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Screening for Therapeutic Targets of Vorinostat by SILAC-based Proteomic Analysis in Human Breast Cancer Cells

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Abstract

Histone deacetylases (HDACs) play critical roles in silencing tumor suppressor genes. HDAC inhibitors reactivate tumor suppressor genes and inhibit tumor cell growth in vitro and in vivo, and several HDAC inhibitors are currently being evaluated in clinical trials for cancer therapy. A comprehensive analysis of proteins regulated by HDAC inhibitors would enhance our ability to define and characterize their essential therapeutic targets. Here, we employed SILAC (stable isotope labeling with amino acids in cell culture)-based quantitative proteomics to identify acetylated proteins in human breast cancer cells. Treatment with the clinically relevant HDAC inhibitor, SAHA (vorinostat), induces lysine acetylation of 61 proteins in MDA-MB-231 human breast cancer cells. SAHA not only induces lysine acetylation in chromatin-associated proteins, but also acetylates previously unrecognized non-histone proteins, including transcriptional factors and regulators, chaperones, cell structure proteins, and glycolytic enzymes in a time-dependent manner. Knowledge of the full repertoire of acetylated proteins will provide a foundation for further defining the functions of HDACs in cancer cells.

Introduction

Lysine acetylation in histones is a key mediator of gene transcription. Acetylated histones usually create transcriptionally active euchromatin, whereas deacetylated histones dictate transcriptionally silent heterochromatin [1]. This reversible process is regulated by the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Three major classes of mammalian HDACs have been extensively described. Class I and II HDACs require zinc to hydrolyze the acetyl-lysine amide bond, which is a critical step for enzyme activity. Class III HDAC enzymatic activity depends on the cofactor nicotinamide adenosine dinucleotide (NAD⁺). In general, class III HDAC activity is not affected by class I and II HDAC inhibitors [1,2]. Recent advances have shown that cancer development is intimately associated with aberrant HDAC expression. It has been recognized that dysregulation of HDAC activity can silence tumor suppressor gene transcription leading to

increased likelihood of neoplastic initiation and progression [3–5]. Given the important role of HDACs in tumorigenesis, a number of class I and II HDAC inhibitors have been developed, and studies have demonstrated that inhibition of HDAC activity leads to hyperacetylation of histones followed by transcriptional activation of certain tumor suppressor genes through reorganization of transcriptionally silent heterochromatin [1]. In various transformed tumor cells, HDAC inhibitors induce cell cycle arrest, cell differentiation and/or apoptotic cell death [2]. Since HDAC inhibitors possess potent antitumor activity with tolerable side effects, several HDAC inhibitors are currently being evaluated in the clinic [2,6]. For example, LBH589, a cinnamyl hydroxamic acid derivative, inhibits class I and II HDAC activities and is in phase II clinical trials. SAHA (vorinostat), a second generation hydroxamate HDAC inhibitor, has entered the clinic for treatment of refractory cutaneous T cell lymphoma.

In addition to the well-documented effects of HDAC inhibitors on epigenetic gene regulation, the scope of lysine acetylation extends to multiple regulatory proteins and diverse signaling pathways [1,2]. Recently, global analyses of lysine acetylation in response to HDAC inhibitors have been initiated, but only a few studies have been reported [7–9]. By using 2-dimensional electrophoresis followed by peptide-mass fingerprinting, Iwabata et al. described organ-specific distribution of acetylated proteins in mice [7]. More recently, immunoaffinity purification of acetylated peptides followed by nano-HPLC/MS/MS has been used to profile acetylated proteins in HeLa cells and mouse liver mitochondria [8]. High-resolution mass spectrometry was used to quantify acetylation changes in response to treatment with HDAC inhibitors SAHA and MS-275 [9]. However, dynamic alteration of lysine acetylation profiles in response to HDAC inhibitors has not yet been reported. Thus, development of a novel method of analysis applied to a cancer cell culture model would greatly enhance our ability to define and characterize the essential therapeutic targets of HDAC inhibitors. We used a SILAC (stable isotope labeling with amino acids in cell culture)-based quantitative proteomic strategy to achieve this goal.

The SILAC-based quantitative proteomic method utilizes stable isotopes to label cellular proteins. We have successfully applied SILAC to the study of tyrosine phosphorylation [10,11]. Herein, we describe an analysis of the acetyl-lysine proteome to identify and quantify dynamic alterations of lysine acetylation in SAHA-treated breast cancer cells. Our results demonstrate that this approach is a powerful method to identify HDAC targets. Data generated from this study will facilitate the future characterization of the biological significance of lysine acetylation in cancer cells.

