Identification and characterization of Alpha tubulin as a non-histone protein substrate/binding partner of hHDAC8 (Human Histone Deacetylase 8) in HeLa cells.

Thesis Submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that the thesis entitled **"Identification and characterization of Alpha tubulin as a non-histone protein substrate/binding partner of hHDAC8 in HeLa cells."** submitted by **G.R.Vanaja** bearing registration number **12LAPH05** in complete fulfilment of the requirements for award of Doctor of philosophy in the **School of Life Sciences** is a bonafide work carried out by her under my supervision and guidance.

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2. International Conference on Genome Architecture and Cell Fate Regulation - GACFR **(International)**.
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Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D

Course Code	Name	Credits	Pass/Fail
1. AS 801	Seminar 1	1	PASS
2. AS 802	Research Ethics & Management	2	PASS
3. AS 803	Biostatistics	2	PASS
4. AS 804	Analytical Techniques	3	PASS
5. AS 805	Lab Work	4	PASS

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DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"Identification and characterization of Alpha tubulin as a non-histone protein substrate/binding partner of hHDAC8 in HeLa cells."** has been carried out by me under the supervision of **Dr. Aruna Sree M.K** and this work has not been submitted for any degree or diploma of any other university earlier.

Dated

G.R. Vanaja

12LAPH05

(Research Scholar)

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To all the great teachers who taught me ….

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ABSTRACT

Although HDAC8 is best understood structurally, its precise subcellular location, function, protein partners and substrates still remain elusive. Here, we show that HDAC8 and its phosphorylated form (pHDAC8) localized predominantly in the cytoplasm in cancerous, HeLa, and normal, HEK293T, cells, although nucleolar localization was observed in HeLa cells. Immunoprecipitation (IP) studies followed by MALDI-TOF analysis identified Alpha tubulin as a novel interacting partner of HDAC8. Further, *in-silico* protein-protein interaction studies, IP and Co-IP experiments, intrinsic fluorescence spectrophotometric analysis, Circular dichroism (CD) and Surface Plasmon Resonance (SPR) studies clearly indicated binding of ac-lys40 tubulin to HDAC8. HPLC-based *in vitro* deacetylation and specific small molecule inhibitor studies demonstrated the deacetylation of acetylated (Lys40 residue of Alpha tubulin. These *in silico* results were further confirmed by IP and Co-IP experiments which indicated alpha tubulin by HDAC8. Differential tissue specific expression of HDAC8 and HDAC6 in various cancer cell lines, followed by expression, activity studies of HDAC8 and HDAC6 (an established tubulin deacetylase) by real time PCR, immunoblot and HDAC enzyme activity in HeLa and HEK 293T demonstrated significant upregulation of HDAC8 in HeLa cells compared to HDAC6. These results suggest the role of HDAC8 as a primary tubulin deacetylase in HeLa when compared to HDAC6. Further, Knockdown by HDAC8 siRNA or inhibition studies clearly explains ac-alpha tubulin as its potential substrate for deacetylation. Functional significance of this interaction was demonstrated by cell-migration, cell morphology and cell cycle analysis which suggested the role of HDAC8 as tubulin deacetylase. These findings have paved a path towards studying and understanding the molecular regulation of tubulin by HDAC8 in cervical cancer.

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ABBREVATIONS

µl: Microliter

µg: Microgram

µM: Micro molar

A549: Human lung carcinoma

ac: Acetylated

ADD3: Adducin 3

AML: Acute myeloid leukemia

ARID1A: AT-Rich Interaction Domain 1A

ATL: Acute T cell Leukemia

BMF: Bcl2 Modifying Factor

CAPRI: Critical Assessment of Predicted Interactions

CD: Circular Dichroism

CdLs: Cornelia de Lange Syndrome

CHCHD3: Coiled coil Helix Coiled Coil Helix Domain Containing 3

ChIp: Chromatin Immunoprecipitation

Co-Ip: Co-Immunoprecipitation

CPNE3: Copine 3

CREB: cAMP Responsive Element Binding Factor

CTCF: CCCTC-binding factor

CTCL: Cutaneous T-cell lymphoma

DAPI: 4', 6 - Diamidino-2-Phenylindole

DEC1: Differentiated embryo-chondrocyte expressed gene 1

DNA: Deoxy ribonucleic acid

DTT: Dithiothreitol

EDTA: Ethylene Diamine Tetra acetic Acid

ERRα: Estrogen Related Receptor Alpha

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GNATs – Gcn5 (general control non-derepressible 5)-related N-acetyltransferases

H2A: Histone 2A

H2B: Histone 2B

H3: Histone 3

H4: Histone4

HATs: Histone acetyl transferases

HDAC8: Histone deacetylase 8

HDACi: Histone deacetylase inhibitors

HDACs: Histone deacetylases

HEK 293T: Human Embryonic Kidney epithelial cells

HeLa: (Henrietta lacks) Human cervical cancer cells

hEST1B: Human Ever Shorter Telomerase 1 B

HMTs: Histone methyl transferases

Hr: Hours

Hsp70: Heat Shock Protein 70

Hsp90: Heat Shock Protein 90

HuT78: Human T cell lymphoma with Sezary Syndrome

Inv (16): Inversion (16)

JAK2/STAT: Janus kinase 2/signal transducer and activator of transcription

Jurkat: Immortalized human T-Cell lymphocyte cells

KDACs: Lysine deacetylases

Lhx1: LIM Homeobox Protein 1

M: Molar

MALDI TOF-TOF: Matrix Assisted Laser Desorption/Ionization

MCF-7: Human breast adenocarcinoma

MDM1: Mitochondrial Distribution and Morphology

Min: Minutes

ml: Milliliter

mM: Millimolar

MMP-9: Matrix metallopeptidase 9

Molt4: Human acute lymphoblastic lymphoma

MTT: [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]

NAD: Nicotinamide Adenine Dinucleotide

NAD^+: Nicotinamide Adenine Dinucleotide Reduced Form

NCOA3: Nuclear Receptor Co-Activator 3

OTX1: Orthodenticle Homeobox 1

p^{53}: Tumor Protein 53.

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PKA: Protein Kinase A

PKG: Protein Kinase G

PMSF: Phenylmethylsulfonyl fluoride

PP1: Protein Phosphatase 1

PRMTs: Protein arginine methyl transferases

PTCL: Pripheral T cell lymphoma

RAI1: Retinoic Acid Induced Protein 1

RKO: Human colon carcinoma

RMSD: Root Mean Square Deviation

RNA pol II: Ribonucleic acid Polymerase II

RNA: Ribonucleic acid

RT: Real time

SAHA: Suberoylanilide hydroxamic acid

SCOs1: Suppressor of Cytokine Signalling 1

siRNA: Short Interfering RNA

SIRT1: Silent Mating Type Information Regulation 2 homolog 1

SMC1A: Structural Maintenance of Chromosome 1A

SMC3: Structural Maintenance of Chromosome 3 Protein

SOX4: Sex Determining Region – Y – Box 4 Transcription Factor

SPR: Surface Plasmon Resonance

STAG2: Stromal Antigen 2

SVIL: Supervillin

TDP-A:Thailandepsin A

THRAP3: Thyroid Hormone Receptor Associated Protein 3

TPM3: Tropomycin 3 Protein

TSA: Trichostatin A

U87: Human Glioblastoma cell line

Unac: Unacetylated

ZRANB2: Zinc Finger RANB2 Type containing 2 Protein

ZSCAN2: Zinc Finger and SCAN Domain Containing 2

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Introduction

1. Epigenetics

The genetic material in eukaryotes is embedded in a structure known as nucleus. This genetic material, DNA, is generally transferred to the offspring, by either undergoing some external modifications on it, or by addition or deletion of DNA sequence, which are heritable. The phenomenon of heritable changes in gene expression, which occur on DNA without changing its genetic makeup, thereby causing a phenotypic change without affecting the genotype, is termed as Epigenetics. Thus, study of epigenetics does not involve with changes of basic DNA, but only with the external additions (which involve post-translational modifications) to either DNA, or DNA associated proteins like histones or even the whole chromatin structure. The effect of these modifications may either last for some cell divisions or even continue for many generations without affecting the basic DNA sequence of the organism (Bird 2007). This condition may arise due to either from hereditary, external environmental/chemicals conditions, or due to other factors like aging, diet etc., These modifications generally include, DNA methylation, chromatin remodeling and histone modifications **(Fig. 1)**.

1.1 DNA Methylation

DNA is subjected to numerous epigenetic alterations in the cells, among which histone modifications and methylation of DNA are widely studied (Baylin and Herman 2000; Strahl and Allis 2000; Tessarz and Kouzarides 2014). Methylation of DNA is a covalent modification which involves in addition of methyl group to the cytosine of CpG dinucleotides (Baylin and Herman 2000). This addition can cause an imbalance in gene expression there by leading to either expression or repression of genes. A special class of enzymes, known as DNA methyltransferases

(DNMTs) control the addition of methyl group. The source of the methyl groups can be either from dietary sources or through chemicals **(Fig. 1)**. The maintenance of the methylation patterns is controlled by three DNMTs, which include DNMT1, DNMT3a and DNMT3b) (Weinhold 2006). Out of these three DNMTs, DNMT1 is responsible for maintaining methylation pattern of already established pattern. DNMT3a and 3b are continuously involved in *de novo* DNA methylation.

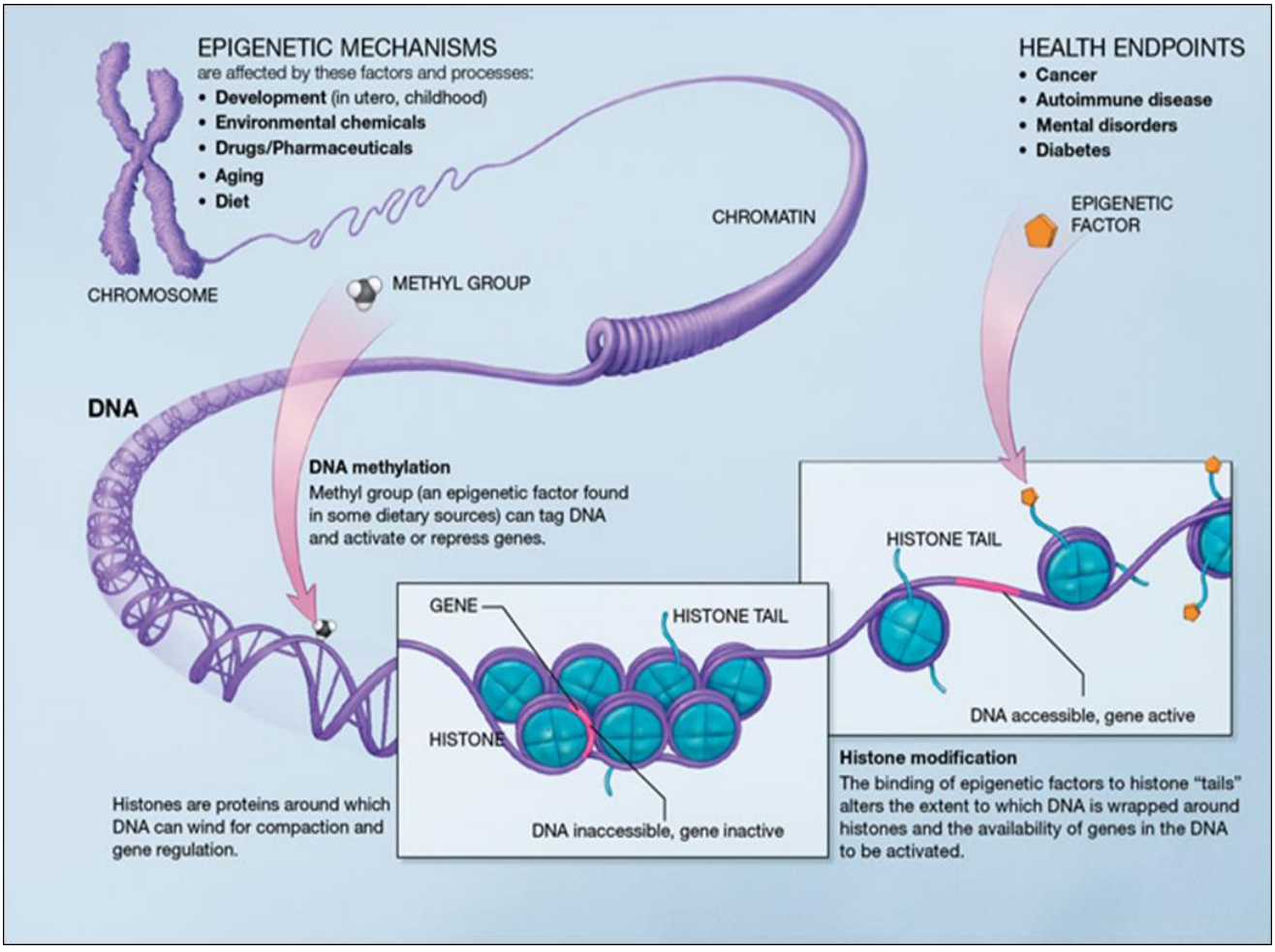


Figure 1. Epigenetics mechanisms: *Leading factors responsible for epigenetics along with various modifications responsible for the outcome i.e., DNA methylation, histone modifications. (Source: https://en.wikipedia.org/wiki/Epigenetics)*

1.2 Chromatin Remodeling

Apart from DNA methylation, chromatin remodeling is considered as another dynamic modification, which involves changes in the gene regulation by providing access to the transcription machinery to alter the chromatin architecture. These changes are triggered by covalent modifications of histones (acetyltransferase's, methyltransferase's, kinases etc.,) or by ATP-dependent remodeling complexes which will ultimately alter the chromatin structure (Teif and Rippe 2009)

Covalent modifications of chromatin are regulated by special group of enzymes known as histone modifying complexes. These enzymatic modifications lead to methylation, acetylation, phosphorylation, ubiquitination etc., on histone N-terminal tails, which are brought about by histone methyltransferases (HMTs), histone acetyltransferases (HATs), kinases etc., These alterations affect the binding affinity of DNA to that of histone proteins, thereby leading to condensed or relaxed chromatin (Wang, Allis et al. 2007). Nucleosome histones are also subjected to numerous post-translational modifications. These include diverse range of events of methylation, phosphorylation, acetylation, ubiquitination, ribosylation, citrullination, sumoylation etc. (Strahl and Allis 2000; Kouzarides 2007; Latham and Dent 2007).

The changes occurring in the nucleosome complexes such as, moving, ejecting or restricting are governed by special group of ATP dependent chromatin remodeling enzymes. These proteins poses ATP domain, the energy generated from this ATP hydrolysis favors to relocate the nucleosome complexes from the DNA, or even by expelling the nucleosomes leading to exposure

of naked DNA and facilitating the gene transcription (Wang, Allis et al. 2007). Five families, SWI/SNSF, ISWI, NuRD/mi-2/CHD, ino80 and SWR1, of chromatin remodelers have been identified till date in eukaryotes using yeast as the model organism. Among the five families, the two remodelers, SWI/SNSF and NuRD/mi-2/CHD, are very well studied so far, (Eisen, Sweder et al. 1995). Thus, both histone modifications and ATP dependent complexes dynamically regulates the gene expression, by controlling the access to RNA pol II **(Fig.2).**

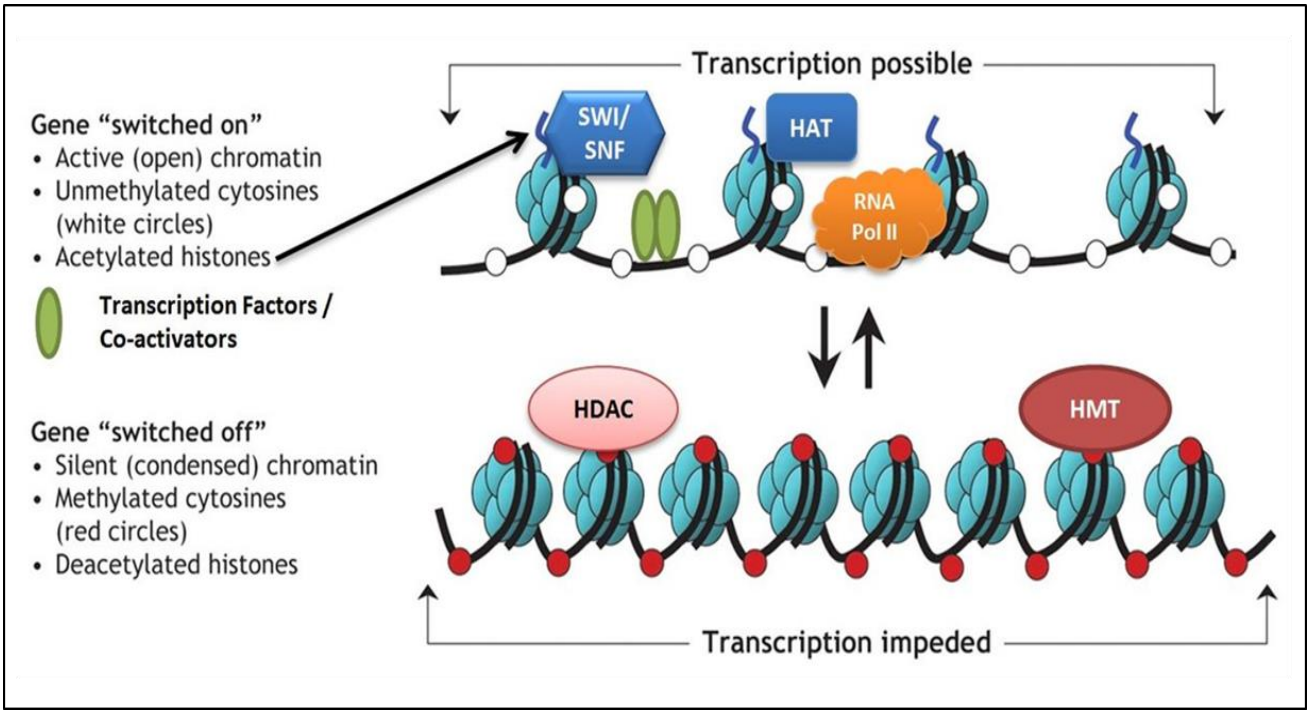


Figure 2. Dynamic regulation of transcription in presence of chromatin remodeling complexes: *Covalent histone modifications (HATs) and ATP dependent chromatin complexes assure transcription by recruiting RNA pol II. Gene repression occurs with HDACs (histone deacetylases) activity. Adapted from Cancer Biol Ther (2003).*

1.3 Histone Modifications

DNA is wrapped around a core of eight histones to form nucleosomes. Nucleosomes, the building blocks of chromatin, consist of 146 base pairs of DNA, which is wrapped around a core

made of histone octamer consisting of two copies of H2A, H2B, H3 & H4 **(Fig. 3)**. Histone H1, considered as linker histone, is involved in linking two neighboring nucleosomes. Structural composition of each histone core protein includes a C-terminal globular domain (70-90 residues) and an N-terminal tail (20-30 residues) (Luger, Mader et al. 1997). Accessibility of RNA polymerase II (Pol II) to DNA and gene expression depends on the disassembly of nucleosomes. Whereas, upon reassembling of nucleosome units it leads to the restricted accessibility, thus resulting in gene repression.

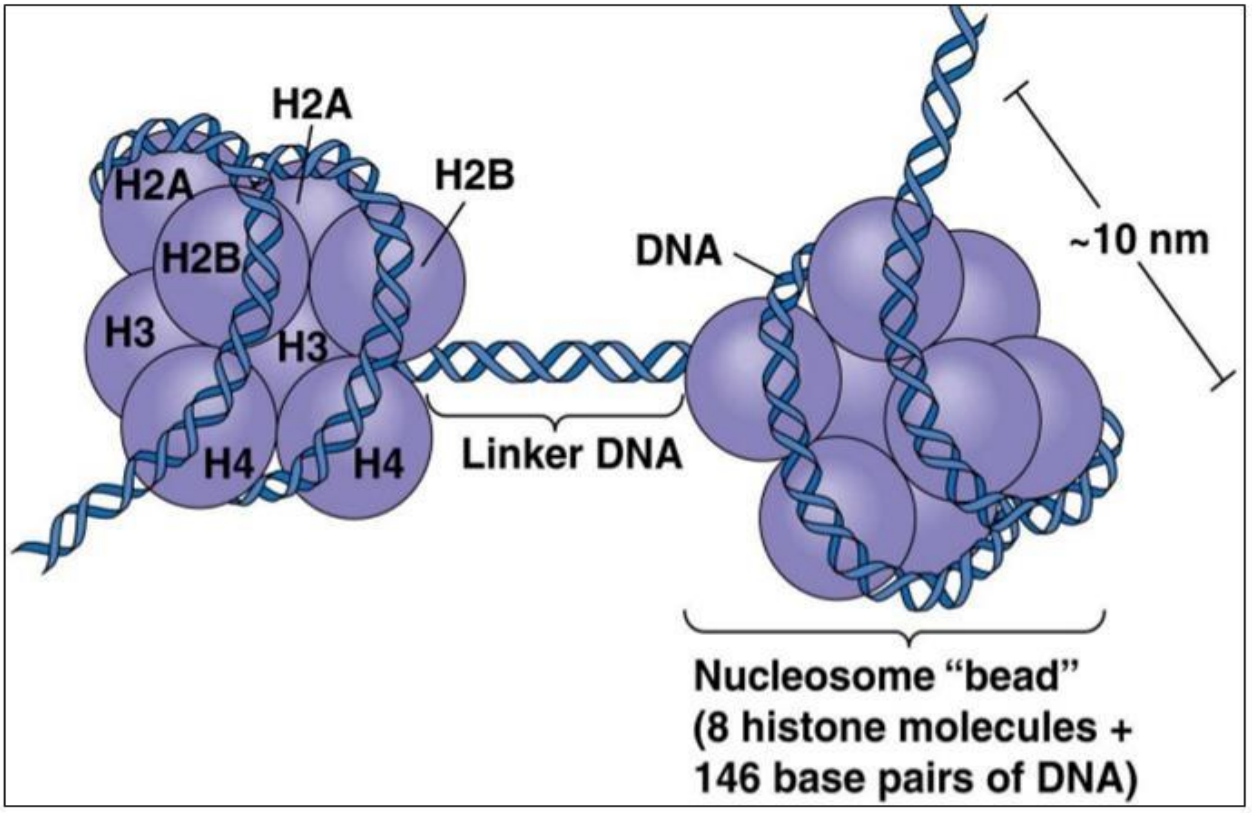


Figure 3*. Schematic representation of nucleosome structural organization along with histone proteins:** *Octamer of histones H2A, H2B, and two tetramers H3 & H4 forms nucleosome unit (146 bp of DNA). Two units of nucleosomes are connected with linker DNA and H1 histone.**

https://www.researchgate.net/figure/263097109_fig2_Schematic-representation-of-the-nucleosome-and-mammalian-core-histone-modifications-A

Dynamic regulation of these chromatin modifications, including chaperones and remodelers of histones are known to modulate the transcription (Venkatesh and Workman 2015). The amino-terminal tails of histones protrude out of the nucleosomes which are constantly subjected to several post-translational modifications. Most studied post-translational modifications in mammals particularly in humans include methylation, acetylation and phosphorylation etc.,(Ropero and Esteller 2007) **(Fig 4)**. Many of these modifications are known to modify the chromatin structure, correlating with gene expression levels.

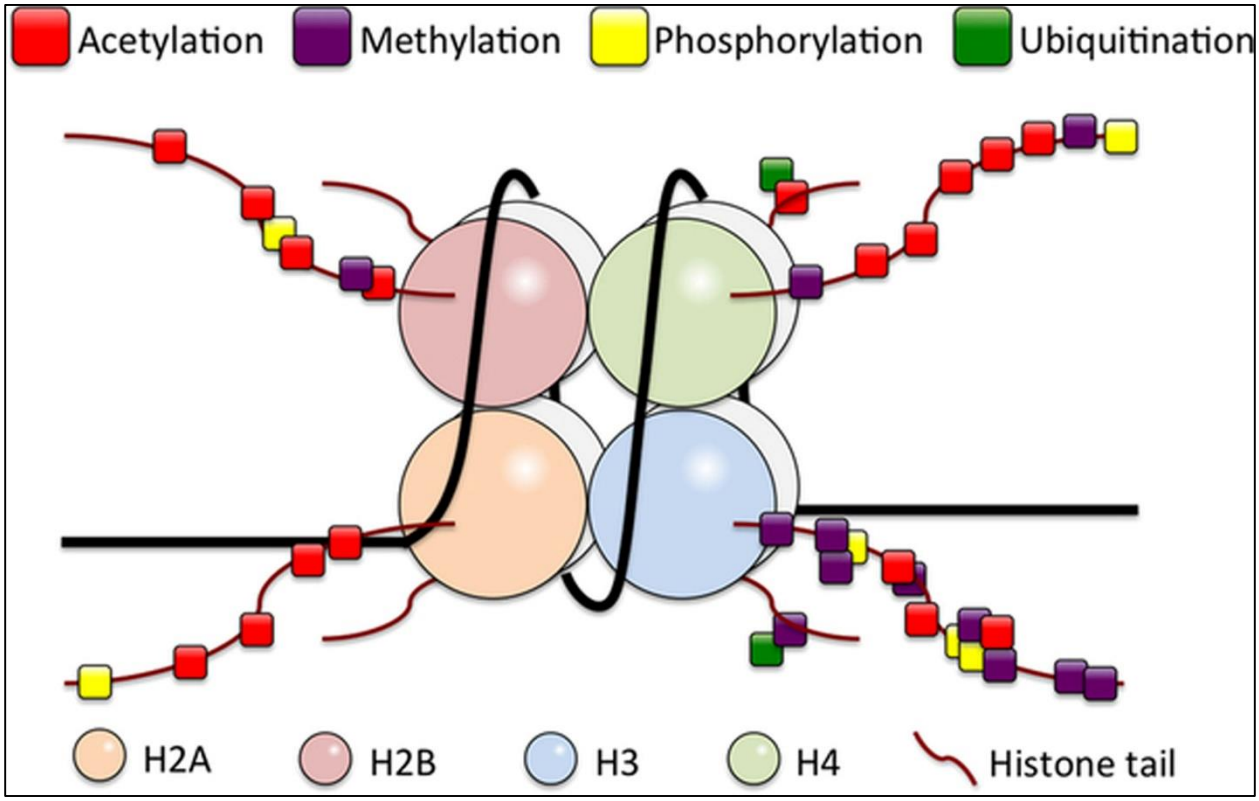


Figure 4. Potential histone modifications: *Addition of different chemical groups to N-terminal tails of histones alters the gene function. Modifications include Ac-acetylation, Me-Methylation, Ub-Ubiquitination, Su-Sumoylation, P-Phosphorylation*

http://www.integratedhealthcare.eu/1/en/histones_and_chromatin/1497

1.3.1 Histone Code Hypothesis

Histone modifications work as a scaffold for recruiting other proteins, by interacting with the specially modified regions either of their tails or globular domain regions along with their interactions with DNA. This in turn will affect the structural integrity of the chromatin, either helping in positive or negative regulation of various genes (Jenuwein and Allis 2001).

1.3.2 Histone Methylation

Histone methylation is considered as a covalent modification involving in addition of one, two or three methyl groups from S-adenosyl-L-methionine to histone proteins by histone methyltransferases (HMTs). DNA methylation is controlled through chromatin dependent transcriptional regulation. Thus, histone methylation is known to regulate the complex formed with DNA and thereby, affecting the activation or silencing of specific genes (Greer and Shi 2012). In mammalian system three known DNA methyltransferases are identified which include, DNMT1, DNMT3A and DNMT3B. A recent addition to this list include DNMT2, which is not considered as a methyltransferase (Rountree, Bachman et al. 2001).

1.3.3 Histone Phosphorylation

Histone phosphorylation is the best studied modification which is known to regulate the cellular homeostasis and other regulatory functions of the cell. Phosphorylation of histones occurs during response of a cell to DNA damage. Extensive studies of histone phosphorylation demonstrate its key role in chromatin remodeling which are linked to transcriptional regulation (Rossetto, Avvakumov et al. 2012).

1.3.4 Histone Acetylation

Acetylation of lysine residues on proteins is a reversible post translational modification (Yang and Seto 2008), which is known to affect the protein-protein interaction, protein–DNA interactions and protein stability. Much focus on acetylation of lysine residues was drawn by the post-translational modifications of the histone proteins, which are known to regulate accessibility of DNA to cellular machinery that in turn, bring out altered protein profiles. Till date over, 3,600 acetylation sites have been discovered in mammalian proteins (Khoury, Baliban et al. 2011), some of which play a vital role in various metabolic activities including gluconeogenesis and DNA damage repair (Choudhary, Kumar et al. 2009; Zhao, Xu et al. 2010). Not surprisingly these aberrant modifications of both histone and non-histone proteins can mount a dramatic proteome variations which can lead to either emergence of a new disease or can even synergize the effects of existing diseases(Drummond, Noble et al. 2005; Haberland, Montgomery et al. 2009).

1.3.5 Histone Acetyl transferases (HATs)

Histone acetyl transferases (HATs) are responsible for addition of acetyl groups to ε-amino group of lysine side chains from acetyl-CoA as a coenzyme to histone tails/non-histone proteins, resulting in more relaxed chromatin and gene transcription activation. Histone acetylation is known to reduce positive charge of histones, and this potentially disrupts the electrostatic interactions between DNA and histones **(Fig. 5)**. This in turn affects the chromatin structure, allowing the DNA accessibility to transcriptional machinery (Sun, Zhou et al. 2012). Histone acetylation has been associated with other genome functions such as chromatin assembly, DNA repair, and recombination (Polo and Almouzni 2005). HATs can be put into two broad categories based on their subcellular localization. Type A-HATs are associated with addition of acetyl groups

to histone proteins, whereas, type B-HATs are involved with cytoplasmic proteins and newly translated histones (Richman, Chicoine et al. 1988).

Based on their structural and functional similarity with the catalytic domain regions there are 30 HATs classified into three major groups. GNATs – Gcn5 (general control non-derepressible 5)-related N-acetyltransferases, MYST HATs, well characterized by presence of MYST domain containing an acetyl-CoA and a zinc finger motif (Avvakumov and Cote 2007) and E1A-associated protein of 300 kDa p300/CREB (cAMP-responsive element binding protein) – binding protein (CBP) (Allis, Berger et al. 2007; Lee and Workman 2007).

1.3.6 Histone Deacetylases (HDACs)

Removal of the acetyl group from histones and other proteins is catalyzed by special group of enzymes known as histone deacetylases (HDACs). Deacetylation leads to condensation of chromatin, leading to gene repression **(Fig. 5)**.

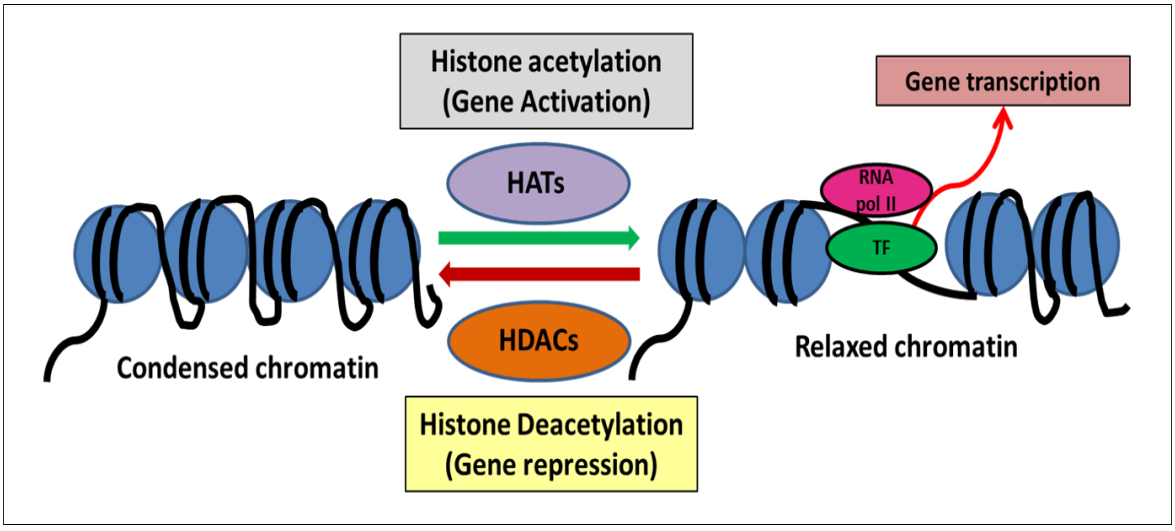


Figure 5. Schematic representation of functional role of Histone acetylation/deacetylation: *Figure illustrates the dynamic states of acetylation/deacetylation of histone proteins regulated by HATs and HDAC enzymes. Addition of acetyl groups to the lysine residues of histone tails allows gene transcription. HDACs remove the acetyl groups leaving condensed chromatin and allowing gene repression. Adapted from The Biochemical Journal. (2003).*

Deacetylation occurs through a charge relay system consisting of two adjacent histidine residues, two aspartic acid residues (located approximately 30 residues away from histidine and are separated by approximately 6 amino acids), and one tyrosine residue (located approximately 123 amino acids downstream to aspartic acid) (Buggy *et. al*.,2000 and Finnin *et. al*.,1999). Zn^{2+} ion is one of the essential components of the charge-relay system; it is bound to the zinc binding site on the bottom of the pocket. This results in more condensed chromatin state and transcriptional gene silencing (Johnstone 2002; Iizuka and Smith 2003). Thus, a critical balance has to be maintained between acetyl transferases and deacetylases. "HDACs are now also called as lysine deacetylases (KDACs)".

HDACs, as the name suggests, have histones as their predominant substrate. However, many HDACs are at least partially cytoplasmic, and there are evidences that some of these HDACs can act on non-histone substrates, including tubulin and p53. The non-histone targets of histone deacetylases include transcription factors, transcription regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators and viral proteins (Glozak *et al.*, 2005; Marks and Dokmanovic, 2005; Rosato and Grant, 2005;Bolden *et al.*, 2006; Minucci and Pelicci, 2006; Zhao *et al.*, 2006).

1.3.7 Histone Deacetylases (HDACs) Classification

Eighteen different mammalian HDACs are been identified till date (Bhalla 2005) (Blander and Guarente 2004) (Marks and Dokmanovic 2005). They are further divided into four different classes depending upon the homology they share with yeast HDACs. Class I HDACs comprises of HDAC 1, 2, 3 and 8 and are known to be homologous to yeast Rpd3 in yeast. Class I HDACs are ubiquitously expressed in all human cell lines and tissues and are localized majorly in nucleus

(de Ruijter, van Gennip et al. 2003). But recent evidences prove that class I HDACs are also localized to cytoplasm (Longworth and Laimins 2006). According to recent phylogenetic analysis, the class I was suggested to be divided into class Ia (HDAC1 and 2), Ib (HDAC3) and Ic (HDAC8) (Gregoretti, Lee et al. 2004). Class II members of HDACs comprise of HDAC 4, 5, 6, 7, 9, and 10 which share homology with Hda1 of yeast. Class II members are known to shuttle between nucleus and cytoplasm, regulating various metabolic processes. This particular group is again divided into class IIa (HDAC 4, 5, 7 & 9) and IIb (HDAC 6 and 10) based on the number of catalytic domains (Bhalla 2005; Marks and Dokmanovic 2005) **(Fig. 6).**

Class I and II are zinc dependent enzymes. SIRTUINS (SIRT1-7) come under class III category, which are NAD+ dependent for their enzyme activity and are orthologs of yeast sir2 family of proteins (Blander and Guarente 2004). HDAC 11 is the only member included in class IV category and found to be homologous to both class I and II **(Fig. 6)** (Gao, Cueto et al. 2002).

