

BIOCHEMICAL CHARACTERISATION OF ABHD14B: A NOVEL LYSINE DEACETYLASE

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

A thesis submitted in partial fulfilment of the requirements of the degree
of Doctor of Philosophy

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2023

DEDICATION

I dedicate this work to my parents

DR. JAYASHREE VAIDYA & DR. RAJENDRA VAIDYA

and to my grandmother

MRS. VRINDA B. JOSHI

CERTIFICATE

I certify that the work incorporated in the thesis titled “Biochemical characterisation of ABHD14B, a novel lysine deacetylase” submitted by Kaveri Vaidya was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other universities or institutions.

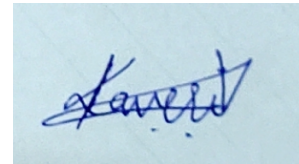
A handwritten signature in black ink, appearing to read 'S. K. Kamat', written over a horizontal line. There are two small dots below the line to the right of the signature.

Supervisor

Date: 4-12-2023

DECLARATION

I declare that this written submission represents my ideas in my own words and where others ideas have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources, which have thus been properly cited, or from whom proper permission has not been taken when needed.



Kaveri Vaidya

Date: 02.12.23

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TABLE OF CONTENTS

DEDICATIONS	2
CERTIFICATES	3
DECLARATION	4
ACKNOWLEDGMENTS.....	5
TABLE OF CONTENTS	6
LIST OF FIGURES.....	8
LIST OF TABLES	9
ABSTRACT	10
Chapters	
I: Introduction	11
II: ABHD14B is a novel lysine deacetylase	
Introduction	20
Materials	22
Methods	22
Results	29
Discussion	40
III: Characterisation of ABHD14B mutants	
Introduction	42
Materials	43
Methods	43
Results	46
Discussion	54
IV: Crystal Structure of S111A ABHD14B	
Introduction	57
Materials	58
Methods	58
Results	60
Discussion	63
V: ABD14B in a Fed/Fasted Paradigm	
Introduction	64
Materials	65

Methods	65
Results	69
Discussion	74
 FUTURE PROSPECTS.....	 76
REFERENCES.....	77
 PUBLICATIONS.....	 85
COPYRIGHT PERMISSIONS LICENCE	86
VITA	87

LIST OF FIGURES

Figure 1.1: Conserved Ser-His-Asp catalytic triad in Serine hydrolases.....	12
Figure 1.2: Conserved catalytic mechanism followed by the serine hydrolase family.....	13
Figure 1.3: The topology of the ABHD fold	14
Figure 1.4: The crystal structure of ABHD14B.....	19
Figure 2.1: mRNA levels of ABHD14B from the Human Protein Atlas.....	20
Figure 2.2: Assays used to study ABHD14B.....	21
Figure 2.3: Coomassie gel showing purification scheme of untagged ABHD14B.....	30
Figure 2.4: Gel-based ABPP assays with WT and S111A human ABHD14B.....	30
Figure 2.5: Colorimetric enzymatic assay with pNP-acetate and its other analogs.....	31
Figure 2.6: Thermal shift assays showing binding of both CoA and AcCoA to S111A (A) and WT (B) human ABHD14B.....	32
Figure 2.7: Colorimetric enzymatic assay with pNP-acetate	33
Figure 2.8: Acetyl transfer/ Deacetylase activity of ABHD14B.....	34
Figure 2.9: Effect of ABHD14B knock down on HEK293T cells.....	36
Figure 2.10: ABHD14B performs a lysine deacetylase reaction on peptide and protein substrates.....	37
Figure 2.11: A- The tunnels calculated by CAVER and MOLEonline.....	39
Figure 2.12: Lysine deacetylase reactions catalysed by ABHD14B in contrast to those catalysed by Sirtuins and HDACs.....	41
Figure 3.1: Sequence alignment of human ABHD14B and ABHD14A. ABHD14B is the query and ABHD14A as the subject.....	43
Figure 3.2: Phylogenetic tree of ABHD14B representing the identified sequences from 697 organism.....	48
Figure 3.3: Conserved residues mapped on the structure of ABHD14B (1imj).....	51
Figure 3.4: Purification gel of W198A ABHD14B showing the two-step purification.....	52
Figure 3.5: Purification of D162A using Ni-NTA His Bind resin.....	52
Figure 3.6: ABPP of the mutants. The upper panel represents the activity gel and the lower panel represents the Coomassie gel used as a loading control.....	53
Figure 3.7: Catalytic activity of ABHD14B mutants to turn over pNP-acetate.....	53
Figure 3.8: Orientation of S75 with respect to the active site. The active site residues are highlighted in green and S75 is highlighted in teal.....	56
Figure 4.1: Covalent inhibition of serine hydrolases by FP-Rh.....	57

Figure 4.2: Initial screen for crystallization of human WT and S111A ABHD14B.....	59
Figure 4.3: human S111A ABHD14B orthorhombic crystal.....	60
Figure 4.4: Structure of human S111A ABHD14B.....	62
Figure 4.5: Structural alignment of S111A ABHD14B and WT ABHD14B.....	62
Figure 5.1: Anti- ABHD14B Western blot to measure protein levels of ABHD14B on Fasting.....	70
Figure 5.2: ABHD14B levels in HEK293T cells in high glucose versus low glucose Media.....	71
Figure 5.3: Cellular localisation of ABHD14B in fed and fasted mouse liver using western blot.....	72
Figure 5.4: Immunoprecipitation of native ABHD14B from mouse liver.....	72
Figure 5.5: Enrichment of native ABHD14B from soluble mouse liver using Sephadex G100.....	73

LIST OF TABLES

Table 1.1: human ABHD proteins and their functions.....	16
Table 2.1: Kinetic constants for WT human ABHD14B against different acylated ester of pNP.....	31
Table 3.1: Fully conserved residues (present in >90% of all the sequences) of ABHD14B...	49
Table 3.2: Functionally conserved residues of ABHD14B.....	50
Table 3.3: List of all the residues mutated.....	51
Table 3.4: Predicted phosphorylation sites on ABHD14B as predicted by GPS6.0.....	56
Table 4.1: Data collection statistics for S111A ABHD14B crystals.....	61

Abstract

Biochemical characterisation of ABHD14B: a novel lysine deacetylase.

Serine hydrolases are one of the largest and most diverse enzyme families. The members of this family play important roles in almost all physiological processes and many are therapeutic targets. Yet many of their members remain unannotated. In this work, I have annotated one such orphan serine hydrolase ABHD14B (alpha-beta hydrolase domain containing protein 14B). This enzyme was first discovered as an interactor of the HAT (histone acetyl transferase) domain of the general transcription factor, TF2D (transcription factor 2D) using a yeast two hybrid cross. On solving its structure, it was found to have a catalytic triad (Serine-Histidine-Aspartate) in its active site. It was also found to have a canonical “alpha-beta hydrolase domain” from where it got its name and its classification as a metabolic serine hydrolase. To study this enzyme in greater detail, I recombinantly expressed and purified the wild type (WT) and S111A mutant of this protein using *E.coli* BL21 (DE3) cells and showed that the S111A mutant was inactive. Using para-nitrophenol (pNP) analogs of different chain lengths as surrogate substrates, I found that this enzyme has a strict preference for shorter substrates and hydrolysed the acetate analog much more efficiently than any of the longer chain length analogs. Using LC-MC methods, I was able to show that this enzyme can transfer the cleaved acetate group from pNP-acetate to a molecule of Coenzyme A (CoA) making acetyl-CoA (AcCoA). I was further able to show that it can also cleave the acetyl group from acetylated lysines on proteins and peptides and form AcCoA. Knocking down this protein in HEK293T cells resulted in lowered AcCoA levels and an increase in protein lysine acetylation. Taking these results together, I annotated ABHD14B as a novel lysine deacetylase. I call it novel because its mechanism of action is very different from that of other known lysine deacetylases, namely HDACs (histone deacetylases) and sirtuins. Using PSI-BLASTS and multiple sequence alignments, I was able to discover that ABHD14B is only found in Chordates and that 40% of its residues were conserved. I made point mutations of select residues and studied them biochemically. Mutating residues K141 and S75 showed changes in enzyme biochemistry and they emerged as sites for potential post-translational modifications that could cause allosteric regulation of activity. Lastly, I studied the protein expression and cellular localisation of this protein in fed vs fasted mouse livers to see whether a fasted state was a trigger for ABHD14B's activity in vivo. A lot still is unknown about this protein and going ahead, its specific protein substrates and its activity in vivo need to be studied.

CHAPTER 1: INTRODUCTION

Almost a decade ago now, the entire human genome was sequenced for the first time. Titled the Human Genome Project, it was a massive undertaking which saw scientists from all over the world participating for a collective goal. In 2003, it produced for the first time a genome sequence that accounted for more than 90% of the human genome¹. One interesting result from sequencing the human genome was that from the predicted set of proteins, around 41% were proteins for which nothing was known functionally or structurally and were termed as “proteins with unknown function”². This meant that there are thousands of proteins doing important functions in eukaryotic cells, which remain wholly unstudied. In the interest of obtaining a “complete” understanding of the eukaryotic cell, understanding the function of these proteins is as important as understanding the complexities of the interactions of every different component. Some of these proteins may also hold the key to treat certain untreatable diseases like cancers or dementia.

The serine hydrolase superfamily is one of the largest and most diverse known families of enzymes. It gets its name from the highly nucleophilic active site Serine which is at the centre of the functionality of all its members. This nucleophilic Serine is usually present in a highly conserved catalytic triad (or a diad) with a base (Histidine) and an acid in the active site. This family contains a wide variety of enzymes like lipases, peptidases, thioesterases and amidases which all follow the same mechanism of catalysis and whose members make up nearly 1% of the human proteome. Members of this family are known to have very important functions in many pathophysiological processes, like blood clotting, digestion, inflammation and cancer to name a few^{3,4}. Many members are also therapeutic targets and there exist clinically approved drugs (inhibitors) against many Serine hydrolases to treat certain conditions like venous and arterial thromboembolic diseases, acetylcholine esterase (AChE) inhibitors to treat Alzheimer’s disease and dipeptidyl peptidase inhibitors to treat type 2 diabetes⁵.

The first serine hydrolase to be extensively studied was chymotrypsin, a serine protease secreted by the pancreas. It was relatively easy to purify since it has a single subunit structure with one subunit containing one catalytic site. It was initially observed that diisopropyl fluorophosphate (DFP) was able to bind irreversibly to chymotrypsin and inhibit its hydrolytic activity against p- nitrophenyl (p-NP) acetate⁶. On solving the crystal structure of the DFP bound enzyme, they found that a single serine (S195) was bound to the phosphorus of DFP⁷.

They repeated this with several different enzymes like trypsin⁸ and elastase⁹ and found that in those enzymes too, it was a single serine molecule that was bound. This is how it was discovered that the main amino acid in play in these enzymes is a Serine. In similar studies with L-1-tosyl-amido-2-phenylethyl chloromethyl ketone (TPCK III) they found that there was a Histidine molecule present in the vicinity of the active site serine which was important for catalysis¹⁰.

The mechanism of action of serine hydrolases was first elucidated by David M. Blow in 1976¹¹. Blow and co-workers solved the crystal structure of Chymotrypsin to a resolution of 2Å. They identified Histidine 57 (H57) present within 5Å of the sulphur of the tosyl group attached to the active site serine (S195) and concluded that it is involved in the catalysis. They also found that the distance between H57 and Aspartate 102 was close enough to form a stable hydrogen bond, and hence the Serine-Histidine-Aspartate catalytic triad was discovered. Further, the determination of the crystal structures of other serine hydrolases like methylesterase CheB¹², brain acetyl hydrolase¹³, and subtilisin¹⁴ in the following years showed that this catalytic triad was well conserved.

The nucleophilicity of the Serine comes from its position in the active site. The Serine is partially deprotonated by the nearby Histidine of the catalytic triad. Histidine is generally not a strong enough base to deprotonate Serine but Histidine itself is partially deprotonated by the Aspartate in its vicinity. This is called the charge relay system (Figure 1.1). The result of this is that the deprotonated Serine is now able to attack a suitable carbonyl carbon in its active site.

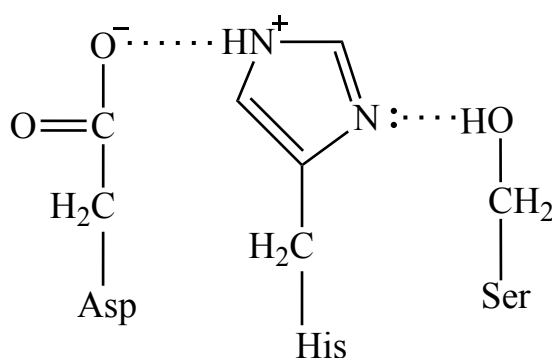


Figure 1.1: Conserved Ser-His-Asp catalytic triad in Serine hydrolases. The dashed bonds show the hydrogen bonding that forms a part of the charge relay system.

This catalytic triad follows a conserved two step mechanism of catalysis (Figure 1.2). At the active site, the Serine is partially deprotonated due to the hydrogen bonding with the nearby Histidine. In the first step of the mechanism, Histidine abstracts a proton from the Serine, causing it to be negatively charged and highly nucleophilic. This oxygen anion attacks an electrophilic centre of the incoming substrate, forming a negatively charged tetrahedral intermediate. Serine hydrolases contain a conserved oxyanion hole which can stabilise this intermediate through hydrogen bonding. The catalytic Histidine (which is now protonated) donates a proton to this tetrahedral intermediate, causing the release of the first product and the formation of the covalent acyl enzyme intermediate. In the second step of the mechanism, a water molecule is activated by the deprotonated Histidine and attacks the electrophilic centre

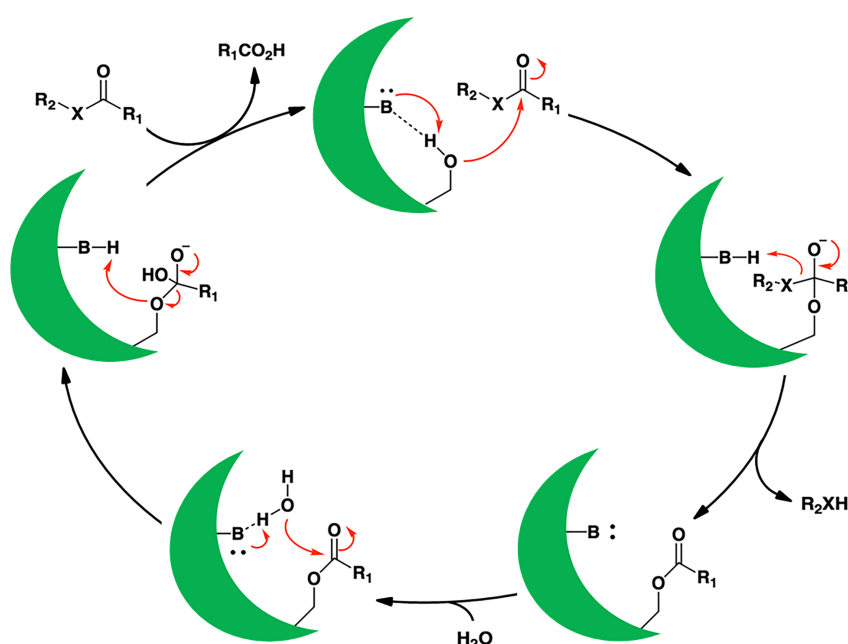


Figure 1.2: Conserved catalytic mechanism followed by the serine hydrolase family.

of the acyl-enzyme intermediate. This causes the formation of yet another tetrahedral intermediate and consequently, the release of the second product from the active site, freeing up the Serine for the next round of catalysis.

Serine hydrolases are broadly categorised into two classes - the Serine proteases and the metabolic Serine hydrolases. The Serine proteases make up for one-third of all known proteases and typically cleave peptide bonds in proteins¹⁴. Examples include trypsin, elastase, plasmin etc. The metabolic Serine hydrolases are a much more diverse group. They are known to cleave ester, amide or thioester bonds in small molecules, lipids or peptides and are involved in

virtually all known pathophysiological processes in mammals¹⁵. This class includes lipases, esterases, thioesterases and amidases. Some examples include small molecule hydrolases like PNPLA2 (patatin-like phospholipase domain containing protein 2) and DAGLA (Diacylglycerol lipase α), neutral lipases like PNLIP (pancreatic lipase) and LPL (lipoprotein lipase), phospholipases like ABHD4 (α/β hydrolase domain containing protein 4) and PLB1 (Phospholipase B), small molecule amidases like FAAH (fatty acid amide hydrolase) and AFMID (arylformamidase), and peptidases like PREP (prolyl endopeptidase) and DPP4 (Dipeptidyl Peptidase 4). With such diverse members, this class of enzymes regulate biochemical pathways in mostly all aspects of physiology. Metabolic serine hydrolases also make good clinical targets and many of their selective inhibitors have been studied and developed and are used in the treatment of diseases such as cardiovascular diseases, obesity, diabetes, neurodegenerative disorders and pain⁵.

With such diverse functions, metabolic serine hydrolases also have very diverse structures, but the majority (>60%) have an α/β hydrolase fold.

The α/β hydrolase fold is a common fold found in proteins in all domains of life. This fold was first discovered in five hydrolytic enzymes that differed widely in their function; namely diene lactone hydrolase, haloalkane dehalogenase, wheat serine carboxypeptidase II, acetylcholinesterase and a lipase from *Geotrichum candidum*¹⁶. These enzymes do not have any significant sequence similarity, neither do they use the same nucleophile, but all have this common fold. Since then, the members of this family have continued to grow in number¹⁷. This fold is characterised by a central beta sheet consisting of eight beta strands connected by alpha helices that flank both sides (Figure 1.3). Generally, the second beta strand runs anti-parallel to the rest.

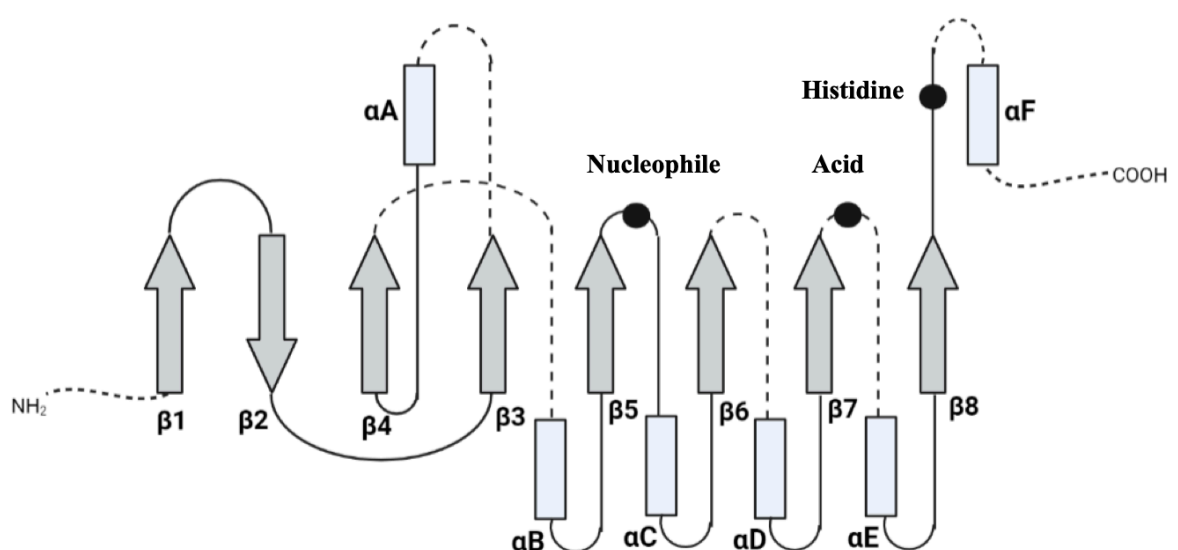


Figure 1.3: The topology of the ABHD fold. Beta strands are represented as arrows and alpha helices are represented as rectangles. Loops are represented as solid lines. The catalytic triad is shown as solid black circles.

The beta sheet also has a left-handed super helical twist, due to which the first and last beta strands are at a 90° angle to each other. This fold is unique in the sense that it tolerates a large number of insertions while maintaining its catalytic machinery, making it very open to adaptation and evolution. The α/β hydrolase fold can provide a stable scaffold for the Serine-Histidine-Aspartate catalytic triad found in most of the metabolic serine hydrolases. The positions of the catalytic triad are highly conserved - the Serine is positioned after strand $\beta 5$, the Aspartate is positioned after strand $\beta 7$ and the Histidine is always positioned after the last β strand (Figure 1.3). The Serine is positioned at a very sharp turn, called the nucleophilic elbow. It is characterised by a consensus sequence GXSXG (where G-Glycine, X-any amino acid and S-Serine) in Serine hydrolases. This tight strand-turn-helix motif makes the active site Serine adopt energetically unfavourable main chain torsion angles and imposes steric restrictions on the flanking residues. This makes sure that the Serine is positioned correctly in the active site to participate in the charge relay system. The geometry of the nucleophilic elbow also helps in the formation of the oxyanion binding site or hole which stabilises the tetrahedral intermediate during catalysis. It is usually formed by two backbone nitrogen atoms, the first one is always the one immediately next to the Serine and the second one is present between strand $\beta 3$ and helix αA . Along with the catalytic domain, most α/β hydrolases also contain a lid or cap domain that helps in substrate specificity¹⁸. These are usually loops or alpha helices inserted at the C-terminal end of the beta strands. Many of the enzymes of this family also have the conserved HXXXXD motif which is an acyl transfer motif. The ESTHER database is a freely available online database dedicated to proteins contained the α/β hydrolase fold¹⁹. Currently, it contains over 30,000 manually curated proteins.

The α/β hydrolase fold is a very good example of divergent evolution. Despite having the same catalytic core and protein structure, serine hydrolases containing this fold are known to catalyse seventeen different reactions²⁰. This is achieved by having a cap or lid domain for substrate specificity and slightly altering the reaction mechanism. Out of the seventeen different reactions, nine of them follow the canonical esterase mechanism described previously and eight of them differ from the canonical mechanism in the formation or release of the acyl enzyme intermediate. A subset of mammalian metabolic Serine hydrolases containing this α/β

hydrolase fold have the nomenclature ABHD (α/β hydrolase domain) proteins¹⁵. They are named so because their structure was either predicted or solved before their biochemical characterisation, and hence they have been named after the α/β hydrolase domain. So far there are 22 known ABHD proteins and are named as ABHD1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 12B, 14A, 14B, 15, 16A, 16B, 17A, 17B, 17C and 18. As seen from Table 1.1, many of them are lipases and play very important roles in lipid metabolism.

