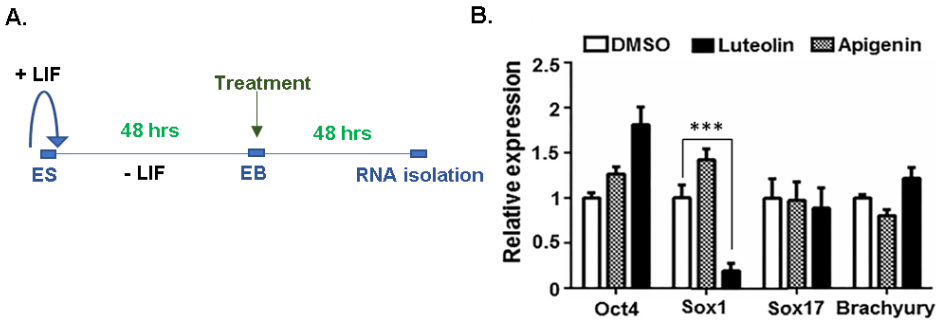


Figure 3.9. Effect of Luteolin in differentiation of embryoid bodies. *(A) Schematic representation of experimental outline. (B) qPCR analysis of expression of different germline markers as indicated upon compound treatment; N=3, Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005 (Swaminathan et al., 2019).*

3.2.6. Luteolin negatively affects differentiation towards neuronal lineages

To exclude the possibility of size related bias towards meso- and endodermal lineage specific differentiation of Luteolin treated EBs, the effect of Luteolin on retinoic acid induced neural differentiation was studied. Transactivation by p300 but not CBP is required for RA-induced differentiation of F9 cells; protein level reduction of p300 was found to impair transcription from RAREs above basal level (Kawasaki *et al.*, 1998).

However, if p300 mediated acetylation affects this differentiation process is not known. Luteolin treatment significantly affected the expression of ectodermal marker Sox1in differentiating EBs and maintained its downregulation until terminal differentiation (Figure 3.10.C). Post differentiation number of cells expressing neuronal marker β-III tubulin were found to be significantly lesser compared to DMSO treated population, the level of expression of β-III tubulin per cell was also found to be reduced in presence of Luteolin (Figure 3.10.D). Interestingly Pax6 and GFAP, early glial differentiation markers were not affected upon Luteolin or Apigenin treatment (Figure 3.10.E and F) highlighting the possibility of involvement of p300-mediated acetylation in regulating ectodermal and neuronal lineage specific differentiation and development.

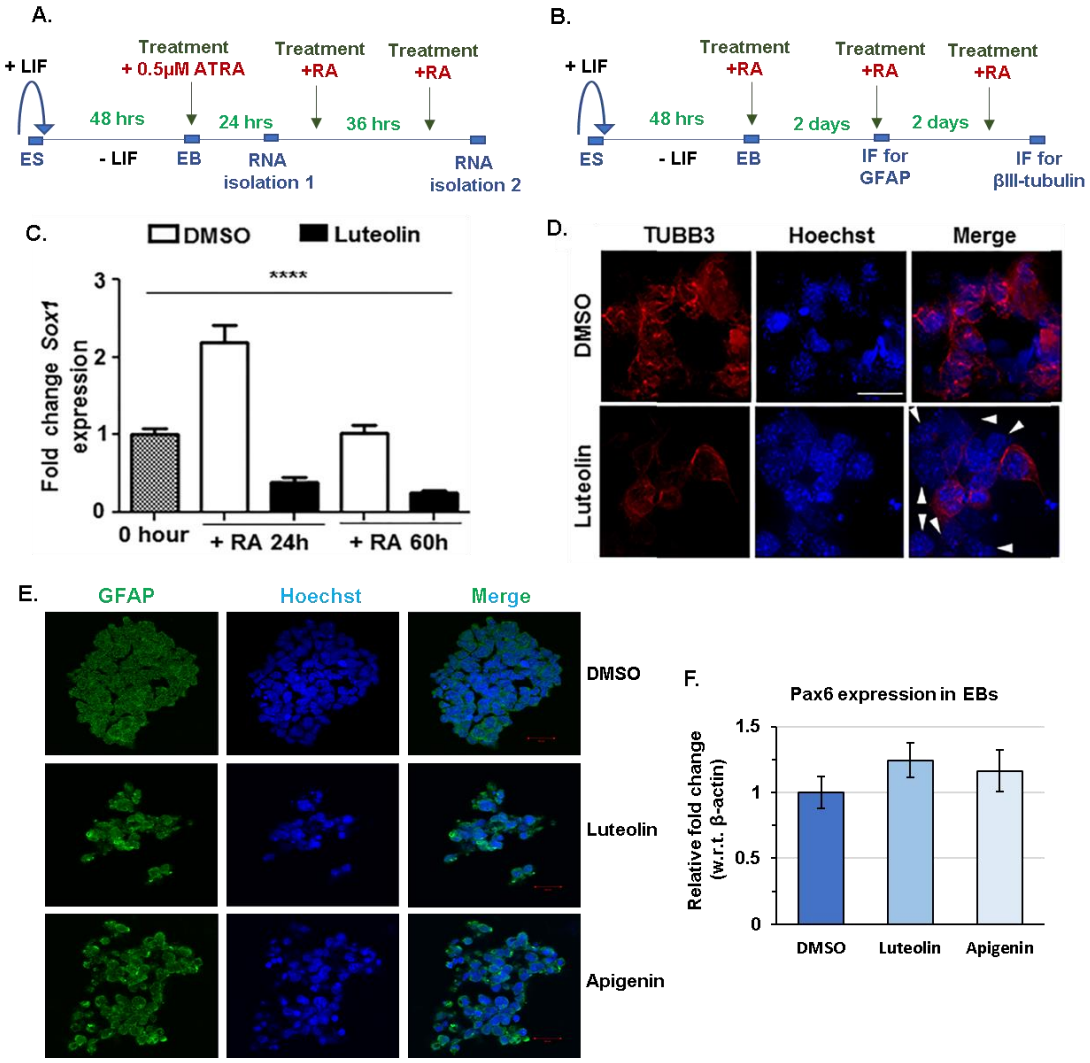


Figure 3.10. Luteolin treatment inhibits neuronal differentiation from EBs. *(A) and (B) Schematic representations for experimental outline of C, F and D, E respectively. (C) qPCR analysis of Sox1 mRNA expression at different timepoints in compound treated*

EBs. N=3, Statistical analysis, ANOVA, P<0.0001(D) and (E) Immunofluorescence images of RA induced differentiation of compound treated EBs stained with β-III tubulin (in D; Swaminathan et al., 2019) and GFAP (in E) antibodies Scale bar: 20µm. Hoechst stains nucleus. Scale bar: 10µm. (F) qPCR analysis of Pax6 mRNA expression after 60 hours of ATRA and compound treatment in EBs. N=3, Statistical analysis, ANOVA P=0.2.

Interestingly, CBP null embryos have impaired neuronal differentiation, PKCγ-mediated phosphorylation of CBP is required for deposition of acetylation marks on the promoters of neuronal and glial genes during differentiation and for maintaining competency of neuronal precursor. Depletion of CBP also affect glial differentiation and generation of astrocytes and oligodendrocytes; besides neuronal marker βIII tubulin, astroglial precursor GFAP expression was also found to be significantly downregulated upon CBP depletion and expression of these genes were rescued by treatment with HDAC inhibitors. CBP binds to promoters of glial precursor gene promoters and deposit acetylation marks to induce transcription (Wang *et al.*, 2010). Hence, in the current study, inhibition of βIII tubulin but not GFAP further highlighted the probability of p300-specific inhibitory action of Luteolin.

3.3.1. Evolution of p300/CBP catalytic activity

Ever since p300/CBP has been characterized as lysine acetyltransferases, numerous studies have been carried out to understand the functional relevance of its enzymatic activity; question remains if the acetyltransferase activity of p300/CBP is the single most important function of these proteins beyond transactivation. p300/CBP sequence is highly conserved in higher eukaryotes. No ortholog of p300/CBP has been found in prokaryotes. Yeast structural homolog of p300/CBP, Rtt109, is an acetyltransferase but bears little sequence or functional similarity with human p300/CBP (Tang *et al.*, 2008). Many different metazoan eukaryotic species such as flies, roundworms, sea-squirt has been found to possess p300/CBP orthologs with poor sequence similarity. In plants Arabidopsis five homologs of p300/CBP has been discovered out of which four has been characterized to possess acetyltransferase activity (Bordoli *et al.*, 2001). During vertebrate evolution the chromosomal region corresponding to p300/CBP along with eight neighbor genes have undergone a duplication resulting in separate p300 and CBP

genes (Giles *et al.,* 1998). Some vertebrates even contain multiple p300 and CBP orthologs. While higher vertebrates like chickens, opossums, mice, human all have one p300 and one CBP genes, lower vertebrates like zebrafish, *Xenopus* have multiple copies of p300 and CBP genes. p300 and CBP has diverged after duplication event and retain 61% sequence identity overall. Interestingly, some domains retain more similarity than others and the highest ratio of sequence conservation is observed in the acetyltransferase domain which retains 86% sequence identity. The divergence of p300 is analyzed in Figure 3.11. A.

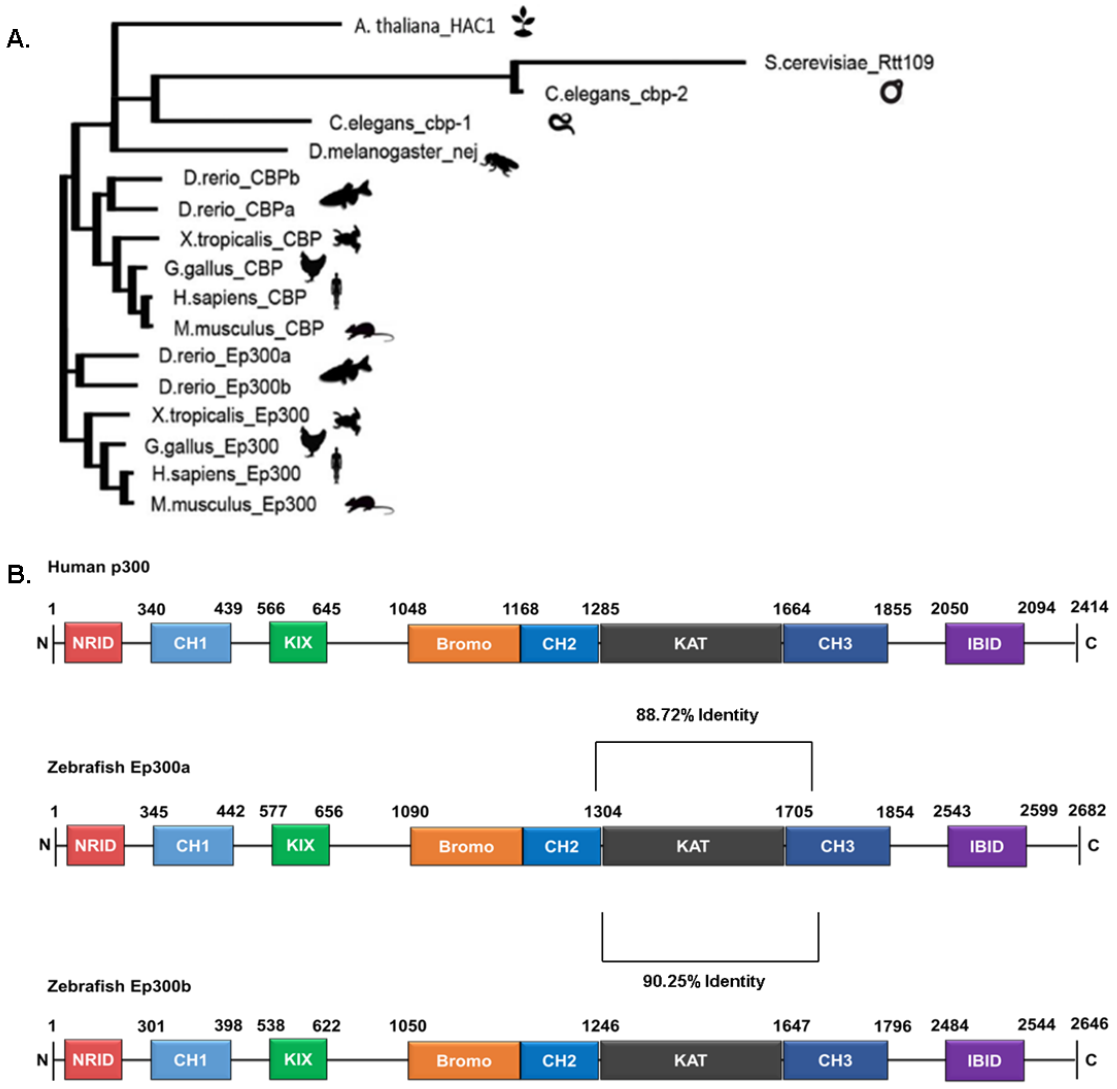


Figure 3.11. Homologs of human p300 in zebrafish*. (A) Phylogenetic mapping ofp300 homologs across species. Distance between two species represents sequence divergence of p300 or CBP in those two species. (B) Domain demarcation based on CDD database search of zebrafish p300a and b as mentioned on the protein schematic and their relative similarity in acetyltransferase domain as compared to that of human p300.*

3.3.2. Zebrafish possesses two coorthologs of human p300

Zebrafish (*Danio rerio*) is a widely used vertebrate model system for development related studies owing to its simple and inexpensive maintenance, short generation time, ability to produce hundreds of externally fertilized embryos that are transparent, easy to manipulate and rapidly develop. The basic morphological features of zebrafish embryo are observable 24 hours post fertilization and major organogenesis completes within 5 days post fertilization (dpf) (Pichler *et al.,* 2003; Kimmel *et al.,* 1995; Sertori *et al.,* 2016; Howe *et al.,* 2013). Zebrafish is also a popular system for screening small molecules for chemical toxicity and drug efficacy for human diseases (Rennekamp and Peterson, 2015; Scholz *et al.,* 2008).

Roughly 70% of human genome has coorthologs in Zebrafish (Howe *et al.,* 2013). Sequence homology search piques two coorthologs of human p300 in Zebrafish; which are enlisted in ENSEMBL database as ep300a (ENSDARG00000100666), ep300b (ENSDARG00000061108). Multiple coorthologs of p300 in zebrafish may have been a consequence of an event of genome duplication; synteny mapping of p300 and neighboring genes in zebrafish and human support this hypothesis (Braasch and Postlethwait, 2012; Barbazuk *et al.,* 2000). A previous study shows that p300 is dysregulated in zebrafish in light-induced retinopathy and pharmacological inhibition acetyltransferase activity of p300 indicates that the protein has protective function for retinal cells (Kawase *et al.,* 2016). Interestingly, human p300 displays similar function in retinal cells and is dysregulated in diabetic retinopathy (McArthur *et al.,* 2011). This indicates the possibility of functional homology between the human and zebrafish p300 homologs. ep300a and ep300b bears 69.8% sequence identity with each other and 65.6% and 65.5% sequence identity respectively with the human counterpart. Using conserved domain database (CDD) search based on sequence homology several domains were identified in ep300a and b; NRID, KIX, CH1, CH2, CH3, bromodomain, acetyltransferase domain and IBID domains in zebrafish p300 coorthologs had similar arrangements as found in human p300. The highest sequence conservation is observed in acetyltransferase domains of ep300a and ep300b which bore ~90% sequence identity with human p300 acetyltransferase domain. The putative conserved domains identified in zebrafish p300 co-orthologs have been discussed in Figure 3.11.B. Interactome study using STRING database predicted similar interactome for both the coorthologs pf p300 in Zebrafish indicating functional redundancy of the two proteins (Data not shown).

3.3.3. Zebrafish ep300a is an active acetyltransferase

Human p300 being a robust acetyltransferase is co-expressed with deacetylase Sirt2 to reduce aberrant acetylation mediated toxicity in expression system (Thompson *et al.*, 2004). Interestingly, it was observed that zebrafish ep300a KAT domain required co-expression of human Sirt2, whereas ep300b KAT domain was expressed without co-expression of any deacetylase. Recombinant ep300a KAT domain acetylated free recombinant histone H3, H2A and H4 robustly whereas ep300b KAT domain could not acetylate any of the recombinant histones (Figure 3.12). Keeping in mind that recombinant histones are most readily accepted as substrates for acetylation by almost all the histone acetyltransferases as compared to other complex substrates such as nucleosomes, or chromatinized templates, ep300b was assumed to be incapable of carrying out acetyltransferase reaction. Similar phenomenon is observed in many other organisms possessing multiple copies of p300/CBP; for example, in *Arabidopsis* out of 5 homologs of human p300, 4 were found to be active and only one ortholog had dominant function and rest possessed redundant subdued level of acetylation (Han *et al.*, 2007).

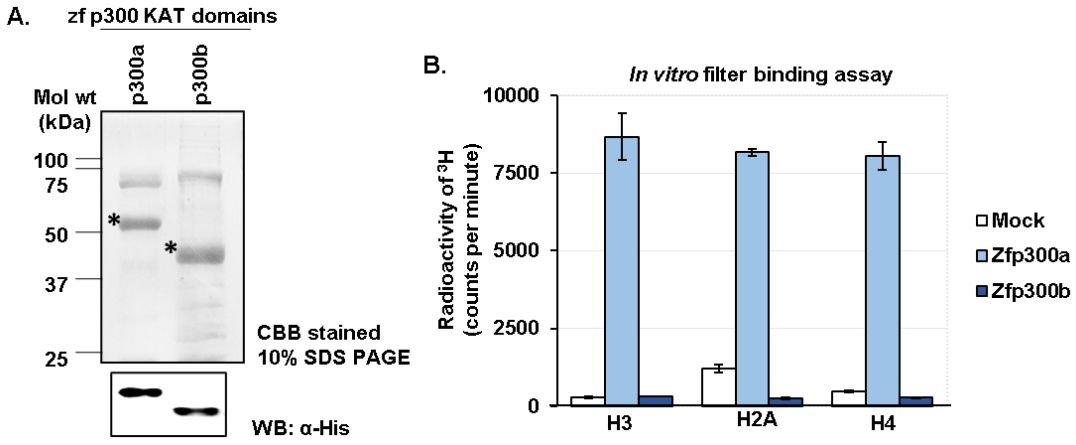


Figure 3.12. Acetyltransferase activities of zebrafish p300a and b KAT domains. (A) *Purification profile of putative acetyltransferase domains of zebrafish p300a and b on 10% SDSPAGE followed by Coomassie staining. Proteins were transferred onto PVDF membrane to immunoblot with polyHistidine tag specific antibody. (B) ~500ng of zebrafish p300a and p300b were subjected to radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi ^{3}H acetyl CoA were used as substrates; Incorporation of ^{3}H-acetyl group (counts per minute) has been plotted along Y axis represents respective enzymatic activity.*

3.3.4. Catalytic activity of ep300a is similar to that of human p300

Even though human p300 KAT domain bears little sequence similarity with KAT domains of the other acetyltransferases, central core domain associated with CoA binding site is structurally similar in the KAT domains. The striking feature in p300 KAT domain is its long, structured, highly acidic and polar substrate binding L1 loop which enables p300 to accept a wide range of substrates as well as small molecule inhibitors (Liu *et al.*, 2008).