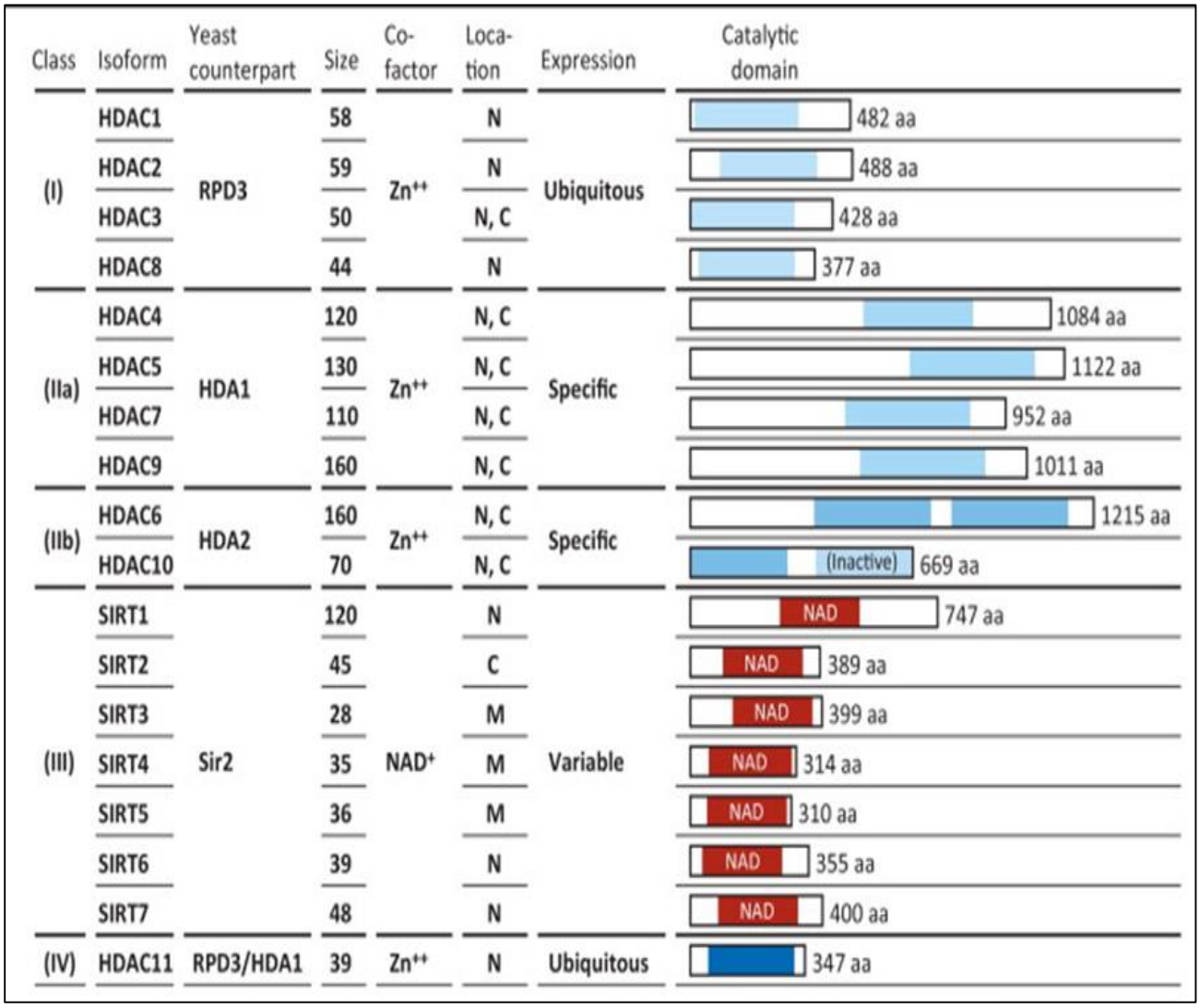


Figure 6. Mammalian HDACs classification: *HDACs are classified into four classes depending upon their homology with yeast counterparts. Class I include HDAC1, 2, 3 & 8, Zn++ dependent. Class II is again subdivided into class IIa, which include HDAC4, 5, 7 & 9. Whereas, HDAC6 & HDAC10 are included in class IIb. Both the subclasses are Zn++ dependent. Classes III is the family exclusively for Sirtuins and are NAD+ dependent. Class IV include HDAC11 and requires Zn++ as a cofactor. Adapted from Trends in microbiology (2013).*

HDACs are known to be involved in the critical signaling networks and known to be linked to numerous disease conditions like neurodegenerative disorders, metabolic dysregulation, various cancers, auto immune and inflammatory diseases etc. (Haberland, Montgomery et al. 2009; Tang,

Yan et al. 2013). They are known to regulate various physiological and pathophysiological functions of the cell, by regulating diverse range of both histone and non-histone proteins involved in growth, angiogenesis, apoptosis etc., **(Fig. 7)**

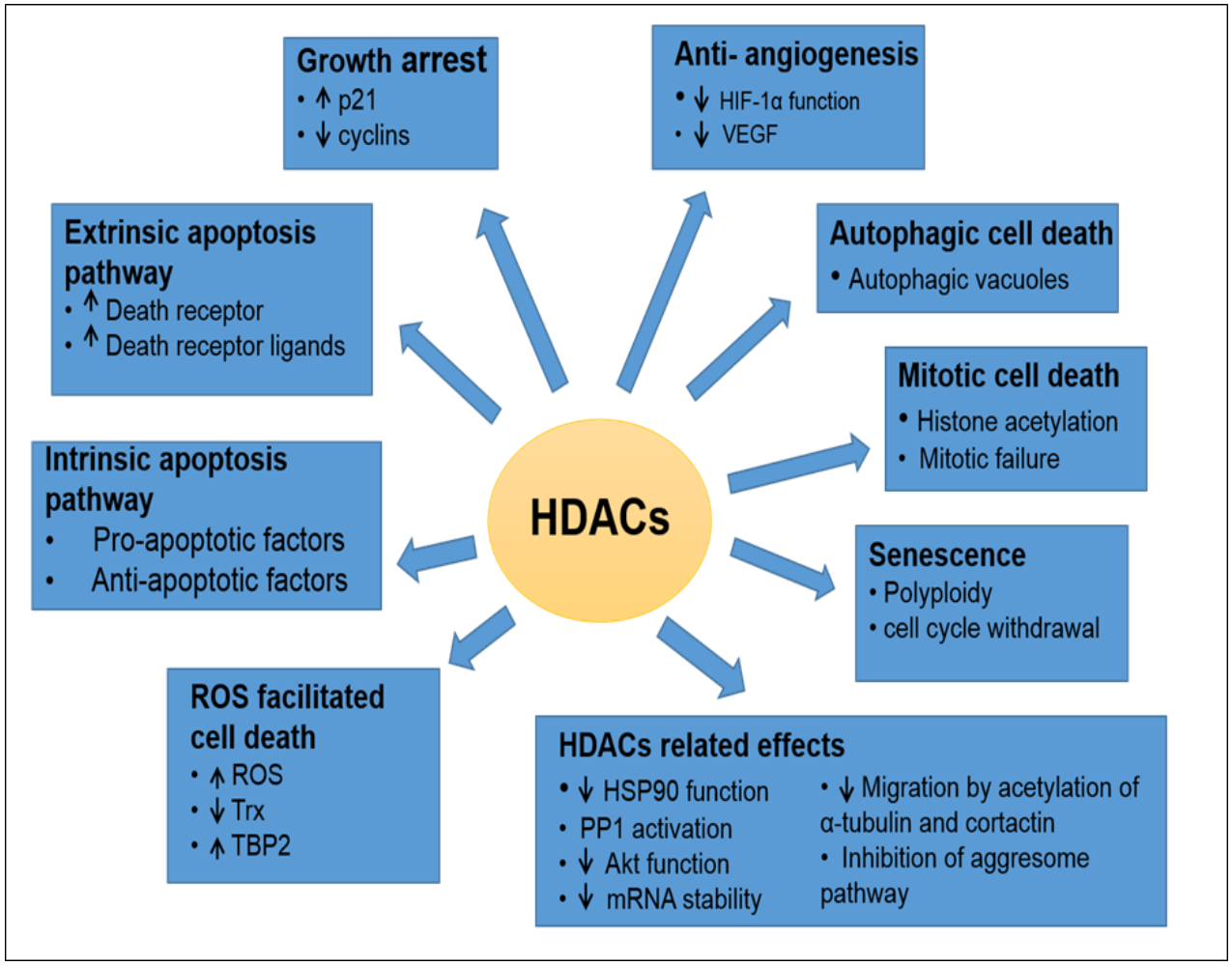


Figure 7. Schematic representation of physiological and pathological functions of HDACs on histone and non-histone substrates: *Adapted from Journal of cellular biochemistry (2009).*

1.4 HDACs and Cancer

Many genes are known to be affected during oncogenesis, due to the effect of HATs and HDACs. Numerous studies performed till date on various cancer cell lines using HDAC inhibitors justify the importance of HDACs in regulating the expression of oncogenes (Glozak et al, 2007). One such important gene reported is p21, which is known to be a target for many of pan-HDAC inhibitors, and been repressed by almost all HDACs (Sambucetti et al., 1999). P53 is another tumor suppressor protein which is a direct substrate for many HDACs as validated by both *in vitro* and *in vivo* studies (Waltregny et al, 2004).

Histone acetylation plays a key role in development of skin cancer, and also in metastasis of gastro intestinal tumors (Fraga et al., 2005) (Yasui et al., 2003). Recent studies of hematological malignancies report that HDACs form complexes with other repressor elements and regulate the normal differentiation and proliferation of myeloid cells. Also, it is identified that aberrant involvement of HDACs with specific promoter regions result in chromosomal translocations like acute promyelocytic leukemia (APL) (Lin, Chen et al. 2006).

Altered expression of respective HDACs in various tumors has been reported from many studies. They include, aberrant expression of HDAC1 in gastric cancer (Choi, Kwon et al. 2001), colon (Wilson, Byun et al. 2006), prostrate (Halkidou, Gaughan et al. 2004), and breast carcinoma (Zhang, Yamashita et al. 2005). Whereas, HDAC2 is involved in cervical, gastric and colorectal cancers (Zhu, Martin et al. 2004; Huang, Laban et al. 2005; Song, Noh et al. 2005). Overexpression of HDAC3 and HDAC6 are mostly reported in colon and breast cancers (Zhang, Yamashita et al. 2004; Wilson, Byun et al. 2006).

Recently sirtuins, which are class III HDACs, have been considered as a part of cancer initiation. They are involved in varied cellular functions including regulating genes, DNA repair, stress response, apoptosis, cell cycle, genome stability etc., inferring their efficient role in tracking normal cell growth and proliferation. SIRT1 a very prominent member of the family, is known to regulate some of the important transcription factors like p300, p53, NF-KB, E2F1 etc., by altering the expression of their target genes by acetylation, leading to tumorigeneses (Vaziri, Dessain et al. 2001; Bouras, Fu et al. 2005; Wang, Chen et al. 2006; Ropero and Esteller 2007) Overexpression of HDAC1, 6 and 8 was associated with matrix metallopeptidase 9 (MMP-9) which is required for invasion (cell migration) of MCF-7 breast cancer cells (Park, Jun et al. 2011).

1.5 HDAC Inhibitors (HDACi)

As discussed previously the key role of HDACs in cancer, they are also noted to be involved in various inflammatory, viral and neurological pathology conditions. With the growing importance of HDACs involvement in various diseases, the demand of HDAC inhibitors usage is increased from past decade. These HDAC inhibitors act on a molecular level by hyperacetylation of their substrates histone and non-histone proteins.

The list of FDA approved HDACi includes Vorinostat 1 (for treatment of refractory cutaneous T-cell lymphoma) (CTCL) along with combination therapy with mTOR inhibitor ridaforolimus for treatment of advanced stage of renal cell carcinoma (Zibelman, Wong et al. 2015), romedopsin 2 for treating CTCL and PTCL (peripheral T cell lymphoma), also combination therapy with tyosinekinase inhibitor eriotinib is in phase 1 trials to treat non-small cell lung cancer (VanderMolen, McCulloch et al. 2011) (Gerber, Boothman et al. 2015), belinostat 3, for treating PTCL(West and Johnstone 2014). Panabinostat was in use from early 2015, which was an oral

drug approved by FDA, along with bortezomib and dexamethasone combination to treat recurrent multiple myeloma (Garnock-Jones 2015). Valproic acid 5 is often combined with DMTi to improve the efficacy for treating DMT upregulated conditions (Blum, Klisovic et al. 2007). Current phase II trials are ongoing for treating multidrug resistant, chronic myeloid leukemia and AML with entinostat 6 (Valente and Mai 2014). Combination therapy of TSA7 with DNMTi, decitabine is known to increase the prognosis of AML (Momparler, Cote et al. 2014). Pan-HDACi AR-42 is known to reduce myeloma in both in vitro and in vivo (primary myeloma mouse model) (Canella, Cordero Nieves et al. 2015). ACY-1215 9 along with bortezomib combination has an improved efficacy in treating lymphoma when treated on mice model (Amengual, Johannet et al. 2015). Thailandepsin A (TDP-A) 10, a recent discovery in desipeptide class of HDACi with lowest concentration of anti-proliferative activity. This is known to induce pro-apoptotic pathway protein Bax, and inturn decreases anti-apoptotic protein Bcl-2 (Xiao, Li et al. 2015) **(Fig. 8).** Apart from anti-cancer activities of HDAC inhibitors, they are also known to possess anti-inflammatory and anti-viral properties.

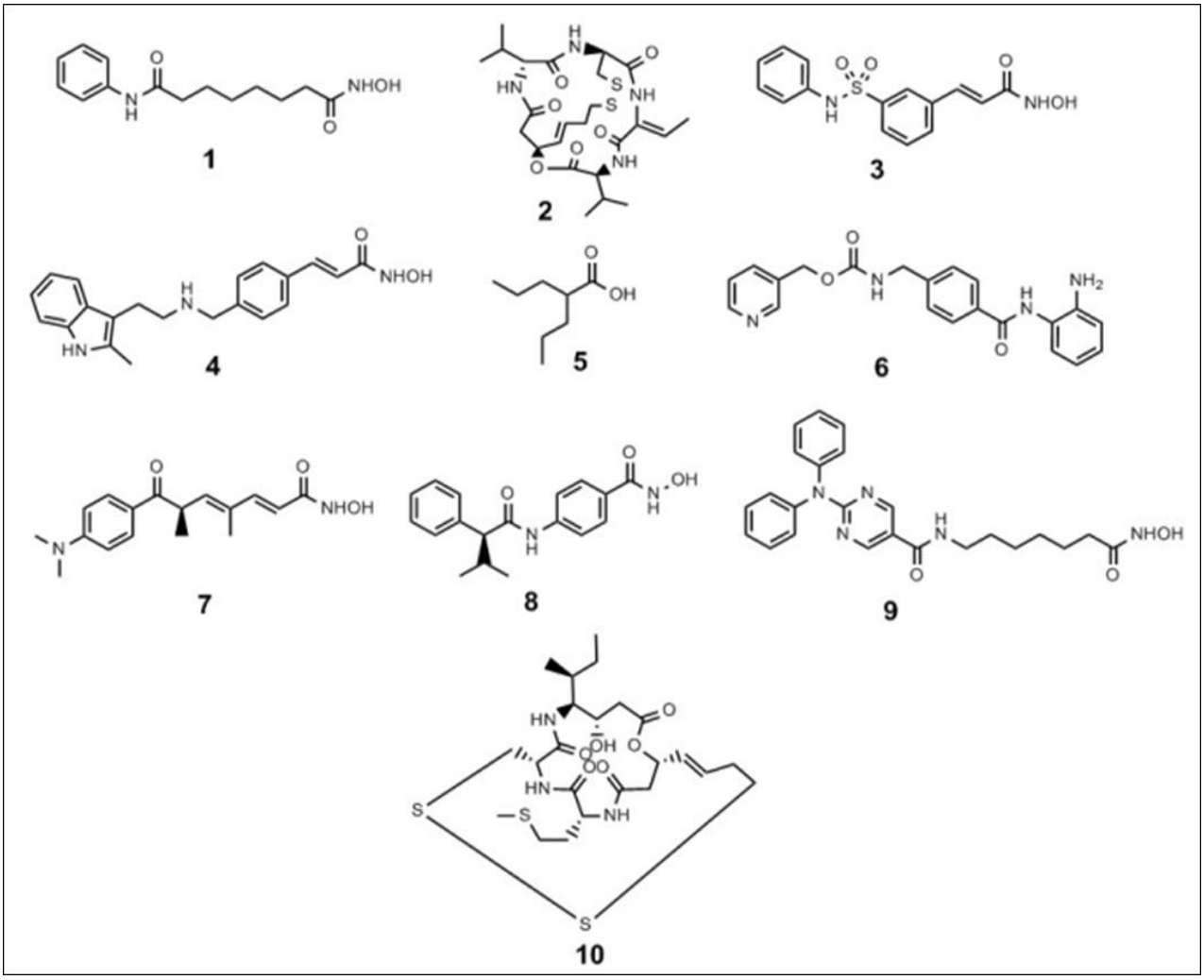


Figure 8. HDAC Inhibitors: *Adapted from Journal of Clinical Epigenetics (2016)*

1.6 Histone Deacetylase 8 (HDAC8)

HDAC8 is the best-studied class I HDAC, both mechanistically and structurally. It was first discovered in the year 2000 where it was involved in catalyzing the deacetylation of number of acetylated histone variants (Hu, Chen et al. 2000; Gantt, Gattis et al. 2006; Dowling, Gattis et al. 2010). HDAC8 is the only deacetylase enzyme known to be crystallized in full length till date **(Fig. 9)** (Vannini, Volpari et al. 2004) and found to be active when expressed and purified from

bacterial system. It is a 377 amino acid (42 kDa) protein and is X-linked in humans, known to function independently without additional co-factors. (Hu, Chen et al. 2000; Gantt, Gattis et al. 2006; Smith and Denu 2007; Dowling, Gantt et al. 2008). HDAC8 is ubiquitously expressed in its primary site i.e. nucleus, as observed in NIH3T3 (Hu, Chen et al. 2000) and HEK 293T cell lines (Buggy, Sideris et al. 2000).

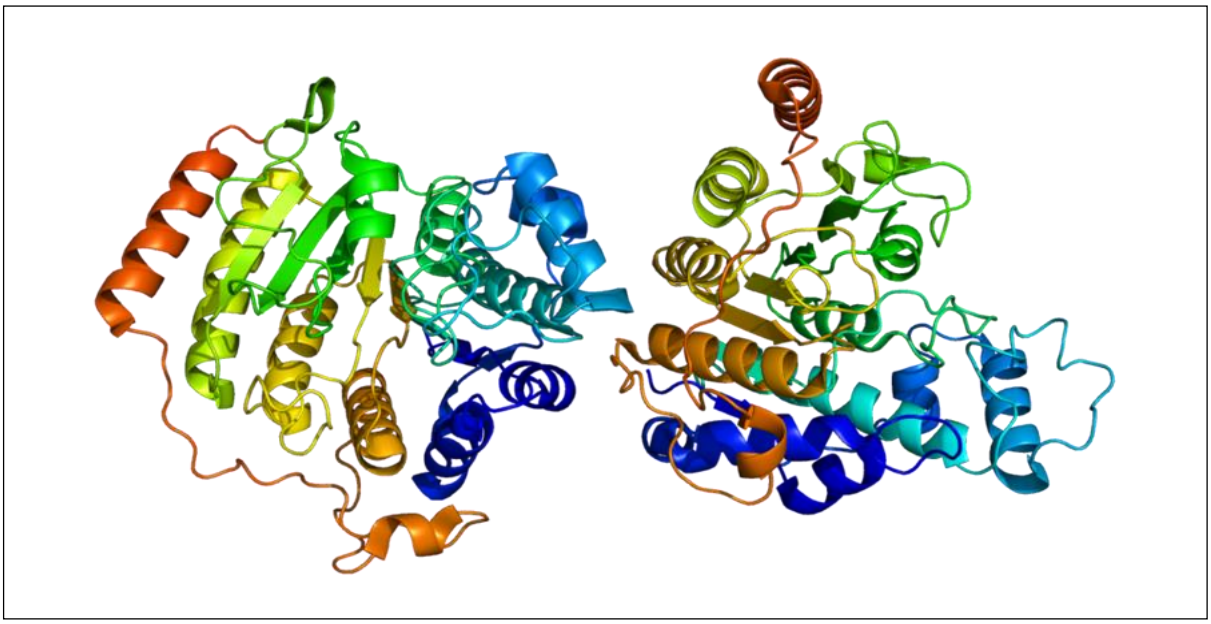


Figure 9. Crystal structure of HDAC8
PDB Ortholog search: PDBe RCSB https://en.wikipedia.org/wiki/HDAC8

Apart from H3 & H4 histone proteins, HDAC8 is able to recognize and deacetylate some of the acetylated non-histone proteins **(Fig. 10)** (Liu, Tarle et al. 1993) (Durst, Lutterbach et al. 2003; Dowling, Gantt et al. 2008). However, the list is incomplete and several of its substrates remained elusive.

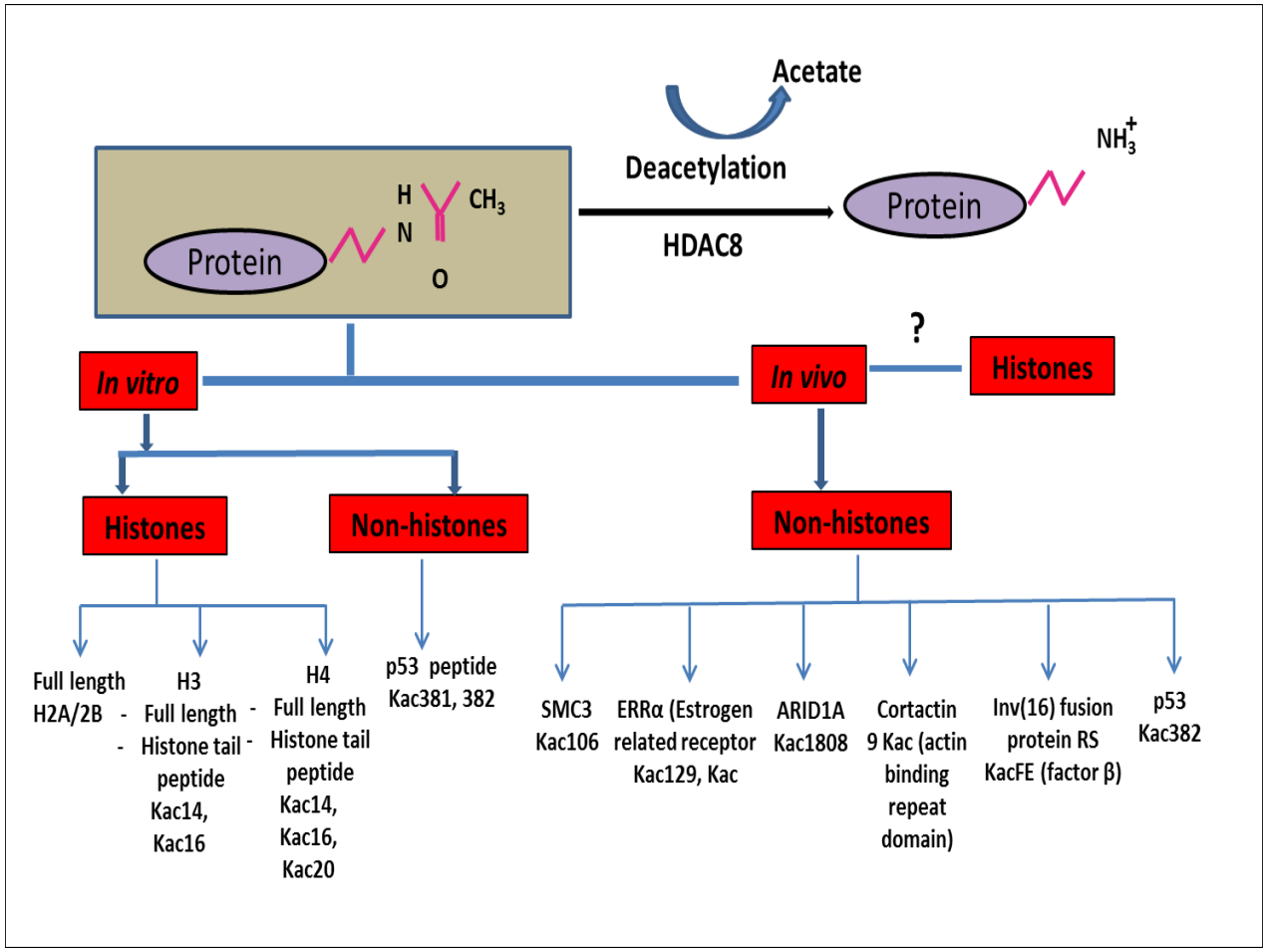


Figure 10. HDAC8 Substrates – Histone & Non-histone: *Deacetylation activity of HDAC8 substrates. HDAC8 is known to deacetylate histones and non-histones in vitro. p^{53} derived peptide is validated tetrapeptide substrate for HDAC8. Substrates for in vivo majorly involve non-histone proteins whereas; in vivo histone substrates remain still controversial. Kac represents for lysine acetylation, RKac represents arginine and lysine RSKacFE, arginine-serine-Kac-phenyl-glutamic acid. Adapted from Trends in pharmacol sci. (2015).*

1.7 HDAC8 and Cancer

Overexpression of HDAC8 is quite aggressive in many cancers like colon, breast, lung, pancreases and also in childhood neuroblastoma (Nakagawa, Oda et al. 2007; Oehme, Deubzer et al. 2009). Knockdown of HDAC8 using siRNA has shown to inhibit human lung, colon, and cervical cancer proliferation (Vannini, Volpari et al. 2004). Whereas, upregulation of HDAC8 is known to inhibit apoptosis in hepatocellular carcinoma (Wu, Du et al. 2013). SOX4 (sex determining region – Y) –box 4 a transcription factor regulates HDAC8 expression by directly activating promoter in adult T cell leukemia/lymphoma (ATL) as supported by the microarray analysis (Schilham, Oosterwegel et al. 1996; Bergsland, Werme et al. 2006; Potzner, Tsarovina et al. 2010). Specific inhibition (PCI-3051) of HDAC8 studies demonstrated calcium-mediated and caspase-dependent apoptosis in T-cell derived lymphoma and leukemic cells (e.g., Jurkat, HuT78, and Molt-4)(Balasubramanian, Ramos et al. 2008) **(Fig. 11)**.

Knockdown of HDAC8 was shown to inhibit the growth of ATL cells lines (Higuchi, Nakayama et al. 2013).

Knockouts of HDAC8 in mice after birth were non-lethal (Haberland, Mokalled et al. 2009). Anti-cancer treatments in humans with pan-HDAC inhibitors are consistently tolerable (Garber 2007). Skull defects are attributed to the overexpression of transcription factors like Otx2 and Lhx1, which are known to be either directly regulated or in complex with HDAC8 (Haberland, Mokalled et al. 2009).

Mutations or truncations in HDAC8 protein is correlated to disability in understanding, mental retardation with severe facial abnormalities (Kaiser, Ansari et al. 2014) (Deardorff, Bando et al. 2012) (Harakalova, van den Boogaard et al. 2012). HDAC8 mutants are not found to be

lethal, but observed with severe facial abnormalities, which justifies the role of HDAC8 as a scaffold for other proteins. (Lee, Sengupta et al. 2006; Gao, Siddoway et al. 2009).

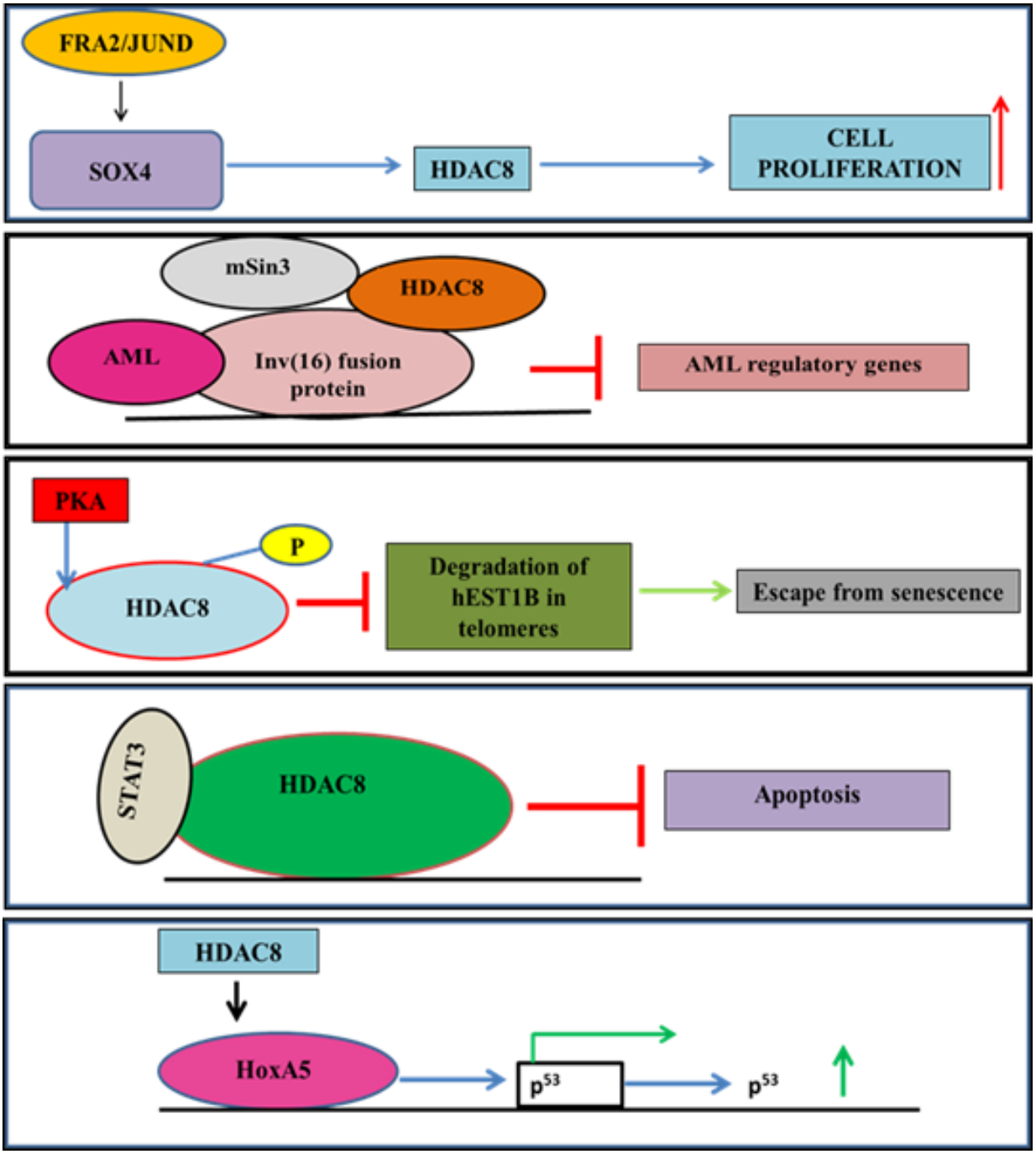


Figure 11. HDAC8 is associated with different protein complexes involved in cancer. *Adapted from Trends pharmacol sci. (2015).*

1.8 HDAC8 and other diseases

Recent studies have identified the role of HDAC8 in enhanced endocytosis, acidification and penetration of Influenza A and Uukuniemi virus (Yamauchi, Boukari et al. 2011). The pathway includes conversion of early endosomes to lysosomes, which resulted in altered endocytosis with insufficient acid conversion of the viruses (de Vries, Tscherne et al. 2011; Yamauchi, Boukari et al. 2011). Knockdown of HDAC8/HDAC3 with siRNA reduced the viral infectivity, (Yamauchi, Boukari et al. 2011) due to decreased HDAC8 levels that in turn alters the dynamics of microtubule networks, causing centrosome splitting, decrease of centrosome associated microtubules which finally leads to improper orientation of centripetal movements of late endo/lysosomes. This signifies the importance of HDAC8 which is known to orchestrate the dynamics of centrosome and cohesion complex and control the endosome mobility. The exact deacetylation mechanism of centrosome or the other regulators of cohesion which leads to entry of influenza virus is quite unclear.

1.9 Known Inhibitors of HDAC8

Specific and selective HDAC8 inhibitor designing along with its characterization in advanced preclinical models is still a questionable challenge. Several examples are available which are selective to HDAC8. Different class of molecules are included in this category, such as “linkerless” inhibitor 1, compound2, others like 12-membered macrocycle-based phenyl hydroxamate 3, and triazole-containing compounds, such as NCC149(5) and 6 (Krennhrubec, Marshall et al. 2007; Tang, Luo et al. 2011; Olson, Wagner et al. 2013; Suzuki, Muto et al. 2014). Reduction in growth of T-cell lymphoma cells was observed with the treatment of Triazoles 5 and 6 and also a dramatic change in the acetylation pattern of cohesin was

demonstrated in HeLa cells. Significant growth inhibition towards many human lung cancer cell lines was observed in vitro with the treatment of Ortho-aryl N-hydroxycinnamides such as 4, which is a very selective inhibitor for HDAC8 (Huang, Wang et al. 2012). Compounds 1, 8, and 9 have an average range of HDAC8 selectivity with micromolar range **(Fig. 12)**.

