# IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF NOVEL SERINE 584 PHOSPHORYLATION OF HISTONE DEACETYLASE 4 (HDAC4)

*Thesis Submitted for the degree of*

**DOCTOR OF PHILOSOPHY**

By

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**Enrollment No. 11LAPH05**

# CERTIFICATE

This is to certify that the thesis entitled ***"Identification and Functional Characterization of Novel Serine 584 Phosphorylation of Histone Deacetylase 4 (HDAC4)"*** submitted by **Shanmukha Kumar D** bearing registration number **11LAPH05** in partial fulfilment of the requirements for award of Doctor of philosophy in the **School of Life Sciences** is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

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| 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 Functional Characterization of Novel Serine 584 Phosphorylation of Histone Deacetylase 4 (HDAC4)"** has been carried out by me under the supervision of **Dr. Arunasree MK** and this work has not been submitted for any degree or diploma of any other university earlier.

Dated:

**SHANMUKHA KUMAR D**

(Research Scholar)

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*Shanmukha kumar D*

*Dedicated to*

*my*

*Mother*

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| aa | Amino acids |
| AFU | Arbitrary Fluorescence Units |
| ALU | Arbitrary Luciferase Units |
| APS | Ammonium per sulphate |
| ATP | Adenosine tri-phosphate |
| bp | Base pairs |
| CaMK | Calcium/Calmodulin dependent protein kinase |
| CaMKIV | Calcium/Calmodulin dependent protein kinase IV |
| DMEM | Dulbecco's modified eagle medium |
| EDTA | Ethylene diamine tetra acetic acid |
| EEO | Electro endosmosis |
| FBS | Foetal bovine serum |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GST | Glutathione S-transferase |
| HDAC4 | Histone deacetylase 4 |
| HDACs | Histone deacetylases |
| IPTG | (isopropyl β-D-1-thiogalactopyranoside |
| m/z | Charge to mass ratio |
| MALDI | Matrix-assisted laser desorption/ionization |
| MEF2C | Myocyte Enhancer Factor 2C |
| nm | Nanometer |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate Buffer Saline |

| | |
|---|---|
| PBS | Phosphate Buffer Saline |
| HEK | Human Embryonic Kidney |
| PCR | Polymerase Chain Reaction |
| PKA | Protein Kinase A |
| PMF | Peptide Mass Fingerprint |
| PTM | Post-translational modifications |
| RIPA | Radioimmunoprecipitation assay |
| RT | Room temperature |
| SDS | Sodium dodecyl sulphate |
| TAE | Tris acetate EDTA |
| TEMED | N, N, N, N - tetramethylethylene diamine |
| Tris | Tris-(Hydroxymethyl) aminoethane |
| TSA | Trichostatin A |
| ERK1/2 | Extracellular signal-regulated kinases 1 and 2 |
| GSK3 | Glycogen synthase kinase3 |

# LIST OF FIGURES AND TABLES

## Figures

## Tables

# INTRODUCTION

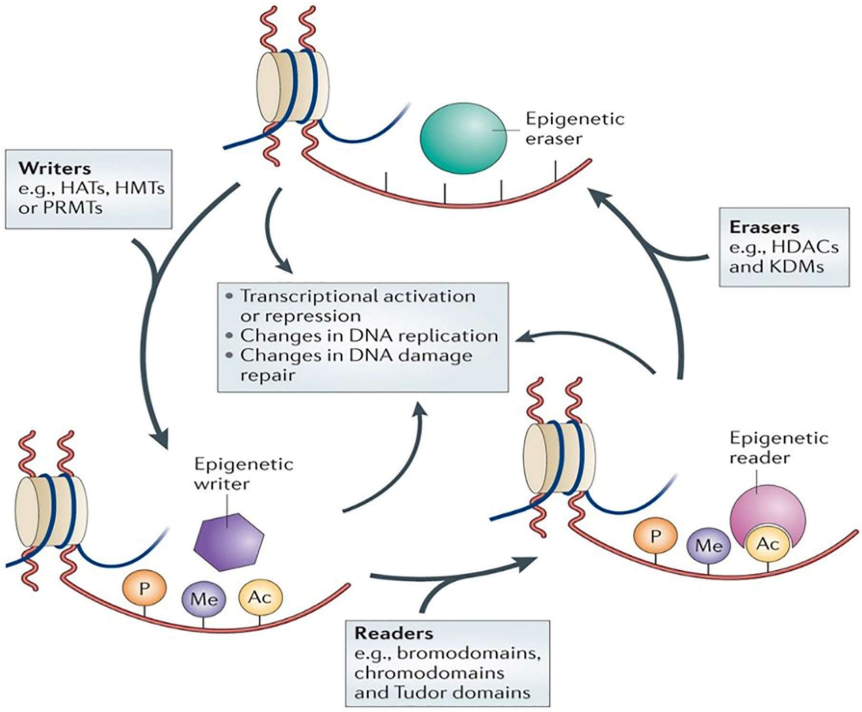
## 1. Introduction:

The term epigenetics was originally coined about a century ago to elucidate the molecular events related to early embryonic growth (Yao, Christian et al. 2016). The term Epigenetics denotes aberrant changes in gene expression pattern that do not arise from DNA sequence alteration (Felsenfeld 2014). Some of the well-known epigenetic mechanisms include DNA methylation, chromatin modifications majorly regulated by miRNAs, long non-coding RNA (lncRNAs) and histone modifications (Yao, Christian et al. 2016). DNA methylations are mediated by set of enzymes known as DNA methyltransferases by transfer of methyl group on 5- position of Cytosine nucleotide present adjacent to Guanine that forms CpG dinucleotide (Lim and Maher 2010). Regions of CpG sites in higher frequency refer to CpG islands. About 70% of gene promoters are known to be associated with CpG islands which includes common housekeeping genes, tissue-specific genes and developmental regulator genes (Zhu, He et al. 2008).

The other type of modifications include histone modifications where histone tails that wrap the DNA are externally modified by the set of enzymes that determine the gene expression pattern. These epigenetic modifications are catalyzed by the set of enzymes which "writes" and "erases" to add or remove the epigenetic marks and "readers" serves as effector molecules. Histone acetyltransferases (HATs) and Histone methyltransferases (HMTs) also known as "writers" add the epigenetic mark to the histone proteins whereas Histone diacetyl transferases (HDACs) form the "erasers" that erase the epigenetic mark. "Readers" form the effector molecules that bind to the epigenetic marks (Falkenberg and Johnstone 2014) (Fig: 1).

Epigenetics in modern terms refers to "outside conventional genetics", implying detailed study of alterations related to gene expression patterns which are stable that arise

during the course of development or cell proliferation (Jaenisch and Bird 2003). Human genome majorly comprises of 20,000-25,000 genes (International Human Genome Sequencing 2004) which encode for approximately 1 million proteins (Jensen 2004).



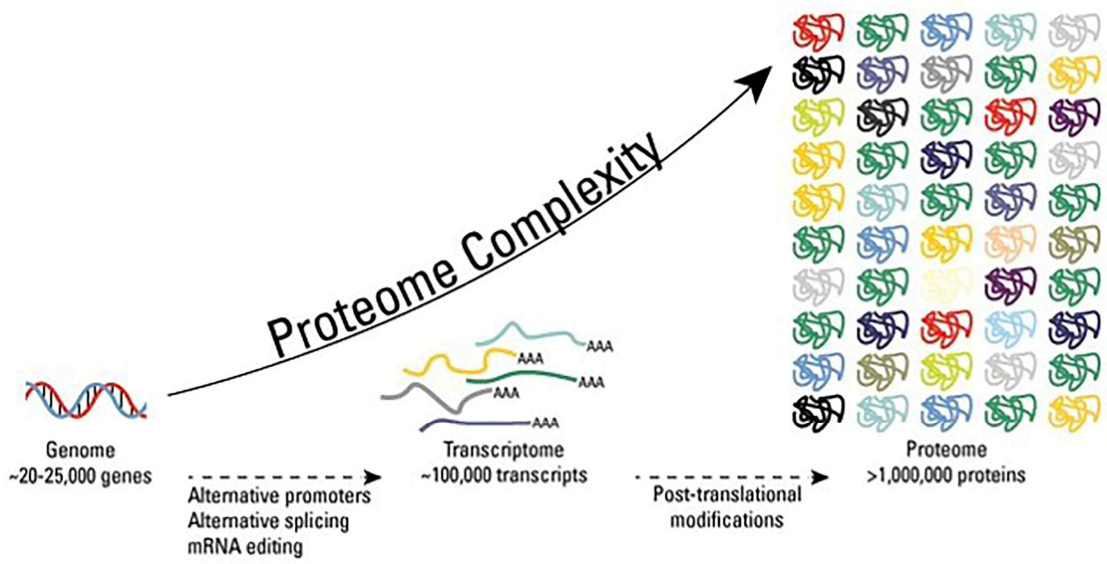

*(Nature Reviews, Drug Discovery, 2014)*

**Figure 1**: *Epigenetic regulation by Writers, Erasers and Readers.*

Transcriptional initiation at the alternative promoter region, differential transcriptional termination, gene recombination, splicing at alternative sites etc lead to the generation of diverse proteins with many functions (Ayoubi and Van De Ven 1996). The first route in the diversification of proteins can be attributed to the "one gene many protein concept" resulting from alternative splicing also known as mRNA splicing (Maniatis and Tasic 2002, Black 2003). Further, the second step forms the post-translational modifications (PTMs), which impart additional complexity to the proteins by modification of the individual amino acids

and their side chain, significantly contributing to the structural and functional diversity of the proteins (Fig: 2).

PTMs contribute majorly to the functional proteomics, the key mediators interacting with the cellular biomolecules such as nucleic acids, proteins, lipids and also cofactors. These PTMs influence the activity of the protein, protein-protein interactions, protein localization, turnover of proteins, signaling cascades, cell division, and cell repair (Karve and Cheema 2011). It is estimated that 5% of the eukaryotic proteins carry out approximately 200 different types of PTMs (Walsh, Garneau-Tsodikova et al. 2005) and these PTMs takes place at the amino acid side chains or at the site of peptide linkages through the enzymatic reaction.




*Source: https://www.thermofisher.com/in/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-post-translational-modification.html*

**Figure 2:** *Proteome complexity. Protein complexity is brought about by the two mechanisms which includes alternative splicing and PTMs, which gives rise to millions of proteins that carry out a wide range of functions in a cell.*

The enzymes which carry out these reactions include various kinases and phosphatases that add or remove a phosphate group respectively, transferases, which transfer functioanl group, ligases that either add or remove a functional group or lipids, sugars,

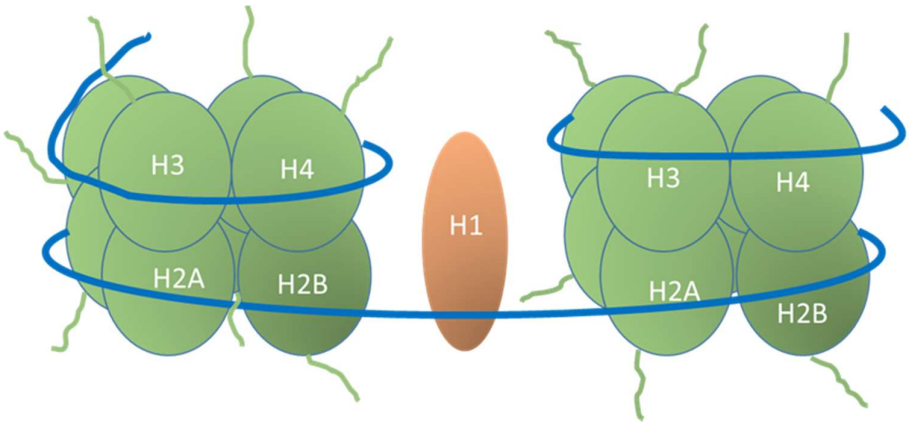
proteins and proteases that cleavage the peptide bonds, remove specific protein sequences or regulatory subunits.

The PTMs occur either at early or later stages of protein life. During the early life, they help in proper folding, stability and in localization to distinct cellular organelles while in later stages i.e., after the translation of the protein, the PTM modified proteins cleave by themselves (autocatalysis) which either may activate or deactivate the function and folding of the protein. Hence the detailed analysis of PTMs is of particular importance as they may help in treating various diseases related to heart, cancer, diabetes, neurological etc. It is always a challenging area to study the post-translationally modified proteins in detail, as they are very difficult to identify and to characterize. Proteomic analysis using Mass spectrometric analysis is the known best means of characterizing the post-translationally modified protein and helps in better understanding the nature of the modified proteins.

Since long time ago, cancer is thought to be the caused by the changes in the aberrant expression pattern of oncogenes and tumor suppressor genes, gene duplications, deletions, translocations and point mutations which lead to uncontrolled growth of cells. It was in the recent times, the link between carcinogenesis with various factors like environmental, dietary factors and disease was identified. This led to a conclusion that indeed epigenetic marks also contribute to a major role in cancer disease (Virani, Colacino et al. 2012). The epigenetic changes majorly observed in mammals are DNA methylations and post-translational modifications of histone tails majorly such as acetylation, deacetylation, phosphorylation, sumoylation, ubiquitination etc. that play a significant role in regulating gene expression (Fischle, Wang et al. 2003, Sengupta and Seto 2004). These epigenetic marks are mostly reversible and hence make an easy choice for the therapy.

## 1.1 Histones:

Histones are the proteins mainly responsible for packaging of DNA to form chromatin. Histones are proteins rich in basic or positively charged amino acids like lysine and arginine and these are highly conserved across species. Nucleosome forms a structural unit of chromatin, which resembles beads present on a string connected by linker DNA (Fig: 3).




*Source: Adapted and modified from https://themedicalbiochemistrypage.org/dna.php*

**Figure 3:** *Structure of nucleosome showing core histone proteins along with DNA.*

A nucleosome typically consists of 145-146 base pairs of DNA wrapped around the histone octamer. Octamer constitutes four different types of histone proteins (H2A, H2B, H3, and H4) arranged as pairs to form tetramer (Ito, Barnes et al. 2000, Strahl and Allis 2000). Another additional protein, H1, forms the linker protein present in between the two successive nucleosomes.

All the histone proteins contain a typical globular C-terminus and an unstructured N-terminus end (Kim, Samaranayake et al. 2009) which carry out a wide variety of functions. C-terminus part of histone is important for histone to DNA and histone-histone interactions whereas the N-terminus part is responsible for DNA-regulatory protein interactions (Marks,

Rifkind et al. 2001) and undergoes a wide variety of post-translational modifications that modify the structure of the chromatin.

Significant growth in the knowledge of acetylation and deacetylation modification of histone tails in cancer has gained importance as these provide additional mechanisms of gene regulation. The presence of acetylated /demethylated lysine at histone tails leads to more relaxed chromatin and thus leads to gene transcription activation, while the deacetylation/methylation of lysine residues is known to be associated with a more condensed chromatin state and transcriptional gene silencing (Johnstone 2002, Iizuka and Smith 2003). Histone acetyltransferases (HATs) add the acetyl group from acetyl-CoA (acetylation) to the histone proteins whereas the Histone deacetyltransferases (HDACs) remove the acetyl group (deacetylation) from histone proteins. (Bernstein, Meissner et al. 2007, Smith and Denu 2009). They act either by alone or in combination which forms the "histone code" majorly recognized by histone and non-histone proteins to form complexes important for gene transcription (Fischle, Wang et al. 2003). HATs and HDACs interact with DNA through multiprotein complexes through corepressors and coactivators rather than binding directly to DNA (Marmorstein 2001, Sengupta and Seto 2004).

## 1.2 Histone Deacetylases (HDACs):

Histone deacetylases (HDACs) are a family of enzymes involved in the removal of the acetyl group from ε- amino position of lysine amino acids (Fig: 4A). Removal of acetyl group enhances the positive interaction of histone proteins with negatively charged DNA backbone thereby contributing to gene repression (Minucci and Pelicci 2006). HDACs deacetylate various histone and non-histone proteins. They co-exist as large complexes interacting with various proteins, thus exerting their functions on gene repression. The PTMs

on HDACs lead to DNA–histone interactions or the binding to proteins, such as transcription factors to chromatin (Buchwald, Kramer et al. 2009, Spange, Wagner et al. 2009).

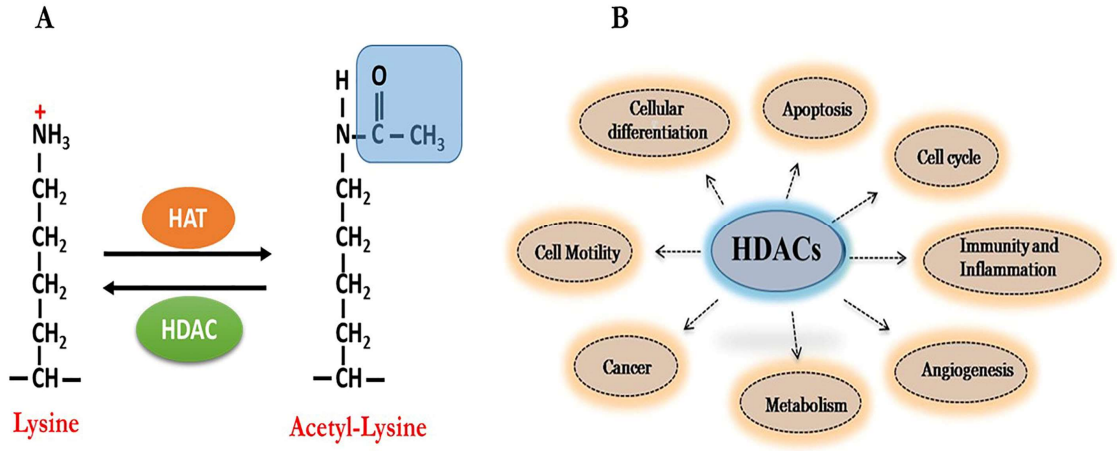



**Figure 4:** Histone deacetylation and its functions. *(A) Acetylation and deacetylation reaction of lysine amino acid catalyzed by HATs and HDACs. (B) Functional roles of HDACs with a depicting physiological and pathological functions.*

Numerous studies have assigned the involvement of HDACs in human cancer showing the altered expression of individual HDACs in tumor samples (Dhordain, Lin et al. 1998, Choi, Kwon et al. 2001, Pandolfi 2001) and the use of specific inhibitors to reduce tumor progression. HDACs affect numerous gene functions related to oncogenesis apart from its normal physiological roles (Fig: 4B). Inhibitors of HDACs are widely applied in cancer therapy.

### 1.3 HDACs classification:

There are 18 mammalian HDACs identified till date and are grouped into 4 different classes based on their respective homology to yeast deacetylases (de Ruijter, van Gennip et al. 2003, Blander and Guarente 2004, Gregoretti, Lee et al. 2004) as seen in figure 5. They are Class I, II, III, and IV. Class I, II and IV require zinc as a cofactor. Class I HDACs

include HDAC 1, 2, 3 and 8 which closely resemble yeast (*Saccharomyces cerevisiae*) transcriptional regulator RPD3. Class II HDACs includes HDAC 4, 5, 6, 7, 9, 10 and share a similarity in domains to HDA1, another deacetylase found in yeast (Bjerling, Silverstein et al. 2002). Based on the domain organization and homology, Class II can be further subdivided into Class IIa (HDAC 4, 5, 7 and 9), which contains highly conserved C-terminal deacetylase catalytic domain homologous to yeast HDA1, but do not share N-terminal domain similarity to other classes of HDACs.

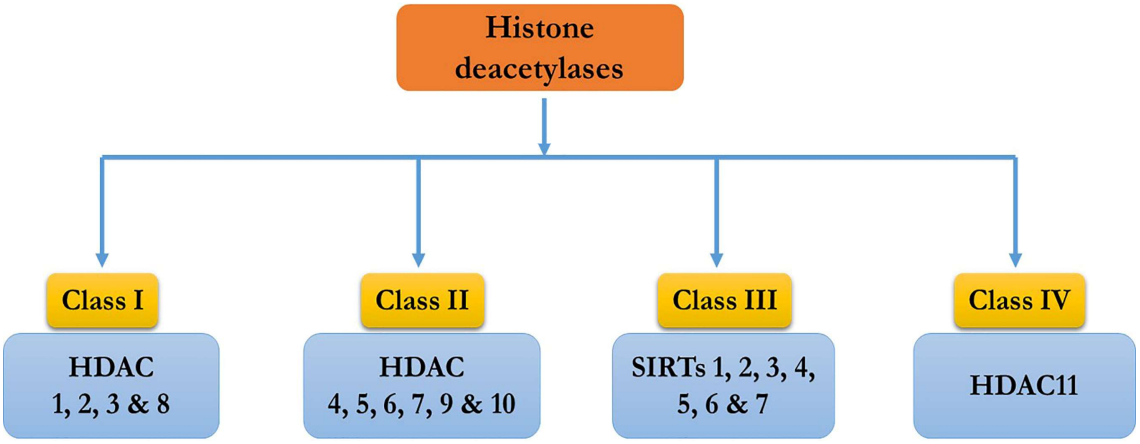



**Figure 5**: *Classification of classical HDAC family and its sub groups.*

Class IIb (HDAC 6 and 8) is characterized by the presence of two catalytic deacetylase domains (Verdin, Dequiedt et al. 2003). HDAC11 is placed under class IV as it has low similarity with class I and II (Gao, Cueto et al. 2002). Class III HDACs require $NAD^+$ as a cofactor and share common sequence homology with a Sir2 protein of yeast (Blander and Guarente 2004). These are also called Sirtuins (SIRTs) starting from SIRTs 1-7 in number.

Localization of HDACs differs from one tissue to other. Their expression can be ubiquitous or in some cases can be restricted to tissues.

### 1.3.1 Class-I HDACs:

Class I HDACs, ubiquitously expressed, play a diverse role of functions in cell differentiation, controlling cell cycle regulation and in the tissue development. The targets of these HDACs include both histone and non-histone proteins. All the class I HDACs lack the DNA-binding motif and bind with the other proteins to get recruited to the chromatin targets. All the known class I HDACs, except HDAC8, are found in subunits in multi-complex proteins and interact with various transcription factors.