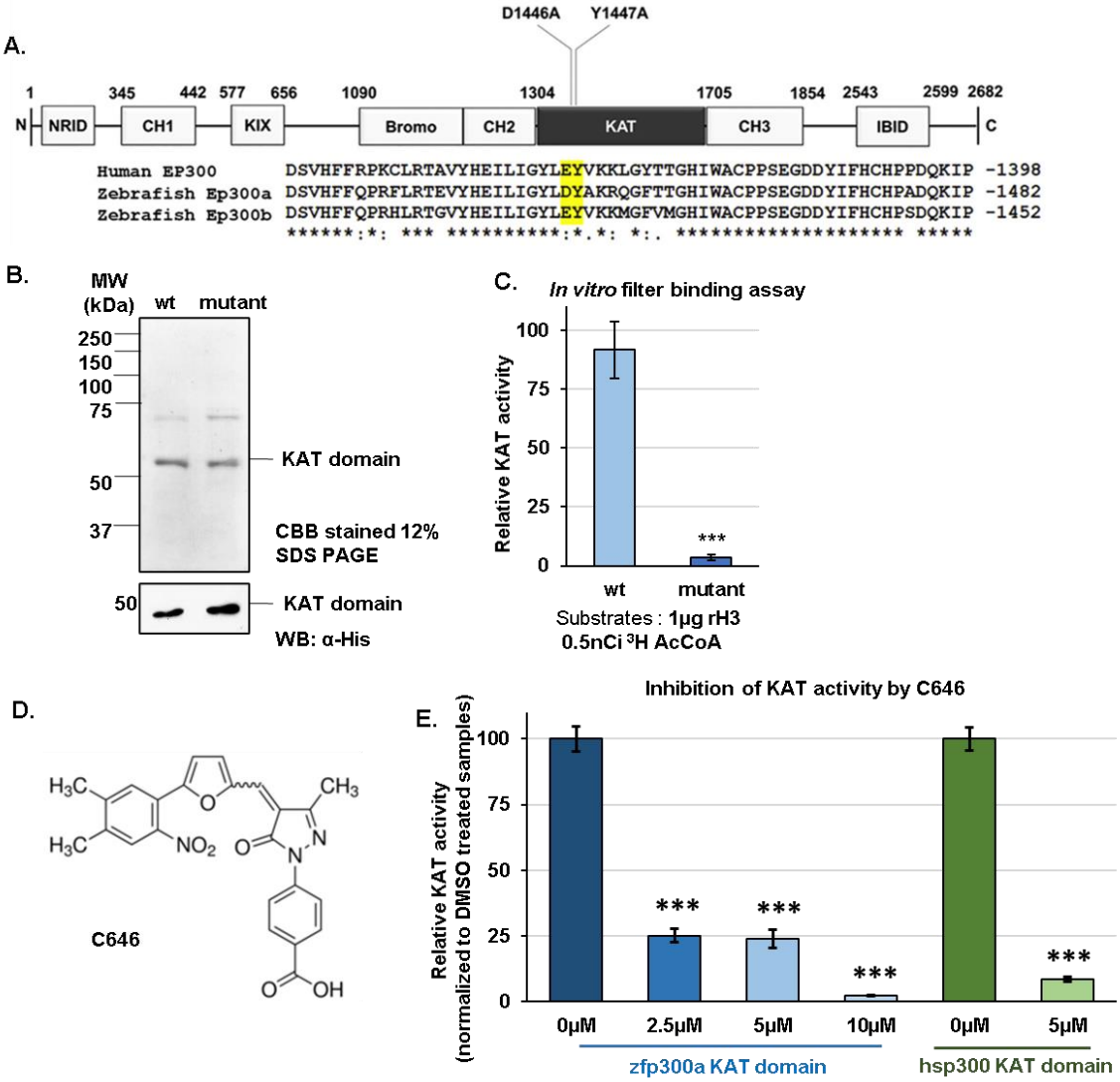


Figure 3.13. Comparison between acetyltransferase activity of human p300 and zebrafish p300a KAT domains. *(A) Relative positions of the conserved putative catalytic residues in zebrafish coorthologs (B)Purification profile of wildtype and D1446A/Y1447A mutant zebrafish p300a acetyltransferase domains on 10% SDSPAGE followed by Coomassie staining. Proteins were transferred onto PVDF membrane to*

immunoblot with polyHistidine tag specific antibody. (C) ~500ng of wild type and D1446A/Y1447A mutant zebrafish p300a acetyltransferase domains were subjected to radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi ^{3}H acetyl CoA were used as substrates; Incorporation of ^{3}H-acetyl group (counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3; Statistical analysis: Students' t-test, P<0.05, P**<0.01, P***<0.005. (D) Structure of C646. (E) ~500ng of wild type zebrafish p300a and human p300 acetyltransferase domains were subjected to radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi ^{3}H acetyl CoA were used as substrates; 0-10µM concentration of C646 (as mentioned) was used as inhibitor in the reactions. Incorporation of ^{3}H-acetyl group (counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3; Statistical analysis: Students' t-test, P*<0.05, P**<0.01, P***<0.005. (Hsp300 represents human p300 KAT domain in all figures).*

Consequently, small molecule inhibitors that selectively binds to p300 seldom inhibit other acetyltransferases. C646 is one such selective competitive inhibitor of p300 with K_i of 400nM; C646 does not inhibit other acetyltransferases such as GCN5, PCAF, MOZ, Sas, SSAT or even yeast p300 homolog Rtt109. C646 establishes interaction with the side chains of multiple residues (Thr1411, Tyr1467, Trp1466, and Arg1410) in the L1 loop of p300 KAT domain; mutation in any of the residues affects catalytic activity of p300 as well as binding of C646 to p300 (Bowers *et al.*, 2010). Interestingly, C646 inhibited purified ep300a KAT domain in a dose dependent manner in a radioactivity based in-vitro assay. At 2.5 and 5µM concentration of C646 ep300a was inhibited by ~85% and at 10 µM concentration complete abrogation of ep300a catalytic activity was observed. the result was comparable to ~90% abrogation of acetylation by human p300 KAT domain at 5µM of C646 (Figure 3.13.D).

This observation indicated that the catalytic site of ep300a must have structural and functional resemblance with human p300. Sequence comparison indicated the presence of two putative conserved catalytic residues in ep300a, Asp1444 and Tyr1445, which are similar to Glu1423 and Tyr 1424 in human p300 (Figure 3.13.A); mutating these two residues in ep300a drastically reduced the catalytic activity of ep300a KAT domain by ~90% (Figure 3.13.B). These observations ascertained the fact that ep300a putative KAT domain indeed possessed intrinsic acetyltransferase activity and the acetyltransferase

domain bore close resemblance to human p300 KAT domain in terms of both sequence and function.

3.3.5. Zebrafish ep300a and human p300 have similar site specificity on histones

To understand the specificity of zebrafish ep300a KAT domain its ability to acetylate different histone acetylation sites in comparison to human p300 was assessed. ep300a KAT domain could acetylate K9, K18 in H3 and K4, K12 in H4 which are common target sites for many other nuclear acetyltransferases. ep300a also acetylated the sites which are majorly recognized and acetylated by human p300/CBP such as H3K14, K27, K56 and H2AK5 (Figure 3.14.B). ep300a KAT domain also recognized mononucleosomes reconstituted from HeLa core histones and robustly deposited acetyl groups onto different lysine residues H3K9, K14, K18, K56 and H4K8 as tested (Figure 3.14. C).

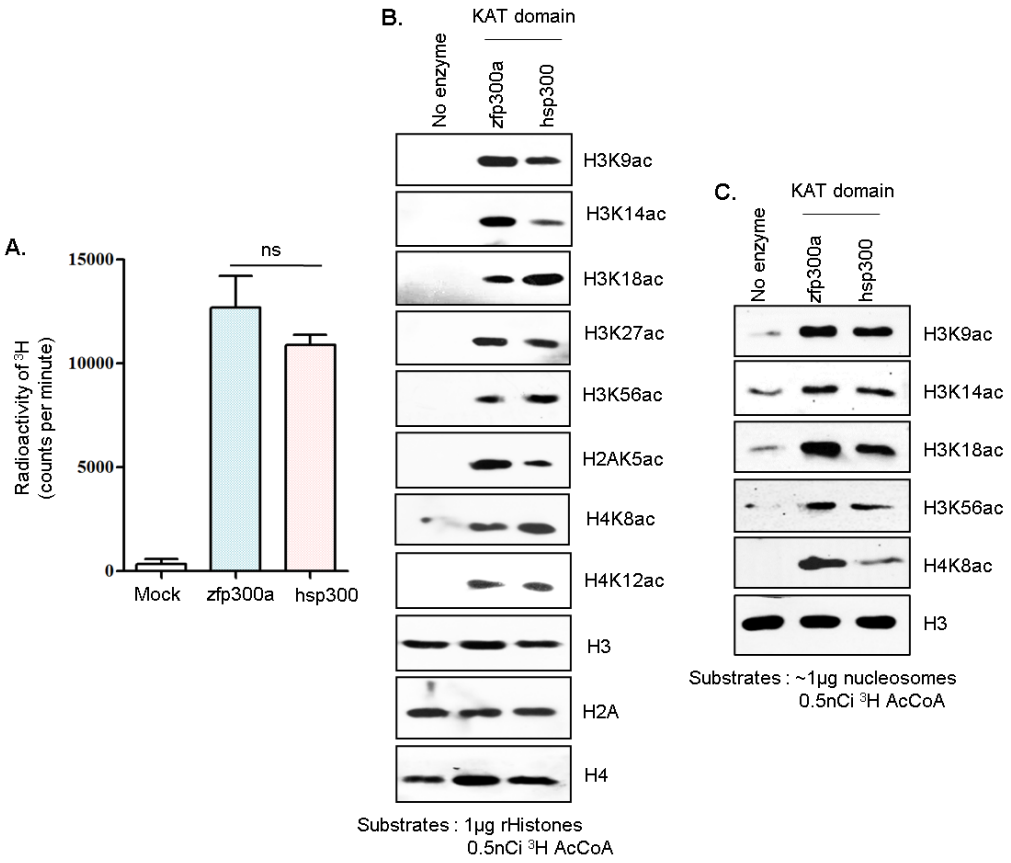


Figure 3.14. Substrate specificity of zebrafish p300a acetyltransferase domain. *(A) Acetyltransferase activities of zebrafish p300a and human p300 acetyltransferase*

domains were normalized using radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi ^{3}H acetyl CoA were used as substrates; Incorporation of ^{3}H-acetyl group (counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3; Statistical analysis: Students' t-test, P<0.05, P**<0.01, P***<0.005. (B) Site-specificity of zebrafish p300a acetyltransferase activity on different core histone. In vitro KAT assay was performed using recombinant H2A, H3 and H4 and then loaded on 12% SDSPAGE and probed with site-specific acetyl-lysine antibodies after transferring onto PVDF membrane. H3, H2A and H4 levels served as the loading control for their respective site-specific lysine acetylation marks. Human p300 acetyltransferase domain was taken as positive control for all the reactions. (C) Site-specificity of zebrafish p300a acetyltransferase activity on reconstituted nucleosomes. In vitro filter binding assay was performed using ~500 ng reconstituted nucleosome and loaded on a 12% SDS PAGE and probed with site-specific acetyl-lysine antibodies. H3 levels served as the loading control. Human p300 KAT domain was taken as positive control for all the reactions.*

3.3.6. Relevance of p300a KAT activity in development of zebrafish

Knockdown of ep300a in zebrafish embryo yielded developmental defects in larvae similar to defects observed when embryos are treated with C646. In both the cases, larvae had smaller eyes, smaller heads, shortened jaws, absence of swim bladders, pectoral fins, defect in cartilage formation, presence of pericardial edema and smaller body frame. These developmental defects closely resemble Rubinstein Taybi syndrome (RSTS2), an autosomal dominant dysmorphic syndrome in human which is characterized by microcephaly, craniofacial defects, dysmorphic limbs and cardiac anomaly. A closer observation pointed out reduced expression for Sox9b (homolog of human Sox9) tbx5a, and, NeuroD responsible for defects in cartilage formation, pectoral fin generation, neuronal differentiation respectively in both ep300a deficient and C646 treated zebrafish. None of the defects were observed in larvae deficient in ep300b which showed close to normal developmental phenotype ascertaining our observation of ep300b being catalytically inactive. Interestingly, overexpression of ep300a KAT domain RNA but not mutant KAT domain RNA and treatment with deacetylase Sirt1 inhibitor, CHIC35 could partially rescue the defects caused by the loss of ep300a. Majorly the craniofacial defects,

Sox9b expression followed by cartilage defect, pectoral fin, heart defects were rescued by the presence of ep300a catalytic activity (Babu *et al.*, 2018). This study demonstrated the functional relevance of zebrafish p300a catalytic activity during larval development and supports our observation on in-vitro catalytic activities of zebrafish p300 coorthologs.

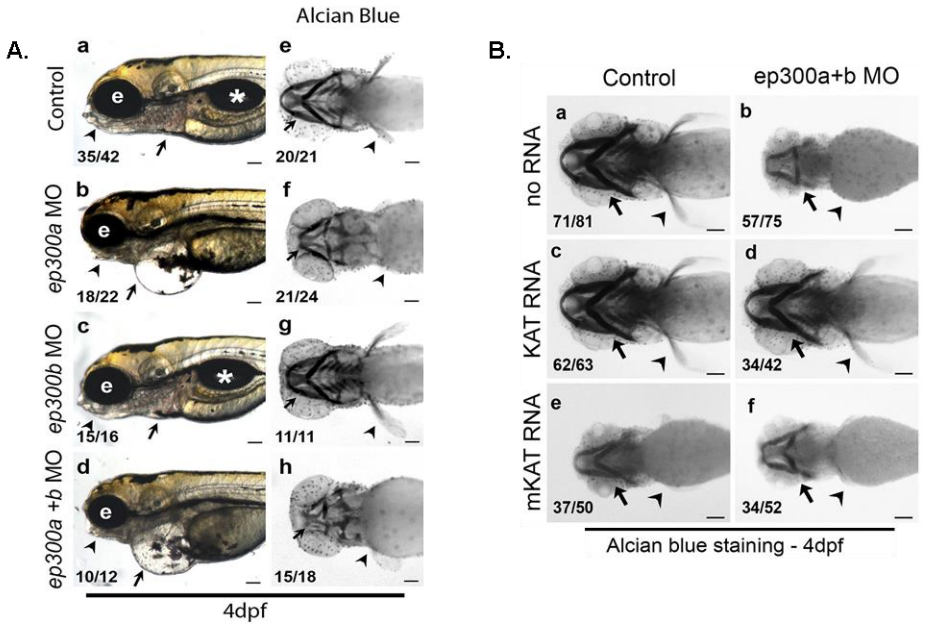


Figure. 3.15. Role of p300 acetyltransferase activity in zebrafish larval development (Babu et al., 2018). *(A) Zebrafish larvae development after zfp300 co-ortholog specific morpholino (4ng ep300a or 8ng p300b or combined) treatment. Unstained or alcian blue stained (for visualizing cartilage) larvae were observed under light microscope. Visible defects in morpholino treated larvae were indicated with arrows, letters or, symbols. (B) Morpholino (combined) treated zebrafish developed in presence of active or mutated p300a KAT domain RNA was observed under light microscope, defects and rescued phenotypes were indicated with arrows.*

3.4. Summary

Collectively, these set of studies demonstrated the relevance of p300 mediated acetylation in vertebrate embryonic development. In the first part, perturbation of p300-mediated acetylation using a small molecule inhibitor, Luteolin affected embryonic stem cell differentiation without affecting cellular viability. Inhibition of acetyltransferase activity of p300 led to a higher accumulation of Oct4 in protein level leading to meso-

and endodermal lineage specific differentiation. Interestingly, inhibition of p300 mediated acetylation negatively affected expression of neuronal marker but not the glial markers indicating a possibility of neuronal lineage specific function of p300-mediated acetylation.

In the second part, the acetyltransferase activity of human p300 homolog in zebrafish, p300a was characterized. Zebrafish p300a and b possessed significant sequence conservation with its mammalian counterpart and the maximum identity was observed in the acetyltransferase domain. Yet, only p300a was found to be a functional homolog in zebrafish as it could acetylate free and nucleosomal core histones *in vitro*. A parallel study on p300 deficient zebrafish embryonic development indicated manifestation of abnormalities in the specific ectodermal and cardiac development which validated our observation on the requirement of p300 mediated acetylation in neuronal lineage specification in differentiating embryonic stem cells. Additionally, rescue of the ectodermal defects in p300 deficient zebrafish by overexpression of functional catalytic domain of zebrafish p300a clearly demonstrated that the acetyltransferase function but not the transactivation function of p300 is required for proper ectodermal development in vertebrates.

Chapter 4

Characterization of acetyltransferase activity of TFIIIC220

This chapter provides evidences that suggests human TFIIIC220 possesses intrinsic acetyltransferase activity. This chapter begins with in-vitro characterization of the enzymatic activity of purified recombinant TFIIIC220, followed by identification of the putative acetyltransferase domain of the protein. Preliminary investigation points out that enzymatic activity of TFIIIC220 may be H3K18 residue specific. Knocking down of TFIIIC220 reduces H3K18 acetylation in global level in cells. Further analysis shows that TFIIIC220 may also participate in cellular stress response pathways independent of its RNAPIII-dependent functions. TFIIIC220 is upregulated in various stress conditions and may act in a p53-dependent manner.

4.1. Background

Conventionally acetyltransferases are categorized as transcriptional coactivators that are not included in basal transcription machineries of eukaryotic system. Recent works have identified association of acetyltransferase activities with components of basal transcription machineries of RNAPII and RNAPIII. In case of RNAPII system, general transcription factors TFIID subunit TBP associated factor 250 (TAFII250 or, TAF1), TFIIB and TFIIF have been found to possess acetyltransferase activities, the full spectrum of functional relevance of which are yet to be characterized. The effects of TAF1-mediated H3 and H4 acetylation (Mizzen *et al.,* 1996), and, autoacetylation of TFIIB and TFIIF are assumed to be limited for induction of RNAPII-mediated transcription (reviewed in Torchia *et al.,* 1998; Choi *et al.,* 2004). On the other hand, general transcription factor for RNAPIII, TFIIIC was found to possess acetyltransferase activity that was thought to be involved in relieving chromatinized repression of RNAPIII target sites fundamentally (Kundu *et al.,* 1999). Intriguingly, functional relevance of TFIIIC complex is not limited to RNAPIII-mediated transcription initiation, but, TFIIIC, often independent of RNAPIII co-occupancy, exert a wide range of effects starting from nucleosome remodeling to chromatin organizing and modulating RNAPII transcription as discussed in chapter 1. Undoubtedly this opens up a wide prospect for

acetyltransferase activity by TFIIIC to be involved multiple cellular processes beyond RNAPIII transcription. Out of six subunits in TFIIIC2 complex at least three subunits (p220, p110 and p90) were shown to acetylate core histone (H3, H4 and H2A) through radioactivity based in-gel assay. Among these subunits acetyltransferase activities of TFIIIC110 (Kundu *et al.*, 1999) and TFIIIC90 (Hseih *et al.*, 1999) have been further characterized through recombinant expression of complete or partial protein followed by *in vitro* characterization of acetyltransferase activities. Similar to TAF1, acetyltransferase activities of TFIIIC110 and 90 were found to be limited for H3 and H4. The details of acetyltransferase activity of the largest DNA-binding subunit of TFIIIC, TFIIIC220 remain elusive, and has been investigated in this chapter.

4.2.1. TFIIIC220 possesses intrinsic acetyltransferase activity

TFIIIC220 comprises of 2109 amino acids and corresponds to a total molecular weight of ~240kDa (calculated on web.expasy.org). Due to large size of the protein full length human TFIIIC220 (isoform 1) was cloned and expressed using baculovirus expression system. Recombinant full-length TFIIIC220 (here onwards mentioned as TFIIIC220fl) was purified from baculovirus infected Sf21 cells. TFIIIC220fl was affinity-purified as an intact single protein (Figure 4.1.A), and did not copurify the components of insect TFIIIC complex highlighting the intrinsic structural difference between mammalian and insect TFIIIC complex subunits. The presence of the protein in the eluted fraction was confirmed by immunoblotting with TFIIIC220 specific antibody (Figure 4.1A). Intrinsic catalytic activity of purified full-length TFIIIC220 was investigated by subjecting the protein to *in vitro* radioactivity based assay with recombinant histone H3 and as compared to mock purified eluted fraction, TFIIIC220 could deposit significant amount of tritiated acetyl group onto H3 (Figure 4.1.B). Though p300KAT domain and TFIIIC220fl were not used in equimolar ratio, the reactions were performed in presence of limited substrates over a prolonged period, yet, radioactive product generated by TFIIIC220fl was found to be much lesser compared to that of p300 KAT domain. Since acetylation (histone) is known to be a fast reaction (Waterborg, 2002), this observation indicated a probability of TFIIIC220 having limited site specificity on histone H3. Since TFIIIC-holocomplex was shown to acetylate H3, H2A and H4 among four core histones (Kundu *et al.*, 1999), the specificity of TFIIIC220 for these three histones were tested in

radioactivity-based gel assay. TFIIIC220 could acetylate all three histones *in vitro* (Figure 4.1.C).

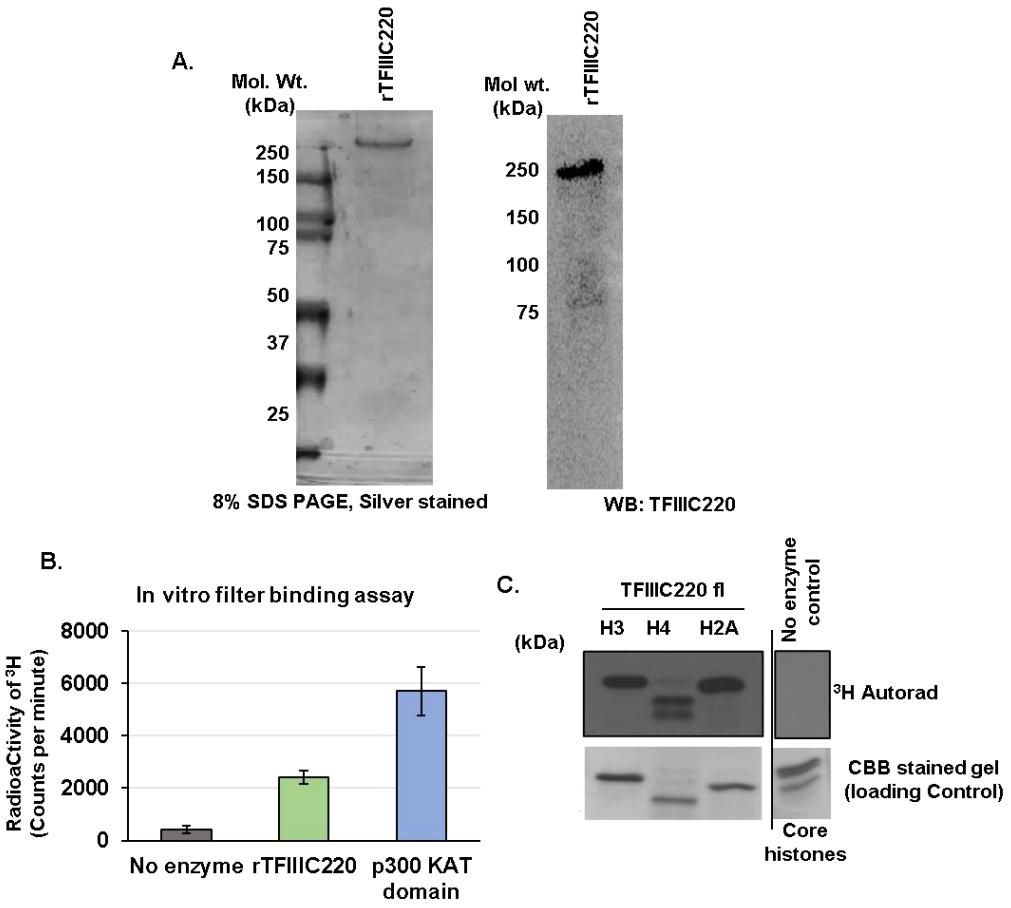


Figure 4.1. Acetyltransferase activity of full-length TFIIIC220. *(A) Purification profile of affinity-purified eluted fraction from insect cells infected with baculovirus containing TFIIIC220 construct on 8% SDS-PAGE. Protein on gel was visualized after silver staining. Eluted fraction was transferred onto PVDF membrane and probed with antibodies specific for human TFIIIC220. (B) ~500ng of full-length TFIIIC220 and 50ng of human p300 KAT domain were subjected to radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi 3H acetyl CoA were used as substrates; Incorporation of 3H-acetyl group (measured as counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3. (C) ~500ng of full-length TFIIIC220 was subjected to radioactivity-based gel assay; 1µg of recombinant histone H3, H4 and H2A and 0.5nCi 3H acetyl CoA were used as substrates and reaction mixture was run onto 12% SDS-PAGE; Incorporation of 3H-acetyl group onto histones*

were traced by radiography on X-ray film.1µg of calf-thymus type-IIa core histones was used as negative control for the reaction.

