Conformational Plasticity in Histone Deacetylases as a Source of New Discoveries

BY

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THESIS

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Chicago, Illinois

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This thesis is dedicated to my mother, Afaf Mukahul, without whom I would never have been able to achieve any of my accomplishments.
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I wish to thank, first and foremost, my advisor, Dr. Pavel A. Petukhov for his support, encouragement, and guidance through the past years of my graduate life. I still remember when I came to his office asking for a rotation in his lab, and told him that I had minimum research skills in my undergraduate studies. He did not hesitate to accept me in his lab and to take the risk of training me the necessary research skills. As a mentor, he always encouraged me to think out of the box and to try new things. As an advisor, he always directed me toward improving my research and writing skills by sharing his own research and grant/papers writing experience. Dr. Petukhov also permitted me to design and troubleshoot experiments independently by enforcing active learning and critical thinking skills. He encouraged me to apply for grants and scholarships by showing me how to think scientifically when approaching any writing assignment. As principal investigator, he always found a balance between achieving career goals and meeting with me regularly to discuss experiments related to my projects, or new ideas that I would like to implement or share. He also involved me in various collaborative projects that have improved my communication skills, provided good networking opportunities, and enabled me to learn more about other disciplines and diseases.

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I would like to thank my wife, Jenine Krakra, and her family for their love and support. Since Jenine became part of my life, she has always been there for me and have helped in every possible way.

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I would like to give the proper attention to my life idol and inspiration, my mother Afaf Nimer Mukuhal. She has inspired me through my life with her dedication, integrity, and attention to small details. She has been there for me whenever I needed help and support. I will never be
able to thank her and there are no words that can describe her lifelong passion towards me. She has sacrificed everything to see me fulfill and pursue my dreams and to support my decisions. I would not be of any significance in life without her presence and attention. I acknowledge proudly and undoubtedly that my mother, Yousef, and Ibrahim have greatly molded my life, and they are my partners and the true heroes behind any current or future successes of mine.

Finally, I would like to thank Allah for being a Muslim and for the blessings and treats he bestowed on me. I thank Allah for making this inspiring and enriching journey smooth and pleasant.
CONTRIBUTION OF AUTHORS

Chapter 1 and 2 are a literature review that places my dissertation project in the context of the larger field and highlights the significance of my research question. Chapter 3 gives a brief description of experimental methods that have been used in the dissertation project. Chapter 4 represents a published manuscript (Abdelkarim H., Brunsteiner M., Neelarapu R., Bai H., Madriaga A., van Breemen R. B., Blond S. Y., Gaponenko V., and Petukhov P. A. (2013) Photoreactive "nanorulers" detect a novel conformation of full length HDAC3-SMRT complex in solution, ACS Chemical Biology 8, 2538-2549) for which I was the primary author and a major driver of the research.

Dr. Pavel A. Petukhov and I contributed to the writing, editing, collecting, and analysing the results for the majority of the paper content. Dr. Michael Brunsteiner performed the computational model shown in Figure 31. Dr. Raghupathi Neelarapu performed the synthesis of probes 1, 6, 7, and 8. Dr. He Bai optimized the conditions for the HDAC3 activity assay and assisted with the photolabeling experiments with probe 1 shown in Figure 23, 24, and 27. Ms. Antonett Madriaga performed the acetylation studies shown in Figure 29 and assisted in the photolabeling experiments shown in Figure 30. Drs. Richard van Breemen and Sylvie Blond laboratories have provided an initial set of certain enabling data that are not part of this thesis or the publication in the ACS Chemical Biology journal. The STD-NMR experiments shown in Figure 36 were performed by me and supervised by Dr. Vadim Gaponenko. The sections in the text related to describing and discussing the results of Figure 36 were written and edited by Dr. Vadim Gaponenko, Dr. Pavel A. Petukhov, and myself. Dr. Vadim Gaponenko also helped in editing the final version of the
CONTRIBUTION OF AUTHORS (CONTINUED)

published manuscript. The biological model depicted in Figure 37 was proposed by Dr. Pavel A. Petukhov and me.

I have generated Figures 22 and 37 and performed experiments shown in Figures 20, 21, 23, 24, 25, 26, 28, 30, 32, 33, 34, 35, and 36 (15 of 19). I have also performed the experiments shown in Table 1. All these experiments were critical to the conclusions of this manuscript. Chapter 5 represents my synthesis of the research presented in this thesis/dissertation and my overarching conclusions. The future directions of this dissertation research are also discussed in chapter 4 and chapter 5. The abstract, appendices A and B were adapted from the published manuscript.
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<tbody>
<tr>
<td>ABPP</td>
<td>Activity based protein profiling</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>BEProFL</td>
<td>Binding ensemble profiling with (F) photoaffinity labeling</td>
</tr>
<tr>
<td>BLA</td>
<td>Boc-Lys(Ac)-AMC</td>
</tr>
<tr>
<td>CHD</td>
<td>Chromodomain helicase DNA binding</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>CADD</td>
<td>Computer assisted drug design</td>
</tr>
<tr>
<td>CU</td>
<td>Connecting unit</td>
</tr>
<tr>
<td>CoREST</td>
<td>Corepressor of the transcription factor REST</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Cu catalyzed azide–alkyne 1, 3-dipolar cycloaddition</td>
</tr>
<tr>
<td>CTCL</td>
<td>Cutaneous T cell lymphoma</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>DAD</td>
<td>Deacetylation activating domain</td>
</tr>
<tr>
<td>DBC-1</td>
<td>Deleted in breast cancer 1</td>
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</table>
LIST OF ABBREVIATIONS (CONTINUED)

Ins(1,4,5,6)P$_4$ D-myoinositol-1,4,5,6-tetraphosphate

DNA Deoxyribonucleic acid

FDA Food and Drug Administration

GPS2 G-protein suppressor 2

HSC70 Heat shock cognate protein 70

HSP90 Heat shock protein 90

His Histidine

H Histone

HATs Histone acetyl-transferases

HDACs Histone deacetylases

HID Histone interacting domain

imTAG Image tag attachment group

ID Interacting domain

kD Kilo Dalton

K Lysine

KATs Lysine (K) acetyl transferases
LIST OF ABBREVIATIONS (CONTINUED)

KDACs Lysine (K) deacetylases
MTA Metastasis associated gene
MBD Methyl CpG binding domain
mRNA Messenger RNAs
mM Milimolar
nM Nanomolar
ND Neurodegenerative Diseases
NAD+ Nicotinamide adenine dinucleotide
NRRE NR response element
NCoR Nuclear co-repressor complex
NES Nuclear export signal
NLS Nuclear localization signal
NMR Nuclear magnetic resonance
NR Nuclear receptor
NURD Nucleosome remodeling and deacetylase
PUMA p53 up-regulated modulator of apoptosis
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PRG</td>
<td>Photoaffinity reactive group</td>
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<td>PRP</td>
<td>Photo-reactive probes</td>
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<td>PAL</td>
<td>Photoaffinity labeling</td>
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<tr>
<td>PP4</td>
<td>Protein phosphatase 4</td>
</tr>
<tr>
<td>RRL</td>
<td>Rabbit reticulocyte</td>
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<tr>
<td>RD</td>
<td>Repression domain</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>Research and development</td>
</tr>
<tr>
<td>RbAp</td>
<td>Retinoblastoma associated proteins</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>STD</td>
<td>Saturation transfer detection</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid or thyroid-hormone receptor</td>
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<tr>
<td>SAP</td>
<td>Sin3 associated proteins</td>
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<tr>
<td>SAHA</td>
<td>Suberanilohydroxamic acid</td>
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<tr>
<td>SBG</td>
<td>Surface binding group</td>
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<tr>
<td>SANT</td>
<td>SWI3, ADA2, NCoR, TFIIB</td>
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<tr>
<td>TRiC</td>
<td>TCP-1 ring complex</td>
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<td>TBL1</td>
<td>Transducin like protein 1</td>
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<td>TBL1-R</td>
<td>Transducin like protein 1 related protein</td>
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<tr>
<td>TF</td>
<td>Transcription Factors</td>
</tr>
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<td>TSA</td>
<td>Trichostatin A</td>
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<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>ZBG</td>
<td>Zinc binding group</td>
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SUMMARY

Histone deacetylases (HDACs) have emerged as promising targets for various therapeutic applications. The design of selective inhibitors of a particular HDAC isoform is necessary to enhance in vivo potency, reduce toxicity of currently available inhibitors, and to broaden the therapeutic scope of HDAC inhibitors. As most of the HDAC isoforms assemble with other proteins to form multi-protein complexes with unique functionalities, the structures of these complexes may hold the key for ways to manipulate the epigenetic machinery in tissue/cell-specific manner.

In this proposal, the goal is to investigate conformational plasticity within the HDAC3/SMRT complex using a multidisciplinary approach that integrates knowledge from areas such as medicinal chemistry, photolabeling experiments, biological tests, and NMR studies. Specifically, the project tells the story of novel photoreactive HDAC probes - “nanorulers” - that are designed and applied to measure the distance between the binding site of HDAC3 and SMRT-DAD in solution using a variety of enzymatic, photocrosslinking, and STD NMR experiments. These experiments were conducted, for the first time, with full-length HDAC3 in complex with both the recombinant SMRT-DAD as well the full-length SMRT from cell lysates.

Our study shows that under physiologically relevant conditions in solution SMRT-DAD is positioned approximately 10-13 Å closer to the binding site of HDAC3 than in the X-ray. Multiple independent lines of evidence presented in the project suggest that Ins(1,4,5,6)P₄, a negatively charged molecule, can cause a conformational switch without affecting the enzymatic activity of the HDAC3-SMRT-DAD complex. To the best of our knowledge, this project represents the first
example where photoreactive probes were used to detect conformational changes that are occurring in transcriptional complexes in response to chemical stimuli.

This dissertation will be divided into five chapters. Chapter 1 will provide a general overview of the HDAC isoforms; their co-partners; mechanism of action; types of inhibitors; and possible roles in diseases. In chapter 2, a summary of the key components and structural features unique to HDAC3-SMRT/NCoR complexes are given. Additionally, the potential roles HDAC3 might play in diseases will be discussed. The chapter will also provide examples of drug discovery efforts in finding HDAC3 potent or/and selective inhibitors. In chapter 3, a brief description of the fundamental principles of the key experimental methods used in the thesis is explained and rationale for the choice of the applied method is provided. In chapter 4, we present our findings regarding the full length HDAC3-SMRT complex in solution and show multiple lines of evidence supporting a possible role of conformational plasticity in influencing the epigenetic role of HDAC3/SMRT complex. In conclusion, chapter 5 will briefly highlight the major points discussed in the previous chapters and future directions envisioned for the work presented in this thesis.
CHAPTER 1. INTRODUCTION

For the purpose of this thesis, the “conformational plasticity” term describes any changes in the tertiary structure of histone deacetylase (HDACs), in the quaternary structure of the deacetylase complexes, or in the composition of the deacetylase complexes. These changes may be a result of natural substrates and inhibitors binding or association with other proteins in response to signaling and epigenetic events.

1.1 The Epigenetic Machinery

The word “epigenetics” was initially introduced by Conrad Waddington, a prominent developmental biologist and geneticist, in the 1950s as part of his epigenetic landscape theory. Epigenetics can simply describe the cellular events regulating gene expression without directly altering the sequence of deoxyribonucleic acid (DNA). In eukaryotic cells, chromatin is found in the nucleus and it is highly organized in units called nucleosomes that consist of DNA coiled around proteins called histones.

These nucleosomes connect with each other to form two distinct regions of chromatin: 1) the heterochromatin, a region that is inaccessible to transcription and contains inactive genes, and 2) the euchromatin, a more open and easy accessible region to transcription and usually contains active genes. The interplay between these regions is orchestrated by various epigenetic enzymes in cell/tissue specific manner, through covalent modifications, to control and determine the fate of cellular processes such as DNA repair; chromatin remodeling; gene expression patterns; cell proliferation; and cell survival and death.
A single nucleosome unit usually consists of 147 base pairs of DNA wrapped non-covalently (via the negatively charged phosphate side chains) to a highly positively charged octamer of four histone pairs, namely H2A, H2B, H3, and H4 (Figure 1). The chemical modifications are usually confined either to the DNA or to the histones and can influence the nature of the interaction within and between these units, making the task of gene expression either easier or difficult to achieve.\(^{(3, 4)}\) Methylation, acetylation, phosphorylation, and ubiquitination are well-known examples of such modifications. These alterations are under a very tight homeostatic control to maintain a healthy and natural state of cells and tissues. Any irregularities that disturb the homeostatic balance can result in the up/down regulation of certain genes and/or pathways that may result in undesired pathological outcomes, such as inflammation, uncontrolled growth of cells, and cell death.\(^{(3)}\)

In this thesis, the focus will be on the family of enzymes that mediate the acetylation/deacetylation events inside the cells. As the acetylation typically occurs at the lysine side chains in proteins, the enzymes that catalyze such a reaction are called lysine (K) acetyl transferases (KATs), and their counterparts the lysine (K) deacetylases (KDACs). When these enzymes exert their catalytic activity on histones, they are termed histone acetyl-transferases (HATs) and histone deacetylases (HDACs), respectively. The direct outcome of acetylation in histone is a relaxed nucleosome that is ready for transcription. On the other hand, the deacetylation of histones, by HDACs, or more precisely by deacetylase complexes containing HDACs, results in a more condensed nucleosome and transcriptional repression. The imbalance in the functions of one of these enzymes, in particular HDACs, has been proposed to be directly correlated to a variety of diseases, such as cancer, multiple sclerosis, and Alzheimer’s disease. (Figure 1)\(^{(4-7)}\)
In this chapter, an overview of HDACs isoforms classes; their related complexes; their mechanism of action; classes of inhibitors; and their roles in diseases will be discussed.
1.2 HDAC Classification and Catalytic Mechanism

HDACs, as part of the epigenetic machinery, are considered among the promising therapeutic targets by both academic and industrial researchers.\(^8\) A PubMed search with the word “HDAC” yielded more than 5000 articles, among them 800 scientific reports in 2013 alone. The roles of HDACs vary from chromatin remodeling and gene expression, to regulation of many cellular processes (Figure 1).\(^9\)

According to phylogenetic analysis and sequence homologies with yeast proteins, HDACs belong to four major classes (I–II–III–IV).\(^9\) Class I, II, and IV of HDACs share a common enzymatic mechanism, the Zn\(^{2+}\)-catalyzed hydrolysis of the acetylated lysine amino acids,\(^10\) whereas class III HDACs, sirtuins, are nicotinamide adenine dinucleotide (NAD\(^+\))-dependent enzymes (this group is not the focus of this thesis and will not be discussed further in the thesis).\(^11\)

The eleven Zn\(^{2+}\)-dependent HDACs are grouped in class I - HDAC1, HDAC2, HDAC3 and HDAC8,\(^12-18\) class IIa - HDACs 4, 5, 7, and 9, class IIb - HDAC6 and HDAC10,\(^16, 19, 20\) and class IV - HDAC11.\(^21\) The HDAC isoforms are believed to localize in different regions within the cells. For instance, HDAC1 and HDAC2 are known to present only in the nucleus, whereas HDAC3 and HDAC8 - in the nucleus and cytoplasm. Class II and IV have been found in the nucleus, mitochondria, and the cytoplasm (Figure 2).\(^3, 22, 23\)

The structural and mechanistic features of HDACs (conformational plasticity of tertiary structures) has been the subject of active research field. X-ray crystallography coupled with mutagenesis studies, and biochemical, spectroscopic and computational methods have resolved some of these features.\(^23, 24\) To date, the crystal structures of six out of eleven HDACs isoforms
HDAC1, HDAC2, HDAC3, HDAC4, HDAC7, and HDAC8 have been resolved with or without inhibitors or substrates. The first x-ray structures showed a conserved catalytic site of the class I HDACs that contains a Zn$^{2+}$ ion, located at the end of a 9-11 Å tunnel, in coordination with histidine (His) and two aspartate (Asp) amino acids. Later crystal structures of HDAC2 and HDAC8 have revealed conformational changes in the topology of the active site or the whole protein. Bressi et al. have shown that the HDAC2 binding site to adapt a foot pocket like structure upon binding of N-(2-aminophenyl) benzamide. While Wiest et al. have analyzed the HDAC8 crystal structures and observed the ability of HDAC8 to exist in at least three conformations: A) one pocket conformation represents the typical topology of the HDAC active site with a narrow channel, termed pocket by the authors, leading to the Zn$^{2+}$, B) a two pocket conformation that include an additional pocket close to the active site, and C) one wide groove conformation that feature a more shallow binding site. The first two conformations of HDAC8 were detected in solution by us using a SAHA-like photoreactive probe coupled with in silico analysis and proteomic data.

Several research groups have described the mechanistic roles of Zn$^{2+}$ and the amino acids involved in the catalysis using a set of molecular dynamics and site mutagenesis studies. It has been hypothesized that the first step is chelation of the oxygen atom in the acetylated lysine to Zn$^{2+}$, resulting in an increase of the nucleophilicity of the carbon of the carbonyl group. A tyrosine (Tyr), near the active site, abstracts a proton from water to form a hydroxide ion that chelate with Zn$^{2+}$. The hydroxide ion attacks carbonyl carbon that forms an acyloxanion intermediate. A charge relay system, which consists of His and Asp dyad, initiate chemical rearrangements that lead to the hydrolysis of the acyloxanion into acetate ion and the free deacetylated lysine (Figure 2). Further details into the mechanism have revealed that the acetate ion exits the HDAC binding site.
through a 14 Å internal channel termed as the “acetate release or escape channel.” \(^{(38, 39)}\) The knowledge of HDAC catalytic mechanism and the crystal structures of various conformations have significantly contributed to the design and discovery of different HDAC inhibitors, as it will be discussed later in this chapter.
Figure 2. A representation of the zinc dependent HDAC family. An animated version of HDACs active site along with the proposed catalytic mechanism.
1.3 HDACs Complexes: Components and Possible Roles in Diseases

Multiple regulatory mechanisms can influence the activity and localization of HDACs either by direct chemical modifications (phosphorylation or ubiquitination) and/or forming distinct multi-protein complexes.\(^\text{(22)}\) HDACs (except HDAC8) are mainly found in macromolecular co-repressor complexes, such as CoREST, NuRD, Sin3, NCoR, and SMRT. These complexes are responsible for regulation of gene expression.\(^\text{(40-42)}\) The repression effects are initiated by interaction with: A) a liganded or unliganded nuclear receptor (NR) to the region of the DNA called the NR response element (NRRE) B) DNA interacting protein that can recognize (de)methylated cytosine-phosphate-guanine (CpG) islands of the DNA; C) with transcription factors, such as p53 and p21. These proteins recruit either one or more co-repressor complexes to silence the gene expression (Figure 3).\(^\text{(41, 42)}\) In this chapter a brief description of these complexes will be provided for the known complexes of HDACs, except those of HDAC3 that will be discussed in details in the next chapter.