Materials and Methods

Cell culture and SILAC-labeling

MDA-MB-231 breast cancer cells were grown in Dulbecco's Modified Eagle media (DMEM, Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS, HyClone, Logan, UT) and 2 mM L-glutamine at 37°C in a humidified incubator with 5% CO₂-95% air atmosphere. The cells grown in natural arginine and lysine containing DMEM were used as a control (normal Arg/Lys). For SILAC labeling, arginine- and lysine-free DMEM were obtained from Invitrogen. The Arg/Lys-free DMEM was supplemented with light (¹³C₆ Arg/D4 Lys; +6 Da and +4 Da shift for labeled arginine- and lysine-containing peptides, respectively), and heavy (¹³C₆ ¹⁵N₄ Arg/¹³C₆ ¹⁵N₂ Lys; +10 Da and +8 Da shift for labeled arginine and lysine-containing peptides, respectively) peptides. The cells were adapted for at least five generations before use in experiments. Detailed SILAC methods used were as described in our previous studies [12].

Lysine acetylation immunoprecipitation

5×10^7 cells from each “state” were treated with 10 μ M SAHA for 24 and 48 h. The cells were lysed in a lysis buffer (10 mM Tris pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, and protease inhibitor mixture tablets (Roche)), briefly sonicated, and then collected by centrifugation. Protein concentrations were measured by the Lowry method (BioRad), and equal amounts of protein from lysates of each state were mixed together. Lysine acetylation immunoprecipitation was performed using anti-acetylated antibodies (ImmuneChem, Burnaby British Columbia, Canada) coupled to protein A agarose at 4°C for overnight with constant mixing. The beads were then washed three times in 1 ml cell lysis buffer followed by boiling with SDS-PAGE sample buffer, and the proteins were stained using colloidal Coomassie stain before gel digestion.

In-Gel digestion and LC-MS/MS analysis

The gel bands were excised and subjected to in-gel trypsin digestion. Extracted peptides were dried down using speedvac, reconstituted in 10 μ l 1% acetic acid and 3% acetonitrile, and analyzed by LC-MS/MS using a reversed phase-LC interfaced with a quadrupole time of flight mass spectrometer (Micromass Q-TOF API-US mass spectrometer). The reversed phase-LC system consisted of trap column (75 μ m $\times$ 3 cm, C18 material 5–10 μ m, 120Å, LC packing) and analytical column (75 μ m $\times$ 10 cm, C18 material 5 μ m, 120Å, LC packing). The peptides were separated using an organic solvent gradient (0–60%) containing 0.1% formic acid and acetonitrile. The MS spectra were acquired in a data dependent manner targeting the four most abundant ions in the survey scan and excluded for MS/MS scan for 60s. Peptide and protein identification was done using the Mascot algorithm (version 2.1.04) to search spectra against the RefSeq (release 22) human protein database. The tolerance used for peptide ions was 0.2 Da and for fragment ions was 0.3 Da. Peptides with a minimum Mascot score of 25 were manually validated and used for generating the list of identified proteins. Relative abundance of proteins was calculated using MSQuant software (version 1.4.3a39) downloaded from <http://msquant.sourceforge.net> [13]. The fold changes were expressed as mean values. Mass spectral data were processed as individual peak list files for every MS/MS spectra and subsequently merged using a Perl script to retain the scan number. The resulting mascot readable file was used for mascot search. The mascot search results were parsed with LC-MS/MS instrument data file using MSQuant. The new_MSQ_quantitationModes.xml files were modified to read the mascot output files and identify the correct light, medium and heavy isotopic MS peaks. The quantitation data was also verified by manual inspection of heavy- and light-peptide-derived MS and MS/MS spectra in MSQuant.

Confirmation of acetylated proteins identified from proteomic analysis

Cells were grown in normal DMEM medium as described above. After SAHA treatment, whole cell lysates were prepared by lysing the cells with 1% SDS and 10 mM Tris-HCl (pH 7.4). Immunoprecipitations were performed with clear supernatant using antibodies to EF-1 α , Enolase-1, 14-3-3 γ , helicase-like transcription factor (HLTF), and Hsp90) from Santa Cruz Biotechnology (Santa Cruze, CA). The anti-acetylated lysine antibody (ImmuneChem) was used for immunoblotting for detection of lysine acetylation.