Mammalian HDAC1 and HDAC2 are known to be originated from a common ancestor via gene duplication (Gregoretti, Lee et al. 2004) and share 87% sequence identity with mice counterparts. Their C-terminal ends consist of tandem casein kinase-2 (CK2) phosphorylation sites (Pflum, Tong et al. 2001). HDAC3 possess homologous catalytic domain similar to that of HDAC1 and 2, but it consists of only one CK2 phosphorylation site (Zhang, Ozawa et al. 2005). HDAC8 consists of conserved motif for protein kinase A phosphorylation (Gregoretti, Lee et al. 2004, Vannini, Volpari et al. 2004). Out of all known HDACs, HDAC8 is the only HDAC for which crystal structure is available (Vannini, Volpari et al. 2004). HDAC1 and HDAC2 share a close homology and are a part of the repressive complex such as sin3, NuRD, CoREST and PRC2 complex. HDAC3 is known to be associated with N-CoR-SMRT complex (Yang and Seto 2003).

Class I HDACs (HDAC1, 2, 3, and 8) are mainly localized to nucleus and their overexpression is associated with tumor formation in prostate, colon, gastric and breast cancer (de Ruijter, van Gennip et al. 2003).

### 1.3.2 Class II HDACs:

Class IIa HDACs, HDAC4, 5 and 9 expressions were seen more in skeletal muscle, heart, and brain where the biological activities of these proteins might be partially redundant.

(Fischle, Emiliani et al. 1999, Grozinger, Hassig et al. 1999, Verdel and Khochbin 1999, Wang, Bertos et al. 1999, Zhou, Richon et al. 2000, Zhou, Marks et al. 2001). Class IIa HDACs do not bind to the DNA directly rather they interact with the sequence-specific transcription factors and get recruited to the specific promoters (Backs, Song et al. 2006, Zhang, Kohlhaas et al. 2007, Backs, Worst et al. 2011). One of the well-known transcription factors is an MEF2 family transcription factor. Some of the other known transcription factors include CtBP (E1A C-terminal binding protein), 14-3-3 proteins, CaM (calmodulin), heterochromatin protein HP1α and SUMO proteins.

All of the class IIa HDACs are known to shuttle between nucleus and cytoplasm (Miska, Karlsson et al. 1999, Grozinger and Schreiber 2000, McKinsey, Zhang et al. 2000, Wang, Kruhlak et al. 2000, Dressel, Bailey et al. 2001, Kao, Verdel et al. 2001, Miska, Langley et al. 2001, Zhao, Ito et al. 2001). 14-3-3 proteins that are highly conserved acidic proteins bind to class IIa HDACs and export them out of nucleus. Phosphorylation at three serine residues (S246, S467, and S632) at the N-terminal region of HDACs creates binding sites for HDACs and hence are exported out of the nucleus into the cytoplasm (Wang, Kruhlak et al. 2000).

Aberrant expression of Class IIa HDACs has been known to result in Colon cancer, acute myelogenous leukemia (AML), astrocytoma and medullobalstoma. In case of class IIb HDACs have been associated with AML, breast and hepatocellular carcinoma (Mottamal, Zheng et al. 2015) (Ellis and Pili 2010).

### 1.3.3 Class III HDACs:

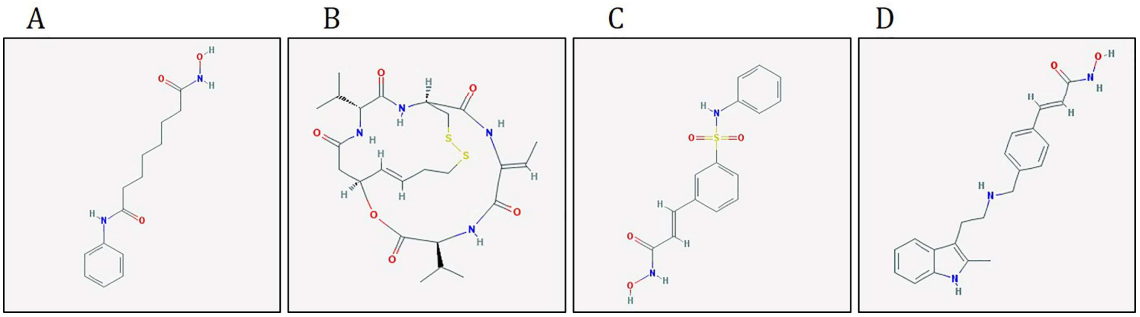
Class III HDACs, also known as Sirtuins (SIRT), are structurally different from class I and class II HDACs. These are homologous to yeast silent information regulator (Sir2)

(Blander and Guarente 2004, Trapp and Jung 2006). These are NAD+ dependent and the activity is regulated by the intracellular concentrations of $NAD^+$/NADH ratio. SIRT1, -2, -6 and -7 expression is seen in the nucleus. Both SIRT1 and -2 are also found in the cytoplasm. SIRT3, -4 and -5 expressions is seen in mitochondria (Michishita, Park et al. 2005, Tennen, Berber et al. 2010). SIRT3 and -6 possess both deacetylase activity and ADP-ribosyltransferase activity. SIRT1, -2, -5 and -7 contain only deacetylase activity. SIRT4 has only ADP-ribosyltransferase activity (Kelly 2010).

### 1.4 HDACs in cancer:

Altered expression of HATs and HDACs have been observed in many of the human cancers (Cress and Seto 2000, Sugita, Taki et al. 2000, Marks, Rifkind et al. 2001, Panagopoulos, Fioretos et al. 2001, Murati, Adelaide et al. 2004, Choi, Elagib et al. 2005, Crowley, Wang et al. 2005, Dokmanovic and Marks 2005, Linggi, Brandt et al. 2005, Bolden, Peart et al. 2006, Ropero, Fraga et al. 2006, Wilson, Byun et al. 2006). Numerous studies signify aberrant expression of HDACs in human cancers like prostate (Panagopoulos, Fioretos et al. 2001), lung (Osada, Tatematsu et al. 2004, Minamiya, Ono et al. 2011), breast (Krusche, Wulfing et al. 2005), liver (Rikimaru, Taketomi et al. 2007), colorectal (Weichert, Roske et al. 2008) and gastric cancer (Weichert, Roske et al. 2008). Classical HDACs are overexpressed in pathological conditions. In the case of the neoplastic state, the tumor suppressor genes such as p53, p21, and gelsolin are repressed (Hoshikawa, Kwon et al. 1994, Van Lint, Emiliani et al. 1996, Saito, Yamashita et al. 1999), whereas tumor activators like HIF-1 (hypoxia inducible factor-1) and VEGF (vascular endothelial growth factor) are up-regulated (Kim, Kwon et al. 2001). One of the main reasons for this abnormal expression of genes can be attributed to the abnormal recruitment of HDACs to the promoters of the target genes. Hence the disruption of HDACs and its complex has evolved as the potent strategy for

the treatment for cancer therapy. This has led to the use or emergence of histone deacetylase inhibitors (HDIs) as promising cancer therapeutic agents. The Food and Drug Administration (FDA) has approved four HDIs for cancer therapy. Vorinostat (suberoylanilide hydroxamic acid, Zolinza) was the first HDI, developed by Merck & Co. Inc., approved for the treatment of cutaneous T-cell lymphoma (CTCL), a rare type of non-Hodgkin's lymphoma of the skin. Romidepsin (FK 228, FR 901228, Istodax, desipeptide), developed by Gloucester Pharmaceuticals, was approved by the FDA for CTCL. Romidepsin has also been approved for peripheral T-cell lymphoma (PTCL) patients who had received at least one prior therapy. The other drug is Belinostat (PXD-101, Beleodaq) developed by Spectrum pharmaceuticals and approved by FDA for patients with refractory peripheral T-cell lymphoma (PTCL). Panobinostat (Farydak), from Novartis, was licensed by FDA for multiple myeloma treatments.




*Source: National Center for Biotechnology Information. PubChem Compound Database.*

**Figure 6:** *HDAC inhibitors. (A) Vorinostat (SAHA) (B) Romidepsin (C) Belinostat (PXD-101) (D) Panobinostat.*

Apart from these, several inhibitors for HDACs are in different stages of clinical trials. Hence research based on the study of HDACs for targeted therapy is widely in use. Furthermore, HDACs are majorly regulated by PTMs and hence targeting the HDACs with respect to PTMs could be the best option for therapy.

### 1.5 Histone deacetylase 4 (HDAC4):

Among the Class II HDACs, Histone deacetylase4 (HDAC4) is a class IIa HDAC which may be described as the potent transcriptional repressor by its ability to interact with different co-repressor complexes with its N-terminal domain especially in the brain (Mielcarek, Zielonka et al. 2015). HDAC4 shares 60-70% sequence identity with HDAC5 and HDAC7 (de Ruijter, van Gennip et al. 2003). The hdac4 expression is ubiquitous in nature with enrichment in the brain, heart, and skeletal muscle (Verdin, Dequiedt et al. 2003, Broide, Redwine et al. 2007).

Human HDAC4 is localized on chromosome 2q37.3 (Wang, Bertos et al. 1999) and produces a transcript of 8980 bp (NM_006037.3). The coding sequence includes 3250 bp and codes for 120 kDa protein. Transcription of the HDAC4 is brought about by direct binding of TFs (transcription factors) such Sp1 and Sp3 to the GC rich consensus sequence. (Liu, Pore et al. 2006). Expression of HDAC4 is also regulated by miRNA, which includes miR-1 (Chen, Mandel et al. 2006), miR-29, miR-206 (Winbanks, Wang et al. 2011), miR-140 (Tuddenham, Wheeler et al. 2006, Miyaki, Sato et al. 2010), miR-155 (Sandhu, Volinia et al. 2012), miR-200a (Yuan, Yang et al. 2011), miR-365 (Guan, Yang et al. 2011) in different cell types.

### 1.6 HDAC4 protein domain architecture:

Human HDAC4 gene codes 1084 amino acids and shares 94% identity with mouse HDAC4 protein. It contains unique binding sites at the N-terminus region for binding with several transcription factors and the zinc catalytic domain at the C-terminus end. The crystal structure of the N-terminus HDAC4 (62-153 amino acids) reveals the protein with glutamine-rich domains containing straight alpha helix that folds as a tetramer (Guo, Han et al. 2007) (Fig: 7). Further, HDAC4 containing PEST1, PEST2, catalytic domain were summarized in figure 8.

The nuclear localization domain responsible for the entry of the protein inside the nucleus from cytoplasm is from 244-279 aa and contain three clusters of arginine-lysine residues in which all the three clusters are required for the nuclear localization of HDAC4 (Wang and Yang 2001).

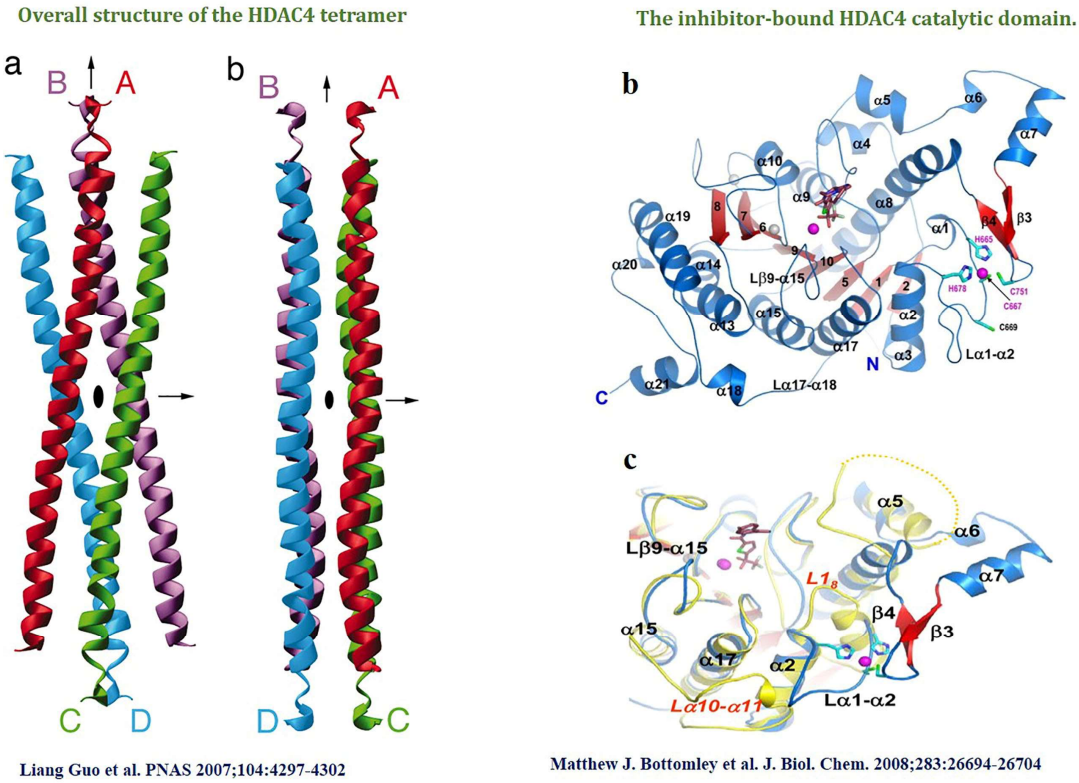



**Figure 7:** *Crystal structure of HDAC4 protein revealing N-terminal rich Glutamine domain and Crystal structure of Catalytic domain bound with trifluoromethylketone (TFMK).(a)N-terminal Glutamine rich HDAC4 folded as tetramer. (b) Crystal structure of HDAC4 catalytic domain bound to inhibitor trifluoromethylketone (TFMK). Potassium and Zinc ions were represented in grey and magenta spheres. (c) Superposition of TFMK bound HDAC4 catalytic domain (blue/red) with HDAC8 (yellow).*

MEF2 transcription factors are well known to bind to HDAC4 protein at the N-terminal end. The mef2-binding domain is present from 118-313 aa and MEF2 binding to

HDAC4 is able to drive HDAC4 into the nucleus in an MEF2-dependent manner rather than nuclear localization domain of HDAC4 (Wang and Yang 2001).

HDAC4 protein undergoes caspase-2 and -3 dependent proteolytic cleavage at the N-terminal region called PEST (proline, glutamate, serine, and threonine rich) domain that helps the protein to regulate the gene expression. PEST1 domain contains (225 to 240 aa) potential cleavage site at aspartate 237 aa and PEST2 domain (275 to 295 aa) contains potential cleavage site at aspartate 289.

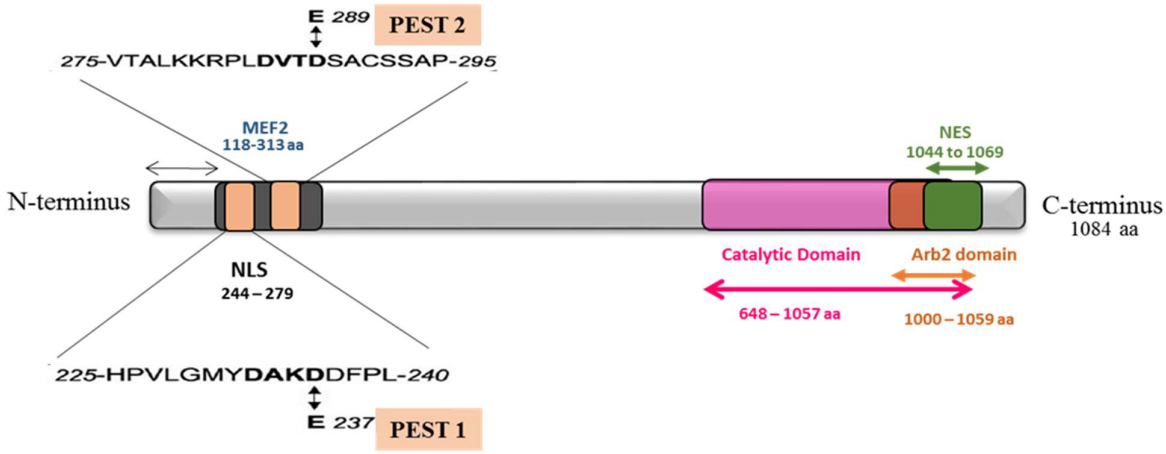



**Figure 8:** *HDAC4 protein domain architecture depicting nuclear localization signal (NLS), MEF2 binding domain, PEST domains (PEST 1 and PEST2), catalytic domain, and nuclear export signal (NES).*

The caspase 2-generated N-terminal HDAC4 triggers release of cytochrome *c* from mitochondria, which in turn induces cell death or apoptosis in a caspase 9-dependent manner and is also a strong repressor of MEF2 transcription factor (Paroni, Mizzau et al. 2004). The catalytic domain responsible for the activity of the protein is from 648-1057 aa (Bottomley, Lo Surdo et al. 2008). The nuclear export signal is present from 1044-1069 aa (Wang and Yang 2001).

## 1.7 Physiological functions of HDAC4 protein:

HDAC4 role is diverse role in regulation of gene expression, cell survival, cell growth and in proliferation. It is also known for the aberrant expression in cancer condition which will be discussed later.

### 1.7.1 HDAC4 in chondrogenesis, Osteoblast differentiation, and chondrocyte hypertrophy:

The role of HDAC4 in bone formation and in chondrocyte hypertrophy came from the knockout studies where HDAC4$^{-/-}$ null mice displayed symptoms of premature developing bone ossification. The reason being for this is the ectopic and former onset of chondrocyte hypertrophy that results from the constitutive expression of Runx2 gene in chondrocyte hypertrophy (Vega, Matsuda et al. 2004). Runx2 is acetylated by p300 acetyl transferase and this acetylated form of Runx2 protects proteins from smurf-intermediated degradation. HDAC4 and 5 deacetylate Runx2 and thus target Runx2 to undergo smurf-mediated degradation (Jeon, Lee et al. 2006). TGF-β is known to repress osteoblast differentiation through HDAC4 and 5, which further interact with Smad3 to form a Smad3/Runx2 complex bind to the Runx2 binding DNA sequence (Kang, Alliston et al. 2005). HDAC4 overexpression is known to promote TGF-β1-mediated synovium-derived stem cell chondrogenesis that prevents stem cell hypertrophy (Pei, Chen et al. 2009).

### 1.7.2 HDAC4 role in muscle development and in cardiovascular diseases:

Myoblasts are formed during myogenesis, which expresses specific transcription factors called MEF2C. Mice lacking MEF2C showed a defect in heart morphogenesis arresting at heart looping stage (Lin, Schwarz et al. 1997). HDAC4 binds to and represses MEF2 function regulating the mesoderm cell specification to cardiomyocytes through inhibition of Nkx2-5 and GATA4 expression. Overexpression of HDAC4 inhibits

cardiomyogenesis, which is shown by the downregulation of cardiac specific muscle gene expression (Karamboulas, Swedani et al. 2006).

During muscle differentiation, HDAC4 forms a complex with MEF2 and binds to the sites of MEF2 promoters and mediates gene repression. This gene repression is relieved by the action of kinase, CaMK, whcih phosphorylates HDAC4 leading to the translocation of HDAC4 into the cytoplasm (McKinsey, Zhang et al. 2000, Zhang, Kohlhaas et al. 2007). Transgenic mouse heart with overexpression of an activated CaMK ie., CaMKIV shows hypertrophy with up-regulation of the embryonal transcripts like atrial natriuretic factor including increased MEF2 activity (Passier, Zeng et al. 2000).

In cardiomyocytes, HDAC4 phosphorylation by CaMKIV results in hypertrophic growth and is blocked by HDAC4 mutant, which is unresponsive to external signals (Backs, Song et al. 2006). In miR-22 null mice, in response to stress created by hypertrophic cardiac growth, miR-22 was found to be essential by directly targeting SIRT1 and HDAC4 (Huang, Chen et al. 2013).

HDAC4 regulates myofilament contraction by upregulating muscle specific LIM protein (MLP) deacetylation. Both HDAC4 and p300/CBP-associated factor (PCAF) associate with the cardiac myofilaments, Z-disc, A-, I- bands of the cardiac sarcomeres. MLP, works as a sensor for the mechanical stretch of the cardiac muscle and is a target for HDAC4 and PCAF. (Gupta, Samant et al. 2008). All the contractions in muscle cells are regulated by the nervous system. The HDAC4 protein is more centralized at the neuromuscular junction (Cohen, Barrientos et al. 2009). Here, neural input signal loss contributes to simultaneous nuclear accumulation of HDAC4 and thus reduced MEF2 transcriptional regulated genes. In case of surgical denervation and in ALS model (Amyotrophic lateral sclerosis), HDAC4 is required for MEF2 dependent repression. Increased HDAC4 expression mimics denervation,

which further activates transcription of acetylcholine (nAChR) receptor across the myofibrils. Thus HDAC4 inactivation inhibits nAChR and MUSK (muscle-specific receptor tyrosine kinase) transcription (Cohen, Waddell et al. 2007).

### 1.7.3 HDAC4 in neuronal maintenance and in mental disease:

The HDAC4 expression in perikaryal cytoplasm of the neurons and localization in the nucleus usually varies (Darcy, Calvin et al. 2010). HDAC4 nuclear translocation leads to stimulation of neuronal cell death observed during low levels of potassium or excitotoxic glutamate conditions. BDNF (brain-derived neurotrophic factor), a neuronal survival factor, treatment prevents nuclear HDAC4 translocation whereas proapoptotic CaMK inhibitor induces accumulation of nuclear HDAC4. Ectopic expression of nuclear-localized HDAC4 contributes to neuronal apoptosis and repress CREB (cAMP response element-binding protein), MEF2 transcriptional abilities (Bolger and Yao 2005).