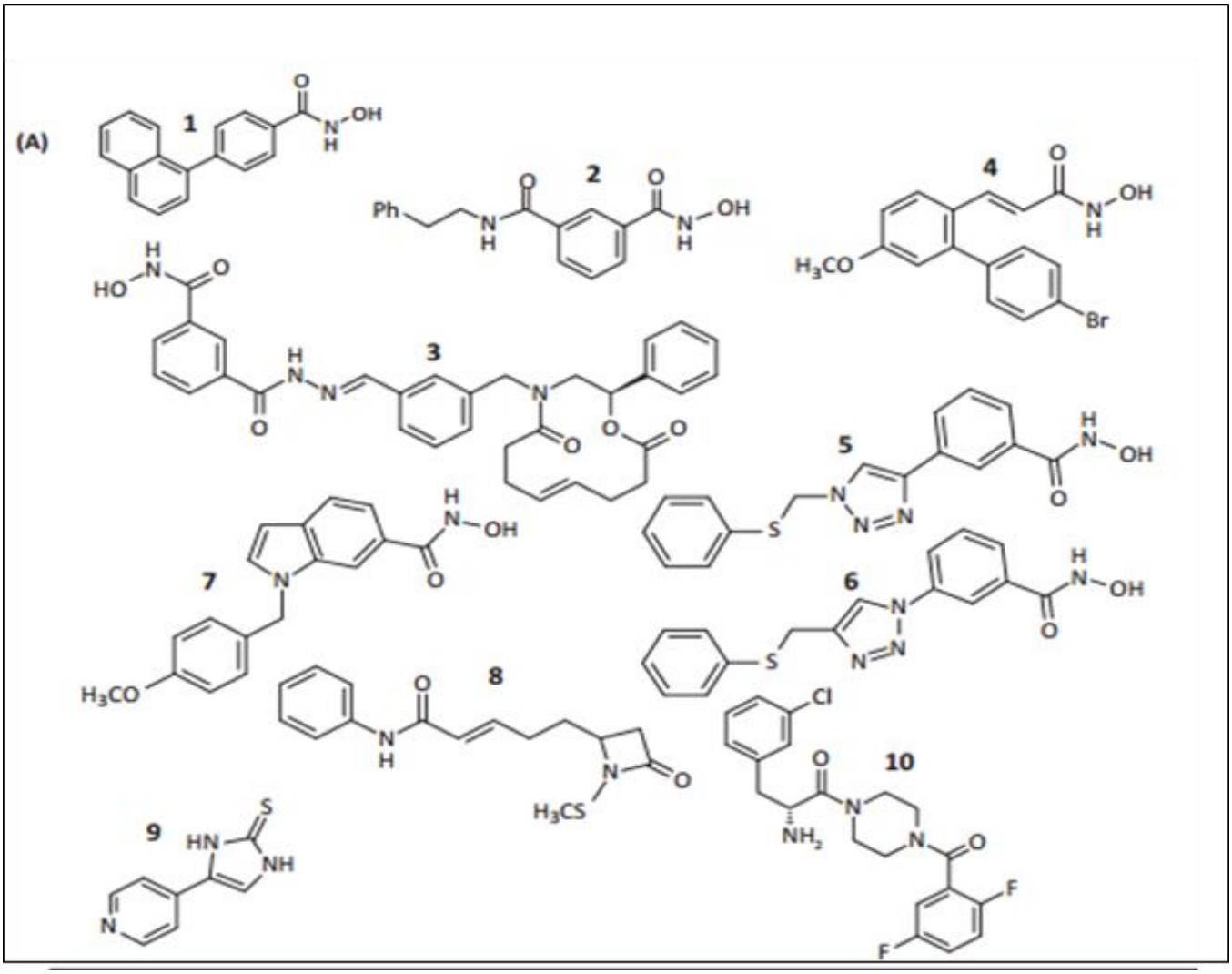


Figure 12. Known specific HDAC8 Inhibitors: *Adapted from Leukemia (2008), Bioorg. Med. Chem. Lett. (2007), J. Med. Chem. (2013, Bioorg. Med. Chem. Lett. (2011), ChemMedChem (2014), ChemMedChem. (2012), ChemMedChem (2009), J. Pharmacol. Exp. Ther. (2003), Bioorg. Med. Chem. (2011)*

Clinical studies with isoform specific inhibitor studies are known to shown promising results, as observed in the case of clinical studies with neuroblastoma and T cell leukemia conditions, where selective HDAC8i was used. PCI-34051 **(Fig. 13)** is a highly selective inhibitor for HDAC8 (>200 fold) is quite effective in anti-proliferative studies with T-cell leukemia, but it did not show any effect in case of solid tumors (Balasubramanian, Ramos et al. 2008) (Huang, Wang et al. 2012).

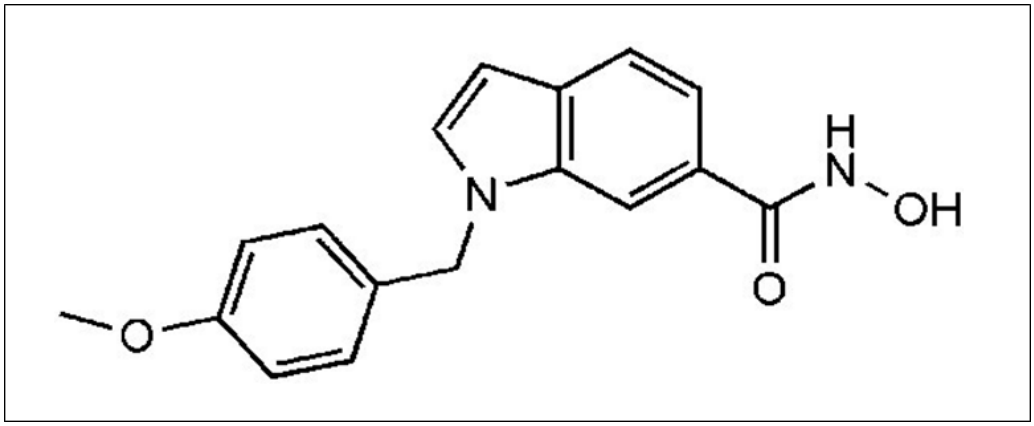


Figure 13. Structure of PCI-34051
http://www.sigmaaldrich.com/catalog/product/sigma/p0062?lang=en&region=IN

Compounds 3 and 10 are considered to be effective at nanomolar concentration. Benzyhydroxamate 2 is also shown to inhibit HDAC8 at nanomolar concentration, but it was also showing its potency in inhibiting HDAC6 at a higher rate. Pan.-HDAC inhibitors like SAHA (Suberoylanilide hydroxamic acid) and TSA (Trichostatin A) show a very weaker affinity for binding with HDAC8. *In vivo* anti-cancer studies have shown promising results, along with adverse side effects like diarrhea, hematological toxicity and cardiac issues. Thus, there is an urgent need in minimizing these side effects by designing inhibitors by differentiating between HDAC8 related specificity to other HDAC isoforms **(Fig. 14)**. This can be more specific with higher standards of safety (Chakrabarti, Oehme et al. 2015).

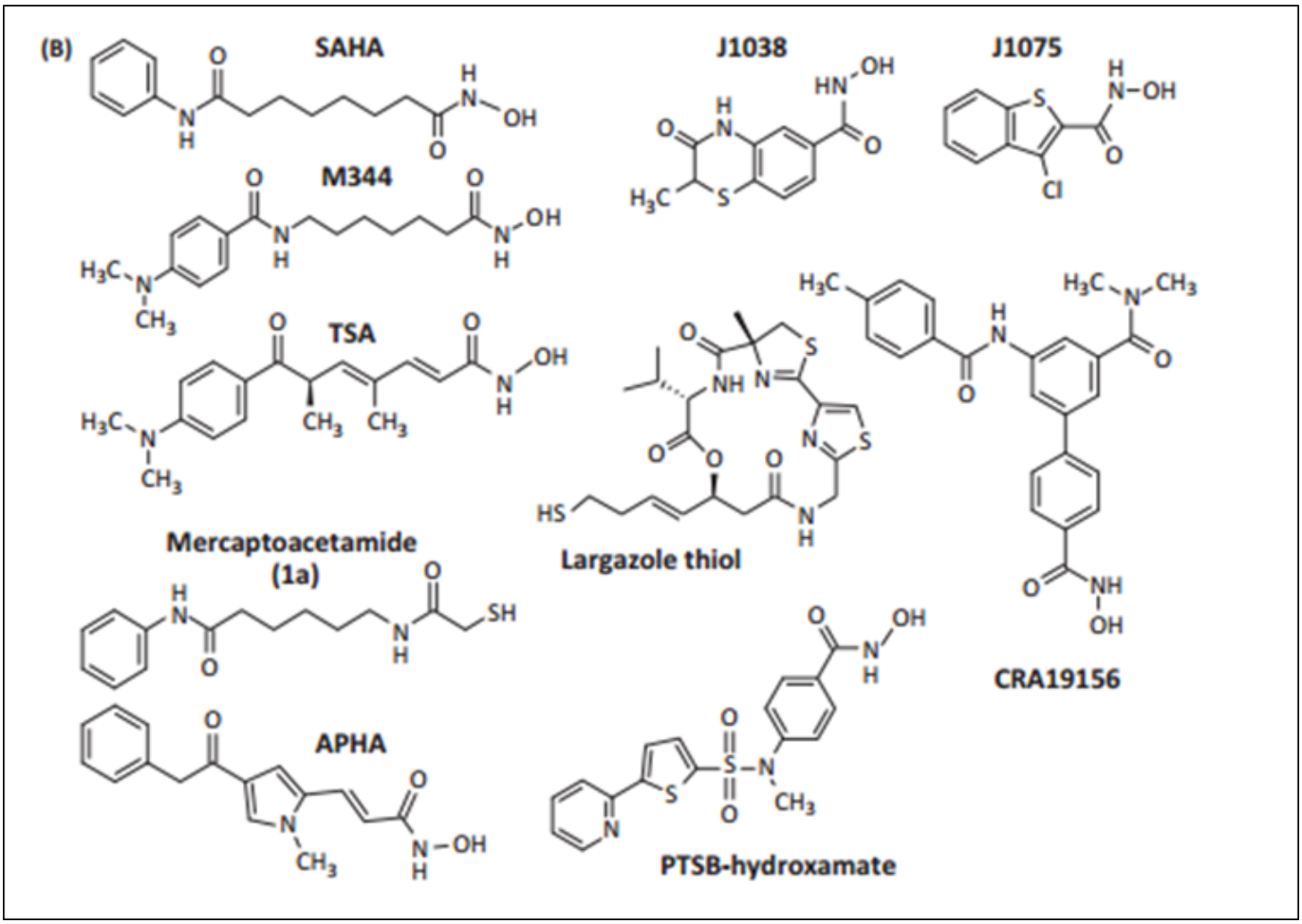


***Figure 14*. Inhibitors co-crystalized with HDAC8:** *Adapted from Structure (2004) PLoS Pathog.(2013), EMBO Rep.(2007), Proc. Natl. Acad. Sci. (2004), ACS Chem. Biol. (2014), Bioorg. Med. Chem. 19, (2011), J. Mol. Biol. (2014), Biochemistry (2008), Biochemistry (2010), J. Am. Chem. Soc. (2011)*

Thus, it is very clear that HDAC inhibitors are drawing attention in current clinical studies for treating various diseases including cancer, viral infections, inflammation and neurological diseases. In general these broad spectrums HDACi are known to have numerous off targets HDACs; this in turn will lead to inhibit the multi protein complexes involved with them. Off target effects are known to cause severe toxicities causing damage to even normal cells. All though

developing specific HDACi is quite chanllenging due to unavailability of crystal structures for all the HDACs. Therefore, it is necessary to develop and use a selective HDAC inhibitor, in order to better understand the molecular effects in a specific cell type.

2.0 Rationale of the study

Although HDAC8 is structurally and mechanistically very well studied, details on its non-histone protein-binding partners are obscure. Furthermore, HDAC8 is X-linked gene and known to be specifically overexpressed in female-specific cancers like cervical, breast, and ovary. Therefore, the rationale of the study was to identify non-histone protein binding partners of HDAC8 in cancerous and normal cells. We have considered HeLa (cervical cancer) cells for our study as cancerous cell line and HEK 293T as normal non-cancerous cell line. Although normal non-cancerous cervical epithelial cell line would have been a better control, due to unavailability of transformed cell line, we used HEK 293 T as a control cell line.

The following are the objectives of the study:

- Identification and characterization of non-histone protein substrate/binding partner of hHDAC8 in HeLa cells.
- Protein-protein interaction studies of HDAC8 & Acetylated (Lys40) alpha tubulin.
- Functional characterization studies of HDAC8 and Alpha tubulin interactions *in vitro.*

Materials & methods

2.1 Materials

Forskolin was purchased from Sigma (USA). PCI-34051 was procured from Cayman chemicals. DMEM, FBS, 1% Penicillin & Streptomycin Antibiotics were obtained from Himedia. Monoclonal antibody of HDAC8 (A-4008) was obtained from Epigenetik, Beta actin ab8227 (Abcam), GAPDH (MA5-15738), Alpha tubulin (B-5-1-2) and anti-acetylated alpha tubulin (Cat no: 322700) were purchased from Thermo scientific Life technologies respectively. Protein A Agarose beads were obtained from Santa Cruz. Poly-L-Lysine (Sigma P8920), 4', 6-Diamindino-2-phenylindole dichloride DAPI as a nuclear stain (Sigma), Alexa Fluor® 555 Dye (Thermo Fischer Scientific). HDAC8 FLUOR DE LYS fluorometric assay kit (BML-AK518-0001) was purchased from Enzo life sciences. Propidium iodide was purchased from Himedia. GST Column (GE Healthcare 17-5132-01), Custom synthesized peptides of alpha tubulin 33-46 amino acids, Acetylated alpha tubulin peptide, at Lys40 DGQMPSDKTIGGGD and Unacetylated Alpha tubulin peptide DGQMPSDKTIGGGD from SIGMA (USA) (resuspended in MilliQ water).

2.2 Methods

2.2.1 Cell culture and Treatments

Human embryonic kidney cells, HEK293T and human cervical carcinoma cells, HeLa, MDAMB 231, A549, K562, Colo and HCT11 were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cell line authentication by STR analysis was performed for 10 genetic loci for all the cell lines using Life code, Genomic technologies Pvt. Ltd., and tested for mycoplasma negative by performing PCR (mycoplasma specific genes) by using EZdetect PCR kit for mycoplasma

detection (CCK009). All the cells were cultured in high glucose DMEM medium containing 10% foetal bovine serum and 1% Penicillin/streptomycin (Himedia). Cells were seeded in 60 mm dishes and upon attaining 80-90% confluency, treatment was given with PCI-34051(Cayman Chemicals) (10µM/20µM) (Bantscheff, Hopf et al. 2011) and tubastatin (5µM) or both (PCI-34051 20µM + Tubastatin 5 µM) for 24 hrs (Bantscheff, Hopf et al. 2011) and Forskolin (Sigma) (10µM) for 45 min independently along with untreated control as described earlier (Lee, Rezai-Zadeh et al. 2004).

2.2.2 Immunofluorescence

HeLa and HEK 293T cells (Control, PCI-34081Forskolin treated), were fixed with 4% formaldehyde prepared in 1XPBS for 20 min at RT followed by permeabilization with 0.25% TritonX-100 in 1XPBS. Nucleolus from HeLa cells were isolated according to the standard protocol (Lam, Trinkle-Mulcahy et al. 2005) and fixed on a slide with Poly-L-Lysine (Sigma P8920) coating. Cells were stained with Monoclonal antibody of HDAC8 (A-4008) from Epigenetik or anti-acetylated alpha tubulin (Cat no: 322700) from Thermo scientific Life technologies respectively, followed with Anti-mouse or Anti-rabbit secondary labelled with Alexa Fluor, and 4', 6-Diamindino-2-phenylindole dichloride DAPI as a nuclear stain (Sigma).

2.2.3 HDAC8 Enzyme Activity Assay

HeLa and HEK 293T cells were cultured in high glucose DMEM medium containing 10% foetal bovine serum and 1% Penicillin/streptomycin (Himedia). Cells were seeded in 60 mm dishes and upon attaining 80-90% confluency, treatment was given with PCI-34051(Cayman Chemicals) (10 µM) for 24 hrs and Forskolin (Sigma) (10 µM) for 45 min (Lee, Rezai-Zadeh et al. 2004) independently along with control (untreated). HDAC8 Enzyme activity assay was performed (HDAC8 FLUOR DE LYS fluorometric assay kit BML-AK518-0001) for Control, PCI-34051, and forskolin

treated Immunoprecipitated (Anti-HDAC8 antibody 2µg for ~500µg of total, cytoplasmic and nuclear fractions) fractions of total protein, cytoplasmic and nuclear fractions of HeLa and HEK 293T cell lines according to the manufacturers protocol.

2.2.4 Protein expression studies/ Western blotting

Total, cytoplasmic or nuclear fractions [Control, PCI-34051 (10 µM/20 µM) & Forskolin (10 µM), Tubastatin 5 µM, or (PCI-34051 20 µM + Tubastatin 5 µM)] treated of HeLa & HEK 293T (or) total protein from HCT11, Colo, K562, A549, MDAMB 231, Hela, HEK 293T (or) HDAC8 siRNA, Scrambled siRNA transfected cells of HeLa and HEK 293T were prepared by lysing the cells with RIPA buffer (total protein) or by using, cytoplasmic extraction with (30 mM Tris p^H 7.5, 10 mM Magnesium acetate, 1% NP-40, 1 mM PMSF, 1X Protease inhibitor cocktail) and Nuclear fraction with (420 mM Nacl, 10 mM HEPES, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, 25% Glycerol, 1 mM PMSF, 1X Protease inhibitor cocktail) of equal conentrations and separated on 10 or 12% SDS-PAGE. Antibodies of HDAC8 (A-4008) from Epigenetik, Beta actin ab8227Alpha tubulin (B-5-1-2) and anti-acetylated alpha tubulin (Cat no: 322700) GAPDH (MA5-15738) from Thermo scientific Life technologies, were used for western blotting.

2.2.5 Immunoprecipitation and MALDI TOF-TOF Analysis

HeLa cells were grown in 100 mm dishes supplemented with DMEM medium containing 10% foetal bovine serum and 1% Penicillin/streptomycin (Himedia) till 90% confluency was attained. Cells were trypsinized, washed with 1XPBS and then lysed with (IP Lysis Buffer 50mM Tris p^H 8.0, 150mM Nacl, 10% Glycerol, 0.5% TritonX-100. 1X Protease Inhibitor Cocktail). In duplicates ~500 µg of total protein with ~2 µg of Anti-HDAC8 (A-4008) from Epigenetik, and Mouse IgG as an isotypic control was set up on a rotaspin at 4°C for overnight. Protein A beads 20 µl were washed

thoroughly three times with lysis buffer, and then added to the Antibody-Lysate mixture and further incubated for two hours. After two hours, beads were washed with wash buffer (MSWB Buffer 50mM Tris p^H 8.0, 150mM Nacl, 1mM EDTA and 0.1%NP-40) for three to five times at 3000 rpm, 2 min 4°C. Samples were prepared in 2X sample buffer, and separated on 10% SDS PAGE along with the input sample lysate. Coomassie staining was performed for the resulting gel, and differentially obtained band in the IP sample was further subjected to MALDI TOF-TOF analysis. Mascot search results confirmed Alpha tubulin-1C as one of the non-histone interacting partner of HDAC8 with a significant score of 56%, 12 matches and ($p<0.05$).

2.2.6 Sequence analysis

The amino acid sequences of tubulins were retrieved from NCBI database (Accession numbers: AAH33064.1, AAH20946.1, and AAF34188.1 for alpha, beta and gamma tubulin respectively). They were further subjected to multiple sequence alignment using Clustal omega (Sievers, Wilm et al. 2011) to infer sequence conservation among the tubulin proteins. The sequences were analyzed for functional conserved regions using Pfam database (Finn, Bateman et al. 2014). This was followed by PDB-BLAST (Altschul, Gish et al. 1990) to search for a suitable template for the homology modelling. The crystal structures for beta-tubulin (PDB ID: 2XRP_A) and gamma-tubulin (PDB ID: 3CB2_A) were available in the database. However, Alpha-tubulin did not have any structure in PDB and therefore its structure was built using homology modelling (described below). In addition to this, Protein BLAST in eukaryotic genomes was also carried out to analyze the sequence homology.

2.2.7 Phylogenetic Tree Analysis of Alpha tubulin

The sequence of human alpha-tubulin C (NCBI ID: AAH33064.1) was used as query against the BLASTP (Altschul *et al.*, 1997) database of Eukaryotes to identify homologues and

the sequences with significant E-value were retrieved. MUSCLE 3.5 (Edgar *et al*., 2004) was used for the multiple sequence alignment. The alignment was edited using AliView (Anders Larsson *et al*., 2015). The Maximum Likelihood tree was constructed using PhyML v. 3.0 (Guindon *et al*., 2010) with LG model.

2.2.8 Homology Modelling

Homology modelling is a well-known methodology for building an atomic-resolution model of a protein from its amino acid sequence (the "query sequence" or "target"). "The technique is based on the identification of one or more known protein structures (known as "templates" or "parent structures") likely to resemble the structure of the query sequence and on the production of an alignment that maps residues in the query sequence to residues in the template sequence. Homology modelling of Alpha tubulin was carried out by I-TASSER, an online webserver for protein structure prediction and structure-based function annotation (Yang, Yan et al. 2015). The tertiary structure prediction was performed by I-TASSER server by using the best align template". The template was selected to analyze 3-D structure because a high level of sequence identity should guarantee a more accurate alignment between the target sequence and template structure.

Out of the generated models of the target sequence, the best template (PDB ID: 4I4T_A) was selected based on the significant sequence, C-Score, TM score and RMSD values of template structure.

The generated predicted models were further analyzed for their quality by I-TASSER using C- score (confidence score) as a reference. "It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is normally in the range of [-5, 2], where a C-score of higher value signifies a model with a

high confidence and vice-versa. TM-score is a scale for measuring the structural similarity between two structures (Zhang and Skolnick 2004). A TM-score >0.5 indicates a model of correct topology and a TM-score<0.17 means a random similarity. This cut-off does not depend on the protein length. With respect to RMSD, it is measured as an average distance of all residue pairs in two structures". The resulting model was further validated by Verify 3D (Luthy, Bowie et al. 1992), ERRAT (Colovos and Yeates 1993) and Ramachandran plot (Ramachandran, Ramakrishnan et al. 1963) programs. PyMOL (Schrodinger Inc.) (DeLano, 2002) was used to visualize the model and generate publishable images.

2.2.9 Protein-Protein Interactions using ZDOCK

Protein-protein interaction studies of tubulins (alpha, beta, gamma) were carried out with HDAC8 (PDB ID: 1W22_A) using ZDOCK (Pierce, Wiehe et al. 2014), which is an interactive online based server for docking protein-protein complexes. "It uses the Fast Fourier Transform algorithm to enable an efficient global docking search on a 3D grid, and utilizes a combination of shape complementarity, electrostatics and statistical potential terms for scoring. It has been shown that ZDOCK achieved high predictive accuracy on protein–protein docking benchmarks, with >70% success in the top 1000 predictions for rigid-body cases in the most recent benchmark version (Pierce, Wiehe et al. 2014). ZDOCK has consistent success (acceptable or better predictions for 22 of the last 35 submitted targets) in the international protein–protein docking experiment, Critical Assessment of Predicted Interactions (CAPRI)" Upon sequence confirmation of the insert, clone was transformed into *Escherichia coli* strain BL21 cells. Protein expression and purification protocols were well standardized. Further confirmation of the purity of the purified protein was carried out by SDS-PAGE with Coomassie staining, which depicts >80% pure followed by Western blot confirmation **(Fig. 32**

a&b). (Wiehe, Pierce et al. 2005, Wiehe, Pierce et al. 2007, Hwang, Vreven et al. 2010, Vreven, Pierce et al. 2013).

2.2.10 Co-Immunoprecipitation and Immunoblotting

Protein Lysates are prepared from control, PCI-34051 and forskolin treated samples of HeLa and HEK 293T cell lines using (IP Lysis Buffer 50mM Tris p^H 8.0, 150mM Nacl, 10% Glycerol, 0.5% TritonX-100. 1X Protease Inhibitor Cocktail). Total protein of ~500 µg was used for incubating with ~3 µg of Anti-HDAC8 antibody or ~3 µg of Anti – α Tubulin Antibody (B-5-1-2) on a rotaspin for overnight at 4°C. Mouse IgG was used as control. Protein A beads slurry of 20 µl was added to each tube after washing three times with IP Lysis buffer and further incubated for two hours at 4°C. The samples were washed three times with wash buffer (MSWB Buffer 50mM Tris p^H 8.0, 150mM Nacl, 1mM EDTA and 0.1%NP-40) and final sample was prepared by boiling the beads in 2X Sample buffer.

Prepared protein samples were resolved on 10 or 12% SDS PAGE and then blotted to PVDF (BioTrace PVDF from PALL life sciences). Antibodies of HDAC8 (A-4008) from Epigenetik, Alpha tubulin (B-5-1-2) and anti-acetylated alpha tubulin (Cat no: 322700) from Thermo scientific Life technologies were used for immunoblotting.

2.2.11 hHDAC8 clone (construct p^{GEX}- 6p1 hHDAC8) and expression of GST-HDAC8 fusion protein

hHDAC8 (Full length) cDNA was amplified by using a polymerase chain reaction (PCR) with forward primer: 5'– GGAATTCATGGAGGAGCCGGAGGAACC-3' and a reverse primer: 5'– CCGCTCGAGCTAGACCACATGCTTCAGATTCCC–3'. The PCR product was then sub cloned into p^{GEX}- 6p1 plasmid. Clone was then overexpressed in *Escherichia coli* strain BL21 (DE3) with

standardized expression conditions as 0.1Mm IPTG, 28°c, 4 hrs. Full length HDAC8 was purified using a GST column (GE Healthcare).

2.2.12 Purification of GST - hHDAC8

Induced culture pellet was lysed in 1XPBS buffer (140mM Nacl, 2.7 mM Kcl, 10 mM Na_2HPo_4, 1.8 mM KH_2PO_4) along with 1X Protease inhibitor cocktail, 1 mM PMSF, and 0.1 mM DTT. To the lysate 1% Triton X 100 is added and centrifuged for 20 min at a speed of 4000 rpm at 4°c. Clear supernatant was used for binding with GST binding buffer (25 mM Tris p^H 7.5, 300 mM Nacl, 1 mM EDTA, 1X Protease inhibitor cocktail) for 3 hrs on a rota spin at 4°c. Binding was followed by collection of unbound and washes with 1X PBS (5 washes). Elutions were collected in 10 mM reduced glutathione, dissolved in 50 mM Tris p^H 8.0. The purity of the protein was then analysed on SDS PAGE, followed by western blot. Activity was then determined by HDAC activity assay.

2.2.13 Surface plasmon resonance studies

All the reagents including GST-HDAC8 and peptides were diluted in HBS-EP buffer. Biacore T200 system (GE Healthcare), with software of Evaluation V 2.0 was used to perform SPR analysis. CM5 chip sensor chip, immobilization and running and regeneration buffers were used according to the methodology described previously (GST capture kit GE healthcare). GST Antibody 25µg/mL was used for immobilization. After attaining the required response units for GST-Ab, GST-HDAC8 binding analysis was standardized with 2, 4 & 6 µg/mL concentrations in HBS_EP buffer. Relative response units (R.U) for both binding and stability were attained to an acceptable range. Binding studies of GST-HDAC8 (6µg/mL) with peptides Acetylated Alpha tubulin peptide at Lys40

DGQMPSDKTIGGGD and Unacetylated Alpha tubulin peptide DGQMPSDKTIGGGD were carried out with following conditions, contact time of 120(s); Flow rate: 30 µl/min; Dissociation time period: 600 (s) regeneration solution Glyc Hcl pH2.0; contact time 30(s); Flow rate: 30 µl/min; stabilization period: 0 (s) analysis temperature: 25^{o}C, sample compartment temperature: 25^{o}C. The experiment was repeated twice.

2.2.14 Fluorescence spectrometry

Binding studies of GST-HDAC8 & alpha tubulin peptides (ac & Unac) forms were carried out using Perkin Elmer precisely LS55 fluorescence spectrometry. Time dependent intrinsic fluorescence decrease in the fluorescence intensities were recorded for blank buffer (Tris pH 8.0 50 mM) GST-HDAC8 (1µM) alone, GST-HDAC8 + acetylated alpha tubulin (500nM) and GST-HDAC8 + unacetylated alpha tubulin (500nM). Pure GST-HDAC8 was incubated with acetylated (500nM) and unacetylated alpha tubulin peptides (500nM) independently, with time dependence from 5-30 min, at 37°C in Tris 50 mM pH 8.0. The decrease in the fluorescence intensity was plotted against the wavelength (nm).

2.2.15 Circular Dichroism Spectroscopy Studies

Binding studies of GST-HDAC8 pure protein of (2µM) concentration (Nacl concentration of 10mM, 50mM Tris p^{H} 8.0) and 500nM concentrations of both acetylated (Lys40) and unacetylated alpha tubulin peptides were carried out on Jasco J-1500 (model L-1500-450). Changes incurred in the secondary structure of GST-HDAC8 upon binding of both the peptides were recorded as the mean ellipticity, with given set of parameters. Start wavelength of 300nm, end wavelength of 190 nm, scan

speed of 100 nm/min, band width of 2nm, cuvette cell size of 2mm and at a temperature of 25°C with two scan accumulations.

2.2.16 HPLC Studies

In vitro deacetylation assay of custom synthesized acetyalated (Lys40) alpha tubulin was carried out on HPLC (Schimadzu) with C18 (4.6x250mm) column. The reaction was carried out in 50mM Tris pH 7.5, 1mM DTT buffer. Initially 500 nM concentrations of both Acetylated (Lys40) and Unacetylated alpha tubulin peptide were incubated individually in the buffer for 5 min or buffer with peptide was incubated for 5 min at 37°C , followed by addition of GST-HDAC8 protein (2µM) and incubation at 37°C for 15 min. The reaction was then quenched by addition of 1% TFA to the final reaction volume of 110µl and injected into HPLC as described earlier (Allis 2004).

2.2.17 RNA interference (siRNA) studies

Source for siRNA oligonucleotides were considered from (Deardorff, Bando et al. 2012) with sequence, HDAC8 Sense: GACGGAAAUUUGAGCGUAUUCUCU and Anti-sense: UAGAGAAUACGCUCAAAUUUCCGU. The oligonucleotides were converted into siRNA by following standard protocol as described earlier (Donze and Picard 2002). HeLa and HEK 293T cells were transfected with HDAC8 siRNA (10-15 µg/100 mm dish) using lipofectamine 2000 (Invitrogen), and considering untransfected as control. Cells were harvested after 72 hrs of post-transfection and processed for total RNA and protein isolation.

2.2.18 Real time Analysis

HeLa and HEK 293T cells treated with PCI-34051 (20 µM) or Paclitaxel (20 µM) for 24 hrs or HDAC8 siRNA (10-15 µg/100 mm dish) transfected HEK 293T and HeLa for 72 hrs, along with control were subjected to total RNA isolation by using TRIZOL (Sigma-Aldrich, USA). As per the manufacturers protocol 1 µg of RNA was reverse transcribed with reverse transcription kit, (Invitrogen). Real-time RT-PCR was performed on Applied Biosystems StepOnePlus™ Instrument using KAPA SYBR® FAST qPCR master mix and gene-specific primers. The experiment was repeated twice, which were performed in duplicates. Fold expression determination, gene-to-GAPDH ratios were determined by using the $2^{-\Delta\Delta Ct}$ method. Details of primers used are given below.

GAPDH: FP 5'-GAGAAGGCTGGGGCTCATTTGC-3',

RP 5'-TGGTGCAGGAGGCATTGCTGATG-3',

HDAC6: FP 5'- GGCTAGGTTATGACTGCCCAGC-3',

RP 5'- CATGATGCCACCCTCCAGACC-3',

HDAC8: FP 5'- TGTCTCTGGAGGGTGGCTACAAC-3',

RP 5'- GGAAACTGAAGCCTGGGCACTC-3'

2.2.19 Cell Migration Assay

HeLa cells are grown in 6 well plates in duplicates till it attains 90% confluency and starved with serum free media for overnight. Using a sterile 200 µl tip wound is created by drawing straight lines at three different points in each well. Detached cells are aspirated and washed three times with 1XPBS. Cells are treated with PCI-34051 (10µM & 20µM) and Paclitaxel (10µM & 20µM) dissolved in 2% FBS respectively along with control (untreated). Migrated cells into the wound were examined by capturing images using Olympus CKX41, ProgRes CT3 phase contrast microscope after 24 and 48 hours.

2.2.20 Cell Morphology Analysis

HeLa and HEK 293T cells are cultured in 6 well plates till attaining 70% confluency. Cells are starved for 12 hours without serum and treated with PCI-34051 (20µM) and Paclitaxel (20µM) along with untreated control for 24 & 48 hrs. Examined difference in the morphology was captured by Olympus CKX41, ProgRes CT3 phase contrast microscope at 10X focussed lens.

2.2.21 Cell Cycle Analysis

Cells (HeLa & HEK 293T) were grown to attain 70% confluency and were serum starved for 12 hours, followed by treatments with PCI-34051 (20µM) and Paclitaxel (20µM) respectively for 48 hrs and processed for cell cycle analysis with propidium iodide staining.

Results & Discussion

Objective: 1

Identification and characterization of non-histone protein substrate/binding partner of hHDAC8 in HeLa cells.

3.1.1 HDAC8 is localized predominantly to Cytoplasm

Modifications occurring on DNA, without altering its genetic makeup and are heritable to daughter cells, is generally considered as an epigenetic modifications. The key modulators of epigenetic regulation include DNA methylation, chromatin remodeling and histone modifications (Weinhold 2006). Among all the histone modifications identified, histone acetylation and deacetylation is very well studied. Regulation of histone deacetylation by HDACs occurs in a very complex and balanced manner. It has been well studied that other than HDAC8, all other HDACs are known to work in multiprotein complexes and regulate many physiological and pathophysiological conditions (Yang and Seto 2003). HDAC8 belongs to class I HDACs that are generally localized to nucleus and regulate gene expression. Although HDAC8 is well-characterized structurally its precise cellular localization, functional role and the substrates are not known completely (Olson, Udeshi et al. 2014). Since HDAC8 is known to be involved in female-specific cancers such as ovary, cervical and breast (Nakagawa, Oda et al. 2007), we have used HeLa cervical cancer cells along with HEK 293T normal cells so as to get an insight on its role in normal and cancer cells. HDAC8 is known to deacetylate acetylated peptide *in vitro* implicating its unique behavior when compared to other class I HDACs that function in protein complexes. In order to better understand this unique nature and identify the protein interacting partners of HDAC8, it is therefore important to first determine the precise cellular localization of HDAC8.

In the present study, the confocal microscopic analysis confirmed the predominant cytoplasmic localization of HDAC8 in both HEK293T and HeLa cells **(Fig. 15a)** unlike other class I HDACs that are known to be majorly nuclear. Results obtained from our localization studies clearly suggest the role of HDAC8 in cytoplasm. Overexpression of HDAC8 was clearly observed

in HeLa, when compared to HEK 293T cells were there was a basal level of expression and confined majorly to cytoplasm. The results obtained are in agreement with the earlier studies (Buggy, Sideris et al. 2000, Hu, Chen et al. 2000, Van den Wyngaert, de Vries et al. 2000, Li, Chen et al. 2014). However, in HeLa cells, nucleolar expression of HDAC8 was also observed which was not seen in HEK 293T cells **(Fig. 15b)**.

