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HDAC1 Substrate Profiling using Proteomics-Based Substrate Trapping

Dhanusha A. Nalawansha, Yuchen Zhang, Kavinda Herath, and Mary Kay H. Pflum*

Department of Chemistry, Wayne State University, 5101 Cass Avenue, Detroit, MI 48202

Abstract

Histone Deacetylase (HDAC) proteins are overexpressed in multiple diseases, including cancer, and have emerged as anticancer drug targets. HDAC proteins regulate cellular processes, such as cell cycle, apoptosis, and cell proliferation, by deacetylating histone and non-histone substrates. Although a plethora of acetylated proteins have been identified using large-scale proteomic approaches, the HDAC proteins responsible for their dynamic deacetylation are poorly studied. For example, few substrates of HDAC1 have been identified, which is mainly due to the scarcity of the substrate identification tools. We recently developed a mutant trapping strategy to identify novel substrates of HDAC1. Herein, we introduce an improved version of the trapping method that uses mass spectrometry-based proteomics to identify multiple substrates simultaneously. Among the substrate hits, CDK1, AIFM1, MSH6 and RuvB-Like1 were identified as likely HDAC1 substrates. These newly discovered HDAC1 substrates are involved in various biological processes, suggesting novel functions of HDAC1 apart from epigenetics. Substrate trapping combined with MS-based proteomics provides an efficient approach to HDAC1 substrate identification and contributes to the full characterization of HDAC function in normal and disease states.

Graphical Abstract

*To whom correspondence should be addressed: pflum@wayne.edu.

Author Contributions

M.H.P. conceived of the project. D.A.N. performed the optimization and trapping experiments. Y.Z. performed the MSH6 validation studies. K.H. performed the Cytoscape analysis. M.H.P, D.A.N., and Y.Z. wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

Repetitive trials, expression levels, abundance data, and an enlarged interactome image. This material is available free of charge via the internet at <http://pubs.acs.org>.

Keywords

histone deacetylase; HDAC1; Vorinostat; substrate trapping

Introduction

Histone acetylation is a key posttranslational modification that affects chromatin structure and transcription regulation.¹ Histone acetyltransferase (HAT) and histone deacetylase (HDAC) proteins regulate the balance between acetylation and deacetylation of histones. Through histone deacetylation, HDAC proteins influence transcription of many genes, including cell cycle kinase inhibitor p21 (WAF1), to control cell cycle progression and proliferation.^{2, 3} Importantly, elevated expression of HDAC proteins is associated with poor prognosis in patients with a variety of cancers, including gastric, ovarian, prostate and multiple myeloma.^{4–6} Overwhelming evidence documents the wide role of HDAC proteins in cell biology and cancer formation.⁷

With their established roles in diseases, HDAC proteins have emerged as therapeutic targets. Four drugs targeting HDAC proteins have been approved by the Food and Drug Administration for treatment of cancer. For example, SAHA (suberoyl anilide hydroxamic acid, Vorinostat, ZolinzaTM) is currently in clinical use for treatment of T-cell lymphoma.^{8, 9} Prior reports document that SAHA augments histone acetylation by inhibiting HDAC activity, which alters histone-mediated transcriptional regulation of critical oncogenes.^{2, 10} The HDAC family comprises eighteen members, including metal-dependent HDAC1 - HDAC11 and NAD⁺-dependent SIRT1- SIRT7.¹¹ The metal-dependent HDAC proteins are sensitive to SAHA and are the focus of this work.

Using large-scale mass spectrometry (MS)-based approaches, thousands of acetylated proteins have been identified.^{12, 13} Acetylation affects protein stability, activity, protein-protein interactions and subcellular localization.¹⁴ With these results, HDAC inhibitors, including SAHA, may be influencing cell biology more globally, beyond histone-mediated epigenetic mechanisms, by deacetylating additional substrates. Unfortunately, the individual substrate profiles of HDAC1–11 remain underexplored. The limited characterization of

HDAC substrates is an obstacle to realizing the full potential of HDAC proteins as therapeutic targets. A substrate profile of HDAC proteins is needed to reveal the non-epigenetic activities of HDAC proteins and HDAC-targeted drugs.

The limited substrate characterization of HDAC proteins is largely due to the lack of simple methods for HDAC substrate identification. In one method, proteomics analysis after treatment with isoform-selective HDAC inhibitors was used to identify possible substrates of HDAC6 and HDAC8.^{15, 16} HDAC6 knockout mice have also been used to identify novel substrates.¹⁷ Most recently, a photocrosslinking unnatural amino acid residue was incorporated into bacterially-expressed HDAC8 near its active site to covalently link potential HDAC8 substrate.¹⁸ Unfortunately, extension of these methods to all HDAC isoforms has not been possible. For example, among the HDAC isoforms, HDAC1 is of particular interest due to its role in multiple cancers, such as breast, prostate, lung and leukemia.⁴ Yet, HDAC1 substrate identification has been hampered by multiple limitations. With genetic methods, HDAC1 knockout mice are embryonic lethal and compensation by HDAC2 makes genetic methods unreliable.^{3, 19} With pharmacological methods, the lack of HDAC1-selective inhibitors hampers mass spectrometry-based methods. New methods are needed to study non-histone substrates to broaden our understanding of HDAC1 function in physiological and pathological conditions, and assist in deciphering the HDAC1 inhibitor mechanism of action.