HDAC1 and HDAC2 were known to be part of three corepressor complexes: Sin3, NuRD, and CoREST.\(^\text{(42)}\) The Sin3-HDAC complex consists of the following components: HDAC1, HDAC2, Sin3a/b, retinoblastoma associated proteins (RbAp) 46 and 48, and Sin3 associated proteins (SAP) 18 and 30 (Figure 4).\(^\text{(43-45)}\) The HDAC1 and 2 mediate deacetylation activity through forming a heterodimer core within the complexes.\(^\text{(45)}\) The RbAp 46 and 48 act as histone chaperones that are responsible for stable binding to core histones (H2A, H3, and H4). Chromatin immunoprecipitation studies showed that SAP 30 interacts with the transcription factors that affect tumor growth and cell cycle such as p53 (a tumor suppressor protein that activate cell cycle arrest and causes apoptosis in cancer), or Swi/Snf chromatin remodeling complex (a multiprotein
complex that suppress cancer growth by destabilizing DNA histone interaction and activate transcription of genes. The Sin3 complex inactivates the actions of these transcription factors by either deacetylating them directly or deacetylating the histones associated with that TF-DNA bound region. Sin3 is capable of forming multiple interaction with other complexes such as NuRD and SMRT/NCoR to form bigger chromatin remodeling complexes.
The nucleosome remodeling and deacetylase complex (NuRD) has some similarities with Sin3 complex because it also includes the HDAC1 and HDAC2 associated with RbAp 46 and 48 proteins (Figure 4). Other components that make the complex unique are a group of proteins, called Mi-2α (or chromodomain helicase DNA binding CHD3), Mi-2β (or CHD4), metastasis associated proteins (MTA1/2), and methyl-CpG-binding domain (MBD2/3). The CHD3/4 domains can mediate chromatin remodeling functionality, through an ATP-dependent mechanism. The MTA1/2 and MBD2/3 are responsible for recruiting the complex to different transcription factors and methylated DNA regions, respectively. The MTA1/2 contains a SANT domain that can interact with HDAC1 to mediate deacetylation. The latter interaction was described by Schwabe et al. in the crystal structure of the HDAC1-MTA complex. The MTA interacts with HDAC1 at a domain called ELM2-SANT. This domain wrap around the active site of HDAC1 in an extended conformation covering the whole protein. The N-terminus of the ELM2-SANT can form a dimer, in which the domain is sandwiched between two HDAC1 proteins. Further investigations revealed the ability of negatively charged small molecule, Ins(1,4,5,6)P₄, in enhancing the activity of the complex by stabilizing the interaction between HDAC1 and the ELM2-SANT domains. These unique components allows NuRD complex to have a more global impact on chromatin remodeling and gene expression in comparison with Sin3 complex, because of the ability of the NuRD complex to bind directly to DNA regions (through CHD3/4 domain), as well to sequence specific DNA binding regions occupied by NR or TF.

NuRD complexes have been shown to play key roles in cancer. For instance, the NuRD complex can stabilize the hypoxia induced factor 1α (HIF1α), by deacetylation of lysine 532, leading to the activation of downstream genes responsible for angiogenesis and cancer metastasis. On the other hand, NuRD complex can silence p53 expression or activity that result
in tumor growth and progression. The process involves two steps found by Luo et al.: A) binding of the p53 via the N-terminus to MTA2; B) deacetylation of the C-terminus of p53 by HDAC1 at multiple lysine residues (Lys 370, Lys 372, Lys 373, Lys 381 and Lys 382) that renders it inactive.\(^{(55)}\)

The co-repressor of the transcription factor REST/NRSF (CoREST) is a unique HDAC1 and HDAC2 complex that contain two SANT domains. Only SANT1 domain is capable of interacting with HDAC1 and/or HDAC2 and is considered essential for activation of these HDACs.\(^{(56)}\) The CoREST complex is unique among those that were discussed so far as it can form “mega-complexes” by interacting with Sin3 containing complexes and other proteins.\(^{(41)}\) The role of CoREST complex in repressing neuronal genes can result in reduced neuronal plasticity and loss of memory formation leading to different neurological disorders.\(^{(57)}\)

The last member of class I HDAC family, HDAC8, has not been reported yet to be part of any repressive complex, but it can interact with proteins involved in cell cycle and protein/ion transport.\(^{(58)}\) Several Studies proposed a possible role of HDAC8 in mediating tumorigenesis in cellular models of neuroblastoma, lymphomas, and leukemia.\(^{(59,60)}\)

Class II and IV HDACs are known to play distinct roles as part of several complexes not only as functional units but also as modulating or scaffolding units capable of stabilizing the complex activity.\(^{(41)}\) For example, HDAC4, class IIa, is known to interact with SMRT/NCoR complexes as a scaffolding unit mediating multiple immunological responses in T-cells.\(^{(28,61,62)}\) On the other hand, HDAC6, class IIb, can interact with heat shock protein 90 (HSP90) affecting many cellular processes in breast cancer.\(^{(63)}\) HDAC6 can also control motility and cell adhesion by the deacetylation of tubulin.\(^{(64)}\) The presence of ubiquitin-binding domain, named ZnF-UBP
domain, on the C-terminal of HDAC6 mediate the ubiquitination of multiple proteins and aggregates leading to their degradation (Figure 4).\(^{(65)}\) HDAC6 is overexpressed in various tumors, such as squamous cell carcinoma, breast cancer stem cells, and acute lymphoblastic leukemia.\(^{(63, 66-68)}\) HDAC6 can mediate its effects through repression of p53 activity or expression.\(^{(69)}\) A summary of some of the known HDAC complexes is shown in Figure 4.

Identification of HDACs within the complexes described was dependent on the ability of HDACs to form strong interactions with their co-partners. A recent study (using a set of proteomics, computational, and biochemical techniques) has reported the ability of HDACs to weakly interact with other proteins to form a more sophisticated and complex interactome.\(^{(58)}\) For instance, a study by Joshi et al., demonstrated for the first time the ability of HDAC11 to interact with SMN complex, an important motor neuronal complex in muscular atrophy, implicating a possible role of HDAC11 in neuromuscular diseases.\(^{(58)}\)
Figure 4. An illustration of currently known deacetylase complexes. Class I HDAC, except HDAC8, are known to be part of different types of repressive complexes. Class IIa are associated with HDAC3 related complexes, and HDAC6 can interact with Ubiquitin.

Although all these reports had advanced our understanding of the component of the HDAC complexes and their roles in diseases, the conformational plasticity in solution within and between these complexes is still unclear.

1.4 Classes of HDAC inhibitors and Their Limitations

A variety of HDAC inhibitors has been described and many are currently under clinical trials. Two HDAC inhibitors, SAHA and FK228, already received FDA approval for the
treatment of cutaneous T cell lymphoma (CTCL) under the names of vorinostat and istodax, respectively.

The vast majority of HDAC inhibitors are designed to bind to the catalytic site of HDACs, and prevent binding of the substrate. A wide range of chemotypes has been identified that are able to inhibit the activity of HDACs.\(^{71, 72}\)

They follow a common pharmacophore model that consist of: (A) a metal binding group which complexes Zn\(^{+2}\), termed as zinc binding group (ZBG), B) a linker domain which mimics the substrate and occupies the active site channel, (C) a connecting unit (CU) that can affect binding on the rim of the active site, for example, interaction of Asp101 in HDAC8 with amide CU in SAHA,\(^{24}\) and (D) a surface binding group (SBG) that interacts with the surfaces of HDAC.\(^{24}\) This latter portion is considered highly important as the surface of HDAC isoform can be viewed as “fingerprint” for that particular isoform and different chemotypes of SBG can influence both selectivity and potency.\(^{23}\) An example of SAHA key interactions with HDAC8 through these groups is provided in Figure 5.

Selective inhibition of HDAC isoforms is considered a desirable approach for development of novel therapeutics, given the variety of recent clinical studies in which non-selective or only moderately isoform-selective HDAC inhibitors have shown limited therapeutic benefit and safety.\(^{73-76}\) On the other hand, Also the inhibition or knockout of a particular HDAC isoform has been shown to be sufficient to elicit a therapeutic effect. For example, inhibition of HDAC1 is typically associated with anti-cancer activity,\(^{77, 78}\) and the development of therapeutics against HDAC2 is considered significant in neurological diseases, such as Alzheimer’s disease\(^ {57}\) and alcohol addiction\(^ {79}\). In addition, non-selective HDAC inhibitors can affect acetylation of more than 3000
sites on more than 1700 proteins leading to difficulties in understanding their effects in multiple cellular processes. \(^{80,81}\)

Figure 5. HDAC inhibitors known pharmacophore model: 1) Surface binding group in red (SBG), 2) Connecting chemical group in green (CU), 3) Linker in orange, 4) zinc binding group in blue (ZBG). The interaction of SAHA with key residues of HDAC8 is shown according to the co-crystal structure of SAHA with human HDAC 8.
Conformational plasticity within the tertiary structures of several HDAC isoforms was shown to be a valuable source for designing potent and selective HDAC inhibitors. The one pocket conformation, found in most HDAC isoforms, has led to the discovery of potent and non-selective inhibitors of class I and II enzymes, such as SAHA, TSA, FK288, and short fatty acids (Figure 6). The foot pocket like binding site of HDAC2 has encouraged further exploration to design novel benzamide based HDAC inhibitors that were found to be active against HDAC1, 2, and 3 but not HDAC8 (Figure 6). The lack of activity against HDAC8 was attributed to the presence of a unique residue (Trp141) in the binding site of HDAC8 that prevented the conformational change into a foot pocket. The two pocket conformation that have been found in HDAC8 has led to the discovery of non-zinc chelating inhibitors with moderate activity against class I HDACs. The ability of HDAC8 to adapt a wide groove conformation has influenced the design and discovery of multiple linkerless and selective HDAC8 inhibitors, such as PCI34051. The unique surface topology of HDAC6 in adapting a bulkier SBG has led to the discovery of a selective HDAC6 inhibitor, tubastatin A (Figure 6).

Although several advances have been achieved on designing selective inhibitors, the widespread of applying HDAC inhibitors as therapeutics in diseases other than cancer is hindered by many factors. As most of the described HDAC inhibitors are based on chelating zinc, they might have off target effects that influence the normal function of ion dependent channels and other metalloenzymes. For example, HDAC inhibitors were found to cause cardiac toxicity by inhibiting potassium channels that are involved in many metabolic and cellular processes integral for cardiac muscle contractions. In addition, HDAC inhibitors can suffer from poor bioavailability, in particular benzamide based HDACs inhibitors, or/and rapid metabolism (for example, SAHA half-life is 30min) limiting their full pharmacological effects. Therefore, it
is of an interest to design selective and/or specific HDAC inhibitors that lack zinc cheating capabilities with enhanced pharmacokinetic profile. The route toward such inhibitors might lie in investigating the dynamic interaction and conformational plasticity within the quaternary structures of HDAC multiprotein complexes. Especially in the presence small molecules (like Ins(1,4,5,6)P$_4$ that is not an HDAC inhibitor) capable of influencing HDACs activity through stabilizing protein-protein interactions within the HDAC1-NuRD complex.
Figure 6. Chemical structures of representative HDAC inhibitors along with their selectivity profiles against class I and class II HDACs.
CHAPTER 2 HISTONE DEACTYLASE 3 COMPLEXES

2.1 Introduction

HDAC3, one of four class I HDAC isoforms, consists of 428 amino acids residues that correspond to a theoretical mass of 49 kDa. HDAC3 is widely expressed and highly regulated isoform that mediates its function through interactions with various proteins.\(^{(93)}\) Since its discovery, multiple scientific efforts have been focused toward exploring these functions and regulatory mechanisms modulating HDAC3 activity and interactions.

In this chapter, a brief view of these efforts will be presented. In particular, the chapter will cover topics related to the components of HDAC3 complex, regulators and proteins that interact with HDAC3 and influence its activity, and potential histone and non-histone HDAC3 targets. The chapter will conclude with a brief description of the possible pathological roles of HDAC3 in diseases, and classes of inhibitors that have been disclosed by several drug discovery groups. These topics will be discussed in the context of conformational plasticity, as it has been done before in chapter 1.

2.2 HDAC3 Complexes: Components and Regulators

Between the year 1997 and 1998, three different groups have announced the identification of HDAC3. It was assigned to class I HDAC family because of its more than 50% amino acid sequence similarity to HDAC1 and HDAC2 and ability to form multiprotein complexes.\(^{(94-96)}\) Unlike HDAC1 and HDAC2, HDAC3 has been shown to possess unique characteristics: HDAC3
C-terminus portion of the protein is different in amino acid sequence from HDAC1 and 2.\(^{(95, 97)}\) HDAC3 has a nuclear import and export signal that allow its localization to both the nucleus and cytoplasm,\(^{(97)}\) and it is the sole functional deacetylase in direct interaction with the nuclear co-repressor complex (NCoR) and the silencing mediator for retinoid or thyroid-hormone receptor (SMRT) complex.\(^{(98, 99)}\) These differences are discussed below.

The first two characteristic features of HDAC3 have been investigated, by Seto et al., through an extensive study exploring the different domains of HDAC3.\(^{(97)}\) It has been found that the deletion of the last 27 amino acids is sufficient to abolish the deacetylation and the transcriptional activity of HDAC3 (Figure 7). To assess the individual contribution of the 27 amino acid residues, several point mutations (one to three amino acids) were performed and did not show a similar effect on the deacetylation or repression activity as did the complete deletion of the 27 residues. The latter observation has been interpreted by the authors as an indication of the involvement of the C-terminus of HDAC3 in global conformational changes that affect the catalytic activity without a direct effect on HDAC3 binding site.\(^{(97)}\)

The second unique feature of HDAC3 was proven by preparing HDAC3 mutants that lack portions of amino acids 188-428. Two domains were identified to be involved in the localization of HDAC3: nuclear export signal (NES) between the amino acids 188-313, which is responsible for the re-localization of HDAC3 to the cytoplasm through the interaction with (hCRM1), a cellular export factor for proteins with leucine-rich NESs (Figure 7)\(^{(97)}\) and a nuclear localization signal (NLS) between the amino acids 313-428, which promote HDAC3 nuclear presence (Figure 7). These domains enable HDAC3 to shuttle between the nucleus and cytoplasm and suggest that
HDAC3 may play a cytoplasmic role through deacetylating non-histones substrates and/or interacting with potential partners.\(^{(97)}\)

It is, however, not known if the HDAC3 nuclear partners NCoR and SMRT can relocalize to the cytosol together with HDAC3 and if HDAC3 can adapt a different conformation from the one in the nucleus.

Next, Seto group sought to determine if HDAC3 is capable of forming oligomers similar to what has been reported in case of HDAC1/2 dimerization in their complexes via their N-terminus.\(^{(51,100)}\) Investigations into the N-terminus portion of HDAC3, more specifically amino acids 1-122, revealed the formation of dimers and trimers of HDAC3. Unlike HDAC1/2, the authors were incapable to elucidate if HDAC3 self-association has an effect on enzymatic activity or can influence a particular cellular response. As they noticed through pull down assays that HDAC3 mutant (that lacks enzymatic activity but contains the N-terminus portion only “oligomerization domain”) were incapable of recruiting fully functional wild-type HDAC3. In addition to that, the N-terminus has been found to be involved in strong and direct interactions with SMRT/NCoR complex that made it more difficult to investigate exclusively the effects of HDAC3 self-association (Figure 7).\(^{(97)}\)
Investigations into the role of SMRT/NCoR complex in activation of HDAC3 isoform was conducted by Lazar et al. They reported that the newly translated HDAC3 is inactive and lacks the ability to interact with SMRT/NCoR, unless it’s properly folded via an energy dependent step\(^\text{101}\). This observation was confirmed by monitoring the activity of immature (newly translated) HDAC3 in the presence of SMRT/NCoR in rabbit reticulocyte (RRL) that were incubated with unhypdrolizable form of ATP. This observation led Lazar’s group to explore the key players responsible for the effect. Two proteins were identified by knockout studies and inhibition with small molecules: ATP dependent TCP-1 ring complex (TRiC) and heat shock cognate protein 70 (HSC70).\(^\text{101}\) The energy dependent step is proposed to proceed with immature HDAC3.
interacting with TRiC and HSC70 in the cytoplasm (Figure 8). Once HDAC3 is properly folded through interactions with these proteins, it is recognized by SMRT/NCoR complex that associates with HDAC3, dissociates the TRiC-HSC70 complex, and promotes the nuclear translocation of HDAC3 to exert its transcriptional repression roles (Figure 8).\textsuperscript{(101)}

Figure 8. ATP-dependent step for the maturation and proper folding of HDAC3, followed by a dissociation step by SMRT/NCoR to displace the TRiC-HSC70 complex and translocate the active HDAC3 to nucleus for repression.
SMRT or NCoR (270 kDa proteins), the two key partners of HDAC3, are co-repressor proteins that mediate complex repression functions through interaction with different liganded and non-liganded NRs. Their cellular localization is mainly nuclear, but it has been reported that SMRT can be exported to the cytoplasm through phosphorylation by MEKK1, a well-known protein of the mitogen activated protein kinases. As in the case of HDAC3, the SMRT translocation was not mentioned to be accompanied with HDAC3.

The SMRT and NCoR can be visualized as a “platform” where thyroid receptor, retinoic acid receptor, and estrogen receptor and various TFs can bind to modulate each other functions. The core components of the complex: are HDAC3, the direct functional deacetylase isoform of complex, transducin like protein 1 (TBL1) and related protein (TBL1-R), and G-protein suppressor 2 (GPS2) (Figure 9). TBL1 and TBL1-R (~55kD) do not interact with HDAC3 directly as proven by pull down studies with HDAC3 or TBL1 containing samples. TBL1 and TBL1-R were reported to be required for the recruitment of the SMRT and NCoR complex to thyroid receptors and other DNA binding proteins to mediate gene silencing. Like TBL1 and TBL1-R, The GPS2 (37kD) has no direct interaction with HDAC3 (Figure 9). GPS2 has been shown to be necessary unit for repressing the JNK signaling through the interaction with DNA binding proteins, providing an additional way for repression by the HDAC3-SMRT/NCoR complex.

Both HDAC1 and HDAC2 can associate indirectly with the SMRT/NCoR complex through Sin3A complex to mediate transcriptional repression of several NRs. Additionally, class II HDACs can interact with the complex to play a non-functional role in regulating the activity and recruitment of the SMRT/NCoR complex for gene repression (Figure
For instance, the nuclear extracts that contain HDAC4 were reported to have a higher deacetylation activity of the HDAC3-SMRT/NCoR than HDAC4 null nuclear extracts.\(^6\)

![Diagram of SMRT/NCoR complex](image)

**Figure 9. Components of SMRT/NCoR complexes involved in the repression of genes.**

The interactions with all these components are mediated through specific regions on the SMRT and NCoR complex as shown in **Figure 10**. The NRs interact at two C-terminal regions of SMRT/NCoR called the interacting domain I and II (IDI and IDII) ([**Figure 10**](image)).\(^9,10,102-105\) Both IDI and IDII were found to be prone to direct phosphorylation by kinases to affect the interaction
between SMRT/NCoR and NR.\(^{106, 112, 113}\) For instance, the phosphorylation of C-terminus of SMRT (where the IDs are located) by protein kinase CK2, mediate and enhance the interaction with thyroid receptors in vitro and in vivo.\(^{112, 113}\)

Three distinctive domains at the N-terminus of the SMRT/NCoR complex termed as repression domain (RD) have been identified through several point mutations and co-immunoprecipitation studies.\(^{112, 113}\) TBL1, TBL1-R, and GPS2 interact with SMRT/NCoR complex at repression domain I (RDI).\(^{104, 105}\) This domain is involved in the interaction with Sin3-HDAC1/2 complex as shown in Figure 10.\(^{110}\)

HDAC3, the remaining core component, interacts with RDI and RDII, through two SANT domains (DNA binding domains found in nuclear proteins, SWI3, ADA2, NCoR, and TFIIB) (Figure 10). A portion of 200 amino acids of the SANT1 domain called the deacetylation activating domain (DAD) is responsible, sufficient, and essential to activate and recruit HDAC3 to its designated histone targets in vitro and in vivo as determined by lazar et al. (Figure 9 and 10).\(^{114, 115}\) An adjacent SANT2 domain termed the histone interacting domain (HID) (Figure 10) is hypothesized to be responsible for interacting with the core histone tail, accelerating the deacetylation activity of HDAC3, and blocking the activity of the HATs.\(^{114}\) The final repression domain of SMRT/NCoR complex, RDIII, interacts with the class II HDACs family (in particular HDAC4, HDAC5, and HDAC7) (Figure 10).\(^{110}\)
As SMRT/NCoR complex can be regulated by its own components (HDAC4, GPS2, TBL1) and/or by kinases, the deacetylation activity and interaction with HDAC3 can provide another route for regulating the complex. One of the interesting examples, described by Zhang et al., is the phosphorylation and dephosphorylation of HDAC3 C-terminal at serine 424 (Ser 424). This particular position was shown to be important for HDAC3 activity, as replacing the Ser424 with alanine resulted in a less active HDAC3 mutant. Further experiments with different mutants and wild type HDAC3 confirmed that the phosphorylation of Ser428 by CK2 kinase enhances HDAC3 activity.