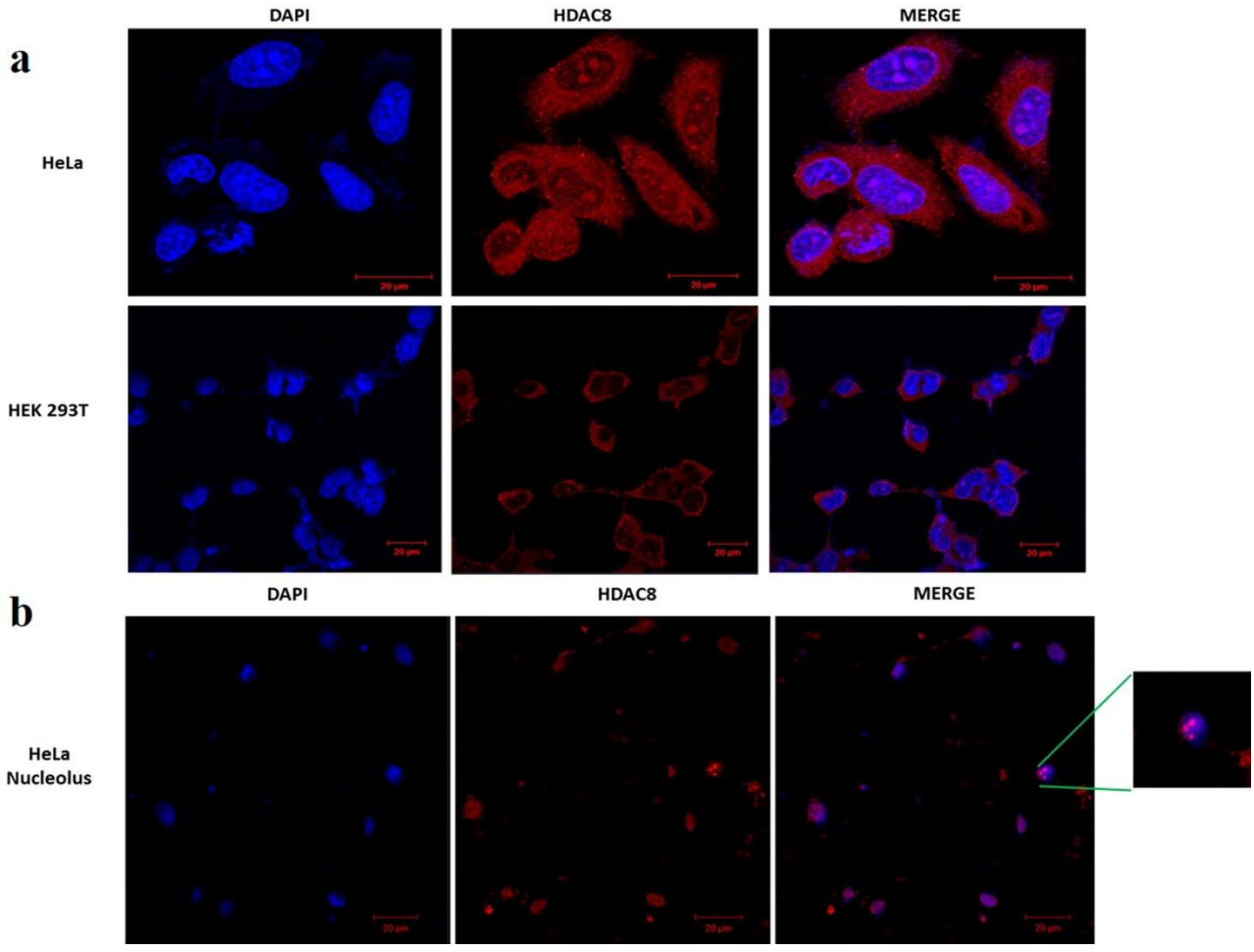


Figure 15. HDAC8 is predominantly cytoplasmic in localization: a. *confocal images of HDAC8 localization in HeLa (upper panel) and HEK 293T (lower panel) (Scale: 20 µm).* ***b.*** *Nucleolar localization of HDAC8 in HeLa (Scale: 20 µm).*

Such nucleolar localization of a protein involved in transcriptional regulation and gene repression in cancer cells was observed with CCCTC-binding factor (CTCF) (Torrano, Navascues et al. 2006). Exploring the role of HDAC8 in nucleolus would be interesting and is not in the scope of this study.

Phosphorylation of HDAC8 is considered as one of the key post translational modification in regulating its enzyme activity. Studies performed with some of the protein kinases like casein kinase II, protein kinase A (PKA) and protein kinase G (PKG) demonstrated that phosphorylation of HDAC8 was known to be catalyzed by PKA and PKG (Tsai and Seto 2002). Predominant phosphorylation of HDAC8 was catalyzed by PKA as demonstrated by *in vivo* studies with H-89, a PKA inhibitor, which was shown to decrease the levels of HDAC8 phosphorylation (Lee, Rezai-Zadeh et al. 2004).

Studies also suggested that Ser39 phosphorylation of HDAC8 leads to fivefold decrease in HDAC8 enzyme activity in an *in vitro* assay with purified histones as the substrate (Lee, Rezai-Zadeh et al. 2004). Furthermore, *in vivo* experiments have suggested that phosphorylated form of HDAC8 has decreased enzyme activity as demonstrated with the increased acetylated levels of Histone 3 and Histone 4 (Lee, Rezai-Zadeh et al. 2004). Phosphorylation of HDAC8 was also known to regulate the protein-protein interactions. Studies confirm that only phosphoHDAC8 was found to be interacting with some of the proteins like hEST1B and also Hsp70. (Lee, Sengupta et al. 2006).

Moreover, Class II HDACs are known to shuttle between cytoplasm and nucleus when phosphorylated. Therefore, we next analyzed the cellular location of p-HDAC8 by treating cells

with forskolin, an activator of PKA. The confocal images revealed no difference in localization of HDAC8 suggesting, HDAC8 and its phospho-form are majorly cytoplasmic in location **(Fig. 16)**.

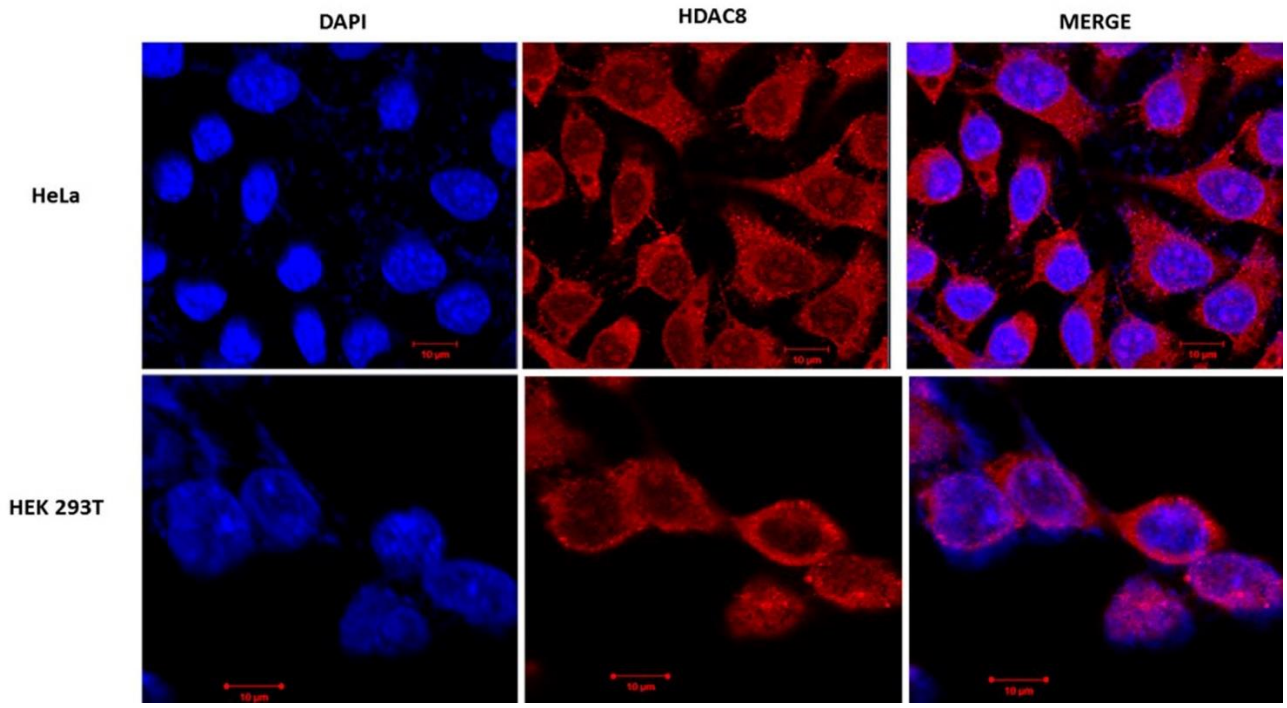


Figure 16: Phospho HDAC8 is majorly cytoplasmic in localization: *Confocal images of HDAC8 localization in Forskolin (10 µM) treated HeLa (upper panel) and HEK 293T (lower panel) (Scale: 20 µm).*

Cytoplasmic localization of HDAC8 was further confirmed by protein expression studies of HDAC8 in total, cytoplasmic and nuclear fractions of HeLa and HEK 293T. Results obtained from immunoblot analysis, clearly supports the localization studies confirming higher expression in cytoplasmic fraction, compared to nuclear in both HeLa and HEK 293T cells **(Fig. 17)**. Results from the activity assay clearly confirms that both cytoplasmic and nuclear fractions of HeLa and HEK 293T are found to be active **(Fig. 18)**. Thus, active form of HDAC8 in both cytoplasm and

nuclear fractions, further implicates the functional importance in regulating both non-histone and histone proteins.

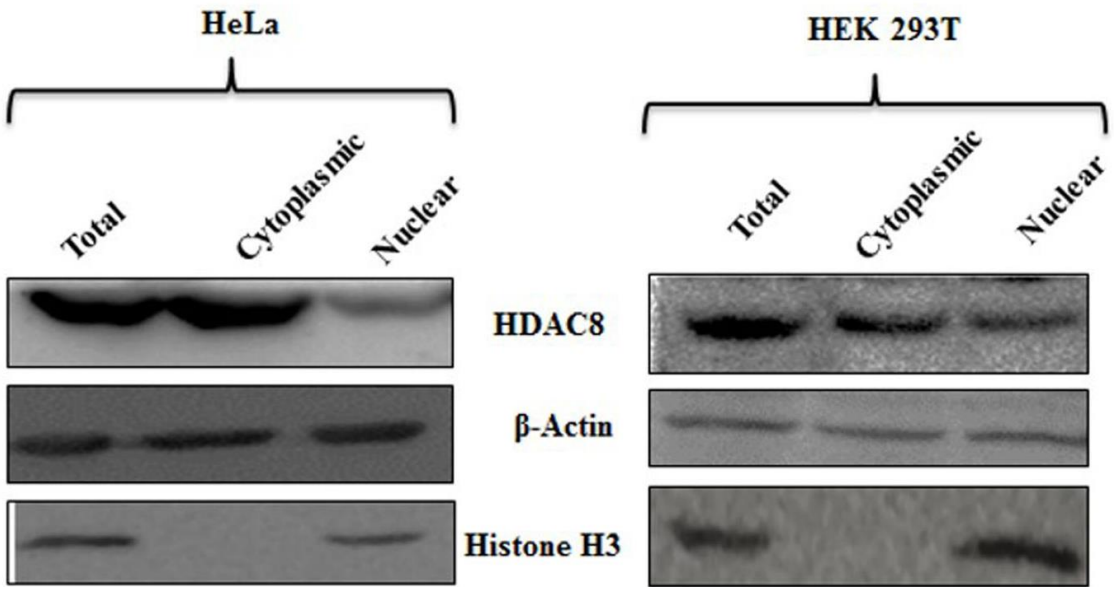


Figure 17. HDAC8 protein expression levels in HeLa and HEK 293T: *HDAC8 protein levels in total, cytoplasmic and nuclear fractions of HeLa and HEK 293T. Beta actin and histone-3 are loading controls.*

Further, we have performed HDAC8 enzyme activity assay using the immunoprecipitated fractions of total, cytoplasmic and nuclear proteins of untreated and PCI-34051(a HDAC8 selective inhibitor).

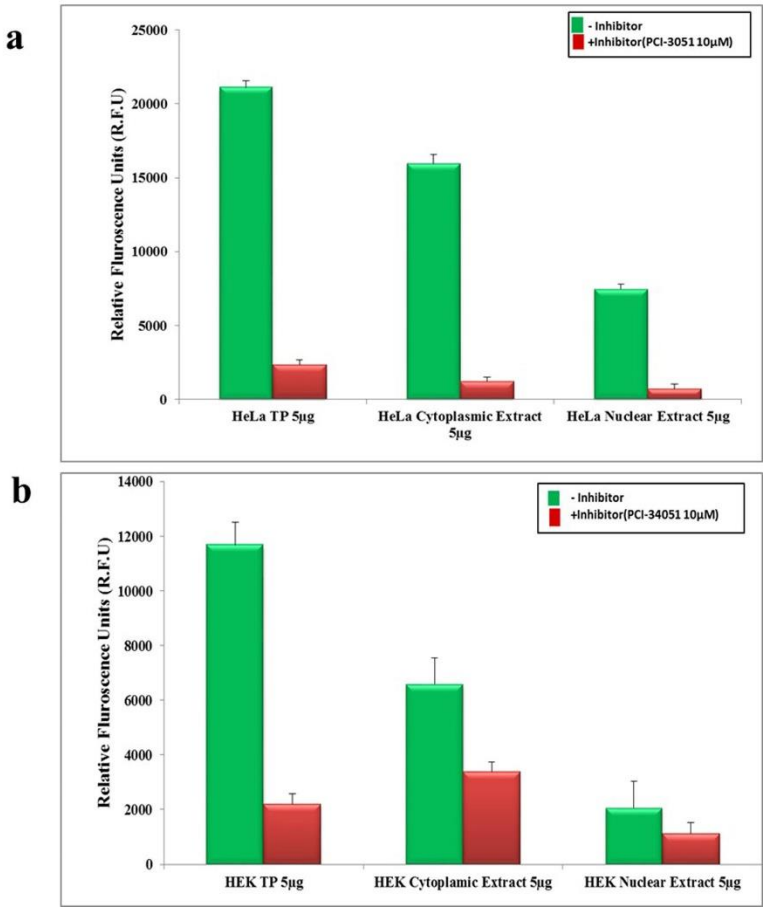


Figure 18. HDAC8 enzyme activity assay: *HDAC8 enzyme activity assay with total (5μg), cytoplasmic (5μg) and (nuclear) 5μg fractions in presence or absence of specific inhibitor PCI-34051(10 μM) in HeLa (a) and HEK 293T (b) respectively.*

Forskolin (PKA activator) treatment is known to decrease HDAC8 activity. In our study, we have confirmed the decreased activity in both total and cytoplasmic fractions of forskolin treated conditions in both HeLa and HEK 293T. But, enzyme activity results from the nuclear fractions in both HeLa and HEK 293T have shown a significant increase in enzyme activity of forskolin treated conditions compared to untreated nuclear fraction indicating that phospho-HDAC8 might have a role in nucleus such as protecting human ortholog of the yeast ever-shorter telomeres 1B (hEST1B) from ubiquitination (Lee, Sengupta et al. 2006). **(Fig. 19)**.

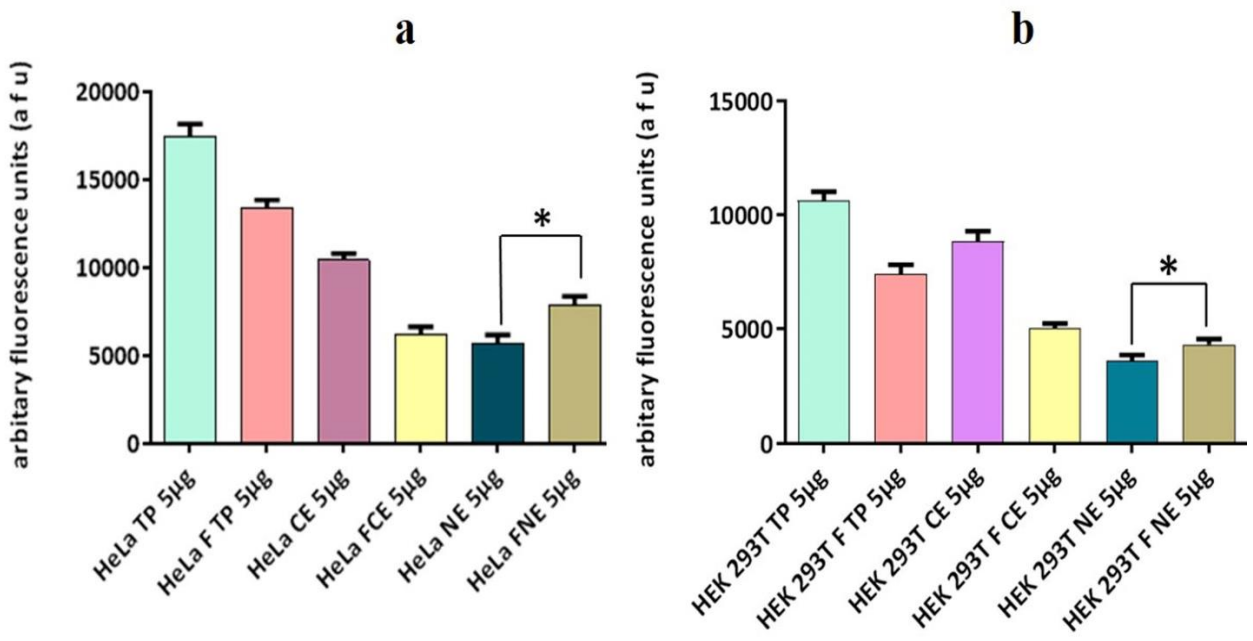


Figure 19. HDAC8 enzyme activity assay with Forskolin (10 µM) treated condition: *HDAC8 Immunoprecipitated fractions of total (5µg), cytoplasmic (5µg) and (nuclear) 5µg fractions, along with untreated control fractions of HeLa (a) and HEK 293T (b) respectively. * indicates p-value <0.001.*

3.1.2 Alpha tubulin is a non-histone substrate of HDAC8

Identification of protein-protein interactions by mass spectroscopy followed by immunoprecipitation (IP) and co-IP experiments is a well-established and very useful technique (Free, Hazelwood et al. 2009). Several interacting proteins have been identified using this approach. α- actin is known to interact with HDAC8 endogenously in smooth muscle cells. The association of these two proteins were validated by co-localization studies, pull down experiments and immunofluorescence staining (Waltregny, De Leval et al. 2004, Waltregny, Glenisson et al. 2005, Karolczak-Bayatti, Sweeney et al. 2011). The results of the study indicated decreased contractions of smooth muscle cells when HDAC8 was knockdown by siRNA. The study has also shown to affect the morphology and structure of smooth muscle cells justifying the role of HDAC8

in modulating the cell cytoskeleton (Waltregny, Glenisson et al. 2005). Actin dynamics were further known to be regulated by association of HDAC8 with proteins like Hsp20 (Dreiza, Brophy et al. 2005), myosin heavy chain (Vicente-Manzanares, Ma et al. 2009), and cofilin (Karolczak-Bayatti, Sweeney et al. 2011). However the direct association of their acetylated forms with HDAC8 is quite unclear which does not consider them into active substrates of HDAC8 (Karolczak-Bayatti, Sweeney et al. 2011).

Furthermore, the levels of acetylation of another cytoskeletal protein, like α-tubulin is known to be altered upon specific inhibition of HDAC8 (Krennhrubec, Marshall et al. 2007) or even by knockdown studies by siRNA(Yamauchi, Boukari et al. 2011) which in turn destabilizes the microtubule dynamics. Explanation for the effects of HDAC8 on the microtubule dynamics may be further understood by the relationship between cohesion activity and microtubules (Tanaka, Fuchs et al. 2000, Wong and Blobel 2008, Chen, Wikramasinghe et al. 2012).

We therefore next tried to identify HDAC8 protein interacting partners by immunoprecipitation followed by MALDI-TOF-TOF analysis. Coomassie staining of the denaturing gel of immunoprecipitated protein samples from HeLa cells showed a very prominent band at ~50 kDa (*) and also above 29 kDa (&) along with HDAC8 at 42 kDa **(Fig. 20a)**. Immunoblot results further confirm HDAC8 protein in IP and input sample **(Fig. 20b)**.

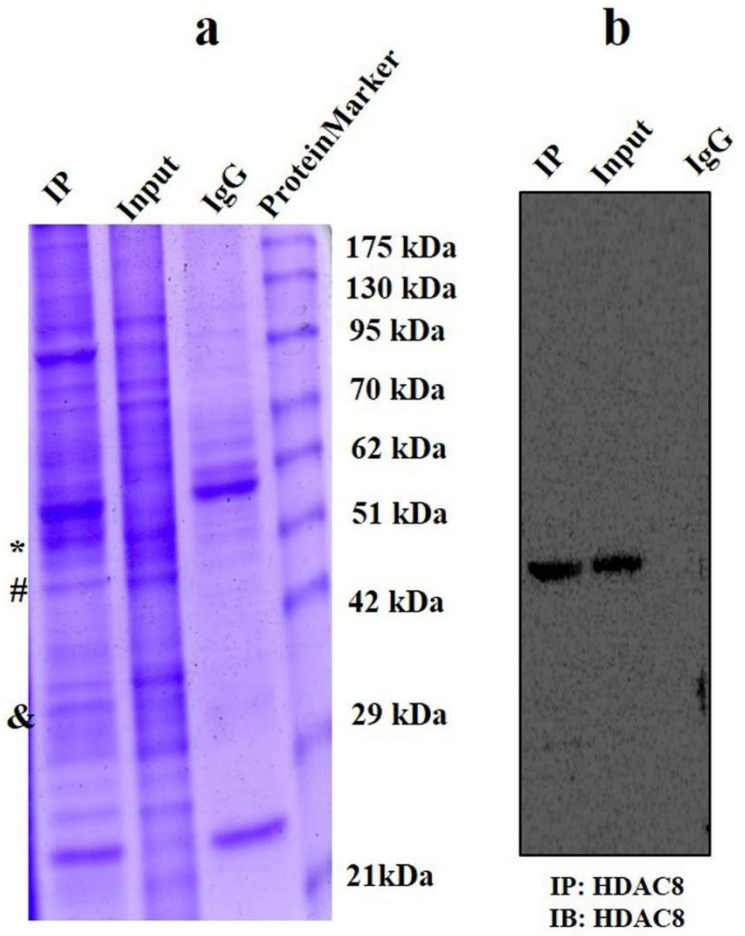


Figure 20. Immunoprecipitation studies of HDAC8 with HeLa cell lysate : a*. Coommassie stained gel with input and HDAC8-immunoprecipitated HeLa cell lysate along with protein marker. Band at (*) ~50 kDa and at ~29 kDa (&) was excised and subjected to MALDI TOF-TOF analysis (#) represents for HDAC8 in coomassie gel at 43 kDa, further confirmed by immunoblot* ***(b).***

The bands were excised from the gel, and subjected to MALDI analysis. Mascot database search results identified protein as Alpha tubulin (~50 kDa) band with a protein score greater than 56, with significant score of $p<0.05$ **(Fig. 21)** and RBGIL (Rab GTPase-activating protein 1-like) with a score of 36 and 12 peptide matches for 29 kDa excised band, along with other proteins which are listed in **Table 1**.