We recently developed a simple trapping mutant strategy to identify substrates of HDAC1.^{20, 21} This approach utilizes an inactive HDAC1 mutant to trap novel substrates. For example, using the mutant trapping strategy, the mitotic protein Eg5/KIF11 was identified as an HDAC1 substrate, which explains the G2/M arrest observed with SAHA.²¹ In addition, the demethylase LSD1 was identified as an HDAC1 substrate and contributes to gene expression changes observed with SAHA.²⁰ These novel substrates revealed a new function of HDAC1 in mitosis and an epigenetic crosstalk between acetylation and methylation in gene expression. As these studies indicate, substrate trapping has the potential to discover new substrates and related biological roles of HDAC1.

Although successful, one limitation of earlier substrate trapping was the low throughput; only one or two substrates were identified in each study.^{20, 21} More powerful would be the ability to identify multiple substrates in one trapping study. Here, the throughput of the trapping method was enhanced by coupling with label-free quantitative proteomics to analyze the full substrate profile of HDAC1 under a cellular condition. This improved proteomics version of substrate trapping indeed identified multiple substrates, which implicate a role for HDAC1 in myriad processes other than transcription. Based on this pioneering study, the proteomics-based trapping method provides an enabling tool to characterize more fully the role of HDAC1 in normal and disease states.

RESULTS AND DISCUSSION

We recently developed a trapping strategy to identify HDAC1 substrates.^{20, 21} In this strategy, an inactive HDAC1 mutant (C151A) binds to substrates more stably compared to wild type HDAC1 (Figure 1A and 1B). As a consequence, the inactive mutant assists in

capturing HDAC1 substrates for subsequent identification by mass spectrometry. In prior work, wild type and C151A mutant HDAC1 were overexpressed in cells before immunoprecipitation from lysates. As a critical control, the mutant was also immunoprecipitated in the presence of the active site inhibitor, SAHA (suberoyl anilide hydroxamic acid, Vorinostat), which distinguished substrates bound through the active sites from associated proteins (Figure 1C). Proteins immunoprecipitated by wild type and mutant were separated by SDS-PAGE to reveal bands present in mutant but not wild type or inhibitor competed samples (Figure 1D). Then, the bands were excised from the gel for identification by LC-MS/MS (liquid chromatography – tandem mass spectrometry) analysis. One disadvantage of this gel-based trapping method is that only highly abundant proteins that are visible by gel analysis and have dramatic changes in binding comparing wild type and mutant will be identified. The requirement of gel-based analysis reduces the throughput of the method, allowing identification of only a few select substrates. We hypothesized that the throughput of substrate identification using trapping would be improved by avoiding gel-based analysis and, instead, moving directly to LC-MS/MS analysis after immunoprecipitation (Figure 1E). By analyzing the full interactome of wild type, mutant, and inhibitor-competed samples with LC-MS/MS, proteins of lower abundance and modest binding differences would be discovered.

Optimization of Trapping Conditions for Proteomics Analysis

As a first step towards developing a proteomics-based trapping study, we optimized the trapping procedure by incorporating a more stringent washing step after immunoprecipitation. The goal of high stringency washing was to remove low affinity or non-specific binders, which could complicate the higher sensitivity LC-MS/MS analysis. In earlier trapping studies, three quick washes with a high salt (500 mM) buffer were used after immunoprecipitation (Figure 2A).^{20, 21} Here, the stringency of washing was enhanced by incubating the immunoprecipitated sample with the high salt wash buffer for 5 min at 4°C with rocking. With high stringency washing conditions, overall proteins levels were reduced compared to samples prepared with earlier lower stringency washing (Figures 2A and 2B, compare lanes 3).²⁰ Regardless of the washing conditions used, several bands were observed in the immunoprecipitates of the C151A HDAC1 mutant compared to the wild type or HDAC inhibitor SAHA-competed immunoprecipitates (Figure 2A, compare lane 3 to lanes 2 and 4; Figure 2B, compare lane 3 to lanes 2 and 5; see brackets). The higher stringency wash conditions were successful at reducing non-specific protein levels after immunoprecipitation, which will facilitate future LC-MS/MS analysis.

To ensure that known substrates can still bind to HDAC1 under high stringency wash conditions, we analyzed the bound proteins from wild type and C151A mutant immunoprecipitates for the presence of the LSD1 substrate, which was recently identified from HEK293 cells using trapping.²⁰ As expected, the C151A mutant trapped more LSD1 than wild type HDAC1 or SAHA-competed samples even with high stringency wash conditions (Figure 2C, compare lane 3 to lanes 2 and 5). The combined data confirmed that the high stringent washing conditions maintained known HDAC1 substrate interactions while reducing non-specific protein levels, in preparation for proteomics analysis.