The authors were able to identify a phosphatase enzyme called PP4 (35 kDa) in the purified HDAC3 samples. They were able to confirm a direct interaction of PP4 with HDAC3 N-terminus.
through co-immunoprecipitation experiments with N-terminus deleted HDAC3 mutants.\textsuperscript{(116)} Further investigations into the role of PP4 revealed that the HDAC3-Ser428 dephosphorylation by PP4 is specific only to HDAC3 among other class I HDACs and results in loss of the catalytic activity of HDAC3.\textsuperscript{(116)}

The discovery of PP4, as a co-partner to HDAC3, has encouraged other research groups to investigate other potential protein-protein interaction that might influence HDAC3 activity. For instance, the deleted in breast cancer 1 (DBC-1) is a nuclear protein that has been shown to be a negative regulator of SIRT1 activity, class III HDACs.\textsuperscript{(117)} Whether DBC-1 can interact with other members of the HDAC family was the research question investigated by Chini \textit{et al.}\textsuperscript{(118)} They have found that DBC-1 can interacts with C-terminus of HDAC3 leading to the inhibition of catalytic activity of HDAC3 through a series of co-immunoprecipitation, site mutagenesis, pull down assays and knockout experiments.\textsuperscript{(118)} Interestingly, the authors mentioned that DBC-1, as tumor suppressor protein and negative regulator of HDAC3 activity, is not expressed in tumors in which HDAC3 activity is implicated in tumorigenesis.\textsuperscript{(118)} Whether the interaction between DBC-1 and HDAC3 is mediated by the NCoR/SMRT complex remains to be investigated.

SMRT/NCoR-HDAC3 complex enzymatic activity has been found, by Holsaka \textit{et al.}, to be regulated by emerin, a protein of the inner nuclear membrane.\textsuperscript{(119)} Emerin has been shown to bind to HDAC3 directly through a domain called the HDAC3-binding domain (95-202 amino acids). Unlike DBC-1 interaction, emerin enhances the catalytic activity of HDAC3 (in the presence of NCoR-DAD) by 2.7 fold and changes the kinetic profile of HDAC3 by increasing $V_{\text{max}}$ and reducing the $K_{\text{m}}$ value for the HDAC substrate. This characteristic kinetic profile has been proposed by the authors to exist due to conformational changes in the quaternary structure of
HDAC3-NCoR-DAD complex to adapt a more active form. Emerin effects on HDAC3 activity in vivo has been investigated in an emerin null mouse model. The knockout of emerin has increased the acetylation and methylation levels of core histones (H4K5, H3K9, and H3K27) in a similar manner to the loss or inhibition of HDAC3 activity. The in vivo model results has led the authors to indicate the possibility that chromatin in emerin null mouse can adapt a more open conformation through loss of HDAC3 recruitment or/and activity in the nuclear lamina which highlight the role of nuclear periphery proteins in gene repression and chromatin remodeling.

Other regulatory mechanisms of HDAC3 have been reported to affect HDAC3 degradation and localization to the cytoplasm, away from its nuclear targets. In a study of apoptosis by Escaffit et al., multiple caspases were found to be able to truncate HDAC3 at the C-terminus resulting in deactivation of HDAC3 and shuttling it into the cytoplasm. The examples provided in this section highlight the significance of protein-protein interactions and conformational plasticity in modulating the activity of HDAC3-SMRT.NCoR complex.

2.3 HDAC3 Substrates and the Feed Forward Model

Multiple targets have been identified as substrates for HDAC3-SMRT/NCoR complexes. They include both the histone and non-histone targets. The deacetylation of these targets regulates their localization, stability, activity and gene transcription.

In vitro studies with truncated histone tails or in cells have shown that HDAC3 can deacetylate H2A, H3, and H4 core histones. The deacetylation of H4 tail has been proposed by Lazar et al. to proceed through a feed-forward model. The model proposes that the deacetylation process starts at the acetylated H4K5, which among other acetylated lysine residues
has the highest intrinsic affinity for HDAC3. After H4K5 is deacetylated, the partially positively charged tail is recognized by the HID domain of SMRT/NCoR complex.\(^{(114, 122)}\) This interaction then leads to a sequential deacetylation of H4K8Ac, H4K12Ac, and H4K16Ac in a feed-forward manner.\(^{(114, 122)}\) However, the model does not provide any insights on a particular mechanism that govern HDAC3 ability to move from one acetylated lysine to next one.

In the non-histone targets, HDAC3 has been reported to control the activity and localization of multiple transcription factors and proteins.\(^{(98)}\) For example, HDAC3 deacetylation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an important transcription factor in cancer and inflammatory diseases, leads to the inhibition NF-κB signaling and translocation of NF-κB to the cytoplasm to be degraded.\(^{(123)}\)

The factors that control selectivity and recruitment of HDAC3-SMRT/NCoR to specific substrates are not clear. For instance, Lazar et al.,\(^{(122)}\) and Johnson et al.\(^{(121)}\) had shown HDAC3 preference to H4 tail in fibroblasts cell lines. While Seto et al. had reported that HDAC3-SMRT/NCoR complex is selectively maintaining the deacetylation status of H3K9 in 3T3 mouse cell lines among other acetylated histones.\(^{(124)}\) Vermeulen et al. had reported the ability of HDAC3 complexes to equally deacetylate both H3 and H4 tails in a variety of \textit{in vitro} assays.\(^{(125)}\) In our opinion, there is enough evidence that suggest that the selectivity of HDAC3 complex to a histone target may be governed by multiple factors including those that express differentially in cellular and tissue specific manner. Additional studies are necessary to determine the nature of HDAC3-SMRT/NCoR complex substrate selectivity in an unambiguous fashion.
2.4 HDAC3-SMRT/NCoR Complex Structural Features

The purification and cloning of HDAC3-SMRT complex by different groups have provided the means to investigate the nature of the interactions that govern HDAC3 complex activity and recruitments.\(^{(104,105,107,108)}\) This aspect was of a great interest to Schwabe research group who initiated a series of structural studies to determine the details of the interactions between HDAC3 and NCoR/SMRT complex. The first of these studies included a study of the SMRT/NCoR-DAD (412-480 amino acids) domain by NMR.\(^{(126)}\) According to the NMR model, the structure of the SMRT/NCoR-DAD is comprised of four helices that interact with each other to form a hydrophobic core and a DAD specific motif (the N-terminus tail).\(^{(126)}\) The DAD domain has been found to be different from that of other SANT domains with respect to the orientation of the helices and the width of the hydrophobic groove formed. The surface character of the DAD is of a mixed nature of acidic and basic regions.\(^{(126)}\) These characteristics were postulated to influence the specific interaction between the SMRT-DAD and HDAC3, since the interacting domain on HDAC3 is largely homologous to the corresponding regions in HDAC1 and 2.

In 2012, the x-ray structure of HDAC3-SMRT-DAD complex was reported by Schwabe et al.\(^{(27)}\) HDAC3 found in the crystal structure represents a product complex with an acetate present in the binding site. HDAC3 was truncated at the C-terminus upon crystallization, a critical region for activity, and SMRT-DAD adapted an extended conformation that was different from the globular NMR conformation for the same portion (amino acid sequence).\(^{(27)}\) Interestingly, D-myoinositol-1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P\(_4\)) was found to occupy a highly basic groove on the interface of HDAC3, and interacts with SMRT-DAD.\(^{(27)}\) The Ins(1,4,5,6)P\(_4\) has been proposed...
to regulate the activity of the complex and other class I HDAC complexes.\(^{25, 27}\) In conclusion, these studies highlight the conformational plasticity of the tertiary (DAD alone) vs. the quaternary structure (DAD in complex with HDACs) and support the possibility that small molecules can interact and modulate the activity and assembly of HDAC complexes.

2.4 HDAC3 \textit{in vivo} Role Beyond SMRT-DAD Interaction

In the previous sections, studies that examined the interaction between SMRT/NCoR and HDAC3 were done \textit{in vitro}, due to the embryonic lethality or serious toxic effects associated with knocking out either HDAC3 or SMRT/NCoR in mouse models.\(^{115, 127-130}\) Lazar \textit{et al.} has been able to present the first animal model to study the interactions between NCoR/SMRT-DAD and HDAC3.\(^{115}\) Lazar research group have shown the necessity of the SMRT/NCoR-DAD portion for the activity of HDAC3 in a mouse model that contained a mutated SMRT/NCoR-DAD not capable of activating HDAC3 due to a single amino acid mutation (Tyr470-> Ala).\(^{115}\) Interestingly, there was no toxic/lethal effects associated with the loss of the interaction in SMRT-DAD defective mice, in comparison with total knock out of HDAC3 \textit{in vivo}.\(^{115, 127-130}\) The loss of interaction between HDAC3 and SMRT-DAD was accompanied by an increased acetylation level of H3K9 and H3K27 markers, but the genomic recruitment of defective SMRT and NCoR complexes was not affected.\(^{115}\) Puzzled by these results, Lazar’s research group investigated the role of HDAC3 in two animal model (SMRT-DAD defective mice and HDAC3 null mice) by monitoring the gene expression patterns in the liver. In general, the HDAC3 null mice had a massive up-regulation of genes than the SMRT-DAD defective mice. Interestingly, the “harmful”
genes expression (related to lipid metabolism in the liver) were upregulated in the HDAC3 null mice but not in the SMRT-DAD defective mice. These results led the authors to the conclusion that HDAC3 possess an independent DAD gene repression activity in SMRT-DAD defective mice. The authors proposed that the less active HDAC3 can mediate these effects either by playing a “scaffolding” role in stabilizing the SMRT/NCoR complex or by interacting with non-histone substrates in the nucleus and/or cytoplasm.\(^{55, 115, 123}\)

The findings by lazar et al. introduce a new role of HDAC3 that has not been known before. The ability to maintain the down regulation of “harmful” genes in the presence of less active HDAC3 support the direction of targeting the complexes rather than the individual HDAC isoforms. It also emphasizes on the significance of conformational plasticity of HDAC3 in providing additional epigenetic roles beyond its deacetylation activity. Additionally, it opens up the possibility of investigating other regulatory players (like emerin and DBC-1) in modulating the activity of HDAC3 independently from SMRT/NCoR complex and studying the possible conformational effects these regulators might have in the absence of SMRT/NCoR complex. However, the ability to detect such conformational plasticity in vitro or in vivo models are lacking. Hence, the development of proper tools to study and identify these changes is extremely important.

### 2.5 HDAC3 an Attractive Therapeutic Target

HDAC3 has been suggested to be a promising drug target for a variety of diseases.\(^{98, 131-139}\) In cancer, HDAC3 overexpression has been correlated to the growth and proliferation of colon tumors by inactivating tumor suppressor proteins, such as p53 and p21.\(^{140}\) In neurological
disorders, HDAC3 was shown to be a key player in regulating long-term memory,\(^{(141)}\) mediating specific toxicity in neuronal cells,\(^{(132)}\) and treatment of cocaine-seeking behavior.\(^{(142)}\) In addition, HDAC3 has been found to play a role in the progression and relapsing episodes in multiple sclerosis,\(^{(133)}\) and diabetes\(^{(143-145)}\) Recently, a selective HDAC3 inhibitor, termed as RG2833, has received an orphan drug designation by FDA for the treatment of Friedreich’s Ataxia that was recently withdrawn according to the FDA website.

Although HDAC3 is involved in many diseases, targeting HDAC3 can result in some unexpected and undesirable outcomes. For instance, down regulation of HDAC3 can lead to cardiac hypertrophy,\(^{(146)}\) reduced bone density, \(^{(147)}\) and significant imbalances between carbohydrate and lipid metabolism \(^{(148)}\) Therefore, the ability to target HDAC3 in a therapeutically relevant way, requires the discovery of new ways to modulate the activity of HDAC3 in cell/tissue specific manner.

The drug design efforts toward the development of potent, selective, and safe HDAC3 inhibitors remains an area of active research. A list of representative classes of selective and active HDAC3 inhibitors disclosed by several groups is shown in Figure 11. The active HDAC3 inhibitors are usually hydroxamic acid bearing different SBGs that most probably bind in the one pocket conformation and, therefore, explains the ability of these scaffolds to inhibit other closely related class I HDACs family members. On the other hand, the selective HDAC3 inhibitors are benzamide-based inhibitors that can induce a conformational change in HDAC3 active site to a foot like pocket. The selectivity and activity toward HDAC3 (and not other classes of HDACs) are not well known or understood, as there are no crystal structures describing the interactions between HDAC3-SMRT complex and any of these inhibitors. Therefore, investigating the nature of the
interactions between HDAC3-SMRT/NCoR complex and the inhibitors in solution is essential to guide rationale drug discovery efforts to design novel inhibitors that can target the quaternary structure of HDAC3 complexes.

In this chapter, we have covered some of the characteristic functional and structural features of HDAC3-SMRT/NCoR complex in vitro and in vivo. We have shown through several examples the significance of protein-protein interactions in modulating the activity of HDAC3-SMRT/NCoR complex. In vitro structural studies have highlighted the conformational plasticity of the complex and the possibility of small molecules to interact and modulate the activity and assembly of HDAC3 complexes. Whereas In vivo studies have shown the ability of HDAC3 to possess a scaffolding role in stabilizing the SMRT/NCoR complex and in the down regulation of undesired genes. Finally, we have shown that current active or selective HDAC3 inhibitors have been designed in relation to the known conformational plasticity models proposed for other HDACs. It is important to mention that conformational plasticity within the quaternary structures of HDAC3 complexes are not well understood and are hard to investigate in vitro and/or in vivo, and this factor can be considered a major limiting step toward the design of inhibitors that can target such complexes.
Figure 11. Examples of active (top) and selective (bottom) HDAC3 inhibitors.
CHAPTER 3 KEY EXPERIMENTAL METHODS

In this chapter, a brief discussion of the key experimental methods used in the dissertation project will be provided.

Method 1. Photoaffinity labeling

The concept of photoaffinity labeling (PAL) was introduced by Frank Westheimer in the early 1960s, to describe small molecules that can label macromolecules.\(^{(149)}\) Since then several research groups has adapted the technique to revolutionize research in the life sciences.\(^{(150-152)}\) Cravatt \textit{et al.} have successfully applied activity based protein profiling (ABPP) via a set of click-chemistry approaches, bioorthogonal probes, and other proteomic techniques to map interactions and identify different targets \textit{in vitro} and \textit{in vivo}.\(^{(33, 153-159)}\) One of the most interesting examples was the identification of members of class I and II HDAC family capable of interacting with an HDAC inhibitor, SAHA-BPyne, in different cancer cellular proteome and living cells.\(^{(159, 160)}\) Adapting the same methodology, Gottesfeld \textit{et al.} has identified HDAC3 as the sole target for class I selective inhibitors in Friedreich's ataxia gene silencing.\(^{(161)}\)

\textit{Suzuki et al.} introduced new bi-functional probes holding two azide groups ,with different photosensitivity,\(^{(162-164)}\) which allowed not only to report the labeled protein and the amino acids participating in the binding, but also to visualize the attached probes with fluorescent markers.\(^{(165-169)}\) A schematic example describing the photoaffinity labeling technique developed by these groups is shown in \textbf{Figure 12}. 
PAL as a tool to explore biological interactions (such as protein–ligand; protein–protein; protein–nucleic acid; and protein–cofactor), has dramatically enhanced our capacity to identify new targets, and to design new ligands for proteins that play a role in pathological processes. \(^{(150-152, 170, 171)}\)

Figure 12. Concept for radioisotope-free photoaffinity labeling using a specific ligand with bi-functional groups. The photoactivation step leads to covalently modify the target protein (step 1) and then click reaction with an imaging tag allows visualization of the captured protein or identification of tagged proteins (step 2).
In general, photo-reactive probes (PRPs) are chemically inert compounds in the absence of ultraviolet (UV) light.\(^{172-174}\) Tandem bioorthogonal (bi-functional) PRPs are a class of ligands that possess three features: A) a target affinity portion which is usually a pharmacophoric chemical scaffold known to bind to the target with high to moderate affinity (Figure 12 and Figure 13), B) a photoaffinity reactive group (PRG) which is activated upon irradiation with UV light to covalently modify the protein (Figure 12 and Figure 13), and C) an image tag attachment group (imTAG) that can chemically react with the imaging tags (biotin or fluorescent tags) to aid in the identification and visualization of the ligand/target complex (Figure 12 and Figure 13).\(^{172-174}\)

The ideal PRGs need to fit certain requirements: A) It has to be activated under mild conditions and must not damage the biological system; B) the lifetime of the excited state of the PRG has to be shorter than the lifetime of a ligand–protein interaction; C) the activated probe has to react with the target protein; D) its introduction into the parent compound must not considerably decrease the biological activity.

Three major types of PRGs are commonly used in PAL: (1) aromatic azides (2) diazirines (3) benzophenones (Figure 13). Benzophenone based PRPs have been widely used to profile HDACs and other targets at cellular level.\(^{33, 153-160}\) However, the benzophenone suffers a number of limitations\(^{159, 160, 175, 176}\): A) the steric effect and bulkiness of this group can alter the activity of resulting probes in comparison with their parent compounds; B) long irradiation periods are needed to fully activate the probes and this might result in non-specific labeling and might damage the cellular system; C) the resulting photocrosslinked product are not stable; D) and the synthetic incorporation of benzophenone into different scaffolds is not straightforward.
Figure 13. A general scheme that describes the characteristics portions of bi-functional PRPs and the available choices for the PRG, imTAG, and different chemical reaction.

The other two PRGs have been based on nitrene or carbene chemistry.\(^{(175)}\) Aromatic azides are by far the most commonly used group in photolabeling, because they are easily synthesized via different chemical routes from available commercial precursors. Also, they can be incorporated easily into ligands with minimum overall effect on activity.\(^{(162-164, 177, 178)}\). The photochemistry of aromatic azides has been extensively studied\(^{(179-185)}\). When an aromatic azide is activated by UV
light (254-300 nm), it generates a singlet nitrene radical that can form a variety of adducts with proteins as shown in Figure 14.\(^{(179, 180, 186)}\) The short UV wavelength and the dehydroazepine intermediate, are a source of photo-induced toxicity and non-specific binding, respectively.\(^{(187-189)}\). These limitations have been resolved, by the introduction of fluorine substitution to the aromatic azides that has resulted in slower rate of ring expansion to the dehydroazepine (ketenimine) intermediate and by short irradiation periods under cold conditions to prevent and/or slow the rate of \textit{in situ} radicals formation.\(^{(190)}\)

Recent literature has favored the application of aromatic diazirines over aryl azides, because they are activated at a longer and relatively safer UV wavelength (360 nm).\(^{(173, 191)}\) Upon UV activation, they form carbene radicals that can insert into different bonds and form more stable products in comparison to nitrene radical inserted products.\(^{(173, 191)}\) Nevertheless, the failure of diazirines to label biological systems has been shown in comparison to aromatic azides.\(^{(192, 193)}\) This can be attributed either to the orientation or to position of the diazirines on the ligand or to their physicochemical properties that make them face the solvent rather their target.\(^{(192, 193)}\) Also, the aromatic diazirines upon photoactivation can form multiple undesired diazo-products that can react nonspecifically similar to dehydroazepine intermediate. The introduction of trifluoromethyl, an electron withdrawing group, has stabilized the diazo-products and diminished their undesired reactivity.\(^{(194)}\) The difficulty to synthesize and incorporate diazirines into ligands and their impact on the overall activity has also favored the utilization of other PRGs over diazirines.\(^{(192, 193)}\)
For the imTAG group’s chemical reaction, several bioorthogonal reactions (chemical reactions that can occur in living cells without affecting natural biochemical processes) has been described in literature by Bertozzi. The alkyne Huisgen cycloligation (click chemistry) and the Staudinger-Bertozzi ligation are among the most commonly used reaction in PAL experiments. The Staudinger-Bertozzi ligation proceeds through a reaction between an azide moiety (attached to a ligand or protein) with an ester-substituted triphenylphosphine (attached to an imaging tag). The
modified version of Staudinger reduction of azides end with an amide ligated product with an imaging tag.⁹⁵ (Figure 15)

![Figure 15. The Staudinger-Bertozzi ligation reaction. A) The formation of aza-ylide, B) intramolecular attack on the ester result in a bicycle product 2, C) hydrolysis and oxidation of the phosphine yield the ligated product 3]

Although the Staudinger-Bertozzi ligation has shown a relatively high bioorthogonal character, the slow kinetics rates of the reaction, the use of high concentration of triphenylphosphine (> 250 µM) that can reduce disulfide bridges within protein targets, and the low yield of the reaction
due to instability of the aza-ylide intermediate has encouraged researchers to look into other azide related reactions.\(^{(195)}\)