MATRIX SCIENCE **Mascot Search Results**

```
User            : VANAJA
Email           : vanajagr.ils@gmail.com
Search title    :
Database        : SwissProt 2013_12 (541954 sequences; 192668437 residues)
Taxonomy        : Homo sapiens (human) (20274 sequences)
Timestamp       : 20 Jan 2014 at 05:25:32 GMT
Top Score       : 56 for TBA1C_HUMAN, Tubulin alpha-1C chain OS=Homo sapiens GN=TUBA1C PE=1 SV=1
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 56 are significant (p<0.05).

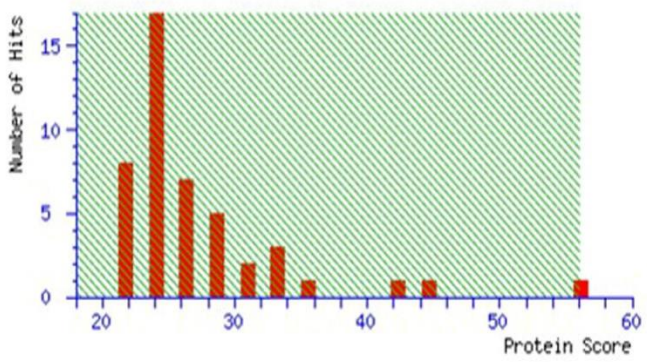


Figure 21. Alpha tubulin as one of the non-histone interacting partner of HDAC8: *MS analysis and Mascot search results confirmed TUBA1-C (Human Alpha Tubulin chain 1 C isoform) with highest top score as the interacting partner of HDAC8.*

Table 1: List of proteins identified from MALDI TOF TOF analysis

S.No	Name of the protein identified	Top Score	Origin	Peptide matches	Mass (Daltons)
1	TBA1C-Tubulin Alpha 1C	56	Homo sapiens	12	50548
2	RBGIL (Rab GTPase-activating protein 1-like	36	Homo sapiens	12	29038
3	NUB1-NEDD8 ultimate buster	11	Homo sapiens	1	71235
4	PRP6 Pre-mRNA processing factor 6	7	Homonsapiens	1	NA
5	F228A Protein FAM228A	7	Homo sapiens	1	23965
6	CHST4 Carbohydrate sulfotransferase 4	6	Homo sapiens	1	45560

3.1.3 HDAC8 co-precipitates with Alpha tubulin

Owing to its abundance in cytoplasm, further results from our immunoprecipitation studies suggested the role of HDAC8 in regulating cytoplasmic proteins (Waltregny, De Leval et al. 2004). MALDI analysis results were further confirmed by immunoprecipitation studies. Co-IP **(Fig. 22a)** and reverse IP **(Fig. 22b)** studies results confirmed the interaction of HDAC8 and alpha tubulin in both HeLa (cervical cancer cell lines) and HEK 2963T (normal cells). Thus, from MALDI results we confirm that Alpha tubulin is one of the non-histone protein interacting partners of HDAC8.

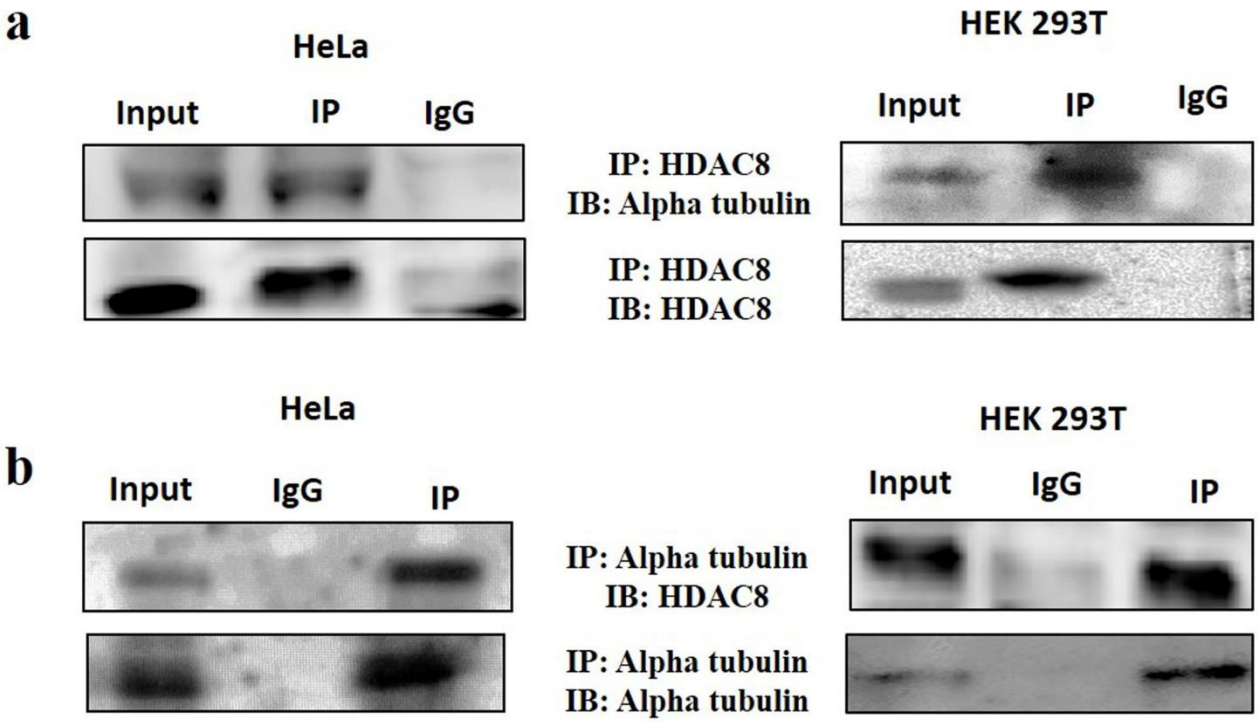


Figure 22. IP & Co-IP confirms HDAC8 & Alpha tubulin: **a.** *Confirmation of HDAC8 and Alpha tubulin by immunoprecipitation using HDAC8 antibody in HeLa and HEK293T cells.* ***b****. Reverse IP using Tubulin antibody confirms HDAC8 & Tubulin interaction in HeLa cells and HEK 293T respectively.*

Objective 2:

Protein-protein interaction studies of HDAC8 & Acetylated (Lys40) alpha tubulin

3.2 *In silico*

3.2.1 Lys40 of Alpha tubulin a conserved residue, interacts with HDAC8

To understand the specificity of HDAC8 towards three isoforms of tubulin proteins, multiple sequence alignment of tubulin proteins using clustal omega analysis was carried out **(Fig. 23)**. The analysis showed that Lysine residue at 40th position (K40) is only present in Alpha tubulin and that other tubulin forms lack this lysine residue. The conserved K40 position in alpha tubulin is known to be acetylated in normal cellular process and is involved in regulation of protein trafficking, cell cycle and cell migration (Sadoul and Khochbin 2016).

Figure 23. Clustal omega sequence alignment of alpha, beta and gamma tubulins: *Clustal omega analysis showing Lys 40 of Alpha tubulin whereas, Beta and Gamma tubulins have serine and alanine.*

Further sequence analysis across different species in eukaryotes revealed the conservation of Lys40 in Alpha tubulin protein indicating its functional significance **(Fig. 24)**. These results are in well-agreement with recently published study (Sadoul and Khochbin 2016).

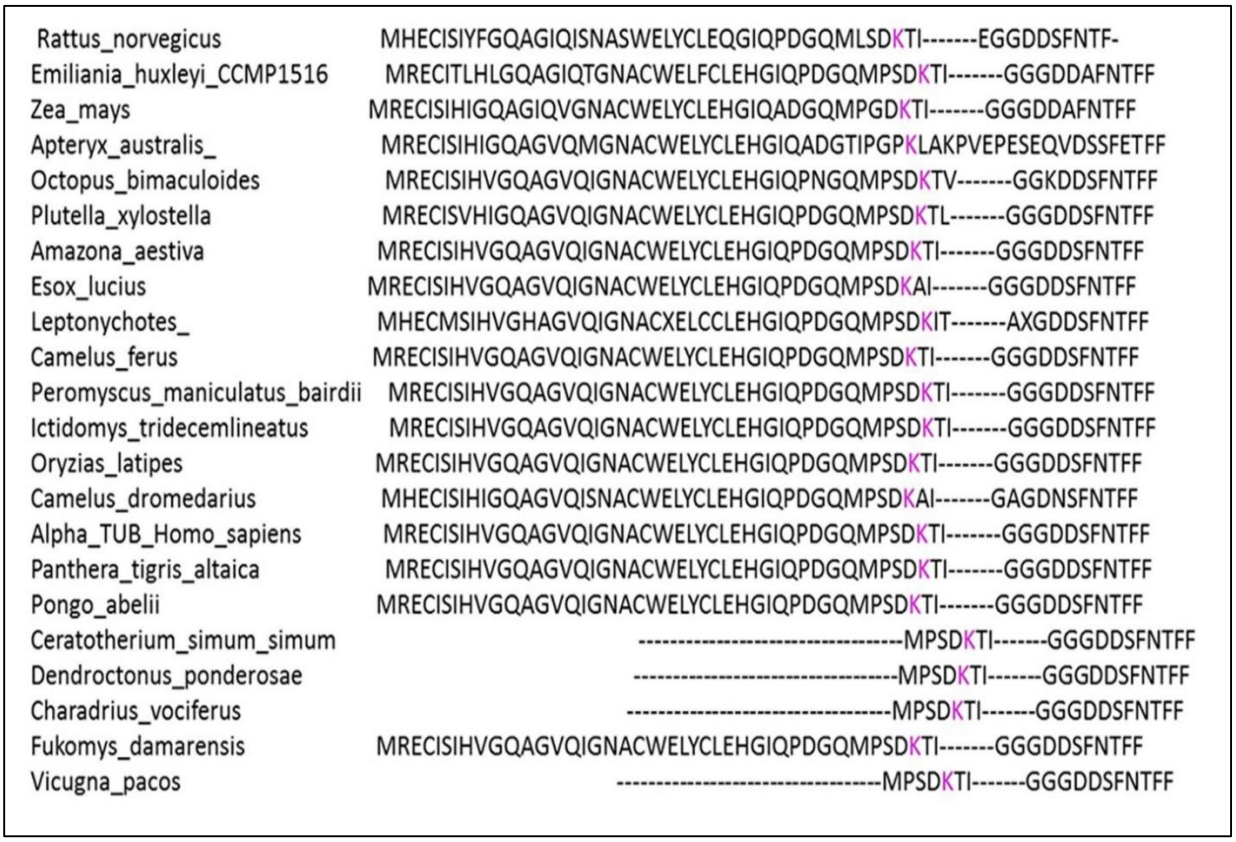
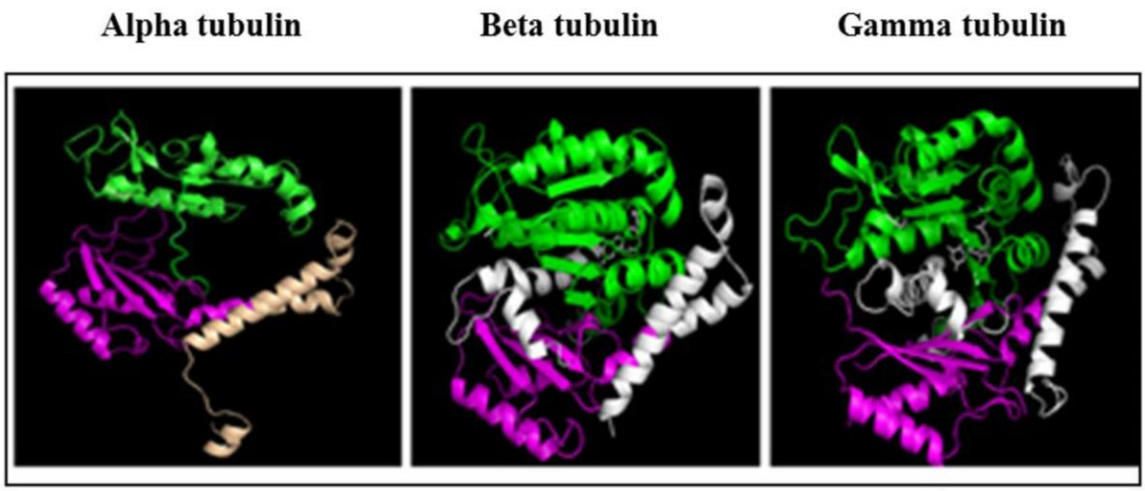


Figure 24. Sequence Analysis of Alpha tubulin in some eukaryotes: *Lys40 position was found to be conserved in eukaryotes.*

The functional conserved domain analysis of tubulins using Pfam database revealed two types of domains: Tubulin and Tubulin_C. Domain identification in protein sequences helps in the classification of proteins into families by predicting the function and structures. It has been observed that Tubulin and Tubulin_C domains in alpha, beta and gamma-tubulins are conserved (**Fig. 25**) and can be involved in similar function.



>gi|21619816|gb|AAH33064.1| TUBA1C protein [Homo sapiens]
MRECISIHVGQAGVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSETGAGKHVPRAVFVDL
EPTVIDEVRTGTYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLDRIRK FDLMYAKRAF
VHFPLATYAPVISAEKAYHEQLTVAEITNACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRT
IQFVDWCPTGFKVGINYQPPTVVPGGDLAKVQRAVCMLSNTTAVAEAWARL DHKFDLMYAKRAF
KSINLCAVLLHSFVLELSYFCSVNVYCRKLLIKLMFPF

2XRP_A(Crystal structure of Human beta-tubulin)
MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPTGSYHGDSDLQLERINVYYNEAAGNKYVPRAILVDLEPGTMDSVRSGP
FGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESESCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYP
DRIMNTFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTCLRFP
GQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMMAACDPRHGRYLTVAAVFRGRMSMKEV
DEQMLNVQNKNSSYFVEWIPNNVKTAVCDIPPRGLKMSATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFT
EAESNMNDLVSEYQQYQDATADEQGEFEEEEGEDEA

>3CB2:A(Crystal structure of Human Gamma-Tubulin)

MPREIITLQLGQCGNQIGFEFWKQLCAEHGISPEAIVEEFATEGTDRKDVFFYQADDEHYIPRAVLLDLEPRVIHSILNS
PYAKLYNPENIYLSEHGGGAGNNWASGFSQGEKIHEDIFDIIDREADGSDSLEGFVLCHSIAGGTGSGLGSYLLERLNDR
YPKKLVQTYSVFPNQDEMSDVVVQPYNSLLTLKRLTQNADCLVVLDNTALNRIA TDRLHIQNPSFSQINQLVSTIMSAST
TTLRYPGYMNNDLIGLIASLIPTPRLHFLMTGYTPLTTDQSVASVRKTTVLDVMRRLLQPKNVMVSTGRDRQTNHCYIAI
LNIIQGEVDPTQVHKSLQRIRERKLANFIPWGPASIQVALSRKSPYLPSAHRVSGLMMANHTSISSLFERT CRQYDKLRK
REAFLEQFRKEDMFKDNFDEMDTSREIVQQLIDEYHAATRPDYISWGTQEQVDVDGGQKLISEEDLLLEHHHHHH

Tubulin domain-green
Tubulin-C domain--magenta

Figure 25. Functional conserved domain analysis of alpha, beta & gamma tubulin with Pfam database: *Tubulin and tubulin_c domains in alpha, beta and gamma-tubulins are conserved and can be involved in similar function.*

3.2.2 Alpha tubulin is conserved in different groups of eukaryotes

As Lys40 position was conserved among eukaryotes, we then analysed for evolutionary relationships of alpha tubulin in eukaryotes. For this, we have carried out phylogenetic analysis. All the major groups of eukaryotes for alpha tubulin were recovered with good bootstrap support. The monophyletic group of Fungi, Telonemids, Cryptophytes, Haptophytes was recovered with >70% of BV at the nodes. Opisthoknots showed a polyphyletic nature. However, most of internal groups were recovered with good phylogenetic signal. Other groups such as Stramenophiles, Discricristates were also found to be polyphyletic. Discricristates were associated with BV= 90% while few phyla in Stramenophiles were associated with BV= 90%. Archaeplastida were associated with Rhizaria with BV >50%.

Alveolus's also formed a sister clade with Archaeplastida and Rhizaria. However, the internal groups were present within the group with BV>50%. Thus, the major groups of eukaryotes for alpha tubulin were recovered and the results are in agreement with previously published studies. The phylogenetic signal is conserved in major groups of alpha-tubulin proteins **(Fig. 26).**

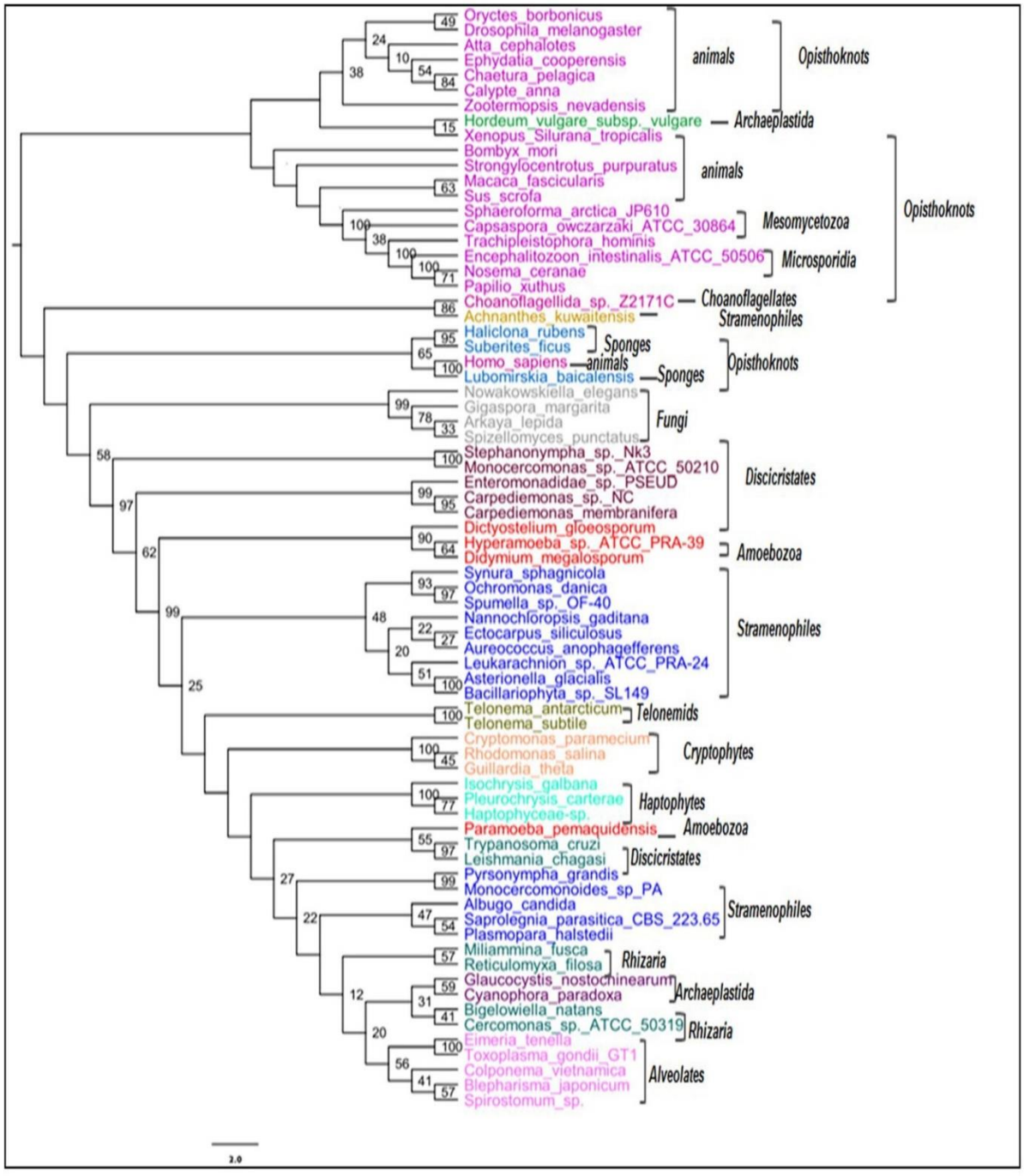


Figure 26. Phylogenetic tree analysis of Alpha tubulin using PhyML v. 3.0*: The phylogenetic signal is conserved in major groups of Alpha tubulin among different groups of eukaryotes.*

3.2.3 Generated 3D structure of Alpha tubulin by homology modelling

The functionally conserved K40 acetylation of Alpha tubulin and lysine deacetylase function of HDAC8 interested us to further evaluate the interaction of alpha tubulin and HDAC8. We used *in silico* approach to first confirm the protein-protein interaction. Since the crystal structure of Alpha tubulin of human was not available, a homology model was generated using I-D TASSER using 4I4T_A as the template structure. 4I4T_A is crystal structure of tubulin-RB3-TTL-Zampanolide complex from *Bos Taurus* (Prota, Bargsten et al. 2013). It is a hexamer; therefore, Chain A was taken for modeling. The overall 3-D (three dimensional) structural arrangement of the crystal structure and model is conserved **(Fig. 27)**.

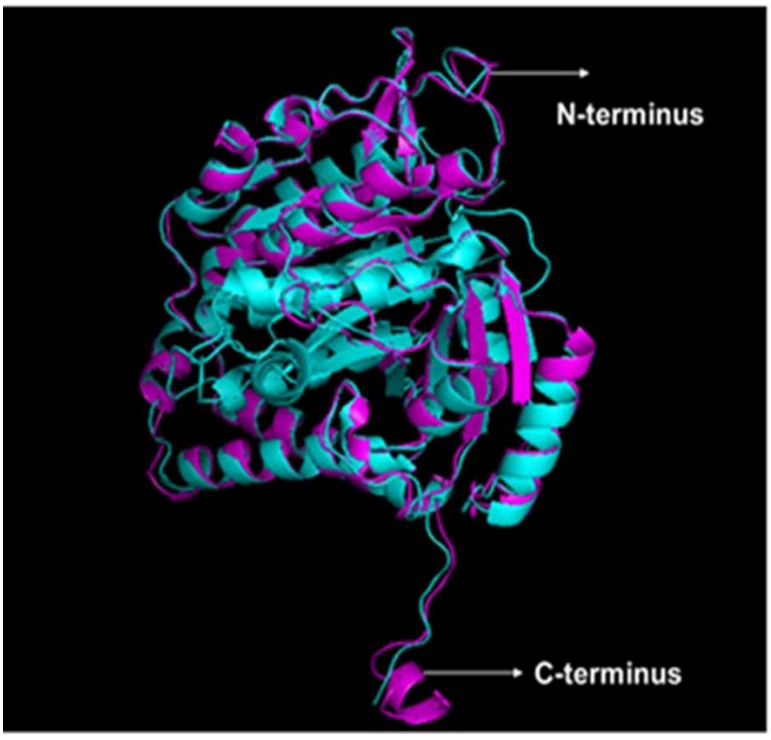


Figure 27. Homology modelling of alpha tubulin: *3D crystal structures (generated by homology modelling) based on its template structure, i.e. (**PDB ID: 4I4T**), which is Crystal structure of tubulin-RB3-TTL-Zampanolide complex from Bos taurus. Superimposition of alpha tubulin-c with 414_t. Alpha tubulin is shown in magenta; 414T_A is shown in cyan.*

More than 75% amino acid residues in the model were in the allowed regions of the Ramachandran plot **(Fig. 28 a & b)** (Ramachandran, Ramakrishnan et al. 1963).

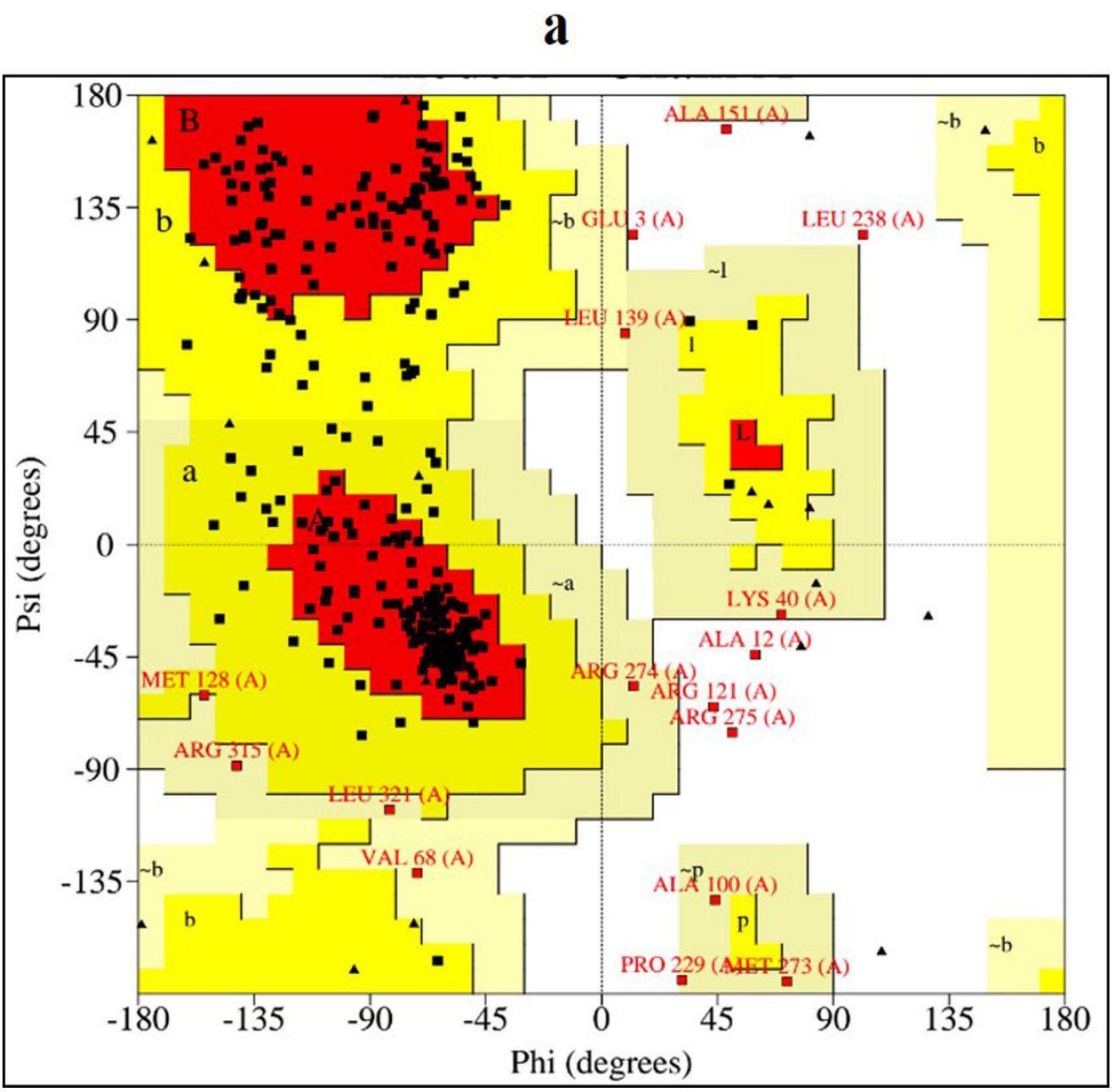


Figure 28. Ramachandran plot: (a). *Ramachandran plot model for chain a – alpha tubulin.* ***(b).*** *More than 75% amino acid residues in model are in the allowed regions of the Ramachandran plot.*

b

Plot statistics

Residues in most favoured regions [A,B,L]	217	75.6%
Residues in additional allowed regions [a,b,l,p]	55	19.2%
Residues in generously allowed regions [~a,~b,~l,~p]	9	3.1%
Residues in disallowed regions	6	2.1%
Number of non-glycine and non-proline residues	287	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	20	
Number of proline residues	16	
Total number of residues	325	

According to Verify-3D, the overall quality of the model was found to be 96.5% and ERRAT program shown that 71% residues had an average 3D-1D score >= 0.2 **(Fig. 29)**. The RMSD between the template structure and model was 1.29 Å. C-score and TM-score were found to be 0.55 and 0.95 respectively within the acceptable ranges validating the model generated.

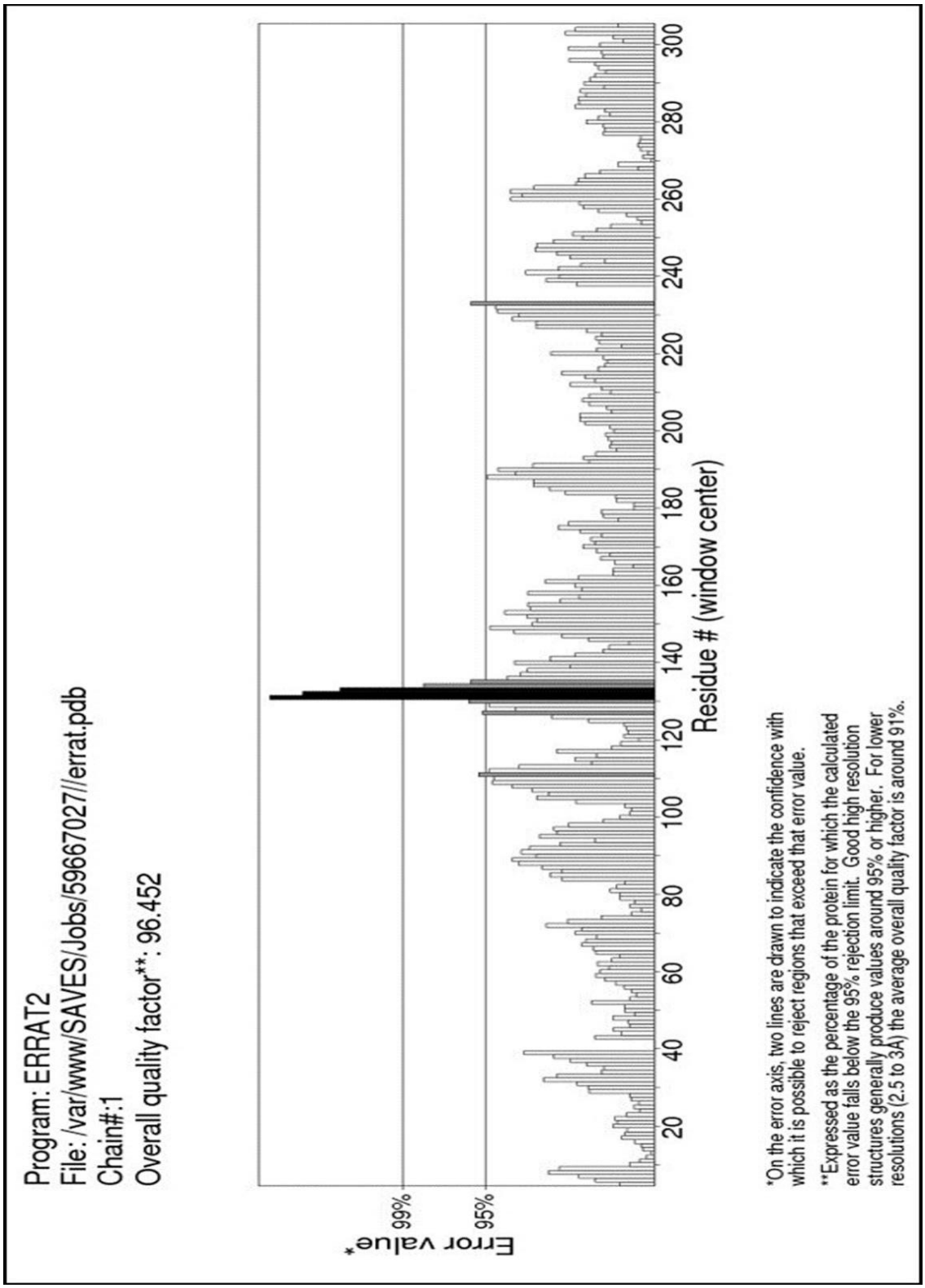


Figure 29. Structural validation by ERRAT: *Overall quality of the model was found to be 96.5% and ERRAT program shown that 71 % residues had an averaged 3D-1D score >= 0.*

3.2.4 Lys40 of Alpha tubulin interacts specifically with Pro205 of HDAC8

Protein-protein interaction studies of the tubulin proteins were carried out using HDAC8 (PDB ID: 1W22_A). The Alpha-tubulin model showed an interaction of Lys40 with Pro205 of HDAC8, including the IIe225, Val223, Tyr227 with Tyr225, Lys374, Asn372 and Tyr368 of alpha-tubulin and HDAC8 respectively. Out of Val223, Ile225 and Tyr227 residues of Alpha tubulin, it was observed that only Ile225 of Alpha tubulin is interacting with Tyr225 residue of HDAC8 (Catalytic region). As per interacting residues of HDAC8, Pro205 and Tyr225 residues belong to histone deacetylase catalytic domain **(Fig. 30 a).**

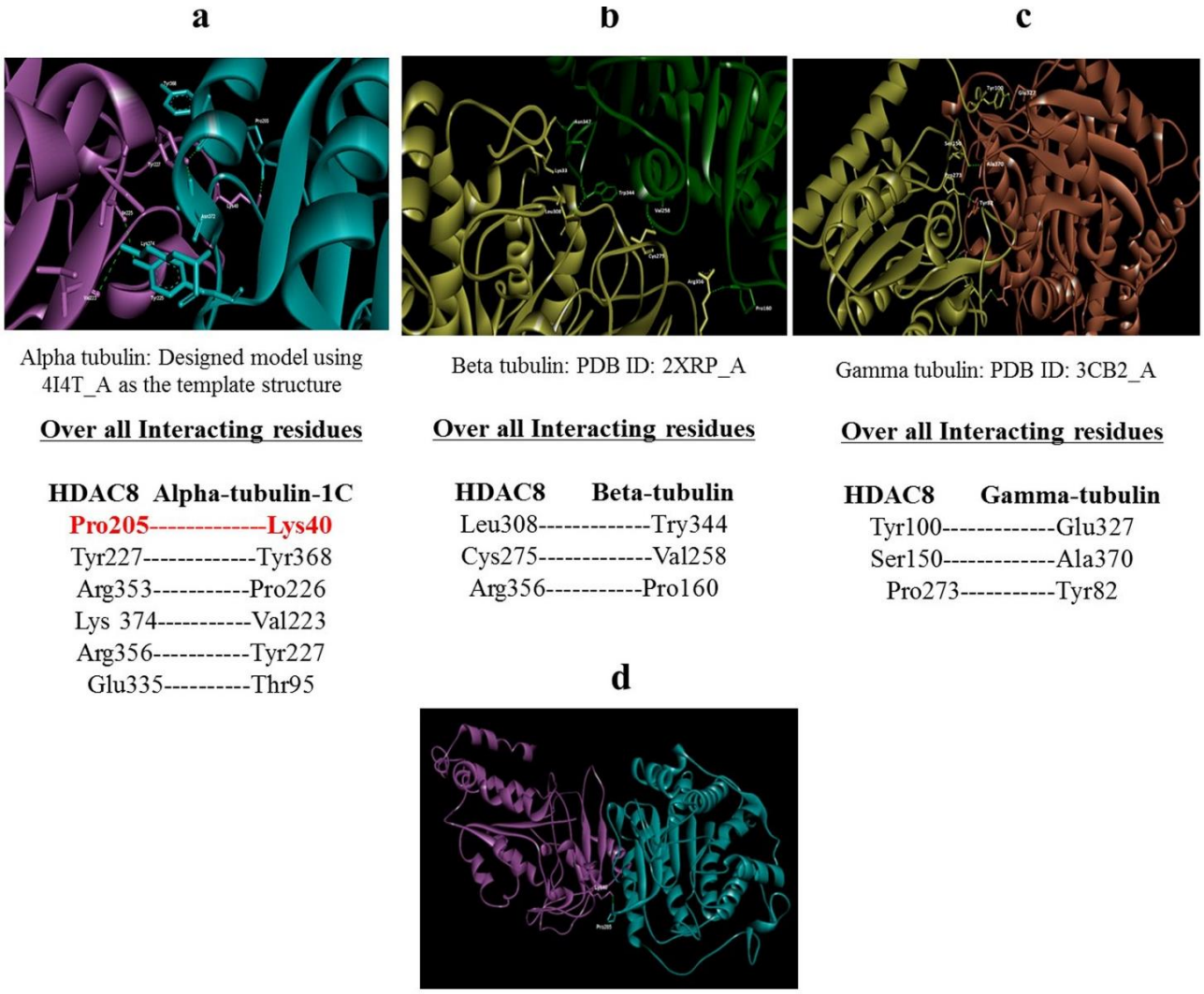


Figure 30. Protein protein interaction studies of HDAC8 with Alpha, Beta and Gamma tubulins: (a, b &c). *H-bonding interactions of HDAC8 with alpha, beta and gamma tubulins. Interacting residues are listed below the figure. Alpha tubulin is shown in pink; HDAC8 in blue; h-bonding shown in green lines, HDAC8 is shown in yellow, beta tubulin in green, HDAC8 is shown in yellow and gamma tubulin in orange brown. (d). Lys40 of Alpha tubulin (Catalytic region of alpha tubulin) interacting with Pro205 of HDAC8 (Catalytic region of HDAC8) Alpha tubulin is shown in pink; HDAC8 in blue.*

With respect to the beta-tubulin (PDB id: 2XRP_A), the residues Pro160, Val258, Trp344, Asn347 of beta-tubulin were found to be interacting with Arg356, Cys275, Leu308, Lys33 of HDAC8 respectively **(Fig. 30 b)**. Gamma-tubulin residues such as Tyr82, Ala370, Glu327, Asn79,

Gln227 were found to be interacting with Pro273, Ser150, Tyr100, Cys352, Gln232 of HDAC8 **(Fig. 30 c)**. Results further demonstrate that catalytic regions of alpha tubulin comprises of Lys40 residue, which is known to interact with Pro205 of HDAC8 **(Fig. 30 d)**. The ZDOCK score of the tubulins are provided in table**.** All the Figures related to protein-protein interaction studies were generated using Discovery Studio Visualizer version 4.1.

Table 2: ZDOCK score for alpha, beta and gamma tubulins interactions with HDAC8

S.No	Protein Name	S_{DS} (kcal/mol)	S_{ELEC} (k Cal/mol)	RMSD (Å)
1	Alpha tubulin	-2.09	-2.73	1.45
2	Beta tubulin	-3.14	-2.73	1.45
3	Gamma tubulin	-2.87	0.10	1.55

Further *in silico* mutational studies were carried out to confirm the interaction between Lys 40 of Alpha tubulin and Pro205 of HDAC8. Mutation of Lys to Arg in Alpha tubulin and Pro to Ala in HDAC8 lead to loss of interaction between the two proteins and confirm their interaction.

Protein-protein interaction studies, with all the three forms of tubulins further validated the importance of alpha tubulin with higher number of interactions, especially Lys40 (a potential

acetylation site) of alpha tubulin (catalytic region) with that of Pro205 of HDAC8 (catalytic region). Importance of Lys40 position for its potential interaction with HDAC8 was further validated by *in silico* mutational analysis (Lys40 to Arg in alpha tubulin & Pro205 to Ala in HDAC8) which revealed loss of interaction between two proteins.

3.3 *In vitro*

3.3.1 Cloned, expressed and purified active hHDAC8 from bacterial system

For *in vitro* binding assays, we have cloned hHDAC8 considering K562 cDNA as a template for HDAC8. The human HDAC8 gene was amplified by polymerase chain reaction (PCR) from human cDNA K562 cell line **(Fig. 31 b)**. Amplified product was cloned into p^{GEX} – 6p1 plasmid. Positive clones (p^{GEX} – 6p1 HDAC8) were further confirmed by colony PCR **(Fig. 31 c)**, followed by restriction digestion for product linearization and product release using Xho1 and EcoR1 restriction enzymes **(Fig. 31 d)**.

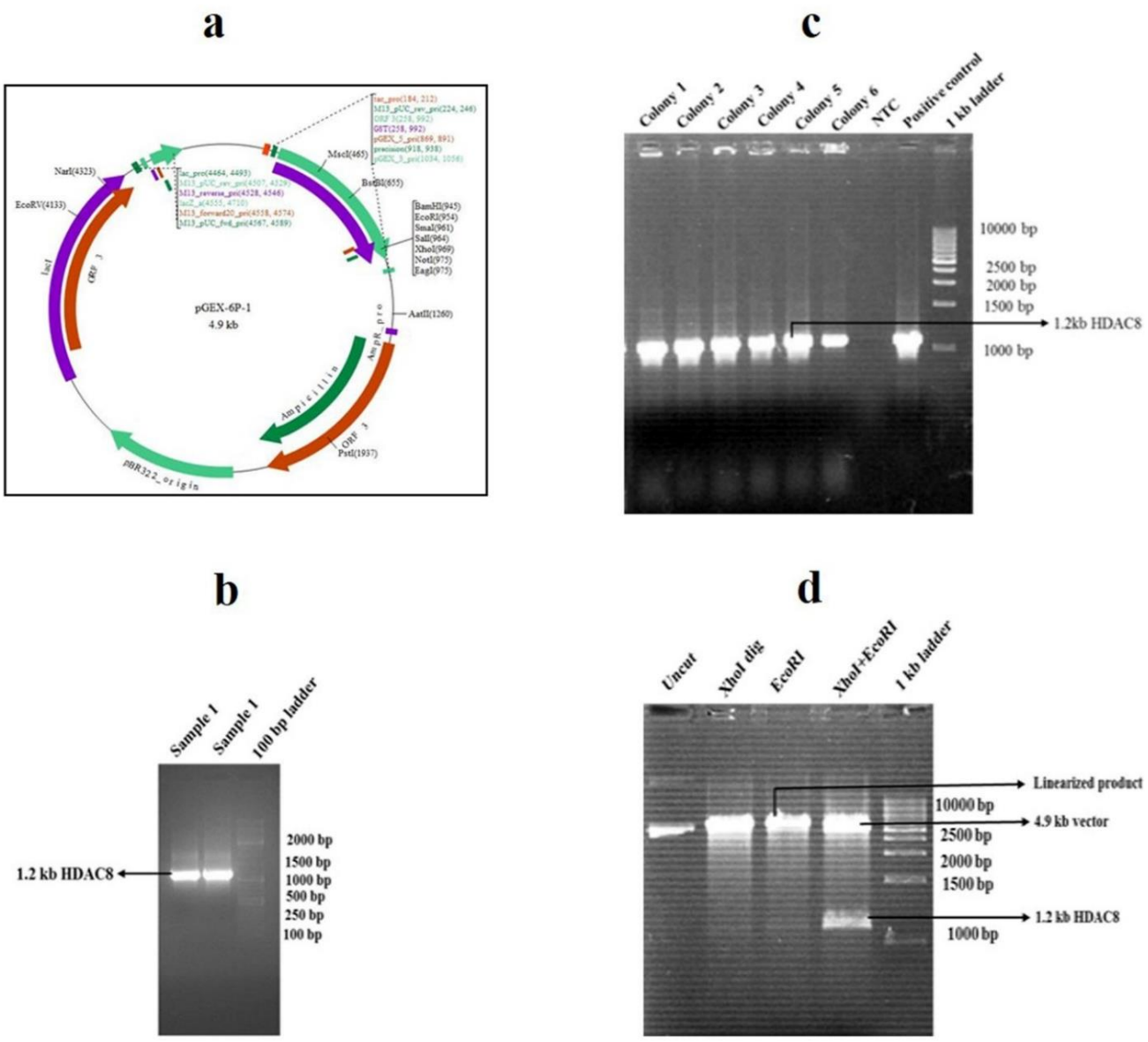


Figure 31. Cloning of hHDAC8 into pGEX-6p1 plasmid: a. *p^{GEX}-6p1 vector (4.9 kb) was used for cloning hHDAC8.* ***b.*** *HDAC8 was PCR amplified at 1.2 kb. Lane 1 represents for PCR sample 1, lane 2 represents for PCR sample 2, and lane 3 for 100 bp DNA ladder* ***c.*** *Clone confirmation by colony PCR, with a product length of 1.2 kb. Lane 1-6 random colonies PCR samples, lane 7 represents for NTC, lane 8 for positive control and lane 9 for 1kb ladder.* ***d.*** *Clone confirmation by restriction digestion. Lane 1 represents for uncut, lane 2 for Xho1 digestion with a linearized product of 6.1 kb, lane 3 for EcoR1 digestion for linearization with 6.1kb length, lane 4 indicated double digestions with insert release at 1.2kb along with 4.9 kb vector, lane 5 1 kb marker.*

Upon sequence confirmation of the insert, clone was transformed into *Escherichia coli* strain BL21 cells. Protein expression and purification protocols were well standardized. Further confirmation of the purity of the purified protein was carried out by SDS-PAGE with Coomassie staining, which depicts >80% pure followed by Western blot confirmation **(Fig. 32 a&b)**.