Substrate trapping with proteomics analysis

With optimized washing conditions established, trapping studies with proteomics analysis were performed using HEK293 cells. Wild type and C151A mutant HDAC1 were overexpressed before immunoprecipitation from lysates in the absence or presence of the HDAC inhibitor SAHA. As a IgG bead negative control, immunoprecipitation of untransfected lysates was also included. After high stringency washing, bound proteins from each sample were analyzed. First, to assure equal protein expression and immunoprecipitation in each sample, the levels of FLAG-tagged wild type or mutant HDAC1 were assessed by western blotting analysis and showed similar proteins in each sample (Figure S2). Next, bound proteins were identified by LC-MS/MS after gel purification and trypsin digestion. The data after LC-MS/MS was further analyzed by label-free quantitation with MaxQuant software.²² A total of 793 peptide hits were observed in four independent trials after LC-MS/MS and label-free quantitation (Table S1).

To generate a list of candidate substrates, the relative intensities of peptides present in the IgG negative control and C151A mutant samples were divided by the intensities of peptides in the wild type HDAC1 samples to generate a fold enrichment value for each quantified peptide (Table S2). To filter out false positives, hit peptides were removed if present at high levels in the IgG negative control sample due to nonspecific binding or in the SAHA competition sample due to interaction outside of the HDAC1 active site. After filtering out false positives, a low confidence hit list was generated that included peptides present with 1.3-fold enrichment in C151A compared to wild type samples in 2 out of 4 trials, which gave 171 candidate substrates (Table S2). Among these 171 proteins were several known substrates of HDAC1, including Histone H2, RuvB-like 2 (RuvBL2), and LSD1 (Table S2, highlighted rows). LSD1 and RubBL2 were identified in an earlier report using the gel-based trapping approach with HEK293 cells.²⁰ The known histone substrate (H2A) was enriched by >2.4 fold in all four independent trials (Tables S2 and S3), making it high confidence hit. The presence of three known substrates of HDAC1 validates that the proteomics-based trapping approach can be used to identify multiple HDAC substrates simultaneously.

To create a higher confidence hit list, peptides with at least 2.5-fold cut-off in C151A mutant compared to WT immunoprecipitates in at least three out of four trials were identified, giving 101 protein hits (Table S3). The list of 101 candidate substrates included both nuclear and non-nuclear proteins because trapping is performed in lysates. Since HDAC1 is exclusively in the nucleus, we further narrowed the hit list by removing non-nuclear proteins, which reduced the list to 35 proteins (Table S4). We note that known HDAC1 substrates, histone H2A and RuvBL2, are present in this higher confidence list. With these 35 hits, a functional analysis of the candidate substrates was performed to gain a picture of their diversity. DAVID Bioinformatics Resources 6.8 software was used to analyze the hits according to localization, cellular processes, and molecular function^{23, 24}. As expected, all proteins are either exclusively nuclear or shuttle between nucleus and cytoplasm (Figure 3A). Some proteins are present in known complexes in the nucleus, such as the NuA4 histone acetyltransferase, SWR1 chromatin remodeling, histone methylating MLL1, and nuclear lamin complexes (Figure 3A). The enriched substrates are also involved in various

cellular events, such as transcription, histone acetylation, ribosome assembly, telomerase localization, DNA repair, protein folding, and translation (Figure 3B). The candidate hits also displayed various molecular functions, such as helicase activity, or DNA/RNA binding (Figure 3C). These molecular functions are consistent with known non-histone-related activities of HDAC1. For example, the activity of the DNA-binding transcription factor p53 is controlled by HDAC1-mediated deacetylation.²⁵ In addition, the downstream effector of cytokine signal transduction pathway, STAT3, is a DNA binding transcription factor inhibited upon deacetylation by HDAC1, 2 or 3.²⁶ The trapped hit proteins suggest that HDAC1 plays a role in cellular events beyond simply histone deacetylation and epigenetic regulation.

A possible advantage of the trapping proteomic approach is discovery of low abundance HDAC1 substrates. To test this possibility, an abundance analysis was performed with the 35 nuclear protein hits.²⁷ Because the quantified peptides from two hits could correspond to two or more protein isoforms, an additional four isoforms were included in this abundance analysis. The 39 proteins showed abundance levels ranging from 0.11 to 5474 ppm (Figure S3), which is similar to the abundance range for all proteins in HEK293 cells (0.01 to 10,000 ppm). Importantly, ten hits (26%) were of low abundance, with levels below 10 ppm (Figure S3). The abundance analysis indicates that proteins hits were captured without dependence on abundance.

Confirmation of trapping hits

To test the success of the trapping study, we sought to confirm the presence of several candidate substrates identified by LC-MS/MS analysis. From the original low confidence hit list (Table S1), the nuclear protein cyclin-dependent kinase 1 (CDK1) was selected for validation. From the higher confidence list of 35 nuclear proteins (Table S4), RuvB like-1 (RuvBL1) and Apoptosis-inducing factor 1 (AIFM1) were selected for validation. Finally, to create an even higher stringency list, peptides showing >5.0-fold cutoff in all 4 trials were selected (Table 1 and Table S5). Within this highest stringency list, MutS homolog 6 (MSH6) was selected for validation.