In 1950, Rolf Huisgen reported the 1,3 cycloaddition reaction of azides with terminal alkynes to form diazoles.\(^{(195)}\) The cycloaddition reaction required high temperature and pressure level to proceed to completion that made it distant from being bioorthogonal.\(^{(195)}\)

In 2001, Meldal and Sharpless laboratories have improved the reaction rates and regioselectivity under mild conditions by using copper (I) as a catalyst, and the reaction was termed as click reaction or copper (Cu) catalyzed azide–alkyne 1, 3-dipolar cycloaddition (CuAAC). (\textbf{Figure 16 and 17})\(^{(197)}\) Interestingly, mechanistic studies have shown that the reaction proceed through a stepwise ligation rather than a concerted mechanism, explaining the regioselectivity and the role of Cu(I) in the reaction.\(^{(196)}\)
Figure 16. The proposed mechanism for the copper (Cu) catalyzed azide–alkyne 1, 3-dipolar cycloaddition “ligation” (CuAAC) reaction

Since then the click chemistry reaction has been considered a well-known bioorthogonal reaction that offers versatility in tagging proteins.\(^{(33, 86)}\) The click reaction was found to be superior to Staudinger-Bertozzi ligation by producing higher yields, stable products, and rapid rates of the reaction.\(^{(195,198)}\) However, the toxicity to cells and living organisms associated with copper sulfate (500 to 1000 µM) and other components of the click reaction have hindered the full scope at cellular level and in vivo and warranted further investigation into a more biocompatible versions of the reaction.\(^{(195)}\) Bertozzi et al. reintroduced and optimized the use of strained alkynes to react readily and with high yields (comparable to CuAAC and better than Staudinger-Bertozzi ligation) with an azide moiety without the need of Cu(I) catalysis.\(^{(195)}\) The Bertozzi research group has provided multiple successful
in vitro and in vivo applications of this reaction and developed a tool box of strained alkyne conjugated reporter tags, in the form of biotin or fluorophore conjugated difluorocyclooctynes.\(^{(195, 199-203)}\) (Figure 17)

The choice of the imTAG group used in photolabeling is diverse,\(^{(204)}\) and, therefore, this section will be limited to discussing affinity tags, in particular biotin, and fluorescent tags. Biotin is by far the most commonly used affinity tag in photolabeling, protein isolation, protein and nucleic acid enrichment, and chromatin Immunoprecipitation (ChIP) assays in vitro or/and tissue extracts.\(^{(204-206)}\) They gained such popularity for multiple reasons: A) their availability and ease of synthesis in different flavors (conjugated alkynes, conjugated strained alkynes, and conjugated azides) are superior to other tags \(^{(156, 195, 206)}\); B) their recognition is mediated by a strong and specific interaction with streptavidin and avidin \(^{(156, 204)}\); C) their detection by a variety of chemiluminescent or/and fluorescent based streptavidin/avidin conjugates provide versatility and facilitate the optimization of labeling process with a sensitivity threshold of pico-molar concentration of labeled protein.\(^{(204-206)}\)
However, a few limitations have been found with the use of biotin as a labeling tag.\textsuperscript{(204)} First, the biotin tag cannot permeate living cells or tissue membranes due to their physicochemical properties, hence their use is restricted to cell lysates and tissue extracts.\textsuperscript{(156, 204)} Second, the dissociation of biotin from streptavidin or avidin occurs under harsh acidic conditions, use of detergents, and high temperatures that might release non-specific background signal and damages the biological target. Third, endogenous biotinylated proteins are found in living cells.\textsuperscript{(156, 204)} Several solutions have been introduced to address these limitations: A) a pre-clearing step of endogenous biotinylated protein from the samples before the addition of the biotin tag can remove
any cross contamination, B) the design of a chemical or biological cleavable biotin tags has helped in removing enriched or isolated protein from streptavidin under mild conditions, and C) running the ligation step with cell lysates or tissues extracts after the labeling with PRPs has helped in profiling living cells and whole tissues without running the \textit{in situ} reaction.

On the other hand, a wide variety of fluorescent tags has been used for labeling proteome \textit{in vitro} and \textit{in vivo}.\cite{206, 207} In general, fluorescent tags offer several attractive properties.\cite{207, 208} First, a fluorescent signal can be acquired very fast in real time, as a typical fluorescent tag can provide approximately $10^7$ photons per second. Second, current methods and instrumentation have increased the sensitivity of detection enormously to enable the monitoring of a single fluorescent molecule over a period of time.\cite{209} Finally, fluorescent tags are also available in multiple colors to allow multiplexed and simultaneous assays and detection, which greatly enhance the applicability in the biological context.\cite{206, 208}

There are two examples of widely used tags. Rhodamine based tags are relatively inexpensive and readily synthesized, but they suffer from rapid photobleaching and high background signal that might hinder their application for imaging and labeling biomolecules.\cite{156, 204} Alexa dyes tags (are synthesized through sulfonation of coumarin, xanthene, and cyanine dyes\cite{210, 211}) which are commercially available with high quantum yields, narrow absorption peaks, and best suited for imaging purposes in living cells and whole tissues, but they are expensive and not easy to synthesize and handle.\cite{210-214}

In our research group, we have followed a rationale that takes into consideration the above criteria for developing successful and cost effective PRPs based on existing HDAC scaffolds combined with compatible click chemistry tags. We took into consideration that our probes should
meet the following: A) maintaining the activity of the PRPs in comparison to their parent HDAC inhibitors; B) maximizing the compatibility of these probes to cells; C) efficiency in crosslinking; and high chemical reactivity with the imTAGs.

In 2009, our research group developed a drug discovery tool that we call “binding ensemble profiling with (E) photoaffinity labeling (BEProFL)” approach. This method combines a set of PAL, proteomics, and computer assisted drug design (CADD) experiments to map the unique surfaces of HDACs to design selective HDAC inhibitors.\(^{(33)}\)

The first successful application of the BEProFL was in determining the available binding positions of SAHA-based PRP on the surface near the active site of HDAC8. The PRP has been found to adapt at least two binding poses while occupying the active site of HDAC8. This observation was confirmed by a series of PAL experiments, molecular dynamic simulations, and proteomic data.\(^{(33)}\) More interestingly, the SAHA based PRP could interact with a pocket close to the active site in an “upside down” pose.\(^{(33)}\) A few modified residues away from the active site of HDAC8 were detected and they appear to be a result of aggregation or non-specific binding associated with SAHA based PRP.\(^{(33)}\)

Further investigations into the upside-down pocket on HDAC8 by using PRP lacking ZBG, revealed that these probes are capable of moderately inhibiting HDAC8 activity and weakly inhibiting other class I HDACs.\(^{(83)}\) Further investigations for acetylation markers in SH-SY5Y demonstrated an increase in acetylation in H4 (a target for class I HDACs) and no effect on α-tubulin acetylation level (a known target for HDAC6).\(^{(83)}\) The BEProFL approach has provided the first set of hits ready to be optimized for the design of potent non-zinc chelating HDAC inhibitors that would be a safer choice as described in chapter 1.
In other publications, we have established a unique BEProFL tool box of PRP-based HDAC inhibitors \(^{33, 83, 177, 178}\) that are diverse in chemical structure, potency, and selectivity.\(^{33, 83, 177, 178}\) A short list of these probes is shown in Figure 18. In chapter 4, we will present a new scope and application of the BEProFL approach in investigating protein-protein interactions within HDAC3/SMRT complex complemented with other methods in more details.

Figure 18. BEProFL toolbox of representative PRPs developed in Petukhov laboratory.\(^{83, 177, 178}\)
Method 2. Fluorescence Based Screening Assay

Several screening assays have been reported in the literature to measure inhibitory activity or binding affinities of potential HDAC inhibitors. The aim of this section is to detail the functional assay that we have optimized in our lab. Therefore, there will be no discussion regarding other types of assays and the reader can refer to the following references for more detailed information. (215-222)

The functional assay used in our laboratory is a fluorescence based method that proceeds through two steps: A) deacetylation catalyzed by HDACs isoforms, B) and hydrolysis catalyzed by trypsin to release the fluorescent substrate that is detected by fluorescence spectrophotometer (Figure 19). (223) The trypsin, a serine protease enzyme, does not recognize acetylated lysine, and hence, the signal source comes from the deacetylation action by HDACs. (223) False-positive results can occur, if the ligands can inhibit trypsin or quench fluorescent signal. To overcome such scenarios, we use a high concentration of trypsin and further validation of inhibitors can be done in the presence of deacetylated substrates. (223)

When evaluating different small molecules for their potency, a range of concentrations (millimolar to sub-nanomolar) of the inhibitor are incubated with the enzyme before the substrate is added. Then, trypsin is added, and the fluorescent signal is measured and recorded in each well. The lower fluorescence measurements correspond to more potency the inhibitor have against a particular HDAC isoform. (Figure 19)
Coumarin based substrates coupled to acetylated lysine (based usually on sequences of H3 and/or H4 tails and other non-histone HDAC targets) were first candidates used to identify HDAC isoforms activity. The simplest of these substrates was Boc-Lys(Ac)-AMC (BLA) (Figure 19).\(^{82,224}\) The latter synthetic substrate has been shown by other groups and us to be a suitable substrate for screening inhibitors against class I HDACs (in particular HDAC1, HDAC2, and HDAC3), class IIb (HDAC6 and HDAC10) and HDAC11.\(^{83,161,177,178,225-229}\) However, the Fluor de Lys (Arg-His-Lys(Ac)-Lys(Ac)), a commercially available substrate provided by Enzo life...
The two-step fluorescent assay was a suitable choice for us, because it provides an economical, robust, sensitive, and rapid way to screen small molecules blocking or influencing the catalytic activity of HDAC isoforms (Figure 19). Our group has successfully optimized conditions for screening inhibitors against HDAC1, HDAC2, HDAC3, and HDAC8.

**Method 3. Saturation Transfer Detection (STD)-Nuclear Magnetic Resonance (NMR)**

STD-NMR is a drug discovery tool that detects interactions between small molecules and large proteins. As the name implies, two proton spectrums are recorded of the protein-ligand sample at different intensities. The first spectrum is termed as the “Saturation-on resonance signal,” in which a narrow pulse, usually between 0 ppm to -1 ppm, is used to excite protons of proteins without affecting the free soluble ligand. The second spectrum is termed as the “Saturation-off resonance signal,” in which a regular proton spectrum is obtained for the ligand and used as a control for the first spectrum. Once the two spectrums are collected, a subtraction of the two spectrums will ideally result in a third spectrum showing the portions of the ligands that have been in contact with the excited protein through spin diffusion via nuclear Overhauser effect.

The STD-NMR is an attractive technique for multiple reasons. It is cost efficient because little amount of the protein is needed. It has a wide range of affinities can be detected millimolar.
to nanomolar). Isotopic enrichment is not required for both the protein and the ligand. The structure of the protein is not required to be known. Lastly, this method is economic and easily applied.\textsuperscript{(233)}

However, some drawbacks of the method are related to its inability detect ligands that either have a fast off rate of binding or have very strong binding. Other drawbacks are related to artifacts introduced by the instability of the protein, choice of solvent, and other related factors such as temperature, length of experiments.\textsuperscript{(233)}

In the next chapter, we will show the inclusion of the STD-NMR method as part of the BEProFL approach as an orthogonal and complementary tool to confirm the binding and to detect the ligand “epitopes” involved in the interaction. To our knowledge, this is the first application of STD-NMR on HDAC isoforms.
4.1 Study Scope

As we have discussed in chapter 2, HDAC3 is found in a large protein complex with the SMRT (also known as the nuclear receptor co-repressor 2 or NCOR2), GPS2, TBL1, and TBLR1.\(^{235}\) This complex recruits additional co-repressors/co-activators and binds to nuclear receptors, \(^{236}\) resulting in modulation of gene expression. Direct interaction of HDAC3 with the deacetylase activating domain (DAD), a segment of approximately 80 amino acids in the SANT1 domain of SMRT,\(^{237, 238}\) is both required and sufficient for HDAC3 activation \textit{in vitro} and \textit{in vivo}.\(^{115, 239}\)

The knowledge of the three-dimensional structure of this complex may not only enable discovery of novel approaches to inhibit the enzymatic activity of the HDAC3-SMRT complex but also gives hints at how HDAC3 may play a deacetylase-independent function \textit{in vivo}\(^{115}\) and possibly even be extended to other deacetylase complexes that contain proteins with similar SANT domains.

Schwabe \textit{et al.}\(^{27}\) has shed light on the structural features and possible regulatory role of Ins(1,4,5,6)P\(_4\) in the HDAC3(Δ379-428aa truncated)-SMRT-DAD complex. Despite the fact that these and other studies by Schwabe and colleagues \(^{240, 241}\) have significantly advanced the understanding of HDAC3-SMRT interactions, much remains to be learned about the way these interactions are achieved.
Our initial photolabeling studies of the full length HDAC3 in complex with SMRT-DAD with small molecule photoreactive HDAC inhibitors, similar to those we published for HDAC2 and HDAC8,\(^{(33, 242, 243)}\) have suggested that SMRT is located closer than that in the X-ray model. The limitations associated with the truncation of amino acids 379-428 at the HDAC3 C-terminal,\(^{(97)}\) the missing “foot pocket”,\(^{(243)}\) and possible differences in the conformations of the HDAC3-SMRT-DAD complex in crystalline and solution states warranted further studies.

Since most widely used methods to study\(^{(244, 245)}\) three-dimensional protein structures and protein-protein interactions in solution\(^{(20, 21)}\) have known limitations,\(^{(246-250)}\) we devise a different approach. Here, we present our observation and results that shows for the first time the use of small molecule photoreactive HDAC probes (“nanorulers”) to characterize the distance between the components in the solution structure of the full-length active form of recombinant and cellular HDAC3-SMRT complex. The findings were then independently validated by STD-NMR experiments.

### 4.2 Results and Discussion

To facilitate the analysis of the HDAC3-SMRT-DAD complex in solution, we designed a series of novel photoreactive inhibitors/probes (Figure 20). The design of these probes included decoration of HDAC ligands with an aryl azide moiety or a 3-azido-5-azidomethylene moiety, a photoaffinity labeling group originally proposed by Suzuki et al.\(^{(162)}\)
Figure 20. The chemical structures of the probes 1-8, the alkyne biotin 9, and TSA and IC50 values of probes 1-8 and TSA against HDAC3-SMRT-DAD complex. IC50 values are expressed as mean ± standard deviation of at least two independent experiments.

We recently demonstrated that the diazide moiety can be successfully incorporated as part of highly potent HDAC inhibitors that can be used in photolabeling experiments with different
HDAC isoforms.\textsuperscript{33, 243, 251} The probes holding the diazide moieties are capable of crosslinking the protein and reacting with the biotin alkyne tag \textsuperscript{9} (Figure 20). The probes \textsuperscript{5} and \textsuperscript{6} with a monoazide moiety are only capable of crosslinking and cannot result in a biotinylated adduct with HDAC protein and, therefore, are used as competitors for the diazide probes \textsuperscript{1-4, 7, and 8}.

The distance between the hydroxamate group and the terminal aryl diazide moiety in the extended conformation varied between 5 Å to 25 Å for the shortest and the longest probes—“nanorulers” \textsuperscript{4} and \textsuperscript{1}, respectively. To evaluate whether the alkylazido moiety is available for copper catalyzed (3+2) cycloligation, we performed a preliminary docking of the probes to a homology model of HDAC3 (the X-ray of HDAC3-SMRT-DAD was not yet published at that time) using a previously published by us procedure.\textsuperscript{251, 252} The probes were also docked to the X-ray of the HDAC3-SMRT-DAD complex (PDB: 4A69) when it became available in PDB. The poses of the probes were found to be comparable to those we obtained with the X-ray model and very similar to the poses of the hydroxamate-based ligands co-crystallized with HDAC8. The probes appeared to be able to extend the alkylazido moiety beyond the relatively narrow well of the active site.

The synthesis of probes \textsuperscript{1, 6}, biotin tag \textsuperscript{9}, and intermediates \textsuperscript{10} and \textsuperscript{11} was described previously.\textsuperscript{33, 251} Probes \textsuperscript{2-5} were synthesized as shown in Figure 21. Substituted benzoic acids \textsuperscript{10, 11, and 14}, were chosen as precursors for the synthesis of the photoreactive probes. 3-Azido-5-(azidomethyl) benzoic acid (\textsuperscript{11}) and its methyl ester (\textsuperscript{10}) were synthesized as reported previously (and a full experimental data is provided in the study by Neelarapu et al.),\textsuperscript{251} whereas 4-azidoaniline hydrochloride (\textsuperscript{14}) was available commercially. The synthesis of the probes \textsuperscript{2-5} and proceeded through a carbodiimide based coupling reaction followed by conversion of the
resulting ester products into the correspondent hydroxamic acids to give the final probes in a 50-70% overall yield.

Figure 21. Synthesis of probes 2-5. Reagents and conditions: (a) 2N NaOH, THF/H2O (8:2), 2 h, rt, 90%; (b) Methyl 7-aminoheptanoate hydrochloride, EDCI, HOBt, DIPEA, CH2Cl2, 0 °C–rt, 90%; (c) Ethyl 4-aminobutyrate hydrochloride, EDCI, HOBt, DIPEA, CH2Cl2, 0 °C–rt, 90%; (d) Suberic acid monomethyl ester, EDCI, HOBt, DIPEA, CH2Cl2, 0 °C–rt, 90%; (e) NH2OH.HCl, KOH, MeOH, 0 °C–rt, 50-70%; (f) NH2OH.HCl, KOH, EtOH, 0 °C–rt, 50%.
The synthesis of the probes 7 and 8 proceeded through a direct reductive amination reaction followed by alkylation of the monoalkylated amino ester with tosylate alcohol of the diazide probe. The ester intermediates were then converted to the hydroxamic acids to give the final probes (Figure 22). A detailed experimental chemical data and procedures can be found in our latest paper by Abdelkarim et al. (253)

Figure 22. Synthesis of probes 7 and 8. Reagents and conditions: (a) Et3N, NaBH(OAc)3, CH2Cl2, rt, 5 h; 50% b) K2CO3, acetone, reflux, 6 h, 70% (c) NH2OH, KOH, MeOH 0 °C—rt, 3 h, 60%. (The synthesis was carried by Dr. Ragupathti Neelarapu, a former member of Petukhov lab)
The IC₅₀ₐₐ of the probes for deacetylase activity of the HDAC3-SMRT-DAD complex were
determined using a competitive fluorescence-based assay reported by us previously and are
provided in Figure 20. The inhibition of HDAC3 was measured using the fluorescent HDAC
substrate Boc-L-Lys(Ac)-AMC and commercially available recombinant human HDAC3-SMRT-
DAD co-expressed in baculovirus expression system in insect cells. The HDAC3 protein contained
a C-terminal His tag and SMRT-DAD protein contained an N-terminal GST tag. The IC₅₀ₐₐs ranged
from double/triple-digit nanomolar for probes 1, 2, 5, 6, 7, and 8 to micromolar for probes 3 and
4.

To study photocrosslinking of the probes 1-8 with the HDAC3-SMRT-DAD complex in
solution, we conducted a series of concentration-dependent and competition experiments (Figure
23-30). The diazide probes 1 and 2 gave a dose-dependent biotinylation of both HDAC3 and
SMRT-DAD (Figure 23). Incubation of probe 3 and 4 at concentration ranging from 0.85 µM to
34 µM (above the IC₅₀ of probe 3) with 1.7 µM HDAC3-SMRT-DAD showed only marginally
detectable background levels of biotinylation and no increase in biotinylation with either HDAC3
or SMRT-DAD at all the concentrations tested (Figure 23). We attributed this faint biotinylation
by probes 3 and 4 to a non-specific binding of the biotin alkyne tag 9 to proteins similar to that
found previously by us and others. (33, 198, 254)
Figure 23. Biotinylation levels and photocrosslinking of probes 1-4 with recombinant HDAC3-SMRT-DAD. (Experiment with probe 1 was performed by Dr. He Bai, a former member of our group)

To confirm the specific binding of probes 1 and 2 and only a non-specific biotinylation in the case of probes 3 and 4, we performed a series of competition experiments with monoazide probes 5 and 6 (Figure 24). Probe 1 was tested with several concentrations of the competitor - monoazide 6, whereas probes 2-4 were only tested with one concentration of the competitor – monoazide 5. For comparison and to validate that the outcome of the competition does not depend on the structure of the monoazide competitor, we included probe 1, as control on the same gel, and tested probe 2 at two different concentrations (Figure 24). We observed a dose-dependent increase in biotinylation of the bands of HDAC3 and SMRT-DAD as the concentration of monoazide 6
decreased from 12 µM to 1.2 µM, with the highest intensity of the bands observed in the absence of monoazide 6 (Figure 24).