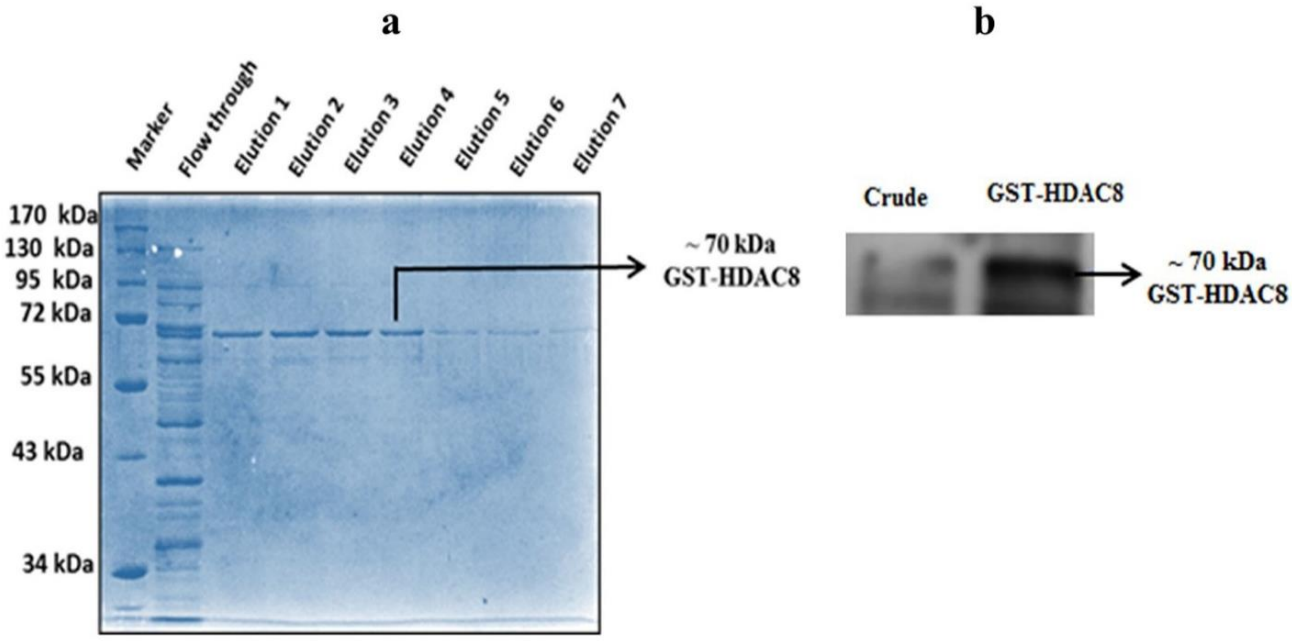


Figure 32. Expression and purification of active hHDAC8 from bacterial system: a. *12% SDS-PAGE Coomassie stained gel for bacterially purified hHDAC8 with GST column. Lane 1 represents for protein marker, lane 2 flow through, lane 3-9 elution's showing ~69 kDa of purified protein (GST-HDAC8).* ***b.*** *Western blots result for crude lysate and purified GST-HDAC8.*

Enzyme activity assay demonstrates the specific activity of purified protein (GST-HDAC8) in presence and absence of specific HDAC8 inhibitor (PCI-34051) **(Fig. 33)**. HDAC8 is the only deacetylase enzyme, which is known to be active when purified from bacterial system.

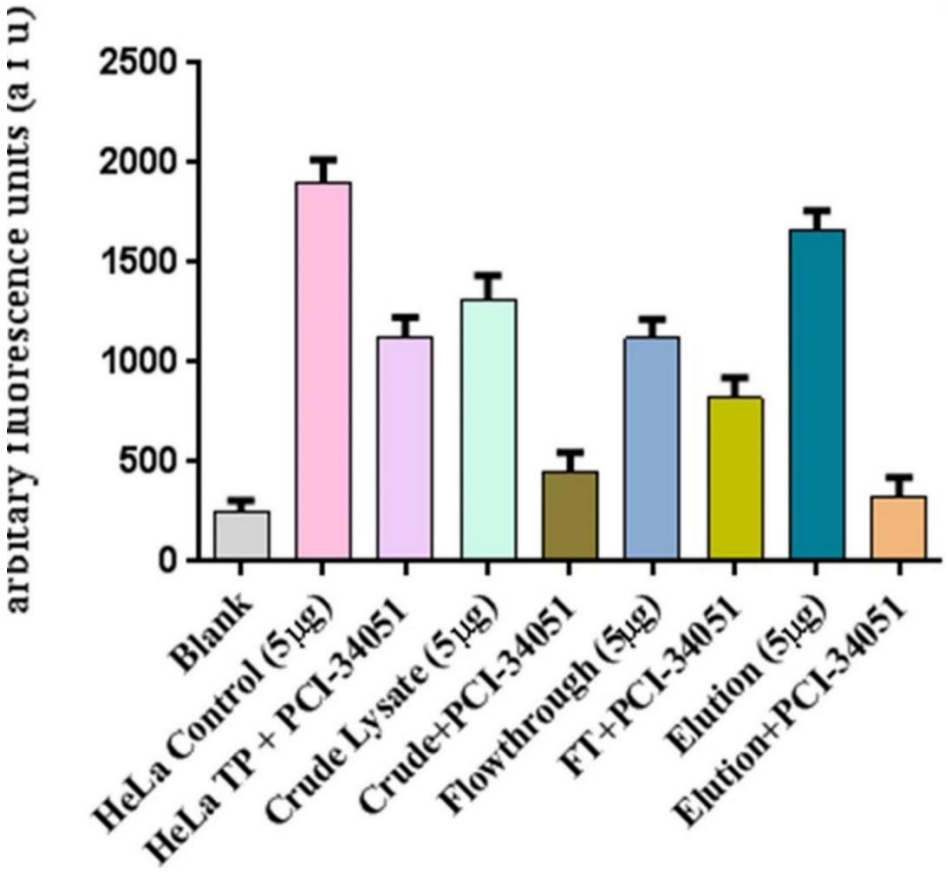


Figure 33. Enzyme activity assay for hHDAC8 purified from bacterial system: *HDAC8 enzyme activity assay representing arbitrary fluorescence units (a.f.u) for blank (only assay buffer), positive control as HeLa nuclear extract, along with crude, flow through and elution samples with/without PCI-34051(10µM). Equal concentration of protein was considered for all the samples (5µg).*

3.3.2 Both acetylated and unacetylated (Lys40) alpha tubulin binds with GST-HDAC8 - Surface plasmon resonance studies

In vitro binding studies were performed for GST-HDAC8 (recombinant purified protein) and alpha tubulin peptides (ac, unac) using SPR (surface plasmon resonance). Surface plasmon resonance is a widely used methodology for studying molecular interactions and binding of proteins and/or small molecule (Schuck 1997, Ramakrishnan, Drescher et al. 2006). SPR allows real time label free detection of biomolecular interactions. It works on the principle of measure of plasmon, which are electron charge density waves, generated due to hitting of polarized light on electrically conducting surface at the interface between two media. It is an optical technique, used to study the interactions between two different molecules, in which one is in mobile phase (analyte), and the other molecule is fixed on the gold film (Schuck 1997).

Initial binding and stability of GST-HDAC8 were significant with good range of response unit values (R.U) in both FC-1 and FC-2 regions of CM5 chip. **(Fig. 34 A, B (a, b &c))** The details of the binding and stability response units for three concentrations of GST-HDAC8 are provided in **Table 3, 4 & 5**. Further binding studies were carried out with the highest concentration of 6 µg/mL of GST-HDAC8.

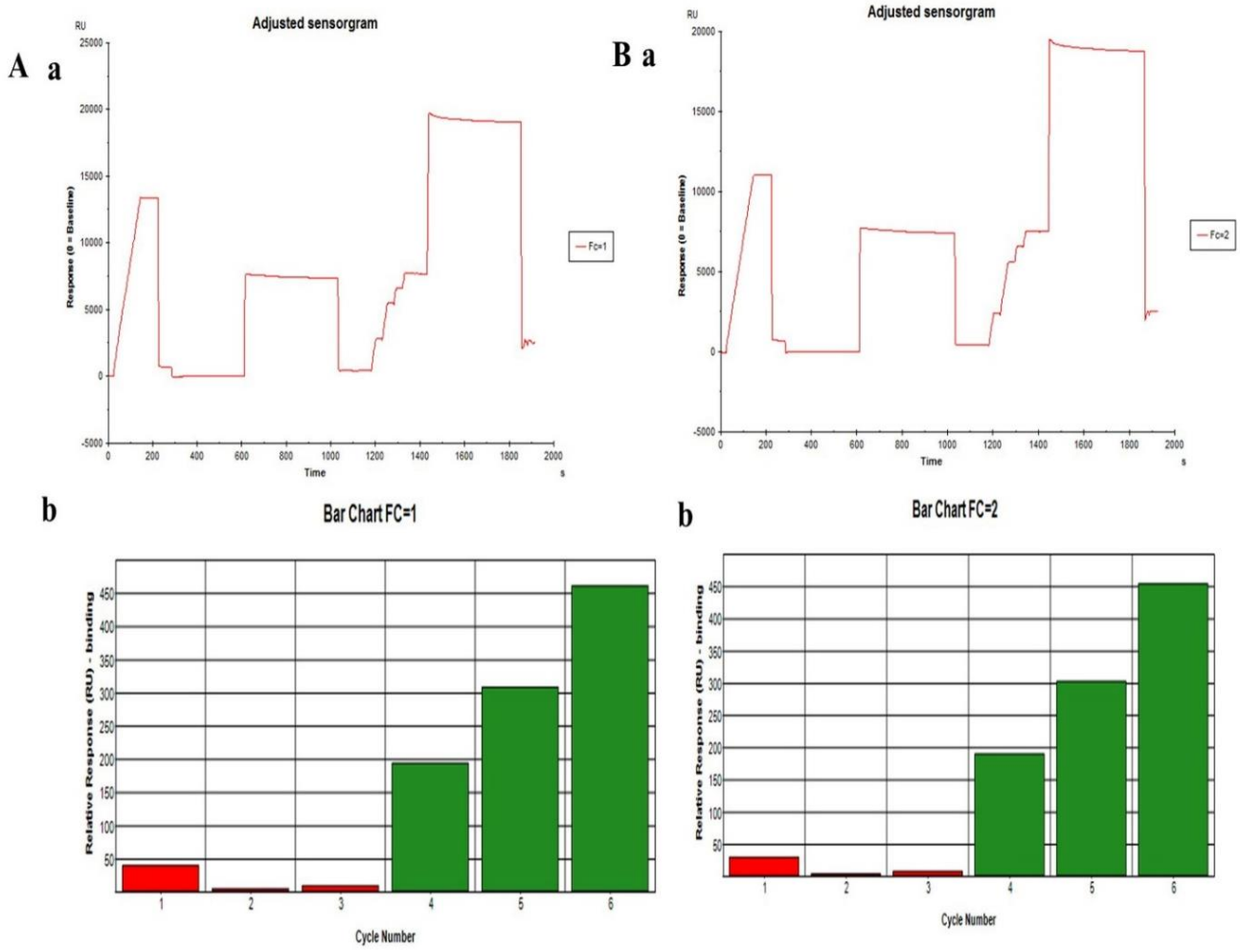
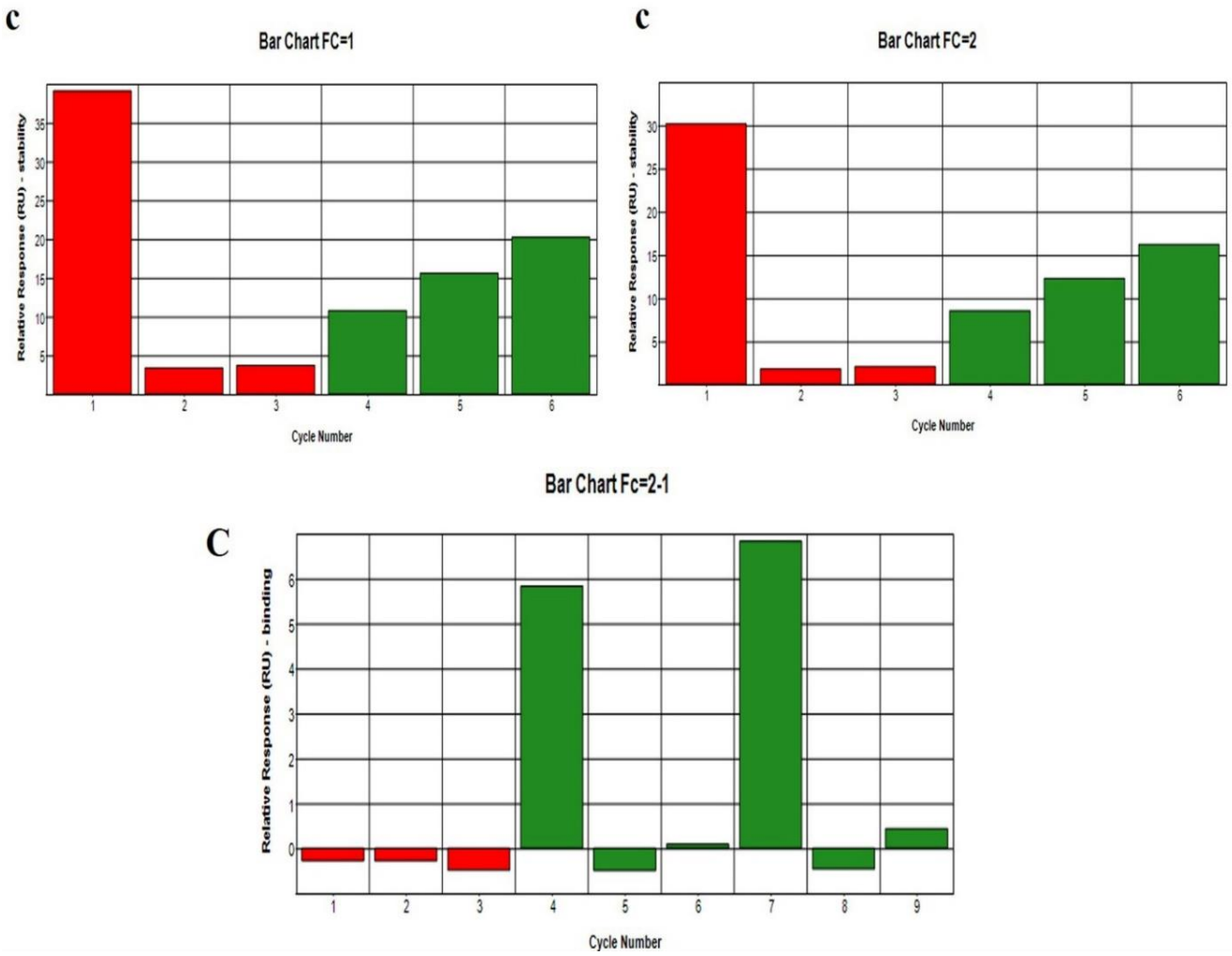


***Figure 34. Binding studies of GST-HDAC8 and alpha tubulin peptides by SPR**: **A, B (a).**GST Antibody (25µg/mL) immobilization in FC-1&2 regions of CM5 chip. **A, B (b).** Binding response for GST-HDAC8 on immobilized GST Antibody with acceptable response units in both FC 1&2 regions.*



A, B(c). *Graphical representation of the stability analysis of GST Ab & GST-HDAC8 protein (2, 4 &6 µg/mL concentrations) along with details of response units in the table included.* ***C****. Binding studies of GST-HDAC8 with acetylated and unacetylated alpha tubulin peptides with 500nM, 5 µM, and 50µM concentrations. 500nM concentration from both the peptides displayed acceptable range of binding response units with GST-HDAC8.*

Results from SPR analysis demonstrate the binding of both acetylated (Lys40) alpha tubulin peptide and unacetylated alpha tubulin peptide (with maximum RU units these results gives us a preliminary report for our study that both the forms of alpha tubulins (acetylated and unacetylated) are known to interact with HDAC8.

Table 3: Binding details of GST-HDAC8 in FC-1 region of CM5 chip

Cycle #	Curve	Assay Step Purpose	Sample Name	Report Point	RelResp (RU)
1	FC=1	Start-Up	HBS-EP	binding	39
2	FC=1	Start-Up	HBS-EP	binding	4.5
3	FC=1	Start-Up	HBS-EP	binding	8.7
4	FC=1	Sample	GST HDAC8 2 µg/mL	binding	193.2
5	FC=1	Sample	GST HDAC8 4 µg/mL	binding	307.9
6	FC=1	Sample	GST HDAC8 6 µg/mL	binding	460.5

Table 4: Binding details of GST-HDAC8 in FC-2 region of CM5 chip

Cycle #	Curve	Assay Step Purpose	Sample Name	Report Point	RelResp (RU)
1	Fc=2	Start-Up	HBS-EP	binding	29.1
2	Fc=2	Start-Up	HBS-EP	binding	2.8
3	Fc=2	Start-Up	HBS-EP	binding	6.9
4	Fc=2	Sample	GST HDAC8 2 µg/mL	binding	189.3
5	Fc=2	Sample	GST HDAC8 4 µg/mL	binding	301.9
6	Fc=2	Sample	GST HDAC8 µg/mL	binding	453.3

Table 5: Stability details of GST-HDAC8 in FC-2 region of CM5 chip

Cycle #	Curve	Assay Step Purpose	Sample Name	Report Point	RelResp (RU)
1	FC=2	Start-Up	HBS-EP	stability	30.2
2	FC=2	Start-Up	HBS-EP	stability	1.8
3	FC=2	Start-Up	HBS-EP	stability	2.1
4	FC=2	Sample	GST HDAC8 2 µg/mL	stability	8.5
5	FC=2	Sample	GST HDAC8 4 µg/mL	stability	12.3
6	FC=2	Sample	GST HDAC8 6 µg/mL	stability	16.2

Table 6: Binding details of Unac & ac- alpha tubulin peptides with immobilized GST-HDAC8

Cycle #	**Curve**	**Assay Step Purpose**	**Sample Name**	**Report Point**	**RelResp (RU)**
1	Fc=2-1	Start-Up	HBS-EP	binding	-0.3
2	Fc=2-1	Start-Up	HBS-EP	binding	-0.3
3	Fc=2-1	Start-Up	HBS-EP	binding	-0.5
4	Fc=2-1	Sample	Unacetylated peptide 500nM	binding	5.8
5	Fc=2-1	Sample	Unacetylated peptide 5uM	binding	-0.5
6	Fc=2-1	Sample	Unacetylated peptide 50uM	binding	0.1
7	Fc=2-1	Sample	Acetylated peptide 500nM	binding	6.8
8	Fc=2-1	Sample	Acetylated peptide 5uM	binding	-0.5
9	Fc=2-1	Sample	Acetylated peptide 50uM	binding	0.4

3.3.3 Binding of ac-alpha tubulin to HDAC8 decreases intrinsic fluorescence

Further we have performed fluorescence spectroscopy analysis to understand the binding of GST-HDAC8 and peptides time dependently (5-30 min, conditions similar to that of HDAC8 enzymatic assay). Fluorescence spectrometry is widely used in studying the molecular (binding information) and structural changes in the protein, which is affected by either protein-protein interaction or protein with small molecule interactions. The significant change which occurs around the microenvironment of the aromatic amino acids like Trp, Tyr and Phe are known to induce changes in the intrinsic fluorescence emission properties of the protein, which are measured and analyzed (Ying, Huang et al. 2015). HDAC8 has Trp-4, Phe-13, and Tyr-21 residues respectively. Results clearly demonstrate decrease of intrinsic fluorescence in GST-HDAC8 in presence of acetylated alpha tubulin peptide (Lys40), which is due to change in its amino acid microenvironment thereby leading to decrease the intrinsic fluorescence of GST-HDAC8. The decrease in the fluorescence clearly defines the HDAC8 enzyme activity reaction in *in vitro* conditions, when compared to unacetylated alpha tubulin where there was no change in the fluorescence **(Fig. 35 a&b)**.

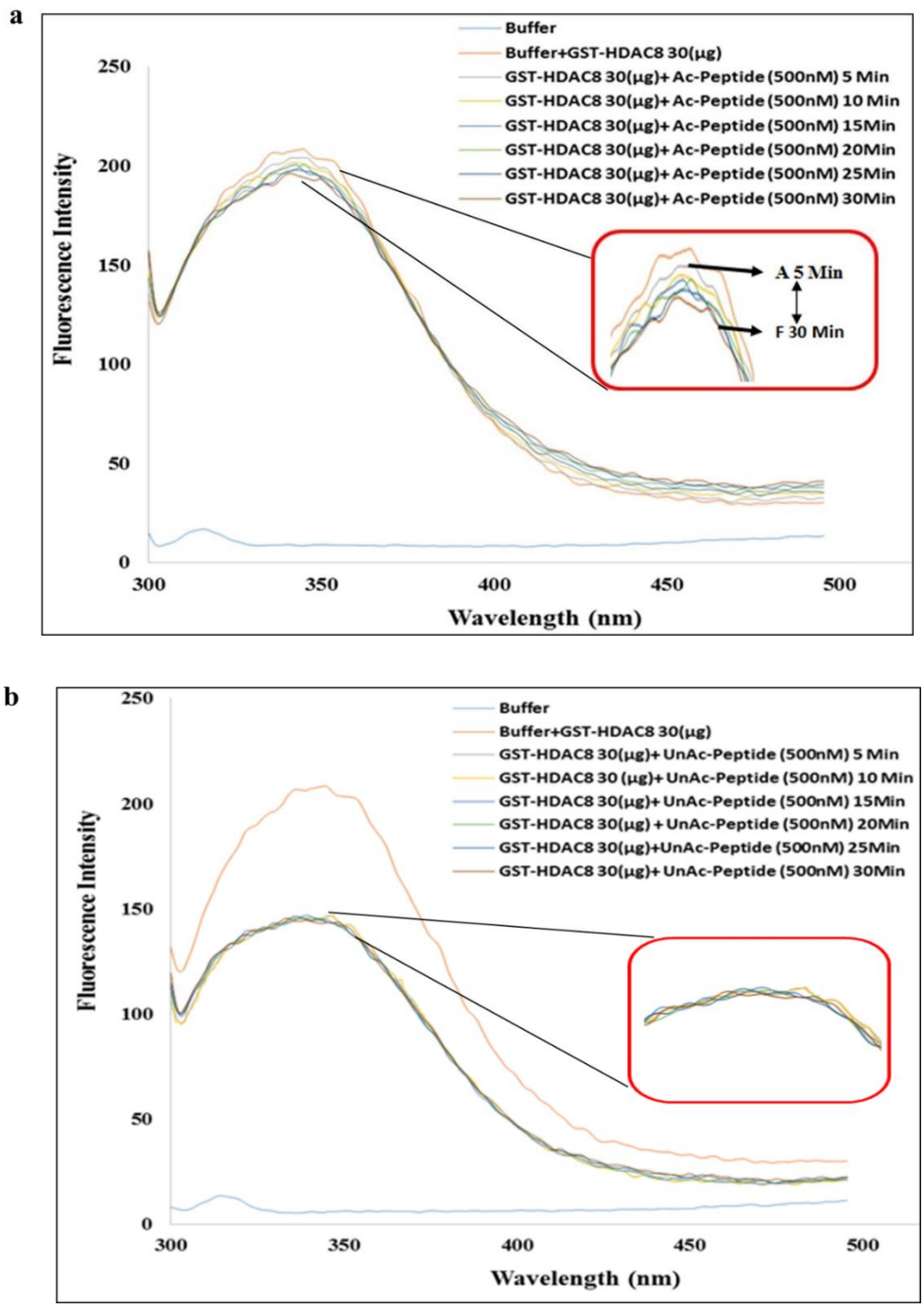


Figure 35. Binding studies of HDAC8 and Alpha tubulin peptides (ac & Unac forms)*: **a.** Fluorescence emission spectra analysis. **(a)** Change in fluorescence intensities of GST-HDAC8 with acetylated alpha tubulin peptide at different time points from A-5min, B-10min, C-15min, D-20min, E-25min, F-30min in MilliQ water. Arrow indicates decrease in intrinsic fluorescence upon increased time of incubation. **b.** Fluorescence spectra of GST-HDAC8 with unacetylated alpha tubulin peptide with no major changes in the absorption upon increase in time.*

3.3.4 Circular Dichroism studies – Binding of ac-alpha tubulin peptide (Lys40) with GST-HDAC8 leads to conformational changes in the secondary structure of GST-HDAC8.

One of the best spectroscopic methods to understand and study the protein-protein interactions in solution is circular dichroism (CD). It is a very well-known method to address the secondary structure, binding affinities and changes in the conformation of the protein in a solution. It is a quantitative technique; the protein-protein interactions that occur due to the complexes formed is directly proportional to the CD spectra. These changes in the binding affinities are further used to calculate the binding constants of a protein. CD in the far UV region (170-260 nm) arises due to the amide of the protein backbone. Whereas the spectra from 350-260 nm and visible regions arise from aromatic and prosthetic groups (Greenfield 2004). As we have confirmed the binding of GST-HDAC8 with acetylated peptide, then we have performed circular dichroism studies to understand the structural changes of peptides and GST-HDAC8 binding.

Interestingly results from CD spectra analysis demonstrated a significant change in the secondary structure of GST-HDAC8, when incubated with ac-alpha tubulin; however there was an imperceptible change with unacetylated peptide **(Fig. 36).**

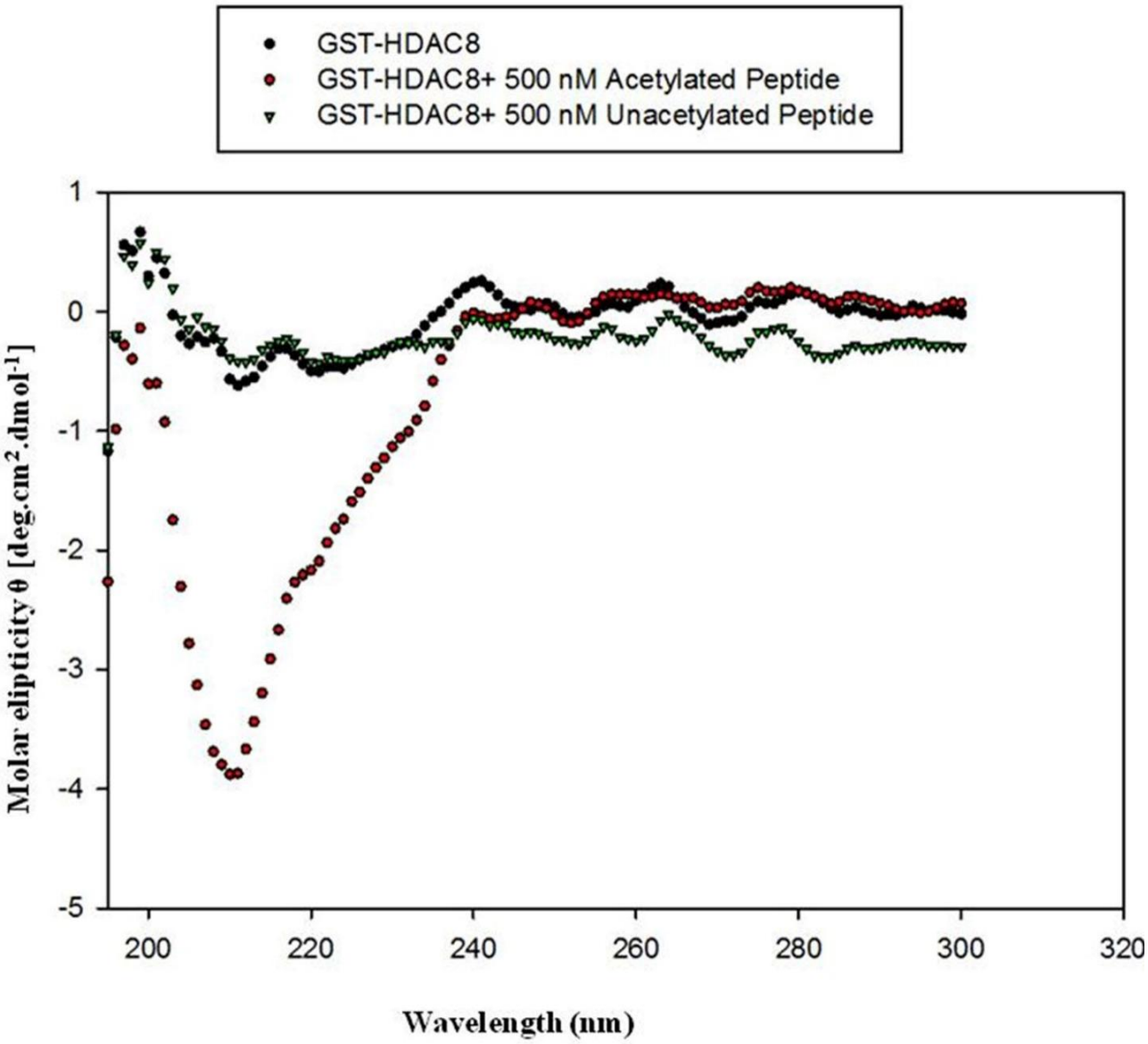


Figure 36. Secondary structure conformational change of GST-HDAC8 upon binding with ac-alpha tubulin: *CD results for change in secondary structure for GST-HDAC8 alone, GST-HDAC8 (2 µM) +ac-alpha tubulin peptide (500nM), and GST-HDAC8 (2 µM) + Unac-alpha tubulin peptide (500nM), significant change in the secondary structure of GST-HDAC8 upon incubating with ac-alpha tubulin clearly signifies the more binding specificity for ac-alpha tubulin but not with unacetylated form.*

3.3.5 HDAC8 co-localizes with acetylated alpha tubulin in HeLa and HEK 293T

Results from our *in silico* and binding studies have confirmed the interaction of HDAC8 with alpha tubulin & acetylated alpha tubulin peptide. Acetylated alpha tubulin form is found to bind more significantly compared to unacetylated form. As evident from the results of circular dichroism studies, there is a clear change in the secondary structure of GST-HDAC8. To further validate the functional interaction we carried out IP and co-IP experiments with HDAC8 and acetylated alpha tubulin (ac-tubulin) antibodies. The immunoblots clearly showed the HDAC8 and ac-tubulin interaction **(Fig. 37)**.

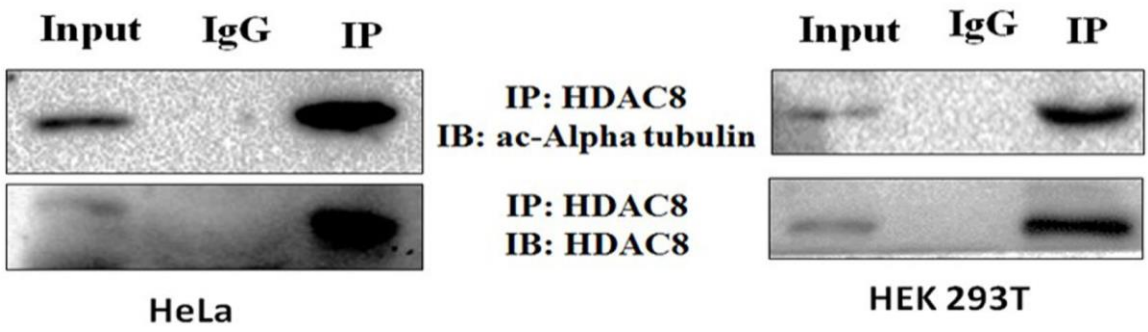


Figure 37. HDAC8 interacts with ac-alpha tubulin*: HDAC8 and Acetylated Alpha tubulin Interaction in HeLa and HEK 293T cells as demonstrated by immunoprecipitation using HDAC8 antibody followed by immunoblot analysis using ac-alpha tubulin.*

Co-localization studies of HDAC8 & ac-alpha tubulin in HeLa and HEK 293T affirms their association and signifies their functional role together. PCI-34051 treatment significantly decreased the levels of HDAC8, which in turn affected the association of acetylated alpha tubulin to a greater extent in HeLa **(Fig. 38)** when compared to HEK 293T **(Fig. 39)**.

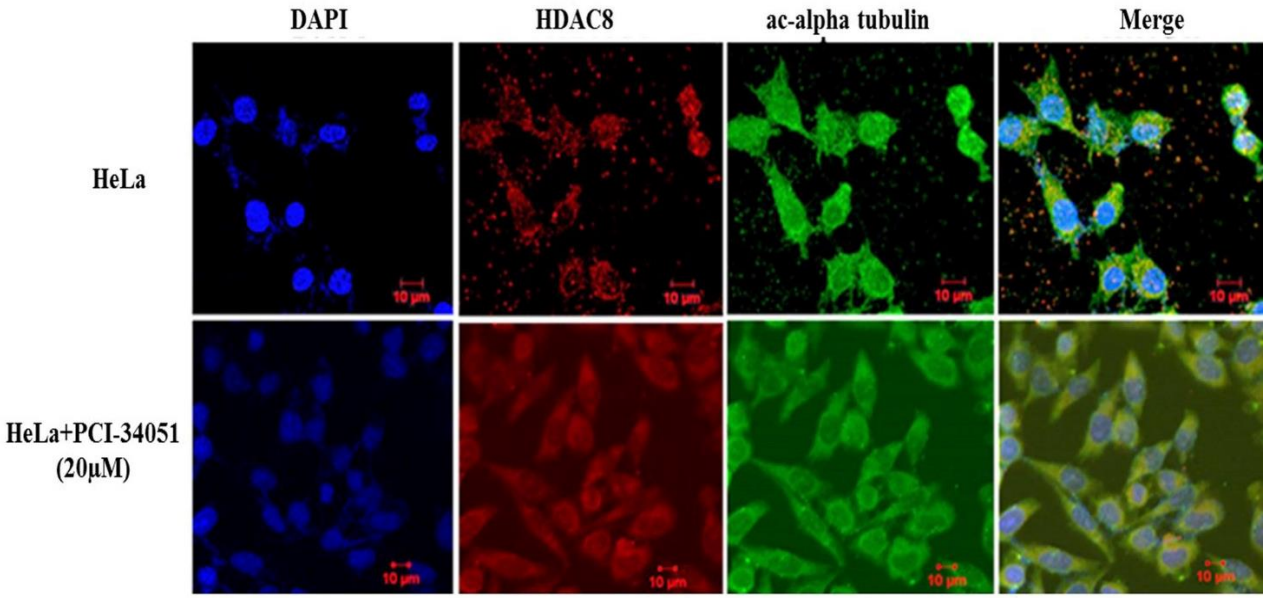


***Figure 38. HDAC8 co-localizes with ac-alpha tubulin in HeLa cells**: Confocal studies demonstrate colocalization of HDAC8 & ac-alpha tubulin in HeLa PCI-34051(20 µM) for 4 hrs treated condition affected the co-localization.*

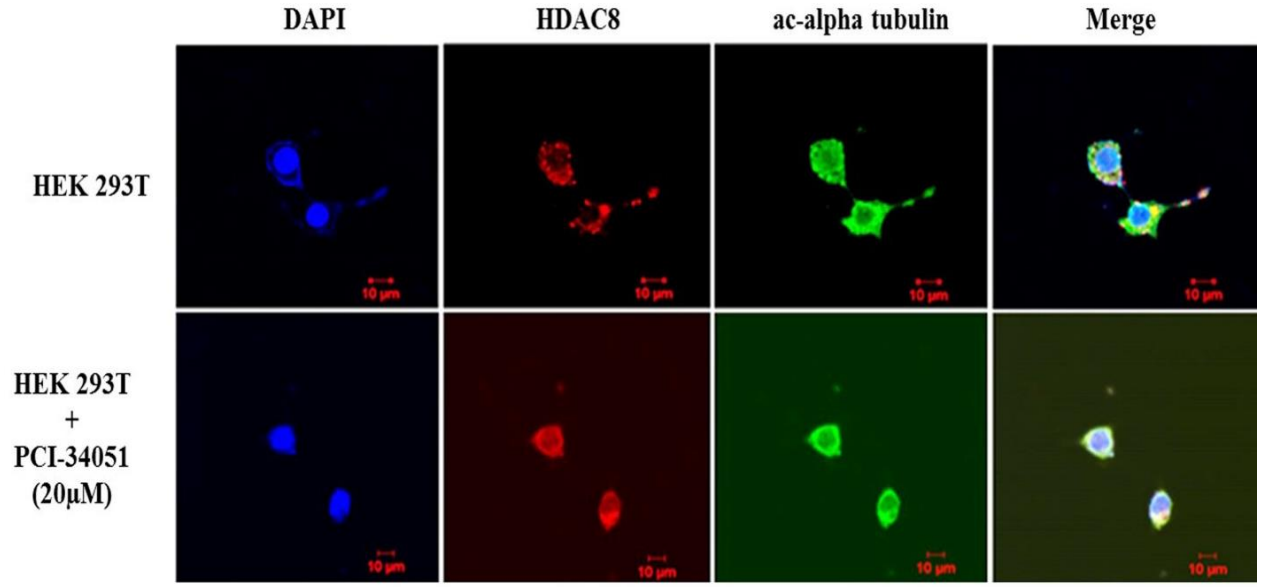


***Figure 39. HDAC8 co-localizes with ac-alpha tubulin in HEK 293T cells**: Confocal studies demonstrate colocalization of HDAC8 & ac-alpha tubulin in HeLa PCI-34051(20 µM) for 4 hrs treated condition affected the co-localization.*

3.3.6 HDAC8 inhibition increases acetylation of alpha tubulin *in vitro*:

Further, we have performed functional characterization of HDAC8 and alpha tubulin interaction. Our results clearly support the data from earlier experiments. Increased levels of ac-alpha tubulin were observed, in forskolin and PCI-34051 treated condition in both HeLa and HEK 293T cells suggesting the importance of HDAC8 in deacetylating acetylated alpha tubulin **(Fig. 40)** further validating it as a novel substrate for HDAC8, in addition to HDAC6, which is an established tubulin deacetylase and known to be a target in many of the cancers (Ding, Ping et al. 2014, Seidel, Schnekenburger et al. 2016).

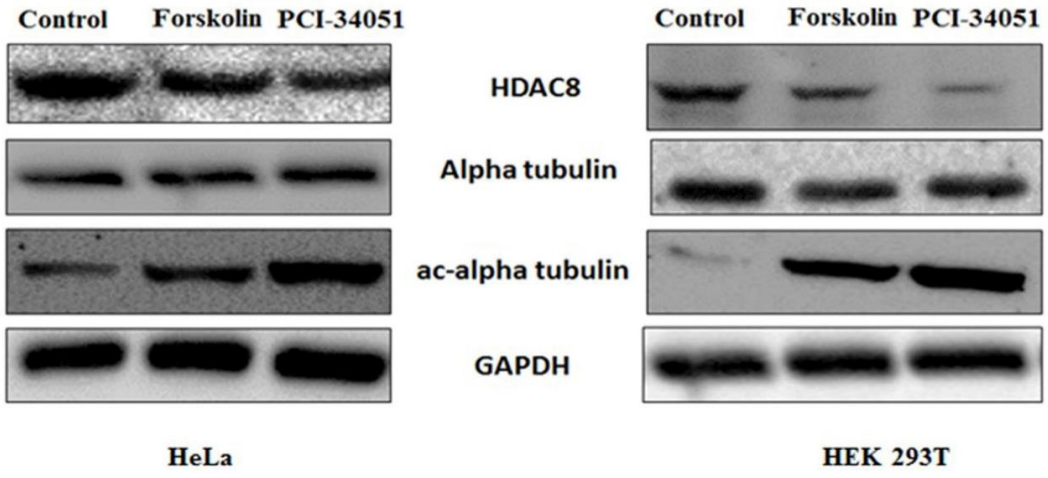


Figure 40. HDAC8 regulates ac-alpha tubulin levels in vitro: *ac-alpha tubulin levels increase when HDAC8 is inhibited with PCI-34051 (20 µM) or when treated with PKA activator, Forskolin (10 µM) and in HeLa and HEK 293T cells.*

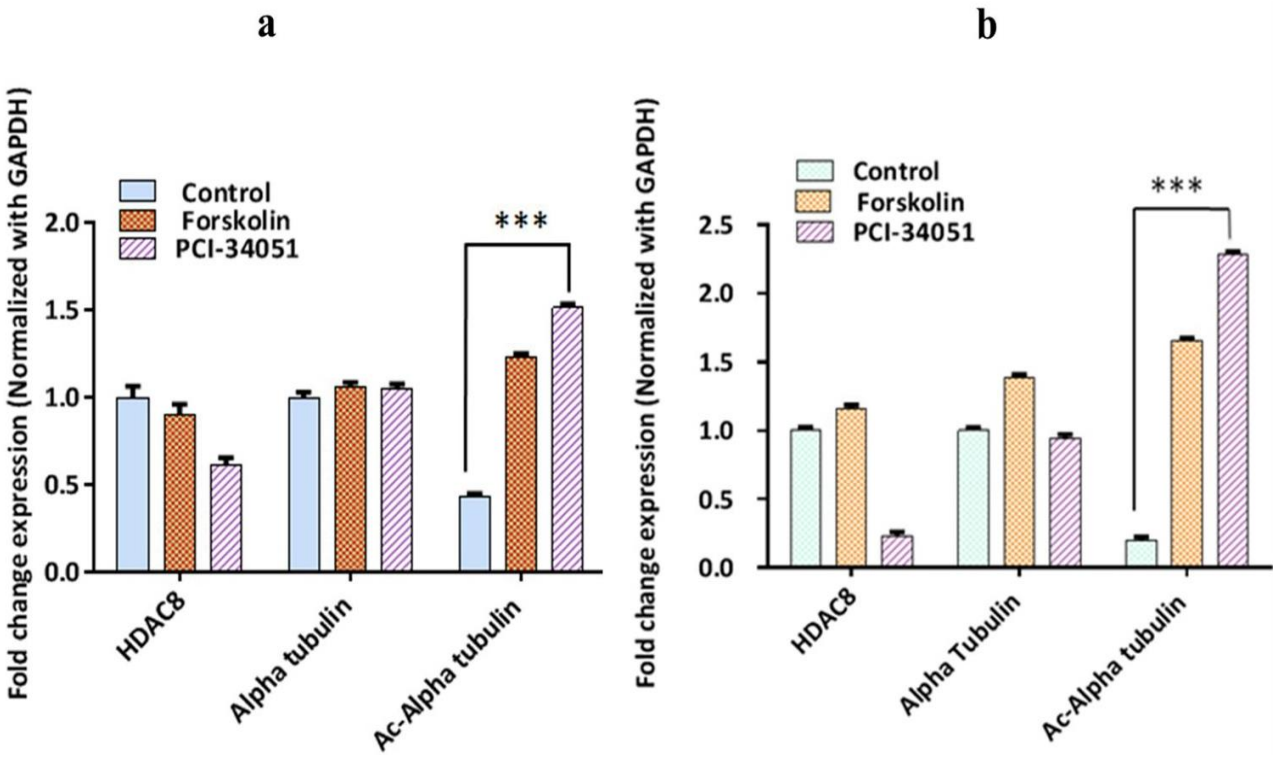


***Figure 40**. Densitometry analysis of the immunoblot bands showing fold change in expression pattern of HDAC8, Alpha tubulin and acetylated tubulin normalized with GAPDH. p value<0.0001 in HeLa (a) and HEK 293T (b).*

3.3.7 GST-HDAC8 deacetylates ac-alpha tubulin *in vitro*: HPLC studies

HDAC8 enzyme activity studies performed with recombinantly expressed and purified protein displayed deacetylation and peptide substrate selectivity even in the absence of additional cofactors (Buggy, Sideris et al. 2000, Hu, Chen et al. 2000, Schultz, Misialek et al. 2004, Gantt, Gattis et al. 2006, Riester, Hildmann et al. 2007, Smith and Denu 2007, Dowling, Gantt et al. 2008, Gurard-Levin and Mrksich 2008, Dowling, Gattis et al. 2010, Gantt, Joseph et al. 2010, Gurard-Levin, Kilian et al. 2010, Haider, Joseph et al. 2011) clearly suggests that HDAC8 has a potential to catalyze *in vivo* deacetylation of other protein in absence of protein complex.

Data from other studies demonstrate that other class I HDAC's like HDAC1, 2, and 3 are known to form complexes and their activity in turn depends on the combination of varied proteins which were incorporated into their complexes (Sengupta and Seto 2004). The specificity and function of these HDAC isozymes are altered by the range of complexes formed by other proteins (Hayakawa and Nakayama 2011).

HDAC8 is phylogenetically most similar to the other class I HDACs, divergent evolution [20] may have altered how HDAC8 interacts with cofactors, possibly allowing this isozyme to function independent of other proteins.

To further confirm the deacetylation of ac-tubulin by HDAC8, we carried out HPLC-based deacetylation assay. Unacetylated alpha tubulin peptide was observed with a retention time of 14.70, acetylated alpha tubulin with 15.37, and GST-HDAC8+acetylated alpha tubulin (Lys40) with a retention time of 14.72, which was equivalent to that of unacetylated peptide. Thus, the significant shift in the peak of acetylated alpha tubulin (Lys40) when incubated with GST-HDAC8 clearly corresponds to the deacetylation and confirms HDAC8 as a tubulin deacetylase **(Fig. 41)**.

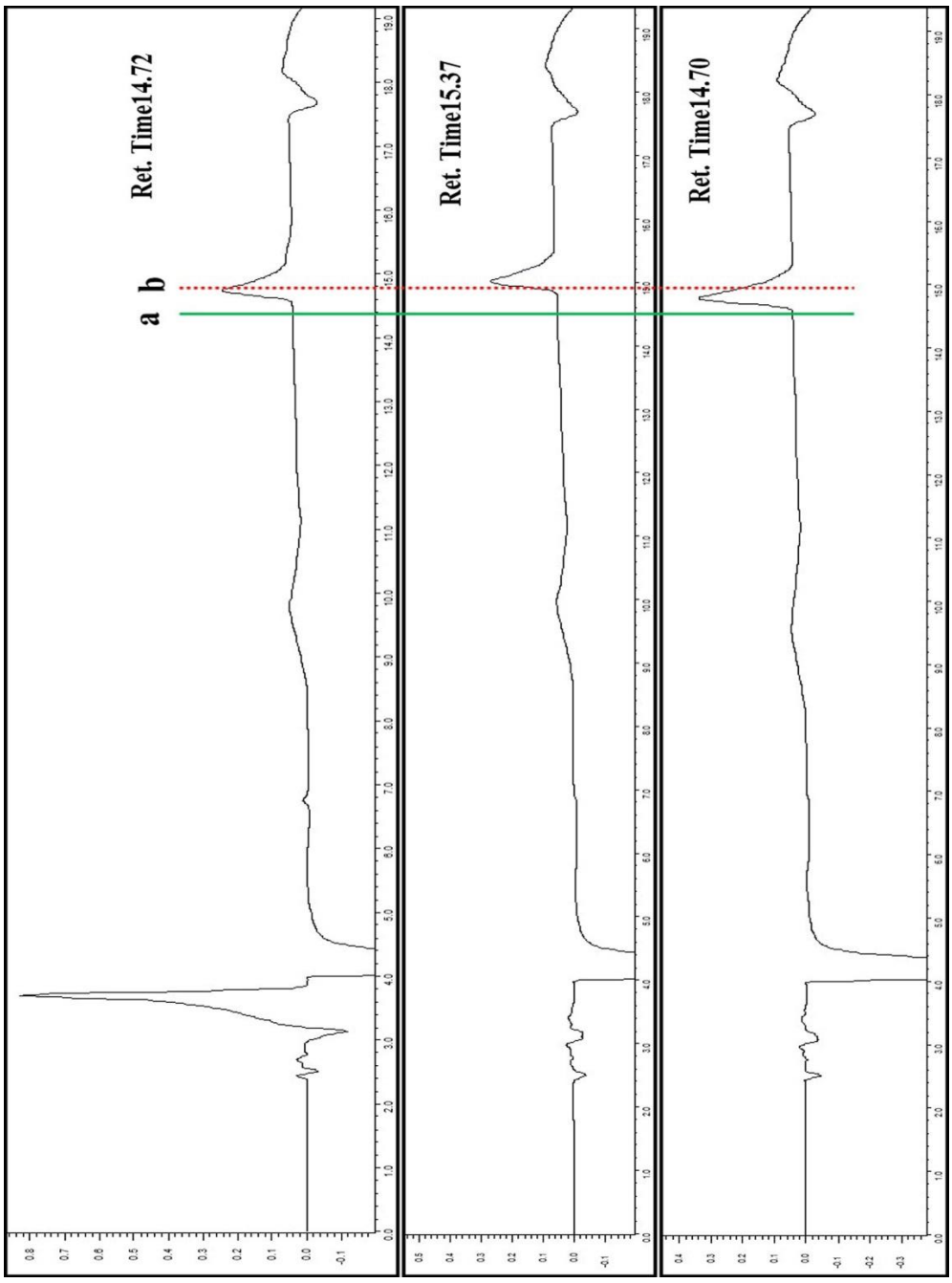


Figure 41. HDAC8 deacetylates ac-alpha tubulin in vitro*: Lower panel of chromatogram represents for Unac-alpha tubulin peptide with a retention time of 14.70 Min. Middle panel of chromatogram for ac-alpha tubulin with retention time of 15.37 Min. Upper panel of chromatogram for GST-HDAC8 (30 µg) + ac-alpha tubulin (500nM) with a retention time of 14.72 Min. Shift in the retention time from a (15.37) to b (14.72) clearly demonstrates the in vitro deacetylation of ac-alpha tubulin by GST-HDAC8*

3.3.8 HDAC8 might be primary tubulin deacetylase in HeLa cells