To confirm the presence of the four selected substrates, trapping studies were performed with subsequent Western blot analysis to observe the presence of the candidate substrates. HDAC1-Flag (WT) or HDAC1-Flag (C151A) were transfected into the HEK293 cells, followed by treatment with SAHA to induce robust acetylation. The wild type and mutant HDAC proteins were then immunoprecipitated either in the presence or absence of competitor SAHA, with elution subjected to western blot to monitor inhibitor-dependent binding to mutant C151A HDAC1 by potential substrates. The first substrate tested, CDK1, is a member of a family of cyclin dependent kinases, which tightly regulates the cell cycle. CDK1 binds to cyclin B1 to form an active complex that drives progression from G2 to M phase and maintenance of mitotic state.²⁸ CDK1 is phosphorylated at T14 and Y15 near the ATP-binding site and are dephosphorylated upon activation.²⁹ CDK1 is also acetylated on K6, as determined from a large-scale proteome study.¹² However, the biological significance of CDK1 acetylation remains unexplored. HDAC inhibitors arrest the cell cycle at both G1/S and G2/M phases.⁴ Given the association of CDK1 and HDAC proteins in cell cycle, the

activity of CDK1 might be influenced by HDAC1. To confirm that CDK1 is trapped by HDAC1, the trapping experiment was performed, followed by western analysis. CDK1 was immunoprecipitated by mutant HDAC1 to a greater extent compared to WT HDAC1 (Figure 4A and Figure S4B, lane 2 versus lane 3), consistent with mutant trapping. Importantly, binding of CDK1 to mutant HDAC1 was significantly decreased in the presence of the active site competitor, SAHA (Figure 4A and Figure S4B, lanes 3 versus 5), indicating that CDK1 interacts through the active site. These results are consistent with the identification of CDK1 in the LC-MS/MS analysis after trapping.

RuvBL1 is a highly conserved protein belonging to the AAA+ family of ATPase.³⁰ RuvBL1 and RuvBL2 form hexamer that is present in a number of high molecular weight complexes, including TIP60 and INO80 complexes. These complexes play important roles in chromatin remodeling, transcription, DNA repair and apoptosis, consistent with the known activities of HDAC1.^{31, 32} A recent study demonstrated that RuvBL1 is methylated at R205 to induce proper DNA repair through homologous recombination.³³ RuvBL1 is also acetylated at lysine 453,¹² although no study has yet demonstrated its biological significance. Using trapping followed by western analysis, RuvBL1 was reproducibly trapped with mutant HDAC1 to a greater extent than WT HDAC1 (Figure 4B and Figure S4D, lane 2 compared to lanes 3). More importantly, the trapped RuvBL1 was sufficiently competed with addition of SAHA (Figure 4B and Figure S4D, lane 3 compared to lane 5), suggesting that RuvBL1 binds the active site of HDAC1. This western blot analysis after trapping is consistent with the presence of RuvBL1 in the MS analysis after trapping.

AIF1 is a flavoprotein with NADH oxidase activity that usually resides in the mitochondrial intermembrane space.³⁴ However upon apoptosis induction, AIF is proteolyzed at the N terminus to create a short form, which is translocated into the nucleus to cause chromatin condensation and caspase-independent cell death, through sequence-independent DNA binding.^{35, 36} Mutagenesis showed that lysine residues in the C terminus are essential for AIF binding to DNA.³⁷ One particular lysine residue, K255, can undergo ubiquitination, which affects its ability to induce chromatin degradation.³⁸ Since lysine residues can also undergo acetylation, it is intriguing to speculate that lysine acetylation might also influence AIF1 function. Using trapping followed by Western analysis, we consistently observed strong binding of AIF with mutant HDAC1 compared to WT HDAC1 (Figure 4B and S4C, lanes 3 compared to lanes 2), with that signal decreased with addition of SAHA competitor (Figure 4B and S4A, lanes 3 compared to lanes 5). In addition to full-length AIF, we also observed the same pattern with truncated AIF (tAIF) (Figure 4B and S4C (tAIF), lanes 2 compared to lanes 3; lanes 5). Our data suggested that both AIF and tAIF might be substrates of HDAC1.

MSH6, is a component of the DNA mismatch repair (MMR) system, heterodimerizing with MSH2 to form MutS alpha. The MutS complex binds at DNA mismatch site to initiate the DNA repair process^{39, 40} MutS alpha can also bind to trimethylated K36 of histone H3 (H3K36me3) to facilitate MMR. MSH2, the binding partner of MSH6, is acetylated at K73, and HDAC10 might regulate DNA MMR through deacetylation of MSH2.⁴¹ Given the HDAC-mediated acetylation of MSH2, we sought to probe the acetylation status of MSH6. Using trapping followed by western analysis, MSH6 reproducibly bound to mutant HDAC1

to a greater extent than WT HDAC1, (Figure 4A and S4A, lane 3 compared to lane 2). SAHA competition resulted in a reduction of MSH6 binding to the mutant (Figure 4 and S4A, lane 3A compared to lane 5), assuring active site binding. These secondary studies suggest that MSH6 is among the trapped hits in the LC-MS/MS analysis.