4.2.2. Screening of specific small molecule inhibitors of acetyltransferases against TFIIIC220

Numerous studies on acetyltransferases have identified plethora of small molecule modulators specific for different families of acetyltransferases which recognize and bind to particular sites on their target acetyltransferase and modulate their functions. Interestingly, specific inhibitors of certain family of acetyltransferases were shown to interact with residues that are structurally conserved in homologous acetyltransferases, any deviation in the conserved binding sites results in non-inhibition or are attributed to weak binding leading to high IC_{50} values. Hence, small molecule modulators are proven to be simple yet dependable measure to gain insight into the structural and functional properties of the catalytic site an acetyltransferase. To understand the similitude of catalytic domain of TFIIIC220, small molecule modulators specific to two different classes of acetyltransferases, p300 and PCAF were chosen. The acetyltransferase domain of p300/CBP is different from that of other known nuclear acetyltransferases (Liu *et al.*, 2008); consequently, specific competitive inhibitors of p300 acetyltransferase seldom inhibits acetyltransferase belonging to a different family. On the other hand, GNAT family acetyltransferase such as PCAF bears moderate similarity with many other nuclear lysine acetyltransferases belonging to GNAT and MYST families. C646, a competitive inhibitor of p300 (Bowers *et al.*, 2010) failed to inhibit recombinant full-length TFIIIC220 at a concentration ranging from 5-25µM, whereas, p300 activity was reduced to almost 17% by 5µM C646 (Figure 4.2.C). This observation indicated that p300 and TFIIIC220 bear little homology in terms of structure and function of their catalytic domains. On the other hand, specific non-competitive inhibitor of PCAF, embelin could inhibit TFIIIC220 in a dose dependent manner. 10 and 25µM embelin inhibited TFIIIC220 mediated acetylation of H3 by 10% and 25% respectively in an *in vitro* assay (Figure 4.2.D). Embelin binds to CoA binding site in the active site tunnel of PCAF and establishes strong interaction with many catalytic residues among which one is C574. Interestingly, CoA binding site of Tip60, a MYST family acetyltransferase, is structurally similar to that of PCAF and docking study shows that embelin is incorporated

the catalytic tunnel of Tip60 yet surprisingly, the binding is destabilized due to steric hindrance by a single residue I647. Accordingly, inhibition of Tip60 mediated acetylation in presence of 10-25µM embelin was insignificant (Modak *et al.*, 2013). Embelin strongly inhibited PCAF mediated acetylation at 10 and 25µM concentrations, the inhibition of TFIIIC220 catalytic activity at similar dosage was not as dramatic as that of PCAF yet it was significant. These results underlined the probable homology between the catalytic functions of PCAF and TFIIIC220.

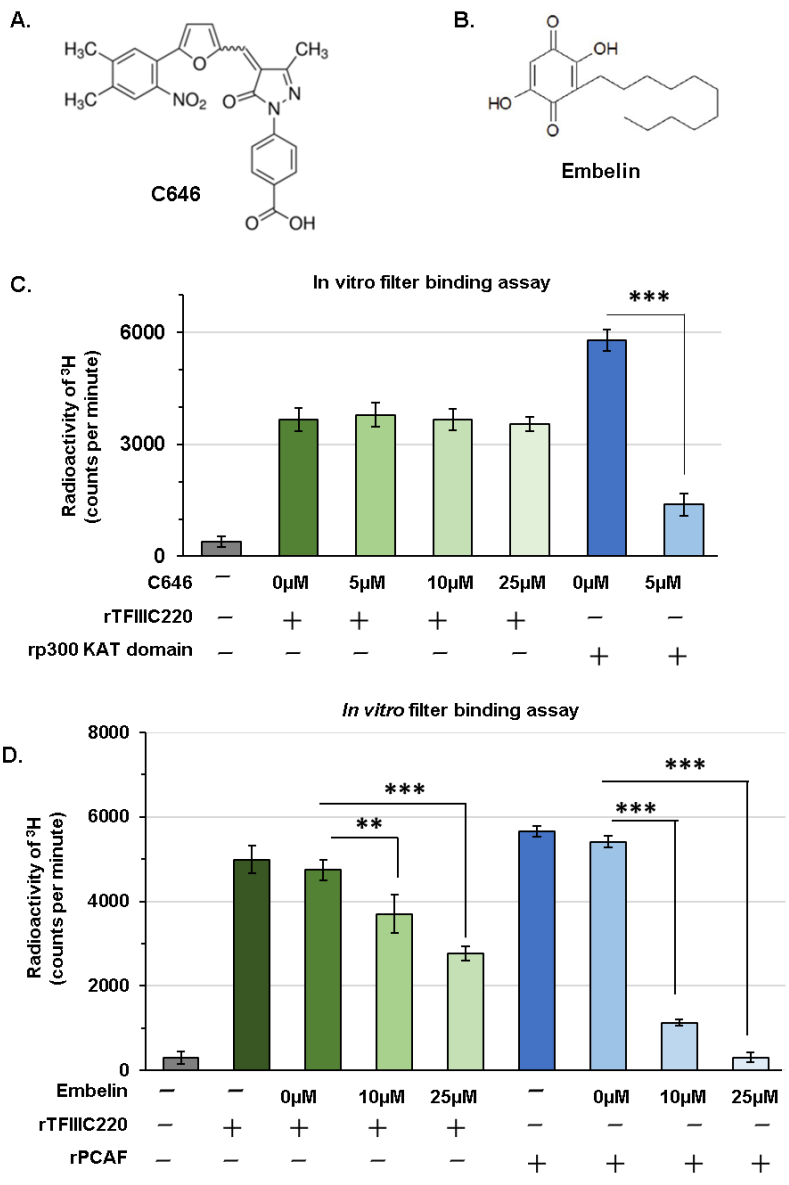


Figure 4.2. Screening of acetyltransferase inhibitors against TFIIIC220fl. *Structure of C646 (in A) and Embelin (in B). (C) ~500ng of full length TFIIIC220 and 50ng of human p300 KAT domain were subjected to radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi 3H acetyl CoA were used as*

substrates; 0-25µM concentration of C646 (as mentioned) was used as modulator in the reactions. Incorporation of 3H-acetyl group (counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3; Statistical analysis: Students' t-test, P<0.05, P**<0.01, P***<0.005. D. ~700ng of full length TFIIIC220 and 200 ng of human recombinant PCAF were subjected to radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi 3H acetyl CoA were used as substrates; 0-25µM concentration of embelin (as mentioned) was used in the reactions. Incorporation of 3H-acetyl group (counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3; Statistical analysis: Students' t-test, P*<0.05, P**<0.01, P***<0.005.*

4.2.3. Identification of TFIIIC220 acetyltransferase domain

Based on the previous observations, the homology between TFIIIC220 and GNAT family acetyltransferases were analyzed in detail. As discussed in chapter 1, GNAT family of acetyltransferases bear very little homology (3-23%) among themselves; but, characteristically possess four conserved regions with higher conservation in an order of C, D, A and B from N- to C- terminal. Domain C is exclusive to GNAT family of acetyltransferases but are not present in two most well studied representative acetyltransferases of this family, i.e., GCN5 and PCAF. the main catalytic domain A is shared between MYST and GNAT family members (reviewed in Salah *et al.*, 2016). Expectedly, pairwise sequence analysis among TFIIIC220, GNAT family KATs (GCN5 and PCAF) and MYST family KATs (MOZ, Tip60) failed to reveal any homology between them (<30% similarity), however, moderate homology was found when individual conserved domains of each protein were analyzed against full-length TFIIIC220. Domain A of GCN5, PCAF, Tip60 and MOZ were aligned to a region corresponding to T875- F1125 in TFIIIC220 (Figure 4.3. A); yet, the paired region yielded significant homology with neither GNAT nor MYST family acetyltransferases. The other conserved motifs D and B of GCN5 and PCAF were poorly aligned with aa1175- 1195 (Figure 4.3.C) and aa 855-1008 (Figure 4.3.D) respectively. Although the putative homology domains were clustered in the mid-region of TFIIIC220 similar to that found on GNAT family acetyltransferases but the order of arrangement from N'- to C- terminal was not the same (Figure 4.3.E.). MYST family acetyltransferases are

identified based on the presence of highly conserved (~90% similarity) MYST domain in them that also encompasses A motif. The MYST domains of Tip60 and MOZ were aligned to a region corresponding to approximately aa800-1200 without significant similarity and poor coverage indicating that TFIIIC220 might not be a member of MYST family acetyltransferase. Both GNAT and MYST family acetyltransferases possess

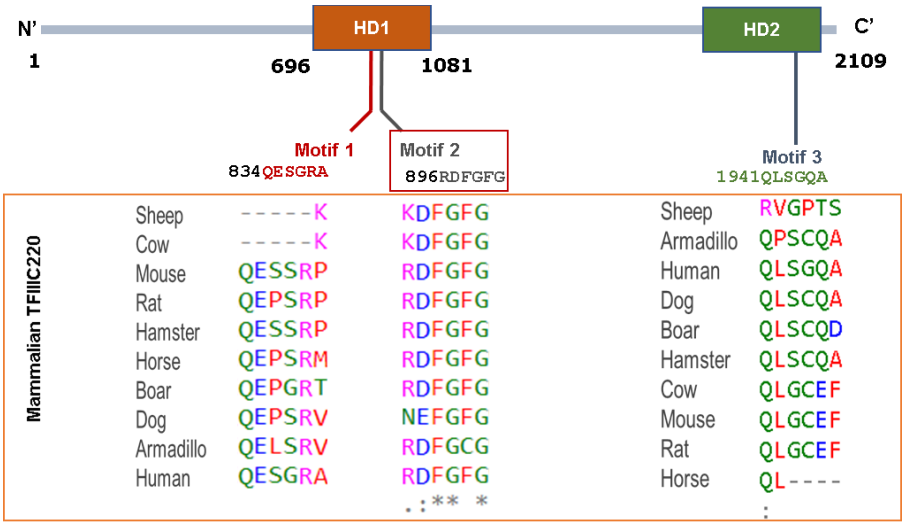


Figure 4.4. Consensus acetyl-CoA binding motifs in TFIIIC220. *Relative positions of putative acetyl-CoA binding motifs and putative KAT domains (HDs) on full length human TFIIIC220 has been represented through a schematic diagram. Sequence alignment of the three motifs across different vertebrate species is represented as Clustal Omega output.*

conserved 'P loop's inside A motif that recognize and bind to acetyl-CoA. 'P loop' has also been identified in acetyltransferases that have not been characterized as GNAT or MYST family (for example, in Brd4) (Devaiah *et al.,* 2016). Interestingly, three motifs on human TFIIIC220 were detected that matched with consensus acetyl-CoA binding motif (R/Q)XXGX(G/A) of P loop. Two of those motifs corresponding to aa 834-839 and aa 896-901 were mapped to the mid-region and motif 3 (aa 1941-1946) was found in the C terminal of isoform1 (AC# Q12789). Splice variant of human TFIIIC220 (isoform 2: AC# NP_001273171) lacked the third motif due to absence of aa1933-1957 stretch. Out of these three motifs, only motif 2 was found to be 100% conserved among mammalian TFIIIC220 (Figure 4.4). Hence, ~50kDa region spanning motif 1 and 2 were

considered to be the putative acetyltransferase domain of TFIIIC220 (represented in figure 4.4)

Recombinant TFIIIC220 putative catalytic domain was expressed with a C-terminal polyHistidine tag and affinity-purified from *E. coli.* The presence of the protein in the eluted fraction was confirmed by immunoblotting with antibodies specific for poly-histidine tag (Figure 4.5. B) and by mass-spectrometric analysis. Additional proteins in the elution corresponding to molecular weights of ~25 and ~75kDa were identified as common histidine rich contaminants from BL21 cells (Bolanos-Garcia *et al.*, 2006) by mass spectrometric analysis. Purified recombinant putative TFIIIC220 KAT domain could acetylate free recombinant histone H3 in radioactivity based *in vitro* assay (Figure 4.5. C).

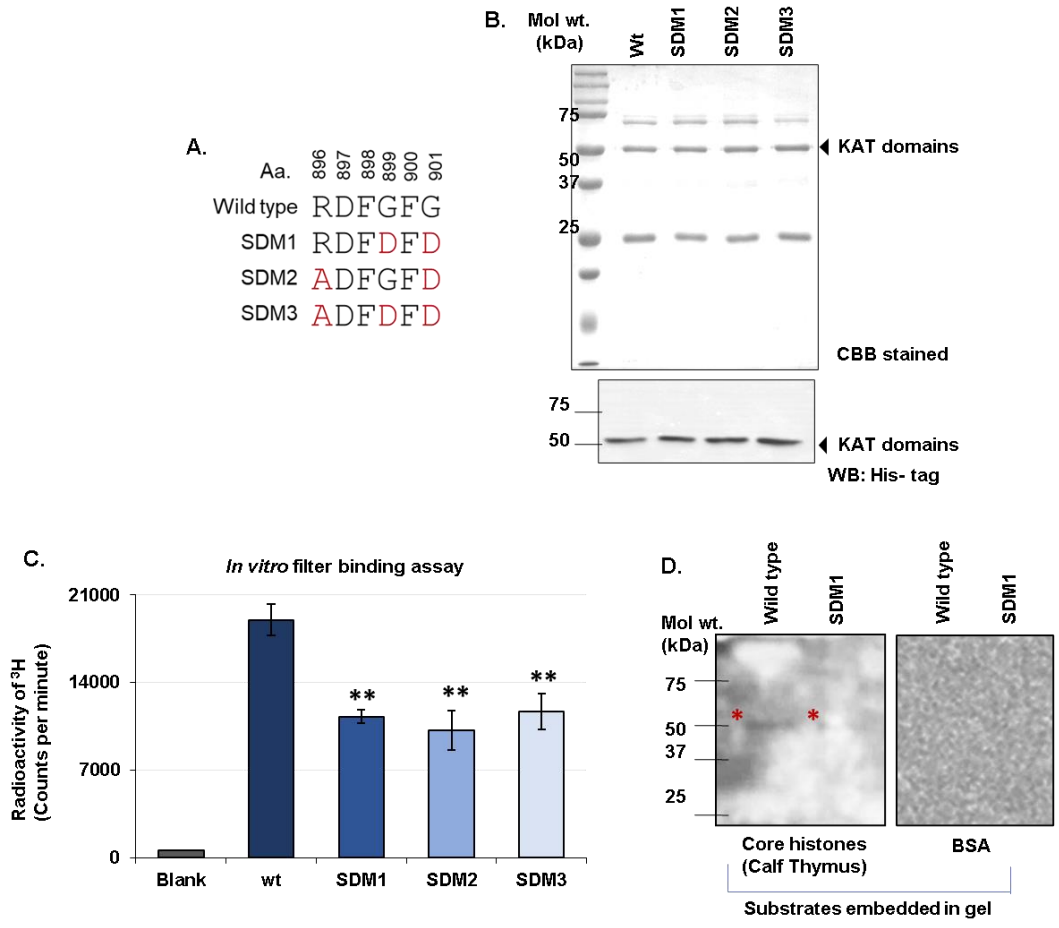


Figure 4.5. Enzymatic activity of putative KAT domain of TFIIIC220. *(A) Representation of respective positions of point mutations generated in different mutated versions of putative TFIIIC220 KAT domain. (B) Purification profile of affinity-purified wild-type and mutated KAT domain proteins from E. coli cells. Eluted fractions were run*

on 12% SDS PAGE and Coomassie stained. Fractions corresponding to putative KAT domains are indicated with black arrow. Eluted proteins were transferred onto PVDF membrane and probed with antibodies specific for poly histidine tag. (C) ~500ng of wild type and mutated KAT domains of TFIIIC220 were subjected to radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi ^{3}H-acetyl-CoA were used as substrates; Incorporation of ^{3}H-acetyl group (measured as counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3. Statistical analysis: Students' t-test, P<0.05, P**<0.01, P***<0.005. All analysis were performed against wt KAT domain value. (D) ~500ng of wild type and mutated (SDM1) KAT domains of TFIIIC220 were run on 10% native gel embedded with 1mg/mL calf thymus core histones or 1mg/mL bovine serum albumin (BSA) and renatured gel was incubated in 0.5nCi/mL 3H acetyl CoA before tracing the in-situ incorporation of ^{3}H acetyl group by radiography on X ray film.*

To confirm that the acetyltransferase activity observed in the *in vitro* assays were associated with the fraction containing our protein of interest and not with the other co-contaminant fractions radioactivity based in-gel assay was performed. Only putative TFIIIC220 KAT domain but not the other fractions could incorporate tritiated acetyl group onto core histones embedded in the gel corresponding to its molecular weight. Putative KAT domain could not acetylate BSA in a separate gel indicating the acetylation of histone observed is not factitious (Figure 4.5.D). To validate the relevance of putative acetyl-CoA binding site in the TFIIIC220 KAT domain, point mutations were generated in the motif to destabilize the interaction with CoA. Numerous reports have suggested that mutation of 4th and 6th conserved glycine residues in P loop results in inactivation of acetyltransferase function of GNAT family acetyltransferases (Salah *et al.*, 2016). Replacing glycine residues with electronegative and bulky aspartic acid and arginine with small neutral amino acid alanine in motif 1 abrogated its acetyltransferase activity by ~40% in an *in vitro* enzymatic assay (represented in figure 4.5.C). Mutated KAT domain showed a decline in rate of acetylation of core histones in an in-gel assay (Figure 4.5.D). Together these observations bolstered the conjecture that TFIIIC220 possessed intrinsic acetyltransferase activity and mid-region of TFIIIC220 might be accountable for its acetyltransferase function.

Another characteristic of GNAT family acetyltransferases is localization of bromodomain in their C-terminal. Pairwise sequence analysis of TFIIIC220 with

consensus bromodomain sequence (Haynes *et al.*, 1992) piqued a region with 62% sequence similarity (and 20% identity) at extreme C-terminus of TFIIIC220 (aa. 2006-2109). Though bromodomain are short conserved stretches and some bromodomains may possess >90% similarity with the consensus motif (for example in case of two bromodomains in Brd4), there are examples of bromodomains sharing lesser homology (e.g., 60% similarity in case of Brm bromodomain) as well. Moreover, bromodomains usually possesses two stretches of amphipathic helices (identified as A and B) that are linked with reverse turn. In case of putative bromodomain of TFIIIC220 two such stretches were found which shared higher relative conservation with known A and B patches of consensus bromodomain (indicated in figure 4.6.A). Interestingly, putative A and B regions of the putative bromodomain of TFIIIC220 corresponded to helical secondary structures connected with a region of undefined structure (Figure 4.6.B).

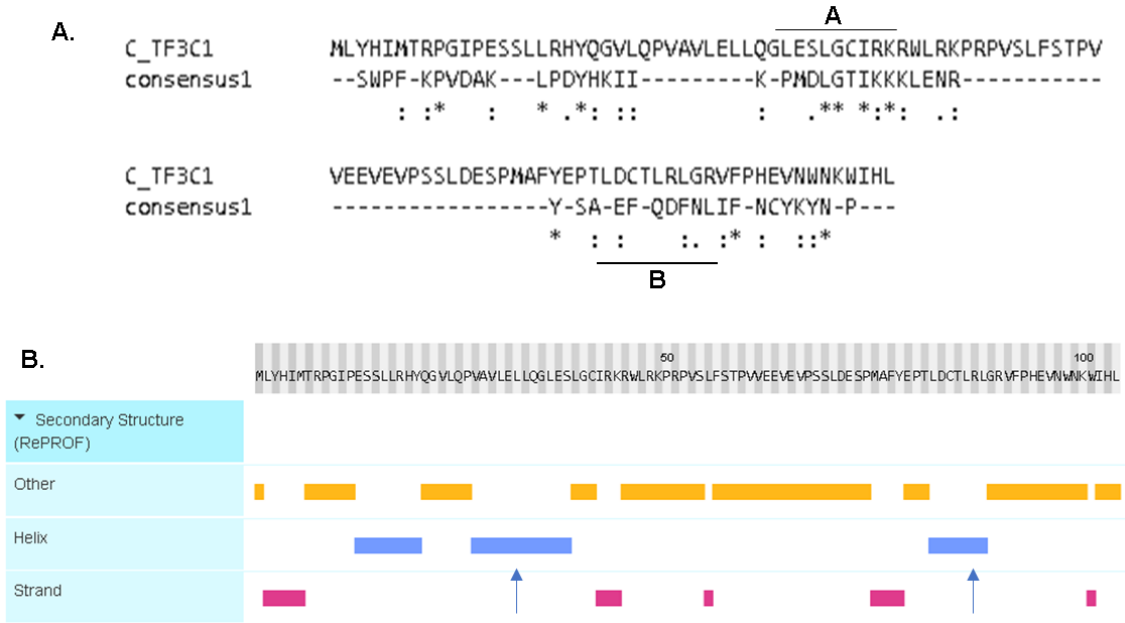


Figure 4.6. Putative bromodomain in TFIIIC220. *(A) Pairwise sequence alignment of C-terminal of TFIIIIC220 (aa. 2006-2109) with consensus bromodomain motif. Conserved A and B amphipathic patches in bromodomains are indicated with bar. (B) Secondary structure prediction for putative bromodomain of TFIIIC220. A and B patches corresponding to regions highlighted in (A) are predicted to be helices (indicated with blue arrows). Analyzed on predictprotein.org (Bernhofer et al., 2021).*

4.2.4. Substrate specificity of TFIIIC220

Difference in substrate specificity of acetyltransferases may result in diversification of functional consequences, hence, the substrate specificity of TFIIIC220 was investigated.