In order to determine the cell type specificity of HDAC8 expression in different cancer cell lines, we have evaluated the expression of HDAC6 and HDAC8 in HEK 293T (human embryonic kidney cells), HeLa (cervical adenocarcinoma), MDA-MB 231(breast adenocarcinoma), A549 (adenocarcinoma human alveolar basal epithelial cells), K562 (chronic myelogenous leukemia), HCT11 (colorectral carcinoma- epithelial) cell lines. Results demonstrated that there was no significant difference in HDAC6 expression, when compared between HEK 293T (Control), HeLa (Cervical cancer) and HCT11 (Colon cancer). Whereas, A549 (Lung cancer), K562 (CML) and MDA-MB 231(Breast cancer) have shown two fold higher expression when compared to control (HEK 293T) **(Fig. 42 a&b)**.

HDAC8 is over expressed in HeLa, A549 and HCT11 compared to control HEK 293T **(Fig. 42 a&c)**. HDAC6 is a very well-known deacetylase for ac-alpha tubulin (Hubbert, Guardiola et al. 2002). Based upon the expression pattern of HDAC6 in different cancer cell lines, we presume its role in deacetylating ac-alpha tubulin in HeLa might be taken over by HDAC8 due to its significant overexpression compared to HDAC6.

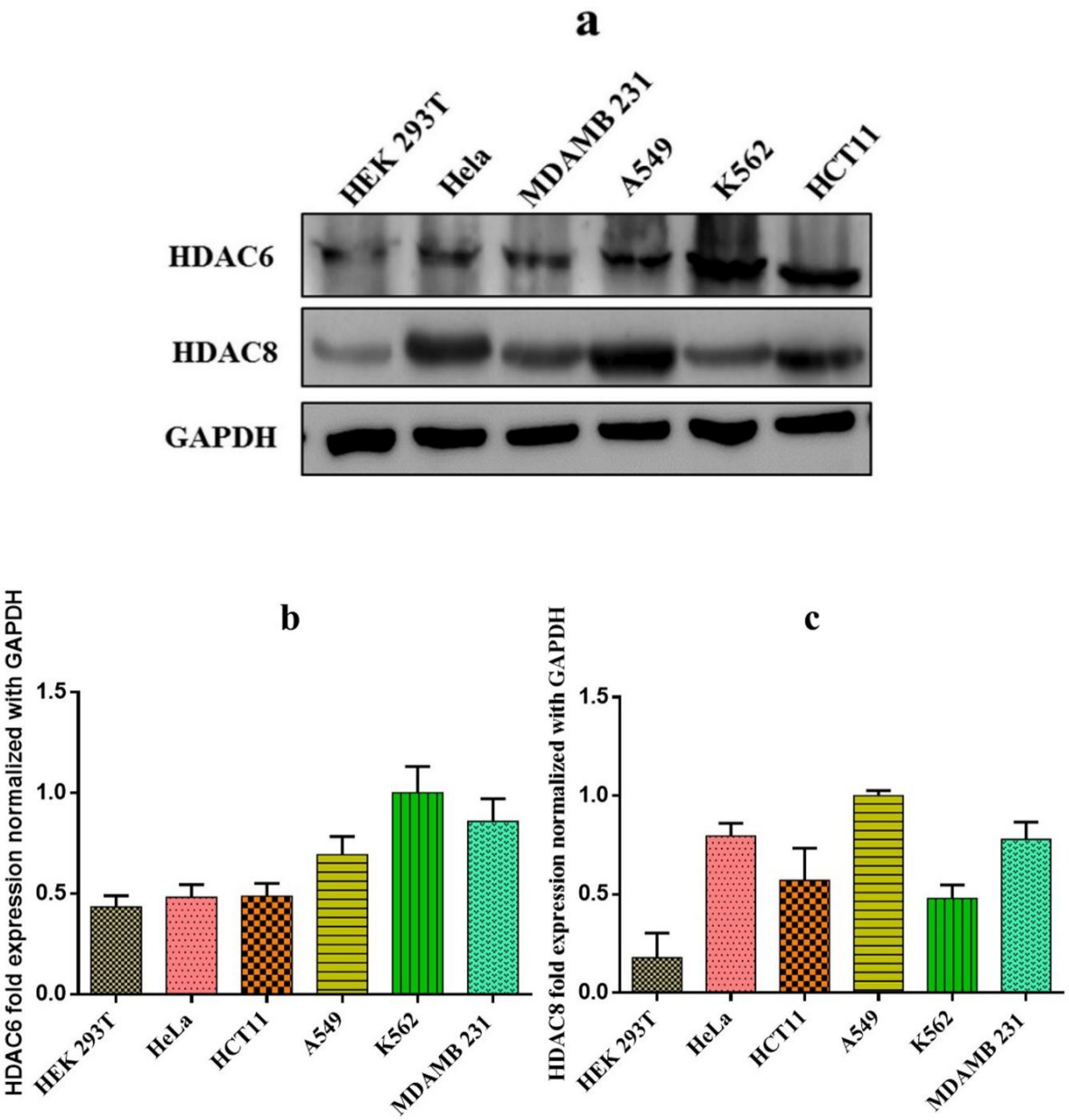


Figure 42. Expression studies of HDAC6 & HDAC8 in different cancer cells: a*. Immunoblot for HDAC6 & HDAC8 expression in HEK 293T, HeLa, HCT11, A549, K562 and MDAMB 231.* ***(b)****. Denistometry graph for HDAC6 expression upon normalization with GAPDH,* ***(c).*** *Graph for fold change expression of HDAC8 protein in different cancer and HEK 293T cells, normalized with GAPDH*

To address the cell type specific role of HDAC6 in HEK 293T and HeLa, we have evaluated mRNA and protein levels and HDAC activity. Real time PCR analysis, immunoblot analysis and HDAC activity assay clearly demonstrated the dominance of HDAC8 in HeLa cells when compared to normal HEK 293T. On the other hand, there was no significant difference in the HDAC6 expression levels in HeLa and HEK 293T cells **(Fig. 43 a, b, c & d)**. These results indicate that HDAC6 might be primary tubulin deacetylase; however, the increased HDAC8 in HeLa cells might be taking over the function of HDAC6 as tubulin deacetylase.

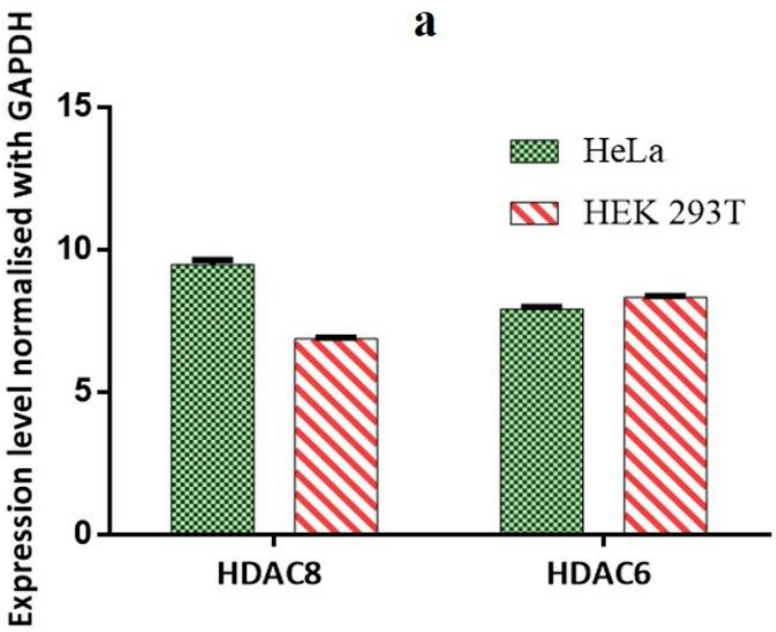
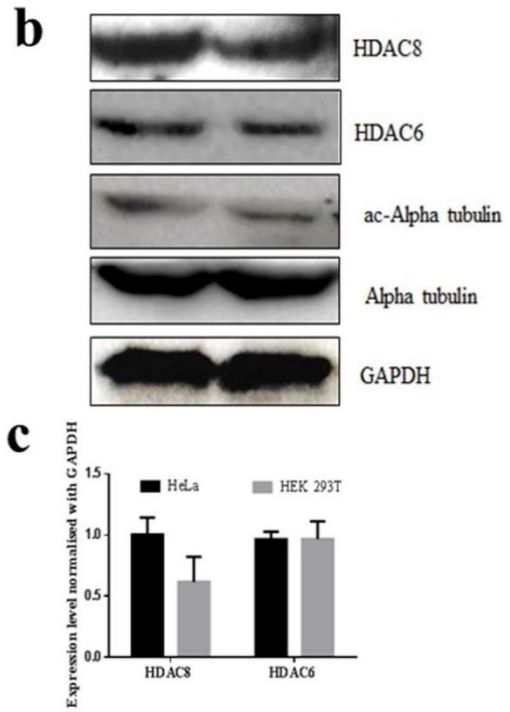
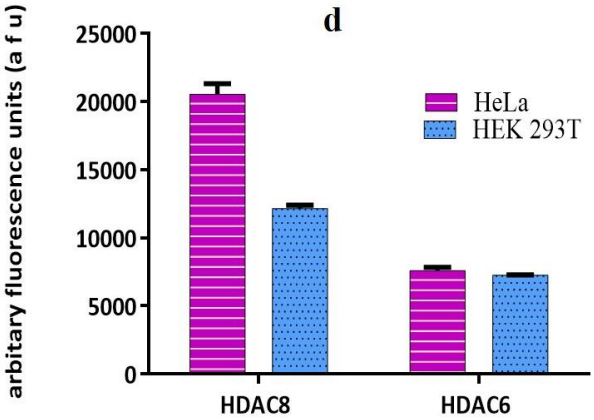


Figure 43. HDAC8 & HDAC6 expression studies in HeLa & HEK 293T cells: a*. Real time analysis confirms higher HDAC8 expression compared to HDAC6 in HeLa and HEK 293T normalized with GAPDH * indicates p-value <0.001.*



***b**. Protein expression pattern of HDAC8 ,HDAC6, ac- alpha tubulin, alpha tubulin along with GAPDH in HeLa and HEK 293T. **c.** HDAC8 and HDAC6 expression was normalized with GAPDH control. HEK 293T has lower expression of HDAC8 compared to HeLa, whereas, there is no difference in the expression pattern of HDAC6 in both the cells.*



***d.** HDAC enzyme activity assay performed with IP Anti-HDAC8 & IP Anti-HDAC6 with HeLa &HEK 293T lysates respectively. * indicates p-value <0.0001.*

Next we tried to determine the effect of HDAC8 and HDAC6 expression on acetylation of tubulin in HEK293T and HeLa cells using specific inhibitor, PCI-34051, Tubastatin and in combination (PCI-34051 + Tubastatin). The increased acetylated alpha tubulin levels in HeLa under PCI-34051 treated conditions when compared to control, clearly demonstrates the possible role of HDAC8 as tubulin deacetylase in HeLa **(Fig. 44 a & b)**.

The combination treatment (upon inhibition of both HDAC6 & HDAC8) have shown a clear impact on acetylation of alpha tubulin, which can be explained due to the synergistic effect of dual inhibition of HDAC6 & 8. In HEK 293T there was no significant increase in PCI-34051 treated condition, whereas dual inhibition of HDAC6 & HDAC8 have shown a positive effect in enhancing the acetylation of alpha tubulin **(Fig. 44 a & c)**.

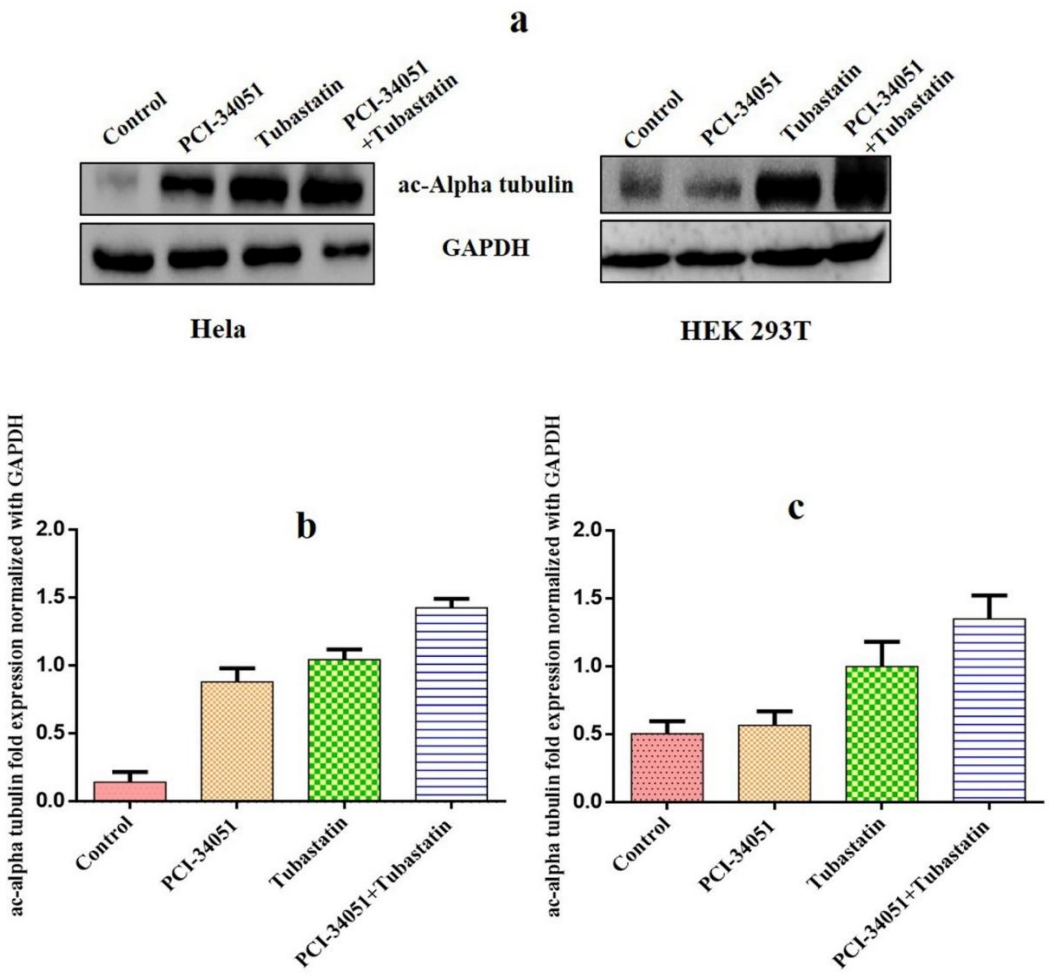


Figure 44. HDAC8 is the primary tubulin deacetylase in HeLa: a. *Significant increase in ac-alpha tubulin levels were observed in presence of PCI-34051 (20 µM), and combination (PCI-34051 20 µM +Tubastatin 5 µM) when compared to control, * indicates p-value < 0.05, (p=0.0004) and tubastatin alone treated condition in HeLa whereas only tubastatin (5 µM) & combination (PCI-34051 20 µM +Tubastatin 5 µM) * indicates p-value < 0.05, (p=0.0118) treatment in HEK 293T increased ac-alpha tubulin levels compared to PCI-34051 (20 µM) which were insignificant.* ***(b&c).*** *Densitometry for ac-alpha tubulin levels normalized with GAPDH in HeLa and HEK 293T.*

Further we examined the effect of acetylation pattern of alpha tubulin by knockdown studies using HDAC8 siRNA in both HeLa and HEK 293T. Real time PCR data confirms the decreased mRNA expression of HDAC8 in both the cell lines **(Fig. 45 a&b)**. Immunoblot results clearly demonstrated the impact on acetylation pattern of alpha tubulin in both the cell lines **(Fig.**

45 c&d). This significant increment in the acetylation of alpha tubulin in HDAC8 KD (siRNA knockdown) samples when compared to control, justifies the role of HDAC8 as one of the possible deacetylase in HeLa but not HEK 293T.

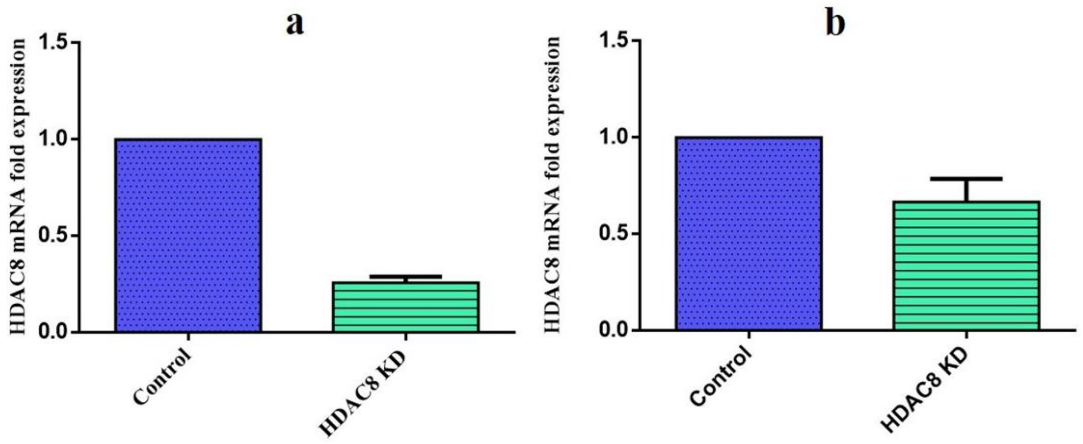
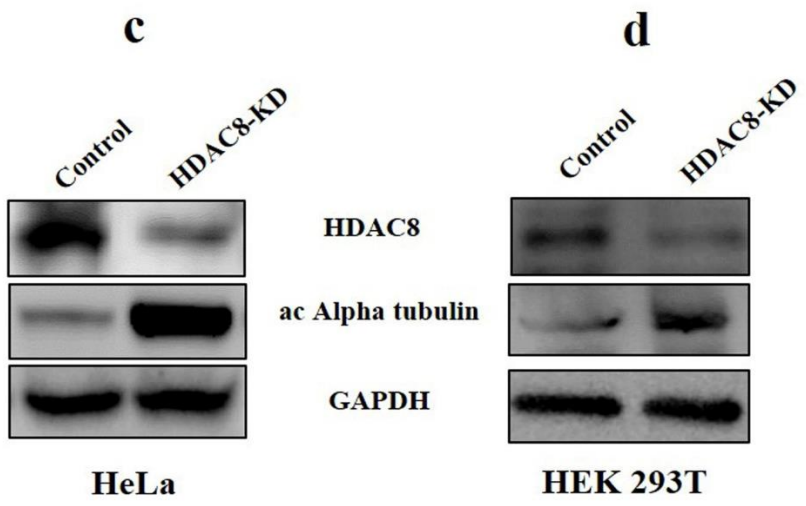


Figure 45. siRNA knockdown of HDAC8 increases ac-alpha tubulin levels: *a&b. HDAC8 Knockdown (siRNA) studies demonstrating significant decrease in mRNA expression of HDAC8 in HeLa along with comparable decrease in HEK 293T.*



(c). Significant (indicates p value = 0.0004 < 0.05) decrease in HDAC8 protein and increase of acetylated alpha tubulin levels in HDAC8 KD (HDAC8 siRNA transfected) samples, when compared to control (untransfected), in HeLa justifies ac-alpha tubulin as one of its potent substrate. (d). Comparable difference in HDAC8 expression and ac- alpha tubulin levels were observed in HEK 293T (HDAC8 KD) samples compared to control (* indicates p value = 0.0296 < 0.05)*

Finally, IP and reverse-IP results of HDAC8 and HDAC6 in HeLa and HEK 293T further demonstrate their independent mode of activity **(Fig. 46)**.

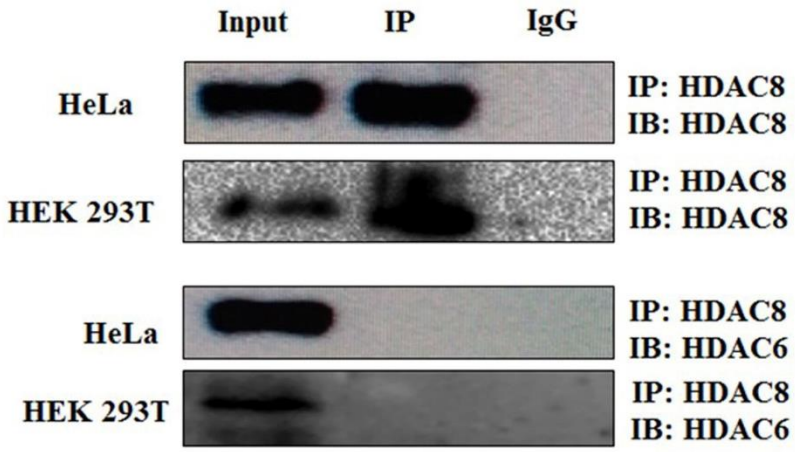


Figure 46. HDAC8 and HDAC6 does not co-precipitate with each other: *IP-Co-IP studies of HDAC8 & HDAC6 in HeLa and HEK 293T.*

Objective 3:

Functional characterization studies of HDAC8 and Alpha tubulin interactions in vitro

3.4.1 HDAC8 alters Alpha tubulin functionality

Tubulins are one of the major cytoskeletal proteins and consist of alpha, beta and gamma tubulin proteins in the family (Williams, Shah et al. 1999, Akhshi, Wernike et al. 2014). Acetylated alpha tubulin is known to stabilize microtubules and effect intracellular transport, cell migration (Akhshi, Wernike et al. 2014) and is the primary determinant for cell morphology (John H. Byrne 2014). In view of alpha-tubulin's role in cell migration and maintaining cell morphology, we further characterized the significant role of HDAC8 and ac-tubulin interaction in cell migration and cell morphology analysis. HDAC8 inhibition impedes the migration property in HeLa in turn affecting the morphology. The effect was quite comparable to that of paclitaxel-treated condition which was considered as a positive control for tubulin inhibition. This can be explained due to the functional role of HDAC8 in deacetylating the acetylated alpha tubulin, which is considered as a mark for stabilization of microtubules. Degree of tubulin acetylation is also known to affect the cell motility by altering the microtubule dynamics (Palazzo, Ackerman et al. 2003). The results clearly demonstrated that when HDAC8 is inhibited with PCI-34051 the cell migration was inhibited up to 48 hrs with a notable decrease in cell size **(Fig. 47 a&b)**.

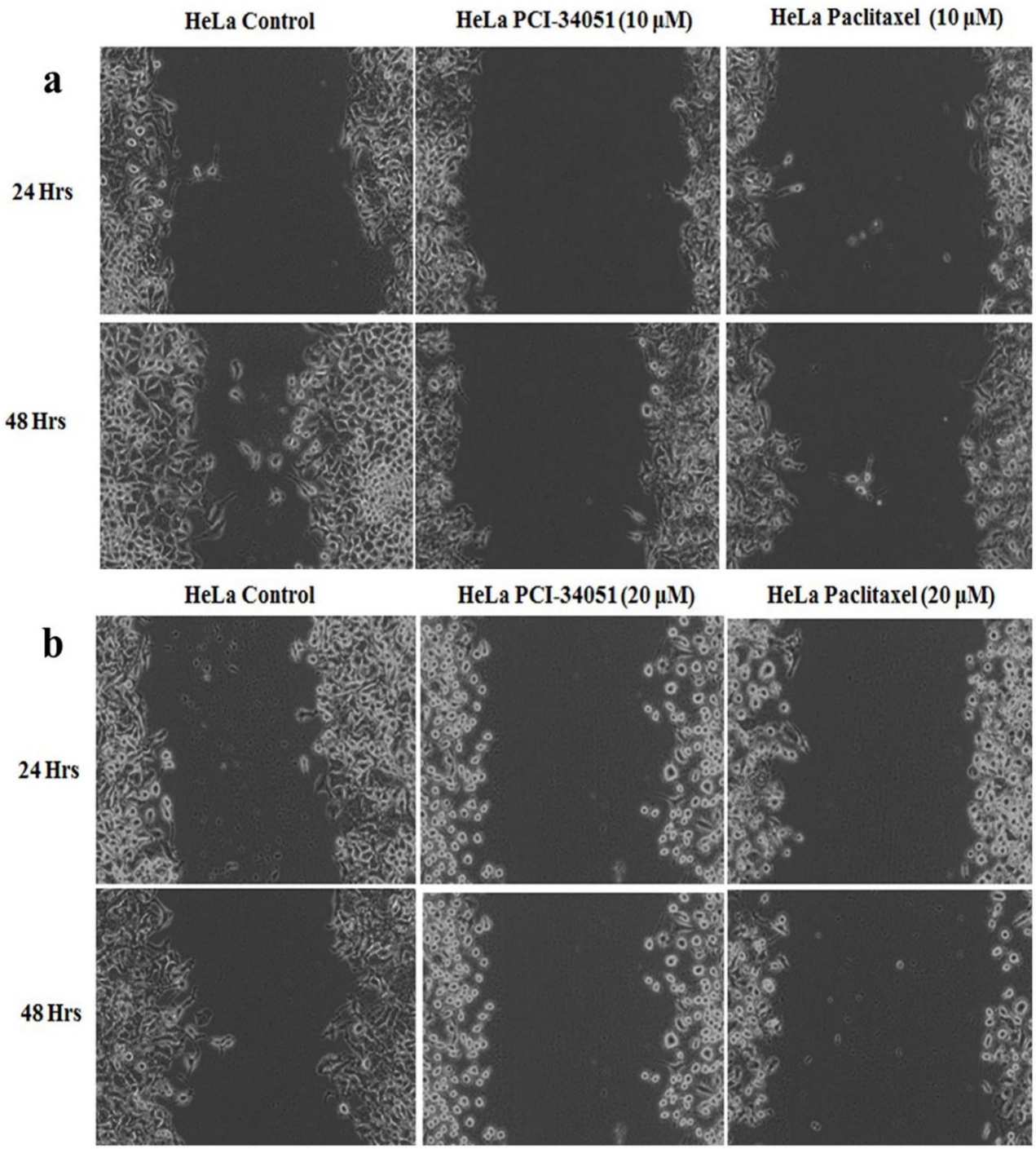


Figure 47. HDAC8 inhibition affects cell migration in HeLa: ***a.*** *Cell Migration Assay in HeLa cells in absence or presence of PCI-34051 (10µM) or Paclitaxel (10µM) Migration efficiency: Control>>Paclitaxel>PCI-34051. b. Cell Migration Assay in HeLa cells in absence or presence of PCI-34051 (20µM) or Paclitaxel (20µM).*

3.4.2 Inhibition of HDAC8 affects the cytoskeletal machinery

Acetylated tubulin is known to stabilize microtubules and effect intracellular transport, cell migration (Akhshi, Wernike et al. 2014) and primary determinant for cell morphology (John H. Byrne 2014). Degree of tubulin acetylation is also known to affect the cell motility by altering the microtubule dynamics (Palazzo, Ackerman et al. 2003). To further validate the difference in the cell morphology, HeLa and HEK 293T cells were treated with PCI-34051 and Paclitaxel for 24 and 48 hrs. A clear distinguished difference in the cell morphology, with decreased cell size (shrinking) in HeLa, and cell aggregation in HEK 293T was observed in both treated conditions when compared with control **(Fig. 48 a&b)**. Shrinkage of cell size in HeLa and clump formation in HEK 293T may be due to disruption in the cytoskeletal machinery in maintaining cell structure. Paclitaxel, an inhibitor of tubulin polymerization, treatment was considered as positive control.

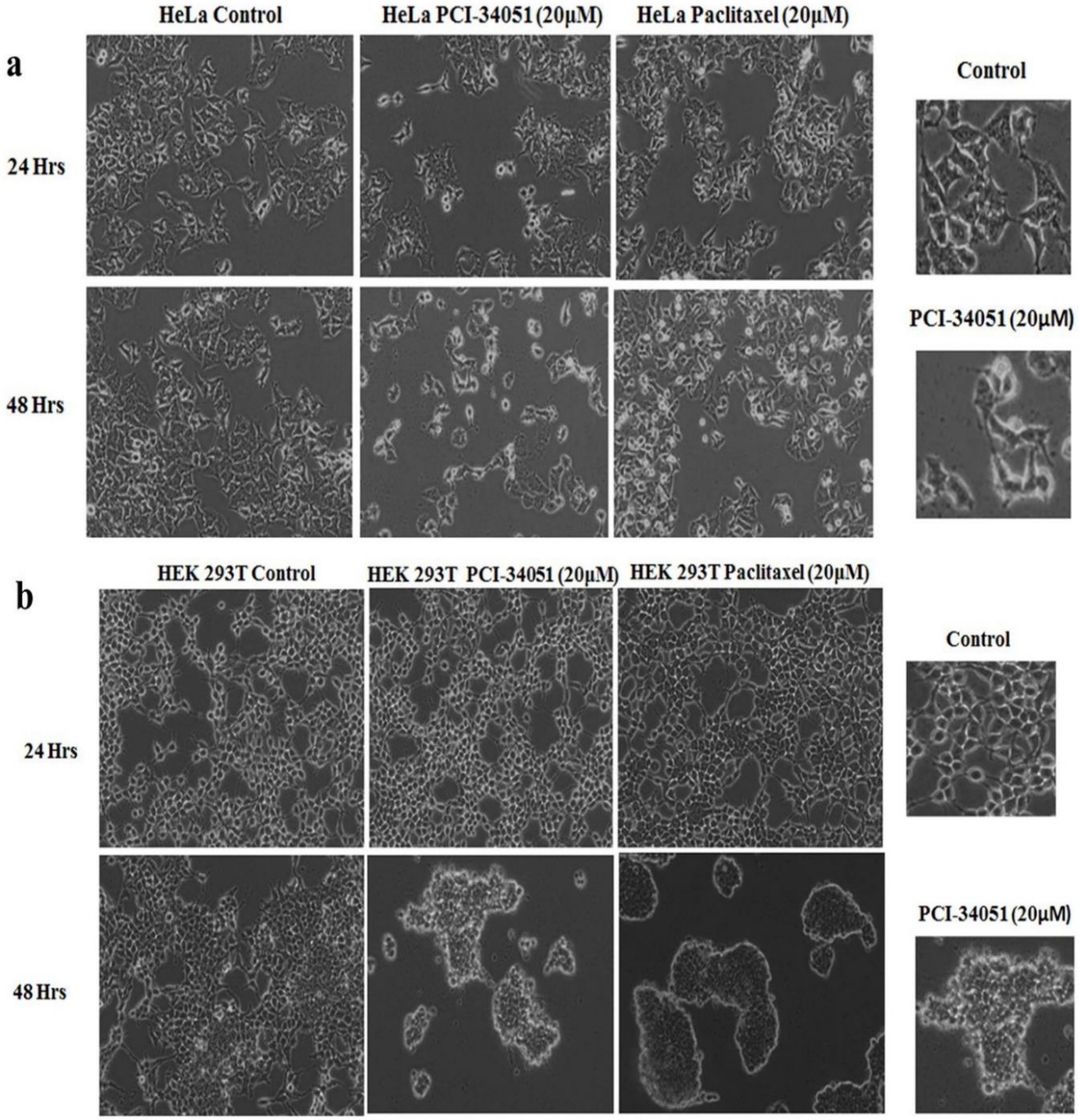


Figure 48. HDAC8 inhibition affects cell morphology in HeLa and HEK 293T: a. *Cell morphology analysis demonstrating reduced (shrinking) cell size of HeLa with PCI-34051 (20µM) or Paclitaxel (20µM) at 24 & 48 hrs. b. Cell morphology analysis in HEK 293T cells representing clump formation with PCI-34051 (20µM) or Paclitaxel (20µM) at 24 & 48 hrs signifying role of HDAC8 in microtubule organization.*

3.4.3 HDAC8 inhibition specifically altered mitotic phase in HeLa

Owing to the important role of microtubules in cell cycle, inhibition of tubulin polymerization with inhibitors such as paclitaxel or inhibition of tubulin deacetylation results in cell-cycle arrest and cell death (Arora, Wang et al. 2009). Therefore we studied the effect of HDAC8 inhibition on tubulin polymerization and cell cycle using PCI-34051. HeLa cells treated with PCI-34051 for 48 hrs showed an increased sub G0/G1 peak along with a significant ($p<0.05$) reduction in mitotic phase of cell cycle when compared to untreated control clearly defining HDAC8 role as primary tubulin deacetylase **(Fig. 49 a&b)**.

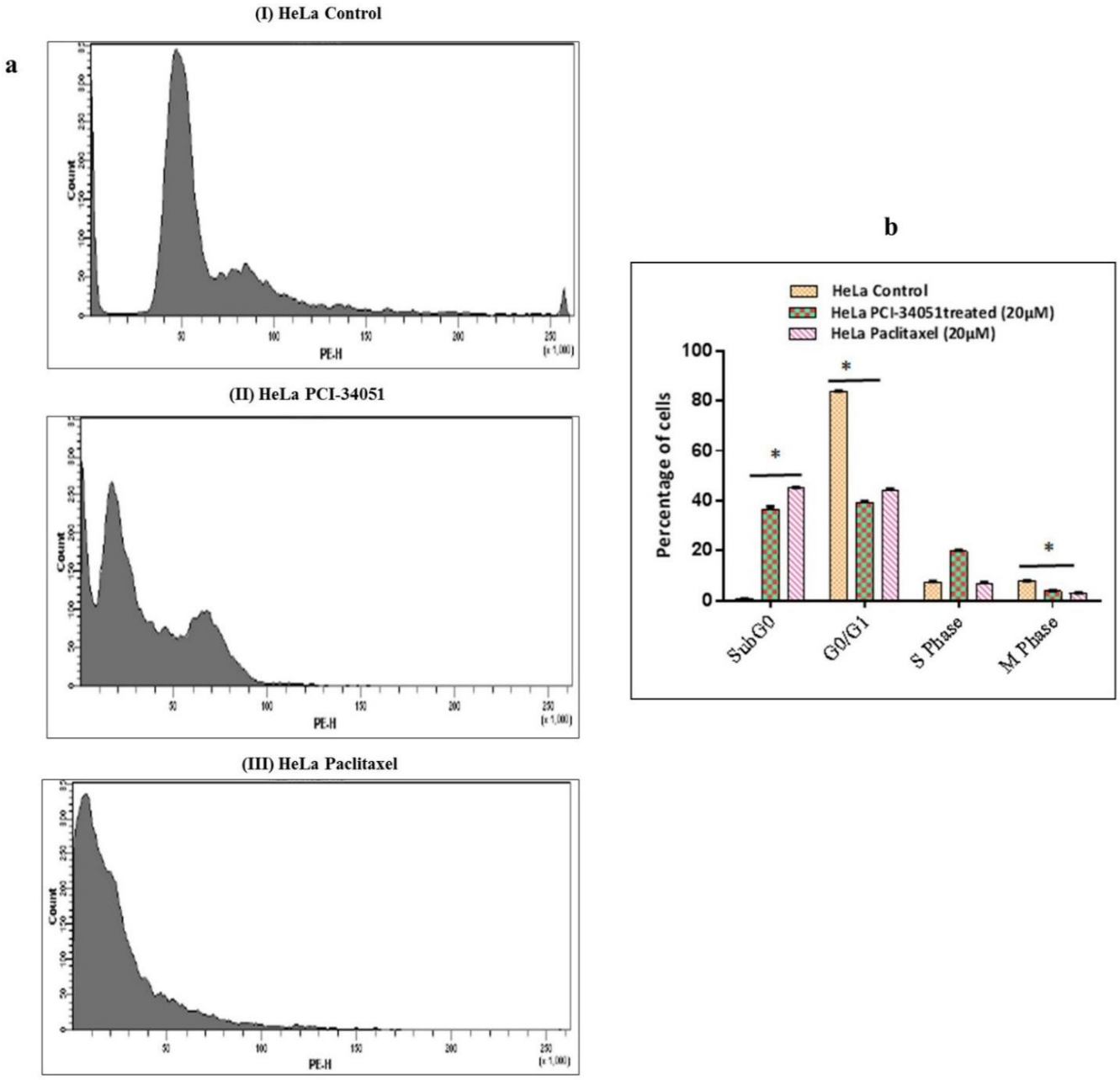


Figure 49. Effect of HDAC8 Inhibition on functional role of Alpha tubulin during M-phase of cell cycle in HeLa cells*: a. Cell cycle analysis of HeLa, showing (I) control, (II) PCI-34051 (20µM) and (III) Paclitaxel (20µM) treated condition. b. Graphical representation of subG0, G0/G1, S and M phase in HeLa, deciphering significant effect of PCI-34051(20µM) on Mitotic phase. * indicates p-value <0.05*

On the other hand, HEK 293T normal cells did not show any mitotic phase reduction when HDAC8 was inhibited indicating that HDAC6 might be the primary tubulin deacetylase. However, a significant increase in the sub G0/G1 phase was observed in HEK 293T cells treated with PCI-34051 which can be attributed to the inhibition of normal physiological functions of HDAC8 **(Fig. 50 a&b)**.

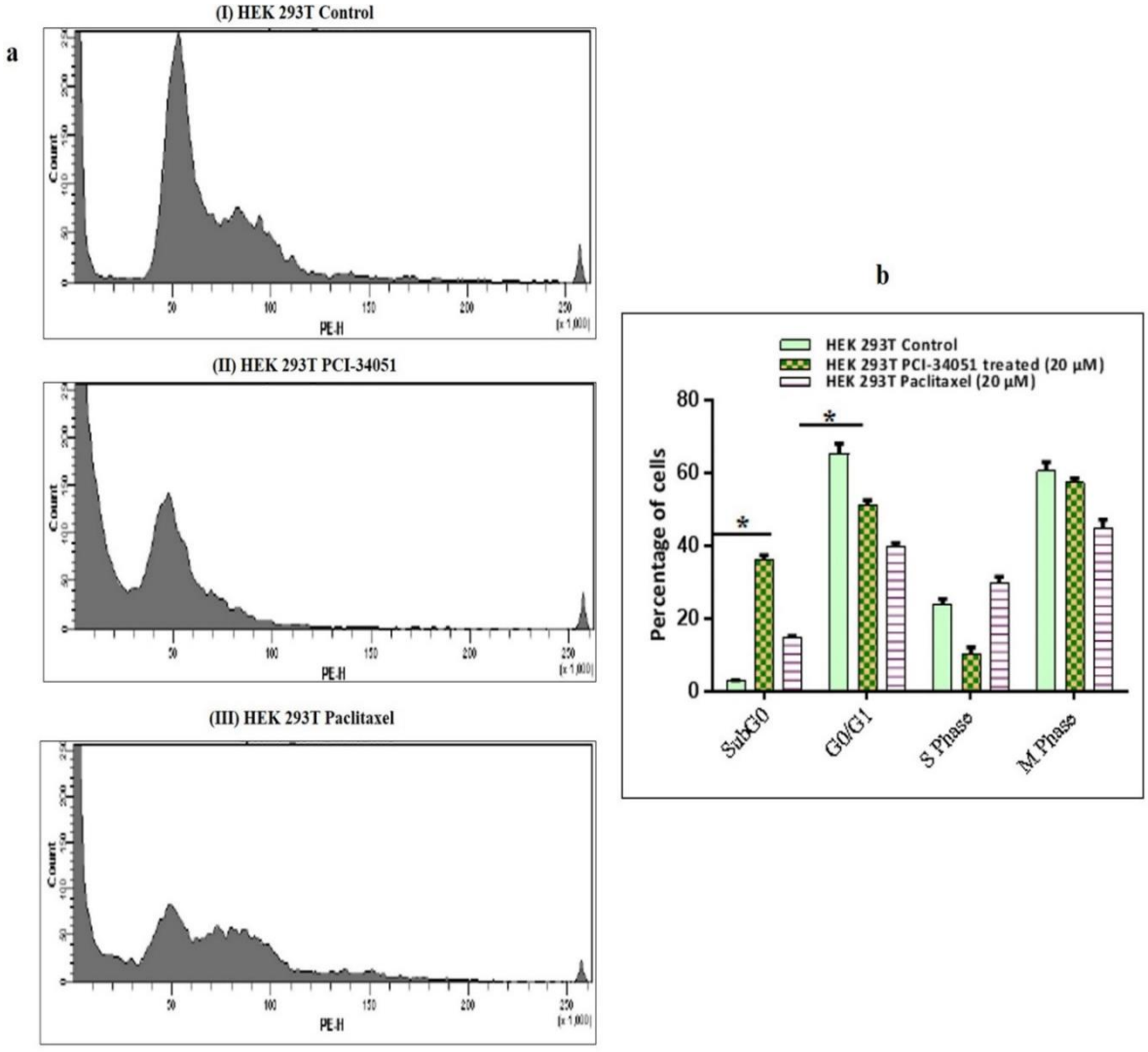


Figure 50. Inhibition of HDAC8 in HEK 293T Inhibition on functional role of Alpha tubulin during M-phase of cell cycle in HEK 293T*: a. Cell cycle analysis of HeLa & HEK 293T, showing (I) control, (II) PCI-34051 (20µM) and (III) Paclitaxel (20µM) treated condition. b. Graphical representation of subG0, G0/G1, S and M phase in HeLa and HEK 293T, deciphering significant effect of PCI-34051(20µM) on Mitotic phase. * indicates p-value <0.05*

Summary & Conclusions

4.0 Summary

4.1 Objective 1: Identification and characterization of Alpha tubulin as a non-histone protein substrate/binding partner of hHDAC8 in HeLa cells.

Although HDAC8 is well-characterized structurally its precise cellular localization, functional role and the substrates are not known completely (Olson, Udeshi et al. 2014). Since HDAC8 is known to be involved in female-specific cancers such as ovary, cervical and breast (Nakagawa, Oda et al. 2007), we have used HeLa cervical cancer cells along with HEK 293T normal cells so as to get an insight on its role in normal and cancer cells.

In our study, we have confirmed major cytoplasmic localization of HDAC8 in HeLa and HEK 293T. However nucleolar localization was also observed in HeLa. Protein Kinase A is known to phosphorylate HDAC8 (pHDAC8) and in the present study we did not see any difference in the localization of pHDAC8 upon activation of PKA by forskolin. Further, enzyme activity and protein expression studies, confirmed active HDAC8 in both cytoplasmic and nuclear fractions. Interestingly, there was an increase in the enzyme activity of HDAC8 of nuclear fraction in both HeLa and HEK 293T, revealing the role of phosphorylated form of HDAC8 in nucleus. Evidence from our immunoprecipitation and MALDI TOF TOF, followed by immunoblotting studies showed Alpha tubulin as a non-histone interacting partner of HDAC8 irrespective of cancerous (HeLa) and normal cells (HEK 293T).

4.2 Objective 2: Protein-protein interaction studies of HDAC8 & Acetylated (Lys40) alpha tubulin.

This objective describes our findings with regard to *in silico* and *in vitro* interaction studies of HDAC8 and Alpha tubulin. Clustal omega and phylogenetic analysis of alpha tubulin revealed its unique Lys40 position conserved across all major groups of eukaryotes. Protein-protein interaction studies, with all the three forms of tubulins further validated the importance of alpha tubulin with higher number of interactions, especially Lys40 (a potential acetylation site) of alpha tubulin (catalytic region) with that of Pro205 of HDAC8 (catalytic region). Importance of Lys40 position for its potential interaction with HDAC8 was further validated by *in silico* mutational analysis (Lys40 to Arg in alpha tubulin & Pro205 to Ala in HDAC8) which revealed loss of interaction between two proteins. Further *in vitro* binding assays were performed by Surface Plasma Resonance, Fluorescence spectroscopy and Circular dichroism. Results clearly demonstrated the importance of acetylated form of alpha tubulin peptide, which is binding, and significantly changing the secondary structure of HDAC8. Whereas, unacetylated form of alpha tubulin was ineffective.

IP and Co-IP results for acetylated alpha tubulin with HDAC8, along with functional regulation studies of acetylation levels of alpha tubulin, in presence or absence of specific HDAC8 inhibitor PCI-34051 and forskolin treated condition revealed significant increase of acetylated tubulin expression, validating it as a novel substrate for HDAC8, in addition to HDAC6, which is an established tubulin deacetylase (Ding, Ping et al. 2014, Seidel, Schnekenburger et al. 2016). Considering the positive results from our binding studies, *in vitro* HPLC-based deacetylation assay was carried out with GST-HDAC8 incubation with acetylated alpha tubulin peptide (Lys40) that clearly admits tubulin as a non-histone substrate of HDAC8.