Interactome Analysis and Validation studies

While the trapping study is expected to identify substrates, it is possible that proteins observed by trapping could be indirectly bound to HDAC1 through association with a true substrate. In other words, trapping has the potential to identify both substrates and the associated proteins bound to those substrates. A challenge is to distinguish true substrates from associated proteins. As a screen to help discriminate true substrates among the hit list (Table S4), we performed an interactome analysis. The expectation was that known interactions between the candidate substrates in the hit list would reveal proteins that could have been immunoprecipitated in the trapping study as either a substrate or associated proteins.

To perform the interactome analysis, the 39 nuclear proteins, including all possible isoforms (Table S4), were analyzed using the GeneMania application in Cytoscape. The analysis showed that several multi-protein complexes exist among the proteins (Figure 5). For example, the RuvBL1 protein, which was confirmed as a trapping hit, is a direct interactor with HDAC1, but also indirectly interacts with HDAC1 via RuvBL2 and PFDN2. Likewise, AIF1 indirectly binds to HDAC1 via multiple intermediary interactions. Given that the presence of RuvBL-1 and AIFM in the trapping study can be explained by indirect interaction, it is possible that they are associated proteins and not substrates. In contrast, the MSH6 protein, which was also confirmed in the trapping study (Figure 3A), has no known direct or indirect interactions with any other protein in the hit list. Therefore, of the four proteins confirmed, MSH6 is the most likely protein to be a true HDAC1 substrate.

To validate MSH6 as a true HDAC1 substrate, the acetylation level of MSH6 after treatment with HDAC inhibitors was analyzed by Western blot. SAHA and SHI-1:2 were used as a pan and HDAC $\frac{1}{2}$ selective inhibitors, respectively, which should both influence HDAC1-mediated MSH6 deacetylation. As a negative control, the HDAC6-selective inhibitor tubastatin A was used, which should not influence HDAC1-mediated deacetylation. HEK293 cells were treated with SAHA, SHI-1:2, or tubastatin A, as well as DMSO as a negative control, to induce acetylation. Endogenous MSH6 was immunoprecipitated and the immunoprecipitates were analyzed using an acetyl lysine antibody. As expected, the pan HDAC inhibitor SAHA and HDAC $\frac{1}{2}$ -selectivec inhibitor SHI-1:2 enhanced MSH6 acetylation (Figure 6A, compare lane 2 to lanes 3 and 5), confirming that HDAC proteins regulate acetylation of MSH6. Importantly, tubastatin A showed no effect on MSH6 acetylation levels (Figure 6A, compare lane 2 to lane 4), consistent with the hypothesis that MSH6 is an HDAC1 substrate. Quantification confirmed reproducibly elevated MSH6 acetylation with SAHA, but not tubastatin A (Figures 6B). These studies with HDAC inhibitor treatment are consistent with HDAC1-mediated deacetylation of MSH6.

Without a systematic tool to identify and monitor HDAC1 substrates, the full biological function of HDAC1 in both normal and disease states has been elusive. We developed

mutant trapping for HDAC1 to fill in this gap and provide an enabling tool to identify substrates and characterize more fully HDAC1-mediated cell biology. In this work, the trapping strategy was combined with proteomics-based analysis to increase the number of candidate substrates identified in a single study. Using HEK293 cells, multiple candidate substrates were identified. Four proteins were observed in the trapping studies with both LC-MS/MS and immunoblotting analysis. Importantly, one candidate substrate, MSH6, showed HDAC1-mediated deacetylation using HDAC inhibitors. This proteomics-based trapping method provides a systematic tool to identify and monitor multiple substrates simultaneously, which will embolden the community to fully characterize the role of HDAC1 in various normal and disease states.

Profiling of acetylated proteins in various mammalian cell lines has revealed thousands of acetylated sites on cellular proteins.¹² Compared to the acetylomics study, 4 of the 6 high confidence candidate substrates contain acetylation sites (PFDN5, MSH6, LMNB1, and AKAP8). In addition, all four proteins validated in this study were also acetylated (MSH6, AIF1, RuvBL1, and CDK1). In a recent study, a click chemistry-based proteomic strategy was applied to profile acetylated substrates of Gcn5 and p300 proteins in HEK293 cells.⁴² Comparing the Gcn5 and p300 substrates to the enriched proteins in the trapping study, 2 of the 6 high confidence candidate proteins (Table 1 and S5) are observed in both studies, including MSH6. In addition, proteins in the less stringent hit lists, including CDK1, AIF1 and RuvBL1, are also among Gcn5 and p300 substrates. The overlap in the acetylomics and trapping data suggest that the candidate substrates identified by trapping are acetylated and could be potential HDAC1 substrates.