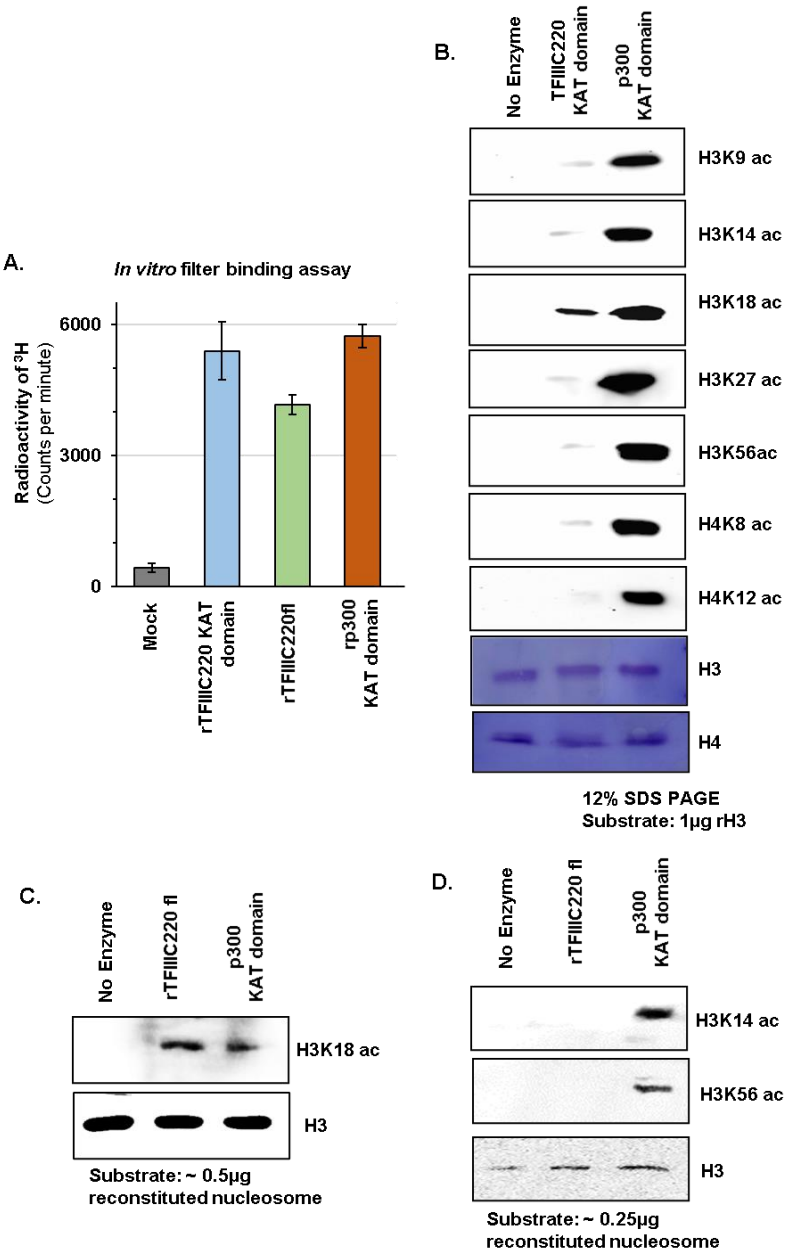


Figure 4.7. Substrate specificity of TFIIIC220. *(A) Acetyltransferase activities of wildtype putative TFIIIC220 KAT domain, full-length TFIIIC220 and human p300 KAT domains were normalized using radioactivity based in vitro filter binding assay; 1µg of recombinant histone H3 and 0.5nCi ^{3}H acetyl-CoA were used as substrates; Incorporation of ^{3}H-acetyl group (counts per minute) has been plotted along Y axis represents respective enzymatic activity. N=3. (B) In vitro KAT assay was performed*

using recombinant H3 and H4 and then loaded on 12% SDSPAGE and probed with site-specific acetyl-lysine antibodies after transferring onto PVDF membrane. H3 and H4 levels served as the loading control for their respective site-specific lysine acetylation marks. Human p300 KAT domain was used as positive control for all the reactions. (C) and (D) In vitro gel assay was performed using ~500 ng (in C) or ~250ng (in D) reconstituted nucleosome containing HeLa core histones. Nucleosomes were incubated with full-length TFIIIC220 or p300 KAT domain and ^{3}H acetyl-CoA and loaded onto a 12% SDS PAGE. After transferring onto PVDF reactions were probed with site-specific acetyl-lysine antibodies. H3 levels served as the loading control for nucleosomes used in the reactions.

In order to characterize the site specificity of TFIIIC220 *in vitro* gel assay was performed using recombinant or nucleosomal histones as substrates. Since p300 is a robust and promiscuous acetyltransferase known to acetylate multiple lysine residues on all the core histones, recombinant human p300 KAT domain was used as positive control for the assays. Based on the previous observation on TFIIIC220 bearing partial homology with GNAT family acetyltransferases, the substrate specificity of TFIIIC220 was expected to match with that of GNAT family members.

As discussed in chapter, mammalian GNAT family acetyltransferases have specificity exclusive for H3 and H4 among core histones. In vitro, recombinant GNAT acetyltransferases show specificity for H3 N-terminal tail residues K9 and 14. Interestingly, TFIIIC220 acetylated none of these residues (K9 or K14) on free histone H3 but it acetylated H3K18 residue (Figure 4.7. B). H3K18ac is widely associated with transcriptional activation (Halasa *et al.*, 2019) and are known to be targeted by p300/CBP, Elp3. Since H3K14 is a site that is targeted by almost all the nuclear acetyltransferases and TFIIIC220 did not acetylate that site indicated that the acetyltransferase activity of TFIIIC220 might be quite specific. Also, TFIIIC220 KAT domain could not acetylate H3K27 (usually targeted by p300/CBP), globular domain residue H3K56 (preferred site for p300/CBP and GCN5). In histone H4, K8 and K12 are two sites that are commonly targeted by multiple KATs (Hat1, GCN5, p300/CBP, Tip60, HBO1 to name a few), TFIIIC220 KAT domain failed to acetylate these two sites on free histone H4 as well (Figure 4.7.B).

Since the putative KAT domain of TFIIIC220 was identified based on homology with consensus acetyl-CoA binding site and inclusion of protein substrate binding sites in KAT domain were not investigated, there lies a possibility that regions outside the putative KAT domain construct of TFIIIC220 might aid in accepting a wider range of substrates. Hence, TFIIIC220fl was used in *in vitro* assay and nucleosomes were used as substrates to simulate *in vivo* conditions. TFIIIC220fl could acetylate H3K18 residue on nucleosomes (Figure 4.7.C) but did not acetylate H3K14 or 56 (Figure 4.7.D). This further emphasized the possibility of acetyltransferase activity of TFIIIC220 being selective for H3K18 residue among histone H3 N-terminal sites.

Next, to understand the role of TFIIIC220 in regulation of H3K18ac dynamics *in vivo* TFIIIC220 knockdown cells were generated.

4.3. 1. Characterization of TFIIIC220 knockdown cells

TFIIIC220 is an essential component of TFIIIC complex that is required for RNAPIII-mediated transcription of non-coding gene products that are directly involved in protein synthesis. Consequently, TFIIIC220 knockout or prolonged knockdown might have deleterious effect on any cell; an inducible knockdown system was used in this study. Doxycycline inducible shRNA targeting exon 33 (CTGATCGTTTCTCTTTCAA) of GTF3C1 was integrated into HepG2 cells through lentiviral transduction. 96 hours of Doxycycline treatment led to ~90% knockdown of GTF3C1 mRNA in TFIIIC220 shRNA transfected HepG2 cells (shTF3C1) as compared to non-silencing control (shNS) transfected HepG2 cells (Figure 4.8.B). Relative fold change was normalized against β-actin mRNA expressed in the cells. No significant change in the expression of other housekeeping genes (GAPDH, α-tubulin) was associated with TFIIIC220 knockdown in HepG2 cells (Figure 4.8.B). Also, TFIIIC220 knockdown or doxycycline treatment did not result in any gross morphological change in transformed HepG2 cells. 70-98% depletion of TFIIIC220 in protein level (maximum knockdown obtained is represented in figure 4.8.C) was observed upon induction of shTF3C1 expression. The expression of TFIIIC220 was normalized against Actin expression; the level of GAPDH, tubulin, histone H3 (as nuclear marker/loading control) remain unchanged upon TFIIIC220 knockdown (Figure 4.8.C). Interestingly, depletion of TFIIIC220 did not result in change in expression of TFIIIC110, another subunit of TFIIIC complex. It remains unclear if

other subunits of TFIIIC complex can assemble and function without its DNA binding subunit TFIIIC220. To understand the effect of TFIIIC220 depletion on RNAPIII-mediated transcription expression of a few target genes were looked into. Upon knockdown of TFIIIC220 the most affected set of RNAPIII targets were found to be tRNAs and 7SLRNA. These genes possess type II internal promoter element where binding of TFIIIC220 to B box element is crucial for recruitment of PIC (preinitiation complex). Next, 5SrRNA transcription was significantly affected as association of TFIIIC and TFIIIA to promoter elements are indispensable for recruitment of TFIIIB and subsequently formation of PIC (Figure 4.8.D).

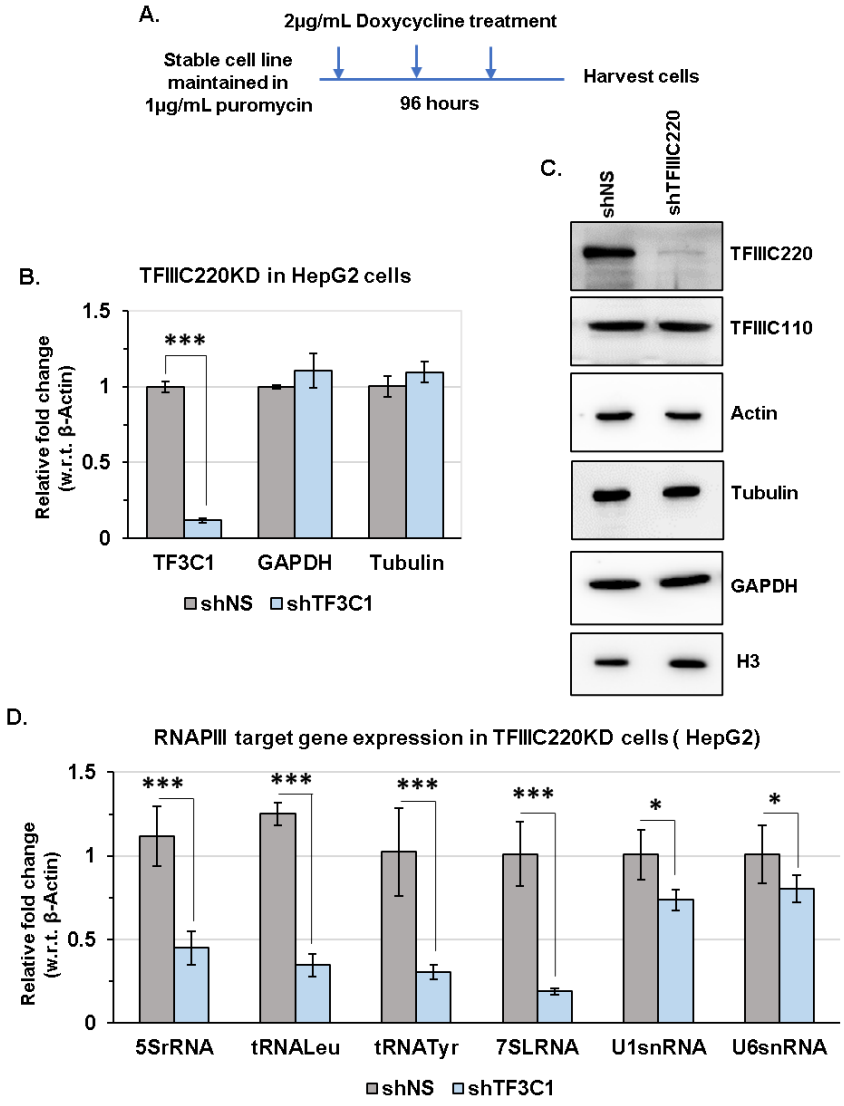


Figure 4.8. shRNA mediated knockdown of TFIIIC220 in HepG2 cells. *(A) Schematic representation of TFIIIC220 knockdown by Doxycycline treatment in HepG2 cells that are stably transfected with shRNA targeting GTF3C1 (shTFIIIC220). Non-silencing*

control shRNA (shNS) transfected cells were used as negative control. (B) qPCR analysis of GTF3C1 and housekeeping genes GAPDH, α-tubulin mRNA expression after 96 hours of Doxycycline treatment. N=3, Statistical analysis: Students' t-test, P<0.05, P**<0.01, P***<0.005 (C) Cell lysates (laemmli) were loaded onto 8 or 12% SDSPAGE and transferred onto PVDF membrane followed by immunoblotting with antibodies specific for TFIIIC220, TFIIIC110 and housekeeping genes β-actin, tubulin, GAPDH and H3 as loading controls. (D) qPCR analysis of RNA polymerase III target gene expression in TFIIIC220 knockdown cells. N=3, Statistical analysis: Students' t-test, P*<0.05, P**<0.01, P***<0.005.*

Oppositely, U6snRNA which contain external promoter element that is directly recognized by TBP, a TFIIIB subunit was moderately affected in absence of TFIIIC220. Though theoretically and experimentally *in vitro* or basal level transcription of U6snRNA strictly does not require TFIIIC, *in vivo* U6snRNA transcription was found to be dependent on the integrity of B-box sequence adjacent to its terminal region and B-box bound TFIIIC complex (Burnol *et al.*, 1993). Surprisingly, the transcription of U1snRNA, a product of RNAPII target gene was also found to be downregulated in TFIIIC220 depleted cells (Figure 4.8.D). TFIIIC is dispensable for *in vitro* transcription of U1snRNA (Gunderson *et al.*, 1990). But, *in vivo*, TFIIIC binding is known to influence transcription of adjacent RNAPII targets in certain cases and primary sequence of U1 snRNA possesses B box, a TFIIIC binding sequence (Galli *et al.*, 1981; Hogeweg and Konings, 1985). If *in vivo* expression of U1snRNA is dependent on TFIIIC complex remains to be investigated.

These observations did not only confirm effective knockdown of TFIIIC220 in GTF3C1shRNA expressing HepG2 cells but also conformed with the notion that in absence of TFIIIC220 the function of TFIIIC complex is negatively affected, and, TFIIIC220 knockdown did not affect expression of other TFIIIC subunit.

4.3.2. TFIIIC220 knockdown affects cellular H3K18ac level

Consistent with previous observation of TFIIIC220 having H3K18 residue specific acetylation, shRNA mediated knockdown of TFIIIC220 led to significant decrease in H3K18acetylation level in HepG2 cells (Figure 4.9.A). To omit the possibility of obtaining cell-type specific bias in the aforementioned observation TFIIIC220 was

depleted in HeLa S3 cells and reduction of TFIIIC220 resulted in decrease of H3K18 acetylation level in HeLaS3 cells as well (Figure 4.9.B).

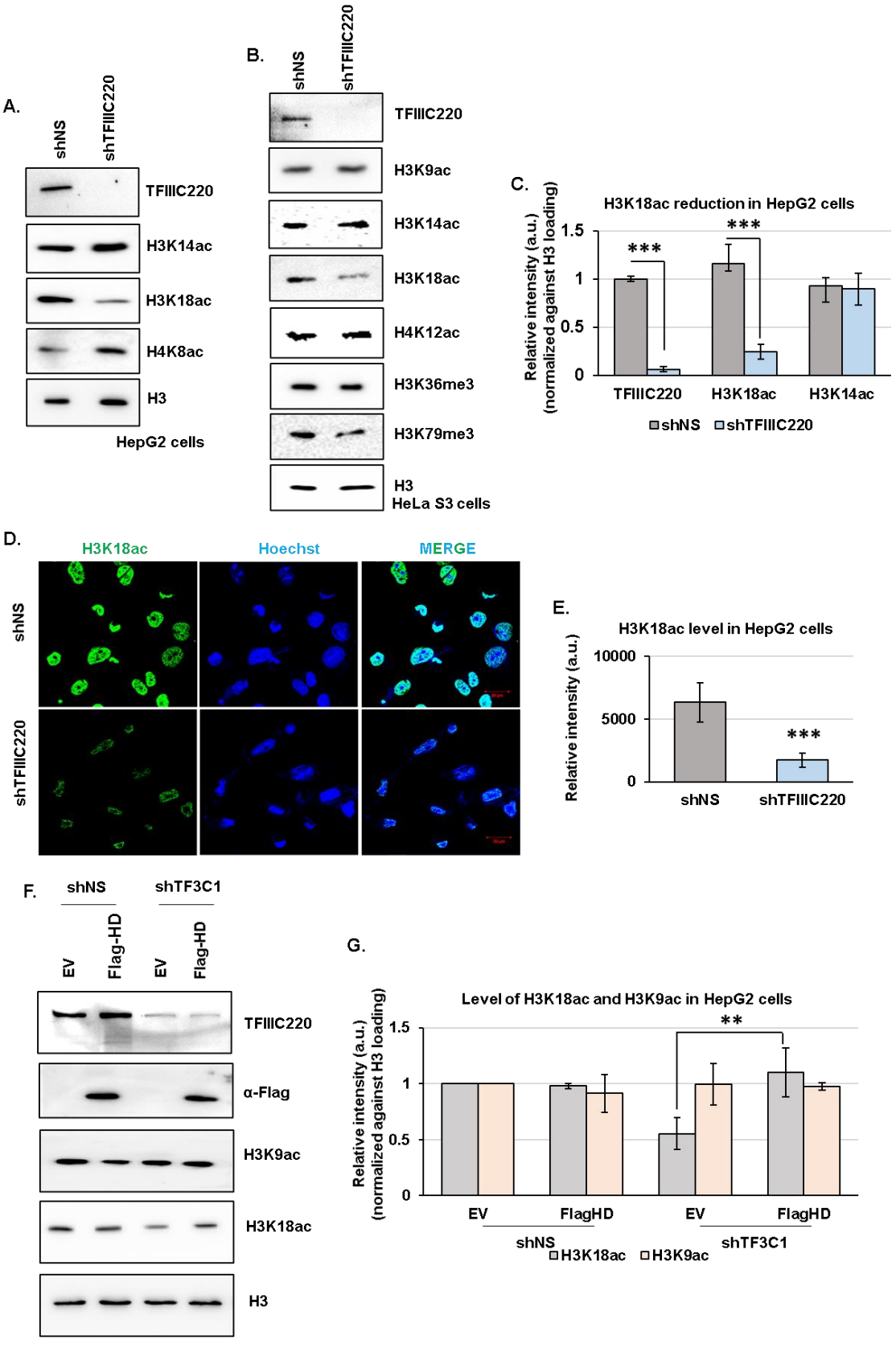


Figure 4.9. TFIIIC220 knockdown led to reduction in H3K18 acetylation level. *(A) and (B) Doxycycline treated lysates (laemmli) of stably transfected HepG2 (in A) and HeLa S3 cells (in B) were loaded onto 8 or 12% SDSPAGE and transferred onto PVDF membrane followed by immunoblotting with antibodies against site-specific acetyl-lysine residues. (C) Quantitation of relative intensities of TFIIIC220, H3 K14, K18 acetylation level obtained from western blotting analysis of TFIIIC220 downregulated HepG2 cells. N=3. Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005. (D) Immunofluorescence images of TFIIIC220 downregulated HepG2 cells stained with H3K18 acetylation specific antibodies. Hoechst stains nucleus. Scale bar: 10µm. (E) Quantitation of relative intensity of acetylated H3K18 staining obtained from immunofluorescence study of TFIIIC220 knockdown HepG2 cells. Average of total intensity per unit area across the cells is plotted along Y axis. N=15. (F) and (G). Doxycycline treated stably transfected HepG2 cells were transfected with empty vector (EV) or TFIIIC220 KAT domain (Flag HD) construct and lysates were loaded onto 8 or 12% SDSPAGE before immunoblotting with TFIIIC220, Flag tag, H3K9ac (negative control), H3K18ac and H3 (loading control) specific antibodies (in H). Quantitation of relative intensities (normalized against individual H3 level) were plotted for H3K18ac and H3K9ac (in G). N=3. Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005.*

As discussed earlier, apart from TFIIIC220, H3K18 site in cell is known to be targeted by other acetyltransferases such as p300/CBP and Elp3, yet, ~95% reduction in TFIIIC220 protein level in HepG2 cells were associated with a shocking 65% decrease in H3K18 acetylation level (Figure 4.8.C). Reduction of H3K18ac in TFIIIC220KD cells was global, throughout nucleus, with no puncta formation or nucleolar staining (Figure 4.9.D). Similar effect of hypoacetylation was not observed in case of other lysine sites on core histones (K9, K14 on H3; K8, K12 on H4); H3K36me3 that is modulated by Set2 methyltransferase was also not affected by TFIIIC220 knockdown (Figure 4.9.A and B). This observation conformed to the data obtained from *in vitro* acetylation assays with full-length and KAT domain of TFIIIC220 (Figure 4.7).