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Figure 24. Biotinylation levels and photocrosslinking of probes 1-4 in the presence of fixed concentration of monoazide probe 5 (42.5 µM) and varied concentration of probe 6 (1.2-12 µM). (Experiment with probe 1 was performed by Dr. He Bai, a former member of our group)
As with probe 1, we observed a concentration-dependent increase in biotinylation of HDAC3 and SMRT-DAD as the concentration of probe 2 increased from 1.7 µM to 8.5 µM (Figure 24). Both probes 1 and 2 show a pronounced difference in biotinylation of HDAC3 and SMRT-DAD bands in the absence or presence of monoazide 5 (Figure 24). No difference in biotinylation of either HDAC3 or SMRT-DAD was observed with probes 3 and 4 in the presence of monoazide 5 despite the 10-fold molar excess of the latter and 990- and 150-fold difference in potency, respectively (Figure 20, 24).

This outcome was predicted based on the preliminary docking. Both probes 3 and 4 are short, and if they are chelated to Zn$^{2+}$, their terminal phenyl ring containing the aryl and alkyl azides is positioned very close to the HDAC3 surface. In such binding poses, accessibility of the alkylazido moieties for the (3+2) cycloaddition is expected to be limited.

To verify this, HDAC3-SMRT-DAD protein complex was subjected to photocrosslinking with probes 2-4 and then denatured and reacted with the alkyne tag 7 in the absence or presence of monoazide 5 (Figure 25). Probes 3 and 4 showed a dose-dependent biotinylation of HDAC3 but not SMRT-DAD. In the same experiment, probe 2 showed a dose dependent biotinylation of both HDAC3 and SMRT-DAD (Figure 25) similar to that observed in non-denaturing conditions, indicating that the outcome of the photocrosslinking and biotinylation steps observed in Figure 24 was not affected by the denaturing process.
Figure 25. Biotinylation levels and photocrosslinking of probes 2-4 under denaturing conditions (2% SDS) and a fixed concentration of probe 5, followed by (3+2) cycloligation with tag 7.

We ran also a densitometry analysis to confirm these observations as shown in Figure 26. Thus, in the case of probes 3 and 4, the accessibility of the alkylazido groups for (3+2) cycloaddition with the alkyne group of the biotin tag 9 is restricted whereas photocrosslinking reaction is not.
Figure 26. Densitometry analysis shows the band density ratios of biotinylation level of HDAC3 or SMRT-DAD to the loading of HDAC3 or SMRT-DAD (normalized to the highest signal) under denaturing condition (2% SDS) and in the presence or absence of probe 5 (42.5 µM) with (a) probe 2; (b) probe 3; and (c) probe 4. Two way ANOVA revealed a significant decrease in the biotinylation signal of HDAC3 and SMRT-DAD with probe 2 in the presence of monoazide probe 5 (*, $p < 0.05$; ***, $p < 0.001$), but only a significant decrease in the biotinylation of HDAC3 with probe 3 (**, $p < 0.01$; ***$, p < 0.001$) and probe 4 (*, $p < 0.05$; ***, $p < 0.01$) and not with SMRT-DAD at the same conditions. The data is plotted as the average of at least 2 independent experiments ± SD.
Next, we determined whether probe 1 can crosslink with SMRT-DAD in the absence of HDAC3 and observed no increase in biotinylation at 2.5, 5, or 12.5 µM concentration of probe 1 (Figure 27). This confirms the ability of the probes to crosslink the SMRT-DAD portion while they occupy the active site of HDAC3.

Next, we investigated if the observed photocrosslinking results can be influenced by the chemical structure of the probes and the competitors. We designed two novel amine-based probes 7 and 8 bearing two surface binding groups (“arms”), where the shorter “arm” was represented by
the photoreactive diazide moiety similar to that in probes 1-4. We included probes 1 and 2 as controls to determine if the trend of crosslinking has been changed and we used TSA, a non-covalent competitor chemically different from monoazides 5 and 6, to investigate whether the chemical structure and non-covalent nature of competition affected the outcome of the photolabeling.

The experiment proceeded using a fixed concentration of the probes 7 and 8 (8.5 µM) in the presence of a fixed concentration of TSA (42.5 µM). Similarly to 1 and 2, probes 7 and 8 photocrosslinked to both HDAC3 and SMRT-DAD and showed marginal biotinylation of both HDAC3 and SMRT-DAD in the presence of TSA (Figure 28).

![Table showing the results of the experiment](image)

**Figure 28.** Photocrosslinking of probes 1, 2, 7, and 8 in the presence of fixed concentration of TSA (42.5 µM)
Finally, we investigated the ability of probe 2 to photocrosslink the cellular form of HDAC3-SMRT complex in the cell lysates of HT-29 cell lines. The choice of this particular cell line was driven by the multiple reports confirming the essentiality and overexpression of HDAC3 in colon carcinoma HT-29 cell line.\(^{137, 255}\)

The concentration of probe 2 used in this experiment was set at the level sufficient to inhibit the deacetylation of HDACs in HT-29 cells and sustain a maximum acetylation of histones 3 and 4 in the range between 3 to 24 h (Figure 29). The effect of U.V. irradiation on formation of possible reactive oxygen species in cell lysates or with recombinant proteins was limited by shortening the irradiation time (3 min) and irradiating the protein at lower temperature (4 °C).\(^{256, 257}\)
Figure 29. Total acetylation of histones H3 and H4 in HT-29 cells treated with probe 2. (a) After 3 h incubation. Densitometry analysis shows the band density ratio of the total acetylation of H3/H4 to expression of GAPDH. One-way ANOVA revealed no significant differences in acetylation at any concentration of probe 2. (b) After 24 h incubation. One-way ANOVA revealed significant differences in acetylation of H3 (***, p < 0.001) and H4 (**, p < 0.01) at concentration of probe 2 in the range of 5 to 25 µM. The data is plotted as the average of at least 2 independent experiments +/- SD. (These experiments were done by Antonett Madriaga)
Probe 2 at 25 µM was able to photocrosslink HDAC3 (ca 49 kD) and two isoforms of SMRT (ca 170 and 230 kD) in a specific manner as the biotinylation signal of these bands disappear in the presence of an excess amount of the monoazide probe 5 (1 mM) (Figure 30). We further confirmed these results by enriching the sample using streptavidin beads in the presence or absence of a different competitor (TSA) at a concentration of 500 µM (Figure 30). The captured biotin adducts on the beads were identified as HDAC3 (ca 49 kD) and SMRT (ca 230 kD). These experiments have shown that similarly to the recombinant HDAC3-SMRT-DAD system, the full length SMRT is in proximity to the binding site of HDAC3 found in HT-29 cells.

Figure 30. Biotinylation levels of cellular HDAC3-SMRT complex with probes 2. (a) Soluble whole cell lysates from the colorectal cancer cell line HT-29 photocrosslinked with probe 2 (25µM) in the presence or absence of monoazide probe 5 (1000 µM). (b) Same as e in the presence or absence of TSA (500 µM) followed by biotin enrichment on streptavidin beads. (The enrichment experiment was done by Antonett Madriaga).
Extensive *in silico* analysis of the protein surface and docking calculations were performed to assist in the interpretation of the experimental data (for more details on the computational works, the reader can refer to our latest publication\(^{(253)}\)). The results suggest that the region proximal to the entrance of the channel leading to the HDAC3 catalytic site is most likely to be involved in protein-protein interactions (Figure 31). The location of SMRT-DAD in the groove next to the HDAC3 active site entrance – *conformation 1* (Figure 31a) appears to be in agreement with the mutation studies conducted by Schwabe and co-authors\(^{(27, 258)}\). The same projection of the HDAC3-SMRT-DAD complex including Ins(1,4,5,6)P\(_4\) found in the X-ray model 4A69\(^{(27)}\) is shown in **Figure 31b** – *conformation 2*. A comparison of *conformations 1* and 2 shows that in *conformation 1* SMRT-DAD occupies roughly the position of Ins(1,4,5,6)P\(_4\) in *conformation 2* (Figure 31). The maximum distance that can be reached by the diazide portion of probes 1 and 2 is rendered as red and green circles, respectively.
Photocrosslinking of the probes to HDAC3 was expected since the probes were bound to the HDAC3 active site at the time of photoactivation. Photocrosslinking with the SMRT-DAD (and full length SMRT), however, can occur only if the SMRT-DAD is positioned such that it can covalently react with the photoactivatable diazide portion of probes 1 or 2 while they are bound to HDAC3. Provided that the residence time of the hydroxamate-based HDAC inhibitors in the binding site of HDACs is substantially longer (ranging between seconds to minutes)\(^{(259, 260)}\) than the half-life of the typical products of the azide photolysis such as nitrenes (nanoseconds)\(^{(261)}\) and
ketenimines (microseconds). \(^{262, 263}\) it is unlikely that the specific photolabeling of SMRT-DAD can occur after dissociation of the probes from the binding site.

The fact that photoactivation of probes \(3\) and \(4\) does not result in photolabeling of SMRT-DAD further confirms that the photolabeling can occur only with the proteins that are in proximity to the probe bound to the active site. In our opinion, the above findings indicate that the only factors affecting the outcomes of the photolabeling of HDAC3 and SMRT-DAD are the distance between HDAC3 and SMRT-DAD and the length of the photoreactive probes—“nanorulers”.

The analysis of the docked poses of probe \(2\) (Figure 31a) shows that its diazide portion is limited to a semi-sphere with a radius of ca 6-7 Å, suggesting that SMRT-DAD is likely to be positioned no further than this distance. We validated this finding with probes \(7\) and \(8\) that had different surface binding groups but otherwise had the same length between the hydroxamic and the photoreactive moieties as in probe \(2\). Despite the difference in the geometry and electrostatics between the amide and amino groups in probe \(2\) and probes \(7\) and \(8\), respectively, and a change in the structure and the nature of the competitor from the covalent monoazides \(5\) and \(6\) to TSA, both new probes showed photolabeling and competition by TSA at HDAC3 and SMRT-DAD identical to those of \(1\) and \(2\).

The photolabeling data obtained with probes \(3\) and \(4\), on the other hand, had a different pattern, which may have two interpretations: 1) these probes may be too short to reach SMRT-DAD in the complex, and 2) they may also adopt poses where the aryl azide group is simply too far from SMRT-DAD but otherwise would be able to reach SMRT-DAD if the probe adopts a different binding pose. The latter interpretation would require SMRT-DAD to be positioned very
close to the active site. Such proximity of SMRT-DAD would interfere with the binding of the vast majority of the HDAC3 inhibitors and, therefore, is unlikely to happen.

To our surprise, in the X-ray structure, the distance between the terminal phenyl ring of probe 2 and the closest amino acid residue in SMRT-DAD is ca 10-13 Å (Figure 31b), which is too far to form a crosslinked adduct between probe 2 and SMRT-DAD. Altogether these findings indicate that in solution the SMRT-DAD is located closer to the binding site of HDAC3 compared to its position in the X-ray structure and may resemble the model shown in Figure 31a. Such close position of SMRT to the active site of HDAC3 may represent an opportunity to disrupt the HDAC3-SMRT complex by the surface binding groups of HDAC3 ligands. For instance, this may provide a rationale for disruption of HDAC4-NCoR complex by HDAC inhibitors FR235222 and FR276457.\(^\text{(62)}\)

Taking into account the photolabeling results and the possibility of SMRT-DAD occupying the position of Ins(1,4,5,6)P_4 suggested by the modeling studies, it was important to determine if Ins(1,4,5,6)P_4, which was reported to be detrimental for activity and stability of the HDAC3-SMRT-DAD complex,\(^\text{(27)}\) affects the enzymatic activity of HDAC3-SMRT-DAD and the photolabeling outcomes in our studies. The same authors reported that at concentrations of monovalent ions K^+ and Na^+ expected for a variety of physiological conditions,\(^\text{(264)}\) the complex between HDAC3-SMRT-DAD was unstable and dissociated, requiring us to explore this aspect as well. If Ins(1,4,5,6)P_4 was solely responsible for activation of HDAC3-SMRT-DAD and assembly of HDAC3-SMRT-DAD into the complex, we should be able to clearly observe the differences in enzymatic activity and photolabeling.
First, we assessed the enzymatic activity of the HDAC3-SMRT complex at three pre-incubation time points (5 min, 3 h, and 24 h) at room temperature. The complex retained activity for at least 3h (the pre-incubation period in which the photolabeling experiments were done), with a complete loss of activity after 24 h (Figure 32).

![Figure 32](image)

**Figure 32.** Enzymatic activity of HDAC3-SMRT-DAD (2.3 nM) at three different pre-incubation times (5 min, 3 h, 24 h) using 25 µM the Boc-Lys(Ac)-AMC.

Based on these observations, we excluded 24 h time point and only monitored the activity at 5 min and 3 h time points. In the next experiment, we monitored the activity of the complex at two time points (5 min and 3 h) in the absence or presence of Ins(1,4,5,6)P₄ at three different
concentrations (0.1, 1, and 10 µM) and at a high 137 mM and low 25 mM concentrations of Na\textsuperscript{+} and 50 mM concentration of K\textsuperscript{+}. The latter conditions are similar to those used by Schawbe et al.\textsuperscript{(27)} We also monitored the activity in the absence or presence of triton X at 0.1% and 0.3% (Figure 33).

The initial conditions (Conditions 2; Figure 33) were the result of the optimization studies conducted by us and others to maximize the activity of the complex, signal to noise ratio, and stability for the experiments to determine IC\textsubscript{50} values of HDAC inhibitors. The conditions were also optimized to minimize possible protein aggregation that may influence the enzymatic activity of the complex as suggested by Bondos et al.\textsuperscript{(265)}

At both pre-incubation times, variation in Ins(1,4,5,6)P\textsubscript{4} and Na\textsuperscript{+} concentrations had little effect on the absolute enzymatic activity of HDAC3-SMRT-DAD, 25000 vs 29000 AFU, respectively. A ca 8-10% drop in activity was observed at 50 mM of K\textsuperscript{+} and 0.1% of triton X and a much more pronounced drop - at 0.3% of triton X after 5 min pre-incubation. The effect was much less pronounced at 3 h pre-incubation compared to 5 min. These experiments demonstrate that Ins(1,4,5,6)P\textsubscript{4} has a rather limited effect on the activity of the HDAC3-SMRT-DAD complex even at very low 25 mM concentration of NaCl. Also these observations indicate that neither Ins(1,4,5,6)P\textsubscript{4} nor the varied concentrations of monovalent ions tested affects the association of the HDAC3-SMRT-DAD complex as otherwise its enzymatic activity would be negligible.\textsuperscript{(18, 237, 258)}
Figure 33. The activity of HDAC3-SMRT complex is investigated with respect to presence of three different concentrations of Ins(1,4,5,6)P_4 (0.1, 1, and 10 µM) and different concentrations of monovalent ions (Na^+ and K) specified below. The data is plotted as the average of at least 2 independent experiments ± SD. Conditions 1 (high monovalent ion concentration): 25 mM Tris-HCl, adjusted pH=8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2. Conditions 2: same as conditions 1 with the addition of 0.1% triton X-100. Conditions 3 (low monovalent ion concentration): 25 mM Tris-HCl, adjusted pH=8, 25 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2. Conditions 4: same as conditions 3 with the addition of 0.1% triton X-100. Conditions 5 (similar conditions to the reconstitution assay in Schwabe, et al. (27)): 50 mM Tris-HCl, adjusted pH=7.5, 50 mM CH_3COOK, 5% glycerol. Conditions 6: same as conditions 5 with the addition of 0.3% triton X-100.
The lower enzymatic activity in the presence of the 3-fold higher concentration of triton X is not surprising taking into account its detergent properties and known binding to hydrophobic channels in proteins.\(^{(266)}\) According to our preliminary docking, triton X-100 fits the binding site and can adopt the binding pose similar to those of the known HDAC inhibitors. A very high 2000-fold excess of acetate ion (in the form of CH\(_3\)COOK), which is the product of the deacetylation of the fluorogenic substrate Boc-Lys(Ac)-AMC and is also known to bind to the active site of HDAC3, may explain the lower enzymatic activity of the HDAC3-SMRT-DAD complex observed in the presence of 50 mM of CH\(_3\)COOK (Conditions 5 and 6, Figure 33). The latter observation suggests that high concentrations of the acetate salts in buffers with HDAC3-SMRT should be avoided.

Next, we characterized the effect of Ins(1,4,5,6)P\(_4\) and monovalent metal ions in a series of photolabeling experiments with probes 2 and 5. Probe 2 (1.7 and 8.5 µM) in the presence or absence of monoazide competitor 5 (42.5 µM) was photocrosslinked with HDAC3-SMRT-DAD complex at high and low concentration of Na\(^+\) and K\(^+\) ions and in the presence or absence of 1.7, 8.5, or 42.5 µM of Ins(1,4,5,6)P\(_4\). The effect of low monovalent ion concentration is shown in Figure 34 and the effect of Ins(1,4,5,6)P\(_4\) is shown in Figure 35.
Figure 34. Investigating the effect of low monovalent ion concentration on the photolabeling of HDAC3-SMRT-DAD-complex, using probe 2 at two different concentrations (1.7 and 8.5 µM) and in the presence or absence of competitor 5 (42.5 µM). The low monovalent ion conditions (25 mM Tris-HCl, adjusted pH=8, 25 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 0.1% triton X-100) had no effect on the photolabeling outcome of probe 2 against HDAC3-SMRT-DAD complex compared with the previously shown high monovalent ion conditions (25 mM Tris-HCl, adjusted pH=8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$).

The complex was pre-incubated for 1.5 h in the buffer with or without Ins(1,4,5,6)P$_4$ before addition of the probes to eliminate any effect the probes might have on the complex formation in solution. Probe 2 was capable of crosslinking both HDAC3 and SMRT-DAD regardless of the concentration of the monovalent ions and it was competed out by probe 5 similarly to that shown in Figure 24 and 28.