Results

SILAC-labeled MDA-MB-231 Cells

Cells were split into three sets and cultured in the presence of DMEM media containing 1) natural L-arginine and L-lysine, 2) light ¹³C₆-L-arginine and ²H₄-L-lysine or 3) heavy ¹³C₆ ¹⁵N₄-L-arginine and ¹³C₆ ¹⁵N₂-L-lysine respectively. The natural amino acid

containing cells were treated with vehicle (DMSO) as a control while light and heavy SILAC-labeled cells were treated with 10 μ M SAHA for 24 and 48 h, respectively. We have previously shown that labeled cells showed no change in biological phenotype as compared with the non-labeled cells [12]. To confirm this conclusion in breast cancer cells, Western blot analysis was used to examine candidate proteins known to be expressed in MDA-MB-231 cells. For example, the labeled and non-labeled cells show similar basal DNMT1 levels (Fig. 1A) and treatment with SAHA has equivalent inhibitory effects on levels of DNMT1 protein in both labeled and non-labeled amino acids. To evaluate effect of HDAC inhibitors on lysine acetylation, acetylated proteins were separated by SDS-PAGE, transferred to a membrane, and detected by using anti-acetylysine antibodies. Fig. 1B shows that treatment of MDA-MB-231 cells with HDAC inhibitors, SAHA and LBH589, leads to modifications in lysine acetylation in numerous proteins by 24 h.

Identification of acetylated proteins

To perform SILAC-based quantitative proteomic analysis, equal amounts of cell lysates from control, 24 h SAHA-treated and 48 h SAHA-treated samples were combined, and acetylated proteins were immunoprecipitated using anti-acetylysine antibody (Fig. 1C). The immunoprecipitation complexes were separated by SDS-PAGE, and visualized by Coomassie Blue staining (Fig. 2, left panel). In parallel, the same immunoprecipitated proteins were immunoblotted, and acetylated proteins were detected using anti-lysine acetylation antibodies. Fig. 2 (right panel) shows that the antibody can detect multiple lysine acetylation targets ranging from 11 kDa to 200 kDa, suggesting that the acetylated proteins can be effectively immunoprecipitated. To proceed to MS/MS analysis, the gel bands were excised and digested with trypsin before LC-MS/MS. Peptide sequences were obtained from the MS/MS spectra, and the MASCOT search algorithm was performed to identify peptide candidates from the human protein database. In the MS spectra, second state labeled Lys ($^2\text{H}_4\text{-Lys}$) and Arg ($^{13}\text{C}_6\text{-Arg}$) show 4- and 6-Da shifts, respectively, while heavy labeled Lys ($^{13}\text{C}_6\text{ }^{15}\text{N}_2\text{-Lys}$) and Arg ($^{13}\text{C}_6\text{ }^{15}\text{N}_4\text{-Arg}$) represent 8- and 10-Da shifts, respectively. The ratio between the intensity of the labeled (light or heavy) peptide peak divided by the intensity of the unlabeled peptide peak is used for quantification of lysine acetylation. Fig. 3 shows representative MS/MS spectra of peptides derived from enolase-1, Hsp90, EF-1 α , and 14-3-3- γ . As an example, an enolase-1 peptide containing TIAPALVSK showed a 2.5-fold difference in the intensity of the light Lys peaks and a 5.4-fold difference in the intensity of the heavy Lys peaks as compared with non-labeling peptides (control sample), indicating that lysine-acetylation in enolase-1 is elevated 2.5- and 5.4-fold after 24 and 48 h of treatment, respectively. Using a predetermined cutoff of 1.5-fold, we identified 61 proteins whose lysine acetylation was increased after 24 h and/or 48 h of SAHA treatment (Table 1). A literature search showed that 14 of these proteins have been previously reported to be acetylated proteins, whilst the remaining 47 proteins are newly described to be acetylated in human breast cancer cells.