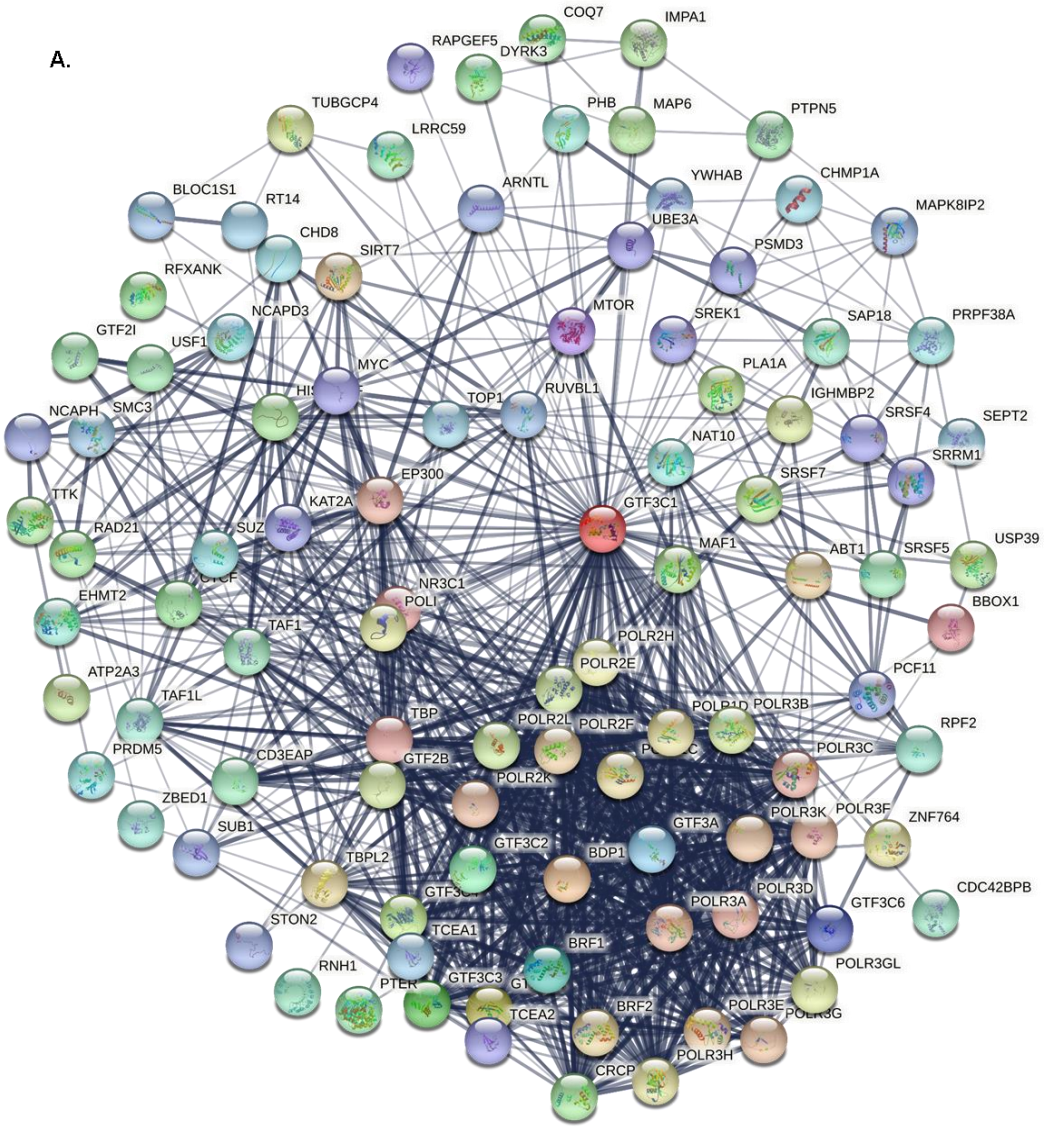
To understand if the reduction in H3K18 acetylation level in TFIIIC220KD HepG2 cells is a direct result of lack of acetyltransferase activity of TFIIIC220, the KAT domain of TFIIIC220 was overexpressed against knockdown background and level of histone acetylation was investigated. TFIIIC220 KAT domain was cloned in mammalian

expression vector with N-terminal Flag tag and C-terminal Myc nuclear localization signal as the NLS present in full-length TFIIIC220 did not correspond to its putative catalytic domain. The rescue in H3K18 acetylation observed upon overexpression of TFIIIC220 KAT domain construct (FlagHD) against a knockdown background was found to be significant as compared to KD cells transfected with empty vector control (Figure F and G). Upon overexpression of TFIIIC220 KAT domain the intensity of H3K18acetylation in TFIIIC220 KD cells was found to be almost equalized with that of control cells expressing non silencing shRNA (Figure 4.9.G). No change in the level of H3K9ac was observed upon overexpression of TFIIIC220 KAT domain in TFIIIC220 KD and control cells (Figure 4.9.G). Interestingly, overexpression of TFIIIC220 KAT domain in cells expressing normal level of TFIIIC220 did not further enrich H3K18ac (in shNS cells transfected with Flag HD as compared to transfection in figure 4.9.G). This might be corollary of tight regulation of H3K18ac by Sirt2 and Sirt7 or high selectivity of acetylation site of TFIIIC220.

4.3.3. TFIIIC220 knockdown accumulates stress-related factors in cells

Like many other acetylation marks, H3K18ac is linked to transcriptional activation. H3K18ac is found to be enriched in regions surrounding TSS of RNAPII as well as RNAPIII and is associated with active transcription (Wang *et al.*, 2008b) and is implicated in nucleolar heterochromatin dynamics, cytotoxicity and poor prognosis in cancer (Juliano *et al.*, 2016; Damodaran *et al.*, 2017; Hiraoka *et al.*, 2013; Ianni *et al.*, 2017). Hence hypoacetylation of H3K18 might be linked to transcriptional remodeling in TFIIIC220 knockdown cells. H3K18ac is also directly implicated in DNA damage repair. On one hand, p300/CBP -mediated H3K18ac along with other histone acetylation marks at DNA damage sites facilitates recruitment of SWI/SNF chromatin remodeling complex (Ogiwara *et al.*, 2011); on the other hand, deacetylation of H3K18 (by Sirt7) has been found to be crucial for 53BP1 recruitment and NHEJ repair (Vazquez *et al.*, 2016). Parallelly, TFIIIC complex is not only associated with RNAPIII-mediated transcription of various essential genes that are involved in protein synthesis, RNA processing and maintenance of cellular homeostasis, but also is involved in modulation of RNAPII gene transcription, chromatin organization along with CTCF, cohesin and condensin (discussed in chapter 1). Cellular transcription levels, a direct readout of

cellular homeostasis is found to be significantly reduced under various stress conditions. RNA polymerases can directly or indirectly sense nutrient rich or stress conditions with the help of various signaling pathways that get activated upon different conditions. In case of nutrient limitation, poor carbon sources and cellular stresses that directly or indirectly affect cell growth such as secretory pathway and cell wall defects, oxidative and ER stress, DNA damage etc. Maf1, a highly conserved protein in eukaryote, acts as a bonafide repressor of RNAPIII mediated transcription upon various stress conditions (Cabart *et al.*, 2008). Negative regulation of RNAPIII mediated transcription upon different stress condition is also brought about by its association with GCN5, TRRAP complex, also p53, ARF, PTEN, BRCA1, hyperphosphorylated Rb can repress RNAPIII mediated transcription under adverse conditions (Dumay-Odelot *et al.*, 2010 and references therein).



B.

pathway	description	count in network	strength	false discovery rate
HSA-76061	RNA Polymerase III Transcription Initiation From Type 1 Promo...	27 of 27	2.29	1.04e-44
HSA-76066	RNA Polymerase III Transcription Initiation From Type 2 Promo...	26 of 26	2.29	1.39e-43
HSA-73780	RNA Polymerase III Chain Elongation	17 of 17	2.29	1.57e-27
HSA-76046	RNA Polymerase III Transcription Initiation	28 of 35	2.19	1.04e-44
HSA-73980	RNA Polymerase III Transcription Termination	17 of 22	2.18	2.95e-26
HSA-76071	RNA Polymerase III Transcription Initiation From Type 3 Promo...	20 of 27	2.16	5.57e-31
HSA-749476	RNA Polymerase III Abortive And Retractive Initiation	28 of 40	2.13	6.41e-44
HSA-1834949	Cytosolic sensors of pathogen-associated DNA	18 of 62	1.75	5.98e-22
HSA-73863	RNA Polymerase I Transcription Termination	8 of 30	1.71	1.24e-08
HSA-8851708	Signaling by FGFR2 IIIa TM	5 of 19	1.71	4.51e-05
HSA-73762	RNA Polymerase I Transcription Initiation	10 of 46	1.62	3.43e-10
HSA-167242	Abortive elongation of HIV-1 transcript in the absence of Tat	5 of 23	1.62	8.97e-05
HSA-203927	MicroRNA (miRNA) biogenesis	5 of 24	1.61	0.00010
HSA-75953	RNA Polymerase II Transcription Initiation	9 of 47	1.57	1.24e-08
HSA-73779	RNA Polymerase II Transcription Pre-Initiation And Promoter O...	9 of 47	1.57	1.24e-08
HSA-73776	RNA Polymerase II Promoter Escape	9 of 47	1.57	1.24e-08
HSA-167162	RNA Polymerase II HIV Promoter Escape	9 of 47	1.57	1.24e-08
HSA-167161	HIV Transcription Initiation	9 of 47	1.57	1.24e-08
HSA-6803529	FGFR2 alternative splicing	5 of 26	1.57	0.00015
HSA-77075	RNA Pol II CTD phosphorylation and interaction with CE	5 of 27	1.55	0.00017
HSA-167160	RNA Pol II CTD phosphorylation and interaction with CE during ...	5 of 27	1.55	0.00017
HSA-167243	Tat-mediated HIV elongation arrest and recovery	6 of 34	1.53	2.18e-05
HSA-167238	Pausing and recovery of Tat-mediated HIV elongation	6 of 34	1.53	2.18e-05
HSA-5250924	B-WICH complex positively regulates rRNA expression	10 of 59	1.52	2.66e-09
HSA-72086	mRNA Capping	5 of 29	1.52	0.00022
HSA-5601884	PIWI-interacting RNA (piRNA) biogenesis	5 of 29	1.52	0.00022
HSA-167290	Pausing and recovery of HIV elongation	6 of 36	1.51	2.75e-05
HSA-167287	HIV elongation arrest and recovery	6 of 36	1.51	2.75e-05
HSA-167158	Formation of the HIV-1 Early Elongation Complex	5 of 33	1.47	0.00035
HSA-113418	Formation of the Early Elongation Complex	5 of 33	1.47	0.00035
HSA-5250913	Positive epigenetic regulation of rRNA expression	11 of 74	1.46	7.80e-10
HSA-167172	Transcription of the HIV genome	10 of 73	1.42	1.24e-08
HSA-73772	RNA Polymerase I Promoter Escape	8 of 59	1.42	1.04e-06
HSA-6781823	Formation of TC-NER Pre-Incision Complex	7 of 53	1.41	1.08e-05
HSA-167200	Formation of HIV-1 elongation complex containing HIV-1 Tat	6 of 46	1.4	8.87e-05
HSA-167152	Formation of HIV elongation complex in the absence of HIV Tat	6 of 48	1.38	0.00010
HSA-427413	NoRC negatively regulates rRNA expression	9 of 74	1.37	2.57e-07
HSA-674695	RNA Polymerase II Pre-transcription Events	10 of 84	1.36	3.56e-08
HSA-72165	mRNA Splicing - Minor Pathway	6 of 52	1.35	0.00015
HSA-212165	Epigenetic regulation of gene expression	13 of 116	1.34	1.78e-10
HSA-168325	Viral Messenger RNA Synthesis	5 of 44	1.34	0.0012
HSA-6782210	Gap-filling DNA repair synthesis and ligation in TC-NER	7 of 64	1.33	2.87e-05
HSA-6807505	RNA polymerase II transcribes snRNA genes	8 of 74	1.32	4.72e-06
HSA-6782135	Dual incision in TC-NER	7 of 65	1.32	3.10e-05
HSA-9670095	Inhibition of DNA recombination at telomere	5 of 48	1.3	0.0017
HSA-112382	Formation of RNA Pol II elongation complex	6 of 61	1.28	0.00032
HSA-9018519	Estrogen-dependent gene expression	11 of 119	1.25	4.40e-08
HSA-6796648	TP53 Regulates Transcription of DNA Repair Genes	6 of 65	1.25	0.00041
HSA-72187	mRNA 3-end processing	5 of 56	1.24	0.0033
HSA-5617472	Activation of anterior HOX genes in hindbrain development dur...	8 of 91	1.23	1.90e-05
HSA-73856	RNA Polymerase II Transcription Termination	5 of 65	1.17	0.0063
HSA-5696398	Nucleotide Excision Repair	8 of 109	1.15	5.46e-05
HSA-72163	mRNA Splicing - Major Pathway	12 of 178	1.12	1.69e-07
HSA-5578749	Transcriptional regulation by small RNAs	5 of 76	1.11	0.0116
HSA-157579	Telomere Maintenance	6 of 92	1.1	0.0025
HSA-162906	HIV Infection	11 of 232	0.96	2.07e-05
HSA-3108232	SUMO E3 ligases SUMOylate target proteins	7 of 166	0.91	0.0064
HSA-5633007	Regulation of TP53 Activity	6 of 159	0.86	0.0391
HSA-3700989	Transcriptional Regulation by TP53	13 of 363	0.84	2.62e-05
HSA-74160	Gene expression (Transcription)	49 of 1455	0.81	6.90e-25
HSA-5663202	Diseases of signal transduction by growth factor receptors and...	12 of 392	0.77	0.00029
HSA-73894	DNA Repair	9 of 309	0.75	0.0078
HSA-1640170	Cell Cycle	15 of 647	0.65	0.00035
HSA-8953854	Metabolism of RNA	15 of 659	0.64	0.00041
HSA-73857	RNA Polymerase II Transcription	25 of 1318	0.57	5.65e-06
HSA-168249	Innate Immune System	19 of 1025	0.56	0.00033
HSA-5663205	Infectious disease	15 of 826	0.55	0.0053
HSA-212436	Generic Transcription Pathway	19 of 1197	0.49	0.0026
HSA-1643685	Disease	21 of 1548	0.42	0.0079
HSA-168256	Immune System	24 of 1956	0.38	0.0097

Figure 4.10. Interactome of TFIIIC220. *(A) The interactome of TFIIIC220 (represented in red node in centre) as predicted from STRING database (string-db.org)*

with medium confidence (0.4). Apart from known interactors like basal transcription machineries, acetyltransferase coactivators, many proteins that are involved in various other cellular pathways are identified. (B) List of REACTOME pathways based on interacting partners of TFIIIC220 as shown in (A).

Apart from some posttranslational modifications deposited on RNAPIII, TFIIIB and TFIIIC can perceive this stress signals for RNAPIII by interacting with various proteins that get activated upon stress or infection. mTORC1, viral protein E1A has been found to interact with TFIIIC220 (Kantidakis *et al.*, 2010; Datta et al, 1991). Recently, TFIIIC gene was found to be differentially expressed under a particular stress condition in plants like Sorghum bicolor (Gelli *et al.*, 2017).

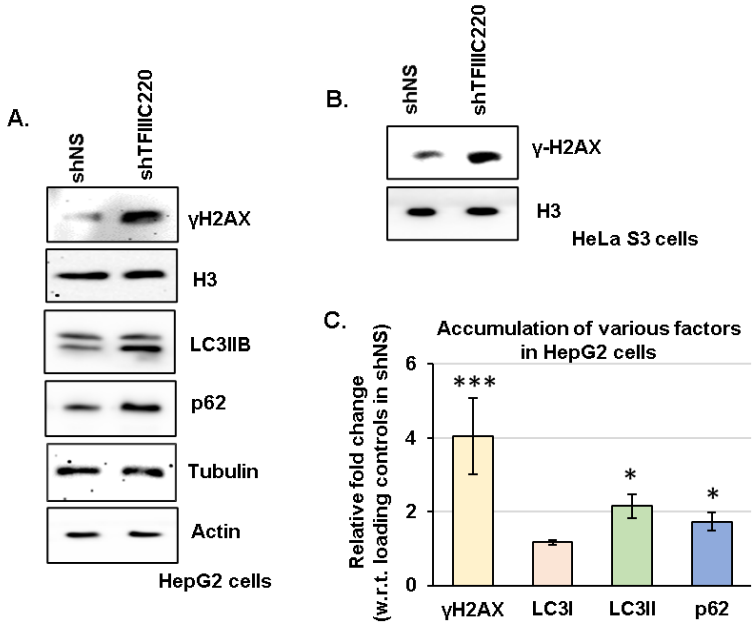


Figure 4.11. Accumulation of stress markers in TFIIIC220KD cells. *shRNA expressing HepG2 cells (in A) and HeLa S3 cells (in B) were lysed after doxycycline treatment and loaded onto 12-15% SDSPAGE to immunoblot with antibodies against γH2AX, LC3IIB, p62, actin, tubulin and H3. (C) Quantitative analysis of relative levels of γH2AX (against H3), LC3I, LC3II, p62 (against Actin) in shTFIIIC220 normalized against that of shNS cells as observed in immunoblot in (A). N=3. Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005.*

In silico interactome study piqued some proteins associated with cellular stress signaling components as potential interactors of TFIIIC220 (Figure 4.10). Considering the effect of TFIIIC220 depletion leading to global hypoacetylation of H3K18 accompanied by

downregulation of RNAPIII-mediated transcription led us to investigate the status of stress related factors in TFIIIC220 knockdown cells.

γ-H2AX (S139 phosphorylated H2AX variant) is one the most well-established chromatin modification mark related to DNA damage and repair. γ-H2AX is an extremely sensitive and reliable marker for DSB (double strand break), and it plays key role in signaling and initiating repair of DSBs. It can increase DNA accessibility through chromatin modification facilitating recruitment and assembly of specific repair proteins. such as BRCA1, 53BP1, Rad51, MDC1 *etc.* It can facilitate DSB rejoining by anchoring broken ends through nucleosome repositioning at damaged site. In case of low level of DNA damage γ-H2AX also signals to checkpoints allowing time for damaged site to get repaired (reviewed in Mah *et al.,* 2010). In fact, cohesins have been found to be recruited through γ-H2AX in damaged sites to prevent loss of large chromosomal regions (Strom *et al.,* 2004). In TFIIIC220KD cells (both HepG2 and HeLa S3) a significant level of accumulation of γ-H2AX was observed (Figure 4.11. A and B). Much lesser level of γ-H2AX was observed in non-silencing shRNA treated cells which negated the probable effect of doxycycline.

Parallelly, microtubule-associated protein 1A/1B-light chain (LC3) is ubiquitously expressed soluble protein that gets recruited to autophagosomal membrane upon conjugation with phosphatidylethanolamine during autophagy. LC3-phosphatidylethanolamine conjugate (LC3-II) is degraded inside autolysosomes by lysosomal hydrolases. Turnover of LC3II is widely used method for monitoring autophagy, autophagy-related processes including cell death (reviewed in Tanida *et al.,* 2008). TFIIIC220 knockdown in HepG2 cells led to accumulation of lipidated form of LC3II (lower band in LC3 blot in Figure 4.11. A), concomitantly the soluble form LC3I was also found to increase slightly but not significantly upon depletion of TFIIIC220 (Figure 4.11.C). A transient increase in LC3II level can indicate accumulation of autophagosomes but does not reflect autophagic degradation (Mizushima and Yoshimori, 2007).

Measuring p62 (SQSTM1/sequestosome 1) degradation is an alternative method for detecting autophagy level in a cell. During autophagy p62 can bind LC3 serving as a selective substrate of autophagy, p62 is also degraded in autolysosomes. As a rule of thumb decrease in p62 level is associated with higher autophagic flux. In TFIIIC220KD

condition in HeLaS3 cells p62 was found to be accumulating (in Figure 4.11.B) indicating a probable case of autophagic suppression. However, the expression level of p62 is not sufficient to estimate autophagic flux as its level can be affected independent of autophagy as well (Nakaso *et al.,* 2004; Bardag -Gorce *et al.,* 2005; Kuusisto *et al.,* 2001).

The aforementioned observations suggested a possibility of TFIIIC220 being involved in stress response (DNA damage and autophagy) pathways, hence, further experiments were carried out to obtain clarity in this regard.

4.3.4. TFIIIC220 knockdown blocks autophagy

To have an apprehension of the status of autophagy in cells with downregulated TFIIIC220, the level of LC3II and p62 accumulation in those cells was compared to that of Bafilomycin treated cells. Bafilomycin inhibits V-ATPase and thus acidification of lysosomes. These lysosomes cannot fuse with autophagosomes thereby leading to blockage of autophagy at a late stage (Yoshimori *et al.,* 1991). Consequently, Bafilomycin treated cells accumulate a large amount of un-recycled cargo adapter proteins such as LC3IIB, p62 *etc.* As observed earlier in Figure 4.11, TFIIIC220 knockdown cells are autophagy competent and depletion of TFIIIC220 led to a significant increase in LC3II and p62 level as compared to non-silencing control cells. After Bafilomycin treatment the level of accumulation of two adapter proteins were further increased. While the increase in LC3II level (Figure 4.12.C) and relative LC3II (Figure 4.12.D) were found to be significant in TFIIIC220KD upon Bafilomycin treatment as compared to untreated cells, accumulation of p62 was increased but the change was not significant (Figure 4.12.D). The accumulation of LC3II and p62 in TFIIIC220KD cells indicated that in absence of TFIIIC220 the degradation of autophagic cargos were affected moderately and Bafilomycin treatment aggravated TFIIIC220KD mediated autophagic suppression. As TFIIIIC220 KD did not show phenotype as dramatic as that observed upon V-ATPase blockage by Bafilomycin, it was assumed that the mild suppression in TFIIIC220 depleted condition stems from blockage of late stages of autophagy, but this in turn might be an effect of low or insufficient supply of late stage autophagosomal and lysosomal components or deficiency in signaling pathway that promotes autolysosome formation.