In the presence of Ins(1,4,5,6)P$_4$, probe 2 was still capable of photocrosslinking both HDAC3 and SMRT (Figure 35), however Ins(1,4,5,6)P$_4$ appears to behave as a competitor of
photolabeling. Unlike the monoazide competitor 5 investigated in this paper as well as other monoazide competitors and HDAC inhibitors, \(^{(33)}\) Ins(1,4,5,6)P\(_4\) showed a unique effect on photolabeling of HDAC3-SMRT-DAD complex not observed with other competitors. Specifically, an increasing concentration of Ins(1,4,5,6)P\(_4\) did not affect the biotinylation of HDAC3 (Figure 3c) whereas the biotinylation of SMRT-DAD was decreased in a dose dependent and a statistically significant fashion (Figure 3c).
C

**Probe 1**

**Densitometry analysis without Probe 5**

<table>
<thead>
<tr>
<th>Condition 1</th>
<th>Condition 2</th>
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<tbody>
<tr>
<td>HDAC3 biotinylation</td>
<td>n.s.</td>
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<tr>
<td>SMRT-DAD biotinylation</td>
<td>n.s.</td>
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**Densitometry analysis with probe 5**

<table>
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<tr>
<th>Condition 1</th>
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<tr>
<td>HDAC3 biotinylation</td>
<td>n.s.</td>
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<tr>
<td>SMRT-DAD biotinylation</td>
<td>n.s.</td>
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**Probe 2**

**Densitometry analysis without Probe 5**

<table>
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<tr>
<td>HDAC3 biotinylation</td>
<td>n.s.</td>
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<tr>
<td>SMRT-DAD biotinylation</td>
<td>n.s.</td>
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**Densitometry analysis with probe 5**

<table>
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<tr>
<th>Condition 1</th>
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<tr>
<td>HDAC3 biotinylation</td>
<td>n.s.</td>
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<tr>
<td>SMRT-DAD biotinylation</td>
<td>n.s.</td>
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</table>
Figure 35. The effect of Ins(1,4,5,6)P_4 on the photocrosslinking of probe 1 and 2 with HDAC3-SMRT-DAD complex. (a) Photocrosslinking of probe 1 and 2 (8.5 µM) with HDAC3-SMRT-DAD, in the presence or absence of monoazide probe 5 (42.5 µM) and in the presence of Ins(1,4,5,6)P_4 at three concentrations (1.7, 8.5, 42.5 µM). Conditions 1 represent the in-house optimized conditions for photolabeling (25 mM Tris-HCl, adjusted pH=8, 25 mM NaCl, 2.7mM KCl, 1 mM MgCl_2, 0.1% triton X-100) and conditions 2 represent the pull down assay conditions from Schwabe et al. paper\(^{(27)}\) (50 mM Tris-HCl, adjusted pH=7.5, 50 mM CH\(_3\)COOK, 5% glycerol, 0.3% triton X-100). (b) Densitometry analysis shows the band density ratios of the biotinylation level of HDAC3 and SMRT-DAD to the loading of HDAC3 and SMRT-DAD at each concentration of Ins(1,4,5,6)P_4. Two-way ANOVA revealed a significant decrease in the biotinylation of HDAC3 and SMRT signal in the presence of monoazide probe 5 (**, p < 0.01) with a significant contribution of Ins(1,4,5,6)P_4 (***, p < 0.001) in further decrease of the biotinylation signal of SMRT only in the case of probe 2. There was no significant interaction between the presence of probe 5 and Ins(1,4,5,6)P_4 (c) Densitometry analysis same as (b). One-way ANOVA revealed significant decrease in biotinylation of SMRT-DAD, only in case of probe 2, at two concentration (8.5 and 42.5 µM) of Ins(1,4,5,6)P_4 (**, p < 0.01; *, p < 0.05), but no significant difference in the biotinylation of HDAC3 at any concentration of Ins(1,4,5,6)P_4 (p < 0.336) in the presence or absence of probe 5. The data is plotted as the average of at least 2 independent experiments +/- SD.

To investigate the effect different experimental conditions may have on inhibition of the HDAC3-SMRT-DAD enzymatic activity by probe 2, we determined its IC\(_{50}\)s in the presence or absence of Ins(1,4,5,6)P_4, potassium acetate, and triton X (Table 1). We found that in all cases the effect of Ins(1,4,5,6)P_4 on IC\(_{50}\) value of probe 2 was comparable or even smaller than that of the other components of the buffers, such as monovalent metal ions, acetate ions, and triton X. However, we observed an interesting trend - regardless of the nature of other components of the buffers, IC\(_{50}\) values of probe 2 with Ins(1,4,5,6)P_4 were 18-26% better than those without Ins(1,4,5,6)P_4 in otherwise identical conditions.
Table 1: IC$_{50}$ values of probe 2 against HDAC3-SMRT-DAD in the presence or absence of 10 µM of Ins(1,4,5,6)P$_4$.\textsuperscript{1}

<table>
<thead>
<tr>
<th>Assay conditions\textsuperscript{2}</th>
<th>Pre-incubation time</th>
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<tr>
<td></td>
<td>5 min</td>
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<tr>
<td>Conditions 1</td>
<td></td>
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<tr>
<td></td>
<td>26.0 ± 1.74</td>
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<tr>
<td>Conditions 1 (Without Ins(1,4,5,6)P$_4$)</td>
<td>32.1 ± 5.1</td>
</tr>
<tr>
<td>Conditions 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52.8 ± 0.95</td>
</tr>
<tr>
<td>Conditions 2 (Without Ins(1,4,5,6)P$_4$)</td>
<td>64.2 ± 3.79</td>
</tr>
<tr>
<td>Conditions 3</td>
<td></td>
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<tr>
<td></td>
<td>44.4 ± 0.88</td>
</tr>
<tr>
<td>Conditions 3 (Without Ins(1,4,5,6)P$_4$)</td>
<td>55.8 ± 2.52</td>
</tr>
</tbody>
</table>

\textsuperscript{1}The IC$_{50}$ values (nM) are expressed as mean ± SD of at least two independent experiments, HDAC3-SMRT concentration 2.3 nM. \textsuperscript{2}Conditions 1 (in house conditions): 25 mM Tris-HCl, adjusted pH = 8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$. Conditions 2 (similar conditions to the reconstitution assay in Schwabe, et al.): 50 mM Tris-HCl, adjusted pH = 7.5, 50 mM CH$_3$COOK, 5% glycerol. Conditions 3: same as conditions 2 with the addition of 0.3% triton X-100.

Any other deviation from the “in-house” conditions resulted in at least 1.2-fold (at 5 min) higher IC$_{50}$ values, a trend opposite to that observed with Ins(1,4,5,6)P$_4$. Considering that Ins(1,4,5,6)P$_4$ does not inhibit the enzymatic activity of HDAC3-SMRT-DAD by itself (Table 1),
it appears that the presence of Ins(1,4,5,6)P<sub>4</sub> has a small synergistic effect on inhibition of the complex by probe 2, which may be expected if Ins(1,4,5,6)P<sub>4</sub> causes the distance between the binding site of HDAC3 and SMRT-DAD to increase.

Puzzled by these findings, we decided to investigate the effect Ins(1,4,5,6)P<sub>4</sub> may have on the photolabeling with probe 1, the longest in this series (Figure 35). Our docking studies have shown that unlike probe 2, probe 1 can reach SMRT-DAD in both our model shown in Figure 31a and in the X-ray model shown in Figure 31b. Since both conformations can be reached by probe 1, we anticipated that the decrease in biotinylation of SMRT-DAD by probe 1 in response to increasing concentration of Ins(1,4,5,6)P<sub>4</sub> will be not as pronounced as in the case of probe 2 even if there is a change in the conformation of the HDAC3-SMRT-DAD complex. Consistently with this model, we found that Ins(1,4,5,6)P<sub>4</sub> had no statistically significant effect on photolabeling of either HDAC3 or SMRT-DAD with probe 1 at all the concentrations tested in the presence or absence of the competitor, monoazide 5 (Figure 35).

To provide further corroborating evidence that the position of SMRT-DAD in the complex with HDAC3 in solution is in agreement with the photocrosslinking results, we performed a series of NMR saturation transfer difference (STD) experiments with probe 7 and/or Ins(1,4,5,6)P<sub>4</sub> in a buffer solution with HDAC3-SMRT-DAD. This method allows investigation of binding epitopes of small ligands that are in contact with high molecular weight proteins. (267, 268) Probe 7 was chosen because of its intermediate affinity for the HDAC3-SMRT-DAD complex that allows favorable detection by STD. The results of the experiment are shown in Figure 36. When probe 7 (100 µM) was added to the HDAC3-SMRT-DAD complex (1 µM) we observed an STD response from its aromatic and alkyl portions, indicating that they participate in binding to the complex (Figure
36a). Addition of 25 µM Ins(1,4,5,6)P₄ prevented detection of the aromatic groups in the STD spectra. Only the STD signals most likely corresponding to the methylenes adjacent to the amine nitrogen atom were observed (Figure 36b). More precise assignment was not possible due to chemical shift changes. We also detected the STD response when 25 µM of Ins(1,4,5,6)P₄ was incubated with the HDAC3-SMRT-DAD complex without probe 7 (Figure 36c).

Figure 36. Saturation transfer difference (STD) spectra of (a) 100 µM probe 7 with 1 µM HDAC3-SMRT-DAD complex without Ins(1,4,5,6)P₄ (blue), (b) same as a with 25 µM Ins(1,4,5,6)P₄ (red), (c) 1 µM HDAC3-SMRT-DAD complex with 25 µM Ins(1,4,5,6)P₄ (green). Signal assignments are marked on the spectra.
There are several important conclusions that can be derived from these experiments. First, they confirm the previous finding by Schwabe et al. that Ins(1,4,5,6)P₄ binds to the HDAC3-SMRT-DAD complex. The fact that the STD response for Ins(1,4,5,6)P₄ was observed only after its addition to the HDAC3-SMRT-DAD complex indicates that Ins(1,4,5,6)P₄ was not present in the complex during purification in the amounts sufficient to generate an STD response and its binding is relatively weak but perhaps comparable to that of probe 7. Second, addition of Ins(1,4,5,6)P₄ leads to conformational changes in the complex between the HDAC3-SMRT-DAD and probe 7. Third, the nature of the STD signals for probe 7 without and with Ins(1,4,5,6)P₄ is consistent with the conformation of the HDAC3-SMRT-DAD complex in our model (Figure 31a) and the X-ray model (Figure 31b), respectively. In the X-ray model the aromatic ring of probe 7 would not be in direct contact with SMRT-DAD and is not expected to give the STD response. Clearly, both the STD NMR and the photocrosslinking data are in agreement.

The data presented raise an important question, can the HDAC3-SMRT-DAD complex in solution adopt both the conformation proposed in our model (conformation 1, Figure 31a) and the one observed in the X-ray model (conformation 2, Figure 31b)?

It certainly appears so based on the experimental evidence provided above. The next important question is whether Ins(1,4,5,6)P₄ is as important for the complex with the full-length HDAC3 as it was reported for the truncated one? The outcomes of our enzymatic, photolabeling, and NMR studies clearly indicate that Ins(1,4,5,6)P₄ may not play the same role. Although according to the STD NMR experiments it binds HDAC3-SMRT-DAD complex, it is not present in the purified HDAC3-SMRT-DAD complex. Moreover, despite a considerable 43- to 4300-fold excess in the concentration of Ins(1,4,5,6)P₄ to that of the HDAC3-SMRT-DAD complex in our
experiments, Ins(1,4,5,6)P₄ has displayed negligible effect on the enzymatic activity of the complex and is not capable of dissociating HDAC3-SMRT-DAD. Instead, the increase in the concentration of Ins(1,4,5,6)P₄ appears to result in switching between conformation 1 where the photoreactive probes can crosslink with SMRT-DAD and conformation 2 where SMRT-DAD is too far for the photocrosslinking to occur.

In conformation 1 SMRT-DAD abstracts the positively charged site containing His17, Lys25, Arg265, and Arg301 and prevents Ins(1,4,5,6)P₄ from binding to HDAC3. As the concentration of Ins(1,4,5,6)P₄ increases, it starts to compete with SMRT-DAD for the positively charged site on HDAC3. The equilibrium between the two conformations shifts toward conformation 2 where the distance between the binding site of HDAC3 and SMRT-DAD is longer, which explains why in the case of probe 2 biotinylation of SMRT-DAD decreases whereas biotinylation of HDAC3 does not and in the case of longer probe 1 biotinylation of both SMRT-DAD and HDAC3 remains constant upon addition of Ins(1,4,5,6)P₄. The switch between conformations 1 and 2 is also consistent with the small synergistic effect of Ins(1,4,5,6)P₄ on the inhibition of HDAC3-SMRTR-DAD complex by probe 2. The STD NMR experiments strongly support both the phenomenon of the conformational switch and the differences in the distances in the conformations occurring in the absence and presence of Ins(1,4,5,6)P₄.

On the basis of this data we propose that strongly negatively charged molecules, such as Ins(1,4,5,6)P₄, or DNA, or even the C-terminal portion (truncated in the X-ray structure amino acids 379-428) of HDAC3, which is rich in aspartic and glutamic acids, may play a regulatory role in switching between the two (or more) conformations of HDAC3-SMRT complex as shown in Figure 37.
It is tempting to speculate that changes in the HDAC3 and SMRT relative orientation, geometry of the binding site, and charge distribution associated with *conformations 1* and 2 may be necessary to appropriately position HDAC3 for the unidirectional deacetylation of K5Ac→K8Ac→K12Ac→K16Ac in histone H4, a so-called feed-forward model of deacetylation (Figure 6, step 1), to provide substrate specificity for histone and non-histone targets, to stabilize the co-repressor complex, and to block access to HATs.

Considering proximity of K16 in H4 to DNA in nucleosomes (for instance in PDB: 1AOI), strong electrostatic attraction between the positively charged HDAC3 residues His17, Lys25, Arg265, and Arg301, the binding site for Ins(1,4,5,6)P₄ in the X-ray model, and DNA would be expected after HDAC3-SMRT deacetylates K16. Such binding may then cause HDAC3-SMRT-DAD to adopt *conformation 2* (Figure 37, step 2) much like it happens in the presence of Ins(1,4,5,6)P₄.
Figure 37. Proposed regulatory role of the conformational switch in the HDAC3-SMRT complex. Step 1 - the feed forward model of histone deacetylation in conformation 1,\(^{236}\) step 2 – the conformational switch model where deacetylation of histone H4 is terminated by adopting conformation 2. The HDAC3-SMRT-DAD binding interfaces in conformations 1 and 2 and the HDAC3 catalytic site are rendered by green, gray, and yellow, respectively. The histone tail, the acetylated lysine residues, and the phosphate groups on the deoxyribose sugars on the DNA are rendered by black line, yellow hexagons, and blue circles, respectively.

In fact, re-organization of the SMRT complex is required to release the interaction between HID, a histone interaction domain located in the second SANT domain of SMRT,\(^{238, 271}\) and the deacetylated H4 histone tail to allow it to bind to DNA. Upon release, the mostly positively charged SANT2 domain (PDB: 2LTP)\(^{272}\) would then be expected to engage in the interaction with DNA (Figure 37, step 2). Structural similarity of the SANT domains with Myb\(^{258}\) and SLIDE\(^{273}\) DNA binding domains further supports this scenario.
The switch between *conformations* 1 and 2 upon binding of HDAC3 and/or SANT2 to DNA may, therefore, serve as a termination signal for the histone deacetylation machinery as a result of re-orientation of the catalytic site of HDAC3 away from the histone tail (*Figure 37, step 2*). In concert with the other components of the histone code, such conformational switch may not only be responsible for maintaining the proper order of deacetylation but may also provide a “failsafe” mechanism if the deacetylation proceeds in an incorrect order.

Despite the difference in the protein components of the deacetylase complexes involving HDAC1/2 and HDAC3, many of the HDAC3 and SMRT structural features discussed above are also found in the HDAC1 and HDAC2 deacetylase complexes. For instance, similarly to SMRT, CoREST contains two SANT domains, and both HDAC1 and HDAC2 contain the same positively charged pocket formed by His17, Lys25, Arg265, and Arg301 that is found in HDAC3. Such conservation of the features suggests a possibility that the observations presented in this manuscript may have implications beyond the HDAC3-SMRT complex.

### 4.3 Conclusions and Future Directions

In summary, six novel photoreactive HDAC probes - “nanorulers” - were designed and applied to measure the distance between the binding site of HDAC3 and SMRT-DAD in solution using a variety of enzymatic, photocrosslinking, and STD NMR experiments.

Unlike previous studies, the experiments were conducted with the full-length HDAC3 in the complex with both the recombinant SMRT-DAD as well the full-length SMRT from cell lysates. Our study finds that under physiologically relevant conditions in solution SMRT-DAD is positioned ca 10-13 Å closer to the binding site of HDAC3 – *conformation* 1 – than in the X-ray
model – *conformation* 2. Multiple independent lines of evidence with the photoreactive probes presented in the manuscript suggest that Ins(1,4,5,6)P₄ can cause a switch between *conformations* 1 and 2 without affecting the enzymatic activity of the HDAC3 and SMRT-DAD complex.

To the best of our knowledge this is the first example where the photoreactive probes were used to detect conformational changes occurring in transcription complexes in response to chemical stimuli. We propose that the observed conformational switch plays an important role in “interpreting” the histone code.

The possibility of SMRT-DAD to be located at different distances from the active site of HDAC3, highlight the importance of quantitation of photocrosslinked adducts to determine the ratios of *conformation* 1 and 2 that exist in solution. With the tools at hand and the current status of the experimental techniques available to us, quantification of the conformational 1 and 2 was not possible. Although the relative contributions of conformation 1 and 2 is not known, there is no doubt that a change in the quaternary structure of the HDAC3-SMRT-DAD complex does occur in response to the increase in concentration of Ins(1,4,5,6)P₄. Interestingly, this change has no effect of the enzymatic activity of HDAC3 toward the substrate commonly used in in vitro studies with recombinant HDACs. Whether this observation remains to be true in vivo is an important scientific question to investigate. It is plausible that such conformational change(s) is the Nature’s approach to modulate substrate specificity/affinity to deacetylation of different types of substrates in response to chemical stimuli, signaling molecules, or additional components of the deacetylase complex.

We consider the quantification of various possible conformations of HDAC3-SMRT complex especially important for the future drug design efforts to rationally target the
physiologically related conformations of HDAC3-SMRT-DAD in cell/tissue specific manner. Therefore, I envision that the developed methods in this proposal can form a basic infrastructure for collecting quantitative data, using *in vitro* and *in vivo* models, on the conformations and overall quaternary structure HDAC3-SMRT-DAD can adapt in solution. In this context, future studies based on these findings are directed toward: the design of “nanoprotractors” to determine the portions of SMRT-DAD and HDAC3 involved in the interaction with other probes; optimization of proteomic protocols to identify and quantify photocrosslinked adducts; and the design of small molecules that can target one or more physiologically relevant conformations of this complex.
CHAPTER 5. CONCLUSIONS AND OUTLOOK

In this dissertation, I have studied several novel aspects of HDAC conformational plasticity. Specifically:

In chapter 1, I gave examples of HDACs multiprotein complexes and interactome that are unique in their components, localization, and possible role in diseases. We believe that conformational plasticity of HDACs is a part of naturally evolved mechanism to control a large variety of events with only a handful of HDAC isoforms. Studying HDAC conformational plasticity is a challenging task as the methodology and tools capable of interrogating these events are limited.

In chapter 2, I have focused our discussion on the function and structural features unique to HDAC3-SMRT/NCoR complex. Possible conformational changes in the tertiary and quaternary structure of HDAC3-SMRT/NCoR complex have been extensively documented in multiple publications. The most significant observation of all is that conformational plasticity of HDAC3 can influence its function, activity, localization, and interactions with substrates and proteins.

In chapter 3, the BEProFL approach was reviewed and shown to be a useful tool for characterizing ligand-protein interactions. The approach and the photoreactive probes are versatile, flexible, portable, and are capable of interrogating conformational plasticity in solution.

In chapter 4, I studied conformational plasticity of HDAC3-SMRT complex using the photoreactive probes - “nanorulers” - and the techniques originally developed for BEProFL. Specifically, I measured the distance between the HDAC3 binding site and SMRT-DAD and detected at least two conformations of HDAC3-SMRT-DAD complex. It is tempting to speculate
that these conformations are the nature’s way to regulate enzymatic activity of the HDAC3-SMRT complex. Yet, this aspect remains to be investigated.

I envision that these studies will facilitate discovery of novel approaches and tools to detect and study various aspects of conformational plasticity of HDAC complexes. It is quite likely that by inhibiting only a single complex or a subset of HDAC complexes it will be possible to limit the HDAC enzymatic inhibitory or scaffolding effects to a subset of cells and tissues, an important future direction in the epigenetic research.
APPENDIX A CHEMICAL PROCEDURES AND CHARACTERIZATION OF THE COMPOUNDS

General materials and methods

All the reagents and solvents were obtained from commercial sources and used without further purification. $^1$H NMR and $^{13}$C NMR spectra were recorded on Bruker spectrometers at 300/400 MHz and 75/100 MHz, respectively. Chemical shifts were reported on $\delta$ scale in ppm with solvent indicated as the internal reference. Coupling constants were reported in Hz and the standard abbreviations indicating multiplicity were used as follows: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. Mass spectrometry experiment was carried out on Agilent 1100-MSD instrument. The purity and the mass were analyzed by Shimadzu LCMS-2020 with UV detector at 214 and 254 nM using Kinetex C18 column with dimensions 100 x 3.0 mm and stationary particle size 2.6 $\mu$. TLC was performed with Merck 60F$_{254}$ silica gel plates. Chromatography purification was performed on Biotage-Isolera four instrument using pre-filled KP-Sil (normal phase) and KP-C18-HS (reverse phase) SNAP cartridges with UV detection at 254 and 280 nm. Hexanes–ethyl acetate (normal phase) and H$_2$O–MeOH (reverse phase) solvent systems were used as eluent for chromatography unless it is mentioned otherwise.
Experimental:

Synthetic procedures and preparation of compounds have been done according to reported methods.\(^{33, 251, 276, 277}\) The purity of all compounds used for activity assays and photolabeling experiments was found to be ≥ 95% as it was analyzed by reversed-phase LCMS.