In summary, we introduced an improved version of the trapping method to profile multiple HDAC substrates in one experiment. Substrate identification is an efficient way to broaden the understanding of HDAC function in physiological and pathological conditions. The proteomics-based trapping method can be applied to other HDAC isoforms, given the strict conservation of many amino acids, including C151, among the HDAC family.⁴³ This proteomics-based mutant trapping strategy for HDAC proteins provides a foundation to fully characterize HDAC activities in normal and disease states, which will facilitate development of more effective therapeutics and encourage a complete understanding of HDAC inhibitor mechanism of action.

METHODS

Antibodies and reagents

FLAG antibody (F3165), LSD1 (L4418), and proteomic grade trypsin (T6567) were purchased from Sigma. Sypro ruby total protein stain was obtained from Thermos Fisher. Proteomic grade formic acid (LC6201) was purchased from ProteoChem. Antibodies to MSH6 (A300–023A-T), CDK1 (A303–663A-T), RuvBL1 (A304–716A-T) and AIF1 (A302–783A-T) were purchased from Bethyl Laboratories. The antibody against acetyllysine (S9441) was obtained from Cell Signaling Technologies.

Cell culture, plasmids and transient transfections

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life technologies) supplemented with 10% Fetal bovine serum (Life technologies-FBS) and 1% antibiotic/anti-mycotic (Gibco) at 37°C in a 5% CO₂ environment. HDAC1 single point mutants were generated using pBJ5HDAC1-FLAG expression plasmid in prior work.⁴³ Transfection of WT or C151A expression plasmids (5 µg) into HEK293 cells (20 X 10⁶) was performed using jetprime reagent (VWR) according to manufacturer instructions. After 48h of recovery after transfection, cells were treated with SAHA (10 µM in growth medium containing <2% DMSO) for another 24h to induce robust acetylation, before harvesting, washing with PBS (1 mL) twice, and either storage at -80 °C or use immediately.

Optimization of the Substrate trapping procedure

Substrate trapping was performed as previously described with minor changes.²¹ Cells were either untransfected or transfected with WT or C151A expression plasmids, as described above. Following transfection, cells (20 × 10⁶) were lysed using lysis buffer (500 µL; 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% triton-X-100) containing 1X protease inhibitor (GenDepot) for 30 min at 4°C with rocking. Then, cell debris were removed by centrifugation at 13.2×10³ rpm for 10 min at 4 °C. Anti-FLAG agarose beads (20 µL bead slurry; Sigma) were washed twice with 1X Tris Buffered Saline (TBS, 500 µL; 20 mM Tris-Cl at pH 8.0, 150 mM NaCl) at 4°C prior to immunoprecipitation. Lysates from untransfected or wild type or C151A HDAC1-Flag transfected cells (2.5 mg of total protein) were mixed with pre-washed FLAG beads and rocked overnight at 4°C. For SAHA competition samples, active site inhibitor SAHA (100 µM in <2% DMSO) was included in the immunoprecipitation mixture. After immunoprecipitation, beads were washed three times with trapping buffer (1 mL; 50 mM Tris-Cl pH 8.0, 500 mM NaCl, 10% glycerol, 0.5% triton-X-100). In the old washing procedure, the trapping buffer was added and immediately removed by spinning at 5000 rcf for 1 min at 4 °C to collect the beads. In the optimized washing procedure, samples were rocked for 5 min with the trapping buffer before spinning to collect the beads. Following washing, bound proteins were eluted by boiling for 5 min at 95 °C in 2X SDS loading dye (30 µL; 50 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 0.004% bromophenol blue). β-mercaptoethanol (10% v/v) was added to the samples, and the samples were boiled at 95 °C for 2 min to denature for gel separation. Proteins were separated by 10% SDS-PAGE and visualized by Sypro Ruby total protein stain according to manufacturer's instructions (Molecule Probes) or transferred to a PVDF (Immobilon P, Millipore) membrane and probed with FLAG or an LSD1 antibodies.

Substrate trapping for LC-MS/MS analysis

Trapping for LC-MS/MS analysis was performed as described above with lysates (2.5 mg total protein) containing no protein (untransfected), wild type HDAC1-Flag, or C151A mutant HDAC1-Flag. SAHA competition was performed with lysates expressing the C151A HDAC-Flag by including SAHA (100 µM in lysis buffer) in the immunoprecipitation mixture. The optimized washing conditions described above were used. After elution and denaturation as described, proteins were minimally purified by 10% SDS-PAGE in preparation for LC-MS/MS analysis. In this case, electrophoresis for only 10 min at 100

Volts allowed proteins to enter only the top 1 cm of the separating layer of the gel. Each gel lane (1 cm × 2 cm) was cut into 1 mm small pieces before rehydration and dehydration as described²¹, except with 250 µL volume in each step per sample. Reduction, alkylation, trypsin digestion and extraction were also performed as described previously.²¹