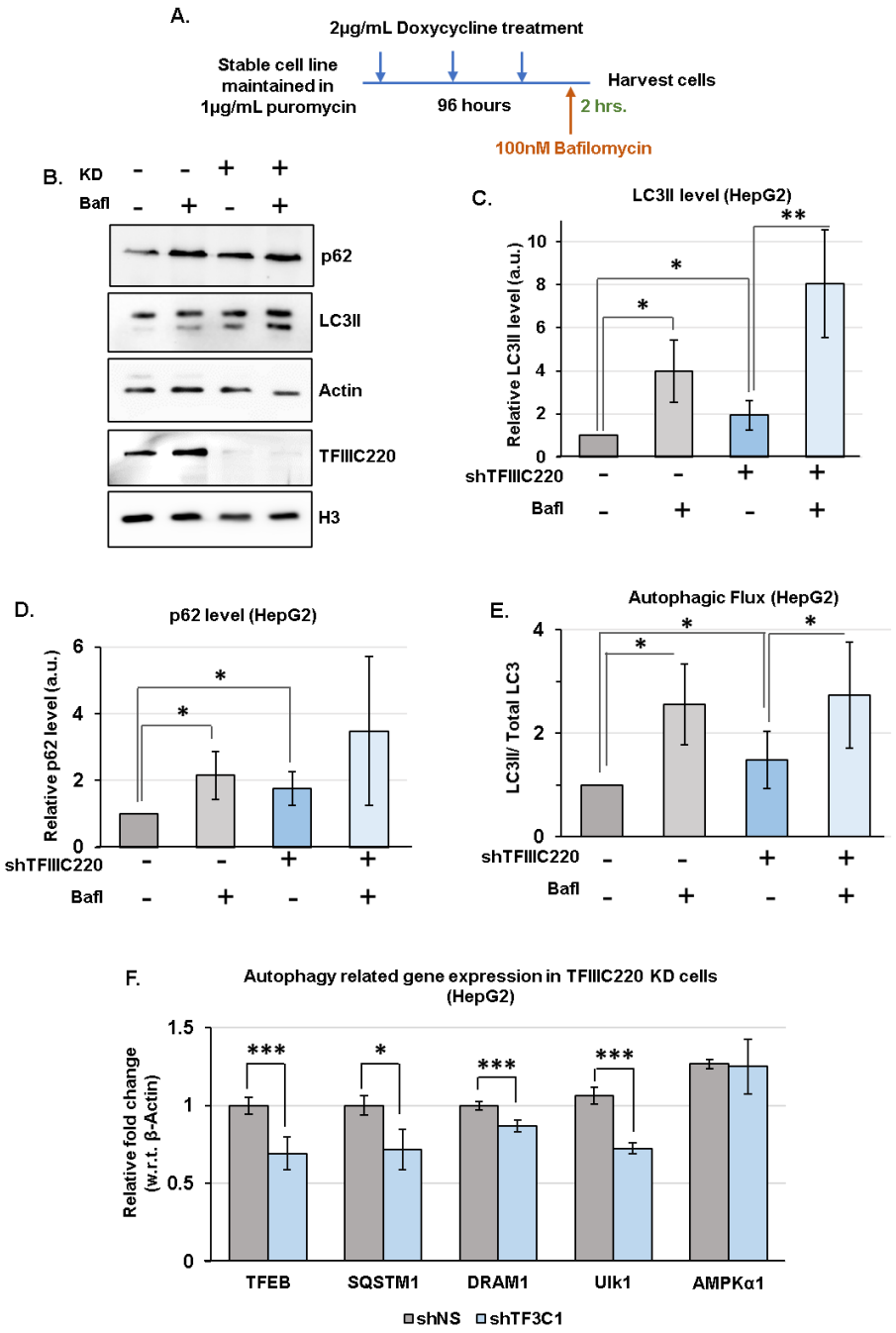


Figure 4.12. Status of autophagy in TFIIIC220 KD cells. *(A) Schematic representation of experimental setup for B-E. (B) Bafilomycin treated HepG2 cell lysates (Laemmli) were run onto 12% SDSPAGE and transferred onto PVDF membrane before probing with antibodies specific for proteins as indicated. C-E. Quantitation of relative intensities of LC3II (in C), p62 (in D) and fraction of LC3II in total LC3 (in E) obtained from western blotting analysis of TFIIIC220 KD cells; N=4, Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005. (F) qPCR analysis of autophagy related gene*

(TFEB, SQSTM1, DRAM1, Ulk1, AMPKα1) in TFIIIC220KD cells. N=3, Statistical analysis: Students' t test P<0.05, P**<0.01, P***<0.005.*

To test the first hypothesis, the expression of various genes related to autophagy in TFIIIC220KD cells were studied. In HepG2 cells downregulation TFIIIC220 was found to be associated with significant downregulation of TFEB (transcription factor EB), p62 (SQSTM1), DRAM1 (DNA damage regulated autophagy modulator 1), and Ulk1 (Unc like kinase 1) (Figure 4.12.F). The expression of AMPKα1 was not modulated. Interestingly, TFEB is a master regulator of lysosomal biogenesis that coordinates expression of lysosomal hydrolases, membrane proteins (GABARAP, MAP1LC3B, and ATG5), genes involved in autophagy (BECN1, WIPI1, ATG9B, NRBF2), genes important for substrate capture (SQSTM1) and for autophagosomes trafficking and fusion with lysosomes (UVRAG, RAB7) (Settembre *et al.*, 2011). Hence, downregulation of TFEB alone is sufficient to suppress autophagic process. Ulk1 is another protein that promotes fusion of autophagosome to lysosome (Wang *et al.*, 2018). However, AMPK mediated phosphorylation of Ulk1 is required for its association with ATG13 and autophagy related function and increase in transcription of AMPK was not observed. AMPK was found to be a regulator of not only Ulk1 but many other nodes in autophagy such as PI3K, forkhead box proteins, TFEB translocation etc. (Tamargo-Gomez and Marino, 2018). However, AMPK activity is dependent on posttranslational modification (Thr 172 phosphorylation by LKB1, CAMK2 *etc.*) rather than on its transcript level. If TFIIIC220 KD leads to deregulation of autophagic signaling as well remains to be investigated.

4.3.5. TFIIIC220 is upregulated in autophagy

Following previous observation on autophagic suppression in TFIIIC220KD cells the next logical step was to understand the relevance of TFIIIC220 in autophagy regulation. 24 hours incubation of HepG2 in low serum (0.5%) or no serum induced autophagy which was measured by higher LC3II level. TFIIIC220 was found to be increased in protein level (Figure 4.13.B). The increase in TFIIIC220 in protein level upon serum starvation appeared to be synchronous with increase in LC3II level (Figure 4.13. C).

As TFIIIC220 as a component of TFIIIC complex is known to regulate RNAPII transcription from TSSs located adjacent to ETCs (discussed in detail in Chapter 1), the presence of TFIIIC220 binding B -box elements were investigated

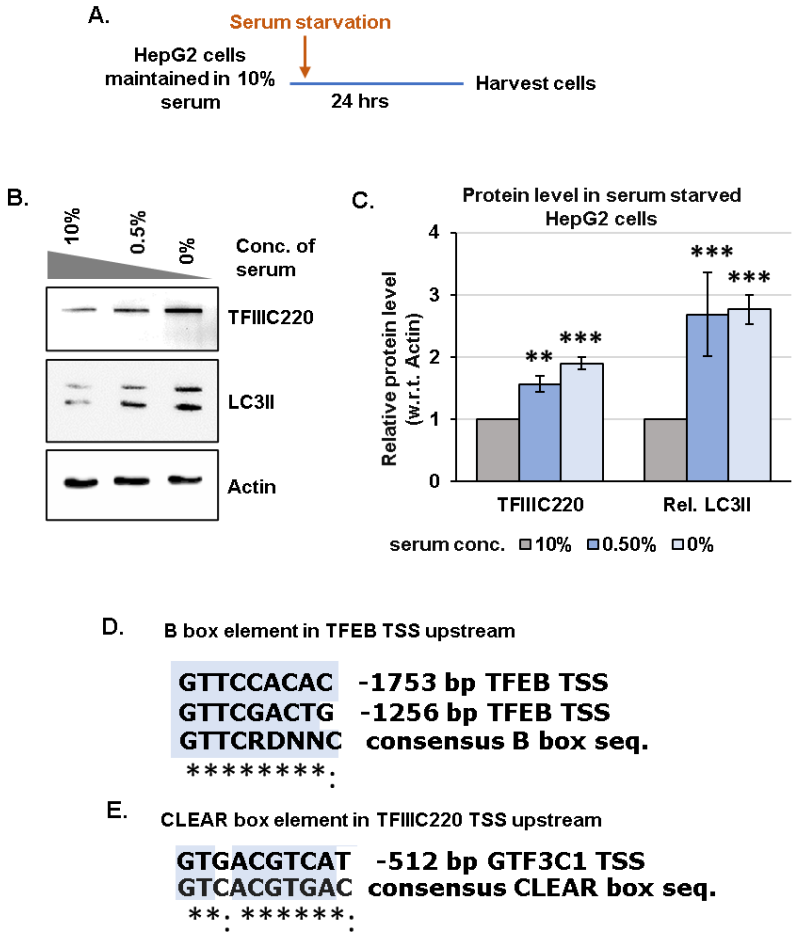


Figure 4.13. TFIIIC220 and autophagy. *(A) Schematic representation of experimental setup for B. (B) HepG2 cells were incubated in media supplemented with 10, 0.5 and 0% FBS for 24 hours and lysates were run on 10 and 12% SDSPAGE before transferring onto PVDF membrane. Blots were probed with antibodies specific for TFIIIC220, LC3II and Actin. (C) Quantitation of relative intensities of LC3II and TFIIIC220 (normalized against loading control Actin) obtained from western blotting analysis serum starved HepG2 cells; N=3, Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005.(D) and (E) Alignment of consensus B and CLEAR box motifs with best matches obtained from TFEB (in D) and TFIIIC220 (in E) upstream elements (within 2kb upstream of TSS) respectively.*

Further speculation into the probable scenario of autophagy related genes being transcriptionally regulated by TFIIIC220 and vice versa, it was observed that TFIIIC220 promoter region (within 1kb upstream of TSS) contained a consensus sequence for CLEAR box motif that recruits TFEB and TFEB upstream element (within 2K upstream of TSS) possessed a consensus B-box motif that is recognized by TFIIIC220. However, if TFIIIC220 and TFEB controls the expression of each other in reality in different cellular context remain to be elucidated.

It is noteworthy that acetyltransferases are commonly known to be involved in downregulation or impairment of autophagy; GCN5 overexpression in mammalian or Drosophila model increased TFEB acetylation inactivating its function leading to impaired autophagy (Wang *et al.,* 2020). During fasting decline in acetyl-CoA level in brain reduced p300 mediated acetylation of mTORC1 decreasing its activity to stimulate autophagy (Son *et al.,* 2019; Son *et al.,* 2020). Hence, apart from transcriptional regulation if TFIIIC220 regulate TFEB expression through acetylation as well remains to be investigated.

4.3.6. TFIIIC220 is upregulated in various cellular stress conditions

Early reports and our previous findings indicated a possibility of TFIIIC220 expression being regulated by various stress factors. To elucidate the premise the level of TFIIIC220 in cells that were subjected to genotoxicagent-mediated stress was examined. 5-Fluorouracil (5FU) or Adrucil is an anti-metabolite cytotoxic drug widely used for solid tumors, including of the breast, gastrointestinal system, head and neck, and ovary (Holland *et al.,* 2006). 5-FU and its metabolites can exert anti-proliferative effects through inhibition of thymidylate synthetase (TS) and/or incorporation of uridine and thymidine into RNA and DNA (Longley *et al.,* 2003). Sublethal dosage of 5FU treatment (150-750µM) over different time points (8-24 hours) led to a concomitant increase or stabilization of TFIIIC220 protein level along with p53, a multifunctional stress-related protein (Figure 4.14.C). Induction of DNA damage did not lead to TFIIIC220 foci formation unlike stress-granule related proteins or aberrant cytoplasmic or nucleolar translocation of TFIIIC220 (Figure 4.14.E).

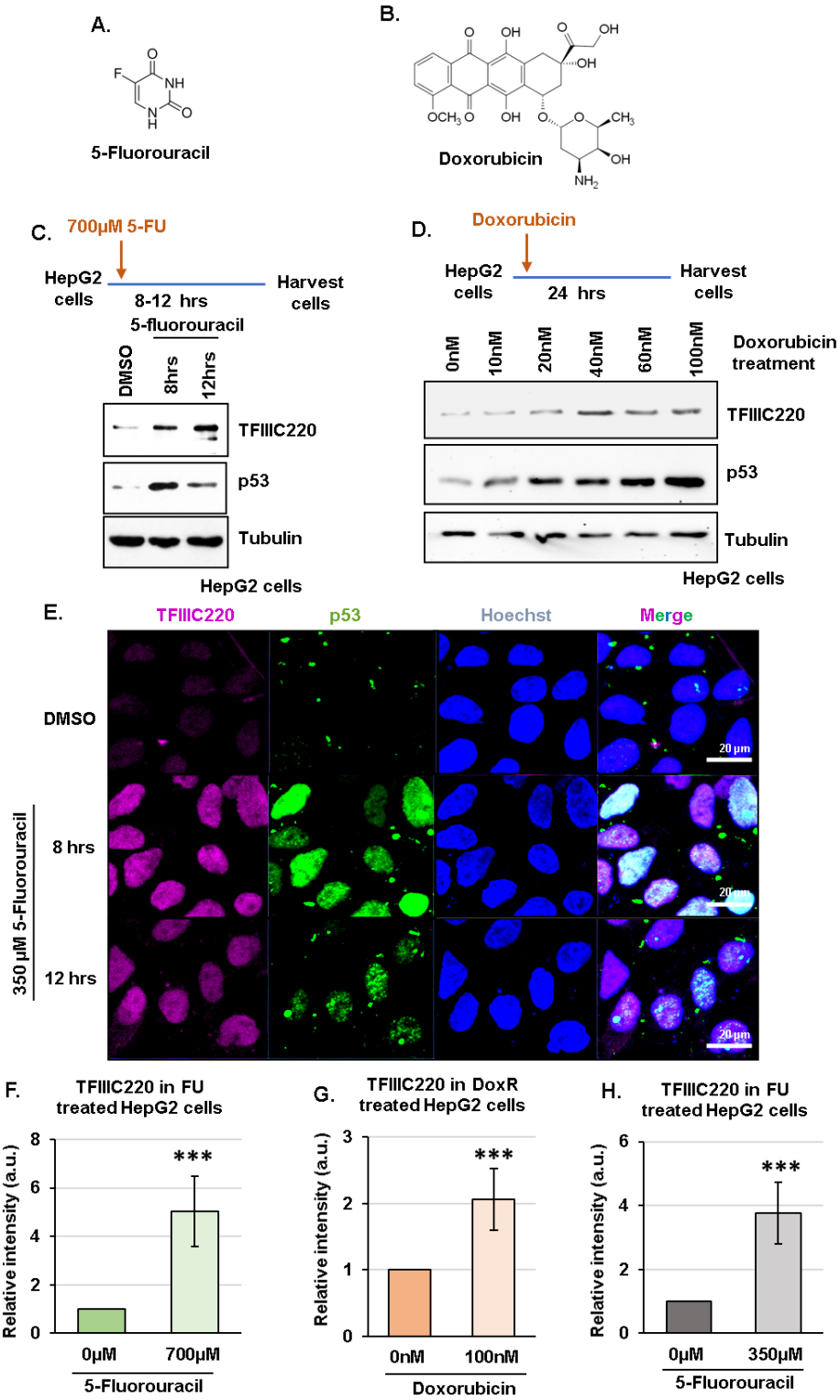


Figure 4.14. Upregulation of TFIIIC220 upon genotoxic insult in HepG2 cells. *(A) and (B) Structures of 5 fluorouracil and Doxorubicin. (C) and (D) HepG2 cells were treated with 350μM 5-FU for 8-12 hours (in C) or 10-100nM concentration of Doxorubicin for 24 hours (in D). Cell lysates were run onto 8-12% SDS PAGE and immunoblotted with antibodies specific for TFIIIC220, p53 and α-tubulin (loading*

control). (E). Immunofluorescence images of HepG2 cells treated with 350µM 5-FU for 8-12 hours, stained with TFIIIC220 and p53 specific antibodies. Hoechst stains nucleus. Scale bar: 20µm. (F-H) Quantitation of relative intensities of TFIIIC220 obtained from experiments performed in C-E respectively. N=3, Statistical analysis: Students' t test P<0.05, P**<0.01, P***<0.005.*

Doxorubicin or Adriamycin, an anthracycline antibiotic, is another effective and widely used chemotherapeutic agent for treatment of human various malignancies, including metastatic breast cancer, lymphomas and sarcomas, neoplasms etc. but its major adverse effect is cardiotoxicity (Chatterjee *et al.,* 2007; Takemura *et al.,* 2007). Various models have been proposed to elucidate the molecular mechanisms underlying the adverse effects of Doxorubicin on growing cells. It can intercalate with DNA forming adducts and interfering with DNA templated phenomena such as replication, repair, transcription accumulating DNA damage, topoisomerase II poisoning resulting into torsion-induced nucleosome destabilization and ceramide overproduction (Gewirtz *et al.,* 1997; Yang *et al.,* 2014). Different dosage of Doxorubicin for varying time point is shown to have different effects on the cells which ultimately results into cellular toxicity (Lupertz *et al.,* 2010). HepG2 cells when treated with 10-100nM Doxorubicin for 24 hours, accumulated a higher level of TFIIIC220 as compared to DMSO treated cells (Figure 4.14.D).

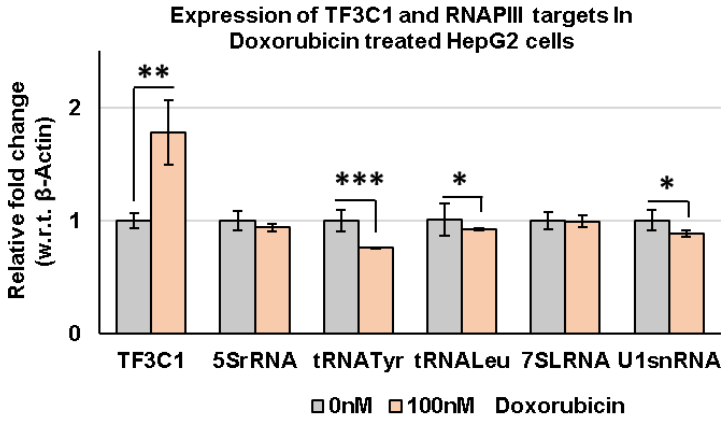


Figure 4.15. GTF3C1 and RNAPIII-targeted gene expression upon genotoxic insult. *qPCR analysis of GTF3C1 and RNAPIII transcripts' (5SrRNA, $tRNA^{Tyr}$, $tRNA^{Leu}$, 7SLRNA and U1 RNA) mRNA expression after24 hours of 0-100nM Doxorubicin treatment in HepG2 cells. N=3, Statistical analysis: Students' t-test, P*<0.05, P**<0.01, P***<0.005.*

To ensure that TFIIIC220 accumulation in stress was not a bystander effect of treating cancer cells (HepG2) with sublethal dosage of genotoxic agents the levels of expression of various RNAPIII target transcripts were checked. The mRNA level expression of TFIIIC220 was found to be almost doubled upon induction of stress for 24 hours which was not accompanied with similar change in transcript levels of 5SrRNA, tRNAs, 7SLRNA and U1snRNA. In fact, transcript levels of tRNAs and U1snRNA were found to be decreased significantly under stress (Figure 4.15).

Interestingly, in unfavorable conditions TFIIIC has also been found to repress RNAPIII mediated transcriptional elongation leading to transcriptional pausing in yeast by tightly binding to cognate binding sites and sequestering TFIIIB components (Brf1 and Bdp1) (Ciesla *et al.*, 2018). If the similar scenario is manifested in human as well remains to be investigated. However, it would not explain the increase in TFIIIC transcript and protein level upon induction of stress as observed in our study.

Collectively these findings substantiate the prospect of TFIIIC220 having implication in stress-related function in cells.

4.3.7. Probable connection between p53 and TFIIIC220

Accumulation of protein level of TFIIIC220 upon induction of both genotoxic stress and serum starvation indicated a possibility of TFIIIC220 having a common role in stress sensing or responding. One such master regulator and transcription factor that integrates multiple stress response pathways in cell is p53. Known as 'guardian of genome' p53 once activated and stabilized can regulate transcription of multiple genes (activating as response element bound transcription factor or with p300/CBP or various components of chromatin remodeler, mediator and elongation complex, repressing with HDAC1/corepressor Sin3 or through sequestration of response elements from other TFs) that leads to cell survival or death in context dependent manner, and, can induce transcription independent pathways promoting apoptosis. Upon genotoxic insult increase in TFIIIC220 protein level was found to be similar to that of stabilization of p53 protein; it is highly likely that p53 might act as a regulator of TFIIIC220 expression or stability. Contrarily, TFIIIC220 might also exert its effect through activation and stabilization of p53. To understand the interrelation (if any) between p53 and TFIIIC220, the level and function of p53 in TFIIIC220 KD cells were probed. In TFIIIC220 KD cells the protein

level of p53 was not found to be different as compared to non-silencing cells (Figure 4.16B) indicating TFIIIC220 might not act upstream of p53 signaling pathways under stress conditions. Also, to find if p53 and TFIIIC220 coregulate downstream pathways through protein-protein interaction or co-recruitment to individual response elements the status of expression of various p53 target genes were checked in TFIIIC220KD cells. Upon induction of DNA damage p53 can induce expression of p21 which in turn blocks CDK 1 and 2 causing G1/S cell cycle arrest. Upon stress p53 induces transcription of pro-apoptotic genes including PUMA, Bax etc. (reviewed in Beckerman and Prives, 2010). The expression of these direct transcriptional targets of p53 *i.e.*, Bax, PUMA(BBC3), and p21 A and B were found to be significantly repressed in TFIIIC220 KD cells (Figure 4.16.C). On the other hand, Survivin, a member of inhibitor of apoptosis protein that directly binds and inhibits caspases thereby blocking apoptosis upon induction by various apoptotic triggers was also found to be significantly downregulated upon TFIIIC220KD (Figure 4.16.C). Survivin and p53 were shown to regulate each other at transcriptional level; p53 activation leads to repression of Survivin expression at both mRNA and protein level (Mirza*et al.,* 2002), and, ectopic expression of Survivin repressed expression of p53 mRNA and overexpression of Survivin counteracts p53 mediated apoptosis (Wang *et al.,* 2004; Mirza *et al.,* 2002). Interestingly, neither downregulation of proapoptotic Bax, PUMA, nor downregulation of anti-apoptotic Survivin reflected onto the cellular viability of TFIIIC220 KD cells. The intake of DAPI (which signifies damaged membrane in apoptotic cells) in TFIIIC220KD cells were found to be equivalent to that of non-silencing control (Figure 4.16.E). The expression of a few other targets and associated factors related to p53 were also investigated in TFIIIC220KD cells. Foxo4 and p53 are known to coordinate various pathways under stress conditions. Both of these TFs share multiple common targets (*e.g.*, p21) and downstream pathways leading to cell cycle arrest, apoptosis, senescence or survival (reviewed in Bourgeois and Madl, 2018). No significant change in the level of Foxo4 transcript was observed in TFIIIC220KD cells (Figure 4.16.C). 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is catabolic enzyme required to maintain a steady-state level of prostaglandins including PGE2, in hepatic cells (carcinoma) PGDH expression led to apoptosis and shunting of tumor growth through p21 dependent pathway and act as tumor suppressor (Lu *et al.,* 2014). Increased expression of PGDH transcript was observed in upon depletion of TFIIIC220 however, the change was not found to statistically significant (Figure 4.16.C).