Though many of the members of the serine hydrolase superfamily are well-characterised in terms of their biochemistry and the physiological pathways they affect in cells, a large portion (~40%) of this family remains uncharacterised. Given that the annotated members play very important roles in nearly all pathophysiological processes, it is imperative that the unannotated members be studied.

NAME	FUNCTION
ABHD2	Triacylglycerol lipase ²¹
ABHD3	Medium chain phosphatidyl choline phospholipase ²²
ABHD4	Lyso- NAPE (N-acyl phosphatidyl ethanolamine) lipase ²³
ABHD5	Lyso- phosphatidic acid acyl transferase ²⁴
ABHD6	Mono-acyl glycerol lipase ²⁵
ABHD7,9	Epoxide hydrolase ²⁶
ABHD10	S - depalmitoylase ²⁷
ABHD11	Diacylglycerol lipase ²⁸
ABHD12	Lyso- and oxidised phosphatidyl serine lipase ²⁹
ABHD16A	Lysophosphatidylserine lipase ³⁰
ABHD17A,B,C	Depalmitoylases ³¹
ABHD1,8,12B, 14A,14B, 18	Unknown

Table 1.1: human ABHD proteins and their functions.

One such orphan serine hydrolase is ABHD14B. It is a 22kDa soluble protein containing the Serine-Histidine-Aspartate catalytic triad and the α/β hydrolase fold, making it a classic member of the metabolic serine hydrolases family. It was first discovered in 2004 by Padmanaban and group³². They were looking for interactors of the general transcription factor

TF2D (transcription factor 2D), specifically for an interactor of its largest subunit, CCG1 (cell-cycle arrest at G1). It is named so, because it was seen that mutations in this subunit caused temperature sensitive ts13 mutant cell line (derived from baby hamster kidney cells) to arrest in G₁ phase of cell cycle³³. This subunit has four different domains with different enzymatic activities as follows - a HAT (Histone Acetyltransferase) domain, a histone H1-specific ubiquitin-activating/ conjugating domain, a protein kinase domain and two bromodomains which bind acetylated tails of histones. The authors were looking for specific interactors of the highly conserved HAT domain of CCG1. For this, they used a yeast two hybrid system. As bait, they used the HAT domain region of CCG1 (447-1112 amino acids) cloned in frame with the Gal4 binding domain and, as prey, human peripheral lymphocyte cDNA library fused to the Gal 4 activation domain. Colonies which contained the cDNA of ABHD14B turned up blue, showing an interaction event between ABHD14B and the HAT domain of CCG1. When they similarly looked for interaction between ABHD14B and the other subunits of CCG1, they found that ABHD14B only bound specifically to the HAT domain. Further, using northern blot hybridisation analysis, they found that the mRNA of ABHD14B was expressed in almost all human tissues, showing its ubiquitous expression. They also observed that ABHD14B, by itself, was able to initiate transcription. When they fused the sequence of ABHD14B to the Gal4 binding domain, it was able to initiate transcription by itself, not requiring the activation domain for the initiation of transcription. They expressed an eGFP-ABHD14B fusion protein in COS (CV1 in origin, carrying SV40) cells and observed that this construct was found both in the nucleus and cytoplasm. Taking all this data together, they concluded that ABHD14B might have a role to play during transcription.

To study this protein further, the group purified, crystallised and solved the structure of ABHD14B using X-ray crystallography^{34,32}. To their surprise, the structure was not like that of any known transcription factors. In fact, the structure was similar to bromoperoxidase, carboxylesterases and a certain soluble epoxide hydrolase. Even though ABHD14B showed very little sequence similarity to any of these proteins, it showed the same α/β hydrolase fold (Figure 1.3A).

ABHD14B has a very canonical α/β hydrolase fold with the second beta strand running anti-parallel to the rest and having five alpha helices, three on one side of the beta sheet and two on the other. The active site is made up of Serine 111, Histidine 188 and Aspartate 162. Serine 111 is present at a sharp turn on a loop connecting the strand β 5 and helix α 3 (Figure 1.4B). Interestingly instead of the canonical nucleophilic consensus sequence “GXSXG”, the Serine

111 in ABHD14B is present in a “SPSLS” sequence. Still, the ϕ and Ψ angles for the nucleophilic Serine fall in the unfavourable regions in the Ramachandran plot, which is characteristic of the nucleophilic elbow. The active site is present in a deep cleft at the carboxyl end of the beta sheets. There is a sulphate present at the active site which could be due to the crystallisation process. The sulphate ion has a tetrahedral structure, which is similar to the tetrahedral intermediate that is formed at the active site during catalysis in serine hydrolases. It is mostly a hydrophobic pocket, as seen from electrostatic surface potential analysis. The analysis also shows that the surface of this protein is a hydrophobic one. ABHD14B lacks the lid or cap domain that most α/β hydrolases have which helps in substrate specificity. From the structure, there are no helpful insights as to how the enzyme attains substrate specificity. The authors also did a few biochemical assays on the purified protein. They found that the enzyme could hydrolyse p-nitrophenyl butyrate to p-nitrophenol in a dose dependent manner. So, ABHD14B has proven to be an active hydrolase but nothing is known about its substrate, what kind of bonds it can break and what is its physiological function within a cell.

ABHD14B is a very interesting enzyme. It is the only known serine hydrolase to interact with a general transcription factor inside the nucleus. Due to this, it could have a potential role in transcription regulation. In the past few years, ABHD14B has been reported to be dysregulated in a few different types of cancers. For example, using quantitative proteomics, ABHD14B was discovered to be downregulated in hepatocellular carcinoma tissues³⁵. It was also reported to be downregulated during ferric nitrilotriacetate induced renal cell carcinoma in rat models using genome wide analysis studies.³⁶ Other genome wide studies have shown that ABHD14B could be a prognostic marker for metastasized gastroenteropancreatic and neuroendocrine tumors³⁷ and also for the differential diagnosis of Ulcerative Colitis and Crohn’s disease³⁸. (For all the diseases mentioned above, further validation is required to study the protein’s potential as a prognostic marker or a pharmacological target). Since ABHD14B could be potentially involved in the pathophysiology of these diseases, it is imperative to elucidate its function and in vivo substrates.

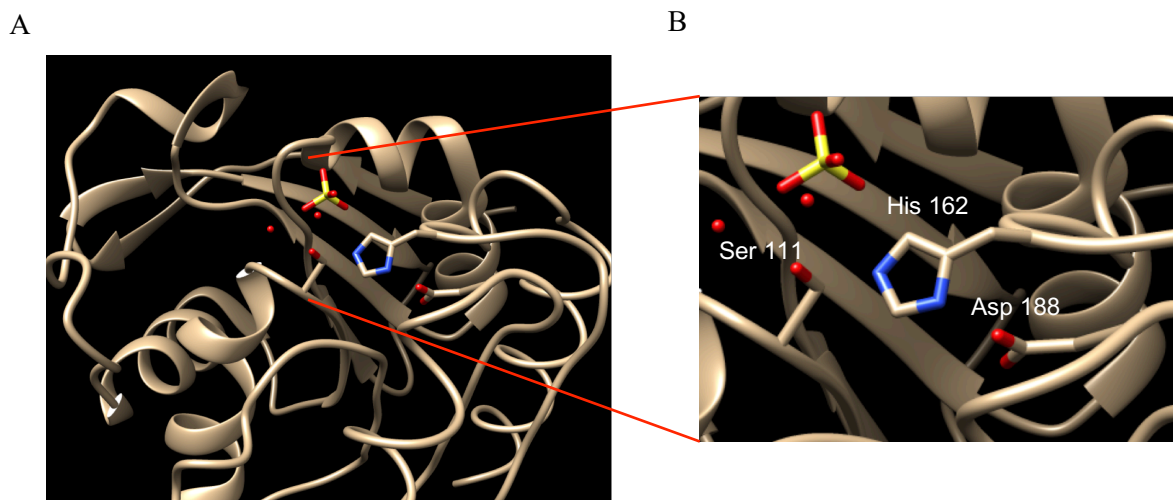


Figure 1.4: The crystal structure of ABHD14B. A- Crystal structure retrieved from Protein Data Bank (PDB ID: 1imj). B- the catalytic triad.

In this thesis, I have attempted to biochemically characterise this orphan enzyme. I have purified human ABHD14B from bacterial cells and using biochemical assays, identified the reaction it can catalyse in cells (chapter 2). Further using bioinformatics tools like PSI-BLASTs and Multiple Sequence Alignments (MSA) I have identified conserved residues of this protein. I have mutated a few key residues and studied their effects on catalysis (Chapter 3). I have managed to crystallize the human S111A ABHD14B mutant and solved its crystal structure to a resolution of 1.5Å (Chapter 4). I have also studied ABHD14B in mouse liver and kidneys in the context of the feeding/fasting paradigm (Chapter 5).

CHAPTER TWO: ABHD14B IS A LYSINE DEACETYLASE.

The crystal structure of ABHD14B was elucidated more than a decade ago, yet its function and biological substrates remain elusive³⁹. In humans the *abhd14b* gene is located on the reverse strand of chromosome 3 and contains three exons and codes for a 22kDa soluble protein. Even though the protein sequence does not have any subcellular localization signal, the protein has been reported to localise in the nucleus³⁹ and extracellular exosomes^{40,41,42,43,44}. From the Human Protein Atlas database, the mRNA of ABHD14B seems to be ubiquitously expressed in all tissues (Figure 2.1). Highest expression is seen in the liver and kidneys.

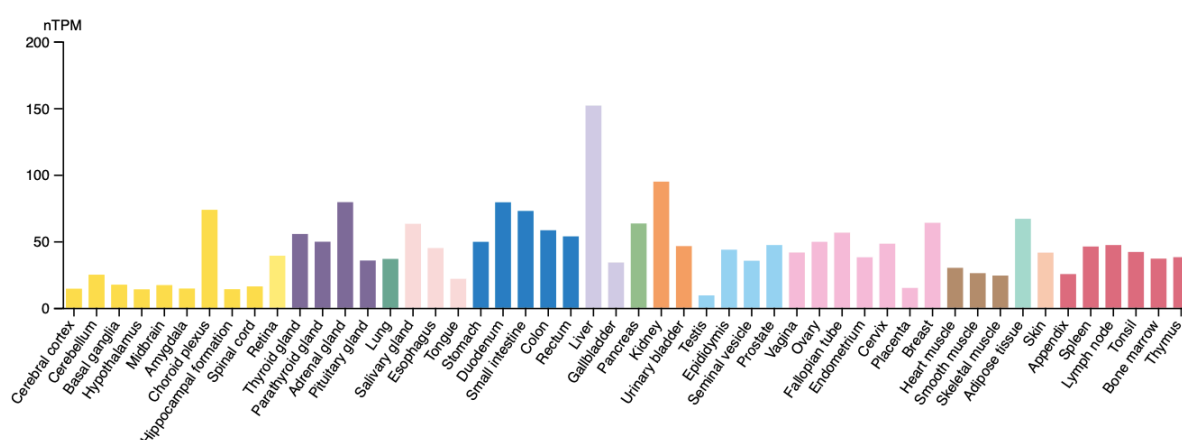


Figure 2.1: mRNA levels of ABHD14B from the Human Protein Atlas.

To study this protein further, I have used a technique called Activity Based Protein Profiling (ABPP)⁴⁴. This is a technique used to study the binding of small molecule probes with proteins and confirm their interaction and activity. The small molecule probe called an activity-based probe (ABP) is made up of three parts – the functional warhead, the linker and the reporter tag (Figure 2.2A). They are used to profile or tag members of that family in a cell or tissue lysate. The functional warheads are small molecules developed to bind irreversibly to the active sites of a particular family of enzymes and generally bind to most members of that family. The reporter tag can vary depending on the application, for example a fluorescent tag like rhodamine is used to assay proteins on a SDS PAGE gel, whereas biotin is used as a reporter tag for proteomics-based profiling. Since ABHD14B is a member of the Serine hydrolase superfamily, I have used flourophosphonate rhodamine (FP-Rh)⁴⁵ to assay it (Figure 2.2B). Flourophosphonate is a warhead specifically designed to bind serine hydrolases. It forms a non-hydrolysable covalent bond with the active site serine. Hence the binding of FP to a serine hydrolase is an indicator of its activity.

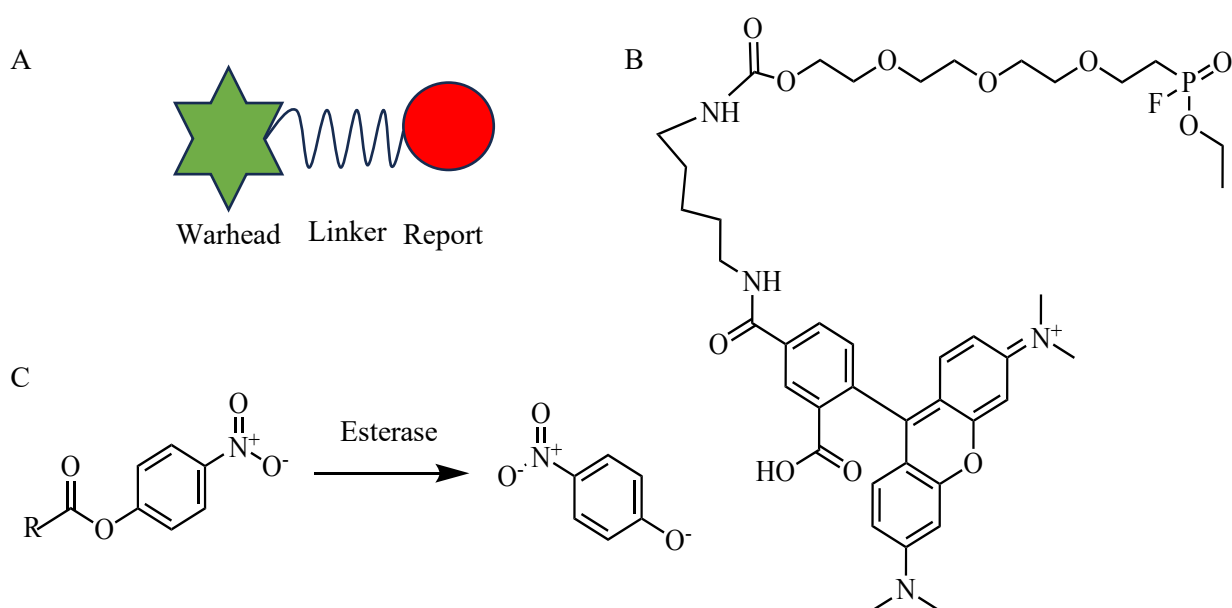


Figure 2.2: Assays used to study ABHD14B; A- Parts of an Activity based probe; B- Structure of FP-Rhodamine; C- p-NP ester hydrolysis assay.

Another assay I have used to study the activity of ABHD14B is the hydrolysis of para-nitrophenol (p-NP) acetate and its analogs. pNP analogs are generally used as substrate mimics to study the hydrolysis activity of esterases and lipases⁴⁶. Their hydrolysis results in the formation of para-nitrophenolate which is yellow in colour and whose absorbance can be measured at 405nm (Figure 2.2C). It is seen that classical esterases show robust activity against shorter chain length analogs (acetate, butyrate) and lipases show robust activity against longer chain length (more lipophilic) analogs⁴⁷. Hence both classes of enzymes show a robust structure-activity relationship against pNP analogs. This makes it a good assay to study whether a particular enzyme has a more esterase-like activity or a lipase-like activity.

In this chapter, I have annotated ABHD14B as a novel lysine deacetylase using biochemical and cellular studies. I have discovered that this enzyme can do an acetyl transfer reaction and have characterised this reaction using a surrogate substrate- pNP-acetate (para-nitrophenol acetate). I have shown that this enzyme can form acetyl coenzyme A (AcCoA) as a product of its acetyl transfer reaction using liquid chromatography followed by mass spectrometry (LC-MS) techniques. Using cellular knock down studies, I have also shown that knocking down this protein in HEK293T cells results in the decrease in the amount of AcCoA in the cell and changes the cellular acetylated lysine profile. Finally, I have shown using LC-MS that ABHD14B is capable of deacetylating acetylated lysine residues on peptides and proteins, thus characterising it as a lysine deacetylase.

Materials

All chemicals, buffers and reagents described in this chapter were purchased from Sigma Aldrich unless specified otherwise. All mammalian cell culture media and fetal bovine serum (FBS) were purchased from HiMedia unless otherwise mentioned. The anti-ABHD14B primary antibody was generated in rabbit using wild type (WT) human ABHD14B purified from *E. coli* as antigen at Bioklone Biotech Private Limited, India (www.bioklone.com). The primary anti-GAPDH antibody (Cat # ab8245) and horse radish peroxidase (HRP) conjugated secondary anti-mouse antibody (Cat # 6ab6789) were purchased from Abcam, while the HRP-conjugated anti-rabbit secondary antibody (Cat # 31460) was purchased from Thermo Fisher Scientific.

Methods

Protein expression in BL21 (DE3) cells: The WT human *abhd14b* gene was synthesized as a codon-optimized construct for expression in *E. coli* (GenScript, USA) and subsequently cloned into the pET-30b(+) vector between the HindIII and NdeI restriction sites, after removing the 6X-His-tag from the plasmid. The S111A ABHD14B mutant was generated using standard Quik-Change site directed mutagenesis protocols as per the manufacturer's instructions (Agilent). The plasmid bearing the desired gene (WT human ABHD14B or S111A human ABHD14B) was transformed into BL21(DE3) *E. coli* competent cells, following which, a single colony was grown in 5 mL of Luria-Bertani (LB) media containing kanamycin (final concentration 50 µg/mL) for 8 hours at 37 °C with constant shaking. This overnight primary culture was used to inoculate 1 L of the same medium, and the cells were allowed to grow at 37 °C with constant shaking until the OD 600 reached ~0.6, at which point protein expression was induced in the cell culture by the addition of 500µM isopropyl β-D-1-thiogalactopyranoside (IPTG). Thereafter, the cells were grown at 30 °C for 16 hours, harvested by centrifugation (6,000 g for 30 mins) and the cell pellets were stored at -80 °C till further use. The protein overexpression was confirmed by SDS-PAGE analysis.

Purification of wild type and S111A14B: The stored cell pellet was thawed on ice and re-suspended in 45 mL of purification buffer (10 mM Tris, 10 mM 2-mercaptoethanol at pH 7.9). The cell suspension was sonicated at 4 °C using a Vibra Cell VCX 130 probe sonicator (Sonics) at 40% pulse amplitude, for 30 min with cycles of 5 sec ON and 10 sec OFF. The resulting lysate was centrifuged at 20,000 g for 45 min at 4°C to pellet the cellular debris and insoluble proteins from the protein of interest (WT human ABHD14B or S111A human ABHD14B) in the supernatant (soluble) fraction. To this resulting supernatant, protamine sulphate (0.05% w/v) was added dropwise at 4°C, over 30 min, and this suspension was centrifuged at 20,000g for 45 min at 4 °C to separate nucleic acids. Post-centrifugation, the supernatant was collected, and ammonium sulphate (40% saturation) was added slowly over 30 min with continuous stirring at 4°C. This mixture was centrifuged at 20,000g for 20 min at 4°C and the resulting pellet was discarded. To the remaining supernatant, ammonium sulphate at 60% and 70% saturation was added slowly over 30 min, the mixtures centrifuged as described earlier, and the resulting supernatant fractions were discarded. The pellets were then resuspended in purification buffer to a final volume of 10 mL and dialyzed in 1 L of purification buffer with 4 buffer changes every 2 hours initially, and the final buffer change was kept overnight for 14 hours to dialyze out any residual ammonium sulphate from the previous purification step. Post-dialysis, the protein fraction (10 mL) was applied to an anion exchange column (GE Healthcare HiTrap CaptoQ, 5 mL), pre-equilibrated with purification buffer, and the protein of interest was eluted with a gradient of NaCl (25 – 250 mM) in purification buffer. Both WT and S111A ABHD14B eluted at 100 mM NaCl concentration as ascertained by SDS PAGE analysis. The fractions containing the protein of interest were pooled and concentrated using a 10-kDa molecular weight cut off filter (Millipore) to a final volume of 6 mL. This concentrated protein fraction (6 mL) was loaded onto a preparative High Load 26/60 Superdex 200 gel filtration column (GE Healthcare) previously equilibrated with the purification buffer containing 100 mM NaCl, and 3 mL fractions were collected from this gel filtration column. The fractions were assessed for purity by SDS-PAGE analysis, and those containing WT or S111A ABHD14B were pooled, concentrated using a 10-kDa molecular weight cut off filter (Millipore) to a final concentration of ~50 µg/µL, flash frozen in liquid nitrogen as 10 µL aliquots, and stored at -80 °C till further use.

Gel-Based Activity based Protein Profiling experiments (ABPP): For all the gel-based ABPP experiments, WT or S111A ABHD14B was treated with the fluorophosphonate-rhodamine (FP-Rh) activity probe⁴⁸, at 37 °C for 1 hour with constant shaking in a final volume of 50 μ L, following which, the reaction was quenched by adding 4X-SDS loading dye, and then boiling at 95 °C for 10 min. For the protein titration experiments, ABHD14B (WT or S111A) concentrations were varied from 0.5 – 10 μ M keeping FP-Rh constant at 5 μ M. For the activity probe titration experiments, ABHD14B (WT or S111A) concentrations were kept constant at 5 μ M, while the FP-Rh concentrations were varied from 0.2 – 5 μ M. Post-quenching the ABPP reactions, the samples were loaded and resolved on a 12.5% SDS-PAGE gel, and the enzyme activity was visualized on Syngene Chemi-XRQ gel documentation system. To ensure accurate protein loading in these gel-based ABPP assays, post-enzyme activity visualization, the gels were stained with Coomassie Brilliant Blue R-250 and imaged on the same system.

Colorimetric Substrate hydrolysis assays: All colorimetric assays were performed in a final reaction volume of 250 μ L in a Varioskan Flash plate reader (Thermo Fisher Scientific), monitoring the release of para-nitrophenolate from substrates by measuring absorbance at 405 nm in a 96 well plate assay at 37 °C. All assays were performed in 10 mM Tris buffer at pH 8 in biological triplicates to ensure reproducibility. For the structure activity assays, 10 μ M of WT ABHD14B was incubated with 500 μ M of para-Nitrophenyl (pNP) analogues of varying chain lengths, namely p-NP acetate, p-NP butyrate, p-NP octanoate, p-NP decanoate, p-NP dodecanoate and p-NP palmitate and absorbance at 405 nm was monitored over 30 min, with plate readings at 10 sec intervals. For the enzyme kinetics assays with p-NP acetate, p-NP butyrate and p-NP octanoate, substrate concentrations of 0 – 1000 μ M were used with all other assay conditions and parameters remaining the same. The resulting data from this experiment was fit to a Lineweaver-Burk equation to yield enzyme kinetic constants for the respective substrate. To study the effects of CoA and acetyl-CoA on ABHD14B catalyzed hydrolysis reaction, 10 μ M WT ABHD14B was pre-incubated with CoA (1 mM) or acetyl CoA (1 mM) at 37 °C in a 96 well plate for 15 min, following which the hydrolysis reaction was initiated by adding 50 μ M p-NP acetate to this mixture, with all other assay parameters remaining the same.