**7-(3-Azido-5-(azidomethyl)benzamido)heptanoic acid methyl ester (10).** To a stirred solution of 3-azido-5-(azidomethyl)benzoic acid (9)\(^{251}\) (0.2 g, 0.91 mmol) in anhydrous CH\(_2\)Cl\(_2\) (5 mL) was added EDCI (0.23 g, 1.2 mmol) followed by HOBt (0.18 g, 1.2 mmol) at 0 °C. After 15 min, a solution of methyl 7-aminoheptanoate hydrochloride (0.24 g, 1.2 mmol) and DIEPA (0.28 mL, 1.2 mmol) in CH\(_2\)Cl\(_2\) (5 mL) was added to the reaction mixture drop-wise at 0 °C. The mixture was allowed to stir at rt and monitored by TLC. Upon completion, the organic layer was washed with saturated aqueous NaHCO\(_3\) solution followed by brine. The organic extracts were dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The crude product was further purified by flash chromatography (95:5 → 40:60, hexanes: EtOAc) to give a pale yellow solid (0.29 g, 90%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 7.46 (s, 1H), 7.42 (s, 1H), 7.10 (s, 1H), 6.29 (s, 1H), 4.42 (s, 2H), 3.46 (q, J = 4, 8 Hz, 2H), 2.33 (t, J = 8 Hz, 2H), 1.66 (m, 4H), 1.40 (m, 4H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 174.2, 166.1, 141.4, 138.1, 137.2, 122.6, 120.9, 117.5, 53.9, 51.5, 40.1, 33.9, 29.3, 28.7, 26.5, 24.7.

**7-((3-Azido-5-(azidomethyl)benzoylamino)-N-hydroxyheptanamide (2).** To a solution of hydroxylamine hydrochloride (3.9 g, 56 mmol) in MeOH at 0 °C was added portionwise powdered KOH (3.3 g, 59 mmol). The solution was stirred for 1 h at rt after the addition was complete. The
precipitate was filtered off and the filtrate containing NH$_2$OH in MeOH was added dropwise to an ice cooled solution of 10 (0.10 g, 0.28 mmol) in MeOH (3 mL). An additional amount of powdered KOH (0.3 g, 5.6 mmol) was added to the reaction mixture and the white solution was stirred and monitored by TLC. MeOH was concentrated at rt under reduced pressure. The residual solid was treated with saturated aqueous NH$_4$Cl solution (20 mL) and extracted with ethyl acetate (3 x 25 mL). The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified on Biotage using reverse phase C-18 column (90:10 → 10:90, H$_2$O: MeOH) to afford 5 as pale yellow solid (0.071 g, 70%).$^1$H NMR (400 MHz, DMSO-d$_6$): δ 10.39 (s, 1H), 8.73 (s, 1H), 8.62 (s, 1H), 7.64 (s, 1H), 7.52 (s, 1H), 7.25 (s, 1H), 4.52 (s, 2H), 3.23 (m, 2H), 1.94 (t, J = 8.0 Hz, 2H), 1.49 (m, 4H), 1.27 (m, 4H).$^{13}$C NMR (100 MHz, DMSO-d$_6$): δ 169.8, 165.3, 140.6, 138.6, 137.1, 124.2, 121.7, 117.7, 53.3, 39.8, 32.7, 29.3, 28.8, 26.6, 25.5.$^{13}$C DEPT-135 (100 MHz, DMSO-d$_6$): δ 124.2, 121.7, 117.7, 53.3, 32.7, 29.3, 28.8, 26.6, 25.5. ESI-MS: [M+H]$^+$ calcd. for C$_{15}$H$_{20}$N$_8$O$_3$ =361.2, found = 361.3; C$_{16}$H$_{21}$N$_7$O$_3$ (MW = 360.3).

4-(3-Azido-5-(azidomethyl)benzamido)butanoic acid ethyl ester (11). Compound 11 (240 mg, 80%) was prepared from 9 (0.2 g, 0.91 mmol) according to the procedure described for 10.$^1$H NMR (400 MHz, CDCl$_3$): δ 7.49 (s, 1H), 7.44 (s, 1H), 7.13 (bs, 1H), 7.06 (s, 1H), 4.37 (s, 1H), 4.13 (q, J = 4, 8, 2H), 3.49 (q, J = 4, 8 Hz, 2H), 2.44 (t, J = 8 Hz, 2H), 1.95 (m, 2H), 1.24 (t, J = 8 3H).$^{13}$C NMR (100 MHz, CDCl$_3$): δ 173.9, 166.1, 141.3, 138.0, 136.9, 122.8, 121.0, 117.4, 60.8, 53.9, 40.1, 32.1, 24.1, 14.2.
APPENDIX A (CONTINUED)

7-((3-Azido-5-(azidomethyl)benzoylamino)-N-hydroxybutanamide (3). Probe 3 (0.048 g, 50% yield) was prepared from 11 (0.10 g, 0.3 mmol) according to the procedure described for 2. \( ^1H \) NMR (400 MHz, DMSO-d6): \( \delta \) 10.38 (s, 1H), 8.7 (s, 1H), 8.54 (s, 1H), 7.83 (m, 2H), 7.51 (m, 1H, 4.53 (s, 2H), 3.26(q, J = 4, 8 2H), 2.02 (t, J = 8.0 Hz, 2H), 1.75 (m, 2H). \( ^13C \) NMR (100 MHz, DMSO-d6): \( \delta \) 169.3, 166.3, 136.3, 135.5, 131.4, 129.1, 127.7, 127.3, 53.8, 39.4, 30.4, 25.7. \( ^13C \) DEPT-135 (100 MHz, DMSO-d6): \( \delta \) 129.1, 127.7, 127.3, 53.8, 39.4, 30.4, 25.7. ESI-MS: [M+H]\(^+\) calcd. for C\(_{12}\)H\(_{14}\)N\(_8\)O\(_3\) =319.1, found = 319.2; [M+Na\(^{+1}\)]\(^+\) calcd. for C\(_{12}\)H\(_{14}\)N\(_8\)O\(_3\) =341.1, found = 341.2 C\(_{16}\)H\(_{21}\)N\(_7\)O\(_3\) (MW = 318.1).

3-Azido-5-(azidomethyl)-N-hydroxybenzamide (4). Probe 4 (0.055 g , 55%) was prepared from compound (8\(^{25l}\) (0.1 g 0.43 mmol) according to the procedure describe for 2. Compound 4 has been detected in NMR as rotamers. \( ^1H \) NMR (400 MHz, methanol-d\(_4\)): \( \delta \) 7.76 (m, 2H), 7.54 (m, 2H), 7.43 (s, 1H), 7.23 (s, 1H), 4.46 (s, 2H). \( ^13C \) NMR (100 MHz, methanol-d\(_4\)): \( \delta \) 166.3, 165.1, 141.2, 138.8, 136.6, 131.1, 128.7, 126.6, 126.4, 122.8, 121.1, 116.8, 53.7, 53.2.\(^{13C \ DEPT-135\)} (100 MHz, methanol-d\(_4\)):131.1, 128.7, 126.6, 126.4, 122.8, 121.2, 116.9, 53.7, 53.3. ESI-MS: [M+H]\(^+\) calcd. for C\(_8\)H\(_7\)N\(_7\)O\(_2\) =234.1, found = 234.1; C\(_8\)H\(_7\)N\(_7\)O\(_2\) (MW = 233.1).

8-((4-Azidophenyl)amino)-8-oxooctanoic acid methyl ester (13). To a stirred solution of Suberic acid monomethyl ester (0.2 g, 1.1 mmol) in anhydrous CH\(_2\)Cl\(_2\) (5 mL) was added EDCI (0.27 g, 1.4 mmol) followed by HOBt (0.19 g, 1.4 mmol) at 0 °C. After 30 min, a solution of 4-azidoaniline hydrochloride (0.24 g, 1.4 mmol) and DIEPA (0.31 mL, 1.4 mmol) in CH\(_2\)Cl\(_2\) (5 mL) was added to the reaction mixture drop-wise at 0 °C. The mixture was allowed to stir at rt and
monitored by TLC. Upon completion, the organic layer was washed with saturated aqueous NaHCO$_3$ solution followed by brine. The organic extracts were dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was further purified by flash chromatography (95:5 → 40:60, hexanes: EtOAc) to afford 13 (0.30 g, 90%). $^1$H NMR (400 MHz, CDCl$_3$): δ 8.07 (s, 1H), 7.52 (d, $J = 8$, 2H), 6.92 (d, $J = 8$, 1H), 3.65 (s, 3H), 2.29 (m, 4H), 1.66 (m, 4H), 1.33 (m, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 174.3, 171.7, 135.5, 135.2, 121.5, 119.3, 51.5, 37.3, 33.9, 28.8, 28.7, 25.4, 24.7. ESI-MS: [M+H]$^+$ calcd. for C$_{15}$H$_{20}$N$_4$O$_3$ =305.2, found = 305.1; C$_{15}$H$_{20}$N$_4$O$_3$ (MW = 304.1).

Octanedioic acid (4-azidophenyl)amide hydroxyamide (5). Probe 5 (0.06 g, 60%) was prepared from 12 (0.1 g 0.33 mmol) according to the procedure describe for 2. $^1$H NMR (400 MHz, DMSO-d$_6$): δ 10.35 (s, 1H), 9.97 (s, 1H), 8.7 (bs, 1H), 7.65 (d, $J = 8$, 2H), 7.05 (d, $J = 8$, 2H), 2.29 (t, $J = 8$, 2H), 1.94 (t, $J = 8$, 2H), 1.51 (m, 4H), 1.27 (m, 4H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ 171.6, 169.6, 137.1, 133.9, 120.9, 119.8, 36.8, 32.7, 28.9, 28.8, 25.5, 25.4. ESI-MS: [M+H]$^+$ calcd. for C$_{14}$H$_{19}$N$_5$O$_3$ =306.2, found = 306.1; C$_{14}$H$_{19}$N$_5$O$_3$ (MW = 305.1).
APPENDIX A (CONTINUED)
APPENDIX A (CONTINUED)
APPENDIX A (CONTINUED)
APPENDIX A (CONTINUED)

N<sub>2</sub>-<sub>4</sub>-O

13

[Chemical structure diagram]

[Graph 1: NMR spectrum with chemical shifts]

[Graph 2: Mass spectrum with m/z values]

APPENDIX A (CONTINUED)
APPENDIX B BIOLOGICAL PROCEDURES FOR COMPOUND SCREENING, PHOTOLABELING EXPERIMENTS, AND STD-NMR

Biological materials

Recombinant HDAC3-SMRT-DAD (catalogue # 50003, lot # 110404) and SMRT-DAD (Catalog #: 50020) were purchased from BPS Bioscience (San Diego, CA) and pan HDAC substrate Boc-Lys(Ac)-AMC was purchased from Chem-Impex (Wood Dale, IL). The primary anti-HDAC3 antibody was from Santa Cruz (Santa Cruz, CA). The anti GST conjugated to horseradish peroxidase (HRP) antibody and the anti-rabbit secondary antibody conjugated HRP were from GE (Piscataway, NJ). Streptavidin and the SuperSignal West Femto Chemiluminescent Substrate were from Thermo Scientific (Rockford, IL). The 96-well plates from Corning (Rochester, NY). Tris(2-carboxyethyl)phosphine (TCEP), CuSO₄, dimethyl sulfoxide (DMSO), tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA), and all other chemicals were purchased from Sigma (St. Louis, MO) if not stated otherwise.

HDAC3 activity assays

The assay proceeded through a simple two-step procedure that can be carried out in half-volume microtitration plates. The in vitro HDAC3 assay was performed with 10 ng (2.3 nM) of HDAC3-SMRT-DAD, 25 μM substrate in HDAC assay buffer 1 (25 mM Tris-HCl, adjusted pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mg/mL BSA). From 1mM DMSO stock of the inhibitors, a three-fold serial dilution using Assay buffer 2 (25 mM Tris-HCl, adjusted pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂) were done.
The enzyme and inhibitor were pre-incubated for 5 min, before initiating the reaction by adding the 10 μL of 125 uM substrate. After 35 min of incubation at rt with different concentrations of inhibitor, 50 μL of developer (1 mg/mL trypsin and 5 μM TSA in 25 mM Tris-HCl, adjusted pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂) was added to quench the reaction and release the fluorophore from the deacetylated product. The fluorescence experiments were conducted at λ<sub>excit</sub> = 360 nm and λ<sub>emis</sub> = 460 nm using SYNERGY H4 Hybrid Reader. The IC<sub>50</sub> values were determined using the GraphPad Prism 5 software. For the activity assay under different condition, buffer 1 and 2 were modified according to the conditions mentioned earlier.

**Photolabeling of HDAC3-SMRT-DAD with probes 1-4 in absence of competitors 5 and 6**

Purified recombinant human HDAC3-SMRT-DAD (500 to 750 ng) in assay buffer 1 supplemented with 0.1% triton X-100 was incubated with various concentrations of probes 1-4 for 2-3 h in the dark, after which the formation of a covalent bond between the azide group present in the ligand and reactive side chains of the HDAC was initiated by UV irradiation (λ=254 nm) for 3 × 1 min with 1 min rest. Then click chemistry was used to attach BT to ligands bound covalently to the protein. The biotin tag 9 was added to all the tubes at concentration of 50 μM, and the chemical reaction was initiated by addition of TCEP (0.5 mM), TBTA (0.1 mM) and CuSO₄ (1 mM). After 1 h incubation at room temperature, protein samples were analyzed by SDS-PAGE and western blot using an anti-biotin primary antibody, streptavidin conjugated to horse radish peroxidase (1:4000). The membranes were then stripped in 0.2 M glycine, pH 2.6 for 10 min, then in 0.2 M glycine, pH 2.3 for another 10 min before being re-blocked with 5% milk and redecorated.
APPENDIX B (CONTINUED)

with an anti-HDAC3 primary antibody (1:2000) and an anti-rabbit-HRP secondary antibody (1:4000).

**Photolabeling of HDAC3-SMRT-DAD with probes 1-4 in the presence of competitors 5 and 6.** Purified recombinant human HDAC3-SMRT-DAD (500 to 750 ng) in assay buffer 1 supplemented with 0.1% triton X-100 was incubated with various concentrations of probes 2-4 for 2-3 h in the dark in the presence or absence of competitor 5 at a concentration of 42.5 µM. The same procedure was repeated with probe 1 at a fixed concentration of 1.2 µM and various concentrations of competitor 6, followed by the UV activation step and the click reaction using the biotin tag 9 as described previously. Protein samples were analyzed by SDS-PAGE and western blot using an anti-biotin primary antibody, streptavidin conjugated to horse radish peroxidase (1:4000). The membranes were then stripped in 0.2 M glycine, pH 2.6 for 10 min, then in 0.2 M glycine, pH 2.3 for another 10 min before being re-blocked with 5% milk and re-decorated with an anti-HDAC3 primary antibody (1:2000) and an anti-rabbit-HRP secondary antibody (1:4000).

**Photolabeling of HDAC3-SMRT-DAD with probes 7 and 8 in the presence of TSA.**

Purified recombinant human HDAC3-SMRT-DAD (750 ng) in assay buffer 1 supplemented with 0.1% triton X-100 was incubated with probes 7 and 8 (8.5 µM) for 2-3 h in the dark in the presence or absence of TSA at a concentration of 42.5 µM. Followed by the UV activation step and the click reaction using the biotin tag 9 as described previously. Protein samples were analyzed by SDS-PAGE and western blot using an anti-biotin primary antibody, streptavidin conjugated to horse radish peroxidase (1:4000). The membranes were then stripped in 0.2 M glycine, pH 2.6 for 10
min, then in 0.2 M glycine, pH 2.3 for another 10 min before being re-blocked with 5% milk and re-decorated with an anti-HDAC3 primary antibody (1:2000) and an anti-rabbit-HRP secondary antibody (1:4000).

**Photolabeling experiments with probes 2-4 under denaturing conditions.**

Purified recombinant human HDAC3-SMRT-DAD (750 ng) in assay buffer 1 supplemented with 0.1% triton X-100 was incubated with probes 2-4, at two different concentrations, for 2-3 h in the dark in the presence or absence of competitor 5 at a concentration of 42.5 µM. Then we added sodium dodecyl sulphate (final concentration is 2% w/v; stock solution was prepared in assay buffer 1 and 0.1% triton X-100), mixed the solution gently by means of a pipette, and incubated the reaction vials at 37 °C for 30 min. Then we proceeded with the UV activation step and the click reaction using the biotin tag as described previously. Protein samples were analyzed by SDS-PAGE and western blot using an anti-biotin primary antibody, streptavidin conjugated to horse radish peroxidase. The membranes were then stripped in 0.2 M glycine, pH 2.6 for 10 min, then in 0.2 M glycine, pH 2.3 for another 10 min before being re-blocked with 5% milk and re-decorated with an anti-HDAC3 primary antibody (1:2000), followed by the addition of anti-rabbit-HRP secondary antibody (1:4000) to visualize HDAC3. The membranes were stripped again using the same reagents, described earlier, and then re-blocked with 5% milk and re-decorated with anti-GST HRP (1:2000) conjugate to visualize GST tagged SMRT-DAD.
APPENDIX B (CONTINUED)

Photolabeling of HDAC3-SMRT-DAD with probe 2 under low monovalent ion concentration

Purified recombinant human HDAC3-SMRT-DAD (750 ng) in buffer (25mM Tris-HCl, adjusted pH=8, 25 mM NaCl, 2.7mM KCl, 1mM MgCl₂) supplemented with 0.1% triton X-100 was incubated with various concentrations of probe 2 for 2-3 h in the dark in the presence or absence of competitor 5 at a concentration of 42.5 µM. Followed by the UV activation step and the click reaction using the biotin tag 7 as described previously. The membranes were then stripped in 0.2 M glycine, pH 2.6 for 10 min, then in 0.2 M glycine, pH 2.3 for another 10 min before being re-blocked with 5% milk and redecorated with an anti-HDAC3 primary antibody (1:2000), followed by the addition of anti-rabbit-HRP secondary antibody (1:4000) to visualize HDAC3. The membranes were stripped again using the same reagents, described earlier, and then re-blocked with 5% milk and redecorated with anti-GST HRP (1:2000) conjugate to visualize GST tagged SMRT-DAD.

Photolabeling of HDAC3-SMRT-DAD with probe 1 and 2 in presence of D-myo-inositol-(1,4,5,6)-tetrakisphosphate (Ins(1,4,5,6)P₄).

Purified recombinant human HDAC3-SMRT-DAD (750 ng) in buffer (50 mM Tris-HCl, adjusted pH=7.5, 50 mM CH₃COOK, 5% glycerol) supplemented with 0.3% triton X-100 was incubated first with various concentrations of Ins(1,4,5,6)P₄ for 1.5 h in the dark, then the addition of probe 1 or 2 (8.5 µM) in the presence or absence of competitor 5 at a concentration of 42.5 µM for another 1.5 h. Followed by the UV activation step and the click reaction using the biotin tag 7 as described previously. The membranes were then stripped in 0.2 M glycine, pH 2.6 for 10 min, then
in 0.2 M glycine, pH 2.3 for another 10 min before being re-blocked with 5% milk and redecorated with an anti-HDAC3 primary antibody (1:2000), followed by the addition of anti-rabbit-HRP secondary antibody (1:4000) to visualize HDAC3. The membranes were stripped again using the same reagents, described earlier, and then re-blocked with 5% milk and redecorated with anti-GST HRP (1:2000) conjugate to visualize GST tagged SMRT-DAD. Densitometric analysis of each spot was measured using Image J software and were plotted using GraphPad Prism. Relative intensities were calculated by setting the maximum signal to 100% and normalizing the rest of the values to the maximum signal. The data is plotted as the average of at least 2 independent experiments with the standard deviation. Statistical analysis was performed using the one-way ANOVA (Null concentration of Ins(1,4,5,6)P$_4$ was used as control).