MS and data analysis

Mass spectrometry was performed as previously described.²¹ Briefly, dried peptide digests were resuspended in a solution of 5% acetonitrile, 0.1% formic acid and 0.005% trifluoroacetic acid. Samples were separated by ultra high pressure reverse phase chromatography using an Acclaim PepMap RSLC column and an Easy nLC 1000 UHPLC system (Thermo). Peptides were analyzed with a Fusion mass spectrometer (Thermo) with a 120,000 resolution orbitrap MS1 scan over 375–1600 m/z, followed by iontrap resolution MS2 scans using a 1.6 m/z window and 30% normalized collision energy for HCD. Peak lists were generated with Proteome Discoverer (ver 1.4; Thermo), and peptides scored using Mascot (ver 2.4; Matrix Science) and Sequest (ver 2.1). The search parameters included parent and fragment ion tolerances of 10 ppm and 0.6 Da, respectively, fixed modification of +57 on C (carbamidomethylation), variable modifications of +16 on M (oxidation), +42 on K and N-termini (acetylation), +1 on NQ (deamidation), and a tryptic digest with up to 2 missed cleavages. MS2 spectra were searched against a consensus human protein database from Uniprot (downloaded on 2016-04-07, 20,145 entries), and simultaneously against a scrambled database to calculate the false discovery rate (FDR). Protein identification was considered to be positive if at least 1 unique peptide was scored as 1% FDR, and the protein threshold was 1% FDR.

Label free quantitation was performed using Maxquant software.²² The intensities of peptide signals found in the wild type samples were set to 1.0. Fold changes in intensities were calculated by dividing the intensity of each peptide in IgG control, C151A mutant without SAHA, and C151A mutant with SAHA by the intensity of that peptide in the wild type sample. To filter out false positives due to nonspecific binding to the IgG beads during immunoprecipitation, proteins were removed if peptide intensities in the IgG control was higher than that in the wild type HDAC1 immunoprecipitate (>1.0-fold enrichment value). To filter out false positives that bind to HDAC1 outside of the active site, proteins were removed if peptide intensities in the C151A mutant immunoprecipitates in the presence of the active site competitor SAHA were equal or higher (>1.0-fold enrichment value) than in the absence of SAHA. After filtering for false positives, candidate substrates were selected if peptides showed at least 1.3-fold enrichment in the C151A mutant (no SAHA) compared wild type in at least two out of four trials (Table S1). As a more stringent analysis, peptides enriched in C151A mutant with >2.5 fold cutoff in 3 out of 4 trials are listed in Table S2. These more stringent hits were then filtered down to 45 hits by removing non-nuclear proteins manually (Table S3). As even higher stringency lists, peptides with >4-fold in C151A mutant in at least 3 out of 4 trials are shown in Table S4, and peptides with >5-fold enrichment in C151A mutant in all 4 trials are shown in Tables 1 and S5.

Functional annotation of hits in Table S3 were performed using DAVID Bioinformatics Resources 6.8 software (<https://david.ncifcrf.gov>).^{23, 24} The abundance of the protein hits

were analyzed using paxdb database.²⁷ The known physical protein-protein interactions among the trapped hits (Table S3) were mapped using the GeneMANIA application in Cytoscape 3.3.0.⁴⁴ Hits were then manually colored in the Cytoscape map according to confidence level (Tables S3–S5).

Substrate trapping for immunoblotting confirmation

Trapping was performed as described above. After elution from the beads and denaturation with SDS loading dye as described above, proteins were separated by 10% SDS-PAGE, transferred to a PVDF (Immobilon P, Millipore) membrane and probed with FLAG antibody to detect HDAC1 protein levels or antibody specific for each individual candidate substrate protein for validation.

Validation using HDAC inhibitor treatment

HEK293 cells were grown for 48 h in growth media containing DMEM, 10% FBS and 1% antibiotic to 70% confluency. Then, the cells were treated with DMSO (0.2 %), SAHA (10 μ M), tubastatin A (TubA, 10 μ M) and SHI-1:2 (10 μ M or 20 μ M) for 24 h. Cells were harvested, washed with PBS (1 mL) twice, and lysed in lysis buffer containing protease inhibitors, as described above, at 4 °C for 40 min with rocking followed by centrifugation at 13.2×10^3 rpm for 10 min at 4 °C. The supernatant was collected and endogenous proteins were immunoprecipitated using specific antibodies by incubating at 4 °C overnight with rotation. Then the proteins were immunoprecipitated using the prewashed agarose beads by again incubating at 4 °C for 3 h. After immunoprecipitation, beads were washed three times with lysis buffer (1 mL), and bound proteins were eluted using 2 $\times$ SDS loading dye (35 μ L), separated by 10% SDS-PAGE, transferred to a PVDF membrane (Immobilon P), and immunoblotted with acetyllysine and MSH6 antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

HDAC	histone deacetylase
HAT	histone acetyltransferase
SAHA	suberoyl anilide hydroxamic acid
LC-MS/MS	liquid chromatography-tandem mass spectrometry
SDS-PAGE	sodium dodecylsulfate- polyacrylamide gel electrophoresis

CDK1	cyclin-dependent kinase 1
RuvBL1	RuvB like-1
AIFM1	Apoptosis-inducing factor 1
MSH6	MutS homolog 6

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Figure 1. Proteomics-based substrate trapping.