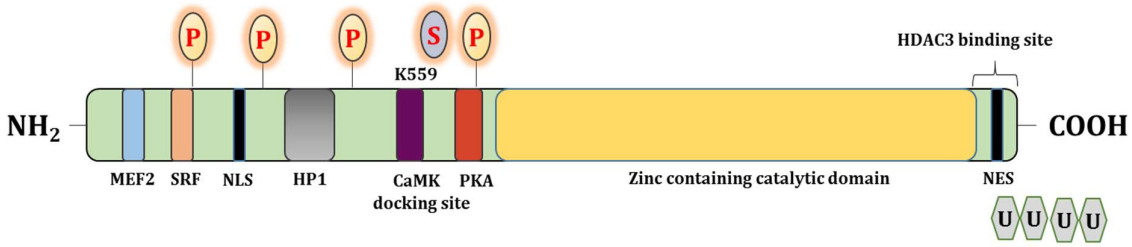
HDAC4 plays a crucial role in retinal development. Downregulation of HDAC4 expression in retina development leads to apoptosis of bipolar interneurons and rod photoreceptors. Overexpression of HDAC4 in these cells leads to a reduction in naturally occurring cell death. In mouse model, overexpression of cytosolic HDAC4 during retinal development elongated survival capacity of photoreceptors (Chen and Cepko 2009).

Ataxia-telangiectasia (neurodegenerative), is caused due to the mutation of Atm gene is well characterized. HDAC4 nuclear accumulation in neurons of Atm deficiency mice leads to neurodegeneration (Li, Chen et al. 2012).

## 1.8 Post-translational modifications:

HDAC4 function, activity, and stability are controlled mainly by post-translational modifications (PTMs). It is also known that PTMs of HDAC4 help to interact with other

proteins, thereby regulating the gene expression. PTMs of HDAC4 influence its subcellular localization and interaction partners, which mediate the transcriptional regulation through repression via chromatin condensation. Understanding the regulatory networks of HDAC4 helps us to unravel the importance of these proteins in various cellular, physiological, development and pathological processes.




*(Adapted and modified from Wang et al. 2014)*

Figure 9: *Some of the PTMs of HDAC4 protein and its domains. Phosphorylated (P), Sumoylated residues (S) residues are marked with a circle. Polyubiquitylation (U) is depicted by multiple hexagons.*

HDAC4 is the protein that undergoes extensive PTMs such as phosphorylation, sumoylation, carbonylation, ubiquitinylation and proteolytic cleavage. Some of the known PTMs of HDAC4 are summarized below:

### 1.8.1 Phosphorylation:

HDAC4 undergoes reversible phosphorylation/dephosphorylation regulatory mechanism for its function. Acidic proteins such as 14-3-3 family are good binding partners of HDAC4 that specifically bind to the phosphoserine conserved motifs. HDAC4 Phosphorylation at Serine 246, 467 and 632 leads to creation of binding sites for the 14-3-3 proteins to bind HDAC4 that escorts the phospho-HDAC4 into the cytoplasm with the simultaneous activation of HDAC4 target genes (Grozinger and Schreiber 2000, McKinsey,

Zhang et al. 2000, Wang, Kruhlak et al. 2000). Phosphorylation of HDAC4 is carried out by particular kinases present inside the nucleus such as CaMK, ERK1/2, PKA, and GSK3.

CaMKII causes phosphorylation of S246, S467, and S632 by binding to HDAC4 through unique docking site and drives the nuclear export of HDAC4 affecting the HDAC4 target genes (Nakagawa, Kuwahara et al. 2006). Myogenesis is promoted by CaMK by disrupting MEF2-HDAC4 complex thus leading to nuclear export (Backs, Song et al. 2006). Protein kinase A specifically phosphorylates at tyrosine 207 resulting in proteolytic cleavage, thus antagonizing CaMKII-mediated activation of MEF2 (Backs, Worst et al. 2011). Further, the proteolytic cleaved product of N-terminus HDAC4 specifically inhibits MEF2 activity, and not SRF (Serum response factor) thus counteracting CaMKII prohypertrophic actions without affecting cardiomyocytes survival. GSK3 phosphorylates HDAC4 on 298 and 302 and thus leading to proteasome-mediated degradation of HDAC4 and regulating its stability (Cernotta, Clocchiatti et al. 2011).

### 1.8.2 Carbonylation:

Carbonylation, also known as Alkylation, is a post-translational modification that takes place inside the cell majorly after the oxidative stress. Carbonylation may be defined as the covalent addition of reactive carbonyl group on cysteinyl thiol on the proteins. Cysteine amino acid at 667 and 669 in HDAC4 protein and cysteine amino acid at 274 and 276 in DNAJb5 undergo oxidation thus forming intramolecular disulfide bonds in response to the ROS (reactive oxygen species), which simultaneously generates the cardiac hypertrophic stimuli. These stimuli are further reduced by thioredoxin -1. DNAJb5 and HDAC4 interaction leads to nuclear export inhibition and hence HDAC4 is retained inside the nucleus (Ago, Liu et al. 2008). Cardiac-specific Nox4 knockout mice show the attenuation of both HDAC4 oxidation and cardiac hypertrophy (Matsushima, Kuroda et al. 2013).

### 1.8.3 Sumoylation:

Sumoylation may be defined as the covalent addition of SUMO proteins to the lysine residues that targets the proteins to degradation. HDAC4 is sumoylated at lysine 559 by the addition of SUMO-1 protein, which is further modified by SUMO-2 *in vivo* (Tatham, Jaffray et al. 2001). Lysine 559 sumoylation of HDAC4 performed by E3 SUMO-protein, a ligase also called as RANBP2, has not affected subcellular distribution of HDAC4 and its binding partners interaction (Kirsh, Seeler et al. 2002) but however, the mutant form displayed significant transcriptional repression and also decreased enzymatic activities when compared with the wild-type protein. It is also well established that this HDAC4 sumoylation was prevented by the CaMK4 phosphorylation.

### 1.8.4 Ubiquitination:

Ubiquitination may be defined as the conjugation of small molecules called ubiquitins to the lysine amino acids of the target proteins by the formation of isopeptide bond. The ubiquitin molecules may be added to the target proteins in the form of a single monomer or in the form of a polyubiquitin chain. Monoubiquitination of proteins acts as the signal for the signaling pathway leading to different biological processes, whereas polyubiquitination targets the proteins for degradation *via* proteasomal pathway. Ubiquitination is mediated majorly by E1 activating enzyme, E2 conjugating enzyme and E3 ubiquitin ligases.

HDAC4 is said to undergo ubiquitination *via* proteasomal pathway majorly regulated by GSK3β. The mechanism of action and the biological significance of the ubiquitination mechanism on HDAC4 needs to be understood clearly (Cernotta, Clocchiatti et al. 2011).

### 1.8.5 Proteolytic cleavage:

HDAC4 undergoes dynamic changes with the proteolytic processing of caspases, which takes place during apoptosis in a cell. Caspase-2 and Caspase-3 particularly cleave HDAC4 protein on aspartate 289 into two fragments of C-terminal end 97 kDa and N-terminal end 34 kDa. Caspase -3 further processes 34 kDa protein to yield smaller fragments. Caspase -2 generated N-terminal 34 kDa fragment moves into the nucleus to induce cell death and repressess MEF2 transcription factor. Both, caspase 2 and 3, cleave HDAC4 protein *in vitro,* but caspase-3 specifically cleaves HDAC4 *in vivo* upon UV-induced apoptosis (Paroni, Mizzau et al. 2004).

## 1.9 Subcellular distribution of HDAC4:

As discussed earlier the subcellular distribution of HDAC4 is majorly regulated by phosphorylation and dephosphorylation. Phosphorylation of S246, S467 and S632 leads to creation of binding sites on 14-3-3 proteins that export HDAC4 outside of the nucleus into the cytoplasm. HDAC4 protein translocation is also mediated by interaction through CRM1 (Importin 1), thus leading to nuclear export of proteins containing leucine-rich nuclear export signals like HDAC4. Nucleoporin 155 (Nup 155) a nuclear pore complex (NPC) component mediates nuclear-cytoplasmic HDAC4 protein transport (Kehat, Accornero et al. 2011). Since HDAC4 cannot bind directly to DNA, these enzymes are recruited to their specific promoters with the help of sequence-specific DNA binding proteins. Inside the nucleus, HDAC4 functions in gene expression regulation through transcription factors, repressor proteins etc., in different tissues.

## 2.0 Regulation of non-histone protein deacetylation by HDAC4:

HDAC4 is known to deacetylate non-histone proteins apart from histone proteins, thereby regulating the gene function. Some of the known non-histone partners of HDAC4 are Runx2 and p53 HIF-1α, MLP, MEKK2 and STAT1, DNAJb8.

HDAC4 deacetylates lysine amino acids near the C-terminal end of p53 protein, thereby decreasing transcriptional activity during DNA damage (Basile, Mantovani et al. 2006). HDAC4 also deacetylates Runx2 protein, which is a major target of the BMP (Bone morphogenic protein) pathway, which causes osteoblast differentiation and increased bone formation. Signaling pathway of BMP-2 stimulates p300-mediated Runx2 acetylation leading to increased transactivation and thus inhibiting the Smurf1-mediated degradation. Deacetylation of Runx2 by HDAC4 targets the protein for Smurf-mediated degradation thereby inhibiting osteoblast differentiation (Jeon, Lee et al. 2006). Muscle LIM protein (MLP) is a Z-disc associated protein. Acetylation of MLP is mediated by PCAF (p300/CBP-associated factor). HDAC4 deacetylates MLP thus contributing a major role in the regulation of muscle contraction.

HIF-1α protein is mainly involved in angiogenesis, metabolism and in cancer development. It is the major component of the HIF-1 transcriptional complex. Using HDAC4 shRNA it was demonstrated that increased HIF-1α acetylation but not with the HDAC1 and HDAC3 shRNA. Also, there was decreased HIF-1 transcriptional activity and hypoxia-induced target gene expression with simultaneous decrease in the resistance to docetaxel chemotherapy (Qian, Kachhap et al. 2006, Seo, Kim et al. 2009, Geng, Harvey et al. 2011).

HDAC4 is known to interact with MEKK2, a MAP3 kinase thus leading to the activation of muscle atrophy program. Also, HDAC4 stimulates AP1 activity leading to the MAP kinase cascade by interacting with a MAP3 kinase, MEKK2. Deacetylation of MEKK2

by HDAC4 leads to activation of the MEKK2 and thus promotes neurogenic muscle atrophy (Choi, Cohen et al. 2012).

HDAC4 also interacts with STAT1 (Signal transducer and activator of transcription 1). Upon exposure to chemotherapy HDAC4 overexpressing tumor cells causes deacetylation of STAT1 thus promoting ovarian cancer cell survival (Stronach, Alfraidi et al. 2011).

## 2.1 HDAC4 role in cancer:

Importance of HDACs role in cancer is well known by the fact that inhibitors of these HDACs are used for treating cancers. Most of the HDAC inhibitors available are pan-inhibitors leading to several undesired side effects due to over-lapping functions of HDACs. However, development of class-specific and isoform-specific HDAC inhibitors has become difficult in absence of crystal structures of full-length HDACs.

BCL6 belongs to POZ/zinc finger family, involved in differentiation and/or survival of B-cell lymphoma during chromosomal alteration. microRNAs are the important regulators of initiation and progression of cancers. One such is miR-150 which is highly expressed in most of the solid tumors and in hematological malignancies. microRNA miR-150 specifically binds to 3’ UTR region of HDAC4 and inhibits its translocation. HDAC4 ectopic expression in DLBCL (human-activated B-cell type diffuse large B-cell lymphoma) leads to reduced miR-150 induced proliferation, increased apoptosis and clonogenic potential (Sandhu, Volinia et al. 2012).

Expression of HDAC4 is observed maximally in the proliferative compartment of normal colon and also in the small intestinal epithelium. Its downregulation is observed during differentiation process. HDAC4 along with Sp1 decreases H3 acetylation at the

Sp1/Sp3 binding site-rich p21 (WAF1/Cip1) proximal promoter. In human glioblastoma model, p21 (WAF1/Cip1) induction mediated through HDAC4 silencing leads to growth arrest in cancer cells and inhibition of the tumor growth (Wilson, Byun et al. 2008, Mottet, Pirotte et al. 2009). In breast cancer samples, FOXP3 (forkhead box P3) protein (X-linked tumor suppressor) is essential for p21 downregulation. FOXP3 inhibits binding of HDAC4 which leads to histone H3 acetylation (Liu, Wang et al. 2009). HDAC4 is known to play role in hepatocellular carcinoma (HCC). Upregulation of HDAC4 was seen in miR-22 downregulated HCC tissues leading to HCC cell proliferation and increased tumorigenicity (Zhang, Yang et al. 2010). In HCC cells, miR-200a is known to target HDAC4. In turn, HDAC4 also inhibits miR-200a expression *via* histone H3 deacetylation at the miR-200a promoter region mediated through the Sp1-dependent pathway (Zhang, Yang et al. 2010, Yuan, Yang et al. 2011).

Resistance to platinum chemotherapy is commonly observed in ovarian cancer patients making it difficult to increase patient survival. Biopsy samples of ovarian cancer patients before and after resistance to platinum chemotherapy displayed increased HDAC4 expression (Stronach, Alfraidi et al. 2011).

Tasquinimod (antiangiogenic drug) is an oral drug, which is employed for treatment of the castration-resistant prostate cancer is currently in the phase III clinical trials. This drug is known to bind directly to HDAC4 protein thereby leading to inhibition of deacetylation of histone proteins and also other HDAC4 related transcription factors such as HIF-1α (Isaacs, Antony et al. 2013).

## 2.2 Rationale of the Study:

Our major area of interest has been to understand the diverse regulation of the Histone Deacetyalse (HDACs) enzymes. These proteins are widely deregulated in different cancer conditions and also some of these show cell type specific expression.

Of these HDACs, class IIb forms a majorly important group of enzymes as these proteins have a unique regulation system i.e. they shuttle between nucleus and cytoplasm for additional regulatory functions. In particular, we have focused on HDAC4, a class IIb enzyme, which is known to play diverse functions required for both physiological as well as pathological processes.

Other important fact for selecting HDAC4 includes regulation by post-translational modifications (PTMs). HDAC4 PTMs help to interact with other proteins and also known to undergo proteolytic cleavage or ubiquitination required for cellular signaling. PTMs also help to shuttle between nucleus and cytoplasm. They also show restricted expression and differ in different cell types as evident in the earlier studies to function in cell differentiation and development. Hence a detailed understanding of the PTMs of HDAC4 will help to uncover the functional regulatory aspects of the protein.

With these observations, we aimed to unravel PTMs of the HDAC4 protein, in particular, we have focused on the phosphorylation that dynamically regulates HDAC4 protein.

## 2.3 Objectives:

### Objective 1

Identification of novel HDAC4 PTMs.

a. *In silico* prediction of probable PTMs of HDAC4.

b. Identification of PTMs of HDAC4 by MALDI-TOF/TOF analysis.

### Objective 2

Functional characterization of the identified PTM of HDAC4.

# MATERIAL AND METHODS

## 2.4 Materials and Methods

### 2.4.1 Materials

HDAC4 clone was obtained from Addgene library (#30485), USA. HDAC4 (#PA5-29103) and His tag antibodies (#MA121315) were purchased from Thermo Scientific, USA. Site-directed mutagenesis kit was purchased from Agilent Technologies, USA. Cobalt resin was purchased from Clontech, USA. Dual Luciferase® Reporter Assay System kit (#E910) was purchased from Promega, USA. Tri-reagent for RNA isolation was purchased from Sigma Aldrich, USA. cDNA synthesis kit was porcured from Fermentas, USA. Kit for HDAC activity assay was purchased from Enzo Life Sciences, Inc. USA. H-89 Dihydrochloride (#9844) inhibitor was purchased from CST, USA. SYBR Green procured from KAPA Biosystems, USA to perform real-time PCR.

### 2.4.2 Bioinformatic prediction of probable PTMs of HDAC4:

Bioinformatic prediction of post-translational modifications were carried out using in silico tools available online. The tools includes NetPhos, Sumoylation, NetNGlyc, YingOYang, BDM-PUB. The analysis predicted some of the reported and some unreported sites of modifications. Out of them, the best score of them were taken and summarized in a table format in results section.

**Methods:**

### 2.4.3 Cloning of hHDAC4

The complete sequence of 3.25 kb of Human HDAC4 was amplified from clone purchased from Addgene library HDAC4 (#30485). HDAC4 was cloned into bacterial expression vector pET-28a(+) using gene specific primers as mentioned in the below table 1. HDAC4 was also simultaneously cloned into mammalian expression vector pcDNA3.1/His C vector using gene primers mentioned in table 1. The total reaction volume includes 50 µl each consisting of final concentrations of 10 pmol primers, 0.3 mM dNTP each, and 1 µl Pfu

DNA polymerase enzyme (KAPA HiFi). PCR amplification was carried out in Eppendorf® Mastercycler. The reaction conditions include initial denaturation 95 ºC: 3 min, and is followed by 35 cycles of 95 ºC for 45 sec, 64 ºC: 45 sec, 72 ºC: 2 min 10 sec. The final extension of 72 ºC: 10 min was kept and the amplified product was run on 1 % agarose gel and the bands were visualized by UV Transilluminator (Major science, USA). The separated amplified products were extracted using Gel extraction method (Thermo scientific, USA) as per standard protocol.

Table 1: Cloning primers used for the PCR amplification of hHDAC4:

| Primer Name | Primer sequence (5'----- 3') |
|---|---|
| **Primers for pET-28a** | |
| Fwd primer *EcoRI* | GCAT GAATTC ATG AGC TCC CAA AGC CAT CC |
| Rev primer *Not-I* | ATAAGAAT GCGGCCGC CTA CAG GGG CGGC TCC TC |
| **Primers for pcDNA3.1/His C** | |
| Fwd primer *EcoRI* | GCAT GAATTC ATG AGC TCC CAA AGC CAT CC |
| Rev primer *Not-I* | ATAAGAAT GCGGCCGC CAG GGG CGG CTC CTC TTC |

The amplified PCR product and the vector were digested separately using EcoRI and Not-I restriction enzymes in a 30 µl reaction. The reaction includes 3 µl of 10X Buffer O (Fermentas, USA), 3 µg of PCR product/Vector, 0.5 µl each of *EcoRI* and *Not-I*. The reaction was carried out in a water bath (EQUITRON) for 12 h at 37 ºC. The digested products were loaded on 1% agarose gel, separated and extracted by gel extraction method (Thermo scientific, USA) according to standard protocol.

The digested insert and vectors were mixed in a 4:1 ratio using ligase from T4 DNA ligase (Fermentas, USA). The reaction was performed in 15 µl volume containing 1.5 µl of

10X Ligase buffer, 250 ng DNA, 60 ng vector, 1 µl of T4 DNA ligase and kept at 4 ºC for overnight incubation.

XL-Blue cells/Rosetta™ cells were employed for plasmid transformations and for protein expression. Briefly, ligation mixture/ 50 ng plasmid were added to the cells. The reaction was incubated on ice for 30 min. Luria-Bertani (LB) broth of 1ml was then added to the cells after heat pulse of 2 min at 42 ºC and incubated in an orbital shaker (ORBITEK) for additional 1 h at 160 rpm. The culture pellet was spun down for 5 min at 5000 rpm and plated on Ampicillin/Kanamycin LB agar plate. Following the next day, the appeared colonies were screened for the insert presence by performing PCR reaction with gene specific primers and re-confirmed by Sanger sequencing.

### 2.4.4 Bacterial expression and purification:

HDAC4 pET-28a(+) was expressed as his tag protein in Rosetta™ cells. The protein is purified using TALON® beads (Clontech Takara, USA). The plasmid containing the gene of interest was transformed into Rosetta™ cells. A single colony was added to LB broth with kanamycin (final 50 µg/µl) for overnight at 37 ºC at 160 rpm (primary culture). Next day morning, 1% of primary culture was inoculated into 500 ml of fresh LB with kanamycin and allowed to grow until the $OD_{600}$ reaches 0.4 - 0.6. After culture reached appropriate $OD_{600}$, a 0.3 mM IPTG (final concentration) was added to the culture and incubated at 28 ºC temperature for period of 5 h at 160 rpm. The culture pellet obtained after centrifugation at 10,000 rpm for 10 min and sonicated (SONICS Vibracell™) (10 sec ON and 15 sec OFF) in lysis buffer (50mM phosphate buffer pH 7.4, 100 mM NaCl, 10 mM Imidazole, 5 mM β-mercaptoethanol) including protease inhibitor cocktail. Sonicated lysate was cleared by centrifugation at 12,000 rpm for half an hour and was later allowed to bind to His beads for 1 h, washed with wash buffer (50mM phosphate buffer pH 7.4, 100 mM NaCl, 20 mM Imidazole, 5 mM β-mercaptoethanol). The protein was eluted using elution buffer (500 mM

Imidazole in Wash buffer). Elutes were dialyzed in phosphate buffer (PBS) and protein concentration was measured using Bradford method and stored at -20 ºC.

### 2.4.5 Cell culture:

HEK 293T cells were purchased from NCCS (National Centre for Cell Science) repository, Pune. Cultures were grown in DMEM (Dulbecco's Modified Eagle's medium) (Invitrogen, USA) containing 10% FBS and 1X antibiotic in incubator maintained at 37 ºC with 5% $CO_2$. All the transfections reactions were carried out using Lipofectamine® 2000 procured from Invitrogen, USA.

### 2.4.6 Mammalian expression and purification:

HDAC4 pcDNA3.1/His C plasmid was transfected into HEK293T cells and purified using His tag TALON® beads (Clontech Takara, USA). HEK293T cells were seeded in T-75 flasks until the confluency reached 70% and incomplete DMEM was added to the cells before transfection. The protocol includes: 10 µg of plasmid and 10 µl of Lipofectamine® 2000 was individually mixed with 200 µl of the incomplete DMEM medium. After incubation of 5 min, plasmid DNA was mixed with lipofectamine and further incubated for 30 min. These contents were added to the HEK 293T cells and kept in an incubator maintained at 37 ºC with 5% $CO_2$ for 5 h. After 5 h, complete DMEM (DMEM+ 10% FBS+ 1% antibiotic) was later added to the cells and allowed to grow for 48 h. The cells were collected washed in 1X PBS and lysed in RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris pH 8.0) including protease inhibitor cocktail. The lysate was allowed to bind to TALON® beads for 3 h time period in endorotation at 4 ºC. The beads were further washed with wash buffer (50mM phosphate buffer pH 7.4, 100 mM NaCl, 20 mM Imidazole, 5 mM β-mercaptoethanol). Elution was carried out using elution buffer (500 mM Imidazole in wash buffer). The protein was

dialyzed in wash buffer to remove imidazole and the protein concentration was assessed by Bradford assay kit. The samples were stored at -20 ºC.