Thermal Shift Assays: Thermal shift assay was performed on a CFX-Real Time RT-PCR system (BioRad) using the Sypro-Orange dye as per manufacturer recommended protocols (Sigma-Aldrich) in a 96 well plate⁴⁹. Briefly, 10 μ M of WT or S111A ABHD14B was incubated with CoA (10 mM) or acetyl-CoA (10 mM) at 37 °C for 15 mins, following which, 1X Sypro-Orange dye was added to the reaction mixture, to make up the final volume to 25 μ l in 10 mM Tris at pH 8. The fluorescence was measured in the FRET channel with an excitation and emission wavelength of 470 and 569 nm respectively, over a temperature range of 45 °C to 70 °C. All thermal shift assays were done in biological triplicates to ensure reproducibility of data. The raw fluorescence data from the thermal shift assays was converted into % Response using the following equation:

$$\% \text{ Response} = [100*(X-A)]/(B-A)$$

where X = fluorescence at any temperature, A = fluorescence at 45 °C, and B = fluorescence at 70 °C. Thus, based on this equation, the % response at 45 °C will always be 0, while that at 70 °C will always be 100.

Mammalian Tissue culture: All mammalian cell lines (HEK293T, HeLa, A549, THP1 and MCF7) described in this chapter were purchased from ATCC and cultured in complete medium [RPMI1640 supplemented with 10% (v/v) FBS, and 1% (v/v) penicillin-streptomycin (Pen-Strep) (MP Biomedicals)] at 37 °C with 5 % (v/v) CO₂ unless otherwise media. The cell cultures were stained routinely with 4',6-diamidino-2- phenylindole (DAPI) and assessed by microscopy to ensure that they were devoid of any mycoplasma contamination using established protocols⁵⁰. All cell lines were cultured in 15 cm tissue culture dishes (Eppendorf), and upon 80% confluence were harvested by scrapping, washed with Dulbecco's phosphate buffer saline (DPBS) (3x times), centrifuged at 200g for 5 mins to remove excess DPBS, and stored at -80 °C till further use.

LCMS method to Measure AcCoA and CoA: For measuring the cellular levels of CoA and acetyl-CoA, the cell pellets were re-suspended in 150 μ L of extraction buffer (1:1 methanol and water with 5% glacial acetic acid) by vortexing and incubated on ice for 10 mins. This was followed by the addition of 1.5 μ L of 5 M ammonium formate, vigorous vortexing and a further 5 mins incubation on ice. Finally, the extracts were centrifuged at 16,000 g for 5 mins at 4 °C,

the supernatant was transferred in 1 mL glass vials, dried down under vacuum and stored at -40 °C until the LC-MS analysis⁵¹.

The dried extracts were re-suspended in 35 µL of solvent A (2% acetonitrile in water with 100 mM ammonium formate) by vortexing and/or water bath sonication. To remove any particulate impurities, the re-suspended extracts were centrifuged at 16,000g for 5 mins at 4 °C, and 20 µL of the supernatant was injected on a Phenomenex Gemini. C18 column (50 X 4.6 mm, 5 µm, 110 Å) fitted with a Phenomenex guard column (4 X 3 mm) using an Exion UHPLC system. The temperatures of auto-sampler and column oven were set at 8 °C and 42 °C, respectively and the flow rate for the LC-MS run was 0.2 mL/min. A typical LC run consisted of 30 mins, and had the following gradient sequence: (i) 0% solvent B (98% acetonitrile in water with 5 mM ammonium formate) for 2 min; (ii) linear increase in solvent B from 0% to 60% for the next 6 min; (iii) linear increase of solvent B from 60% to 90% in the next 1 min; (iv) maintaining 90% solvent B for 10 min; (v) linear drop in solvent B from 90% to 0% in 1 min; and (vi) re-equilibrating column with 0% solvent B for 10 min⁵¹. The UHPLC was coupled to X500R Quadrupole Time-Of-Flight (QTOF) mass spectrometer (Sciex), which was operated in high resolution mass spectrometry (HRMS) positive ionization mode with the following sources parameters: ion source gas 1 at 40 psi, ion source gas 2 at 50 psi, curtain gas at 30 psi, CAD gas at 7 psi, temperature at 500 °C, spray voltage at +5500 V, declustering potential (DP) at 90 V, DP spread 20 V, collision energy 20 V. The m/z ([M+H]⁺) of CoA and acetyl-CoA are 768.1225 and 810.1331, respectively. All data was collected and analysed using SCIEX OS-Q (version 1.4.0.18067) software as described earlier⁵². For the ABHD14B catalysed in vitro acetyl-transfer assays, the final reaction volume was 100 µL. In this assay, 10 µM pNp-Acetate was incubated with CoA (1 mM), and the reaction was initiated by 10 µM of WT or S111A ABHD14B, and allowed to proceed for 5 mins at 37 °C. To reduce the rate of non-enzymatic acetyl-transfer reaction between pNp-Acetate and CoA, this assay was done at pH 7. The reaction was filtered through a 3-kDa molecular weight cut off filter (Millipore), and the resulting flow through from this experiment was subjected to LC-MS analysis describe above.

For the in vitro acetyltransferase assays with acetylated histone 3 peptide, the final reaction volume was 50 µL. In this reaction, 2.5 µM peptide was incubated with CoA (25 µM), and the reaction was initiated with 2.5 µM WT or S111A ABHD14B and allowed to proceed for 5 min at 37 °C and pH 7.0. To directly compare the relative rates of the acetyltransferase reaction between acetylated lysine-containing peptides and pNPAcetate, the aforementioned assay was

also performed with pNP-acetate (2.5 μ M) in a 50 μ L volume with the same enzyme and CoA concentrations. For the in vitro acetyltransferase assays with histones from calf thymus, the final reaction volume was 50 μ L. In this reaction, 100 ng of the histone preparation was incubated with CoA (25 μ M), and the reaction was initiated with 2.5 μ M WT or S111A ABHD14B and allowed to proceed for 5 min at 37 °C at pH 7.0. At the end of the experiment, all reactions were filtered through a 3 kDa molecular weight cutoff filter (Millipore) by centrifugation to remove the enzyme, and 20 μ L of the resulting flow-through from these experiments was subjected to LC–MS analysis described above. (*A part of this experiment was done by Dr. Abinaya R.*)

Generation of ADBH14B Knock down cell lines: Three human abhd14b lentiviral shRNA knockdown constructs and a non-targeting (control) construct were purchased from TransOMIC, USA. These constructs have puromycin resistance and ZsGreen fluorescent reporter coding genes. The abhd14b targeting 5' sequences are KD_1: ACATCTGCACTGACAAAATCAA, KD_2 : ACCCATGGGTCAGACCAGCTTA, and KD_3 : CGAAGACTCCAGCTCTGATTGA. The lentivirus cDNAs were generated by co-transfecting HEK293T grown in complete media, with shRNA constructs, plasmids psPAX2 and pMD2.G (1:1:0.5) using the transfection reagent PEI 40,000 using established protocols⁵⁰. 2 days post-transfection, the lentiviral particles were filtered through a 0.45 μ m filter and used to transduce HEK293T cells along with 4 μ g/mL polybrene. 1-day post-transduction, the lentiviral containing media was replaced with fresh complete media containing puromycin (5 μ g/mL), and the cells bearing the desired constructs were thereafter selected on puromycin. The complete media + puromycin (5 μ g/mL) was changed every 2 days and cell selection was checked for, by the amount of green fluorescence in cells. The puromycin selection strategy was continued until all the nongreen fluorescent cells were removed (> 99%) from the cell population. Once this was achieved, the cells were harvested and lysed in DPBS by sonication, and the whole cell lysates was used to confirm knockdown of ABHD14B by western blot analysis. All the knockdown HEK293T cell lines were revived and maintained in complete media containing 1 μ g/mL puromycin, to ensure that desired constructs are not competed by any other cell populations. (*This experiment was done by Dr. Abinaya R.*)

Western Blot analysis: For cells harvested from aforementioned mammalian cell culture section, the cell pellets were re-suspended in 1 mL of cold sterile DPBS, and lysed by sonication using an earlier reported protocol⁵³. Thereafter, all the lysates were separated into soluble and membrane proteomic fractions by ultracentrifugation at 100,000 g for 1 hour at 4 °C, and the soluble fraction was used for western blot analysis. The protein concentration of the soluble fractions was estimated using Pierce BCA Protein Assay kit (Thermo Fisher Scientific). 70 µg of cellular soluble proteome were loaded and resolved on a 12.5% SDS-PAGE gel and subsequently transferred onto a nitrocellulose membrane at 70 V for 3 hours at 4 °C. The efficiency of the transfer was assessed by Ponceau staining of the membrane. The membrane was then blocked with 5% (w/v) milk powder in PBST (1X phosphate buffer saline (PBS) with 0.1% (v/v) Tween-20) for 1 h at room temperature and probed with a primary antibody (dilution 1:1000 to 10,000) overnight (12 – 14 h) at 4 °C. The membrane was then washed with PBST (3x times) and probed with an appropriate HRP-conjugated secondary antibody (dilution 1:10,000) for 1 h at room temperature, and any unreactive secondary antibody was washed off using PBST (3x times). Finally, the western blots were visualized by SuperSignal West Pico PLUS Chemiluminescent substrate (ThermoFisher Scientific) and imaged on a Syngene Chemi-XRQ gel documentation system. The ImageJ software (NIH, USA) was used for any densitometric analysis of the western blot images^{54,55,56}.

Identification of substrate binding channel in ABHD14B: The tunnel analysis was conducted using the CAVER v1.0 online server^{57,58}. In brief, the structure of the monomer of human ABHD14B (PDB: 1IMJ)³⁹ was loaded onto the server, and the catalytic triad sequence (S111, D162, H188) was manually input to provide a starting point for the analysis. Using the default server settings, two tunnels were found, one of which was a subset of the other (essentially forming one, unified tunnel). In parallel, tunnels were calculated using the MOLEonline server^{59,60} and yielded a near identical result. The tunnel-defining residues (entrance, exit, and bottleneck) were visualized using PyMol and found to be basically identical for Caver and MOLEonline. An electrostatic calculation using the APBS plugin to PyMol^{61,62,63} was also performed on the native monomer of human ABHD14B (PDB: 1IMJ). First, the required PQR files were generated using the PARSE force-field and these PQR files were subsequently used to calculate the electrostatic surface diagrams^{61,62,63}. Software packages for analysis and visualization of ABHD14B were compiled by SBGrid⁶⁴. (*This experiment was*

done by Mr. Johnny Mendoza and Dr. Jennifer Bridwell-Rabb, University of Michigan, Ann Arbor.)

Data Plotting: All graphs and plots presented in this paper were made using the GraphPad Prism 7 (version 7.0b) software for MacOS-X. Unless otherwise mentioned all data in bar format is presented as mean $\pm$ standard deviation for three or more biological replicates from independent experiments.

Results

Purification and Biochemical Properties of human ABHD14B: The WT enzyme and the active site-directed S111A mutant of human His-ABHD14B were successfully expressed and purified from *E. coli* in high purity (>99%). The purification scheme typically afforded 10-15 mg of ABHD14B/L of culture (Figure 2.3). To determine, whether the S111A mutant was catalytically inactive, we first resorted to established gel-based ABPP assays⁴⁸ and found that as a function of increasing enzyme concentration, WT human ABHD14B, but not S111A human ABHD14B, displayed robust dose dependent activity against the FP-Rh probe, which was kept constant in this assay (Figure 2.4A). Next, I found that as a function of increasing the FP-Rh probe concentration, and keeping the enzyme concentration constant, that WT human ABHD14B again, showed robust dose dependent activity, while the S111A mutant showed no activity at all in this gel-based ABPP experiment (Figure 2.4B). To complement these gel-based ABPP assays, I also performed substrate hydrolysis assays, as previous studies have shown that ABHD14B has hydrolase activity against acylated-para-nitrophenol derivatives³⁹. Towards this, I incubated WT and S111A human ABHD14B, against pNP-acetate (500 μ M), and found that WT human ABHD14B robustly produced para-nitrophenolate from pNP-acetate, while S111A human ABHD14B had very little activity against this substrate (Figure 2.5A). Taken together, these results suggested that the active site directed S111A mutant of human ABHD14B was catalytically inactive.

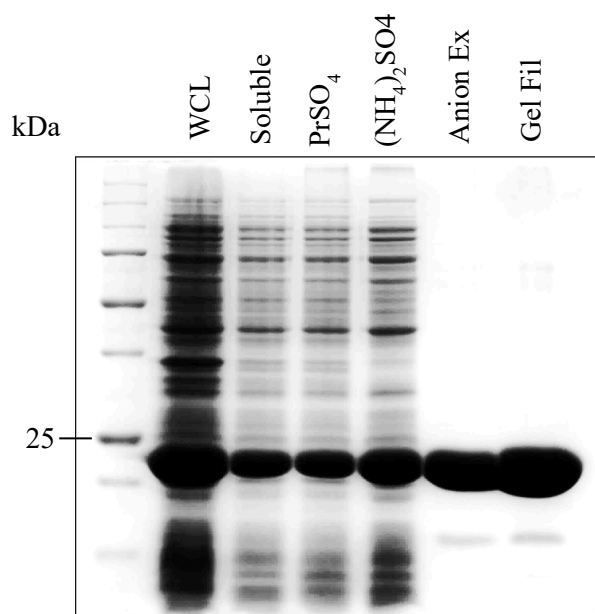


Figure 2.3: Coomassie gel showing purification scheme of untagged ABHD14B from *E. coli* BL21(DE3) cells. WCL -Whole Cell lysate, Soluble- soluble fraction of lysate, PrSO₄ – Protamine sulphate, Anion Ex- Anion Exchange chromatography, GelFil- Gel Filtration through SuperDex200.

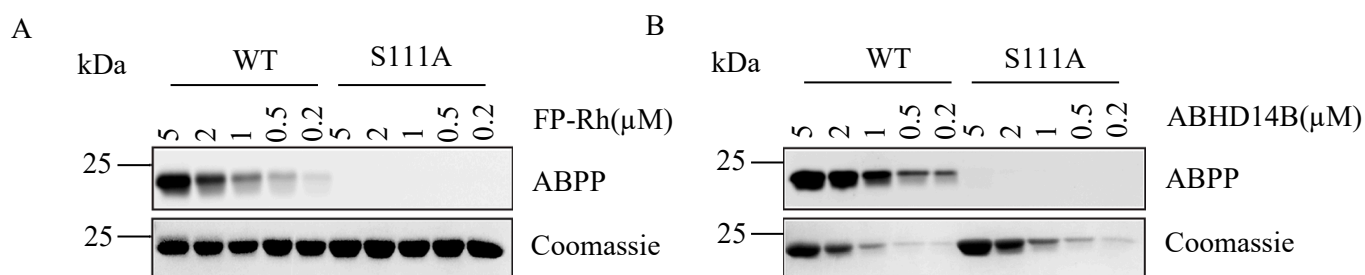


Figure 2.4: Gel-based ABPP assays with WT and S111A human ABHD14B, showing robust dose dependent activity of WT ABHD14B, but not S111A ABHD14B, as a function of increasing: (A) enzyme concentration (0.5 – 10 μ M), and (B) activity probe (FP-Rh) concentration (0.2 – 5 μ M), while keeping the other constant (activity probe concentration (5 μ M) in A; enzyme concentration (5 μ M) in B). The gel based ABPP experiments were done three independent times with reproducible results each time.

Since WT human ABHD14B was able to turnover acylated-para-nitrophenol derivatives, I decided to perform structure activity studies with this class of surrogate substrates, to assess whether this enzyme has any preference for the esterified acyl-group to pNP. Towards this, I incubated WT human ABHD14B (10 μ M) with 500 μ M of different pNP-analogues of varying chain lengths, ranging from acetate (two carbon atoms, C2) to palmitate (16 carbon chain, C16), and monitored the release of para-nitrophenolate produced from these surrogate substrates. From these studies, I found a distinct trend, where WT human ABHD14B preferred

smaller acylated esters of pNP as substrates, with pNP-acetate being the best substrate (Figure 2.5B). Further, I found that this enzyme sluggishly turned over pNP-octanoate (C8) but was unable to turnover acylated-esters of pNP having more than 10 carbon atoms. To ascertain this substrate preference profile, I performed enzyme kinetics assays on the three pNP-substrates that WT human ABHD14B was able to turnover, and from this experiment it was further confirmed that pNP-acetate was indeed the best substrate for this enzyme (Table 2.1). Albeit sluggish due to the unnatural nature of the pNP-acetate substrate, these substrate profiling results taken together, suggest that ABHD14B might function as a deacetylase or acetyltransferase in biological settings.

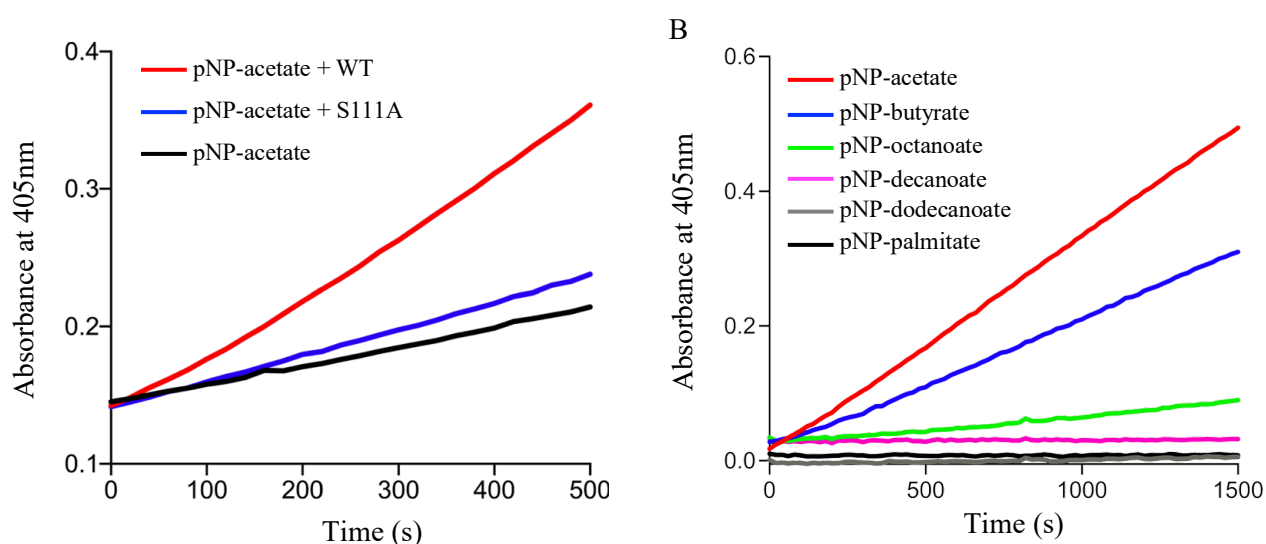


Figure 2.5: Colorimetric enzymatic assay with pNP-acetate and its other analogs. A-Colorimetric enzymatic assay showing robust activity of WT human ABHD14B, but not S111A human ABHD14B, against pNP-acetate (500 μM). B- same assay done with longer chain length analogs of p-nitrophenol.

Substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($\text{M}^{-1}\text{s}^{-1}$)
pNP- acetate	2.2 ± 0.8	1.8 ± 0.6	20.4 ± 2.5
pNP-butyrate	0.6 ± 0.2	3.1 ± 0.6	3.2 ± 0.4
pNP-octanoate	0.2 ± 0.1	3.9 ± 0.7	0.9 ± 0.2

Table 2.1: Kinetic constants for WT human ABHD14B against different acylated ester of pNP.

Coenzyme A (CoA) or Acetyl CoA (AcCoA) might be potential substrates for ABHD14B:

In findings reported by Padmanabhan et al., describing the three-dimensional structure and preliminary biochemical characterization of ABHD14B, the authors also speculate that WT human ABHD14B likely binds AcCoA, because of its association to the HAT domains of important transcription factors³⁹. Given this literature precedence, I decided to test whether human ABHD14B binds AcCoA and/or CoA using established thermal shift assays⁴⁹, and chose the S111A mutant for these studies, as I hypothesized that binding interactions (if any) of AcCoA and/or CoA would be much longer for a catalytically inactive mutant of an enzyme. From these assays, where S111A human ABHD14B (10 μ M) was incubated with AcCoA or CoA (both at 10 mM), I saw a left-ward (lower temperature) shift in the fluorescence profiles (% Response) for both these compounds, with the shift being more prominent for CoA (~ 5 $^{\circ}$ C) compared to AcCoA (~ 2.5 $^{\circ}$ C), suggesting that CoA most likely binds to the enzyme tighter than acetyl-CoA (Figure 2.6). I also saw similar leftward shift, albeit to a much lesser extent (~ 2 $^{\circ}$ C), in thermal shift assays after incubating WT human ABHD14B with AcCoA and CoA without a distinct change in binding for AcCoA or CoA.

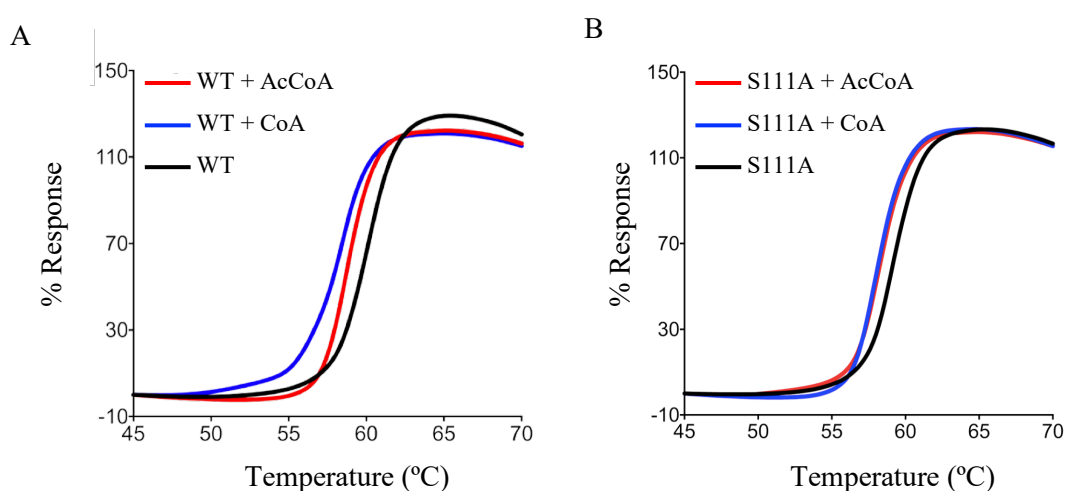


Figure 2.6: Thermal shift assays showing binding of both CoA and AcCoA to S111A (A) and WT (B) human ABHD14B.