A) With substrate trapping, wild type (WT) HDAC1 binds to substrates (purple triangle) transiently due to deacetylation and release. B) In contrast, inactive C151A mutant HDAC1 binds to the substrates more stably without the ability to deacetylate. C) Substrates bound to the active site of the C151A mutant will be competed away in the presence of an active site inhibitor, such as SAHA (red triangle). D) In prior work, immunoprecipitated proteins were separated by SDS-PAGE, with proteins observed in mutant but not WT or inhibitor-competed lanes excised from the gel and analysed by LC-MS/MS to identify candidate substrates. E) In this work, immunoprecipitates from WT, mutant, and inhibitor-competed samples were directly analyzed by LC-MS/MS with label-free quantitation to identify multiple candidate substrates in a single study.

Figure 2. HDAC1 substrate trapping with low and high stringency wash conditions.

Wild type and C151A mutant HDAC1 were expressed as Flag-tagged proteins in HEK293 cells. After 48h, cells were treated with SAHA for another 24h to induce robust acetylation. WT and C151A mutant HDAC1 were immunoprecipitated in the absence or presence of SAHA and either A) low stringency or B) high stringency washing conditions were used after immunoprecipitation. After washing, bound proteins were eluted, separated by SDS-PAGE, and visualized with Sypro Ruby stain to visualize all proteins. Western blotting was also used with FLAG antibodies. Arrows indicate the location of HDAC1, while brackets indicate the presence of candidate substrates in mutant but not wild type or SAHA-competed samples. C) Western blotting was also used with LSD1 antibody to show substrate trapping. Repetitive trials of are shown in Figure S1.

Figure 3. Functional classification of candidate substrates.

The 35 nuclear protein identified from substrate trapping (Table S4) were classified according to cellular compartment (A), cellular processes (B), and molecular function (C) using DAVID Bioinformatics Resources 6.8 software (<https://david.ncifcrf.gov>).^{23, 24}

Figure 4. Confirmation of Trapping Hits using Western Blot analysis.

HEK293 cells were transfected with HDAC1-Flag (lanes 2 and 4) or C151A HDAC1 mutant-Flag (lanes 3 and 5). After 48h, cells were treated with SAHA for another 24h to induce robust acetylation and then subjected to immunoprecipitation (IP) using anti-FLAG agarose beads, followed by immunoblot (IB) analysis of bound MSH6, CDK1, RuvBL1 and AIF1 proteins. As controls, anti-FLAG beads were incubated with lysates without overexpression of wild type or mutant HDAC1-Flag (IgG, lane 1), and untransfected lysates were loaded as a migration standard (lysates, lane 6). As an input control, lysates from each condition were probed with individual antibodies to demonstrate equal level of protein expressed and loaded. Repetitive trials are shown in Figure S4.

Figure 5. Interactome map of the hit proteins.

Known physical protein-protein interactions among 38 trapped hits (Table S4) were analyzed using GeneMANIA in Cytoscape. UBC was omitted due to its global association with almost all other proteins, which complicated the analysis. Colored shapes represent proteins in the lower (blue, 2.5-fold enrichment, Table S4) and higher (red, 5-fold enrichment, Table S5) stringency hit lists (Tables S3–S5). Proteins with a diamond shape were tested in confirmation or validation studies. Lines between proteins indicate a known physical interaction, with the thickness indicating the confidence in that interaction. Proteins encircling HDAC1 (black lines) are known direct interactors, whereas the other proteins bind HDAC1 indirectly through one (red lines), two (blue lines), three (purple lines), or four or more (green lines) associated proteins. The remaining proteins (right) show no known interactions. An enlarged version of this image is available as Figure S5.

Figure 6. Validation of HDAC1-dependent MSH6 deacetylation.

A) HEK293 cells were treated with DMSO, SAHA (10 μ M), tubastatin A (TubA, 10 μ M), or SHI-1:2 (10 μ M) for 24 hr. After cell harvesting and lysis, endogenous MSH6 was immunoprecipitated (IP) with an MSH6 antibody and Protein A/G beads. Immunoprecipitates were analyzed by immunoblotting (IB) with acetyl lysine (AcK) and MSH6 antibodies. B) Quantification of the Ac-Lys western blot from part A. Mean and standard error of three independent trials are shown (Figure S6). * p <0.05

Table 1-Highest stringency hit list from HDAC1 substrate trapping^a

Protein ID	Protein name	Gene name	Localization ^b
Q8N4J0	UPF0586 protein C9orf41	C9orf41	N/C
O75190	DnaJ homolog subfamily B, member 6	DNAJB6	N
Q99471	Prefoldin subunit 5	PFDN5	N/C
P52701	DNA mismatch repair protein Msh6	MSH6	N
P20700	Lamin-B1	LMNB1	N
O43823	A-kinase anchor protein 8	AKAP8	N/C

^aThe six proteins observed in C151A mutant immunoprecipitates with at least 5-fold enrichment compared to wild type immunoprecipitates in all four independent trials (Table S4).

^bN-Nucleus, C-cytoplasm.