### 2.4.7 Western blot:

Protein samples that were separated on SDS-PAGE (10, 12 or 15%) were subjected to western blot for the identification of the protein. Briefly, the proteins were transferred to Nitrocellulose (NC) membrane in the presence of phosphate buffer (90 mM $NaH_2PO_4$, 190 mM $Na_2HPO_4$) with 400 mA at 4 ºC for 3 h. The transfer of the proteins was analyzed by staining the nitrocellulose membrane with ponceau S stain (0.1% (w/v) in 1% (v/v) Acetic acid). The nitrocellulose membrane was then blocked with TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween20) having 5% milk solution for 1 h at RT. The membrane was incubated with 1:1000 primary antibody (pierce Rabbit HDAC4) made in TBS-T overnight at 4 ºC followed by 3 washes of TBS-T 10 min each. The membrane was subjected to secondary antibody (Goat anti-rabbit HRP; 1:10,000) for 1 h at RT followed by 3 washes of TBS-T each for 10 min. The membrane was developed using ECL substrate (Thermo SuperSignal® West Pico Chemiluminescent substrate) and Kodak Image station.

### 2.4.8 HDAC activity assay:

HDAC4 isolated from the bacterial and mammalian system were compared for the HDAC4 activity assay by using fluorescent substrate *FLUOR DE LYS*® purchased from Enzo as per the manufacturer's protocol. Briefly, 1/10 µg of purified recombinant HDAC4 was incubated along with the HDAC assay buffer and 60 µM (final) of the fluorescent substrate to a final volume of 50 µl 37 ºC for 15 min. Reaction was stopped with the addition of 50 µl developer (trypsin final 0.1 mg/ml) followed by incubation for 30 min at 37 ºC and the Arbitrary Fluorescent Units (AFU) were recorded (Excitation 330 nm and Emission 395 nm).

### 2.4.9 In Gel digestion, Mass spectrometry and Database search:

Excised protein gel bands were minced into small pieces using sterile surgical blade and were washed with 25 mM Ammonium bicarbonate (ABC) in vortex for 15 min at RT, then followed by another wash with ABC for 30 min. Later 1:1 ratio of 25 mM ABC and acetonitrile (ACN) and was then added to minced gel pieces and further vortexed for 15 min at RT. The same washing step was repeated again to clean the gel bands. Next, 50 µl of 10 mM Dithiothreitol (DTT) (made in 100 mM ABC) was added and kept for 1 h at 60 ºC with vortexing. After removing excess liquid, 50 µl of 50 mM Iodoacetamide (made in 100 mM ABC) was added and kept in dark for 30 min at RT. Excess amount of liquid was removed and samples were washed with 1:1 ratio of 25 mM ABC and ACN and then incubated with 40 µl of Trypsin (20 µg/µl) (Trypsin Gold Mass Spectrometry Grade, Promega, USA) and kept overnight at 37 ºC. Peptides were extracted using extraction buffer (5% TFA, 50% Acetonitrile). For the extraction of peptides from the minced gel pieces, 100 µl of extraction buffer was added and vortexed for 45 min at RT and supernatant was collected. 50 µl of extraction buffer was later added and vortexing was done for 45 min and the supernatant was collected. The collected supernatant was speedvac concentrated and finally dissolved in 10 µl of 5% acetonitrile containing 1% TFA (trifluoroacetic acid). Finally, Zip tip (Millipore, USA) was performed to remove salts. The peptide samples were finally dissolved in 5% acetonitrile (0.1% TFA). 1:1 ratio of peptide protein sample and 5 mg/ml HCCA matrix (5 mg/ml) (α-cyano-4-hydrocinnamic acid) of each 1 µl was spotted on MALDI plate. The plate was subjected to Applied Biosystems 4800 MALDI TOF/TOF™ analyzer. Mass spectrometry (MS) and MS/MS spectra were acquired in a data-dependent manner and later search was carried out with the SWISS-PROT database in the Protein Pilot 4.0 software.

### 2.4.10 Site-directed mutagenesis:

Site-specific point mutations were introduced at the respective positions using Quick change lightning multisite-directed mutagenesis kit (Agilent technologies, USA). The mutated site specific primer was used as a forward primer (mentioned in table 2). The total reaction volume is 25 µl which includes 10 pmol of mutated forward primer, 2.5 µl of 10X buffer, 1 µl of 10 mM dNTPs, 0.5 µl of a quick stop, 1 µl of enzyme and 70 ng of template DNA (HDAC4 - pcDNA3.1/His C). The reaction conditions includes: initial denaturation of 95 ºC for 2 min, then followed by 30 cycles of 95 ºC for 30 sec, 60 ºC for 40 sec, and 72 ºC for 5 min. The final extension of 65 ºC for 5 min. After PCR reaction, 1 µl of DpnI enzyme was added to the tube and kept in incubator maintained at 37 ºC for 1 h.

15 µl of sample was then transformed into the ultracompetent XL-10 Gold cells and transformation procedure followed was as mentioned earlier. The colonies obtained were screened for the presence of insert and mutation was confirmed by sequencing.

Table 2: List of site specific mutated primers employed for site-directed mutagenesis

| Primer Name | Primer sequence (5'----- 3') |
|---|---|
| H4 S265A Forward | GCC GAA AGA CGG **GCC** AGC CCC CTG TTA CGC |
| H4 S266A Forward | GCC GAA AGA CGG AGC **GCC** CCC CTG TTA CGC |
| H4 S265/266A Forward | GCC GAA AGA CGG **GCC** **GCC** CCC CTG TTA CGC |
| H4 S584A Forward | GAG AGA CTC **GCC** CTT CCC GCC CTC |

### 2.4.11 Confocal microscopy:

Localization of HDAC4 protein was monitored using antibody against His tag. HDAC4 wild-type and mutant proteins (HDAC4 S584A, S265/266A) were transfected individually at 60 % confluency into HEK 293T cells grown on cover slips. Cells were

changed to complete DMEM medium after 5 h and allowed to grow for additional time of 48 h. Cells were later washed with 1XPBS and fixation was carried out in 4 % paraformaldehyde for 15 min. Cells were washed with 1XPBS and permeabilization was carried out in 1 % Triton X-100 for 5 min at RT. Non-specific binding of antibody is prevented by blocking the cells with 3 % BSA (made in PBS) and incubated for 1 h at RT. 1:100 dilution of primary antibody (Anti-his, Invitrogen, USA) was added to cells and allowed to incubate for 2 h at RT, then followed by 3 washes of 0.2 % triton X-100. Alexa Fluor 488 (Invitrogen, USA) secondary antibody was added at 1:500 dilution and then kept for 1 h at RT, followed by 3 washes with 0.2 % Triton X-100. Mounting medium along with DAPI was added and finally sealed with nail polish.

### 2.4.12 Sub-cellular fractionation:

Briefly, HEK 293T cells (~$8X10^6$) cells were washed couple of times with 1XPBS and suspended in 200 µl of ice cold cytosolic extraction buffer (30 mM Tris pH 7.5, 10 mM Magnesium acetate, 1% NP-40 and protease inhibitors) with titurating and allowed to incubate on ice for 5 min and cytosolic fraction was collected by harvesting cells at 12,000g for 5 min at 4 ºC. Cells were additionally washed once with cytosolic buffer and the pellet was suspended in 50 µl of nuclear extraction buffer (420 mM NaCl, 10 mM HEPES pH 7.9, 10 mM $MgCl_2$ pH 7.5, 1 mM EDTA, 25% glycerol, 1 mM DTT including protease inhibitors) and allowed to incubate on ice with occasional vortexing for 30 min. Samples were spun at 16,000g for 30 min and supernatant was collected as nuclear fraction.

### 2.4.13 Protein kinase A (PKA) inhibitor (H-89) treatment:

HDAC4 pcDNA3.1/His C plasmid (10 µg) was transfected using Lipofectamine into HEK 293T cells at 70% confluency. After 5 h of transfection, incomplete DMEM was changed to complete DMEM and 20 µM of H-89 inhibitor was added. The protein was allowed to express for 48 h along with H-89 inhibitor. The protein was then purified using

His tag resin (TALON) and gel excised and subjected to MALDI-TOF-TOF analysis for the identification of sites of phosphorylation.

### *2*.4.14 *in vitro* kinase assay:

In vitro kinase reaction was performed on HDAC4-GST fragments containing HDAC4 wild-type and HDAC4-S584A spanning 529-635 amino acids. Approximately, 5 µg of purified HDAC4-GST fusion protein and GST alone was treated with Protein Kinase A (PKA) (50 units) and incubated in kinase reaction buffer consisting of 2 µCi of [γ-$^{32}$P] ATP, 25 µM ATP, phosphatase inhibitor, for half an hour at 30 ºC. Reaction was halted using SDS loading dye, samples were resolved on SDS-PAGE, transferred to a nitrocellulose membrane and finally exposed to phosphoimager (PharosFX$^{TM}$ Plus system, Biorad, USA).

After the kinase reaction, a subset of samples were transferred to Nitrocellulose membrane by Western blot (as mentioned earlier) and probed with 2 µg/ml phosphoserine antibody (#ab9332, Abcam, USA), followed by secondary Goat anti-rabbit HRP antibody (1:1000) (#31460 Thermo Scientific, USA).

### 2.4.15 Gene Reporter assays:

For the gene reporter assays, the 4XMEF2-luc promoter is used which is a kind gift from Ron Prywes Columbia University, NY, USA. All Transient transfections were performed in HEK 293T cells by using dual luciferase assay kit from Promega as per the standard protocol. 3 µg of plasmid DNA (4XMEF-luc), 600 ng of HDAC4 WT/HDAC4 S584A/ HDAC4 S265/266A, 600 ng MEF2C and 60 ng of PLRTK (firefly) was used in a 6 well format as triplicates and allowed to grow for 48 h. The cells were then washed with 1XPBS and lysis was carried out in 500 µl of passive lysis buffer for 30 min on a rocker. Cells were collected into individual tubes without spinning. 20 µl of lysate was added followed by 40 µl of LARII and the reading was recorded (X) in a luminometer. 40 µl of

Stop glow was added to the same tube and the reading was recorded (Y).The ratio of firefly (X) and renilla (Y) for each sample was calculated to obtain relative luciferase units (RLU).

### 2.4.16 RNA isolation:

Briefly, 1.2 x $10^6$ HEK 293T cells were taken for the total RNA isolation. Cells were first washed with 1XPBS and Tri-reagent® (Sigma-Aldrich, USA) of 300 µl was added and mixed thoroughly until homogeneous mixture is formed and kept for 5 min at RT. 60 µl of chilled chloroform was then added, mixed by vigorous shaking and incubated at RT for 5 min. Samples were centrifuged at 12000g, 4 ºC for 15 min. The uppermost aqueous layer was transferred into the fresh tube without the disturbing middle layer. Two volumes of chilled isopropanol were added mixed slowly and incubated in -80 ºC for 1 h. Centrifugation at 12000g 4 ºC for 15 min. The supernatant was discarded and the pellet was washed with 70% ethanol for 12000g 4 ºC for 10 min. The RNA pellet was finally dissolved in 60 µl of nuclease free water.

### 2.4.17 cDNA synthesis:

cDNA synthesis was performed with the cDNA synthesis kit purchased from Invitrogen™. Briefly, 1 µg RNA was used for the conversion of mRNA to cDNA. The reaction volume includes 1 µg total RNA, 1 µl of 10 mM dNTPs, 1 µl of oligo $(dT)_{18}$. The total volume was adjusted to 12 µl by nuclease-free water. The reaction was initiated by incubation at 65 ºC for 5 min. Next, 4 µl of the 5X buffer, 1 µl of 0.1 M DTT, 1 µl of RNase OUT (40 U/µl), 1 µl of M-MLV (200 U/µL) reverse transcriptase were added and PCR was performed at 42 ºC for 60 min and the reaction was stopped at 70 ºC for 5 min.

### 2.4.18 Real-time quantitative PCR analysis:

Quantitative real-time PCR was performed for the MEF2C target genes. The MEF2C target genes selected for the real-time PCR are Anxa8, Irs1, Klf2, Klf4, and RhoB.

The primer sequence for the genes are summarized in below table.

Table 3: List of primers used for the quantitative Real-time PCR

| Primer Name | Primer sequence (5'----- 3') | Amplicon length (bp) | Tm ºC |
|---|---|---|---|
| Anxa8 Fwd | TTCATCACCATCCTGTGCACGC | 171 bp | 60.2 |
| Anxa8 Rev | GTAGCTGTGGAGGTTTTGGGTGC | | 60.5 |
| Irs1 Fwd | CACGGATGATGGCTACATGCCC | 169 bp | 60.4 |
| Irs1 Rev | CATGTAGCCATTGGGGTCCACTC | | 59.8 |
| Klf2 Fwd | GAGTTCGCATCTGAAGGCGCATC | 156 bp | 60.5 |
| Klf2 Rev | CACAGATGGCACTGGAATGGC | | 58.9 |
| Klf4 Fwd | CCCAATTACCCATCCTTCCTGCC | 166 bp | 60 |
| Klf4 Rev | CGTAATCACAAGTGTGGGTGGCG | | 60.5 |
| RhoB Fwd | GCATGAAGCAGGAACCCGTG | 176 bp | 59.2 |
| RhoB Rev | GTTGATGCAGCCGTTCTGGGA | | 60.2 |

cDNA template was diluted to 20 fold for the real-time analysis. The SYBR green master mix from KAPA Biosystems was used. 1 µl of cDNA (1:20), 0.4 µl of primer (forward), 0.4 µl of primer (reverse), 5 µl of 2X SYBR green was added and the total reaction volume was made up to 10 µl using nuclease-free water.

# RESULTS

# 3. Results

## 3.1 Objective 1: Identification of novel HDAC4 PTMs

### (a) *In silico* prediction of probable PTMs of HDAC4

*In silico* prediction of probable PTMs of human HDAC4 was performed using available online bioinformatics tools. The tools used for the analysis have been summarized below. The probable peptide sequence and sites of post-translational modifications along with the score values were briefed in Fig. 10.

| PTM | Software | Predicted sites | Peptide | Score |
|---|---|---|---|---|
| Sumoylation | SUMO | 559 | AQAGVQVKQEPIESD | 32. 222/100 |
| Serine Phosphorylation | Net Phos2.0 | 209<br>265<br>266<br>584 | KTQHSSLDQ<br>ERRSSPLLR<br>ERRSSPLLR<br>QRQPSEQEL | 0.995/1<br>0.517/1<br>0.995/1<br>0.995/1 |
| Tyrosine Phosphorylation | Net Phos2.0 | 201<br>540 | SDPRYWYGK<br>LDEPYLDRL | 0.927/1<br>0.747/1 |
| Threonine Phosphorylation | Net Phos2.0 | 525 | HPEETEEEL | 0.968/1 |
| N-linked Glycosylation | NetNGlyc 1.0 | 353 | NITL | 0.6850/1 |
| O-linked Glycosylation | YingOYang 1.2 | 215<br>628 | S (OH)<br>S (OH) | 0.6461/1<br>0.6931/1 |
| Ubiquitination | BDM-PUB | 162 | LKNKEKGKESAVAST | 2.80/1 |

**Figure 10:** ***in silico analysis of the probable post-translational modifications.*** *The post-translational modifications of HDAC4 protein depicting software, predicted the site of modification, peptide sequence, and score value. The high score value indicates the highest probability of prediction of the modification. The red highlighted amino acid in the peptide sequence is the one likely prone to modification.*

The analysis predicted the sites that are already known and some unreported modifications. Sumoylation of HDAC4 is predicted at K559 and earlier studies also reported lysine 559 modification by SUMO-1 protein and mutant HDAC4 exhibited transcriptional

repression and decreased enzyme activity (Tatham, Jaffray et al. 2001), (Kirsh, Seeler et al. 2002). Serine phosphorylation analysis using NetPhos2.0 predicted sites of phosphorylation at 209,265,266, and 584 along with other sites. Of these, Serine 265/266 phosphorylation have been reported earlier by Liu and his coworkers (Liu and Schneider 2013). Tyrosine phosphorylation analysis predicted sites 201, 540 and others. Tyrosine phosphorylation at 201 by Protein Kinase A has been known to proteolytically cleave HDAC4 counteracting CaMKII mediated activation of MEF2 (Backs, Worst et al. 2011). The other PTMs such as Threonine phosphorylations, Glycosylation (N-linked and C-linked) and Ubiquitination are predicted, but are not reported and need to be further validated. Since phosphorylation of HDAC4 is widely known and the *in silico* analysis also predicted several novel sites, further studies were carried out to identify novel phosphorylations of HDAC4.

**(b) Identification of PTMs of HDAC4 by MALDI-TOF/TOF analysis.**

### 3.1.1 Cloning, expression, and purification of Human HDAC4 from bacterial expression system:

To characterize the sites of phosphorylation in human HDAC4 protein, the full-length gene was cloned and expressed in a bacterial expression system. Theoretically, we assume that no phosphorylation takes place in bacterial purified HDAC4 and hence is used as a PTM (-)ve control. HDAC4 was cloned into bacterial expression vector pET-28a (+) under a T7 promoter. The primers were designed in both 5' and 3' ends by inserting *EcoRI* and *Not-I* restriction enzyme sites. HDAC4 gene was amplified from a commercial plasmid pcDNA-HDAC4-FLAG from Addgene (Addgene plasmid # 30485) (Fig: 11 A). Restriction digestion of both insert and vector were carried out and ligated using DNA ligase. The ligated product was added to XL-1 Blue cells by transformation procedure and the presence of HDAC4 insert was confirmed through colony PCR. Double digestion with *EcoRI* and *Not-I* enzymes (Fig: 11 B) and DNA sequencing also confirmed the presence of HDAC4 insert

This positive clone was later transformed into Rosetta™ (DE3) expression cells. The expression of the protein was standardized using different temperatures (including 37 ºC, 28 ºC and16 ºC), IPTG (isopropyl β-D-1-thiogalactopyranoside) (0.3, 0.6 and 0.9 mM) conditions to obtain protein expression in the soluble fraction. Finally, protein expression was induced at 28 ºC with 0.3 mM IPTG (Fig: 11 C) and the lysate was bound to His beads (TALON™, Clontech) and eluted using imidazole (Fig: 11 D). The eluted protein was confirmed using antibodies against His-tag and HDAC4 (Fig: 11 E). The detailed procedure of the expression and purification was mentioned in materials and methods.

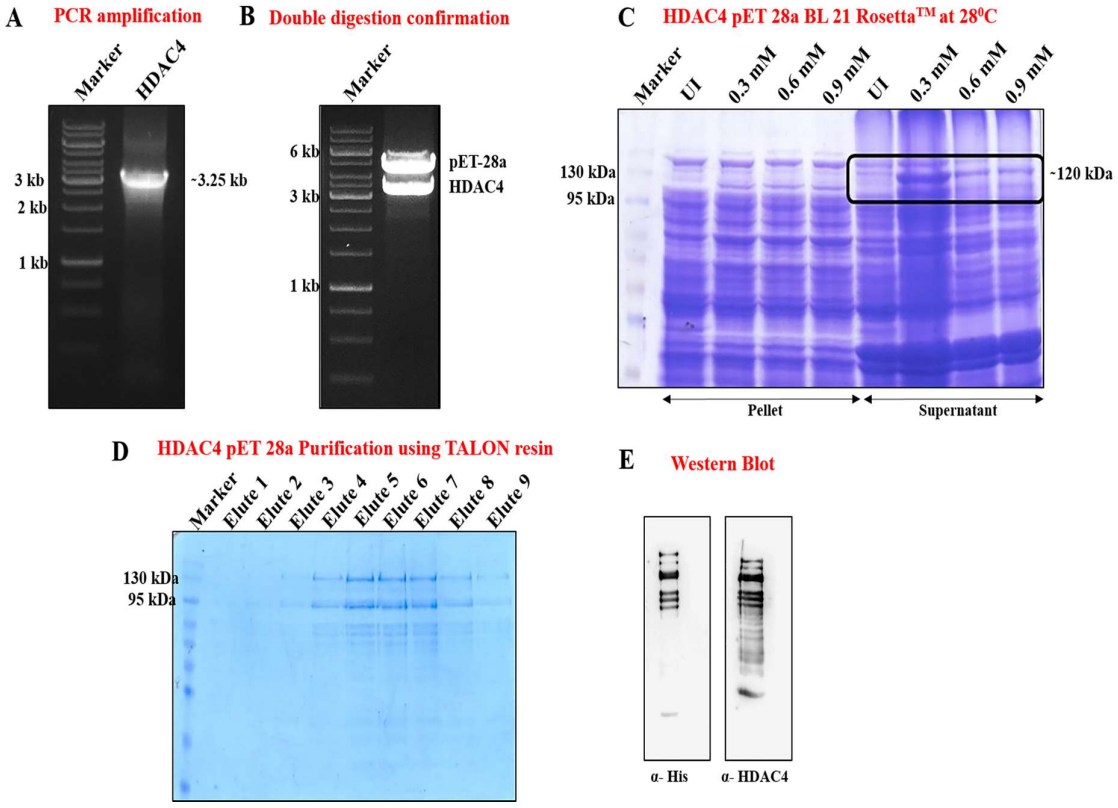



**Figure 11:** ***Cloning, Expression and Purification of HDAC4 from bacterial expression host (Rosetta™): (A)*** *PCR amplification of full-length human HDAC4 from HDAC4-pcDNA FLAG vector on 0.8% agarose gel.* ***(B)*** *Double digestion of the plasmid construct using EcoRI and Not-I restriction enzymes confirming the presence of HDAC4.* ***(C)*** *Standardization of the expression conditions of HDAC4 in 10% SDS-PAGE gel at 28 ºC with different IPTG conditions.* ***(D)*** *Purification of the recombinant HDAC4 protein using cobalt beads (TALON ™) run on 10% SDS-PAGE gel.* ***(E)*** *Western blot of the HDAC4 protein purified from cobalt resin probed with His-tag and HDAC4 antibody.*

### 3.1.2 Cloning, expression, and purification of Human HDAC4 from mammalian expression system:

Cloning of HDAC4 into a mammalian expression vector, pcDNA 3.1/His C was similar to that of bacterial system. HDAC4 cDNA sequence was PCR amplified (Fig: 12 A), cloned into the vector, the colonies were confirmed by the release of the insert (Fig: 12 B) followed by DNA sequencing.