Effect of CoA/AcCoA binding on p-NP acetate hydrolysis: Next, I wanted to assess whether the binding of either AcCoA or CoA to ABHD14B, has any effect on the p-NP acetate hydrolysis reaction catalysed by WT human ABHD14B. In this assay, WT human ABHD14B (10 μ M) was pre-incubated with either AcCoA or CoA (1 mM each), and the reaction was initiated by adding p-NP acetate (50 μ M). AcCoA had no effect on the kinetics of the reaction,

however CoA significantly enhanced the rate of this hydrolysis reaction (Figure 2.7). I performed another enzyme kinetic assay for WT human ABHD14B against p-NP acetate substrate in the presence of excess CoA (1 mM), and obtained the following kinetic constants: $k_{cat} = 9.1 \pm 1.4 \text{ min}^{-1}$; $K_m = 1.7 \pm 0.4 \text{ mM}$; $k_{cat}/K_m = 89.2 \pm 12.3 \text{ M}^{-1}\text{s}^{-1}$, which further showed that the rate of the hydrolysis reaction becomes almost 4.5 times faster (from k_{cat} and k_{cat}/K_m values) in the presence of excess CoA (also see Table 2.1).

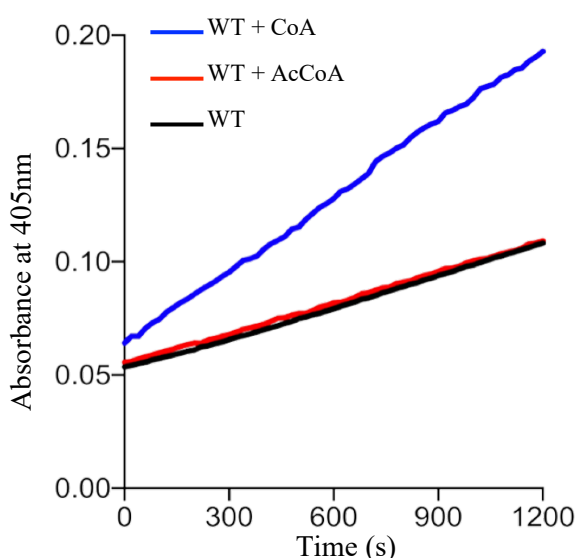


Figure 2.7: Colorimetric enzymatic assay with pNP-acetate, showing increase in rate of enzymatic reaction following incubation of ABHD14B with CoA, but not AcCoA.

Can ABHD14B Hydrolyse AcCoA: The authors of the same aforementioned paper also speculate that the binding of AcCoA to ABHD14B might result in its hydrolysis, to yield CoA and acetate³⁹, and I also tested this hypothesis, looking for the formation of CoA from acetyl-CoA by LC-MS analysis. However, I did not find any turnover of AcCoA in the presence of WT human ABHD14B, suggesting that this enzyme does not possess any deacetylase (or HAT-type) activity for AcCoA. (Figure 2.8A).

Deacetylase activity of ABHD14B: The fact CoA binds more tightly to ABHD14B and accelerates the enzyme catalysed p-NP acetate hydrolysis reaction, and the inability of this enzyme to hydrolyse AcCoA, suggested that ABHD14B might, contrary to previous speculation, function as a deacetylase. To confirm if AcCoA is produced when WT human ABHD14B was incubated with p-NP acetate and CoA, we performed LC-MS analysis⁵¹. I

found from this experiment, that this was indeed the case, where WT human ABHD14B, but not S111A human ABHD14B, robustly produced substantial AcCoA, when incubated with p-NP acetate and CoA (Figure 2.8B). Taken together, these results conclusively show that ABHD14B performs as an acetyl-transfer reaction, where CoA is the eventual acetyl-group acceptor from an acetylated-substrate, and AcCoA is a product of this enzyme catalysed reaction.

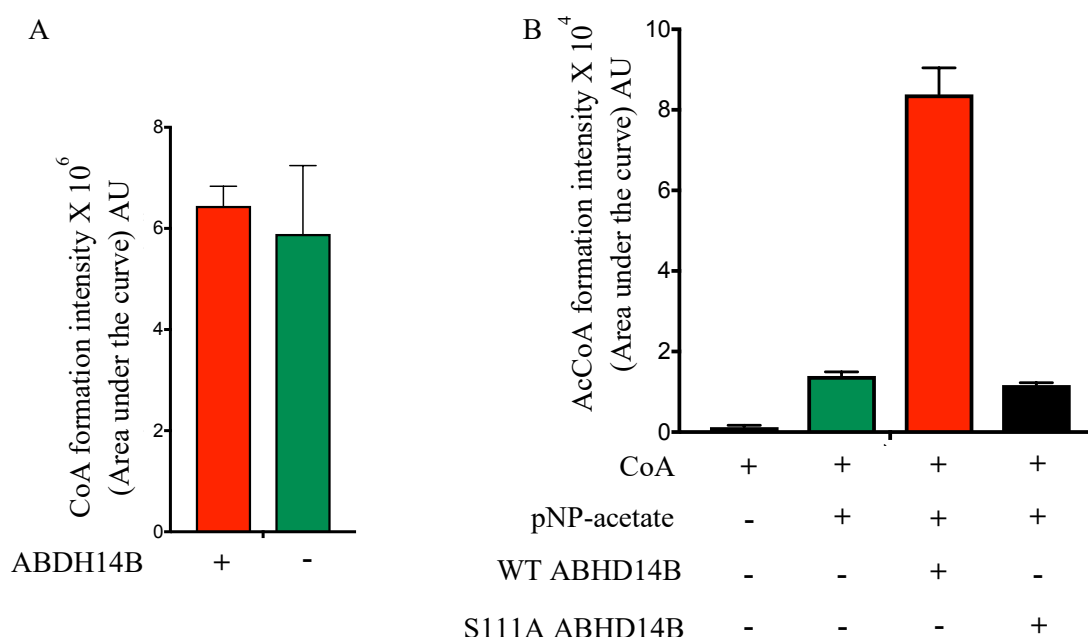


Figure 2.8: Acetyl transfer/ Deacetylase activity of ABHD14B. A- WT human ABHD14B cannot hydrolyse AcCoA to form CoA. B- Formation of acetyl-CoA by WT human ABHD14B, but not S111A human ABHD14B, when incubated with pNP-acetate and CoA. The LC-MS data represents mean $\pm$ standard deviation from six independent experiments.

Effects of genetically knocking down ABHD14B in HEK293T cells: To understand the physiological function of ABHD14B, we decided to genetically knockdown ABHD14B in human HEK293T cells using the established RNA interference technology. We found that compared to a non-targeting (scrambled) “control” line, the cell lines generated with shRNA-targeting ABHD14B (KD_1, KD_2 and KD_3) showed good knockdown of ABHD14B in HEK293T cells (Figure 2.9A). Since the lines KD_2 and KD_3 showed greater than 90%

knockdown of ABHD14B (and KD_1 failed to make this cut off), they were chosen for subsequent studies (Figure 2.8A). First, we found that the knocking down of ABHD14B expression in cells, did not affect cell growth, and we did not observe any obvious phenotypic defects in the knockdown (KD_2 and KD_3) cell lines compared to the control cell line. Since, previous biochemical studies showed that ABHD14B transfers an acetyl-group to CoA, we decided to measure the cellular levels of CoA and AcCoA in the ABHD14B knockdown cell lines using LC-MS analysis. We found that following ABHD14B knockdown, the cellular levels (relative to the control cell line) of CoA, the substrate of the ABHD14B catalyzed reaction remained unchanged (Figure 2.9B). Interestingly however, the cellular levels (relative to the control cell line) of AcCoA, the product of the ABHD14B catalyzed reaction had significantly reduced (~ 50%) following the knockdown of ABHD14B in HEK293T cells (Figure 2.9B). We established the role for CoA and AcCoA in the ABHD14B catalysed acetyl-transferase reaction, but the biological origins of the acetyl-group remained unknown. In cells, conserved and functionally relevant lysine residues on proteins are often acetylated by enzymes using AcCoA (e.g. HAT enzymes), and these post-translationally acetylated-lysines of protein residues serve many functions, important among which are the regulation of gene transcription and controlling cellular metabolism and proliferation. Given its association with important transcription factors, we hypothesized that ABHD14B might be performing a deacetylase-type reaction, transferring the acetyl-group from a post-translationally acetylated- lysine protein residue of a protein to CoA, and making AcCoA in the process. To test this hypothesis, we assessed the lysine acetylation profile of cell lysates prepared from ABHD14B knockdown cell lines (KD_2 and KD_3) and compared them to lysates prepared from the control cell line, by western blot analysis using a commercially available anti-acetylated lysine antibody. Consistent with our hypothesis, we found that ABHD14B knockdown significantly increased the concentration of acetylated-lysine residues in cell lysates (Figure 2.9C), suggesting that in physiological settings, an acetylated-lysine is in fact the other substrate of ABHD14B, and the origin of the acetyl-group of that eventually forms AcCoA. That ABHD14B knockdown increases cellular concentrations of acetylated-lysine residues of protein residues, and concomitantly decreases the levels of AcCoA, shows a clear substrate-product relationship in physiological settings for the ABHD14B-catalyzed deacetylase reaction.

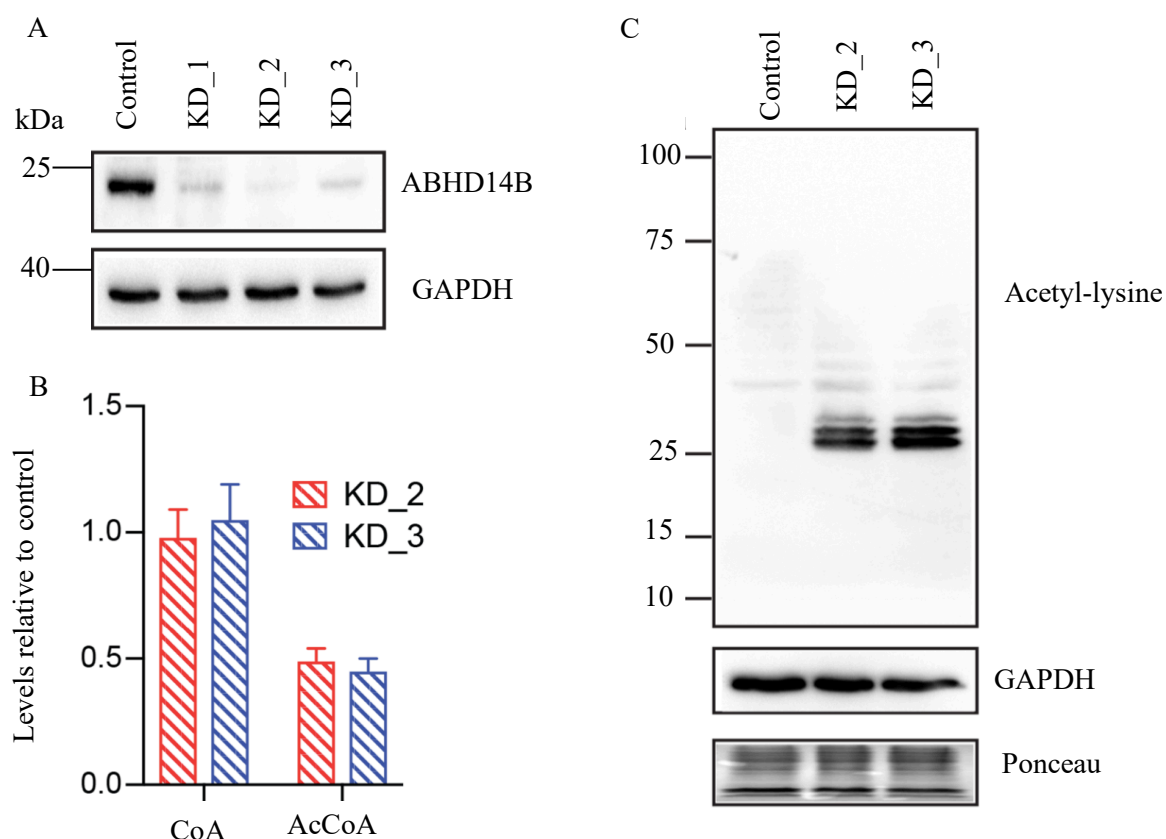


Figure 2.9: Effect of ABHD14B knock down on HEK293T cells: A- Western blot analysis confirming the knockdown of ABHD14B in the KD_1, KD_2 and KD_3 cell lines, relative to the non-targeting control cell line made from HEK293T cells. B- The cellular levels of CoA and acetyl-CoA in the KD_2 and KD_3 cell lines, relative to the non-targeting control cell line measured by LC-MS analysis. The LC-MS data represents mean $\pm$ standard deviation from six independent experiments. C- Western blot analysis, showing increased protein lysine acetylation following ABHD14B knockdown in HEK293T cells. All western blot experiments were done three independent times with reproducible results each time. (These experiments were done by Dr. Abinaya R).

Characterizing the ABHD14B-catalyzed acetyltransferase reaction against acetylated lysine containing substrates: Thus far, I had tested only the acetyl-transfer reaction catalysed by ABHD14B using p-NP acetate as the acetyl donor (Figure 2.8B). but the biological origins of the acetyl group remained unknown. In cells, conserved and functionally relevant protein lysine residues are often acetylated by enzymes using AcCoA (e.g., HAT enzymes),^{65,66} and these post-translationally acetylated lysine residues serve many functions, important among them being the regulation of gene transcription and controlling cellular metabolism and proliferation^{67,68,69}. Given its association with important transcription factors, and in view of the results from genetically disrupting this enzyme in human HEK293T cells, where we find

increased levels of protein lysine acetylation (Figure 2.9C), we wanted to validate if ABHD14B can in fact use acetylated lysine-containing peptides or proteins as substrates for the acetyl-transferase reaction. To test if this was indeed the case, we first chose the commercially available diacetylated histone H3 21-amino acid peptide with an ARTKQTAR(K^{AC})STGG(K^{AC})APRKQLC sequence, where K^{AC} is the acetylated lysine residue. We incubated purified WT and S111A human ABHD14B with equimolar concentrations of the peptide in the presence of excess CoA and assessed the formation of AcCoA in this assay by LC–MS analysis. We found that WT human ABHD14B, but not S111A human ABHD14B, was indeed able to make AcCoA from this acetylated peptide (Figure 2.10A). To compare the relative production of AcCoA from different acetyl donors, we also ran that same assay with equimolar concentrations of p-NP acetate (instead of the acetylated lysine peptide) and found that the level of formation of AcCoA was ~3.5-fold greater when the acetylated lysine peptide was used as the acetyl donor (Figure 2.10A). This result suggests that this enzyme in fact prefers acetylated lysine proteogenic substrates over p-NP acetate. These results taken together show that acetylated lysine containing peptides are better substrates for ABHD14B compared to p-NP acetate, and at first approximation, ABHD14B can perform acetyl-transfer reactions on acetylated lysine-containing peptides, presumably without much sequence consensus.

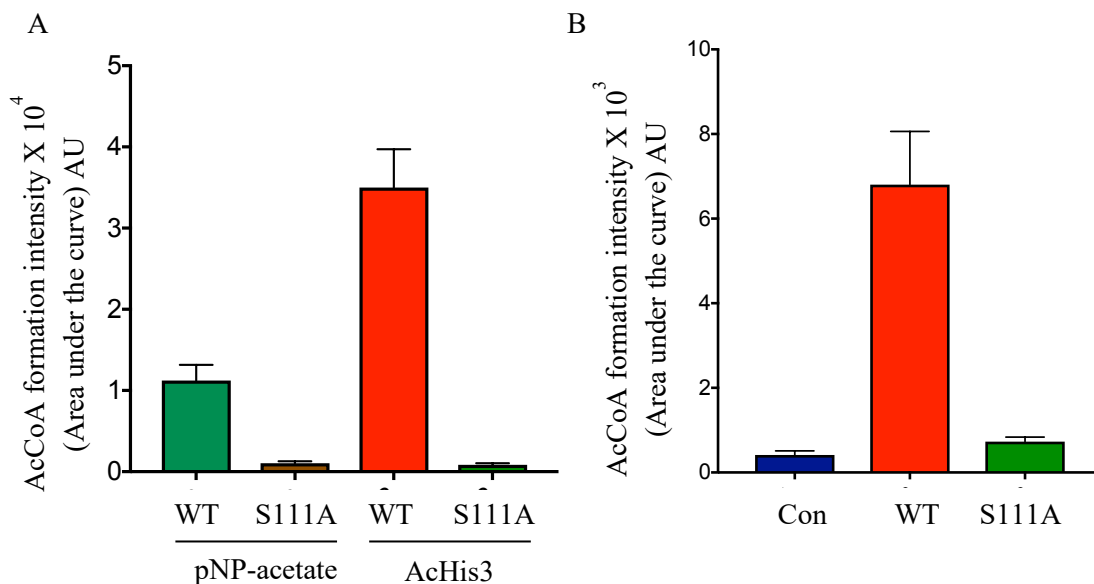


Figure 2.10: ABHD14B performs a lysine deacetylase reaction on peptide and protein substrates. A-Formation of AcCoA by WT human ABHD14B, but not S111A human ABHD14B,

when incubated with an enzyme equimolar concentration of the diacetylated (Lys9/14) histone H3 peptide (1–20) and excess CoA. The control sample for this assay is the entire mixture without the enzyme B -Formation of AcCoA by WT human ABHD14B, but not S111A human ABHD14B, when incubated with histone preparations from calf thymus histones and excess CoA. The control sample for this assay is the entire mixture without the enzyme. All LC–MS data represented in panels are means $\pm$ the standard deviation from three independent experiments. (These experiments were performed by Dr. Abinaya R).

Having shown that WT human ABHD14B can transfer an acetyl group from an acetylated lysine-containing peptide to CoA, we wanted to determine if this enzyme can also perform a similar lysine deacetylase reaction using acetylated lysine containing protein substrates, and we chose histone preparations from calf thymus to test this premise, as previous studies have shown that these proteins are acetylated. In this assay, we incubated WT or S111A human ABHD14B with histone preparations from calf thymus in the presence of excess CoA and assessed whether AcCoA was formed at the end of the reaction by LC–MS analysis. We found from this LC–MS assay that WT human ABHD14B was indeed able to produce AcCoA from histone preparations obtained from calf thymus (that are presumably acetylated), while the catalytically inactive S111A human ABHD14B mutant did not produce any acetyl-CoA of significance and had no enzyme control levels of acetyl-CoA in this assay (Figure 2.10B). Taken together, these results suggest that this enzyme can indeed perform a lysine deacetylase reaction on both peptide and protein substrates containing post-translationally modified acetylated lysine and corroborate the findings from the genetic knockdown of ABHD14B in HEK293T cells shown Figure 2.9.

A putative substrate binding site identified in ABHD14B: To address how substrates could approach the catalytic triad of residues in ABHD14B, we used two computational tools to search for tunnels in the protein^{57,58,59,60}. Both the computational programs calculated similar tunnels that were slightly different in length but superimpose well with one another (Figure 2.11A). These tunnels run from the surface of the protein near the active site and emerge on the opposite site. The combined length of the calculated tunnels is approximately 20 Å, which is appropriate for binding the phosphopantetheine arm of CoA⁷⁰ and positioning the thiol

moiety near the catalytic serine residue (S111). It is important to note that the tunnel entrance shows an overall positive charge (Figure 2.11B) that is likely a key factor in the binding of a sulphate ion in the enzyme active site from the crystallography experiment in its entrance, but could also stabilize formation of the tetrahedral intermediate during the reaction cycle. Importantly, the presence of a tunnel that enters and exits from the surface of the protein serves to explain how this protein could mediate the participation of two substrates (e.g. an acetylated protein residue and CoA) in the reaction. It is also important to note that at the tunnel exit, there are several protein residues (W198, H99, H55, and R56) that could be used to facilitate binding of the pyrophosphate moiety of CoA (Figure 2.11C).

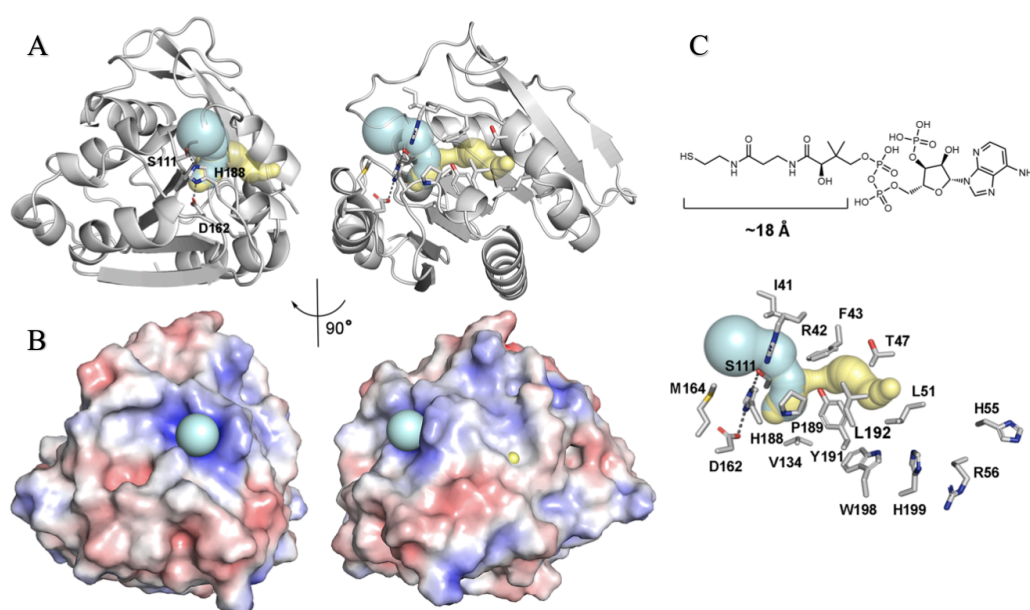


Figure 2.11: A- The tunnels calculated by CAVER (teal) and MOLEonline (yellow) are essentially identical albeit slightly different in length using the available structure of human ABHD14B (PDB: 1IMJ). The entrance of the tunnel is near the catalytic triad (S111, H188, D162). Right panel is the same view rotated by 90°. All of the residues that line the tunnel are shown as sticks. The bottleneck, or narrowest point of the tunnel is S111 of the catalytic triad. B- An electrostatic calculation of the structures shown in panel A. The entrance of the tunnel, which houses the catalytic triad of residues has an overall positive charge, which may assist in catalysis. C- The length of the phosphopantetheine arm of CoA is consistent with the length of the calculated tunnel in ABHD14B. At the exit of the tunnel (yellow) are several candidate residues for interacting with the nucleotide 5'-pyrophosphate moiety of CoA. This view of the tunnel is equivalent to that shown in panel A (right side) and has labels for each of the residues that line the tunnel. (This experiment was done by Mr. Johnny Mendoza and Dr. Jennifer Bridwell-Rabb, University of Michigan, Ann Arbor.)