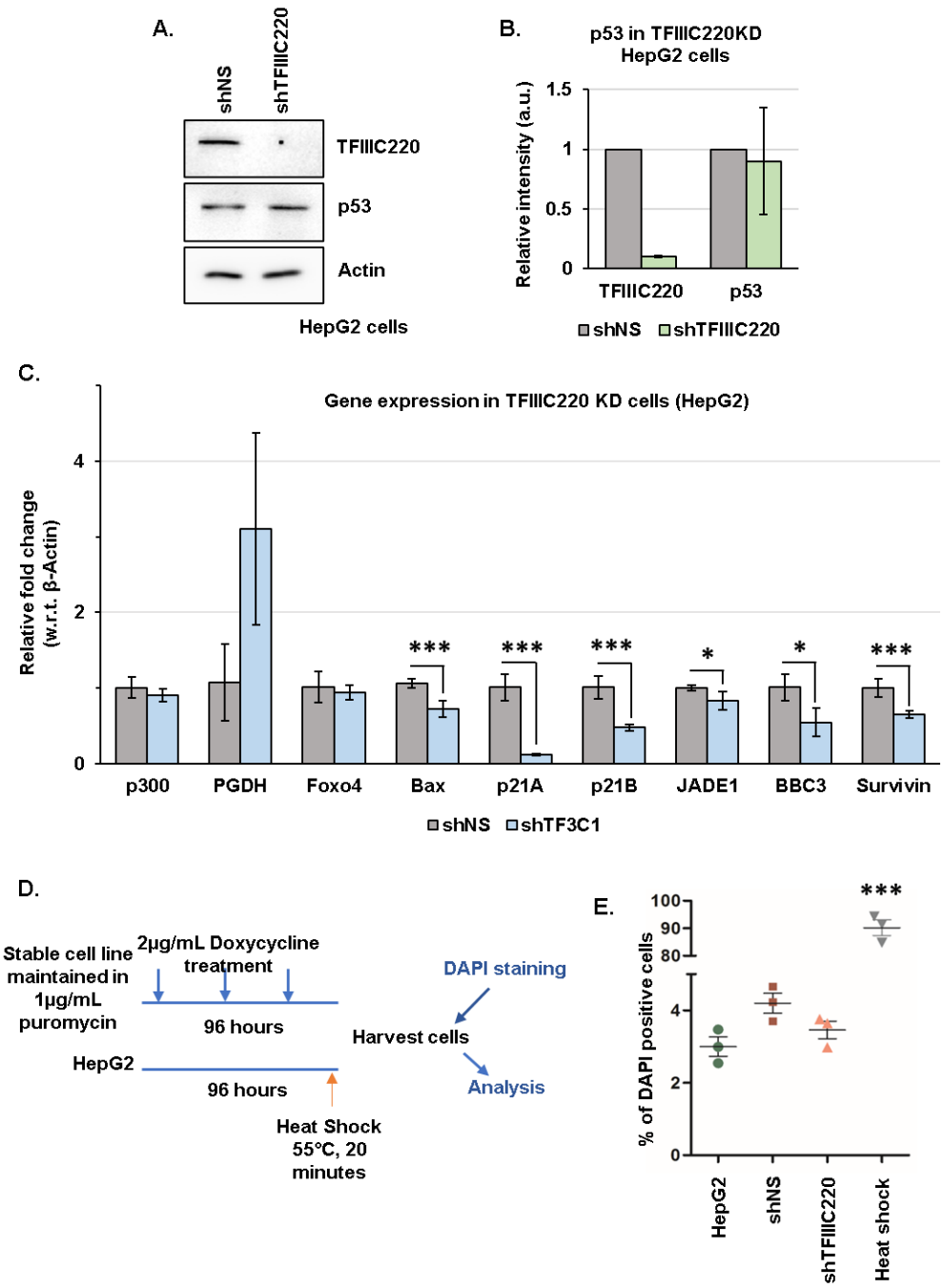


Figure 4.16. Correlation between p53 and TFIIIC220. *(A) shRNA expressing HepG2 cells were lysed after doxycycline treatment and loaded onto 8 and 12% SDSPAGE to immunoblot with antibodies against TFIIIC220, p53 and actin. (B) Quantitation of relative intensities of TFIIIC220 and p53 obtained from immunoblots from A. (C) qPCR analysis of p300, PGDH and p53 targetgenes' (Foxo4, Bax, p21A and B, Jade1, BBC3 and Survivin) mRNA expression in TFIIIC220KD HepG2 cells. N=3, Statistical analysis: Students' t-test, P*<0.05, P**<0.01, P***<0.005 (D) Schematic representation of experimental design for E. (E)Quantitation of percentage DAPI positive (stained)*

population of TFIIIC220KD HepG2 cells after sorting. Untreated and heatshock treated HepG2 cells were used as negative and positive controls respectively.

JADE1 is an acetyltransferase known for its cell cycle control and DNA damage response related function (reviewed in Panchenko *et al.,* 2016). p53 is known to regulate function of JADE1-HBO1 complex via protein-protein interaction upon stress signaling (Iizuka *et al.,* 2008). Surrisingly, JADE1 was found to be significantly downregulated upon TFIIIC220 downregulation (Figure 4.16.C). Finally, the transcript expression of p300, another acetyltransferase involved in H3K18ac and DNA damage foci regulation was not found to be changed upon TFIIIC220 downregulation.

Collectively, this set of observation suggest a probable p53 associated function of TFIIIC220 in transcriptional regulation of downstream genes however, the targets are not involved in universal stress response, instead they might be limited for specific response pathways only.

Next, to understand if p53 stabilization affects the accumulation of TFIIIC220 in stressed cells, the level of TFIIIC in Nutlin 3a treated cells were studied. Nutlin 3a is a small molecule antagonist of Mdm2, it stabilizes p53 in cell by targeted disruption of Mdm2 mediated p53 degradation (Vassilev *et al.,* 2004). 24hours Nutlin 3a or p53 stabilization in HepG2 cells which have wild-type p53 led to an unexpected degradation (a faint signal of degraded product corresponding to molecular weight of ~75kDa was detected) of TFIIIC220 (Figure 4.17.B). Similar effect of Nutlin3a treatment was not phenocopied in p53 null UMSCC-1 cell-line (Figure 4.17.B and E) highlighting that the effect observed was not due to pleiotropic effect brought about by Nutlin 3a treatment but due to a change in p53 level (Figure 4.17. B). Interestingly, Nutlin 3a treatment was rather associated with modest but significant stabilization of TFIIIC220 protein level. Additionally, another component of TFIIIC complex, TFIIIC110 was also appeared to be degraded upon p53 stabilization in HepG2 cells but not in UMSCC-1 cells (Figure 4.17.B). Induction of p53 has been reported to promote degradation of Brf1, a TFIIIB subunit, in TR9-7 fibroblast cells (Eichorn and Jackson, 2001), but not in HeLa, MEF or H1299 cells expressing exogenous p53 (Crighton *et al.,* 2003). If this cell-line specific control of p53 extend to other general transcription factors including TFIIIC as well remains to be investigated. However, unlike TFIIIB, TFIIIC is not known to interact with p53 under various stress conditions (Crighton *et al.,* 2003).

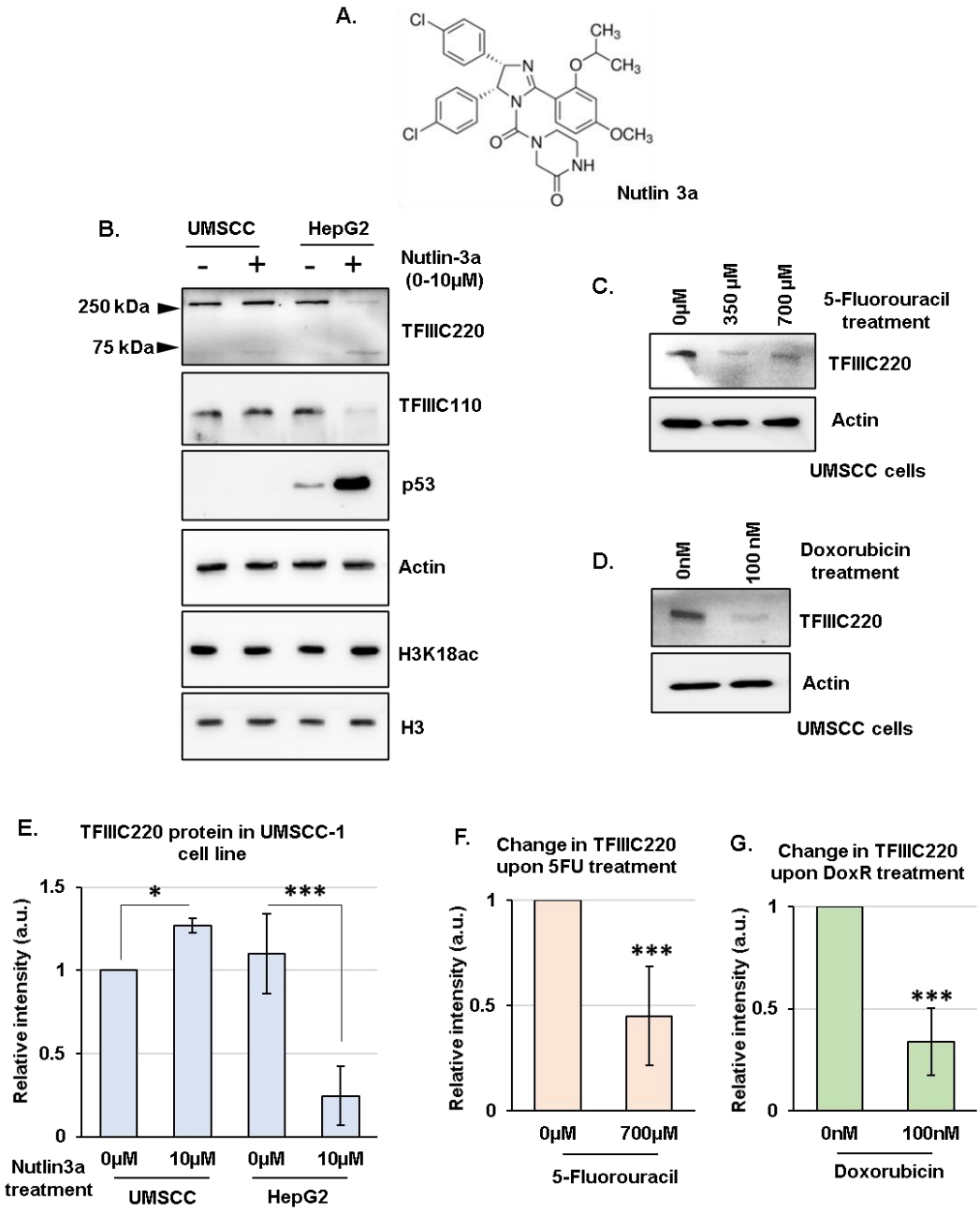


Figure. 4.17. Expression of TFIIIC220 in p53-null cells. *(A) Structure of Nutlin 3a. (B)HepG2 andUMSCC-1cells were treated with 10μM Nutlin3a for 24 hours. Cell lysates were run onto 8-12% SDS PAGE and immunoblotted with antibodies specific for TFIIIC220, TFIIIC110, p53, H3K18ac and actin, H3 (loading controls). (C) and (D) Cell lysates of 0-700μM 5-fluorouracil treated (in C) and 0-100nM Doxorubicin treated (in D) UMSCC-1were run onto 8-12% SDS PAGE and immunoblotted with antibodies specific for TFIIIC220 and actin (loading control). (E-G) Quantitation of relative intensities of TFIIIC220 obtained from experiments performed in B-D respectively. N=3, Statistical analysis: Students' t test P*<0.05, P**<0.01, P***<0.005.*

Finally, to understand if the upregulation of TFIIIC220 protein level upon genotoxic stress is an effect of p53 stabilization, the levels of TFIIIC220 protein in UMSCC-1 was studied upon treatment with chemotherapeutic agents. Neither 5FU nor Doxorubicin treatment could stabilize TFIIIC220 in UMSCC cells, rather the expression was found to be significantly reduced (Figure 4.17. C and D). These results indicated the possibility of p53-mediated transcriptional control or protein level stabilization of TFIIIC220. Parallelly, these results also suggest cell dependent variation in involvement of TFIIIC220 in cellular stress response.

4.4. Summary

This study provided experimental evidences to establish TFIIIC220 as a bona fide histone acetyltransferase. Full length TFIIIC220 could acetylate nucleosomal and free histone H3, H4 and H2A like holo-TFIIIC-complex. Based on the presence of conserved 'P loop' acetyl-CoA binding motif in TFIIIC220 the putative acetyltransferase domain in TFIIIC220 was identified and recombinant putative KAT domain of TFIIIC220 could acetylate H3 and modified H3K18 acetylation *in vitro*. Interestingly, TFIIIC220 knockdown led to a global decrease in H3K18ac level which was rescued by overexpression of functional putative KAT domain against a knockdown background. TFIIIC220 knockdown was also associated with accumulation of DNA damage and autophagy related markers. Autophagy was found to be suppressed upon TFIIIC220 KD as autophagy related genes including master regulator of lysosomal biogenesis *i.e.* TFEB were found to be downregulated in TFIIIC220 KD cells. Upon induction of stress related to genotoxic insult and serum starvation TFIIIC220 was found to be stabilized and this stabilization was found to be dependent on p53. TFIIIC220KD also downregulated the expression of a few p53 target genes.

Chapter 5

Discussion

This chapter reflects on the major findings of the current study. Here the results of the thesis will be critically examined in the light of the known literature and background, emphasizing on the highlights and the probable outcomes of the study.

5.1.1. p300 mediated acetylation is required for proper differentiation of embryonic stem cells

To study the relevance of p300 mediated acetylation in embryonic stem cell differentiation a small molecule modulator of p300 acetyltransferase activity, Luteolin was used. Although an established method of overexpressing catalytic mutant against a null background of acetyltransferase enzyme could have been used as a direct approach to address this issue, a specific small molecule modulator was used simply because of easier manipulation and application. However, both the aforementioned techniques have drawbacks, and that of using small molecule modulator is the pleiotropic non-specific effects exerted by it in the cells. This adjunct effect of Luteolin was handled by using a structural homolog of Luteolin, Apigenin as a negative control in this study. As discussed in chapter 3, multiple reports have established that both Luteolin and Apigenin exert similar range of toxic effects on cancer cells and beneficial effects on normal and stem cells. Luteolin stably bound and inhibited p300 acetyltransferase activity while Apigenin could not. Since p300 and CBP share high structural and functional homology between them and many small molecule inhibitors specific for p300 was also found to inhibit CBP mediated acetylation, there was a possibility of Luteolin affecting CBP-mediated acetylation as well. In terms of embryonic development both p300 and CBP were shown to possess redundant as well as nonredundant function. Ablation of p300 and CBP led to defect in neural tube formation; CBP-mediated acetylation has already been implicated in neural development and RTS disease manifestation (Tanaka *et al.*, 2000). Hence, to pinpoint the relevance of p300-mediated acetylation in embryonic development a p300-specific nontoxic inhibitor was required. Firstly, molecular docking study revealed a lower binding energy for Luteolin and CBP KAT domain as compared to that for

Luteolin and p300 KAT domain indicating the possibility of Luteolin being a specific inhibitor of p300 but not CBP. Secondly, luteolin treatment affected neuronal differentiation but not the expression of glial markers in differentiating EBs. CBP-mediated acetylation has already been established to be crucial for neuronal and glial differentiation (Wang *et al.,* 2010); hence, if Luteolin inhibited CBP mediated acetylation, the expression of glial markers GFAP and Pax6 in differentiating EBs would also have changed. Thirdly, inhibition of p300/CBP mediated acetylation is known to affect brachyury transcription in differentiating stem cells; in Luteolin treated EBs expression of brachyury was not found to be significantly downregulated indicating the presence of an alternative mechanism of deposition of H3K27ac onto brachyury TSS. In Luteolin treated EBs, lack of p300-mediated acetylation might be partially compensated by acetyltransferase activity of CBP.

Parallelly, Luteolin treatment did not affect transactivation property of p300 in stem cells. p300 is employed onto distal regulatory element and mediate long-range chromatin looping through KIX and IBID to induce transcription of pluri-genes in undifferentiated cells (Fang *et al.,* 2014). A direct target of p300 transcriptional coactivator function *i.e.,* Nanog and a pluripotency marker Dppa3 were not found downregulated in Luteolin treated undifferentiated cells indicating the effect of Luteolin to be limited for acetyltransferase domain of p300. Also, in differentiating EBs, Luteolin treatment did not have additional effect on Dppa3 and Nanog expression as compared to DMSO or Apigenin treated EBs indicating that stabilization of Oct4 by Luteolin does not confer stemness under differentiating conditions.

One of the intriguing observations made from Luteolin treated EBs was dramatic accumulation of Oct4 protein. While both Luteolin and Apigenin treatment led to significantly higher level of Oct4 mRNA expression in EBs, Apigenin treatment failed to translate such high produce of mRNA of Oct4 in protein level, luteolin treated EBs accumulated a much higher level of Oct4 protein indicating that p300-mediated acetylation might have negative effect on Oct4 stability. Among various other signals and posttranslational modifications that regulate Oct4 stability, ERK can be fit into a profile that gets activated upon LIF withdrawal (Cherepkova *et al.,* 2016), is associated with degradation of Oct4 (Spelat *et al.,* 2012), and, is known to induce p300 mediated acetylation (Jun *et al.,* 2010). If in differentiating EBs, ERK and ERK-mediated

activation of acetylation by p300 synergistically degrade Oct4 remains to be investigated. The hypothesis is summarized in figure 5.1.

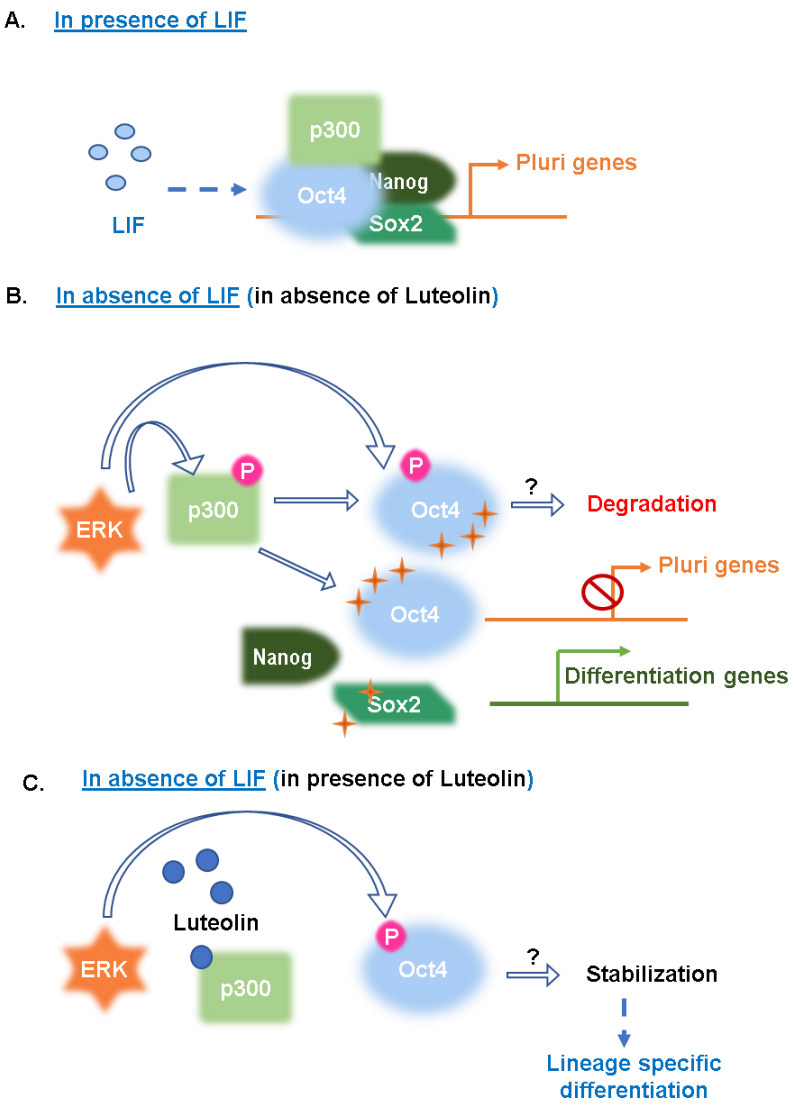


Figure 5.1. Hypothetical model for involvement of p300 mediated acetylation in early differentiation of ES cells. *(A) In presence of LIF (undifferentiating conditions) p300 maintains its transcriptional coactivator function and induces pluripotency-related genes. (B) In absence of LIF (differentiating condition) activated ERK phosphorylates Oct4 and targets it for degradation, in absence of Luteolin p300 acetylates Oct4 that might act cooperatively with ERK mediated phosphorylation of Oct4 thus degrading it. Also, acetylated Oct4 dissociates from Oct4-Sox2-Nanog complex pushing the ES cells for differentiation. (C) In differentiating condition Luteolin inhibits p300 mediated acetylation of Oct4 which might protect it from degradation. Protein level upregulation of Oct4 initiates abnormal differentiation.*

Deficiency in p300-mediated acetylation led to defect in ectodermal lineage specification during early differentiation and neuronal differentiation at a later stage. While the inhibition of ectodermal differentiation can be partially attributed to the smaller size of Luteolin treated EBs, the specific inhibition of later stage neuronal differentiation but not glial inhibition can be attributed to lack of p300-mediated acetylation.