For the expression of the HDAC4 protein, HEK 293T cells were chosen as they have high transfection efficiency and protein production capability. Apart from that, HEK 293T cells are known to have good translational capacity and processing of proteins.

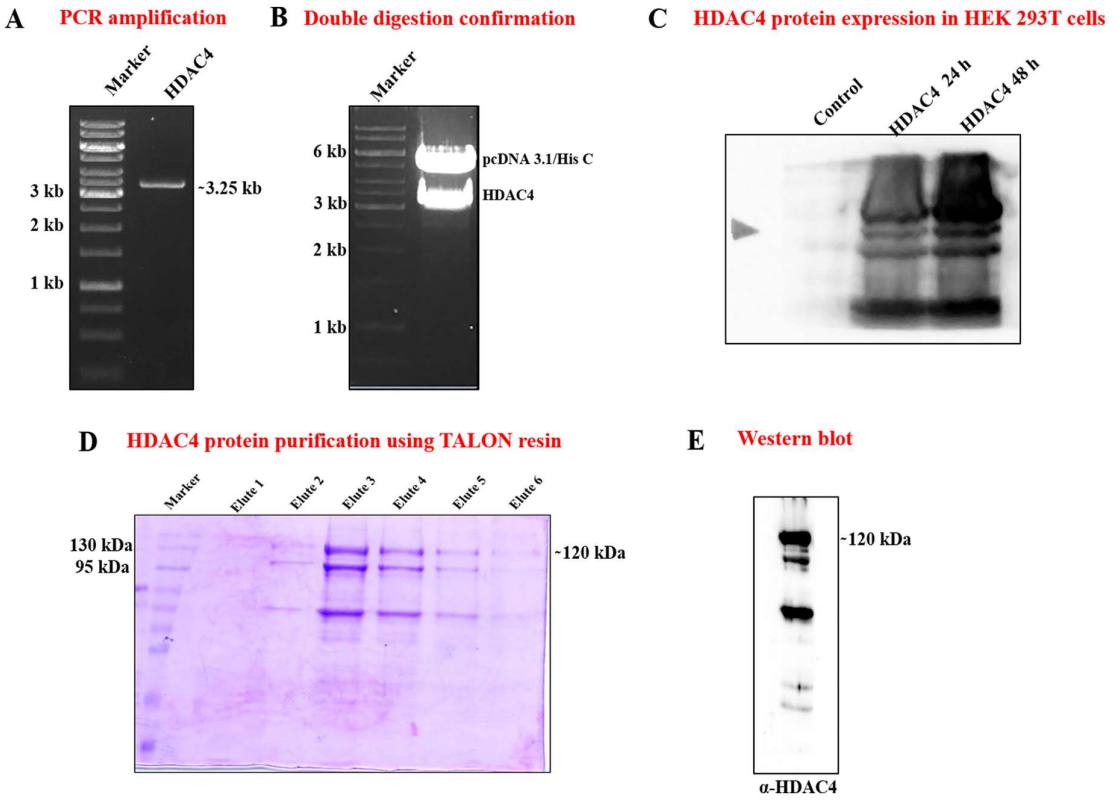



**Figure 12:** ***Cloning, Expression and Purification of HDAC4 from the mammalian system (HEK 293T cells): (A)*** *PCR amplification of HDAC4 gene from HDAC4 pcDNA FLAG construct along with restriction enzyme sites EcoRI and Not-I.* ***(B)*** *Double digestion confirmation of HDAC4 insert using EcoRI and Not-I enzymes.* ***(C)*** *Western blot of the HDAC4 recombinant protein probed with HDAC4 antibody after 24 and 48 h to standardize protein expression.* ***(D)*** *Purification of HDAC4 protein using cobalt resin (TALON ™) specific for the His-tagged protein.* ***(E)*** *Western blot of the purified HDAC4 protein probed with the HDAC4 antibody.*

For the expression of the HDAC4 protein, HEK 293T cells were transfected in T-75 flasks with 10 µg of HDAC4 pcDNA 3.1/His C construct when the cells are at 70% confluency. After 48 h, cells were washed once with ice-cold 1XPBS and the total protein lysate was prepared using RIPA buffer according to the standard protocol. The expression of the recombinant protein was confirmed by Western blot probed specific with the HDAC4 antibody (Fig: 12 C). The lysate was then allowed to bind to cobalt resin (TALON $^{TM}$), which is specific for His tag proteins for 3 h. The protein was eluted using imidazole (Fig: 12 D) and confirmed using HDAC4 antibody (Fig: 12 E).

### 3.1.3 HDAC4 activity assay:

HDAC4 protein was assayed for activity after purification from both bacterial and mammalian systems using the HDAC4 activity kit (Enzo life sciences). HDAC activity is measured by the ability to deacetylate the fluorescent-labeled acetylated substrate. The trypsin enzyme then subsequently cleaves the deacetylated substrate releasing the fluorophore and the resultant fluorescence is read at 395 nm when excited at 330 nm. The graph was plotted using arbitrary fluorescence units (AFU). The results indicated that the HDAC4 purified from HEK 293T cells (mammalian system) was active and that the bacterially purified HDAC4 protein was inactive, as expected (Fig. 13). One explanation for this increase in activity in mammalian purified protein might be due to proper folding of the protein, which is attained with the help of post-translational modifications.

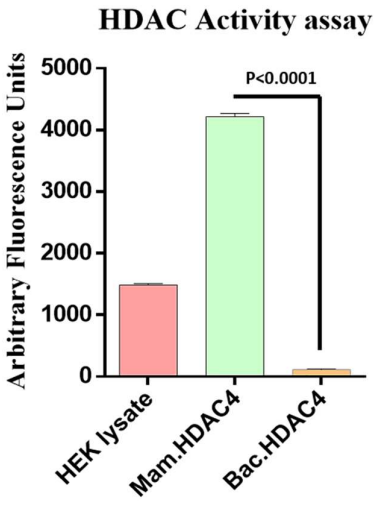



**Figure 13:** ***HDAC4 activity assay:*** *Activity assay of the purified recombinant HDAC4 protein. The activity of HDAC4 purified from the mammalian system was significant when compared with HDAC4 purified from the bacterial system. Error bar represents S.E (n=3).*

### 3.1.4 Identification of phosphorylation sites in mammalian HDAC4 protein:

For the identification of phosphorylation sites in wild-type HDAC4 purified from HEK 293T cells, MALDI-TOF/TOF analysis was performed. The results identified HDAC4 protein with a sequence coverage of 24% (Fig: 14 A). After the successful identification of HDAC4, the peptides were scanned for the phosphorylations that confer the difference of 98 ($PO_4^{2-}$) or 80 ($HPO_3^-$) daltons between non-phosphorylated and phosphorylated peptides. Interestingly, two phosphorylated peptides were observed, one corresponding to Serine 265/266 and the other at Serine 584 position. Serine phosphorylation at 265/266 is observed corresponding to peptide 265-270 aa (SSPLLR) with a PMF of m/z 832.4401and its counterpart peptide with PMF 672.3869 purified from the bacterial system.

The Serine phosphorylation corresponding to 584 position is novel with peptide 582-591aa (QPSEQELLFR) of PMF 1326.6869 and its counterpart peptide with 1246.4459 was identified in bacterial purified HDAC4 protein (Fig: 14 B).

A comparison was made with the peptide peaks of HDAC4 purified from bacterial and mammalian system obtained from MALDI-TOF/TOF analysis (Fig: 14 C)

Conclusively, we have identified three phosphorylation sites in HDAC4 protein isolated from mammalian system i.e., HEK 293T cells of which one is novel Serine 584 and the other two are already reported serine phosphorylations at 265/266 positions. Our initial *in silico* prediction also identified Serine 584 phosphorylation along with Serine 265/266.

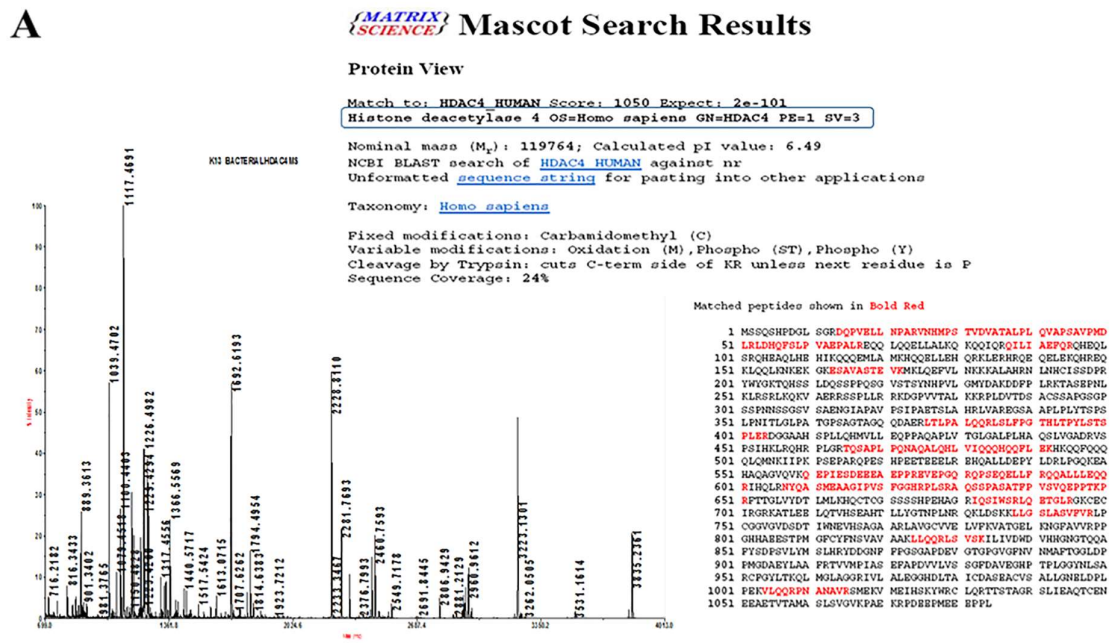



**Figure 14 A**: *PMF spectrum of HDAC4 purified from bacterial expression system and right panel denotes aligned peptide region of full-length HDAC4 protein.*

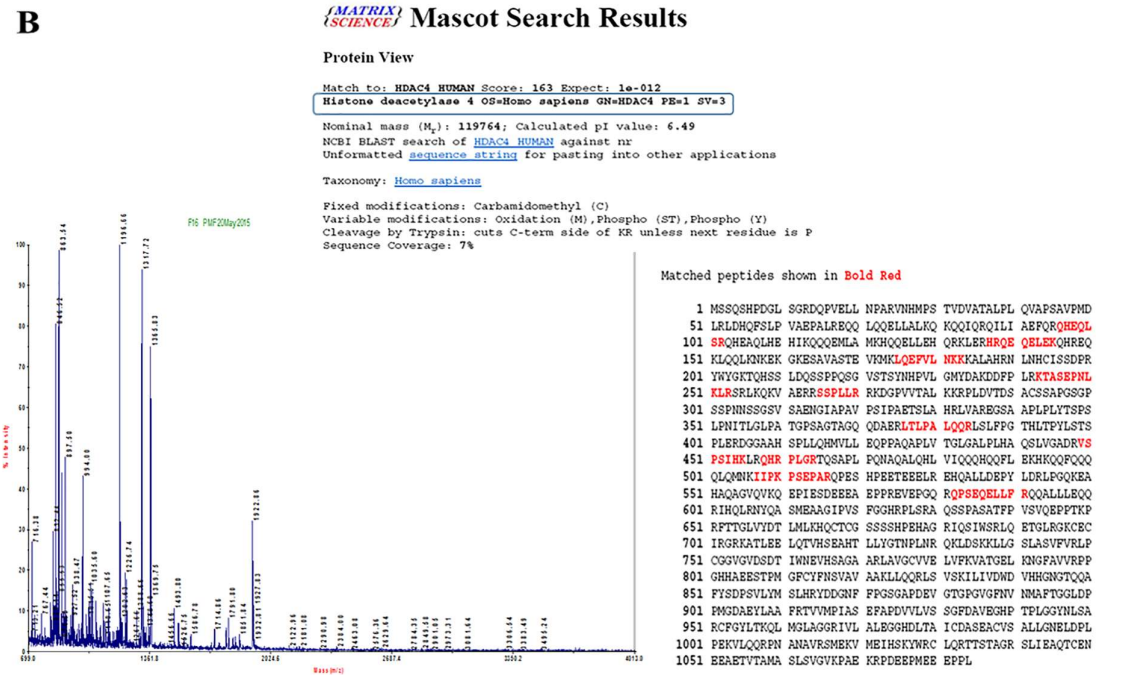
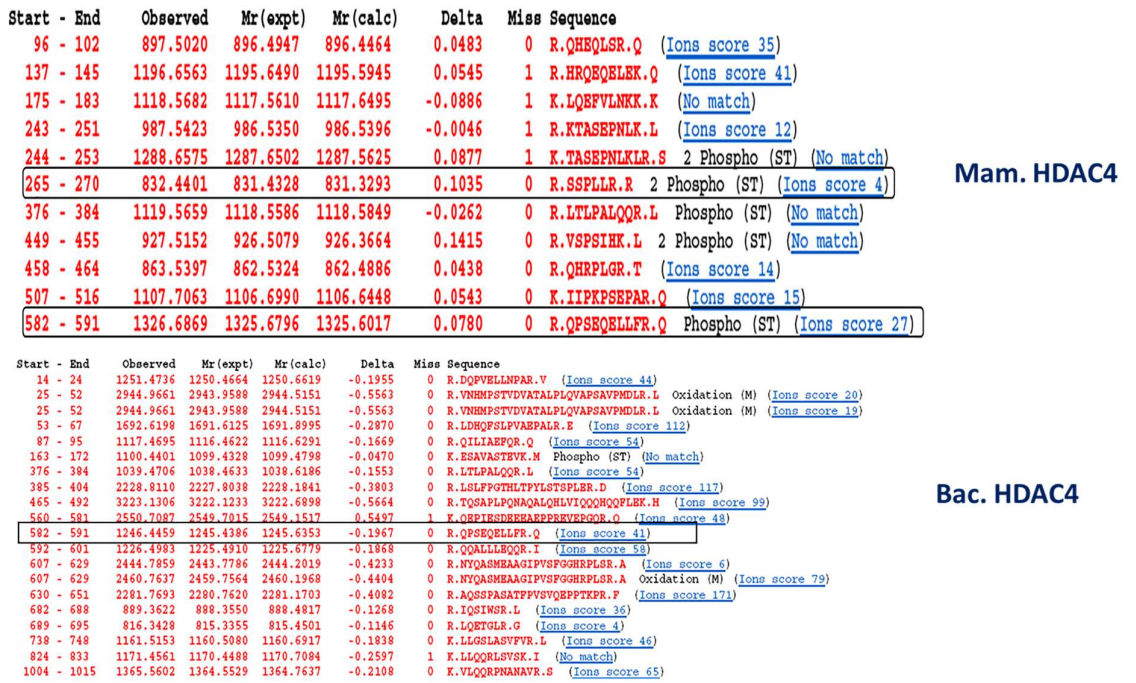



**Figure 14 B:** *PMF spectrum of HDAC4 purified from HEK 293T cells and right panel depicts aligned peptide regions.*



Mam. HDAC4

| Start - End | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Sequence |
|---|---|---|---|---|---|---|
| 96 - 102 | 897.5020 | 896.4947 | 896.4464 | 0.0483 | 0 | R.QHEQLSR.Q (Ions score 35) |
| 137 - 145 | 1196.6563 | 1195.6490 | 1195.5945 | 0.0545 | 1 | R.HRQEQELEK.Q (Ions score 41) |
| 175 - 183 | 1118.5682 | 1117.5610 | 1117.6495 | -0.0886 | 1 | K.LQEFVLNKK.K (No match) |
| 243 - 251 | 987.5423 | 986.5350 | 986.5396 | -0.0046 | 1 | R.KTASEPNLK.L (Ions score 12) |
| 244 - 253 | 1288.6575 | 1287.6502 | 1287.5625 | 0.0877 | 1 | K.TASEPNLKLR.S 2 Phospho (ST) (No match) |
| 265 - 270 | 832.4401 | 831.4328 | 831.3293 | 0.1035 | 0 | R.SSPLLR.R 2 Phospho (ST) (Ions score 4) |
| 376 - 384 | 1119.5659 | 1118.5586 | 1118.5849 | -0.0262 | 0 | R.LTLPALQQR.L Phospho (ST) (No match) |
| 449 - 455 | 927.5152 | 926.5079 | 926.3664 | 0.1415 | 0 | R.VSPSIHK.L 2 Phospho (ST) (No match) |
| 458 - 464 | 863.5397 | 862.5324 | 862.4886 | 0.0438 | 0 | R.QHRPLGR.T (Ions score 14) |
| 507 - 516 | 1107.7063 | 1106.6990 | 1106.6448 | 0.0543 | 0 | K.IIPKPSEPAR.Q (Ions score 15) |
| 582 - 591 | 1326.6869 | 1325.6796 | 1325.6017 | 0.0780 | 0 | R.QPSEQELLFR.Q Phospho (ST) (Ions score 27) |

Bac. HDAC4

| Start - End | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Sequence |
|---|---|---|---|---|---|---|
| 14 - 24 | 1251.4736 | 1250.4664 | 1250.6619 | -0.1955 | 0 | R.DQPVELLNPAR.V (Ions score 44) |
| 25 - 52 | 2944.9661 | 2943.9588 | 2944.5151 | -0.5563 | 0 | R.VNHMPSTVDVATALPLQVAPSAVPMDLR.L Oxidation (M) (Ions score 20) |
| 25 - 52 | 2944.9661 | 2943.9588 | 2944.5151 | -0.5563 | 0 | R.VNHMPSTVDVATALPLQVAPSAVPMDLR.L Oxidation (M) (Ions score 19) |
| 53 - 67 | 1692.6198 | 1691.6125 | 1691.8995 | -0.2870 | 0 | R.LDHQFSLPVAEPALR.E (Ions score 112) |
| 87 - 95 | 1117.4695 | 1116.4622 | 1116.6291 | -0.1669 | 0 | R.QILIAEFQR.Q (Ions score 54) |
| 163 - 172 | 1100.4401 | 1099.4328 | 1099.4798 | -0.0470 | 0 | K.ESAVASTEVK.M Phospho (ST) (No match) |
| 376 - 384 | 1039.4706 | 1038.4633 | 1038.6186 | -0.1553 | 0 | R.LTLPALQQR.L (Ions score 54) |
| 385 - 404 | 2228.9110 | 2227.8038 | 2228.1841 | -0.3803 | 0 | R.LSLFPGTHLTPYLSTSPLER.D (Ions score 117) |
| 465 - 492 | 3223.1306 | 3222.1233 | 3222.6898 | -0.5664 | 0 | R.TQSAPLPQNAQALQHLVIQQQHQQPLEK.H (Ions score 99) |
| 560 - 581 | 2550.7087 | 2549.7015 | 2549.1517 | 0.5497 | 1 | K.QEPIESDEEEAEPPREVEPGQR.Q (Ions score 48) |
| 582 - 591 | 1246.4459 | 1245.4386 | 1245.6353 | -0.1967 | 0 | R.QPSEQELLFR.Q (Ions score 41) |
| 592 - 601 | 1226.4983 | 1225.4910 | 1225.6779 | -0.1868 | 0 | R.QQALLLEQQR.I (Ions score 58) |
| 607 - 629 | 2444.7859 | 2443.7786 | 2444.2019 | -0.4233 | 0 | R.NYQASMEAAGIPVSFGGHRPLSR.A (Ions score 6) |
| 607 - 629 | 2460.7637 | 2459.7564 | 2460.1968 | -0.4404 | 0 | R.NYQASMEAAGIPVSFGGHRPLSR.A Oxidation (M) (Ions score 79) |
| 630 - 651 | 2281.7693 | 2280.7620 | 2281.1703 | -0.4082 | 0 | R.AQSSPASATFPVSVQEPPTKPR.F (Ions score 171) |
| 682 - 688 | 889.3622 | 888.3550 | 888.4817 | -0.1268 | 0 | R.IQSIWSR.L (Ions score 36) |
| 689 - 695 | 816.3428 | 815.3355 | 815.4501 | -0.1146 | 0 | R.LQETGLR.G (Ions score 4) |
| 738 - 748 | 1161.5153 | 1160.5080 | 1160.6917 | -0.1838 | 0 | K.LLGSLASVFVR.L (Ions score 46) |
| 824 - 833 | 1171.4561 | 1170.4488 | 1170.7084 | -0.2597 | 1 | K.LLQQRLSVSK.I (No match) |
| 1004 - 1015 | 1365.5602 | 1364.5529 | 1364.7637 | -0.2108 | 0 | K.VLQQRPNANAVR.S (Ions score 65) |

**Figure 14 C:** *Comparison of HDAC4 PMF peptides obtained by MALDI-TOF/TOF analysis from both bacterial and mammalian expression system. Detailed analysis revealed two Serine phosphorylation sites, one at Serine 265/266 and other novel at 584. The box indicates the peptide sites of phosphorylation.*

### MS/MS analysis of the Serine 584 and 265/266 phosphorylation:

MS/MS analysis of the peptides QPSEQELLFR, corresponding to amino acids 582-591, and SSPLLR, corresponding to 265-270, was performed to understand the phosphorylation signatures at 584 and 265/266 positions. The MS/MS fragmentation of peptide peak QPSEQELLFR 1246.4459 corresponding to Serine at 584 position was observed in bacterially purified HDAC4 protein (Fig: 15 A). Similarly, the fragmentation peak QPSEQELLFR of 1326.6869 was observed in HDAC4 purified from mammalian HDAC4 (Fig: 15 B). Both the spectra were shown depicting the presence of b type ($b_1$ to $b_{10}$) and y type ($y_1$ to $y_{10}$) ions. The $b_1$ ions corresponds to Glutamine and $b_{10}$ forms the Arginine amino acid, whereas, the $y_1$ forms the Arginine and $y_{10}$ forms the Glutamine amino acid respectively. Examining the mass difference between between $b_3$ and $b_4$ and $y_7$ and $y_8$ ions suggest us a difference of 98 Dalton shift which is attributed to the presence of phosphorylation ($PO_4^{2-}$) at 584 position.