Discussion

In this chapter, I aimed to annotate the function of ABHD14B, an orphan enzyme from the serine hydrolase family. I show here that ABHD14B catalyzes an unprecedented lysine deacetylase (KDAC) reaction, transferring an acetyl group from a post-translationally acetylated lysine residue of a protein to CoA, and in the process makes AcCoA and regenerates the amine of the lysine residue of proteins (Figure 2.9), and we provide several lines of compelling evidence toward this annotation. First, I showed by substrate hydrolysis assays using surrogate acylated p-NP analogues that ABHD14B has a very tight structure activity relationship and strongly prefers p-NP acetate as a substrate and, as the chain length of the acylated group of pNP increases, the efficiency of enzymatic hydrolysis of ABHD14B concomitantly decreases (Figure 2.5 and Table 2.1). Second, I showed that ABHD14B binds CoA, that this binding of CoA significantly increases the level of p-NP acetate hydrolysis, and that this enzyme can in fact transfer the acetyl group from p-NP acetate to CoA to form acetyl-CoA in the process (Figure 2.8). Third, I showed that genetically knocking down ABHD14B in human HEK293T cells results in increased levels of protein lysine acetylation and decreased cellular concentrations of AcCoA, thereby confirming in cell physiological settings that ABHD14B indeed functions as a novel lysine deacetylase (Figure 2.9). Fourth, and most importantly, I validated these cellular findings by complementary in vitro acetyltransferase assays using acetylated lysine-containing peptide and protein substrates and showed that ABHD14B can in fact produce AcCoA from these substrates (Figure 2.10). Interestingly, I also found in these assays that in comparison to the “surrogate” p-NP acetate substrate, the “native” acetylated lysine-containing peptide and protein substrates produce more AcCoA for similar substrate concentrations, suggesting that the latter are preferred substrates for ABHD14B (Figure 2.10). Finally, I have identified an active site tunnel in the WT human ABHD14B that extends from the surface of the protein near the catalytic triad to the far end of the protein, suggesting that indeed this enzyme’s active site can accommodate two different substrates and more specifically is comparable in length to the phosphopantetheine arm of CoA, which eventually accepts the acetyl group (Figure 2.11).

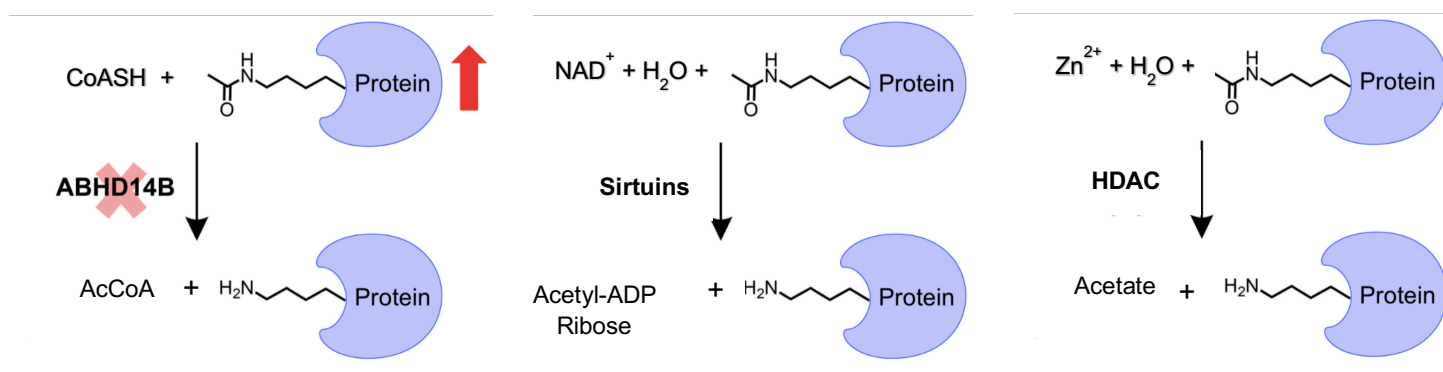


Figure 2.12: Lysine deacetylase reactions catalysed by ABHD14B in contrast to those catalysed by Sirtuins and HDACs.

Why is the ABHD14B catalysed lysine deacetylase novel? There are currently two known enzyme families that can perform a lysine deacetylase reaction: the sirtuins^{71,72} and the HDACs^{69,73}. Both of these enzyme classes have distinct enzymatic mechanisms for deacetylating lysines. The sirtuins use a molecule of NAD⁺ to perform the deacetylase reaction, and in the process, generate acetyl-ADP-ribose, the eventual fate of the acetyl group from the post-translationally acetylated lysine (Figure 2.12). In contrast, the HDACs, have conserved acidic (Aspartate) and basic (Histidine) residues in the enzyme active site that act in tandem to activate a water molecule, and perform a cofactor-less hydrolysis reaction. The fate of the acetyl group in the enzymatic reaction catalysed by HDACs, is the formation of a free acetate molecule that is eventually recruited back into metabolism (Figure 2.12). Here, by functionally characterizing ABHD14B, I present a third mechanism for deacetylating lysine residues, using the consensus serine hydrolase mechanism, where in the first step the acetyl group from lysine is transferred to the nucleophilic serine residue of ABHD14B to form an acetyl-enzyme covalent intermediate. In the second step, a molecule of CoA binds to the enzyme, and the acetyl group from the acetyl-enzyme covalent intermediate is transferred to CoA to yield AcCoA. The unique and distinguishing features of the ABHD14B catalysed reaction in comparison to sirtuins and HDACs, is that, the reaction catalysed by the former involves the formation of a covalent acetyl-enzyme intermediate, and the eventual fate of the acetyl-group is the formation of AcCoA.

CHAPTER THREE: Characterization of ABHD14B mutants.

ABD14B is a novel lysine deacetylase and the first one discovered of its kind and its mechanism of action is very different from that of the other two known classes of deacetylases, the sirtuins and the HDACs. Both the sirtuins and HDACs are highly conserved evolutionarily and can be found in most kingdoms of life. There are more than 15,000 known sirtuins identified in more than 6000 species spanning all domains of life from bacteria to plants and animals⁷⁴. HDACs are also equally conserved having members in all eukaryotic organisms and homologs in bacteria and archaeobacteria⁷⁵. This tells us that these proteins play a very vital role in all cellular life^{73,72}. Since ABHD14B is also a lysine deacetylase, I wanted to investigate its conservation across all domains of life, and whether this information can shed any light on the nature and importance of its function.

The *abhd14b* gene is located on the reverse strand in chromosome 3 in humans and on the forward strand in chromosome 4 in mice. In both these organisms, it is flanked upstream by the gene *pcbp4*. Another gene *abhd14a* is present on the opposite strand of *abhd14b* almost completely complimentary to it. This gene encodes for a protein ABHD14A, which is unannotated. From its sequence, it appears to be a metabolic serine hydrolase and a homolog of ABHD14B. They both have high sequence similarity (Figure 3.1) and a 47% identity, making them homologs. One main difference is that ABHD14A contains an N-terminal alpha helix which is absent in ABHD14B and could be responsible for its anchorage to the membrane. In one study it was found that in the developing mouse cerebellum the expression of ABH14A was controlled by the presence of Zic1 and its expression peaked at day 17.5⁷⁶. Apart from this, an anomalous expression of ABHD14A has been associated with certain neurodegenerative diseases^{77,78,79,80} and cancers^{81,82}. This almost complimentary arrangement of two homologous proteins is unusual and could have implications in their evolution.

In this chapter I have identified ABHD14B sequences across different domains and used them to build a phylogenetic tree. I have also done multiple sequence alignments (MSA) with the sequences and identified conserved residues. I have picked certain important conserved residues and made point mutations to biochemically study the effects of the mutagenesis further.

This work was done in collaboration with Dr. M. S. Madhusudhan. The phylogenetic tree building and the MSAs were done by his student Mr. Golding Rodrigues.

Score	Expect	Method	Identities	Positives	Gaps	Frame
167 bits(423)	3e-56()	Compositional matrix adjust.	91/195(47%)	121/195(62%)	9/195(4%)	
Query 19		LFFREALPGSGQARFSVLLHGI	RFSSETWQNLGTLHRLAQAGYRAVAIDLPGLGH---	S		75
Sbjct 81		IFYREVLPLNQAH	RVVLLHGKAFNSHTWEQLGTLQLLSQ	RGYRAVALDLP	PGFGNSAPS	140
Query 76		KEAAAPAPIGELAPGSFLAAVVD	ALELGPPVVISPSLSGMYSLPFLTAPGSQ	LPGFVPVA		135
Sbjct 141		KEAST-----EAGRAALLER	ALRDLEVQNAVLVSPSLSGHYALPFLMRGHHQLHGFVPIA			195
Query 136		PICTDKINAANYASVKTPALIVYGDQDP	-MGQTSFEHLKQLPNHRVLIMKGAGHPCYLDK			194
Sbjct 196		PTSTQNYTQEQFWAVKTP	TLILYGELDHILARESRLRHLPHNSVVKLRNAGHACYLHK			255
Query 195		PEEWHTGLLDLFLQGL	209			
Sbjct 256		PQDFHLVLLAFLDHL	270			

Figure 3.1: Sequence alignment of human ABHD14B and ABHD14A. ABHD14B is the query and ABHD14A is the subject.

Materials

All chemicals, buffers and reagents were bought from Sigma Aldrich unless specified otherwise. Human *abhd14b* gene was synthesized as a codon optimised construct from Genscript for expression in *E.coli*, pET45b(+) vector (Millipore) with a N terminal 6X His tag, Pfu DNA polymerase was bought from Promega (Cat# M7741) and Luria Bertini broth was purchased from HiMedia in a powdered form (Cat#M1245). Ampicillin (Cat# 69-52-3) and isopropyl- β -D-1-thiogalactopyraniside (IPTG) (Cat# 376-93-1) were bought from MP Biomedicals. His-Trap High Performance column (Cat #17-5248-02) and HiLoadTM16/60 Superdex200 were bought from GE Healthcare, Amcon Ultra-15 Centrifugal Filter units (Catalog # C7715). Ni-NTA His Bind Resin was bought from Merck (Catalog #70666). The Chemical probe Flourophosphonate rhodamine (FP-Rh) was a generous gift from Prof. Benjamin F. Cravatt (The Scripps Research Institute).

Methods

Bioinformatics searches and analysis: To investigate the prevalence of ABHD14B protein sequences across all organisms, PSI-BLAST searches were done⁸³, using the reference sequence for human ABHD14B (RefSeq: NP_001139786.1, Uniprot: Q96IU4). This was done against standard non-redundant databases with a expect threshold of 0.00005 and a maximum

return of 5000 hits, for three iterations. For further sorting of the sequences, a home-made script was written to determine the presence of the conserved catalytic triad, across the length of the sequence by carrying out global pairwise alignments using the Needleman-Wunsh algorithm. For the MSAs, one sequence was taken from each representative organism and aligned using MAFFT⁸⁴. The alignments were further used in MEGA-X⁸⁵ to build maximum likelihood trees and the trees were annotated and visualized in iTol⁸⁶. The fully conserved residues were identified using pypisviz and Bio.Align packages in Python and functionally conserved residues were identified manually.

(The PSI-BLAST searches and tree-building was done by Mr. Golding Rodrigues and the MSA was done by Mr. Archit Devrajan.)

Cloning, Expression and Purification of ABHD14B mutants: The human *abhd14b* gene (codon optimised) was cloned into the pET45b (+) vector, with a N-terminal 6X-His tag. For generating single mutations, Dpn1 dependant site-directed mutagenesis was carried out using Pfu DNA polymerase. The resulting colonies were sequence verified and the plasmids containing the desired mutations were individually transformed into competent *E.coli* BL21 (DE3) cells. A single colony from this transformation was inoculated into 5 ml of Luria Bertani (LB) medium containing Ampicillin (working concentration 100 µg/ml) and grown overnight (12-14 hours) in an incubator at 37 °C with 180 rpm shaking. This primary inoculum was transferred into 1 L of the same media and grown under similar conditions till its absorbance at 600 nm reached a value of ~0.6. After this, the expression of the desired protein was induced by adding 500 µM of IPTG to the culture. The culture was kept at a temperature of 18 °C for the next 16 hours with 180 rpm shaking. The culture was then pelleted by spinning at 6000 g for 20 minutes at 4 °C. The pellet was washed once with 50 mM Tris (pH 8) and subsequently stored at -80 °C until further use. Over expression was checked by SDS- PAGE followed by Coomassie staining. Upon confirmation of robust expression, the pellet was used further for protein purification.

ABHD14B mutants were purified using Ni-NTA affinity chromatography using a His-Trap High Performance column. A cell pellet (containing cells from 1 L BL21 culture which has confirmed overexpression of desired protein) was thawed and resuspended in 50 ml of Binding Buffer (50 mM Tris pH 8 10 mM imidazole). The cells were lysed using a Vibra Cell VCX 130 probe sonicator (Sonics) at 60% amplitude for 30 minutes with 2 seconds “ON” and 3

seconds “OFF” cycle on ice. The lysate was then centrifuged at 30,000 g for 30 minutes to pellet down the insoluble fraction. At the same time, a His-Trap High Performance column was equilibrated with 10 Column Volume (CV) of Binding Buffer. The resulting soluble fraction from the spin (which contained the ABHD14B mutants) was applied to the column and the flow through collected. The column was then washed with 10 CV of binding buffer. The protein was eluted from the column using an increasing gradient of imidazole (50 mM – 500 mM) in 50 mM Tris pH 8. The purity of the fractions collected was checked using SDS-PAGE. A few of the mutants eluted cleanly from the column and fraction containing the protein were pooled and set up for dialysis overnight (with one buffer change after 3 hours initially) to remove the imidazole. After the dialysis, the mutants were concentrated using Amicon Ultra-15 Centrifugal Filter unit with a molecular weight cut-off filter of 10 kDa. After sufficient concentration, aliquots of 10 µl were made, flash frozen and stored at -80 °C till further use.

For the mutants that did not elute out cleanly an additional step for purification was done.

The fractions containing the ABHD14B mutant were pooled and concentrated to a volume of 5 ml using a Amicon Ultra-15 Centrifugal Filter unit with a molecular weight cut-off filter of 10kDa. Approximately 4ml of this concentrated protein was loaded onto a HiLoad™16/60 Superdex200 gel filtration column which had been pre-equilibrated with 50 mM Tris pH 8. All the mutants eluted in the range of 87-92 ml. The fractions containing the proteins were again concentrated in a similar way and aliquots of 10 µl were made, flash frozen in liquid nitrogen and stored at -80 °C till further use.

For the D162A mutant, after sonication, most of the protein was getting separated into the insoluble fraction, therefore a column could not be used for purification. Instead, Ni-NTA His Bind Resin was used. The cell pellet was lysed as described earlier, after which the lysate was given a short spin at 6000 g for 15 minutes at 4 °C to remove unlysed cells. 1 ml of the Ni-NTA resin was used for one purification. 1 ml of the beads were taken in a 2 ml Eppendorf tube, spun at 700 g for 2 minutes and the supernatant discarded. The beads were then given two 2 ml washed with the Binding Buffer. The lysate from before was added to the beads and kept at 4 °C for 30 minutes with constant gentle mixing using an end over end rotor. After 30 mins, the mixture was spun at 700 g for 2 minutes. The supernatant was collected as the flow through. The beads were then washed twice using binding buffer. The mutant protein was eluted in 1 ml fractions and an increasing gradient of imidazole was used. The beads were incubated with the elution buffer for 10 minutes with gentle shaking before spinning at 700 g for 2 minutes and collecting the supernatant. Two fractions were collected for each

concentration of imidazole. All the fractions were run on a SDS-PAGE gel and fractions containing the protein were pooled. Dialysis and concentration were done as previously mentioned. **(This was done with help from Ms. Mihika Yeolekar)**

Gel Based Activity based protein profiling (ABPP): All gel-based ABPP assays were performed as described in Chapter 2 (Page 24). In these assays, the reaction volumes were 50 μ l and both the protein and FP-Rh were had concentrations of 5 μ M. All samples were resolved in a 12.5 % SDS PAGE gel and the activity was imaged using an iBright1500 (Invitrogen) gel documentation system.

Colorimetric Substrate Hydrolysis Assays: All colorimetric substrate hydrolysis assays were performed using p-NP acetate as described in Chapter 2 (Page 24). The buffer used was 50 mM Tris pH 8 and the reaction volume was 250 μ l and the reaction was carried out for 30 min. The concentration of ABHD14B mutants used was 10 μ M and the concentration of p-NP acetate was 100 μ M. For calculating the activity relative to WT, the 500 second time point was chosen since the assay was found to be linear at this time point with a good signal to noise ratio.

Data plotting: All data in this chapter was plotted using GraphPad Prism 9 version (9.5.1) software for Mac OS -X. All data is represented as the mean $\pm$ the standard deviation for at least three independent experiments (biological replicates).

Results

Identification of ABHD14B sequences: Since ABHD14B and ABHD14A have a high sequence similarity, we noticed that across various protein databases, these two enzymes were mis-assigned to each other. Certain ABHD14B sequences were assigned as ABHD14A and vice versa. To distinguish one sequence from the other, we had to set down certain criteria to classify a sequence as one or the other. A bioinformatics study done on mammalian ABHD enzymes identified two conserved sequence motifs for mammalian ABHD14Bs – the

nucleophilic motif of VVISPSLSSGMY sequence (containing the SXSXS motif) and an acyl transfer motif of GAGHPCYLDKPE sequence (containing the HXXXXD motif)⁸⁷. Thus, the sequences containing the catalytic triad, the nucleophilic motif (allowing for two mismatches)

and the acyl transferase motif (allowing for two mismatches) was classified as an ABHD14B sequence.

From the initial 5000 hits obtained from using the human ABHD14B sequence (RefSeq: NP_001139786.1, Uniprot: Q96IU4), 1012 sequences contained the nucleophilic motif (of which 792 were perfect matches), 1265 sequences contained the acyl transferase motif (of which 217 were perfect matches) and 2929 sequences contained the catalytic triad. In total 847 sequences from 697 organisms met the filtering criteria and were classified as ABHD14B sequences.

Phylogenetic classification of ABHD14B sequences: From the obtained sequences, it was clear that ABHD14B was almost exclusively found in the phylum *Chordata* (695 of the 697 organisms). It was also clear that quite a few organisms contained more than one isoform of ABHD14B, especially in class *Mammalia* and *Aves*, which is why the number of sequences was significantly more than the number of organisms. While studying the sequences further, we chose the longest ABHD14B sequence from any particular organism. As seen from Figure 3.2, the most represented classes in phylum *Chordata* were *Aves* (~ 45%, 313 organisms of the 697 organisms identified) and *Mammalia* (~ 27%, 189 organisms of the 697 organisms identified) followed by *Actinopterygii* (Bony Fish) (~ 22%, 156 organisms of the 697 organisms identified) and a small representation seen in *Reptilia* (~ 5%, 33 organisms of the 697 organisms identified). Based on the analysis, the avian sequences were most closely related to the mammalian sequences and sequences from *Actinopterygii* and *Reptilia* were closely related to each other and together were more closely related to the mammalian sequences than the avian ones. Another interesting finding was that 263 out of the 697 organisms that were found to have the sequence for ABHD14B also contained the sequence for ABHD14A.

Conservation of residues within the ABHD14B sequences: We wanted to see the extent of conservation in the identified ABHD14B residues. To find the conserved residues, we performed MSA analysis on the sequences and found that 86 residues were highly conserved

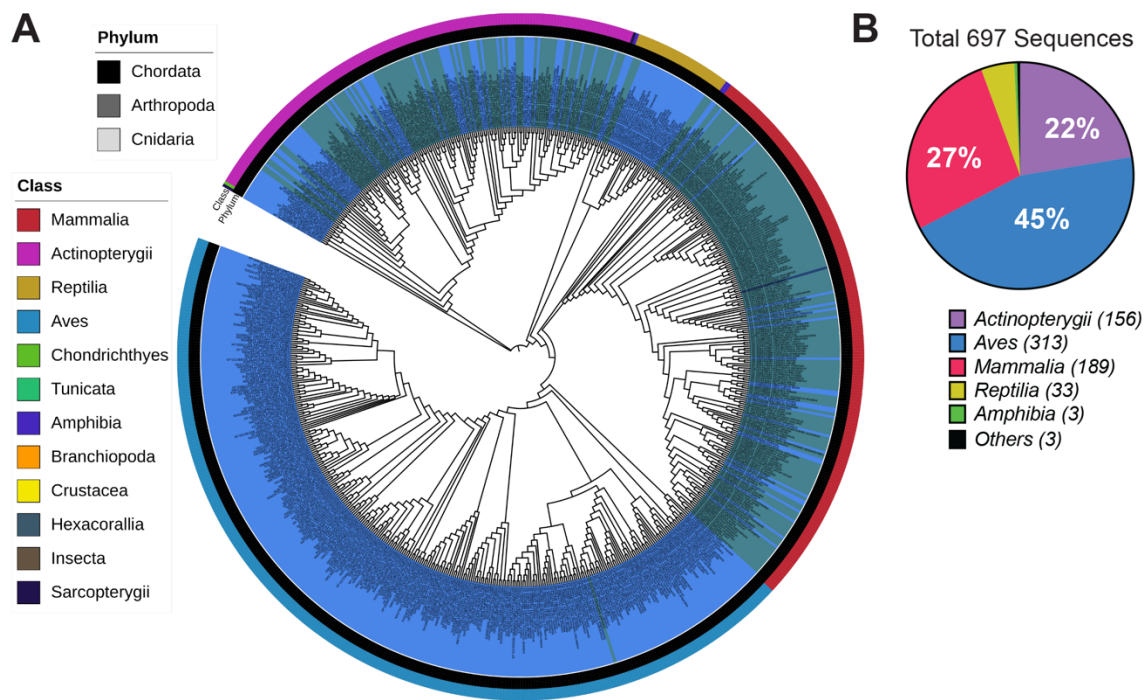


Figure 3.2: A -Phylogenetic tree of ABHD14B representing the identified sequences from 697 organisms. The outer and middle circular colouring represents the class and Phylum to which the sequence belongs (see the legend) while the inner circular colouring (blue or green) represents whether the organism has the sequence for only ABHD14B (blue) or for both ABHD14B and ABHD14A (green). B- The pie chart represents the ABHD14B sequence data from various organisms. (This was done by Mr. Golding Rodrigues).