SAHA induction of lysine acetylation occurs in a time-dependent manner

Lysine acetylation is a reversible process, and dynamic alterations of lysine acetylation are likely to contribute to differences in gene transcription and biological outcomes. Our study design afforded us the opportunity to monitor alterations in the profile of lysine acetylation at two time points during SAHA treatment of MDA-MB-231 cells. As shown in Table 1, only six proteins are acetylated after 24 h of SAHA treatment, principally histones (H2 and H3 family). We found that 19 proteins are persistently acetylated after both 24 and 48 h of SAHA treatment. These include acetylated histones (H1, H4), chaperones (Hsp70 and Hsp90), and transcription factors/regulators (translational elongation factors, 14-3-3 and sequestosome 1). We also observed that the majority of proteins (n=36) had unaltered lysine acetylation at 24 h, and showed increases in acetylation after 48 h of SAHA treatment. Many

of these proteins are involved in cell structure maintenance, chromosome and transcription regulation. As previous reports indicate that SAHA reactivates silent tumor suppressor genes, and induces cell apoptosis by 48 h in vitro [14,15], this therapeutic effect is correlated with the prolonged increase in acetylated histones, transcription factors/regulators, and cell structure maintenance proteins, suggesting that sustained lysine acetylation contributes to the pharmacological effects of SAHA over time.

Verification of SILAC-based proteomic analysis

To validate our findings, selected candidate proteins were immunoprecipitated, and their lysine acetylation status was examined by immunoblotting. As shown in Fig. 4, this independent analysis confirmed the MS/MS results in non-SILAC labeled cells. For example, we confirmed that EF-1 α , Hsp90, 14-3-3- γ , and enolase-1, were hyperacetylated at 24 and 48 h in SAHA treated cells. We also confirmed that lysine acetylation in HLTf (helicase-like transcription factor) was markedly increased at 24 h of SAHA treatment, whereas total HLTf protein levels remained unchanged. Collectively, these data confirm the accuracy of the mass spectrometric method for identification of acetylated proteins.

Discussion

Prolonged SAHA treatment can lead to a significant accumulation of lysine acetylated proteins

Given the extensive roles for HDACs in gene transcription, DNA repair and signal transduction during neoplastic transformation, a global screening of lysine acetylation is essential for understanding the molecular mechanisms of HDAC inhibitors in cancer therapy. Recently, several studies profiled lysine acetylation in human tumor cells and animal tissues. Iwabata et al. generated anti-acetylysine mouse monoclonal antibodies and identified organ specific distribution of acetylated proteins in mice. They found that α -enolase, NF-L, Hsc70, α -tubulin, and β -actin are specifically acetylated in mouse brain, whereas α -actin and creatine kinase can be lysine-acetylated in mouse skeletal muscle [7]. Kim et al demonstrated that treatment of HeLa cells with a combination of class I, II and III HDAC inhibitors (TSA and sirtinol) for 24 h led to identification of 75 acetylated proteins (37 in the cytosol and 38 in the nucleus) [8]. Inhibition of HDAC induces acetylated proteins in mouse liver mitochondrial protein, suggesting that HDACs play key roles in regulation of activities of metabolic enzymes [8]. Most recently, Mattias Mann's group found that lysine acetylation not only alters chromatin remodeling and nuclear transcription, but also regulates cytoplasmic protein-protein interaction. For example, acetylated 14-3-3 disrupts interaction of 14-3-3 with cyclin dependent kinase Cdc28 [9]. Although these studies identify a broad scope of lysine acetylation, the dynamic alterations of lysine in response to clinically relevant HDAC inhibitors in cancer cells have not been investigated.

We observed 36 proteins that were lysine acetylated only after 48 h of SAHA treatment, which include those controlling cell structures (tubulin) as well as chaperone proteins. We also found that SAHA treatment led to persistent lysine acetylation of 19 proteins after 24 and 48 h. Thus, SAHA treatment enhances lysine acetylation in time-dependent manner that is distinct for different sets of proteins. This is consistent with the clinical observation that an increase in acetylated histones can be maintained over a longer period of SAHA treatment coincident with antitumor activity of SAHA that can be achieved over time [16]. The time dependent acetylation may be important in regulation of protein functions. It has been known that HDAC4 and HDAC5 interact with ankyrin-repeat domain protein (ANKRA), the Aryl hydrocarbon receptor repressor (AhRR) binding protein, leading to inhibition of AhRR activity [17]. The ANKRA inhibitory effect can be reversed by treatment with HDAC inhibitors [17]. We identified that SAHA induces acetylation of

ANKRA at 24 h but not 48 h, suggesting that early lysine acetylation may be important in inhibition of ANKRA biological function. ATP-dependent chaperone Hsp90 α interacts with multiple oncogenic proteins to promote tumor cell proliferation and invasion [18–20]. We observed that Hsp90 is persistently acetylated for up to 48 h. The prolonged acetylation inhibits ATP-dependent Hsp90 chaperone function leading to degradation of DNMT1, a key oncogenic protein in maintaining DNA methylation [21]. Furthermore, we showed that acetylation of mitochondrial translational release factor 1-like (MTRF1L) only occurs after 48 h of SAHA treatment. MTRF1L is an essential mammalian mitochondrial factor involved in the initiation and elongation phases of translation, and this delayed post transcriptional modification implicates a role of HDACs in the translational termination process in mammalian mitochondria.