As shown in figure 15 C, the fragmentation of the peptide SSPLLR m/z of 672.3869 which is a bacterially purified HDAC4 is shown as a mass spectra indicating the presence of b type and y type ions. The fragmentation peak of peptide SSPLLR m/z 832.4401 is shown in Fig: 15 D. Examining fragments of $b_n$ ion series between $b_1$ and $b_2$, $b_2$ and $b_3$ give us the mass difference of 180 Da each which corresponds to the phosphorylation present on successive Serine amino acids present on 265 and 266 amino acid. Similarly, the y type ions are also observed for the presence of phospho signatures. The mass difference of 196 Da is observed between $y_4$, $y_5$ and $y_6$ respectively.

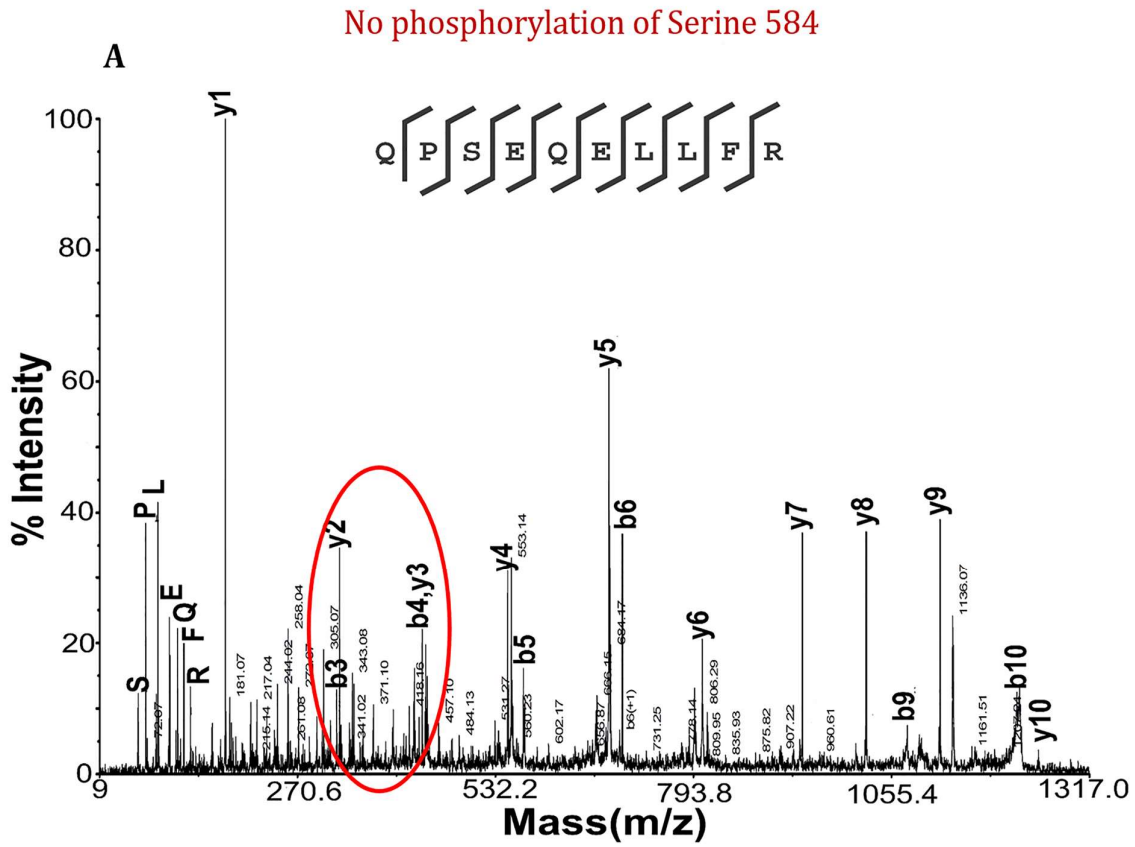



**Figure 15 A:** *MS/MS spectrum of HDAC4 peptide QPSEQELLFR with m/z 1246.4459 (582-591 amino acids) purified from a bacterial expression system. No mass difference was identified between $b_3$ and $b_4$ ion.*

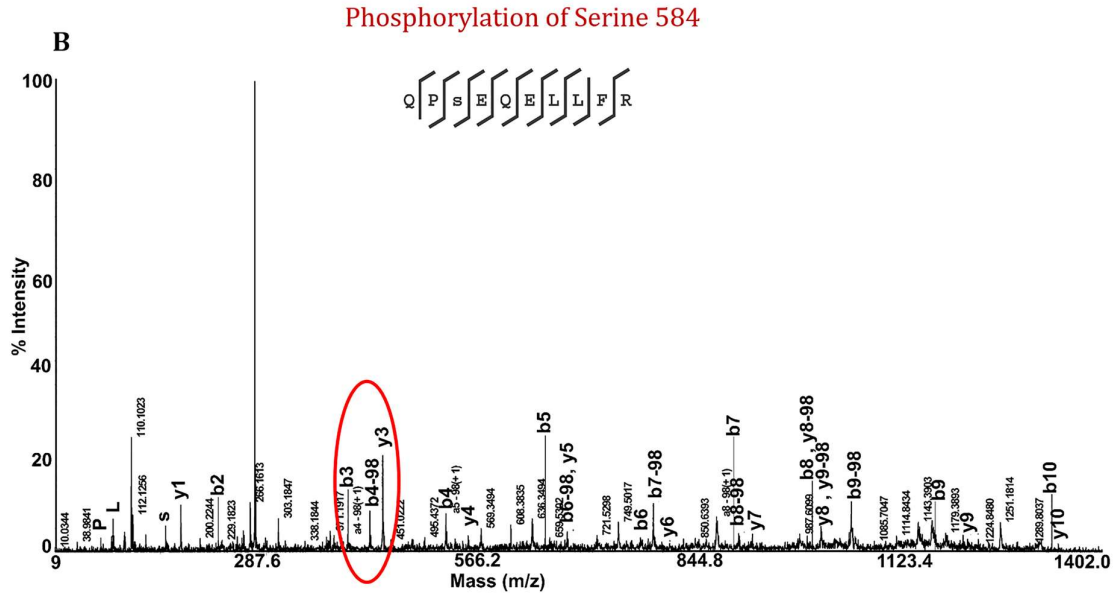



**Figure 15 B:** *MS/MS spectrum of HDAC4 peptide QPSEQELLFR 1326.6869 (582-591 amino acids) purified from a mammalian expression system. The difference between $b_3$ and $b_4$, $y_7$ and $y_8$ suggests us a mass difference of 98 daltons which confirms the presence of Serine phosphorylation at 584 position.*

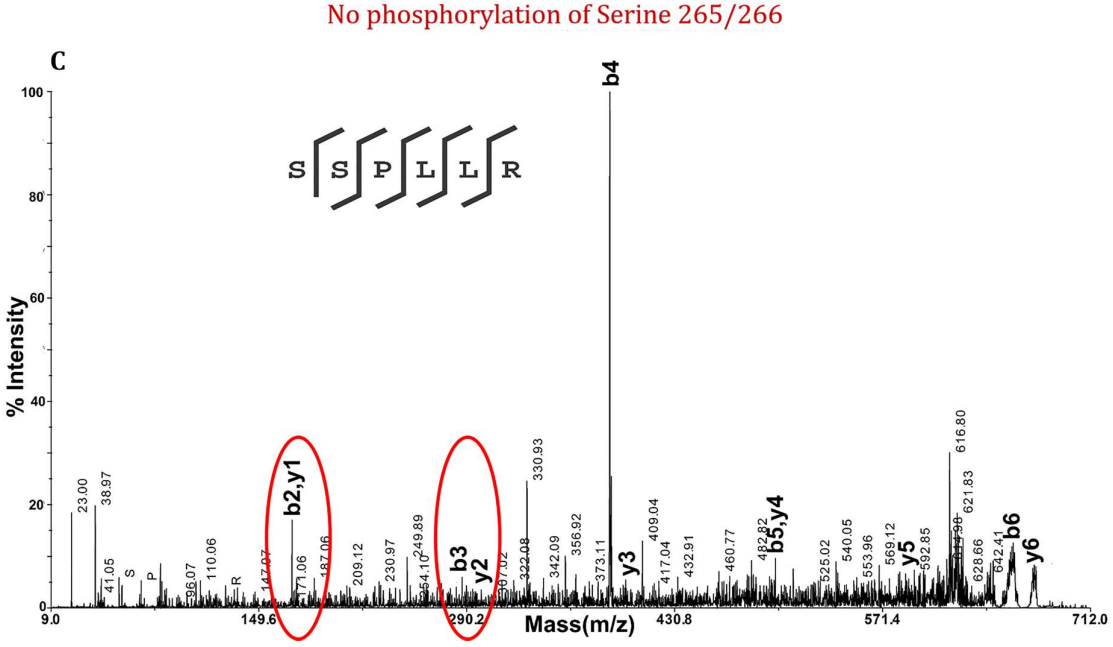



**Figure 15 C:** *MS/MS spectrum of HDAC4 peptide SSPLLR 672.3869 (265-270 amino acids) purified from a bacterial expression system. The red box indicates no significant mass difference between $b_1$, $b_2$ and $b_3$ ions.*

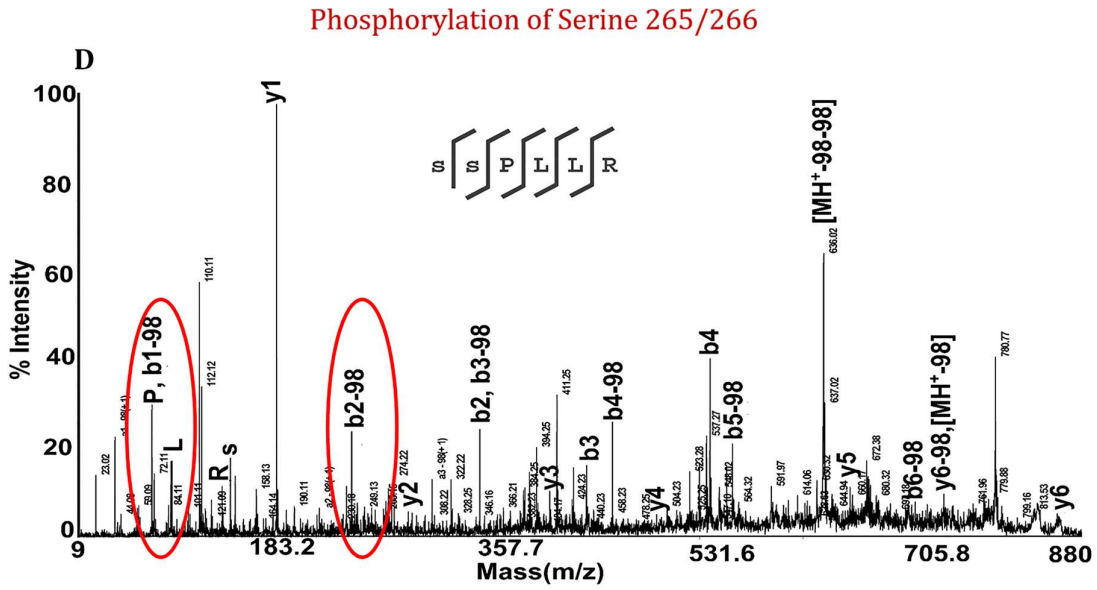



**Figure 15 D:** *MS/MS spectrum of HDAC4 peptide SSPLLR 832.4401 (265-270 amino acids) purified from a mammalian expression system. The difference between $b_1$, $b_2$ and $b_3$ suggests us a mass difference of 196 daltons which confirms the presence of double Serine phosphorylation at 265 and 266 positions.*

Taken together, we confirm the presence of novel Serine phosphorylation at 584 and along with already known serine 265/266 dual phosphorylation.

## 3.2 Objective 2: Functional characterization of the identified PTM of HDAC4

### 3.2.1 Site-directed mutagenesis of Serine 584 and 265/266 to Alanine amino acid:

To understand the significance of identified novel 584 phosphorylation of HDAC4, site-directed mutagenesis of serines at 584, 265 and 266 positions to alanine was carried out by using Quick change lightning multisite-directed mutagenesis kit (Agilent Technologies, USA). Point mutations were also performed for individual Serine at 265 and 266 to Alanine amino acid. (Fig: 16).


**Figure 16:** ***Site-directed mutagenesis.*** *HDAC4 wild-type sequence showing two Serine amino acids in black color. S265A sequence shows the conversion of Serine at 265 positions to Alanine amino acid. Similarly, S2666A and S265266A depicts the conversion of Serine at respective positions to Alanine amino acids.S584A depicts the Serine to Alanine mutation.*

### 3.2.2 HDAC4 activity assay for the mutated 584 and 265/266 positions:

To emphasize the activity of the mutated HDAC4 protein (both S584A and S265266A), histone deacetylase (HDAC) activity was performed. Mutated HDAC4 (S584A and S265/266A) and wild-type protein were overexpressed in HEK 293T cells and after 48 h, the protein was affinity purified using TALON resin. The purified proteins were thus evaluated for the deacetylase activity using fluorescently labeled HDAC substrate (Biomol).

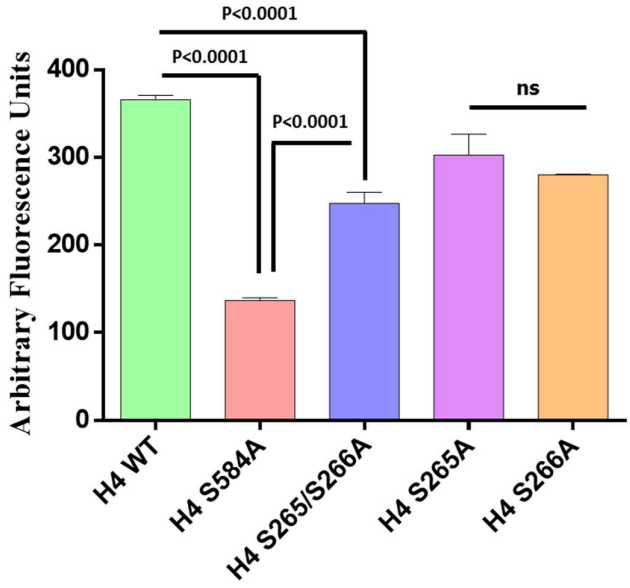



**Figure 17:** ***HDAC4 activity assay of wild-type and mutants.*** *HDAC4 activity assay using fluorescent substrate FLUOR DE LYS®. 1 µg of purified each HDAC4 purified proteins were accessed for the deacetylase activity. The mutant proteins Serine 584 and double mutant S265/266A were able to significantly downregulate the activity of the HDAC4 protein when compared with wild-type protein. Error bar represents S.E (n=3).*

The results identify that the HDAC4 mutants, both S584A and S265/266A, were defective in the activity of the protein significantly when compared to the wild-type. HDAC4 S584A displayed a significant decrease in HDAC activity when compared with the already reported double mutant S265/266A and wild-type protein (Fig: 17). HDAC activity of the point mutants at 265 and 266 also revealed decreased HDAC activity (less significant) of the protein. Thus, the activity assay results indicate the significance of S584 phosphorylation in regulating HDAC4 activity. One of the plausible answers as to why S584A showed a decreased activity is that 584 is close to the catalytic domain and probably phosphorylation of 584 is affecting the protein conformation and thus activity.

### 3.2.3 Localization of HDAC4 wild-type protein and mutant proteins:

HDAC4 protein is majorly regulated by reversible phosphorylation as reported in several studies. Phosphorylation and dephosphorylation of HDAC4 protein signal to undergo dynamic changes in subcellular distribution between nucleus to cytoplasm thereby regulating gene function. Hence we sought out to understand whether these subcellular changes in HDAC4 wild-type protein and its mutant proteins i.e. both S584A and S265/266A do take place or not.

Immunofluorescence analysis using His-tag antibody revealed no significant localization difference between wild-type and its mutant proteins (Fig: 18 A). The amount of HDAC4 protein observed in the cytosol and nuclear fractions were more or less the same in both wild-type and in mutant proteins. Further to support this observation, we have transfected HDAC4 wild-type and its mutants (S584A and S265/266A) individually into HEK 293T cells and after 48 h, cytosolic and nuclear fractions were isolated, transferred to nitrocellulose membrane and probed using HDAC4 specific antibody (Fig: 18 C) and the

results identify no significant difference between cytosolic and nuclear fractions supported by GAPDH (Cytosolic control) and Histone H3 (Nuclear control).

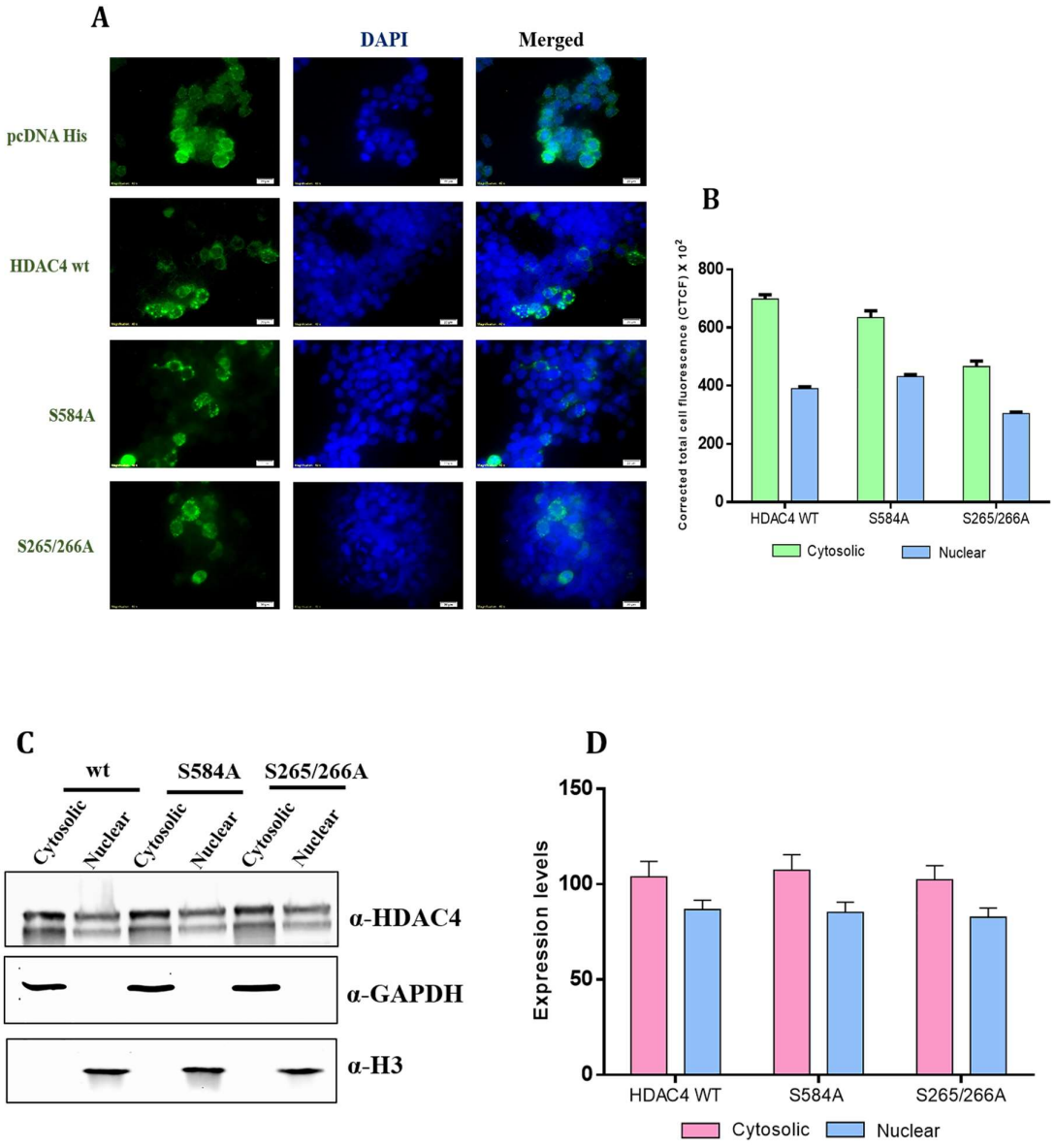



**Figure 18:** ***Confocal microscopy of HDAC4 wild-type and its mutants:*** ***(A)*** *HDAC4 wild-type and its mutants (S584A and S265266A) were transfected into HEK 293T cells. Protein was probed using anti-his antibody and detected using secondary antibody Alexa Fluor 488.* ***(B)*** *Quantification of mean intensity fluorescence in cytoplasm and nucleus.* ***(C)*** *Sub-cellular fractionation of HDAC4 wild-type and its mutants S584A and S265/266A. HDAC4 wild-type and its mutants were transfected into HEK 293T cells and after 48 h, and sub cellular fractions were prepared and probed using HDAC4 antibody. GAPDH was used as cytosolic marker whereas Histone H3 was used as a nuclear marker.* ***(D)*** *Image J quantification of the sub-cellular distribution of HDAC4 wild-type and its mutants. Error bar represents S.E (n=3).*

### 3.2.4 Bioinformatic prediction of the probable kinase at Serine 584 and 265/266 positions:

To further identify the probable kinase responsible for the phosphorylation of HDAC4 at Serine 584 and 265/266 positions, a bioinformatics approach was followed using Net Phos 3.1 from Centre for Biological Sequence Analysis (CBS), the University of Denmark, which specifically identify kinases involved in the phosphorylation based on conserved motifs. Analysis of the results revealed several kinases being involved in the phosphorylation of these sites (Fig. 19). In the case of Serine 265 positions, the analysis predicted PKA (Protein Kinase A) as the potential kinase with high score. But in the case of 266, unspecified kinase was identified with the highest score and other probable kinases like Ribosomal s6 kinase (RSK) and Protein kinase A (PKA) with the similar score. However, earlier studies from Liu et al (Liu and Schneider 2013) identified PKA as the kinase that phosphorylates HDAC4 at 265 and 266 in skeletal muscle fibers. Leading to MEF2-dependent gene regulation. Also, when we analyzed for the PKA-conserved motif, the analysis revealed that the PKA motif (RRXS/T) is more conserved in 265/266 position than unknown RSK motif.