(present in >90% of all sequences) (Table 3.1). This means the overall sequence conservation of ABHD14B across all organisms was around 40% which is high. And within the class *Mammalia* It was found to be significantly higher (~80%).

Apart from the overall conserved residues, we also found that 20 residues were functionally conserved (presence of functionally similar amino acid type at that position) (Table 3.2). To study them further I mapped these residues on the structure of ABHD14B (1imj) (Figure 3.3). From the structure I found that a lot of the conserved residues were clustered around the active site. The flexible loop present near the active site also seems to be well conserved. Another region of high conservation was the putative CoA binding tunnel which consists of many hydrophobic residues.

Residue	Location
I41, R42	Present near the edge of the active site cleft, near the entrance of the putative CoA tunnel
F43, S44, S45, W48	Part of the putative CoA tunnel
T 53	Present near the putative CoA tunnel
L 54, L57, A58, G61	Present on alpha helix A
A 64, V65, A66, D68	Present on beta strand 4
L 69, P70, G71, L72, G73, S75, K76, A78, A80, P81, A82, G85, P89	Present on loop connecting beta strand 4 and alpha helix B
F 92, L93, V96, L100	Present on alpha helix B
V 106, V107	Present on beta strand 5
S 109, P110	Present in the nucleophilic elbow
S 111	Active Site Serine
L 112	Present in the nucleophilic elbow
S 113, G114, M115, Y116, S117, L118, P119, F120, L121	Present on alpha helix C
Y 131, P133, V134, A135	Present on beta strand 6
P 136, I137, C138, T139, K141	Present on loop connecting beta strand 6 and alpha helix D
Y 147	Present on alpha helix D
L 155, I156, V157, Y158, G159, D160	Present on beta strand 7
Q 161	Present on the loop connecting strand 7 and alpha helix E
D 162	Part of the catalytic triad
G 165, S168, L172	Present on alpha helix E
L 175	Present on loop connecting alpha helix D and beta sheet 8
H 178	Present on beta sheet 8
V 180	Present on beta sheet 9
G 185, A186, G187	Present in the loop connecting beta sheet 8 and alpha helix F
H188	Base of the catalytic triad
C 190, Y191, L192	Present in the loop connecting beta sheet 8 and alpha helix F
D 193, P195, W198, H199, L202, F205, L206, L209	Present on alpha helix F

Table 3.1: Fully conserved residues (present in >90% of all the sequences) of ABHD14B

Residue	Location
E46, L51	Present on alpha helix A
I67	Present on beta sheet 3
I84, E86, A88	Present on loop connecting beta strand 4 and alpha helix B
D98	Present on alpha helix B
L102	Present in loop connecting alpha helix B and beta strand 5
I108	Present on beta strand 5
P129, G130, V132	Present on beta strand 6
D140	Present on loop connecting beta strand 6 and alpha helix D
A144, N146, V150	Present on alpha helix D
M164	Present on alpha helix E
L181, I182, M183	Present on beta sheet 9

Table 3.2: Functionally conserved residues of ABHD14B.

Mutagenesis and Biochemical characterisation of mutants: From bioinformatics, I was able to find the conserved residues of ABHD14B. I created point mutations of certain important residues using site-directed mutagenesis and studied their effects on the biochemical activity of the enzyme (Table 3.2). The residues chosen were the following – H188 and D162: active site residues, R42 and K141: present at the edges of the active site cleft and could potentially interact with the incoming substrates, H55, R56 and H199: positively charged residues in the CoA tunnel could help CoA binding by neutralising the negative charges on the phosphate moieties, W198 and Y191: present in the CoA binding tunnel, might be involved in pi stacking with CoA, S75: conserved residue on the surface of the protein. All the residues were mutated to an Alanine. They were cloned with a 6X His tag and the same was used for purification. Certain mutants like R42A, H55A and K141A were easy to purify and required only a single step Ni-NTA purification. All the other mutants did not elute out cleanly and required a two-step purification. For these mutants, after the first step of Ni-NTA purification, we passed the fractions containing the protein through a preparative Superdex 200 column to obtain clean protein. Figure 3.4 shows the purification gel of W198A as a representative. Using this method, we were able to obtain pure samples of R56A, S75A, H188A, Y191A, W198A, D193A and H199A. It was also observed that these mutants required a lower temperature to be expressed in high amounts and eluted from the Ni-NTA column with a much lesser concentration of imidazole (100-200 mM) as opposed to the wild type (500 mM). This could be because a lot of these residues that are mutated are present in the interior of the protein and mutating them to an Alanine could cause a disruption in the normal folding of the enzyme. If the protein is not

folded very well, its binding to the Ni-NTA column is also affected. For the D162A mutant, best results were obtained by purifying the protein using Ni-NTA beads rather than a column.

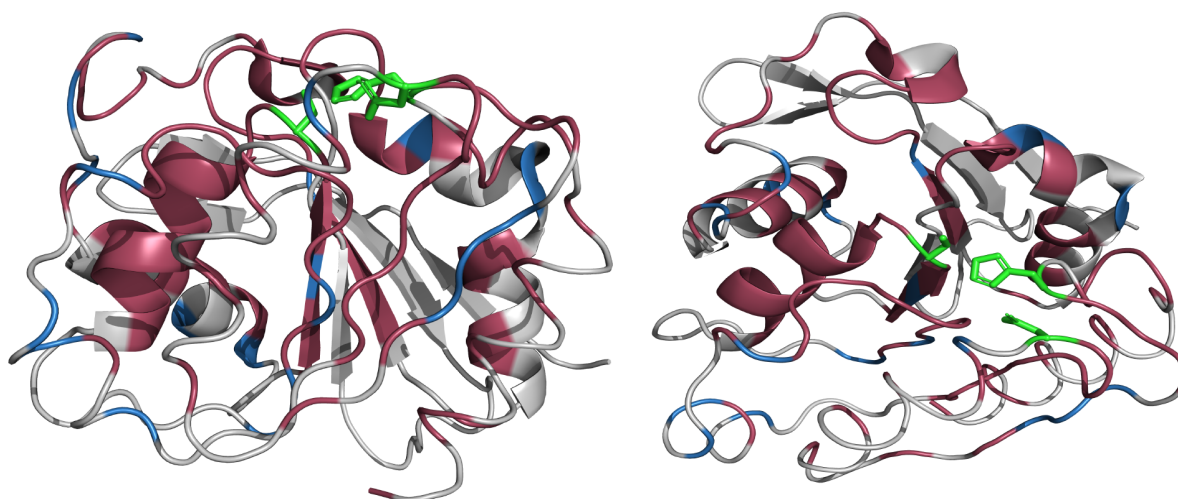


Figure 3.3: Conserved residues mapped on the structure of ABHD14B (1imj). The pink residues are fully conserved, and the blue residues are functionally conserved. The residues in green are the catalytic triad.

Residue	Description
R42	Present close to the active site, might interact electrostatically with the substrate.
H55, R56, H199	Present in the CoA tunnel, might be involved in pyrophosphate charge neutralisation
S75	Fully conserved residue near the surface, might be a site for PTM
K141	Predicted to interact electrostatically with the substrate
Y191, W198	Found in the CoA binding tunnel and might be involved in pi stacking with CoA
D193	Part of the acyl transfer motif
D162A, H188A	Part of the catalytic triad

Table 3.3: List of all the residues mutated.

To study the effect of the mutations on the activity of the enzyme, I used a gel based ABPP approach. I used the FP-Rh (Fluorophosphonate rhodamine) probe⁴⁸. As seen in Figure 3.6, the active site mutants, namely S111A, D162A and H188A were unable to interact with the probe and showed no signal on the activity gel. This was expected since the probe binds to the active site Serine and the binding depends on the nucleophilicity of the Serine. Mutating the two gate-keeping residues of the active site tunnel- R42A and K141A did not seem to affect the binding of FP-Rh. Reducing the positive charges in the CoA tunnel by mutating H55 and R56 did not affect FP-Rh binding but mutating the positive charge on H199A seemed to reduce binding as compared to the wild type. Mutating D193 which is part of the acyl transfer motif HXXXXD drastically reduced the activity of ABHD14B towards FP-Rh. Interestingly, the S75A mutant also showed significant reduction in the binding of FP-Rh as compared to the wild type.

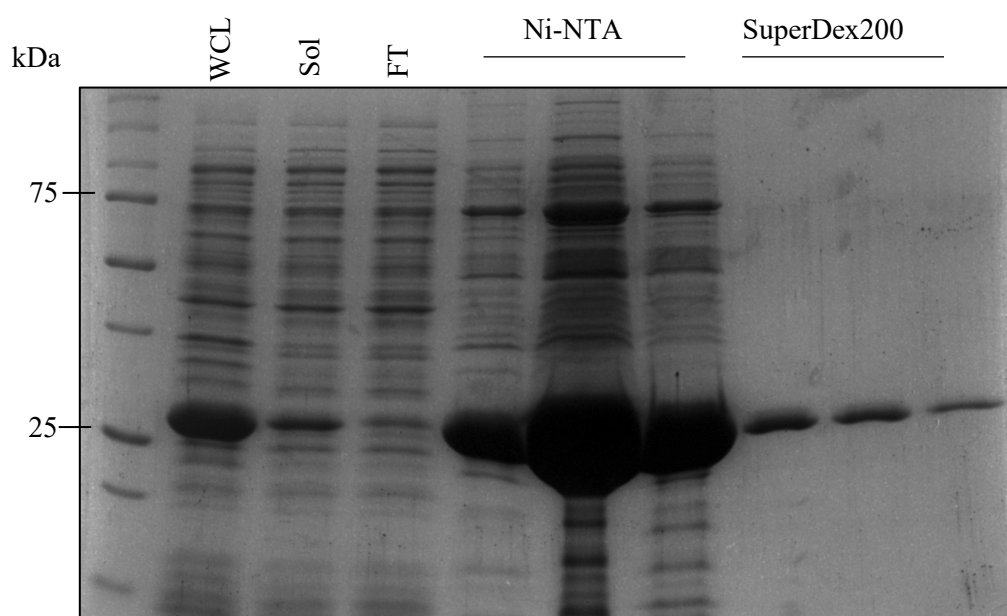


Figure 3.4: Purification gel of W198A ABHD14B showing the two-step purification. WCL – whole cell lysate, Sol – Soluble fraction, FT- Flow Through

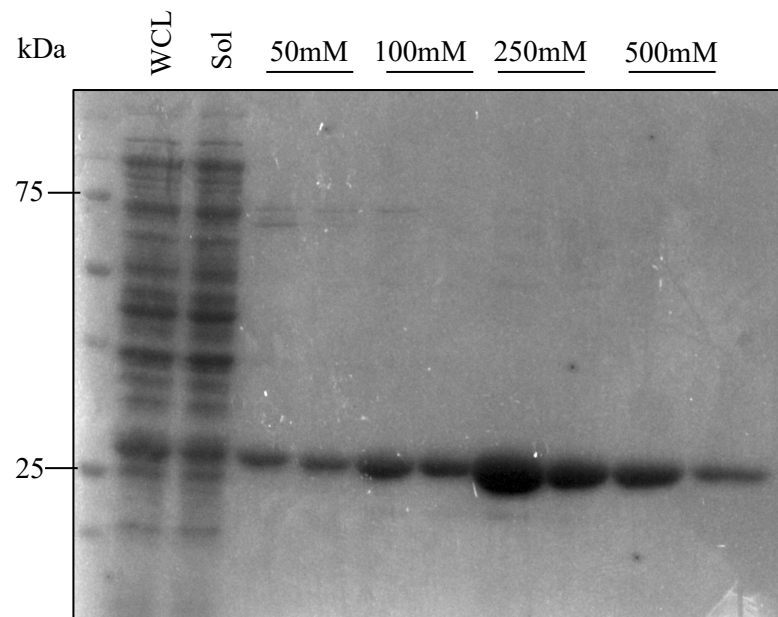


Figure3.5: Purification of D162A using Ni-NTA His Bind resin. The concentrations indicate the concentration of imidazole used for elution; WCL- whole cell lysate, Sol- Soluble fraction.

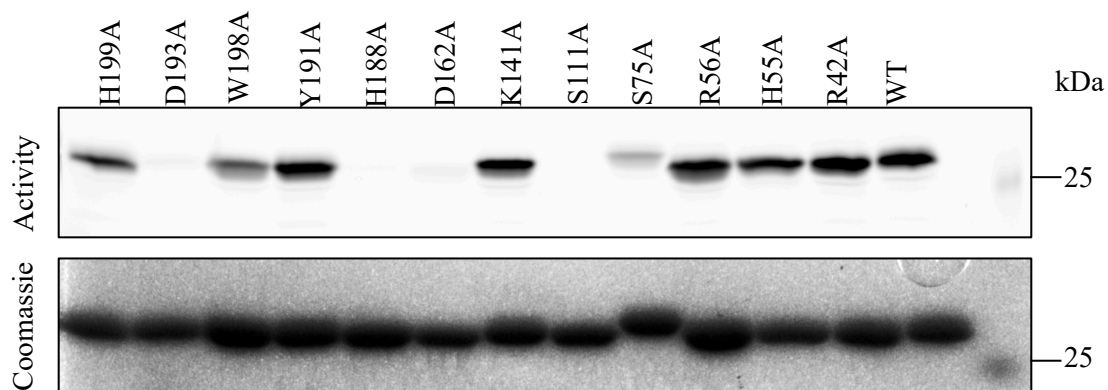


Figure 3.6: ABPP of the mutants. The upper panel represents the activity gel and the lower panel represents the Coomassie gel used as a loading control.

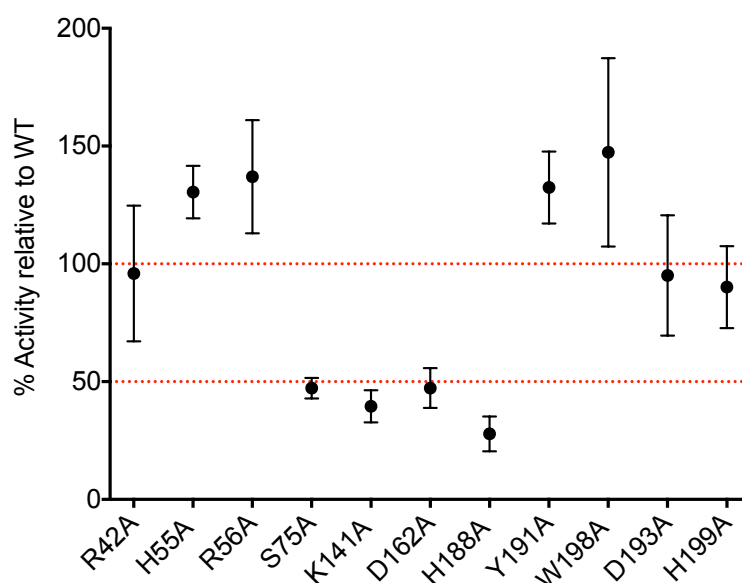


Figure 3.7: Catalytic activity of ABHD14B mutants to turn over pNP-acetate. The activities have been normalised to that of the WT.

To study the catalytic activity of the mutants I used the surrogate substrate p-NP acetate since WT ABHD14B can hydrolyse p-NP acetate as described earlier (Figure 3.7). As expected the active site mutants H188A and D162A were very sluggish in the hydrolysis. The hydrolysis capacity of S75A was also significantly diminished as compared to the WT. So was the hydrolysis capacity of K141A. The other mutants' catalytic activity was comparable to that of the WT.

Discussion

Through extensive bioinformatic analysis, we have identified 847 sequences from 697 organisms belonging to ABHD14B (Figure 3.2). This novel lysine deacetylase seems to be present only in *Chordates* with no sequences found in bacteria, Protista, plants or fungi. This seem to be very different from other known deacetylases namely the HDACs and Sirtuins, which are highly conserved across all domains of life and perform very vital functions inside the cell. This does raise the question whether the main function of ABHD14B is only required by *Chordates*. Maybe ABHD14B evolved later in time to solve a problem that only persisted

in *Chordates*. There are no known homologs of this protein in any other phylum. Though this can only remain a speculation till the main targets for this enzyme are elucidated.

Through MSA, we identified residues that were conserved across all the sequences. 86 out of the 210 residues were conserved (found in >90% of all sequences) (Table 3.1) and 20 were functionally conserved (Table 3.2). Out of these conserved residues, I picked a few important ones to study further.

K141 lies at the edge of the deep cleft in which the active site sits. From the structure, it is hypothesized that this residue can electrostatically interact with the substrate moiety, since the active site region is predominantly a hydrophobic pocket. By mutating K141 into an Alanine, the ability of the enzyme to turn over p-NP acetate was greatly reduced (<50% of the WT) yet, it was still able to bind FP-Rh (Figure 3.6 and 3.7). FP-Rh is an irreversible covalent inhibitor whereas p-NP acetate is a substrate mimic. The fact that the catalysis was significantly reduced indicates that this residue might be important in the proper orientation of the first substrate (acetylated peptides/proteins) for optimal catalysis. In recent acetylome studies, K141 was found to be acetylated in MEF cells⁸⁸. Though it requires some amount of validation, K141 has emerged as an interesting residue to study further.

D193 is a conserved residue found in the acyl transfer motif HXXXXD. It is very close to the active site Histidine H188. In some acetyl transferases, the Aspartate in the HXXXXD motif is important in keeping the Histidine in place in the active site⁸⁹. And placement in the active site is very important for a Serine hydrolase because of the charge relay system. In ABHD14B, when you mutate D193 to an Alanine, it causes drastic reduction of its binding to FP-Rh, though its capability to turn over p-NP acetate remains largely unchanged (Figure 3.6 and 3.7). This could be due to a lowered nucleophilicity of the active site serine, since it is much easier to attack the carbonyl carbon in p-NP acetate than to attack the phosphorous atom in FP-Rh. The lowered nucleophilicity of the active site Serine could indicate a slightly disturbed charge relay system. Hence D193 might be important in structurally placing the active site Histidine correctly for an efficient charge relay. Since this residue is part of the acyl transfer motif, further studies are required to see the effect of this mutation on an acetyl transfer assay involving CoA. Mutating residues found the CoA binding tunnel i.e. H55, R56, H199, Y191 and W198 did not show significant change in enzyme activity. The Y191A and H199A mutants did show slightly reduced binding to FP-Rh, but all of them were able to robustly turn over p-NP acetate (Figure 3.6 and 3.7). Since the overall structure of the tunnel is quite rigid, it is possible that mutation of more than one residues together might be required to see significant change in catalysis. Further experiments need to be done to study the binding capacity of these mutants to CoA.

S75 is a conserved residue found on the surface of ABHD14B. It is present on a flexible loop at a distance of $\sim 17\text{\AA}$ from the active site (Figure 3.8). Since it is so far away from the active site, it does not have any predicted role in catalysis. Intriguingly, mutating this residue had far reaching effects on activity. The S75A mutant showed greatly reduced binding to FP-Rh and showed significantly lower ($<50\%$) turnover of p-NP acetate (Figure 3.6 and 3.7). Since the lack of the -OH group of S75 seems to affect catalysis, it is possible that this residue might be a site for PTM associated allosteric regulation⁹⁰. This residue seems to be a good candidate for a PTM like phosphorylation because it is present on the surface of the protein and is exposed. To check whether ABHD14B has any potential kinase recognition sites I used an online tool called Group-based Prediction system (GPS) 6.0 which predicts protein-kinase specific phosphorylation sites on eukaryotic proteins⁹¹. This tool uses sequence-based predictions to identify phosphorylation sites and factors in the sequence features around the phosphorylation site. In the prediction results of human ABHD14B, there were six sites recognised as potential phosphorylation sites as listed in Table 3.4. Out of the six sites, three are fully conserved, namely Serine 75, Tyrosine 147 and Tyrosine 191. Serine 75 is a predicted site of the class of CMGC kinases, which comprise of cyclin dependant kinases, mitogen activated kinases, glycogen synthase kinases and CDC-like kinases. More studies need to be done to verify and identify this PTM in-vivo and its effect on the activity of this protein.

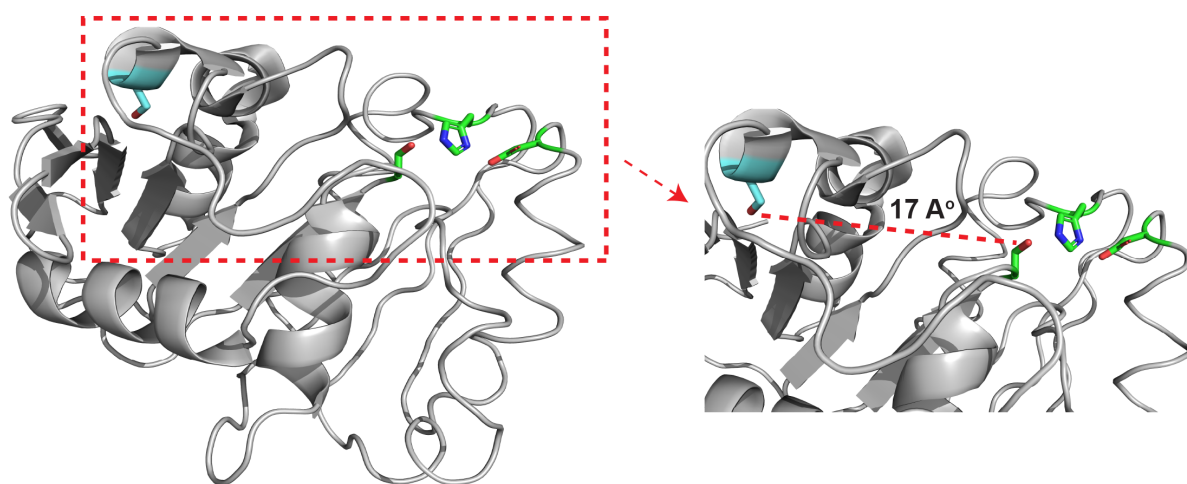


Figure 3.8: Orientation of S75 with respect to the active site. The active site residues are highlighted in green and S75 is highlighted in teal.

Position	Code	Kinase	PSP	Score	Cutoff
167	T	CK1	DQDPMGQTSFEHLKQ	0.1254	0.0532
28	S	CMGC	FREALPGSGQARFSV	0.0504	0.0403
75	S	CMGC	DLPGLGHSKEAAAPA	0.0683	0.0403
167	T	CMGC	DQDPMGQTSFEHLKQ	0.0411	0.0403
11	T	TKL	SVEQREGTIQVQGQA	0.6824	0.5041
147	Y	TK	DKINAANYASVKTPA	0.9599	0.7641
191	Y	TK	KGAGHPCYLDKPEEW	0.8335	0.7641

Table 3.4: Predicted phosphorylation sites on ABHD14B as predicted by GPS6.0. CK1-Caerin kinase-1; CMGC-Cyclin dependant-, mitogen activated-, glycogen synthase- kinases; TK- Tyrosine kinases; TKL – tyrosine kinase-like kinases.