**Photolabeling of SMRT-DAD with probe 1.** Purified recombinant human SMRT-DAD (500 ng) in assay buffer 1 supplemented with 0.1% triton X-100 was incubated with various concentrations of probe 1 for 2-3 h in the dark, followed by the UV activation step and the click reaction using the biotin tag 9 as described previously. Protein samples were analyzed by SDS-PAGE and western blot using an anti-biotin primary antibody, streptavidin conjugated to horse radish peroxidase (1:4000). The membranes were then soaked in coomassie brilliant blue and de-stained to visualize SMRT-DAD protein.

**Photolabeling of HDAC3-SMRT with probes 2 and 5 in HT-29 cell lysates.**

In microcentrifuge tubes, 50 μg of HT-29 cell lysate was aliquoted. To the reaction vials, probe 2 (25 μM) in lysis buffer was added in the presence or absence of competitor 5 (1 mM). The reaction
vials were incubated at 4°C for 3 hours shaking. Then, the vials were UV irradiated (254 nm) 3 x 1 min with 1 min rest in between. To the vials, 2 μL of Acetylene Biotin (1 mM), 1.5 μL of TCEP (25 mM), 2 μL of TBTA (2.5 mM), and 2 μL CuSO4 (25 mM) were added. Reaction vials were stirred on magnetic stirrer for 90 minutes RT. To prepare protein for denaturation, 4X LDS Sample Buffer (Invitrogen) with 8% β-mercaptoethanol and was added. The vials were boiled for 3.5 minutes. Reaction vials were separated by gel electrophoresis (7.5% polyacrylamide) at 100V until 25 kD marker ran to the bottom of the gel.

Saturation Transfer Difference (STD) NMR

STD experimental conditions: The STD experiments were performed with 100 μM probe 7 and 1 μM HDAC3-SMRT-DAD complex without Ins(1,4,5,6)P4 (blue) and with 25 μM Ins(1,4,5,6)P4 (red) or with 25 μM Ins(1,4,5,6)P4 only (green). The spectra were acquired as previously described, at room temperature on an 800 MHz Bruker NMR spectrometer in the interleaved fashion with and without saturation. For saturation the methyl region was irradiated at -1 ppm.

STD Sample preparation: A 10 mM solution of probe 7 dissolved in DMSO was used to prepare 1 mM stock solution of the probe dissolved in DMSO-d6. A 1 mM stock solution of Ins(1,4,5,6)P4 in D2O was used as a stock solution. Total volume of the sample was 200 μL and D2O and DMSO-d6 levels did not exceed 10% of the total volume.

Sample 1 preparation (blue): to 150 μL 1X PBS was added 20 μL of probe 7, 10 μL of HDAC3-SMRT-DAD stock (1.6 μg/μL), and 20 μL of D2O
Sample 2 preparation (red): to 150 µL 1X PBS was added 20 µL of probe 7, 10 µL of HDAC3-SMRT-DAD stock (1.6 µg/µL), 5 µL Ins(1,4,5,6)P4, and 15 µL of D2O.

Sample 3 preparation (green): to 150 µL 1X PBS was added 20 µL of DMSO-d6, 10 µL of HDAC3-SMRT-DAD stock (1.6 µg/µl), 5 µL Ins(1,4,5,6)P4, and 15 µL of D2O.
This thesis is a presentation of my original research work done under the guidance of my advisor, Dr. Pavel A. Petukhov, at the University of Illinois at Chicago (UIC). The material, described in the abstract, chapter 4, and appendices A and B, has been published in the ACS Chemical Biology journal and it is cited as follow: Abdelkarim H., Brunsteiner M., Neelarapu R., Bai H., Madriaga A., van Breemen R. B., Blond S. Y., Gaponenko V., and Petukhov P. A. (2013) Photoreactive "nanorulers" detect a novel conformation of full length HDAC3-SMRT complex in solution, ACS chemical biology 8, 2538-2549.

Dr. Pavel A. Petukhov (the corresponding author) and me (first author) have been responsible for writing, editing, collecting, and analysing the results for the majority of the paper content. Wherever contributions of others are involved, every effort is made to indicate this clearly in the text, with due reference to the literature, and acknowledgement of collaborative research. A license agreement provided in appendix C allows the first author the full usage of figures, tables, and written material and grants the right to copy material into this thesis.

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APPENDIX C (CONTINUED)

We thank Dr. Karol Bruzik (UIC) for providing us with D-myo inositol (1,4,5,6) tetrakisphosphate sodium salt. We also thank Dr. Jonna Frasor (UIC) for careful reading of the published paper and for providing many helpful comments.
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CITED LITERATURE


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photoaffinity labeling studies of novel series of photoreactive benzamide probes for histone deacetylase 2, Bioorganic & Medicinal Chemistry Letters 22, 5025-5030.


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AWARDS, HONORS, AND FELLOWSHIPS

Professor Ludwig Bauer Award 2012, 2014
University of Illinois at Chicago, Chicago, IL
Sponsored Registration to the AAAS Annual Meeting 2014
The AAAS Section of Pharmaceutical Sciences
Protein Society - Finn Wold - Travel Award 2013
The 27th Annual Symposium of The Protein Society, Boston, MA
Phi Kappa Phi Honorary Membership 2013
University of Illinois at Chicago, Chicago, IL
Student Presenter Award 2012, 2013
University of Illinois at Chicago, Chicago, IL
Graduate Student Council Travel Award 2013
University of Illinois at Chicago, Chicago, IL
Chancellor’s Student Service Award 2012
University of Illinois at Chicago, Chicago, IL
Provost's & Deiss Award for Graduate Research 2012
University of Illinois at Chicago, Chicago, IL

AAAS/Science Program for Excellence in Science 2012
The American Association for the Advancement of Science, Washington, DC

Rho Chi Honorary Membership 2012
University of Illinois at Chicago, Chicago, IL

Top 12 Poster Presenter Award 2012
Society for Laboratory Automation and Screening(SLAS), San Diego, CA

Future of Science Fund Scholarship 2012
Keystone Symposia (F1), Tahoe City, CA

ADDF Young Investigator Scholarship 2012
The 6th Drug Discovery for Neurodegeneration Conference, New York city, NY

Chancellor's Graduate Research Fellowship 2010-2012
University of Illinois at Chicago, Chicago, IL

Tony B. Academic Travel Award 2011
Society for Laboratory Automation and Screening(SLAS), San Diego, CA

W.E. van Doren Scholar 2010
University of Illinois at Chicago, Chicago, IL

Academic Excellence Award (Top GPA Award) 2006
Hikma Pharmaceuticals, Amman, Jordan

Certificate of Merit for Academic Excellence 2006
University of Jordan, Amman, Hashemite Kingdom of Jordan

Biopharmaceutics & Clinical Pharmacy Department Scholarship 2004
University of Jordan, Amman, Hashemite Kingdom of Jordan
PUBLICATIONS


  Contributions:
  - Second author position.
  - Expression, purification, and biochemical characterization of recombinant HDAC8.
  - Activity assay testing of compounds against HDAC1, HDAC3-SMRT-DAD complex, and HDAC8.

  Contributions:
  - Forth author position.
  - Biological testing of benzamide based HDAC probes against recombinant HDAC1 and HDAC3-SMRT-DAD complex.
  - Expression, purification, and biochemical characterization of recombinant HDAC8.

  Contribution:
  - Fifth author position.
  - Synthesis of the clickable biotin tag with a terminal alkyne.
  - Expression, purification, and biochemical characterization of recombinant HDAC8.
  - Biological testing of ligands against recombinant HDAC1.
MANUSCRIPTS IN PREPARATION/UNDER REVIEW


- **Abdelkarim H**, Petukhov P, Conformational Plasticity in Histone Deacetylases as a Source for New Discoveries. *(In preparation)*


POSTER PRESENTATIONS

**The 33rd Midwest Enzyme Chemistry Conference (MECC), October 13th, 2013**

Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu, Antonett Madriaga, Richard van Breemen, Sylvie Y. Blond, Pavel A. Petukhov. Photoreactive Chemical Rulers Detect a Novel Conformation of HDAC3-SMRT Complex.

**The 11th Annual CBC Symposium, October 13th, 2013**

Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu, Antonett Madriaga, Richard van Breemen, Sylvie Y. Blond, Pavel A. Petukhov. Photoreactive Chemical Rulers Detect a Novel Conformation of HDAC3-SMRT Complex.

**The 27th Annual Symposium of The Protein Society, July 20-23th, 2013**

Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu, Antonett Madriaga, Richard van Breemen, Sylvie Y. Blond, Pavel A. Petukhov. Photoreactive Chemical Rulers Detect a Novel Conformation of HDAC3-SMRT Complex.

**The 2012 AAPS Annual Meeting and Exposition, October 14–18, 2012**

Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu, Richard van Breemen, Sylvie Y. Blond, Pavel A. Petukhov. Mapping the binding sites of HDAC3/NCoR using photoaffinity chemical probes as molecular rulers.
The 32nd Midwest Enzyme Chemistry Conference (MECC), October 13th, 2012
Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Richard van Breemen, Pavel A. Petukhov. Photoreactive HDAC3 inhibitors act as “nanorulers” probing the HDAC3/NCoR2-DAD complex in solution.

The 10th Annual Discovery on Target, October 1-2nd, 2012
Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Richard van Breemen, Pavel A. Petukhov. Photoreactive HDAC3 inhibitors act as “nanorulers” probing the HDAC3/NCoR2-DAD complex in solution.

Keystone Symposia (F1), March 19-24th, 2012
Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Richard van Breemen, Pavel A. Petukhov. The development and implementation of nanorulers as a novel drug discovery tools to characterize HDAC3/NCoR2 interface.

The 3rd Annual College of Pharmacy Research Day, March 8-9th, 2012
Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Richard van Breemen, Pavel A. Petukhov. The development and implementation of chemical nanites to probe HDAC3/NCoR2 interface.

The 6th Drug Discovery for Neurodegeneration Conference, February 12-14th, 2012
Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Richard van Breemen, Pavel A. Petukhov. The implementation of photoreactive chemical probes in characterizing HDAC3/NCoR complex: a step toward neurodegenerative compatible HDAC inhibitors.

The 1st Annual SLAS Meeting, February 4-8th, 2012
Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Richard van Breemen, Pavel A. Petukhov. The development and implementation of chemical nanites to probe HDAC3/NCoR2 interface.

The 9th Annual CBC Symposium, October 21st, 2011
Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Richard van Breemen, Pavel A. Petukhov. Mapping the binding sites of HDAC3/NCoR2 complex using photoreactive chemical probes.
The 49th Annual MIKI Meeting, April 8-10th, 2011

Hazem Abdelkarim, Michael Brunsteiner, Bai He, Raghupathi Neelarapu Yong Soo Choi, Subash Velaparthi, Richard van Breemen, Sylvie Y. Blond, Pavel A. Petukhov. Mapping the binding sites of HDAC3/NCoR2 complex using photoreactive chemical probes.

SCIENTIFIC TALKS

Dissertation Seminar

March, 2014

‘Conformational Plasticity in Histone Deacetylases as a Source of New Discoveries’

The 50th annual MIKI meeting

April, 2012

‘Mapping the Binding Sites of HDAC3/NCoR2 Complex Using Photoaffinity Chemical Probes’

Keystone symposia (F1) -Invited Talk-

March, 2012

‘The Development and Implementation of Nanorulers as a Novel Drug Discovery Tool to Characterize HDAC3/NCoR2 Interface’

College of Pharmacy, University of Illinois at Chicago

April, 2010

‘Utilization of Non-conventional Interactions in Drug Design’

ACADEMIC SERVICE

52nd MIKI Meeting

2014

Role: Oral session chair

Graduate Student Council, Graduate College, UIC

2011-2014

Role: Medicinal Chemistry graduate student representative

2nd -4th annual College of Pharmacy Research Day

2011-2013

Role: Volunteer

American Association of Pharmaceutical Scientists UIC student chapter

2009-2012

Role: Treasurer
48th MIKI Meeting 2010
Role: Volunteer
Webster-Sibilsky lectureship in Medicinal Chemistry 2010
Role: Committee member

PROFESSIONAL AFFILIATIONS

Academic Drug Discovery Consortium (AD₂C) 2013-present
Phi Kappa Phi Honor Society 2013-present
The Protein Society 2013-present
International Chemical Biology Society (ICBS) 2012-present
Rho Chi Honor Society 2012-present
The American Association for the Advancement of Science (AAAS) 2011-present
Society for Laboratory Automation and Screening (SLAS) 2011-present
American Association of Pharmaceutical Scientists (AAPS) 2010-present
American Chemical Society (ACS) 2009-present
The Alzheimer Research Forum 2009-present
Jordan Pharmaceutical Association (JPA) 2006-present

NOMINATIONS AND RECOGNITIONS

Student Researcher, UI Campus Insight Presentation 2014
ACS Chemical Biology, Introducing Our Authors, v.8, iss.11 2013
Student Research Highlight Presentation 2013
Dean Scholar, University of Illinois at Chicago 2013
Chicago Biomedical Consortium (CBC) Scholar 2012
PROFESSIONAL AND TEACHING EXPERIENCE

Graduate Research Assistance 2010-2014

A. Prof. Pavel A. Petukhov laboratory, UIC

➢ Grant Name (R01): Photoaffinity Labeling Probes for Development of Novel Isoform Selective HDAC Inhibitors (5R01CA131970)

Duties:

- Synthesis of photoreactive based HDAC inhibitors.
- Synthesis of amine based HDAC inhibitors.
- Synthesis of biotin alkyne reporter tag.
- Purification and assay development of HDAC8.
- Screening compounds against HDAC1, HDAC3, and HDAC8.
- Photolabeling with HDAC1, HDAC3, and HDAC8.
- Photolabeling with MCF-7 and HT-29 cell lysates.

➢ Grant Name (R21): Small Molecule Inhibitors of Malate Synthase Against M. Tuberculosis (5R21AI083661)

Duties:

- Synthesis of Malate synthase G inhibitors.
- Purification of Malate synthase G.
- Assay development for screening compounds.

➢ Grant Name: Mapping the Binding Site of HDAC2 for the Design of Novel HDAC inhibitors Lacking Zinc Binding Groups (ADDF; category: Drug discovery, Neuroprotection, grant # 20101103)

Duties:

- Screening compounds against HDAC1 and HDAC3.
- Purification and assay development of HDAC8.

B. Grant proposals written under Dr. Pavel Petukhov’s supervision

➢ Preliminary examination-R01 grant proposal format

   Title: The implementation of photochemical probes to map HDAC3/NCoR complex
Department of Defense – Breast Cancer Research Program (BCRP) – concept award
Title: Photoreactive Chemical Probes for Direct Analysis of HDAC Isoform Inhibition in Triple-Negative Breast Cancer

Department of Defense – Breast Cancer Research Program (BCRP) – idea award
Title: Chemical Probes for Profiling of HDAC Isoform-Selective Inhibition in Breast Cancer

Department of Defense – Multiple Sclerosis Research Program (MSRP) – concept award (considered as an alternate funding application)
Title: Selective HDAC4 Inhibitors to Regulate Immune Response and Battle Neuronal Death in Multiple Sclerosis

C. Collaboration Projects (as a member of Dr. Petukhov Laboratory)

- **Prof. Gregory R. Thatcher Laboratory, UIC**
  Project code name: SERM – HDAC Hybrids.
  Duties:
  - Tested the inhibitory activity of a small set of SERM – HDAC Hybrids against HDAC1, HDAC3, and HDAC8

- **Prof. Subhash C. Pandey Laboratory, UIC**
  Project code name: HDAC Inhibitors and alcohol Addition.
  Duties:
  - Provided and synthesized several photoreactive probes and HDAC inhibitors for the project.
  - Provided procedures and protocols for the photolabeling experiments with recombinant proteins and whole cell lysates.
  - Provided a proof of principle and procedures for in gel detection with HDAC8.
  - Provided HDAC inhibitors to be biologically evaluated in alcohol addiction *in vivo* models.

- **Prof. Primal de Lanerolle Laboratory, UIC**
  Project code name: HDACs/Actin interactions.
  Duties:
  - Provided procedures and protocols for the photolabeling experiments with recombinant proteins and whole cell lysates.
  - Guided, provided, and supervised activity assays experiments with class I HDACs.
Prof. Vadim Gaponenko Laboratory, UIC
Projects code name: Eb3 potential inhibitors.
Duties:
- Run 2D NMR experiments with recombinant Eb3
Project code name: STD-NMR of HDAC inhibitors.
Duties:
- Run STD-NMR experiments of several HDAC inhibitor with HDAC1, HDAC3, and HDAC8

Prof Nadim Mahmud Laboratory, UIC
Project code name: HDAC inhibitors effects in stem cell growth and/or inhibition.
Duties:
- Provided and synthesized several HDAC inhibitors for biological evaluation in stem cell in vitro models

Prof. Douglas Feinstein Laboratory, UIC
Project code name: HDAC inhibitors and Multiple sclerosis.
Duties:
- Provided and synthesized several HDAC inhibitors for biological evaluation in glial cell in vitro models

Graduate Teaching Assistance 2009-2010, 2013

College of Pharmacy at UIC

Courses:

PHAR408: Principles of Drug Action and Therapeutics VIII (Spring 2013)
PHAR 403: Principles of Drug Action and Therapeutics III (Spring 2010)
PHAR 402: Principles of Drug Action and Therapeutics II (Fall 2009)
PHAR 342: Experiential I – IPPE (Spring 2009)

Duties:

Helped in Material Preparations.
Proctored and graded exams and quizzes.
Technical support - uploaded materials and grades to Blackboard.

Pharmacist 2006-2008

Community Pharmacy, Amman, Jordan
Medical Representative 2006-2007

Roche Pharmaceuticals, Jordan

Area of Specialization: Antibiotics

Pharmacy Trainee 2002-2006

College of Pharmacy, University of Jordan

In Patient: 100 hours in practice of clinical pharmacy in nephrology and cardiology departments

Out Patient: 1440 hours in practice of community pharmacy

Summer Intern 2005

Wyeth Pharmaceuticals, Jordan

Area of Specialization: Antibiotics

CERTIFICATES

Certificate of Accomplishment 2013

Writing in the Sciences - Stanford online course organized by Dr. Kristin Sainani

Certificate of Appreciation 2010-2011

American Association of Pharmaceutical Scientists

International Computer Driving License (ICDL) 2006

ECDL/ICDL Foundation

RESEARCH EXPERIENCE AND LABORATORY SKILLS

Chemistry

- Executing multi-step synthesis – Reactions: coupling reactions, heterocyclic chemistry, reductive amination, etherification, hydrolysis, click reactions, oxidation/reduction reactions, and azides related reactions…etc.

- Well versed in many purification techniques

Manual
Flash chromatography (ISOLERA purification system)
High performance liquid chromatography (HPLC)
- Related software and search engine: ChemDraw, Chemaxon (Marvin), SciFinder, and Reaxys.

Biology
- Molecular Biology: protein expression, cell lysis (French press, sonication), protein purification (FPLC, affinity, ion exchange, and size exclusion columns), enzyme Kinetics, fluorometric Based Assay for IC50 and Ki determination, luminescence-Based Assay for IC50 and Ki determination.
- Immunochemistry: SDS-polyacrylamide gel electrophoresis, Western blots, and in gel detection.
- Photo-labeling with photo reactive chemical probes applied to recombinant proteins and cell lysates samples.
- Instruments: synergy H4 hybrid multi-mode microplate reader, BMG Optima microplate reader, iBlot transfer system, charge coupled device (CCD) camera, Bio-Rad transfer system (semi dry and wet), and Bio-Rad 1-D Electrophoresis Systems… etc.

Advanced spectroscopic techniques
- Structure elucidation using 1D/2D NMR experiments.
- Determination of ligands binding “epitopes” using saturation transfer difference (STD-NMR) experiments.
- Determination of purity and mass for several HDAC and malate synthase G inhibitors using MS and LC-MS experiments.
- Instruments: Bruker (400, 500, and 800 MHz), and Shimadzu 2020.

Computer-assisted drug design (CADD)
- Basic knowledge in using CADD software: Molecular operating environment (MOE), Chimera (visualization software), Suite of computational informatics software (SYBYL), and PyMOL.