The Net Phos predictions for S584 were similar to that of S266 where an unspecified kinase was with high score followed by RSK and PKA with similar score. Furthermore, the PKA conserved motif was almost conserved at this site with a slight change of one amino acid. The amino acid sequence at 584 was identified as RQPS. PKA motif (RRSS) differs from this motif (RQPS) by change of Glutamine (Q) rather than Arginine (R). Because Serine 584 phosphorylation was identified along with already reported Serine double phosphorylation (Serine 265/266) and the conserved motif analysis depicts PKA, we hypothesized PKA might be probable kinase responsible to phosphorylate serine at 584 position also.

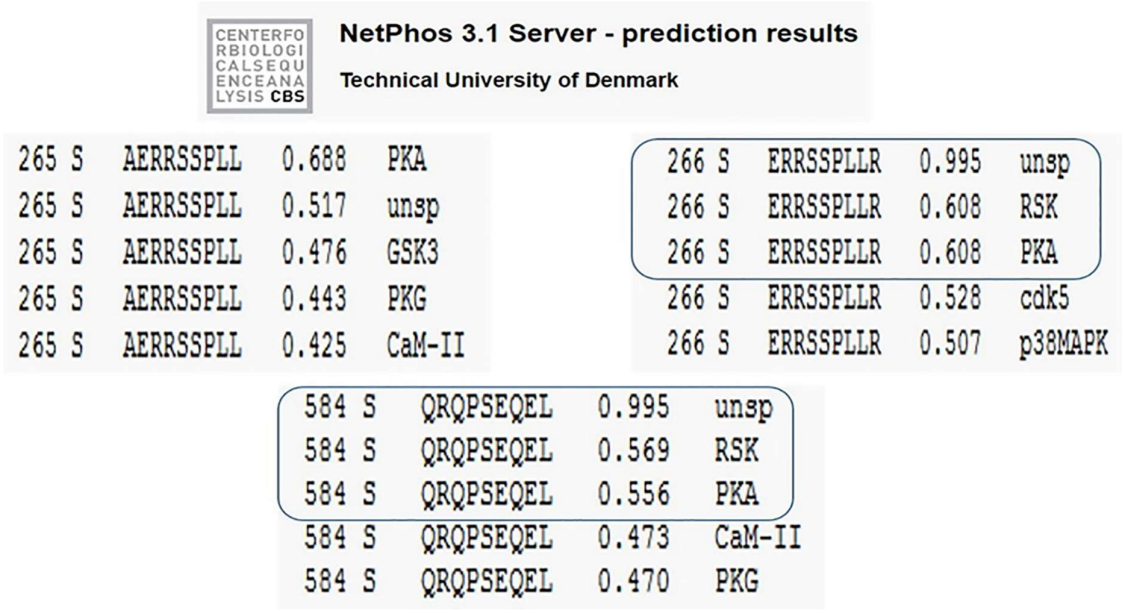



**Figure 19:** ***Bioinformatic prediction of probable kinase using NetPhos 3.1 Server:*** *Kinase responsible for the phosphorylation at the sites 265, 266 and 584 were analyzed using NetPhos 3.1 server. The figure depicts the probable kinase responsible for phosphorylation.*

### 3.2.5 Identification of Protein Kinase A (PKA) as the target kinase:

To identify PKA as the kinase responsible for the Serine phosphorylation at the sites 584 and 265/266, we have employed inhibitor-based assays where we used well-known PKA inhibitor H-89, Dihydrochloride (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) (CST, USA), a reversible and competitive inhibitor for PKA. Several reports highlight the importance of H-89 to be used as PKA-specific inhibitor (Chijiwa, Mishima et al. 1990, Johannes, Prestle et al. 1995, Engh, Girod et al. 1996, Meja, Catley et al. 2004) for protein kinase A (PKA). Hence we asked whether PKA phosphorylation of Serine 584 and 265/266 can be prevented by H-89 (PKA inhibitor), which indirectly suggests us PKA phosphorylation on these sites. To test the hypothesis, HDAC4 wild-type protein was overexpressed in HEK 293T cells in the presence of 20 μM H-89 and the resulting peptides were analyzed for the presence of phosphorylated peptides corresponding to 584 and 265/266 positions.

The results identified the peptides corresponding to the negative phosphorylation of HDAC4 on 584 and 265/266. These peptides were similar to those obtained from bacterially purified HDAC4 protein. The peptide QPSEQELLFR corresponding to PMF 1246.7189 (Fig: 20 A) was obtained in H-89 treated sample which suggests the negative phosphorylation on Serine 584 and the similar negative phosphorylation peptide corresponding to PMF 1246.4459 was obtained in the case of bacterially purified HDAC4 thus indicating the phosphorylation on Serine 584 amino acid was mediated through PKA enzyme.

Similarly, the peptide (SSPLLR) corresponding to the negative phosphorylation at 265/266 was identified with PMF corresponding to 672.01 (Fig: 20 B). Identification of the similar negative phosphorylation in the bacterially purified HDAC4 at 672.3869 suggests us that the phosphorylation was inhibited by PKA inhibitor (H-89) which indirectly suggest us that the PKA as the target kinase responsible for the phosphorylation at both 265/266 position.

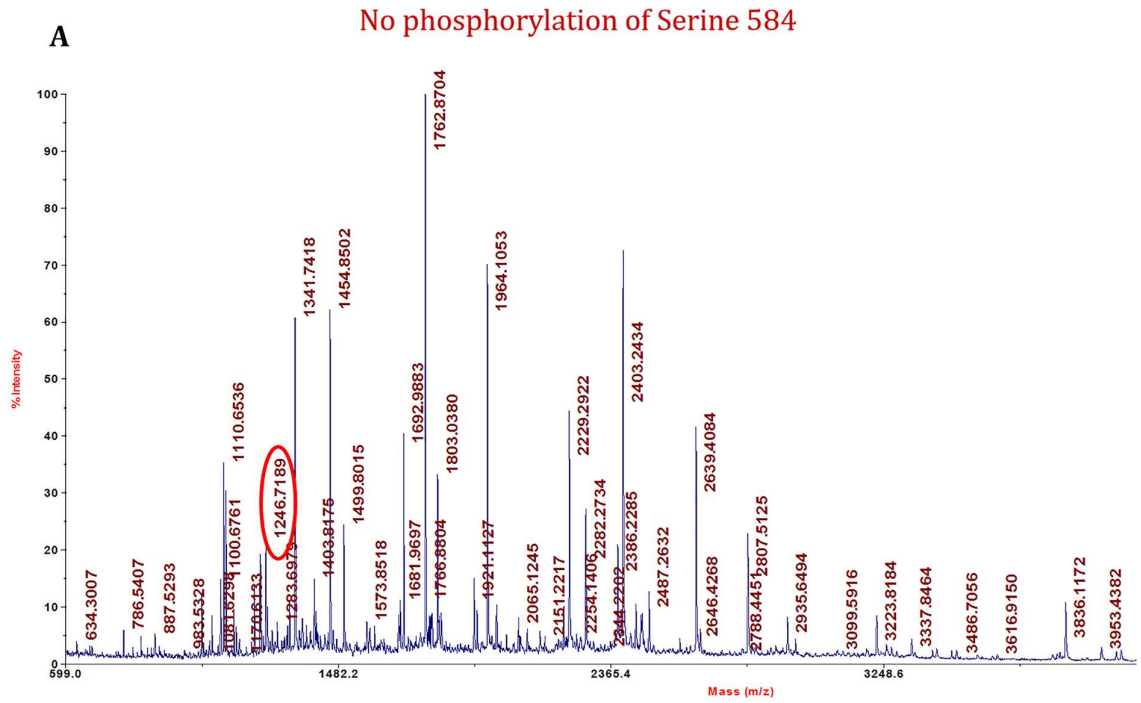



**Figure 20:** ***(A) PMF spectrum of HDAC4 peptide QPSEQELLFR (582-591 amino acids).*** *The wild-type HDAC4 protein was overexpressed in the presence of 20 µM and the protein was subjected to MALDI-TOF/TOF analysis. The presence of no phosphorylation peptide (m/z 1246.7189) corresponding to Serine 584 suggests PKA as a probable kinase.*

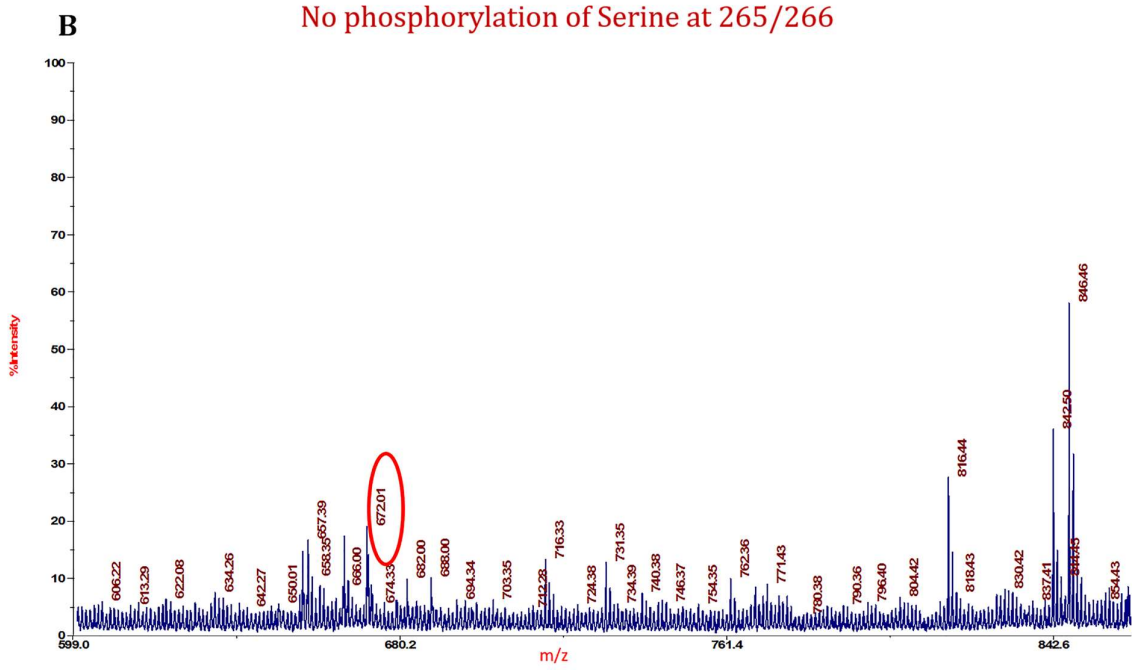



**Figure 20:** ***(B) PMF spectrum of HDAC4 peptide SSPLLR (265-270 amino acids).*** *The wild-type HDAC4 protein was overexpressed in the presence of 20 µM and the protein was subjected to MALDI-TOF/TOF analysis. The presence of no phosphorylation peptide (m/z 672.01) corresponding to Serine 265/266 suggests PKA as a probable kinase.*

As the Serine 265/266 phosphorylation by PKA was well documented by Liu et al., (Liu and Schneider 2013) we have focused our further studies only on novel phosphorylation of Serine 584. To achieve this, we have used *in vitro* kinase phosphorylation assay. A fragment of HDAC4 protein (530-635 amino acids) spanning wild type Serine and mutated Serine (to Alanine) at 584 region was cloned individually into GST tag vector pGEX-4T1 and purified as GST-tag protein. Also, GST protein was purified and used as a control. This purified HDAC4 wild-type and mutant were incubated with PKA enzyme along with [$\gamma$-$^{32}$P]-ATP. The samples were resolved by 15% SDS-PAGE. The proteins were then transferred to nitrocellulose membrane. The membrane was later dried and subjected to phosphorimager (PharosFX$^{TM}$ Plus, Biorad, USA). The results identify positive signal for the wild-type protein incubated with PKA which subsequently displayed low signal in the mutant protein incubated with PKA enzyme (Fig: 21 A). Further, no signal was observed in the wild-type

Serine and mutated Serine without PKA enzyme. No signal was also detected in GST control protein. This confirms the Serine phosphorylation on 584 position by Protein Kinase A (PKA).

Additionally, a similar result was obtained by probing with phosphoserine antibody (Fig: 21 B), which also supports PKA-mediated phosphorylation of Serine 584.

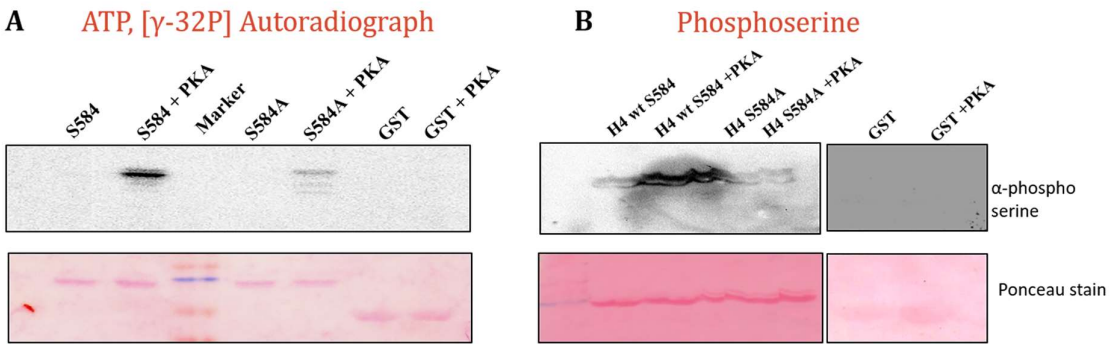



**Figure 21:** ***PKA phosphorylation of Serine 584 position.*** ***(A)*** *Autoradiogram of the in vitro kinase assay of the HDAC4 fragment spanning 584 region.* ***(B)*** *in vitro kinase assay of HDAC 584 fragment probed with phosphoserine antibody.*

### 3.2.6 Luciferase assay of the MEF2C promoter:

A family of transcription factors, MEF2 (Myocyte enhancement factor 2) is mainly involved in the differentiation of various cell types which include cardiac, skeletal, smooth muscle, chondrocytes, endothelial cells, neurons and neural crest cells (Pon and Marra 2016). HDAC4 is known to repress MEF2-related gene transcription. HDAC4 along with MEF2 forms complex and this complex binds to the promoter of the target genes thereby causing gene repression (Zhao, Sternsdorf et al. 2005). The repressive activity of HDAC4 and its mutants (S265/266A and S584A) on MEF2 gene targets, was assessed the luciferase signal from the MEF2 promoter fused to the luciferase gene. The luciferase MEF2 promoter construct (4X-Luc promoter) was a gift from Prof. Ron Prywes, University of Columbia, USA. Trichostatin A (TSA) (HDAC inhibitor) was used as control to evaluate the reaction efficiency.

The results identified that HDAC4 mutants, both S265/266A and S584A, were able to relive the transcription of luciferase gene indicating that the mutants were less repressive than the wild-type HDAC4 protein (Fig: 22).

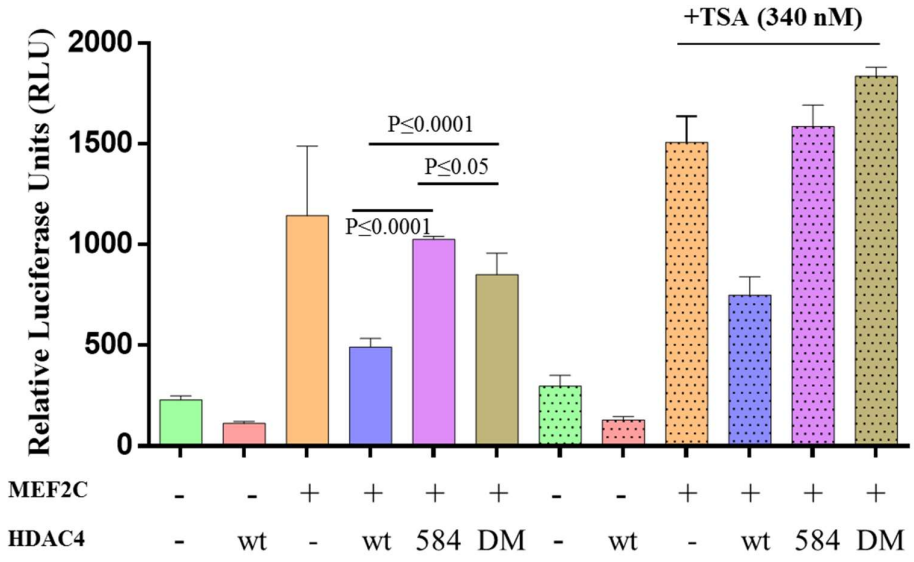



**Figure 22:** ***Luciferase assay of the MEF2C promoter in combination with the HDAC4 wild-type and the mutants.*** *Wild-type HDAC4 and its mutants (S265/266A and S584A were co-transfected along with MEF2C plasmid into HEK 293T. After 48 h, cells were lysed and reading was recorded in a luminometer. The HDAC4 mutants both S265/266A and also S584A were able to relieve the transcriptional ability of the MEF2-luc promoter than the wild type protein. Error bar represents S.E (n=3).*

The double mutant S265/266A was also able to relive the luciferase expression but not as effective as the S584A mutant.

### 3.2.7 Real-time PCR of the MEF2C target genes:

After examining the HDAC4 mutants efficacy at the promoter level, we next want to understand the expression levels of the MEF2C target genes. The selected gene targets include Anxa8, Irs1, Klf2, Klf4 and RhoB as mentioned by Giorgio et al. in 2013 (Di Giorgio, Clocchiatti et al. 2013) and qPCR was carried out to quantify the gene transcription. As shown in the figure (Fig: 23) the target genes Anxa8, Klf2 and RhoB were upregulated

significantly in S584A mutant when compared with S265/266A and wild-type. This might be due to the fact that the mutant HDAC4 S584A was not able to form a stable complex with MEF2C which further cannot bind to the promoter of the MEF2C target genes and repress them. The other genes such as Irs1 and Klf4 showed no significant difference between all the three cases (wild-type, S265/266A, and S584A). Hence we conclude that the mutant forms of HDAC4 in particular S584A was able to decrease the activity of HDAC4 protein thus proving the fact that the phosphorylation at these sites is important for the function of the protein.

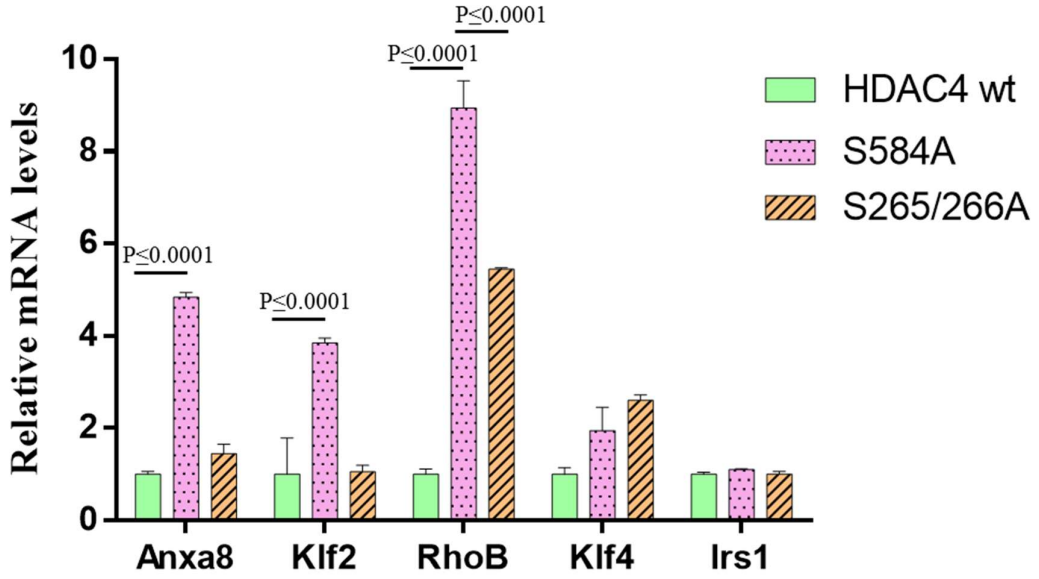



**Figure 23:** ***qPCR of the MEF2C target genes.*** *HDAC4 (wild-type/S584A/S265266A) along with MEF2C were co-transfected. After 48 h, total RNA was prepared and converted to cDNA was employed as a template in real-time PCR. The target genes (Anxa8, Klf2, and RhoB) were upregulated in S584A and S65/266 mutants. Klf4 and Irs1 did not show any significant upregulation. GAPDH was used as an internal control. Error bar represents S.E (n=3).*

# DISCUSSION

## 4. Discussion:

Epigenetic regulation of gene expression is mainly brought about by three factors such as histone modifications, DNA methylation and chromatin structure modification (Kim 2014) . A major pool of enzymes involved in gene expression and repression brings about histone modifications. Histone modifications are performed by HATs (histone acetyltransferases) and HDACs (histone deacetylases) which adds or remove acetyl groups from histone proteins as well as non-histone proteins (Bernstein, Meissner et al. 2007).