As our results clearly demonstrate the role of HDAC8 in deacetylating acetylated alpha tubulin, question arises for the cell type specificity of HDAC6 and HDAC8. We first analyzed the expression of HDAC6 and HDAC8 in normal and cancerous cervical tissue samples in The Human Protein Atlas database and learned that HDAC6 expression is low in cervical cancer tissues when compared to normal cervix (medium expression) and that HDAC8 is overexpressed (low-medium expression) in cancerous cervix when compared to undetectable expression in normal cervix [37]. Initially we have confirmed the expression of HDAC6 in different cancer cell lines of various origins i.e., HeLa, HCT11, A549, K562 & MDAMB 231. There was no significant change in the expression of HDAC6 in between HEK 293T (normal), HeLa and HCT11 cell lines, when compared to A549, K562 & MDAMB 231. Whereas, HDAC8 expression was observed to be high in HeLa, A549, HCT11 & MDAMB 231. These immunoblot results are in comparison with human protein atlas data, suggesting tissue specific expression pattern of HDAC6 & HDAC8 in different cancer cell lines, assigning their specific/independent roles in deacetylating their target substrates. Further, results from our studies state dominant expression of HDAC8 in HeLa cells, to that of HEK 293T, when compared to HDAC6 expression which was not significant further confirms with the human protein atlas database. This increases the probability of considering HDAC8 in taking over the function of a tubulin deacetylase in HeLa, compared to HDAC6. HDAC8 inhibition (PCI-34051) contrastingly increased the acetylation of alpha tubulin in HeLa, when compared to HEK 293T. Dual inhibition of both HDAC6&8 further increased the acetylation attributing to their independent role in deacetylating acetylated alpha tubulin. HDAC8 knockdown studies, confirms the importance of HDAC8 role as one of the tubulin deacetylase in HeLa.

4.3. Objective 3: Functional characterization studies of HDAC8 and Alpha tubulin interactions *in vitro.*

This part of the study clearly focuses on importance of HDAC8 in regulating the microtubule functions. The study highlights on three major functional roles of microtubules - cell migration, maintenance of cell morphology and regulation of cell cycle. Our results from cell migration assay, under HDAC8 inhibitory condition clearly defined the role of HDAC8 in regulating microtubule machinery to impede the migration in HeLa cells. Apart from significantly effecting the migration, we have also demonstrated that cell morphology of HeLa (shrinkage of treated cells) and HEK 293T (clump formation in treated cells) was affected with HDAC8 inhibition condition, equally to that of positive control paclitaxel. This can be explained due to the functional role of HDAC8 in deacetylating the acetylated alpha tubulin, which is considered as a mark for stabilization of microtubules. Degree of tubulin acetylation is also known to affect the cell motility by altering the microtubule dynamics (Palazzo, Ackerman et al. 2003). Owing to the important role of microtubules in cell cycle, inhibition of tubulin polymerization with inhibitors such as paclitaxel or inhibition of tubulin deacetylation results in cell-cycle arrest and cell death (Arora, Wang et al. 2009). Our results demonstrate the significant ($p<0.05$) reduction in mitotic phase in HeLa treated with PCI-34051, when compared to HEK 293T. This justifies the role of HDAC8 in HeLa as predominant deacetylase of alpha tubulin, which is in turn involved in regulating cell cycle in G2/M phase. Whereas, no major significant change in the mitotic phase of HEK 293T condition can be explained due to the availability of HDAC6 even upon HDAC8 inhibition might be replacing the role of tubulin deacetylase.

Diagrammatic representation of overall summary

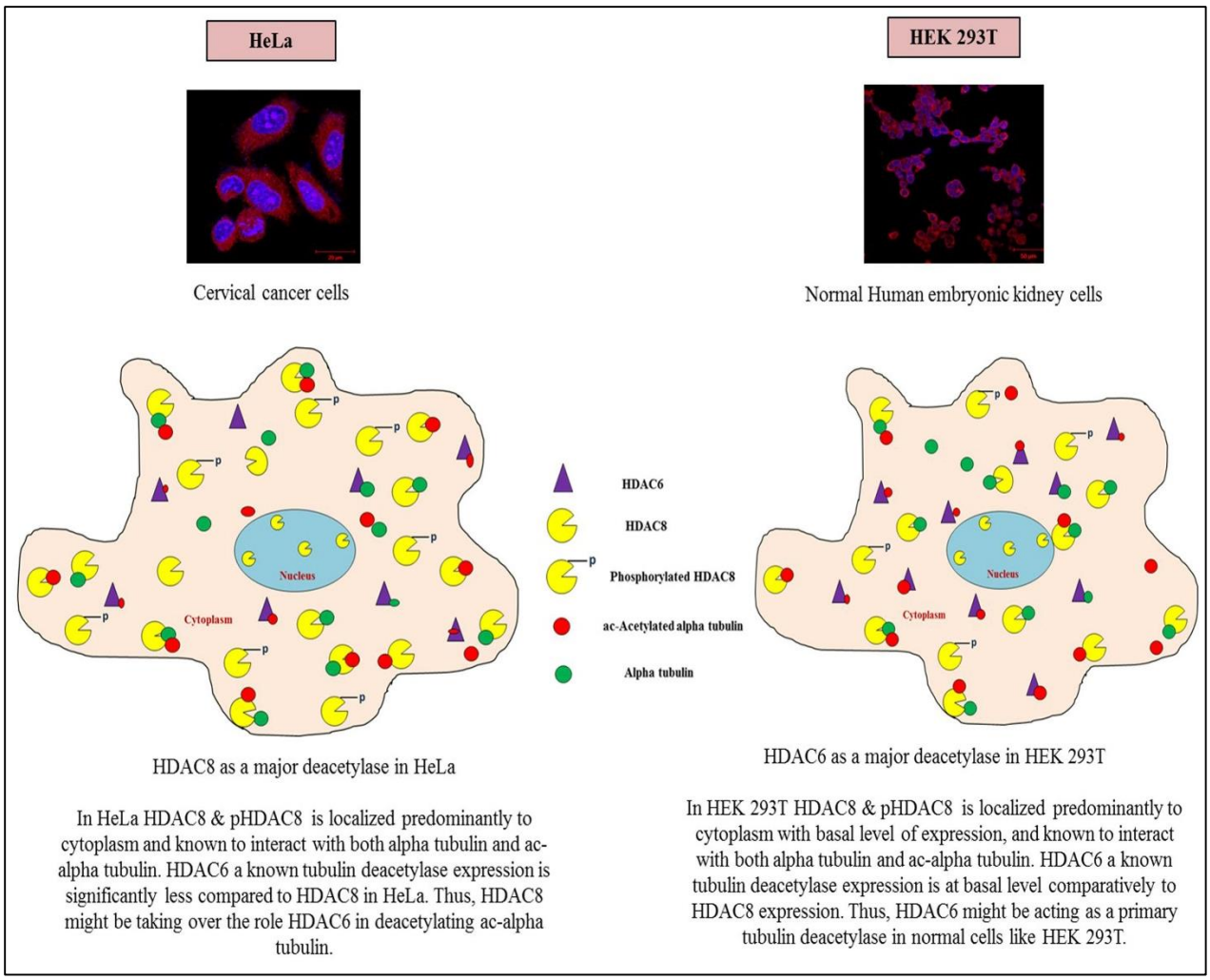


Figure 51. Schematic representation of overall summary

5. Conclusions

- Localization of HDAC8 and pHDAC8 is majorly cytoplasmic.
- Identified Alpha tubulin-1C (Top score match of >56%) as one of the non-histone protein interacting partners of HDAC8 by MALDI TOF-TOF. Immunoprecipitation and Reverse Immunoprecipitation studies have confirmed the interaction of HDAC8 with alpha tubulin in both HeLa and HEK 293T cells.
- *In silico* studies demonstrated that Lys40 in alpha tubulin was unique conserved residue present only in alpha tubulin and known to be acetylated in cellular process. Tubulin domains in alpha, beta and gamma are conserved and can be involved in similar function. Significant zdock score confirms the interaction of HDAC8 with alpha tubulin. Lys40 of alpha-tubulin belong to tubulin catalytic domain (N-terminal region) which is interacting with Pro205 of HDAC8 catalytic domain.
- HDAC8 interacts and regulates the levels of acetylated alpha tubulin as revealed by binding and interaction studies using SPR, fluorescence spectroscopy and Circular dichroism.
- Human cancer protein atlas data analysis followed by expression studies clearly indicated dominant expression of HDAC8 in HeLa cells compared to HEK293T cells
- Further inhibitory studies of HDAC8 by PCI-34051 in HeLa cells affected cell migration and cell morphology. HDAC8 inhibition significantly altered the subG0, G0/G1 and Mitotic phase of cell cycle in HeLa cells compared to HEK 293T cells signifying the role of HDAC8 as tubulin deacetylase in HeLa.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Anti-inflammatory profile of *Aegle marmelos* (L) Correa (Bilva) with special reference to young roots grown in different parts of India

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ABSTRACT

Introduction: *Aegle marmelos* (Bilva) is being used in Ayurveda the Indian systems of medicine for the treatment of several inflammatory disorders. The plant is a member of a fixed dose combination of Dashamoola in Ayurveda. However, the usage of roots/root bark or stems is associated with sustainability concerns.

Aim of the study: The present study is aimed to compare the anti-inflammatory properties of different extracts of young roots (year wise) and mature parts of Bilva plants collected from different geographical locations in India, so as to identify a sustainable source for Ayurvedic formulation.

Materials and methods: A total of 191 extracts (petroleum ether, ethyl acetate, ethanol and aqueous) of roots, stems and leaves of *A. marmelos* (collected from Gujarat, Maharashtra, Odisha, Chhattisgarh, Karnataka and Andhra Pradesh region)were tested for anti-inflammatory effects *in vitro* on isolated target enzymes cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX), lymphocyte proliferation assay (LPA), cytokine profiling in LPS induced mouse macrophage (RAW 264.7) cell line and *in vivo* carrageenan induced paw edema in mice.

Results: Of 191 extracts, 44 extracts showed COX-2 inhibition and 38 extracts showed COX-1 inhibition, while none showed 5-LOX inhibition. Cytokine analysis of the 44 extracts showing inhibition of COX-2 suggested that only 17 extracts modulated the cytokines by increasing the anti-inflammatory cytokine IL-2 and reducing the pro-inflammatory cytokines like IL-1β, MIP1-α and IL-6. The young (2 and 3 years) roots from Gujarat and young (1 yr) roots from Odisha of Bilva plants showed the most potent anti-inflammatory activity by suppressing the pro-inflammatory cytokines and inducing anti-inflammatory cytokine. These three extracts have also shown *in vivo* anti-inflammatory activity which were comparable to those in adult stem and root barks.

Conclusion: The present study reveals that young roots of Bilva plants from Gujarat and Odisha region could form a sustainable source for use in the Ayurvedic formulations with anti-inflammatory activities. The present study also indicates that the region in which the plants are grown and the age of the plants play an important role in exhibiting the anti-inflammatory effect.

1. Introduction

Aegle marmelos (L.) Correa is a member of Dashamoola (10 root drugs) group. This combination is widely used in generic Ayurvedic formulations such as *Daśamūlarishta*, *DaśamūlaKaśāya*, and *Daśamūlakaṭutrayādi*Kvātham. *The* plant grows wild in dry forest in outer Himalayas and Shivaliks. Bilva is a medium to large sized

Abbreviations: LPS, lipopolysaccharides; Con A, Concanavalin A; PE, petroleum ether; EA, ethyl acetate; ET, ethanol; AQ, aqueous.

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Thieno[3,2-*c*]pyran-4-one based novel small molecules: Their synthesis, crystal structure analysis and in vitro evaluation as potential anticancer agents

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ABSTRACT

Novel thieno[3,2-*c*]pyran-4-one based small molecules were designed as potential anticancer agents. Expeditious synthesis of these compounds was carried out via a multi-step sequence consisting of few steps such as Gewald reaction, Sandmeyer type iodination, Sonogashira type coupling followed by iodocyclization and then Pd-mediated various C–C bond forming reactions. The overall strategy involved the construction of thiophene ring followed by the fused pyranone moiety and then functionalization at C-7 position of the resultant thieno[3,2-*c*]pyran-4-one framework. Some of the compounds synthesized showed selective growth inhibition of cancer cells in vitro among which two compounds for example, **5d** and **6c** showed IC_{50} values in the range of 2.0–2.5 μM. The crystal structure analysis of an active compound along with hydrogen bonding patterns and molecular arrangement present within the molecule is described.

With more than 10 million new cases every year, cancer has become one of the most devastating diseases worldwide. According to WHO, in the year 2000, 5.3 million men and 4.7 million women developed a malignant tumor and 6.2 million died from the disease. The number of new cases is expected to grow by 50% over the next 20 years to reach 15 million by 2020. Among various types of cancers hepatocellular carcinoma (HCC) has emerged as the fifth most common cancer worldwide.[1] The chronic myelogenous leukemia (CML), a slowly progressing cancerous disease of blood and bone marrow usually occurs in the middle aged adults,[2] is the leading cause of leukemia mortality. It is therefore necessary to identify new and more effective anticancer agents for the potential treatment of HCC and CML. All these reasons and our continued interest in the identification of novel anticancer agents[3] prompted us to design novel scaffold based on a known anticancer agent that is, 8,9-dihydroxy-6*H*-benzofuro[3,2-*c*]chromen-6-one[4] or **A3** (Fig. 1). While the compound **A3** possess flexible structural features its structural modification for the identification of new anticancer agent has not been explored earlier. The structures **D** of target molecules were arrived via **B** by (i) replacing the furan moiety of **A3** by a thiophene ring and (ii) incising the benzene ring fused with the pyranone moiety. The substituents R^1 and R^2 were chosen to introduce diversity into the basic structure of **D**. Our design was essentially based on the fact that the central thienopyranone core of **D** would mimic an isocoumarin[5] moiety that is known to be integral part of several pharmacologically active agents and drugs including a clinical candidate NM-3[6] (**C**, Fig. 1).

While, thiophene moiety is common in many bioactive agents and drugs[7] as a class of compounds thienopyranones however are rather unusual. Only a few number of thieno[2,3-*c*]pyran-4-ones were synthesized and evaluated for their antileishmanial and antifungal activities.[8] In 2006, we reported regioselective synthesis of various mono and disubstituted thieno[2,3-*c*]pyran-7-one, and thieno[3,2-*c*]pyran-4-ones some of which showed anticancer properties in vitro.[9] In further continuation of this work we undertook the synthesis of thieno[3,2-*c*]pyran-4-one based target molecules represented by **D** (or **6**, Scheme 1). To the best of our knowledge, most of these compounds were unknown in

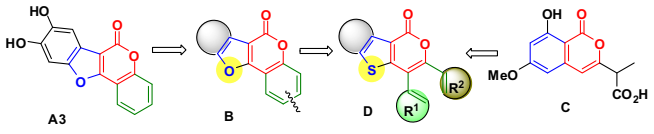

Figure 1. Design of thieno[3,2-*c*] pyran-4-one based novel and potential anticancer agents (**D**) from a known coumestan derivative **A3** and a isocoumarin derivative NM-3 (**C**).

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Appendix

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Conferences/Workshop/Symposiums

1. Poster presentation: Presented part of Ph.D work as a poster with title "A novel non-histone protein substrate for hHDAC8 is Alpha tubulin" at 85th Annual meeting of society of biological chemists (India), held at CFTRI-Mysuru, India on 21-24th Nov, 2016 – **Best Poster Award.**
2. Poster presentation: Bioquest 2016, Oct 20-21, conducted at School of Life sciences, University of Hyderabad, India. – **Best Poster Award.**
3. GIAN (Global Initiative of Academic Networks) -Workshop on Lipid Signalling in Health and Disease in Plant and Animals Dec 05-15 2016.
4. GIAN (Global Initiative of Academic Networks) -Workshop on Immunologicals in Animal and Human Health. July 04-16 2016
5. Poster presentation: Bioquest 2015 held on 23-24th Sep, 2015 at University of Hyderabad.
6. Attended Hands on training and workshop on basic flow cytometry, held during 1st & 2nd Dec, 2014, at University of Hyderabad, India.
7. International Conference on Genome Architecture and Cell Fate Regulation, held from 1st – 4 th December, 2014, at University of Hyderabad, India.
8. 82nd Annual Meeting of the Society of Biological Chemists (India) and International Conference on "Genomes: Mechanism and Function" held from 2nd - 5 th December, 2013 at School of LifeSciences, University of Hyderabad, Hyderabad, India.
9. Poster presentation: UOH-AS joint workshop on Frontiers in Biological Sciences, held on April 8-9 2013, at University of Hyderabad, India.
10. 16th ADNAT convention conference on animal Genetics and Genomics organized by National Institute of Animal Biotechnology, held on 16th-19th December 2012.
11. Indo-German Symposium on System Biology organized during Nov. 27-29 2012 at University of Hyderabad, Hyderabad, India.
12. Poster Presentation- XXXII All India Cell Biology Conference & International Workshop on Cell Cycle Regulation-2009. Title: "Lysine-specific demethylase 1: Recombinant bacterial expression and activity".

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