CHAPTER FOUR: CRYSTAL STRUCTURE OF S111A HUMAN ABHD14B.

We annotated ABHD14B as a novel lysine deacetylase which uses the canonical ping-pong mechanism of catalysis followed by all serine hydrolases to do an acetyl transfer reaction from an acetylated lysine residue on proteins or peptides to a molecule of CoA, forming AcCoA. This mechanism of deacetylation is novel and ABHD14B is the only known deacetylase of its kind. It contains the highly conserved α/β hydrolase domain found in many hydrolases. Unlike a lot of other α/β hydrolases, ABHD14B lacks a lid or cap domain¹⁶. This domain is found close to the active site and has implications in substrate specificity. ABHD14B shows a very strong structure activity relationship, as shown from biochemical studies done previously (Chapter 2, Figure 2.4B). It prefers the shorter acylated esters of pNP (para nitrophenol) as substrates, as opposed to the longer ones. But from its structure, there are no clues as to how it achieves substrate specificity. It is possible that it does so by a global change in its structure after binding to a substrate. To study its substrate specificity and better understand its mechanism, I wanted to solve the structure of ABHD14B in its inhibitor bound state. The inhibitor chosen was FP-Rh which, from previous studies, was known to bind robustly to the protein (Chapter 2, Figure 2.3). Bound FP-Rh mimics the tetrahedral intermediate during the first step of the catalytic process⁴⁸. Elucidating its structure could give us insights into the structure of the intermediate and show us how the structure changes during the first step of catalysis.

I also wanted to crystallise the human S111A ABHD14B with CoA since we have shown that CoA binds to S111A better than WT ABHD14B from thermal shift assays (Chapter 2, Figure 2.5).

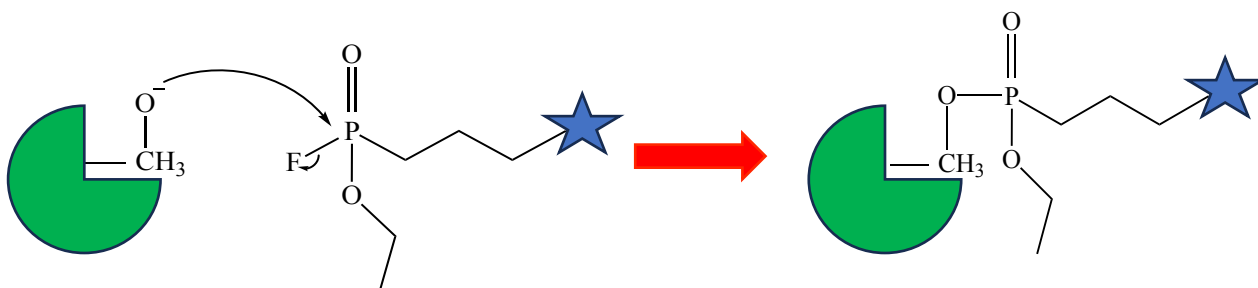


Figure 4.1: Covalent inhibition of serine hydrolases by FP-Rh. The blue star represents the fluorescent rhodamine group, the green object represents a serine hydrolase protein.

In this chapter, I have attempted to crystallize human WT ABHD14B, human S111A ABHD14B, WT ABHD14B bound to FP-Rh and S111A ABHD14B bound to CoA. I was successful in crystallizing the S111A ABHD14B mutant and solving its three-dimensional structure using X-Ray crystallography. I was unfortunately not able to crystallize the WT enzyme, or the enzyme bound to FP-Rh or CoA.

Materials

All chemicals mentioned in this chapter were bought from Sigma Aldrich. 12-well plates were bought from ThermoFischer (Catalog #150200). The chemical probe Flourophosphonate rhodamine (FP-Rh) was a generous gift from Prof. Benjamin F. Cravatt (The Scripps Research Institute).

Methods

Purification of recombinant human ABHD14B (WT and S111A): Recombinant human ABHD14B (WT and S111A) was cloned and purified as described earlier (Chapter 2 Figure 2.3). I chose to crystallize the tag-free version because it has been previously reported that the His-tagged version does not crystallize very well.³²

Crystallization of WT and S111A ABHD14B: The purified protein was first dialyzed into the crystallization buffer containing 10 mM Tris, 10 mM 2-mercaptoethanol, 100 mM NaCl and 10 % (v/v) glycerol³⁴. Crystal trays were set up using the hanging drop technique⁹². The initial screen was based on the conditions mentioned in literature for the crystallization of wild type human ABHD14B, with an added component – urea³. Different concentrations of 80 mM Tris (pH 7.5 and pH 8), Urea (5 mM and 12 mM) and (NH₄)₂SO₄ (1.2 M, 1.55M and 2 M) were used in the screen (Figure 4.2). The screen was set up in the 12-well plate, having 600 µl of reservoir solution and a drop composition of 2 µl reservoir solution and 1 µl 5 mg/ml protein at two temperature 18 °C and 4 °C. After the initial screen, standardisation was done with respect to protein concentration (5 mg/ml – 20 mg/ml), drop size, and crowding agent (glycerol, PEG). The S111A ABHD14B crystals were frozen in 20% glycerol as the cryoprotectant in the

parent condition and frozen using liquid nitrogen. Data was collected at two facilities, Elettra Sincrotrone facility at Trieste and Diamond light source at the Harwell Science and Innovation campus, Oxfordshire. The data collection statistics for all the crystals is listed in Table 4.1. I went ahead with data obtained from the K6 crystal since it had the highest resolution at 1.5 Å, a good completeness of data and a large number of unique reflections.

Tris-Cl	pH 7.5			pH 8			
(NH ₄) ₂ SO ₄	1.2 M	1.55 M	2 M	1.2 M	1.55 M	2 M	
12 mM Urea							A
5 mM Urea							B
	1	2	3	4	5	6	

Figure 4.2: Initial screen for crystallization of human WT and S111A ABHD14B.

Structure determination of S111A ABHD14B: The data for crystal K6 was collected at Diamond light source at the Harwell Science and Innovation campus, Oxfordshire. The crystal diffracted until a resolution of 1.5 Å. Reduction of data was done using IMOSFLM⁹³ and scaling was done using AIMLESS⁹⁴. Molecular replacement was done using PHASER⁹⁵ accessed through CCP4⁹⁶ package. Refinement was done using PHENIX⁹⁷ package and model building using COOT⁹⁸. S111A ABHD14B structure was solved using WT ABHD14B structure (PDB no.1imj) as a model for molecular replacement³².

Crystallization of WT and S111A ABHD14B with interactors: WT ABHD14B was incubated with an equimolar concentration of FP-Rh for 1 hour at 37 °C. Excess FP-Rh was dialysed out by overnight dialysis with one buffer exchange after 3 hours. This solution was

subject to the initial crystallographic screen, but it failed to yield any crystals. This was also repeated with FP-alkyne, since it is a smaller molecule, but still it failed to crystallize.

Similarly, S111A ABHD14B was incubated with equimolar concentration of CoA for 1 hour at 37 °C, CoA dialysed out by overnight dialysis and crystals trays were set up with the initial screen. This too, failed to crystallize.

Results

Crystallization of human WT and S111A ABHD14B: The WT protein formed very small crystals in the initial screen, but I failed to improve the crystal size in further standardisations. The S111A ABHD14B formed well – diffracting orthorhombic crystals in two different conditions: 2 M $(\text{NH}_4)_2\text{SO}_4$ 12 mM Urea 80 mM Tris pH 8 and 2 M $(\text{NH}_4)_2\text{SO}_4$ 5 mM Urea 80 mM Tris pH 7.5 (Figure 4.3). In both these conditions the protein concentration was 15 $\mu\text{g}/\mu\text{l}$ and the drop contained 2 μl reservoir solution and 1 μl of protein and the plates were kept at 18°C. It took approximately 5 days for the crystals to form.

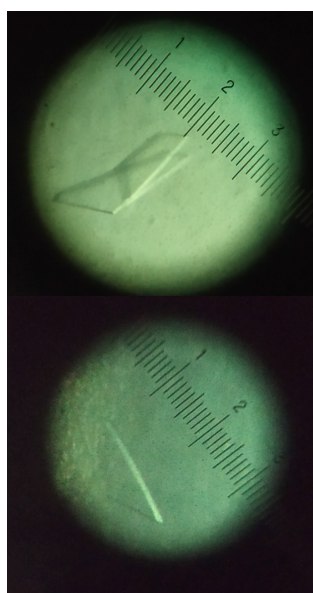
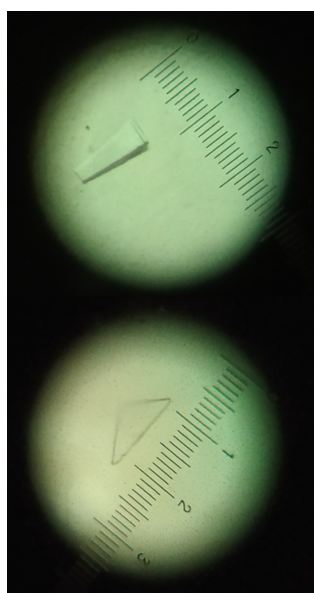


Figure 4.3: human S111A ABHD14B orthorhombic crystals

	K6	G31_1	G31_3	G31_4	G31_5	G31_6
Collected at	DLS	Elletra	Elletra	Elletra	Elletra	Elletra
Wavelength	0.9785	0.9785	0.9785	0.9785	0.9785	0.9785
Space group	P 2 21 21	P 2 21 21	P 2 21 21	P 2 21 21	P 2 21 21	P 2 21 21
a,b,c (Angstrom)	46.77, 75.86, 113.82	46.34, 76.06, 114.60	46.91, 76.15, 114.25	46.67, 76.07, 113.94	47.03, 76.17, 114.20	46.88, 76.09, 114.06
α, β, γ (degree)	90 90 90	90 90 90	90 90 90	90 90 90	90 90 90	90 90 90
Resolution (angstrom)	1.5	1.8	1.7	1.8	1.7	1.8
No. of unique reflections	61879	37414	45875	38357	45849	38619
Rmerge overall (Outer shell)	9.2(84.2)	9.9(62.8)	6.4(30.5)	9.7(40.8)	65.4(109.6)	9.3(44.6)
Rpim overall (outer shell)	6.6(61.4)	4(25.2)	2.4(11.1)	3.4(13.5)	21.5(36.9)	3.6(17.0)
CChalf (outer shell)	0.991(0.355)	0.998(0.894)	0.998(0.828)	0.996	0.936(0.885)	0.998
Mean I/sigmaI (outer shell)	8.5(1.8)	14.7(3)	18.2(-1.4)	14.6	11.9(3.3)	14.8
Completeness (outer shell)	94.6(96.5)	97.9(94.4)	99.9(94.1)	99.8	99.7(100)	99.9

Table 4.1: Data collection statistics for S111A ABHD14B crystals.

Crystal structure of S111A ABHD14B: The crystals belonged to the P₂₂₁₂₁ space group and the a, b and c values were 46.77 Å, 75.86 Å and 113.82 Å. There were two molecules of the protein in one unit cell. I solved the crystal structure of S111A ABHD14B to a resolution of 1.5 Å (Figure 4.5), which was a better resolution than the WT (2.2 Å). The current $R_{\text{work}} = 0.1982$ and $R_{\text{free}} = 0.2169$. There was a SO₄ molecule present in the active site. The structure was very similar to that of the WT enzyme (1imj)³². Mutating the catalytic serine did not drastically change structure of the enzyme, hence there were no new structural features that could be identified.

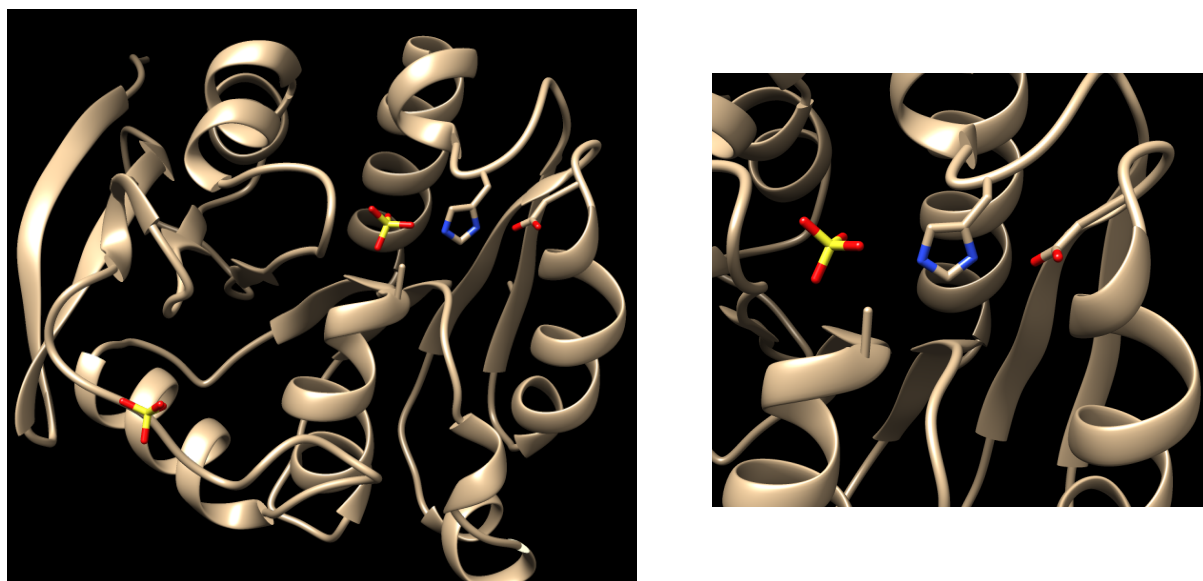


Figure 4.4: A- Structure of human S111A ABHD14B. B- Catalytic triad of S111A ABHD14B



Figure 4.5: Structural alignment of S111A ABHD14B and WT ABHD14B. The blue structure is that of the WT and the sepia structure is the S111A mutant.

Discussion

In this chapter I attempted to crystallize human ABHD14B with an interactor to learn more about its novel mechanism of action. To capture the acylated enzyme, the rate of acylation has to be significantly faster than the rate of diacylation. This can be achieved by mutating certain residues near the active site or changing the reaction conditions of the hydrolysis to slow the

release of the acyl enzyme. From our mutagenesis studies, I have not been able to identify any mutant of ABHD14B which slows down the second step of the reaction.

It is possible to gain insight on the reaction mechanism by studying phosphonates or sulfonates covalently bound to the active site Serine. Phosphonates mimic the first tetrahedral intermediate, whereas sulfonates mimic the second. I failed to crystallize WT ABHD14B bound to FP-Rhodamine or FP-alkyne. Compared to other serine hydrolases, ABHD14B's affinity to FP is much lower. This could be because being a lysine deacetylase, it catalyzes an acetyl transfer reaction and prefers small substrates as shown from the p-NP analogs assay in Chapter 2 Figure 2.5B. FP-Rh is a very large molecule and may not fit perfectly into the active site of ABHD14B, which would hinder its packing and crystal formation. It is possible that ABHD14B might bind better to smaller versions of FP and that is something definitely worth investigating in the future.

From biochemical studies in the lab, we have seen that phenylmethylsulfonylfluoride (PMSF), which is a non-specific inhibitor of serine proteases is also able to inhibit ABHD14B in vitro. Since it is smaller in size than FP-Rh, it could be a better candidate for crystallization studies. I also failed to crystallize a S111A ABHD14B – CoA protein complex. At the start of this project, I was not aware that CoA was the second substrate of ABHD14B. With that knowledge, the number of obstacles for co-crystallization increases. It would be a challenge to firstly irreversibly bind a CoA-like molecule to the protein and then to crystallize it. A lot of the gaps in terms of availability of relevant small molecules need to be filled before such experiments can be carried out.

CHAPTER FIVE: ABHD14B IN A FED-FASTED PARADIGM.

ABHD14B is a novel lysine deacetylase and can make AcCoA by catalysing an acetyl transfer reaction from a post translationally modified acetyl lysine on proteins to a molecule of CoA. AcCoA is the key metabolite of cellular metabolism. It is the product of many catabolic reactions and is also a central substrate for anabolic reactions. It can be derived from glucose, amino acids and fatty acids⁹⁹. It has a high energy thioester bond, which facilitates the transfer of the acetyl group to several different acceptor molecules. Cellular levels of AcCoA are an indicator of the cell's metabolic state¹⁰⁰. To study whether ABHD14B has a role to play in metabolism, another PhD student from our lab Dr. Abinaya Rajendran established a non-viral in-vivo transfection strategy to selectively knock-down ABHD14B in mouse livers¹⁰¹. The knockdown was achieved using In vivo-jetPEI-Gal transfection agent which generated a >90% knock down of ABHD14B exclusively in mouse livers. She investigated the effects of the knock down in a fasting paradigm. On 6 hours of fasting, the following results were obtained - 1. The blood glucose level of knock down (KD) mice did not show reduction on fasting as compared to WT mice. 2. The KD mice had significantly more blood glucose in the Glucose Tolerance test (GTT) and the Pyruvate tolerance test (PTT) 3. The KD mice lost body weight (~15%) while fasting and appeared sluggish 4. The difference between fed-fasted hepatic ATP levels was much more in the KD mice than in the WT mice. 5. Serum glucagon levels were significantly increased in the KD mice as compared to the WT mice¹⁰⁰. It was also interesting to note that in all these cases, the fed levels of blood glucose, hepatic ATP and the body weights remained the same as compared to the WT. The liver transcriptome and proteome undergo significant changes triggered by fasting^{102,103}. I wanted to study whether ABHD14B was one of the proteins whose transcription, expression or activity was changing in this paradigm. In a study from 2011, Zhang and co-workers studied the gene expression profile in mouse livers on feeding, fasting and re-feeding using high throughput sequencing¹⁰⁴. They were able to read ABHD14B transcripts and the transcripts per million in the fed, fasted and re-fed states were 104.42, 84.66, and 118.28 respectively. Since the difference between these numbers is less than 1.5-fold, they considered that the change is not significant and hence concluded that the expression of ABHD14B does not change significantly on fasting⁶.

Yet we still see physiological changes in the ABHD14B KD mice in the fasted state. Towards this goal, I investigated protein levels, localisation and possible post translational modifications of ABHD14B in the fed-fasted paradigm.

Materials

All chemicals, buffers and reagents were bought from Sigma Aldrich unless specified otherwise. Ceria Stabilized Zirconium Oxide beads and Stainless steel beads (SSB32) were obtained from Next Advanced. All mammalian cell culture media was bought from HiMedia unless specified. Magnetic Dynabeads were obtained from Invitrogen (Catalog # 10002D). MS grade trypsin was obtained from Promega (Catalog # V5111).

Methods

Feeding/Fasting paradigm: All animal experiments described have received approval from the Institutional Animal Ethics Committee of IISER Pune (IAEC-IISER Pune) as per guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals constituted by the Government of India. All the mice were housed in the National Facility for Gene Function in Health and Disease at IISER Pune. 10 to 12 weeks old, male, C57BL/6J mice were used in the studies. The mice were divided into two groups, the control group and experimental group with two individuals in each group. The experiment group was not given food for 6 hours and both groups always had ad libitum access to water. After the fasting period, all the mice were ethically sacrificed by initial isoflourane narcosis followed by cervical dislocation. Subsequently, the liver and kidneys of the mice were harvested, flash frozen and stored at -80 °C until further processing.

For one set of experiments, approximately 250 mg of liver tissue was used and one whole kidney. To prepare the tissues for homogenisation, they were first chopped into small pieces using a sharp scissor. They were transferred into 1.5 ml eppendorf tubes and one scoop of beads were added; 5 stainless steel beads for liver and 8-10 zirconium beads for kidney. After adding 500 µl of phosphate buffer saline (PBS), the tubes were kept in a tissue homogeniser (Bullet Blender Tissue Homogenizer Next Advances) at the setting “8” for three minutes, twice. After that the tissues were sonicated in a probe sonicator with a cycle of 2 s “ON”, 2 s “OFF” at an amplitude of 60% for 1 minute. This homogenate was then spun in a tabletop centrifuge at 10,000 g for 15 minutes to remove cell debris. The top layer from this spin was subjected to ultracentrifugation (100,000g) for 1 hour at 4 °C to separate the soluble and insoluble fractions. Since ABHD14B is a soluble protein, for further experimentation, the supernatant from this spin was used.

Bradford Protein estimation assay was performed to estimate protein concentration of the sample and 50 µg of protein was loaded on a 12.5% gel.

HEK High/Low glucose paradigm: HEK (Human Embryonic Kidney) 293T cells were cultured as mentioned before (Chapter 2). They were grown in a high glucose (5 g/L) or a low glucose (2.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) (in 15 cm adherent plates and were harvested at 80-90% confluency and stored as a cell pellet at -80°C until further processing. For processing, 200 µl PBS was added to the cell pellet and sonicated using a probe sonicator with a cycle of 1 s "ON" 3 s "OFF" at an amplitude of 60% for 10 pulses. The lysate was then spun in an ultracentrifuge at 100,000g for 1 hour at 4 °C to separate the soluble and insoluble fraction. Only the soluble fraction was used for further processing. Bradford Protein Estimation assay was performed to estimate protein concentration of the sample and 50 µg of protein was loaded on a 12.5% gel.

Liver Tissue Fractionation: For one experiment, 200 mg of mouse liver tissue was used. The tissue was washed in ice cold 250STM homogenization buffer which had the following composition - 250mM Sucrose, 50mM Tris-HCl (pH7.4), 5mM MgCl₂, 1mM Dithiothreitol (DTT), 25µg/ml Spermine, 25µg/ml Spermidine, 1mM Phenylsulfonyl fluoride (PMSF). The tissue was chopped into smaller pieces using a scissor and added into a dounce homogenizer with 3.5ml of ice cold 250STM buffer. This was dounced for six strokes. The resultant lysate was spun at 200 g for 3 minutes at 4 °C to remove unlysed cells. The supernatant was further spun at 500 g for 5 mins to pellet down the nuclear fraction and the resulting supernatant was used as the cytosolic fraction. Protein estimation was done by Bradford and 50 µg of protein was loaded on a 12.5% gel. The gel was further subjected to Western Blotting.