HDACs remove acetyl group from ε-lysine of histone proteins and non-histone substrates resulting in closed chromatin conformation and thus repressing gene function (Minucci and Pelicci 2006). HDAC enzymes themselves undergo dynamic epigenetic regulation such as post-translational modifications to contribute to diversity in gene function regulation. HDACs are majorly divided into 18 major classes, which induce diverse functions in a cell with a cross-talk between them that explains the redundancy in sharing the common substrates (Zupkovitz, Tischler et al. 2006). The overlapping functions among different classes of HDACs and sharing of common substrates can best be explained by the cell-type specific function of HDACs, which are further regulated by PTMs. Therefore identification of PTMs is an important aspect to understand HDAC regulation. Among the HDACs, our major focus was on Class IIa HDAC i.e. HDAC4 where it is known to be majorly regulated by reversible phosphorylation. Hence we aimed at identifying other PTMs of HDAC4.

HDAC4 phosphorylation has been known to play a diverse range of functions in a cell. Also, HDAC4 protein role in tissue and cellular functions is being well understood in recent times (Liu, Pore et al. 2006). HDAC4 proteins are widely deregulated in several disease conditions (Lemercier, Brocard et al. 2002, Chauchereau, Mathieu et al. 2004, Barrett, Santangelo et al. 2007, Wilson, Byun et al. 2008, Mottet, Pirotte et al. 2009, Zhang, Yang et al. 2010, Geng, Harvey et al. 2011, Stronach, Alfraidi et al. 2011, Xu, Wang et al. 2011, Sandhu, Volinia et al. 2012). Further, regulation of HDAC4 is majorly by reversible

phosphorylation (Wang, Qin et al. 2014) mechanism which mediate wide range of functions pertaining to sub-cellular distribution (Backs, Song et al. 2006), histone protein and non-histone protein deacetylation (Fischle, Dequiedt et al. 2002, Jeon, Lee et al. 2006) etc. Several serine/threonine kinases regulate reversible phosphorylation in response to several stimuli, which facilitate its subcellular distribution (Backs, Song et al. 2006). Apart from known phosphorylation signatures of HDAC4, several of them remain unexplored which render to delineate HDAC4 function. In the present study, we have aimed at identification of novel phosphorylation sites in the HDAC4 protein.

In order to achieve, we have cloned HDAC4 full-length human protein into two different expression systems. One is bacterial expression system and the other being the mammalian expression system. HDAC4 expressed and purified from bacterial expression system *(E.coli)* was considered to be negative for phosphorylation and the other purified from mammalian expression system (HEK 293T) was considered as a test sample. The tryptic digested peptides subjected to MALDI TOF/TOF were scanned for the presence of 80 or 98 daltons shift corresponding to phosphorylation. The analysis revealed two serine phosphorylations: serine 265/266 double phosphorylation and the other serine phosphorylation at 584 position. Of these, Serine phosphorylation at 265/266 has been earlier reported by Liu et al. in 2013(Liu and Schneider 2013) in skeletal muscle cells and it assisted as positive control in our further experiments. The other Serine 584 phosphorylation is novel and has not been reported until now. Our further studies were focused to understand the functional significance of this phosphorylation.

To functionally characterize the S584 phosphorylation, we have mutated serine amino acids present at 265, 266, 265/266 and 584 positions to alanine amino acid, cloned them into mammalian expression vector. These were expressed in HEK 293T and purified using His tag resin. HDAC activity assay of the wild type and mutated proteins indicated a significant decreased activity of S584A mutant compared to S265/266A and wildtype mutant. The other

mutants S265A and S266A did not show any significant decrease in the activity when compared with the wild-type protein.

HDAC4 protein is well known to undergo dynamic changes in sub cellular distribution with respect to post-translational modifications. Hence we asked whether these identified mutants have any role in sub cellular localization of the protein. Immunofluorescence analysis using His tag antibody between HDAC4 wild-type and its mutants (S584A and S265/266A) identified no significant difference between wild-type HDAC4 and mutant proteins. Furthermore, subcellular fractionation established no difference in the subcellular distribution between HDAC4 wild-type and its mutant proteins.

As the serine 265/266 has been reported earlier by Liu et al. in 2013, we have focused on the identification of kinase responsible for the phosphorylation at Serine 584 position. Bioinformatic analysis of probable kinase using NetPhos 3.1 software predicted an unknown kinase (score 0.995), Ribosomal s6 kinase (RSK) (score 0.569) and protein kinase A (PKA) as the target kinase (score 0.566). However, since 584 phosphorylation was identified along with 265/266 and phosphorylation of 265/266 by PKA is reported earlier, we speculated PKA as target kinase. Another reason for this speculation is the presence of PKA-conserved motif RRXS/T with an exception of one amino acid ((ROXS) Glutamine in place of Arginine).

To understand the PKA phosphorylation of Serine 584 phosphorylation, we have used PKA inhibitor i.e. H-89. Several reports have established H-89 as reversible and competitive inhibitor, which is widely known to inhibit PKA activity (Chijiwa, Mishima et al. 1990). Overexpression of HDAC4 wild-type protein in the presence of H-89 and subsequent MALDI analysis of the tryptic digested purified wild-type protein was performed for the presence of phosphorylated peptides corresponding to 584 position. The results indicated negative phosphorylation of HDAC at 584 and 265/266 serine residues suggesting PKA as the kinase.

To further establish PKA as the target kinase for serine 584 phosphorylation, we have used *in vitro* kinase assay using [γ-32P] ATP. HDAC4 protein of 100 amino acids spanning the 584 position (530-635 amino acids) was cloned into PGEX-4T1 plasmid and purified using GST tag protein. These proteins were assayed for the incorporation of the radiolabeled [γ-32P] ATP with and without PKA kinase. Positive signal of the autoradiogram in the wild-type HDAC4 containing Serine at 584 was observed and but not in mutated Serine. This authenticates PKA phosphorylation of HDAC4 on Serine 584 position. This was further supported by using phosphoserine antibody.

In order to identify the functional significance of the 584 phosphorylation, promoter luciferase assay was performed for the MEF2C promoter. HDAC4 is well known to interact with MEF2 group of transcription factors and thus leading to MEF2 transcriptional repression by catalyzing local histone deacetylation (Zhao, Sternsdorf et al. 2005). HDAC4 wild-type and its mutants S584A S265/266A were co-transfected individually along with MEF2C into HEK 293T cells. The wild-type protein displayed decreased luciferase signal as reported in earlier studies, but increased luciferase signal was observed in the case of mutants S584A and S265/266A suggesting mutants were defective in repressing MEF2C mediated gene repression. The S584A mutant displayed significant decrease in activity than S265/266A implying serine 584 is one of the important phosphorylation events required for the activity of the protein.

Further, the important role of 584 phosphorylation in MEF2C-mediated gene repression was delineated by real-time PCR analysis of MEF2C target genes. The genes such as Anxa8, Irs1, Klf2, Klf4 and RhoB are under the influence of MEF2C transcription factor. Real-time PCR analysis demonstrated that the genes Anxa8, Klf2, and RhoB were significantly upregulated in the case of mutant 584 when compared to wild-type and S265/266A. Klf4 and Irs1 showed no significant difference between wild-type and its

mutants. One possible explanation is that these genes might not be under the influence of HDAC4/MEF2C axis in HEK 293T cells.

Hence altogether, we confirm the novel Serine phosphorylation on 584 position in HDAC4 protein, which is catalyzed by PKA kinase. The site Serine 584 is important for the activity of the protein shown by mutational studies. Increased luciferase signal in mutant S584A confirms site is important for the activity of the protein. Real-time PCR analysis of the MEF2C target genes highlight that the Serine 584 phosphorylation mediate repression of genes in HDAC4/MEF2 axis.

# CONCLUSION

## Conclusions:

**The following are the conclusions from the results of the study.**

- *In silico* analysis of the probable post translational modifications predicted novel as well as already reported modifications.
- MALDI TOF/TOF analysis of the mammalian purified HDAC4 protein identified novel Serine 584 phosphorylation along with double serine phosphorylation at 265/266 position.
- Site directed mutagenesis followed by activity assay revealed that phosphorylation of Serine at 584 is important for HDAC4 activity.
- Unlike other known phosphorylations of HDAC4, S584 and S265/266 did not alter the sub-cellular localization of HDAC4 protein.
- Bioinformatic analysis using NetPhos 3.1 identified PKA as potential kinase phosphorylating S584 and S265/266.
- Expression and purification of HDAC4 protein in presence of H-89, PKA inhibitor, followed by MALDI TOF/TOF analysis revealed no unphosphorylated peptides of S584 and S265/266 positions confirming PKA as the responsible kinase.
- *In vitro* phosphorylation assay using [$\gamma$-$^{32}$P]-ATP followed by immunoblot with phosphoserine antibody established PKA as the target kinase for novel Serine 584 phosphorylation.
- MEF2C promoter luciferase assay results clearly defined the functional significance of S584 phosphorylation where the mutant S584A showed significantly less repression of MEF2C promoter compared to wild-type and S265/266A proteins.
- S584 phosphorylation of HDAC4 is important to regulate genes in HDAC4/MEF2C-axis as evident from Real-time PCR analysis of the MEF2C target genes: Anxa8, Klf2, RhoB, Klf4 and Irs1.

# SUMMARY

## 6. Summary

Histone deacetylases (HDACs) being multiprotein enzyme complexes are involved in the removal of an acetyl group from histone proteins. Removal of acetyl group enhances positive and negative interactions between basic histones and negative phosphate groups of DNA thus resulting in gene repression. HDACs act either independently or in a complex deacetylating histone and non-histone proteins. Further, HDACs undergo a wide range of post-translational modifications (PTMs), which lead to alterations in DNA-histone interactions and binding to non-histone targets like transcription factors, repressors, enhancers etc. Numerous studies signify the importance of targeting HDACs in cancer therapy as these group of enzymes are widely deregulated in different types of cancers and inhibitors of HDACs are in various phases of clinical trials.

Mammalian HDACs are majorly 18 in number divided into four different classes with respect to homology with yeast deacetylases (de Ruijter, van Gennip et al. 2003,Blander and Guarente 2004, Gregoretti, Lee et al. 2004). These include Class I, II, III and IV. Of these, Class II is further classified into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC 6 and 8) based on domain organization and homology. Class II HDACs consists of majorly conserved C-terminus catalytic domain homologous to the yeast HDA1, and do not share N-terminus similarity to other classes of histone deacetyalses (Verdin, Dequiedt et al. 2003). Class I, II and IV require $Zn^{+}$ as co-factor whereas class III requires $NAD^{+}$ as co-factor.

HDAC4 belongs to class IIa HDAC and is homologous to yeast deacetylase HDA1 (Wang, Bertos et al. 1999). It shares 60-70% similar sequence identity with other class II HDACs such as HDAC5 and 7 (de Ruijter, van Gennip et al. 2003). The HDAC4 expression is ubiquitous in nature with overexpression in the brain, heart and skeletal muscle (Verdin, Dequiedt et al. 2003, Broide, Redwine et al. 2007). HDAC4 is localized to chromosome

2q37.3. The HDAC4 coding sequence is 3250 bp long with protein of 120 kDa size. Transcription of HDAC4 is brought about by binding of Sp1 and Sp3 transcription factors (Liu, Pore et al. 2006). Crystal structure of N-terminal region of HDAC4 (62- 153 amino acids) reveals the protein with glutamine-rich domains containing straight alpha helix that folds as a tetramer (Guo, Han et al. 2007). The HDAC4 catalytic domain is present in the C-terminal end (648-1057 amino acids) (Bottomley, Lo Surdo et al. 2008). Nuclear localization domain important for nuclear localization is from 244-279 (Wang and Yang 2001). Like other class II HDACs, HDAC4 shuttles between nucleus and cytoplasm.

HDAC4 undergoes extensive PTMs such as phosphorylation, ubiquitinylation, carbonylation and proteolytic cleavage. These modifications lead to altered subcellular localization and its interaction with protein partners. Unraveling these regulatory networks will assist to understand the role in physiological and pathological processes. Further, the HDAC4 expression, PTMs and interacting proteins differ from one cell type to other resulting in wide range of physiological functions such as cell development and differentiation as evident from earlier studies (Wang, Qin et al. 2014). Hence a detailed understanding of PTMs will help to uncover regulatory functional aspects of the protein.

With this background, the aim of the study was to unravel different posttranslational modifications of HDAC4. To achieve this aim, the following objectives were set:

1. Identification of novel HDAC4 PTMs.

    a. *In silico* prediction of probable PTMs of HDAC4.
    b. Identification of PTMs of HDAC4 by MALDI-TOF/TOF analysis.

2. Functional characterization of the identified PTM of HDAC4.

First, to identify the sites of post-translational modifications (PTMs) in HDAC4 protein, bioinformatics prediction of probable PTMs was carried out using various softwares

available at Expasy.org. The analysis predicted some of the earlier reported sites and novel sites of modifications. Therefore, next an attempt was made to identify the predicted modifications in HDAC4 protein expressed and purified from mammalian expression system. Bacterially expressed and purified protein was considered as PTM negative control.

Initially, an effort was made to identify all possible diverse PTMs of HDAC4. But were successful only in identifying phosphorylations that too after Phosphopeptide enrichment followed by MALDI-TOF/TOF analysis. The results identified three phosphorylation sites in the HDAC4 protein: a novel Serine584 phosphorylation and the dual phosphorylations of Serineat 265/266 that were reported earlier. Our *in silic*o prediction results are very much in line with these experimental results.

To further understand the role of S584 and S265/266 phosphorylations, site-directed mutagenesis was carried out. HDAC4 activity assay of the Serine mutants indicated that Serine584 phosphorylation is important for the HDAC4 activity. Also, unlike other sites of HDAC4 phosphorylations which result in nucleocytoplasmic shuttling of protein, the S584 and S265/266 mutants did not show any localization difference.

Next, bioinformatics analysis was performed to identify probable kinase responsible for the phosphorylation at Serine 584 using NetPhosK 1.0 server. The analysis predicted PKA as the target kinase for S584 and S265/266 sites. The phosphorylation of S265/266 by PKA is reported earlier. Since S584 was identified along with S265/266 phosphorylation and the presence of conserved PKA motif at S584 region, we assumed PKA as the probable kinase phosphorylating S584.

To determine PKA-mediated S584 phosphorylation of HDAC4, we purified wild-type HDAC4 protein expressed in presence of inhibitor of PKA, H-89. The protein thus purified was subjected to MALDI-TOF/TOF analysis and the results identified no phosphorylation

peptides corresponding to Serine584 along with reported 265/266 thus suggesting PKA as the responsible kinase. Further, to confirm the PKA phosphorylation of HDAC4 at Serine584, in vitro kinase assay was performed by incubating purified short fragment of HDAC4 (530-635 amino acids) spanning 584 region with PKA pure enzyme and [$\gamma$-$^{32}$P]-ATP. The results identified positive signal for HDAC4 at Serine584 when incubated with PKA, which was subsequently absent in S584A mutant. This altogether confirms PKA-mediated phosphorylation of Serine584.

HDAC4 is known to repress MEF2C related gene transcription. HDAC4 along with MEF2C is known to form a complex and bind to promoters of MEF2 target genes thereby repressing gene function of MEF2C (Backs, Song et al. 2006, Zhang, Kohlhaas et al. 2007, Backs, Worst et al. 2011). To understand the functional role of S584 phosphorylation, the repressive function of wildtype and mutant HDAC4 proteins was assessed using MEF2C promoter luciferase assay. The results were in line with activity assay results where the mutant S584A was not able to repress the MEF2C promoter resulting in increased luciferase activity and suggested that S584 phosphorylation is important for function of HDAC4. This was further confirmed by real time PCR analysis of MEF2C target genes (Anxa8, Irs1, Klf2, Klf4, and RhoB). In conclusion, the study results identify novel Serine phosphorylation of HDAC4 at 584 residue and S584 phosphorylation is important for HDAC4 activity in repressing MEF2C-target genes (Fig. 24).

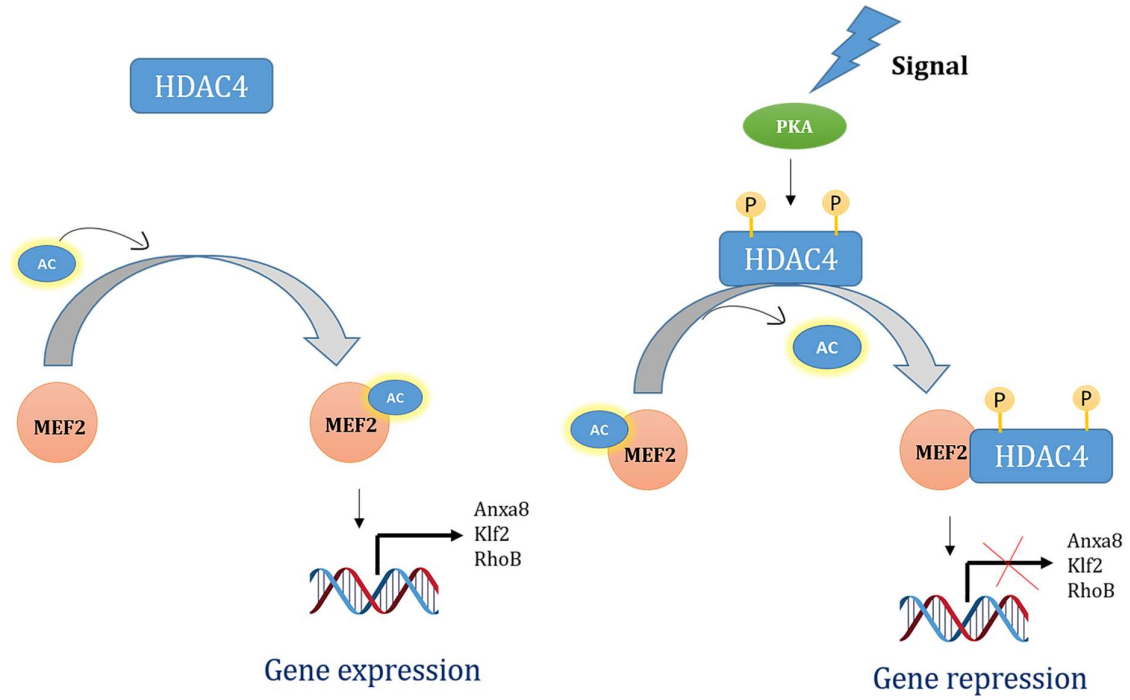



**Figure 24:** *Schematic representation of proposed model for HDAC4 Serine phosphorylation at 584 position leading to MEF2C dependent gene repression.*

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# CPMV-induced synthesis of hollow mesoporous $SiO_2$ nanocapsules with excellent performance in drug delivery†

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Hollow mesoporous-$SiO_2$ nanocapsules have been synthesized at room temperature using unmodified cowpea Mosaic Virus (CPMV) as a template, and without using any catalyst or surfactant during the synthesis. The average size of the capsules synthesized was ~200–250 nm with a 60–100 nm hollow core. The resulting nanocapsules were characterized using high resolution transmission electron microscopy (HRTEM). The biocompatibility of the hollow mesoporous $SiO_2$ nanocapsules was investigated with an MTT assay using the RAW 264.7 cells, HepG2 cells (human liver carcinoma cells), and Hek293 cells (human embryonic kidney cells). The nanocapsules were loaded with fluorescent molecules (rhodamine 6G), doxorubicin (DOX) – an anticancer drug, and chloroquine diphosphate (CQDP) – an antimalarial drug, and their release was studied using a UV-Vis spectrometer. The development of surfactant free, biosafe, hollow and mesoporous $SiO_2$ nanocapsules with CPMV provides a route for the synthesis of porous nanocapsules for drug loading and the sustained delivery of drugs. The synthesis method for hollow mesoporous $SiO_2$ nanocapsules using CPMV is novel, straightforward, and further demonstrates that, in general, nanoformulated capsules can be used for various drug-delivery-based therapeutic applications. To check the *in vitro* efficacy in medical biotechnology, Hek293 and HepG2 cell lines were used to study the cell viability of DOX-loaded hollow silica nanocapsules. The results show that the bio $SiO_2$ nanocapsules synthesized with CPMV present an effective cargo and are suitable for nanoformulating with DOX, with the resultant nanoformulation showing good promise for killing cancer specific cells.

## Introduction

Hollow nanoparticles or nanocapsules[1,2] are emerging as one of the most promising tools in medical biotechnology for a variety of therapeutic applications, such as target drug delivery for treating cancer or tumour cells,[3–8] gene delivery and in molecular bio-imaging.[9–11] To date, an enormous research effort has been devoted to the search for suitable materials and methods to achieve hollow nanocapsules based on polymers,[3,5,6] liposomes,[12] inorganic metals[13–15] and oxides,[4] with specific surface properties, as these are very important for their therapeutic applications, otherwise drug-loaded capsules can fall off the target tissues, due to cell turnover.[16,17] Selective etching based on structural differences has also been employed to produce hollow inorganic and mesoporous core–shell nanocapsules.[18,19] Hollow nanocapsules have been synthesized in various approaches, *e.g.* by template synthesis[6] using nanoparticles (*e.g.* Ag, Au, $SiO_2$, ZnO), by a self-assembly process,[20,21] *etc.* Most of these approaches are restricted by the composition, size and surface properties needed to administrate medicine to the target site and by the biocompatibility of the capsules. The limitations of using nanocapsules in the delivery of medicines are related to the low loading efficiency and sluggish mass transfer to the target sites.[22–24] Although there has been progress in the use of hollow nanocapsules to protect the loaded drugs or genes, they remain very unstable in physiological environments, and are readily excreted *via* the kidneys and also they are non-specifically absorbed through the reticuloendothelial system, where they can then delay/destroy/alter standard cell activities, thus creating unwanted side effects.[16,17,25] A number of research works on the synthesis of hollow organic or inorganic $SiO_2$ have been reported

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†Electronic supplementary information (ESI) available: TEM and SEM images of control $SiO_2$ nanocapsules (without CPMV), TEM images of CPMV, AFM images of CPMV, TEM images of $SiO_2$ with CPMV at different ratios of APTES: TEOS, XRD, DLS and SAED pattern of hollow $SiO_2$ nanocapsules. See DOI: 10.1039/c4dt02549k