However, to establish certainty regarding the involvement of p300-mediated acetylation in neuronal lineage specific development and Oct4 stabilization further experimental evidences are required.

5.1.2. Zebrafish possesses functional ortholog of human p300

In the next part of our study, functional ortholog of human p300 in zebrafish was identified and catalytic activity of the active domain was characterized. Interestingly, out of two orthologs of p300 in zebrafish which did not have any apparent remarkable dissimilarity in terms of sequence conservation and expression pattern across zygote and larvae in zebrafish, only one orthologs, p300a was found to be catalytically active *in vitro*. The acetyltransferase activity of p300a was found to be as robust and non-specific (at least for histone substrates) as its human counterpart. In fact, zebrafish p300a was found to be also sensitive to a highly specific small molecule inhibitor of human p300/CBP i.e., C646 indicating the possibility of possessing high level of functional conservation between the two proteins. Regarding the other apparently non-functional coorthologs in Zebrafish p300b, the protein might be assumed to be a weak acetyltransferase that targets nonhistone substrates (as observed in case of BRCA2 and TFIIB which have been shown to possess acetyltransferase activity only under specific conditions or autoacetylation properties only) that require the aid of a complex or, p300b is simply a nonenzymatic ortholog, a phenomenon that is quite common among organisms possessing multiple co-orthologs of p300/CBP e.g., *Arabidopsis, C. elegans etc.* It will be interesting to study the reason behind abundant expression of this inactive ortholog throughout larval development.

In a parallel study inhibition of acetylation by zebrafish p300a during early embryogenesis by either C646 treatment or morpholino-mediated knockdown manifested severe defects of ectodermal lineage specific larval development, most of which were rescued by overexpression of functional p300a KAT domain RNA but not

inactive p300b (Babu *et al.*, 2019). This set of observation corroborated the finding discussed earlier that p300 mediated acetylation is indispensable for proper ectodermal, neuronal lineage specific differentiation.

5.2. TFIIIC220 possesses acetyltransferase activity

In the second part of our study, TFIIIC, the largest DNA binding subunit of TFIIIC complex was demonstrated to be a functional acetyltransferase that could acetylate free as well as nucleosomal histones. Intriguingly, no other acetyltransferase in vertebrates have been characterized to possess both DNA binding and acetyltransferase function at the same time other than TFIIIC220. While most of the acetyltransferase in eukaryotic system has been linked to transcriptional coactivation from chromatinized template, the functional relevance of TFIIIC220 mediated acetylation may have more pronounced and direct effect on transcription. TFIIIC complex has already been demonstrated to be sufficient for relieving chromatin mediated repression and facilitating RNAPIII mediated transcription *in vitro* (Kundu *et al.*, 1999) Among prominent sites present on N-terminal tail on histone H3 and H4, TFIIIC220 only acetylated H3K18 residue. The H3K18 residue specific activity of TFIIIC220 was validated by *in vivo* knockdown of TFIIIC220. Along with several other acetylation marks, H3K18ac has also been reported to be enriched at TSS of both RNAPII and RNAPIII targets (Moqtaderi *et al.*, 2010). Previous knowledge on H3K18ac dynamics dictates a major share of nuclear H3K18 sites to be acetylated by p300/CBP; hyperacetylation of H3K18ac in cancer is correlated to hyperactivity of p300 (Halasa *et al.*, 2019). In fact, since p300 was identified to tightly associate with TFIIIC complex *in vitro* and *in vivo* it was assumed that weak acetyltransferase activity of TFIIIC is override by the action of p300in the RNAPIII target promoters (Mertens *et al.*, 2008; Park *et al.*, 2017). Yet, we observe a dramatic decrease in global acetylation of H3K18 upon knockdown of TFIIIC220 that was not associated with change in expression level or enzymatic activity of p300. A recent report recently provided experimental evidences supporting the view of TFIIIC controlled H3K18ac sites upon induction of serum starvation without the aid of p300 (Ferrari *et al.*, 2020). Interestingly, Sirt7, a bonafide deacetylase for nuclear and nucleolar H3K18ac was found to interact with TFIIIC220 and TFIIIC complex. Depletion of Sirt7 inhibited tRNA synthesis and upregulated cellular LC3II level (Tsai *et al.*, 2014). It might be

hypothesized that a balance between TFIIIC220 and Sirt7 is required for tRNA synthesis, and also, Sirt7 might counteract acetylation deposited by TFIIIC220 in regulatory regions of autophagy genes. Knockdown of Sirt7 might activate autophagy and upregulate LC3II level and TFIIIC220 knockdown suppresses autophagy and upregulates LC3II level. On the other hand, TFIIIC binding has been shown to have nucleosome positioning and chromatin organizing effect (Donze *et al.*, 2012), since H3K18ac is not known to associate with nucleosome eviction or positioning if TFIIIC220 acetylates other residues on core histones to facilitate this process or recruits other factors through its acetylation activity remains to be elucidated.

Now, the possible association of TFIIIC and its acetyltransferase activity in response to serum starvation has already been partially demonstrated by Ferrari *et al.*, 2020. However, it did not explain the change in TFIIIC level upon induction of cellular stress which was not observed in absence of p53. There might be two explanations for this. Firstly, as a few of p53 target genes were found to be downregulated in TFIIIC220 KD cells it might be hypothesized that TFIIIC acts as a transcriptional coactivator or upstream inducer for a set of p53 target genes. Secondly, p53 might control the steady state of TFIIIC complex subunits upon infliction of genetoxic insult as stabilization of p53 led to degradation of TFIIIC220 and 110. Further study is underway to to explain this dichotomy in the relationship between p53 and TFIIIC.

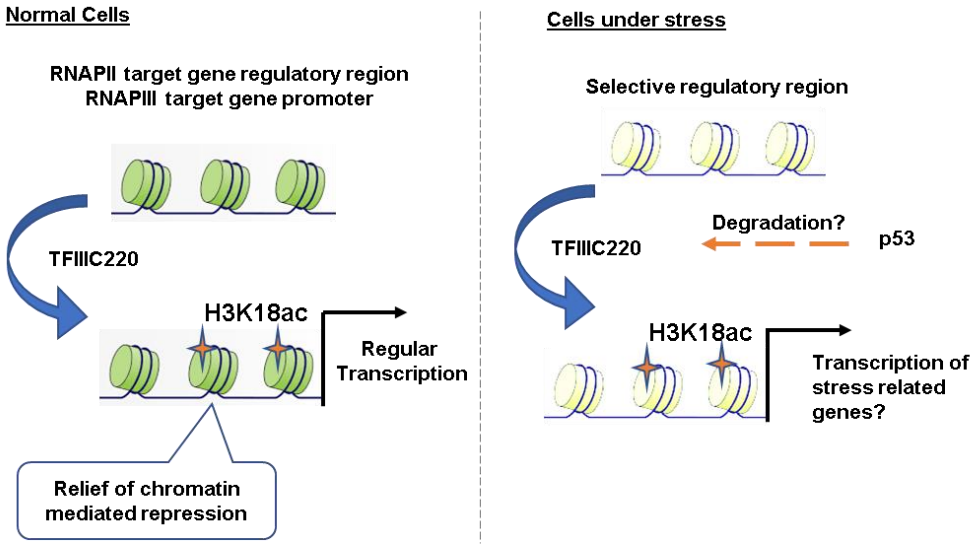


Figure 5.2. Hypothetical model for involvement of TFIIIC220 in cellular stress response. *In favorable condition TFIIIC220 acetylates TSS and induces transcription of its canonical targets. Under unfavorable condition TFIIIC220 might alter its specificity*

of targets, it acetylates regulatory region of genes involved in stress response. p53 might act to maintain steady level of TFIIIC220 by facilitating its degradation.

Finally, knockdown of TFIIIC220 was shown to accumulate a large quantity of γH2AX in cells. This might suggest a genome protective function of TFIIIC but TFIIIC220 knockdown was not associated with any change in p53 protein level. If TFIIIC220 is involved in DNA damage repair machinery or controls the integrity of genome requires more attention.

Another aspect of TFIIIC220 that was observed during this study that requires further investigation is the propensity of TFIIIC220 to autoacetylate. The recombinant full-length TFIIIC220 were found to undergo acetylation under *in vitro* assay conditions (Figure 5.3.A). To exclude the possibility of recombinant protein being acetylated by acetyltransferases in insect cells the status of full-lengthTFIIIC220 in assays utilizing tritiated acetyl-CoA were studied. Both full length and KAT domain of TFIIIC220 were found to incorporate radiolabeled acetyl groups (Figure 5.3.B).

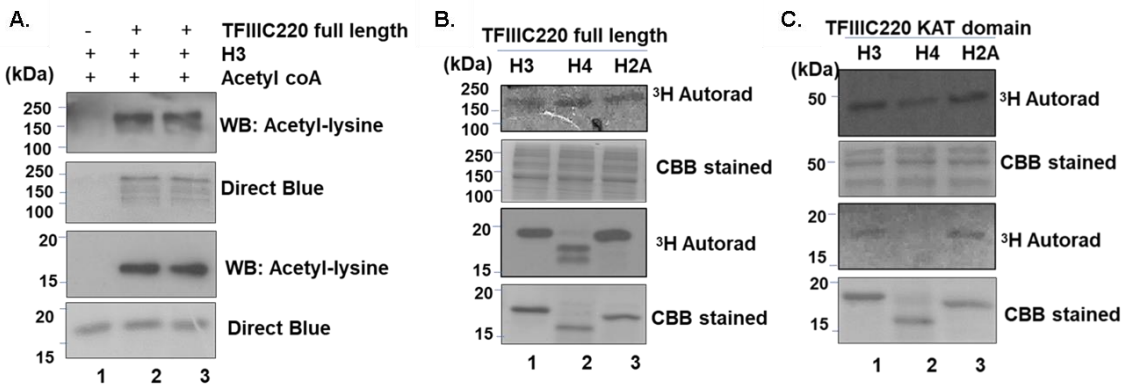


Figure 5.3. TFIIIC220 autoacetylates. *(A) In vitro KAT assay was performed using ~100 ng full length and ~200ng KAT domain of TFIIIC220. 1µg of recombinant core histones (H3, H2A, and, H4) and 40µM acetyl CoA or 50nCi 3H-acetyl CoA were used as substrates. Reaction mixtures were loaded onto 12% SDS PAGE and probed with acetyl-lysine specific antibody (in A) or developed onto X ray films (in B and C)*

Autoacetylation is a common property associated with many acetyltransferases such as p300, MOZ, Tip60, GCN5, PCAF *etc.* Autoacetylation is generally linked to modulation of acetyltransferase activities. In case of GCN5 and PCAF autoacetylation is essential for maintaining its acetyltransferase function in vitro (Herrera*et al.*, 1997). In case of TFIIIC220 if autoacetylation plays a similar role remains to be characterized.

Future perspective:

To elucidate the correlation among TFIIIC220, p53 and cellular stress response, *Drosophila* was chosen as an *in vivo* model system. *Drosophila melanogaster* has served as an excellent model for many diseases, including obesity, diabetes, and hyperglycemia-associated disorders, such as cardiomyopathy or nephropathy. Fat body is an organ analogous to human adipose tissue that also functions as liver. It consists of polyploid and often multinucleate cells that store lipids in lipid droplets. Excess carbohydrates are also stored as glycogen in fat bodies. The fat body consists of polyploid, sometimes multinucleate cells, which store lipids in specialized organelles called lipid droplets. In *Drosophila* fat bodies couple nutrient availability to growth during larval development; these are susceptible to stress induced apoptosis, autophagy *etc.* (reviewed in Gáliková and Klepsatel, 2018) making this organ an ideal organ system in *Drosophila* for studying the role of TFIIIC220 in stress response. On the other hand, insects are the simplest model organism where TFIIIC complex has been characterized to possess acetyltransferase activity (Srinivasan and Gopinathan, 2002). Additionally, in *Drosophila* the RNAPIII independent roles of TFIIIC has been found to be synonymous to that identified in human.

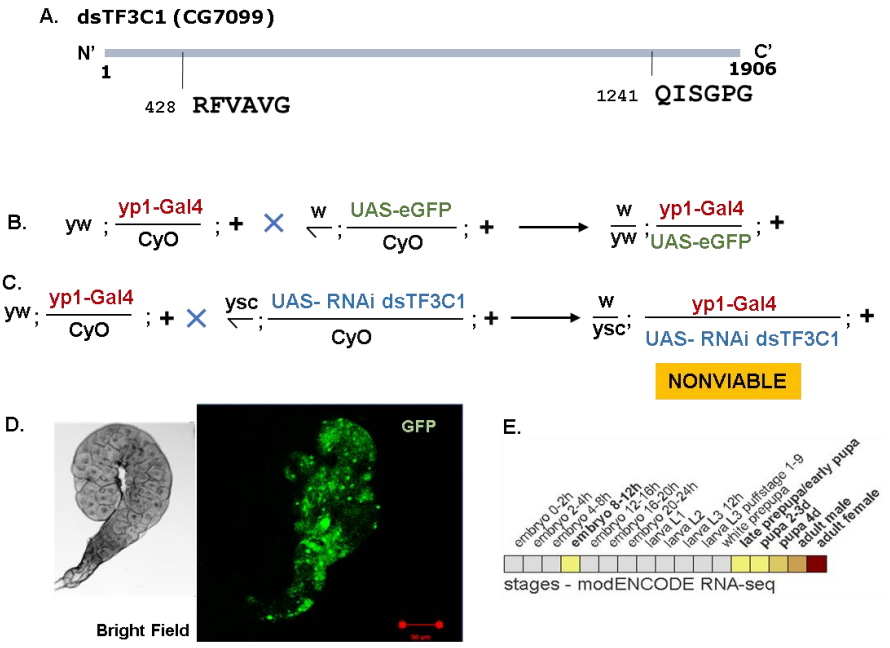


Figure 5.4. Generation of dTF3C1 knockdown fly-lines. *(A) Schematic representation of putative acetyl-CoA binding motifs on dTF3C1. (B) and (C) Genotype of parental and*

F1 fly lines. (D) Expression of GFP in fat body isolated from F1 flies obtained from B. (E) Activation of yp1 across various stages of development in flies.

The Drosophila homolog (CG7099) of human TFIIIC220 is 1906 amino acid long and bears 26% sequence identity with human TFIIIC220. It also possesses two putative acetyl-CoA binding stretches located at N and C-terminal respectively (Figure 5.4.A). The acetyltransferase activity of Drosophila TFIIIC220 homolog (hereon mentioned as dsTF3C1) is yet to be characterized. Interestingly, fat body specific expression of dsRNAi targeting dsTF3C1 under fat-body specific yp1 promoter (using UAS-Gal4 system) was found to be embryonically lethal that did not produce larvae while control population expressing eGFP under yp1 promoter were viable and adult fat bodies expressed eGFP (Figure 5.4. D). dsTF3C1 being a crucial element for production of tRNAs, 5SrRNA and various other products that are required for housekeeping cellular processes such as protein synthesis, RNA processing *etc.* it was assumed that dsTF3C1 depletion at early growth phase might have deleterious effect. To avert larval fatality the process of generating temperature sensitive inducible dsTF3C1RNAi system is underway.

Chapter 6

Summary

In this chapter the major findings of the present study will be summarized.

The present thesis work has focused on investigating functional relevance of two vertebrate acetyltransferases, p300 and TFIIIC220. The main aims of this study were to understand the connection between p300-mediated acetylation and early vertebrate embryogenesis and to characterize acetyltransferase activity of TFIIIC220.

In the first part of our study, the implication of p300-mediated acetylation on vertebrate embryogenesis was elucidated using a small molecule inhibitor of p300, Luteolin. Except for modulating acetyltransferase activity of p300, Luteolin did not downregulate p300 expression, perturbed transcriptional coactivator properties of p300. Also, Luteolin was predicted to not inhibit acetyltransferase activity of CBP, a structural and functional homolog of p300. Through this specific inhibition of p300 acetyltransferase function was found to be essential for maintaining steady level of Oct4 during the course of early differentiation of mouse embryonic stem cells. lack of p300 mediated acetylation results in meso- and endothelial lineage specific differentiation and the end result might be an effect either smaller sizes of embryoid bodies or accumulation of unusually high level of Oct4 in Luteolin treated EBs. Inhibition of p300-mediated acetylation also inhibited expression of neuronal markers but did not affect expression of glial markers following retinoic acid induced differentiation of embryoid bodies. Next, these set of observation was correlated with a parallel study related to characterization acetyltransferase activity of zebrafish p300. Based on sequence homology two orthologs of human p300was identified in zebrafish. The two orthologs did not differ in terms of expression pattern during embryonic development of zebrafish, yet, only one ortholog was found to be active that acetylated free and nucleosomal histones *in vitro*. Mutation in conserved critical residues of functional zebrafish p300 KAT domain abrogated its acetyltransferase activity. Inhibition of p300 acetyltransferase activity in zebrafish by C646 and knockdown of functional ortholog p300 during zebrafish larval development resulted in manifestation of severe development defects that resembles Rubinstein Taybi syndrome in human. A few defects related to ectodermal lineage such as craniofacial features, fin development, cartilage formation etc. were rescued by overexpression of functional KAT domain but not by catalytic mutant version of the domain against a knockdown

background. Together these results confirmed that p300 mediated acetylation is indispensable for proper ectodermal differentiation in vertebrates.

In the second part of the thesis, acetyltransferase activity of human TFIIC220 is characterized in detail. Recombinant full length TFIIIC220 could acetylate H3, H4 and H2A core histone *in vitro*. Among the common acetylation sites of H3 and H4, TFIIIC220 could only acetylate H3K18 residue on free and nucleosomal histones. The catalytic domain of TFIIIC220 possesses conserved P loop that binds to acetyl-CoA and mutation in P loop perturbs the catalytic function of TFIIIC220 KAT domain. Knockdown of TFIIIC220 led to reduction of H3K18ac level significantly which could be rescued by overexpression of putative TFIIIC220 KAT domain. Knockdown of TFIIIC220 was also associated with accumulation of DNA damage marker γH2AX, autophagy related LC3II and p62.further characterization indicated suppression of autophagy in TFIIIC220 KD cells. Remarkably, TFIIIC220 is upregulated in protein and mRNA level in stressed cells upon genotoxic insult and serum starvation. TFIIIC220 protein level stabilization in stressed cells were found to be p53 dependent.

Overall significance of the study:

1. p300-mediated acetylation is dispensable for cellular viability, self-renewal and identity of mouse embryonic stem cells in undifferentiated state (in presence of LIF). Acetylation by p300 is dispensable for viability of differentiating embryoid bodies but is required for proper differentiation during early development. Lack of p300 mediated acetylation leads to decreased enrichment of ectodermal population and accumulation of high level of Oct4 protein in differentiated EBs. Acetyltransferase activity of p300 is crucial for neuronal differentiation but not for glial differentiation.

2. Zebrafish possesses one functional homolog of human p300 which bears significant sequence similarity and similar substrate specificity with its human counterpart. Abrogation of p300 acetyltransferase function in zebrafish can be linked to severe developmental defects mostly related to ectodermal lineage.

3. TFIIIC220 possesses intrinsic acetyltransferase activity and can catalyze H3K18ac *in vitro* and *in vivo*. TFIIIC220 bears partial similarity with GNAT family acetyltransferases. Knockdown of TFIIIC220 reduces H3K18ac level and upregulates

DNA damage marker, blocks autophagy. Upon genotoxic insult and serum starvation TFIIIC220 protein level was increased probably in a p53-dependent manner.

Publications

***Basu M**, Kundu TK. **TFIIIC220 is involved in cellular stress response in p53 dependent manner.** (Manuscript under preparation).*

*Ghosh U, **Basu M**, Pal S, Meena S, Datta D, Ampapathi RS, Kundu TK, Singh G, Chakraborty TK. **Design, Synthesis and Conformational Studies of Cyclic Tetrapeptides having βγ Fused Turns as HDAC Inhibitors.** ChemistrySelect. 2021; 6(31):7887-7893*

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Swaminathan A, **Basu M***, Bekri A, Drapeau P, Kundu TK. **The Dietary Flavonoid, Luteolin, Negatively Affects Neuronal Differentiation.** Front Mol Neurosci. 2019; 12:41.*

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Babu A, Kamaraj M, **Basu M***, Mukherjee D, Kapoor S, Ranjan S, Swamy MM, Kaypee S, Scaria V, Kundu TK, Sachidanandan C. **Chemical and genetic rescue of an ep300 knockdown model for Rubinstein Taybi Syndrome in zebrafish.** Biochim Biophys Acta Mol Basis Dis. 2018;1864(4 Pt A):1203-1215.*

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