Immunoprecipitation of native ABHD14B from mouse liver: Native ABHD14B was immunoprecipitated from mouse liver and checked for activity. Magnetic Dynabeads which are coated with Protein A were used. 25 µl of Dynabeads were used for 7.5 µg of antibody. 25 µl of Dynabeads were taken in a 1.5 ml eppendorf and magnetised with a strong magnetic. The buffer was removed and they were washed with 500 µl of PBST (PBS containing 0.1% TritonX100) three times. They were resuspended in 500 µl PBS and 3 µl (9 µg) of anti-

ABHD14B antibody was added. This was incubated at room temperature for one hour with gentle mixing. After one hour, the beads were magnetised and the remaining buffer removed. They were washed with PBST three times after which 500µl of 20mM DMP (Dimethylpimilimide) in 0.2 M ethanolamine was added. The DMP/ethanolamine solution was used for crosslinking between the primary antibody and the Protein A on the bead so that during elution only the proteins pulled down from the lysate would elute, leaving behind the primary antibody bound to the beads. This was incubated with the beads for one hour at room temperature with gentle mixing. After one hour, the beads were magnetised and the solution removed and a mixture of acetic acid: PBST in a ratio of 1:4 was added to stop the reaction. This solution was incubated with the beads for 5 minutes at RT after which the beads were washed again with PBST three times. After washing, 500 µl of liver lysate was added to the beads and incubated overnight at 4 °C with gentle shaking. Post this incubation, the beads were magnetised and the remaining lysate discarded. The beads were then washed with PBST. After this, either directly 1X SDS PAGE loading dye was added and the samples boiled and ran on a 12.5% gel, or the proteins bound to the beads were eluted using 50mM Glycine at pH 2.8. After elution, 2 µl of 1 M Tris pH 8 was added to adjust the pH and 4X SDS PAGE loading dye was added, the sample boiled and ran on a 12.5% gel.

Isolation of native ABHD14B using a Sephadex G100 column: Native ABHD14B was enriched using a Sephadex G100 column. Filter sterilised 10mM Tris pH 8 was used to pack the column. 0.75 g of dry beads were used. Excess buffer (25ml) was added to the dry beads. The beads were allowed to swell for 3 hours at 30 °C in a water bath. Once the solution cooled down to room temperature, the top layer of only buffer was decanted and fresh buffer added to a volume of 25 ml. Separately a 50 ml burette was set up vertically with two clamps. The tap was opened and a cotton plug was placed inside the burette with the help of a long steel rod. The burette was then filled up with 2 ml of buffer. The prepared Sephadex G100 beads were mixed well and the slurry poured into the burette in a single operation down the inside wall with the help of a glass rod. Approximately 50 ml of buffer was added slowly to facilitate the settling down of the beads. Soluble fraction of mouse liver was prepared as discussed before, the only change being the buffer. The buffer used for this experiment contained 10 mM Tris (pH 8), 10 mM Sodium Vanadate, 10 mM Sodium fluoride, 10 mM β glycerophosphate, 10 mM Sodium pyrophosphate, 10 mM N-acetyl muramic acid 10 µM Trichostatin A and 1X Protease inhibitor complex. These were added to make sure that the post transcriptional marks

on proteins were not cleaved off by deacetylases, phosphatases and demethylases. 200 µl (2.4 mg) of soluble fraction of mouse liver was added to the column using a pipette. Initially 5 ml of flow through was collected, followed by ten 1ml fractions. Fractions containing ADBH14B were identified using Western Blot and fractions from three different preps were pooled to do the proteomics experiments.

Sample preparation for MS-based proteomics to look for PTM marks on ABHD14B: For one set of experiments, 100 µg of liver lysate was used. To the lysate, 9M urea in 10 mM Triethylammonium bicarbonate (TEAB) buffer was added such that the final concentration of urea was 6 M. The volume was made up to 200 µl using 50 mM TEAB. A solution of equal volumes of freshly made 200 mM tris-(2-carboxyethyl) phosphine (TCEP) in TEAB and 600 mM K₂CO₃ was made. This solution was added to the sample such that the final concentration of TCEP was 10 mM. This was incubated at room temperature for 45 minutes. This step is done to reduce disulphide bonds and help linearise the protein. K₂CO₃ acts as a strong base that takes up the H⁺ ions, aiding in the process. A fresh solution of 200 mM Iodoacetamide (IAA) was made in TEAB and added to the sample such that the final concentration of IAA was 20mM. This was incubated in the dark at room temperature for 45 minutes. The IAA alkylates the free -SH groups on the cysteines so that they do not reform the disulphide bonds. This also acts as an important marker for the mass spectrometer. After this, TEAB was added to the sample such that the final concentration of the urea in the sample was reduced to 1-2 M. This is done to make sure that the urea does not cause denaturation of the trypsin. After this, MS grade Trypsin was added to the sample such that the ratio of trypsin:protein is 1:50. This was incubated overnight (16 hours) at 37°C with shaking (800 rpm). Post the trypsinization step, 20% Trifluoroacetic acid (TFA) was added to a final concentration of 0.1%. This is done to increase the pH and precipitate highly hydrophobic peptides. The sample was mixed well and kept at RT for 10 minutes. It was then centrifuged at 15000 g for 10 min and the supernatant used for further processing.

For the desalting procedure, a 200µl tip was packed with 2 discs of C-18 material. This is called a ziptip. Activation: 50 µl of 100% Acetonitrile (ACN) was passed through the ziptip. Equilibration: 60 µl of 0.1% TFA was passed through the ziptip. A little bit was left in the ziptip to ensure that it doesn't dry out. Loading: All the peptides from the tryptic digest were loaded onto the ziptip slowly. Washing: The ziptip was washed with 60µl of 0.1% TFA.

Elution: The peptides were eluted into a fresh tube using 60% ACN in 0.1% TFA. After elution, they were dried in a vacuum centrival.

For all proteomics samples, data was acquired in Information Dependent Acquisition (IDA) mode over a mass range of 300-2000 m/z. All of the runs were performed on a Sciex TripleTOF6600 mass spectrometer interfaced with an Eksigent nano-LC 425. The peptides were passed through an Eksigent C18 trap and eluted on a Eksigent C18 analytical column with a linear acetonitrile gradient. One LC run ran for two hours (after loading) with a constant flow rate of 300 nl/min. Solvent A was water + 0.1% formic acid and solvent B was acetonitrile. The gradient was as follows - 5% (vol/vol) B for 10 minutes, a linear gradient of B from 0% to 80% (vol/vol) over 80 minutes, 80% (vol/vol) B for 15 minutes and equilibration with 5% (vol/vol) B for 15 minutes. Each MS survey scan was followed by MS/MS of the fifteen most intense peptides. Peptide Identification and quantification was carried out by ProteinPilot software from Sciex. A RefSeq mouse protein database (release 86) including contaminant protein sequences was used for peptide identification. A precursor ion and MS/MS mass tolerance were set at 20 and 40 p.p.m., respectively for the peptide searches.

Results

Feeding/Fasting paradigm: To see whether the protein levels of ABHD14B change on fasting, I investigated hepatic protein levels of ABHD14B on fed and 6 hours fasted mice using western blot. Since in the fasted condition I could not use GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as a loading control¹⁰⁵, I used Coomassie staining instead. As seen from Figure 5.1 A, there seems to be no significant changes in the levels of ABHD14B on fasting. I also checked its expression in the kidneys and there seems to be no significant change in the protein levels. This aligns with previously shown data that the hepatic mRNA levels of ABHD14B do not change on fasting⁴. This indicates that the expression of ABHD14B is not induced by a fasted state transcriptionally, or translationally, rather the levels remained unchanged throughout.

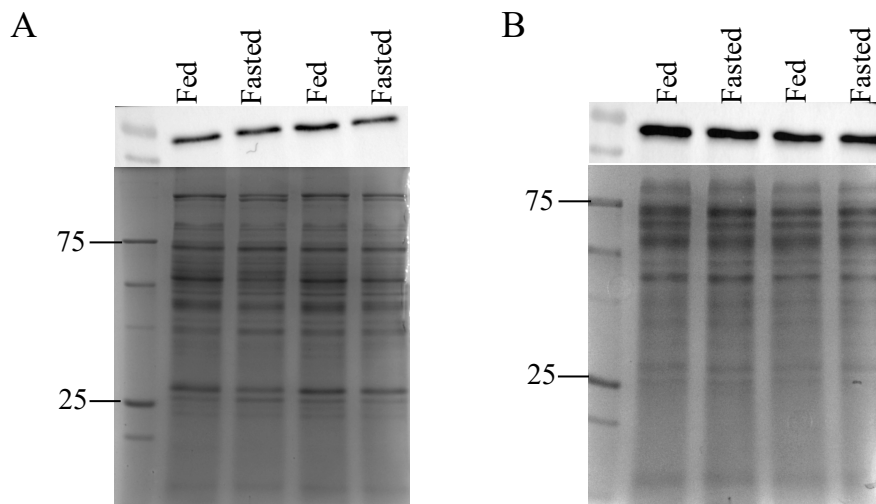


Figure 5.1: Anti- ABHD14B Western blot to measure protein levels of ABHD14B on fasting. The lower panel represents the Coomassie stained gel. A – Western blot on mouse liver soluble fraction. B – Western blot on mouse kidney soluble fraction

High glucose/Low glucose paradigm: Like the fed/fasted paradigm, I wanted to see whether the protein levels of ABHD14B change in HEK293T cells grown in high glucose versus low glucose media. When HEK293T is grown under low glucose conditions the rate of glycolysis decreases and lactate production increases¹⁰⁶. Since the glucose metabolism is altered (just like in the case of fasted mouse liver), we wanted to see whether ABHD14B levels fluctuate. HEK293T cells were grown in high glucose (5g/L) DMEM media or low glucose (2.5 g/L) media for four passages. The protein levels of ABHD14B were measured using western blot. As observed in Figure 5.2, there was no change observed in the levels of ABHD14B in high versus low glucose media, which is similar to the results seen in the fed/fasted paradigm. From these two results, I concluded that even though ABHD14B seems to affect glucose metabolism in the fasted or glucose-scarce state, its protein levels do not change as a function of the amount of glucose in the cell.

Cellular localization of ABHD14B in the fed/fasted paradigm: There are many other possible ways that the function of ABHD14B could be altered/induced in a fasted state. From literature, we know that ABHD14B interacts with TF2D the general transcription factor³². From previous studies in ABHD14B knock down HEK293T cells, it was seen using

transcriptomics that several genes involved in central metabolic pathways, especially those involved with glucose metabolism were altered¹⁰¹. It is also known that in a yeast two- hybrid

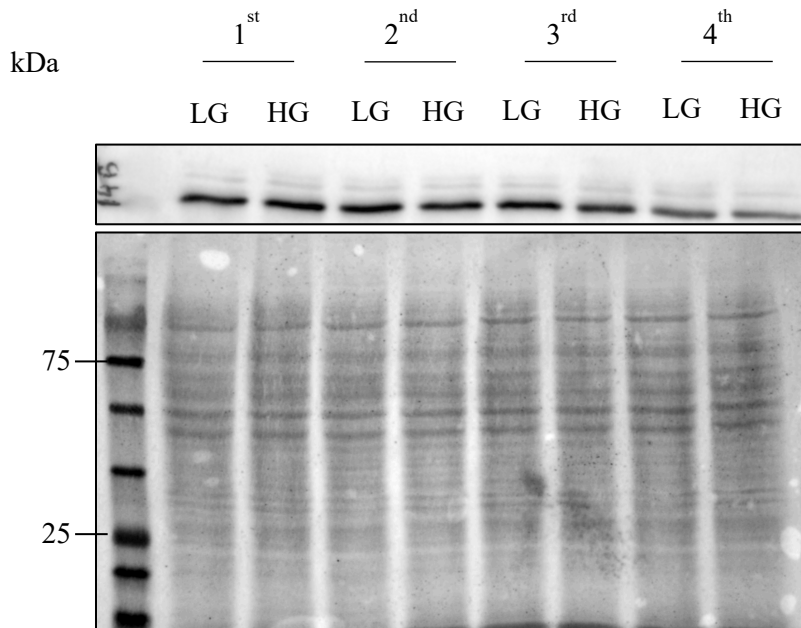


Figure 5.2: ABHD14B levels in HEK293T cells in high glucose versus low glucose media. HG- high glucose, LG – low glucose. The number on the top indicate the passage number of the cell.

system, ABHD14B fused to the Gal-4 binding domain was able to initiate transcription, even in the absence of the activator domain³¹. All this data taken together indicates that ABHD14B could potentially play a role in transcription regulation of genes. It is also known that ABHD14B is expressed equally in the nucleus and cytosol³¹. It is possible that the function of ABHD14B in the fasted state could be induced by a change in its cellular localization. The change in its levels in the nucleus could affect the expression of certain genes. To investigate this, I performed tissue fractionation studies on fed and fasted mouse livers. I separated the cytosolic and nuclear fractions and probed for ABHD14B using Western Blot. I only probed the cytosolic and nuclear fractions because there is no known evidence of ABHD14B localizing to any organelle inside the cell. I used anti- Histone 3 antibody to probe for the nuclear fraction, and GAPDH to probe for the cytosolic fraction. As seen from Figure 5.3, the levels of ABHD14B in the nucleus and cytoplasm do not significantly change in the fed versus fasted state in mouse livers.

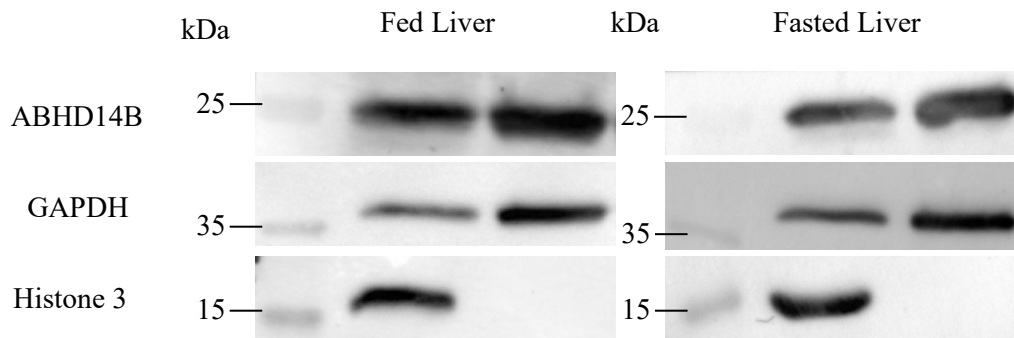


Figure 5.3: Cellular localisation of ABHD14B in fed and fasted mouse liver using western blot. GAPDH was used to probe for the cytoplasmic fraction and Histone3 was used to probe for the nuclear fraction.

Immunoprecipitation of native ABHD14B from mouse liver: To gain more insight into the functioning of ABHD14B in the mouse liver, we decided to isolate it in its native form. One of the main reasons for doing so was that we wanted to check whether it had any post-translational modifications (PTMs). PTMs are one of the main ways in which protein function is regulated dynamically. PTMs can affect protein function, localization and interaction. Recent phosphoproteome and acetylome studies have showed phosphorylation of S91 and acetylation of K141 and K194 in human and mouse livers^{107,88,108,109}. I wanted to see whether we could find any PTMs on native ABHD14B isolated from mouse liver, and whether they differ in fed and fasted mouse liver. I used an anti-ABHD14B antibody to pull down it down from soluble liver fractions. The pull down was done using magnetic Dynabeads. As seen from Figure 5.4, we were successfully able to isolate ABHD14B from the pull down. But when we prepared mass spectrometry samples using this method and sent them for proteomics, we did not get a good coverage of the protein sequence i.e. I picked up very few ABHD14B peptides. To increase protein coverage, the amount of native ABHD14B in the sample needed to be more. To fix this problem, I changed the method of isolating native ABHD14B.

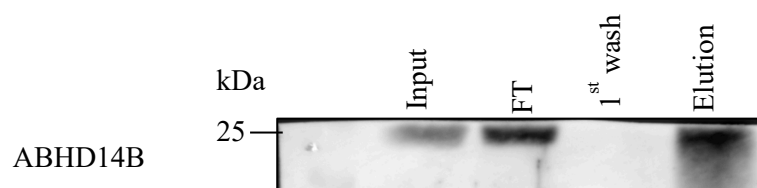


Figure 5.4: Immunoprecipitation of native ABHD14B from mouse liver. FT stands for flow through. ABHD14B is probed by Western blot.

Isolating native ABHD14B using a Sephadex G100 column: To increase the concentration of native ABHD14B in the sample I decided to isolate it using gel filtration. I used a manually packed Sephadex G100 column. And passed approximately 3 mg of soluble liver lysate through it at one time. As seen from Figure 5.5, I collected 20 fraction and did a Coomassie stain as well as a western blot to identify the best fractions. I chose fractions 3 and 4 since ABHD14B was the most enriched in these fractions and they contained a relatively lower number of other proteins. It is important to note here that since the native protein does not have any tag, it was impossible to get highly pure protein. What I was aiming for here was enrichment and not complete isolation. Once I was able to enrich ABDH14B, I went ahead and subjected the enriched sample to mass spectrometry-based proteomics. Even though there were many other proteins in the sample, the machine was able to very robustly pick up ABHD14B peptides. The sequence coverage was more than 50%. Unfortunately, the data analysis software was unable to pick up any PTMs on any of the peptides. This experiment was repeated three times with three different mouse livers and none of the experiments yielded any PTMs.

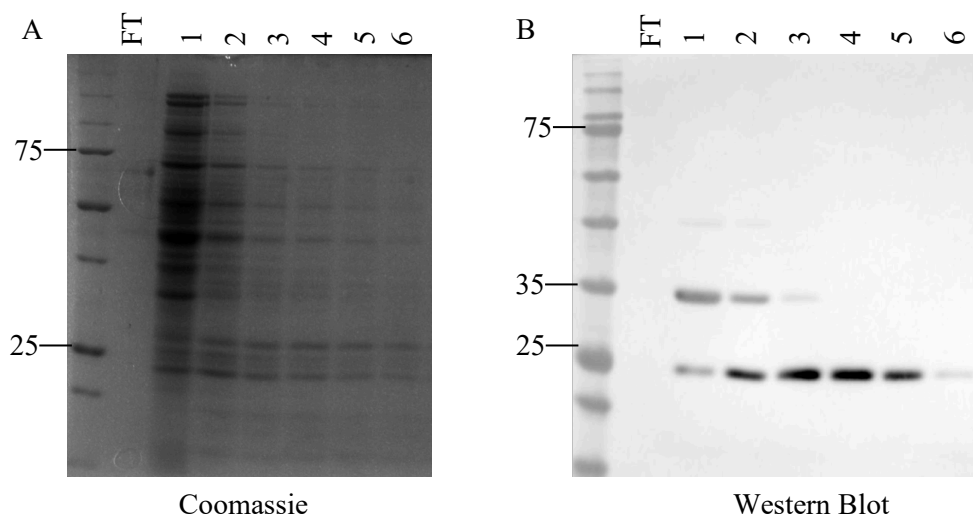


Figure 5.5: Enrichment of native ABHD14B from soluble mouse liver using Sephadex G100. FT stands for Flow Through.

Discussion

ABHD14B is a novel lysine deacetylase. From previous studies in our lab, it was found that on knocking down this protein in mouse livers, certain phenotypes were seen in the fasted conditions. The phenotypes included a higher blood glucose, higher body weight loss, better performance in GTT and PTT tests and heightened serum glucagon levels³. Interestingly, there was no phenotype seen in the fed conditions, which led us to hypothesize that ABHD14B primarily acts in the fasted state in mouse livers and its activity could be triggered in this state. So, in this chapter I set out to find whether there was any quantifiable change in the state of ABHD14B in the fed versus fasted state. From western blot studies I found that there was no change in the protein levels of ABHD14B on fasting. This tells us that the expression of ABHD14B is not induced by the fasted condition but remains the same throughout.

Previous studies with HEK293T knock down lines of ABHD14B showed a dysregulation of gene expression of several different metabolic genes, including those involved with glucose metabolism. It is known that ABHD14B interacts with TF2D and it is possible that it might be regulating these genes from that interaction³¹. So instead of increasing protein expression, the function of ABHD14B could be induced by a change in its localization. To study this I did tissue fractionation studies on fed versus fasted mouse livers and probed for the localization of ABHD14B in the nuclear and cytosolic fractions. I found no change in its localization and the protein remained almost equally distributed in both the fractions. This shows that ABHD14B localization does not change in the fasted state.

Since the protein amount and localization are the same, I decided to investigate whether PTMs are responsible for the activity of ABHD14B in the fasted state. We were able to enrich native ABHD14B using a Sephadex G100 column but were unable to find any PTM using mass spectrometry-based proteomics. One of the reasons for this could be that I did not enrich for any PTM. Generally, when it is known which PTM is being studied, like a phospho-Serine or an acetyl-Lysine, the modification is enriched using antibody or bead-based techniques which selectively isolate proteins/peptides containing the modification which makes it easier for their detection^{110,111}. Since I did not have a specific modification which I was looking for, I could not do enrich for any one modification. Moving forward, it would be worthwhile to individually enrich for specifically modified peptides in different samples and then look for ABHD14B peptides.

Another interesting observation can be made in Figure 5.5B. This blot is probed with an anti-ABHD14B antibody. In lanes marked 1 and 2, there is a faint band at the 35 kDa molecular

weight which is a much higher molecular weight than the protein. ABHD14B is a 22kDa protein and the higher molecular weight band might suggest a modified version of the protein existing in mouse liver. It would not be trivial to fish out this modification since its abundance seems to be much lower than the 22kDa species. Also, it seems to be a very large modification since it is adding almost 15kDa of extra molecular weight on the protein. It remains unclear as to how the functioning of ABHD14B is monitored and how its function is altered/activated in the fasted state.

FUTURE PROSPECTS

From this work, I have annotated ABHD14B as a novel lysine deacetylase and characterized its acetyl-transfer reaction. Using biochemical and molecular biology tools, I have found that this enzyme deacetylates acetylated lysines on proteins and peptides and transfers the acetyl group to a molecule of CoA, forming AcCoA. This is a unique mechanism of deacetylation. Going ahead, it would be interesting to hunt for this enzyme's *in vivo* substrates. It is seen that on knocking down this enzyme in HEK293T cells, the global profile of protein lysine acetylation changes (Chapter 2 Figure 2.8). Mass spectrometry-based proteomics platforms can be used to study the global acetylome profile changes in this knock down system which may point towards direct protein substrates of ABHD14B.^{112,113}

ABHD14B was first discovered as an interactor of the HAT domain of CCG1. It would be interesting to further study this interaction. The roadblock to this is that CCG1 is an insoluble protein and cannot be recombinantly expressed on its own in *E.coli*. Overexpression studies *in vivo* can be used to study the interactions between these two proteins further.

Developing a specific small molecule inhibitor for this protein would provide a great pharmacological tool to study this enzyme. Firstly, solving the structure of the WT protein bound to a small molecule will give us valuable insights into its mechanism and maybe even hint at how it achieves substrate selectivity. Secondly, using a small molecule to inhibit ABHD14B in shorter time periods in cells can give us a much better understanding of how the disruption of this enzyme affects the cell metabolism in real time.

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PUBLICATIONS

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