Previously unrecognized lysine acetylation targets have been identified by using SILAC-based quantitative proteomic analysis

Our study has shown that a therapeutically useful HDAC inhibitor, SAHA, induces lysine acetylation of 61 proteins in MDA-MB-231 human breast cancer cells. These findings suggest that lysine acetylation in histone and nonhistone proteins contribute to the actions of HDAC inhibitors. Among these, it is noteworthy that Hsp70, Hsp90 (α and β), tubulin (α and β), and triphosphatase isomerase 1 have been shown to be lysine acetylated using the SILAC methodology. Identification of these proteins through our proteomic analysis validates our proteomic approach since these proteins are known to be targets from previous proteomic assays [7–9]. Most importantly, we identified previously unrecognized HDAC targets, including chromatin-associated proteins, transcriptional factors, transcriptional regulators, chaperone proteins, structural proteins, cell cycle regulators and tumor antigen. For example, of the acetylated chaperones, we identified lysine acetylation on chaperone proteins including chaperonin containing TCP1 (subunit 3 isoform c) and three subunits of Hsp70 family. Given the critical role of acetylated heat shock proteins in the regulation of oncogenic protein stability and tumor cell invasion [21–24], identification of these members of the chaperone protein family will enhance our ability to further characterize their roles in signal transduction and protein stability. Additionally, several novel modifications of chromosome associated proteins were also identified. These include structural maintenance of chromosomes 3, centrosomal proteins (170 and 290 kDa), and isoform 2 of minichromosome maintenance protein 8. Data generated from our study could greatly advance our understanding of the clinical consequences of SAHA treatment on different cellular pathways.

SAHA induces lysine acetylation in diverse cellular processes

Our data demonstrated that lysine acetylation can occur in several enzymes involved in glucose metabolism, including dihydrolipoamide *S*-succinyltransferase, enolase-1, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase A, and PK3 (pyruvate kinase 3). Lysine acetylation is known to affect these enzyme activities. For example acetylation of glyceraldehyde 3-phosphate dehydrogenase can decrease the key reaction in glycolysis in some types of cancer cells [25]. Other studies have reported that a combination of class I, II and III HDAC inhibitors can target cytosolic metabolic enzymes such as enolase 1 [7,8] and our studies add strong evidence that glycolytic metabolism is connected to class I and II HDACs. It is conceivable that increased lysine acetylation may alter activities of key metabolic enzymes and affect energy homeostasis in response to nutrients in mammals.

Dynamic modification of microfilaments by HDACs has been shown to increase cell motility [26]. Our results identified the previously recognized HDAC targets, tubulin (α and β), and actin [8,27,28]. We also identified previously unrecognized HDAC targets involved

in signal transduction. One representative class is the Rho-specific guanine nucleotide exchange factors (RhoGEFs), which can activate Rho family GTPases, and is required for tumor cell transformation [29]. Mutant or acetylated Rho family proteins may impede their function, and alter cytoskeleton dynamics in transformed cells [8,29]. HDAC inhibition also targeted annexin A2, a member of the annexin family of Ca⁺⁺-dependent phospholipid and membrane binding. As annexin A2 functions as a substrate for protein kinases and mediates cell proliferation, differentiation, inflammation and cytoskeletal interactions [30,31], its acetylation may represent a novel mechanism for regulation of its function.

Transcription factors and regulators are the most prominent proteins modulated by lysine acetylation. The nuclear receptor ER β was acetylated in the ER α negative human breast cancer cell line MDA-MB-231. ER β belongs to ligand-regulated transcription factors, and can form functional homo- or heterodimers with ER α that either enhance or antagonize estrogen response in ER positive tissues [32]. Understanding that ER β is an acetylation target may help further determine its role in breast cancer.

14-3-3 Proteins belong to a family of conserved regulatory molecules which can bind to HDAC 4 and 5 and inhibit HDAC activity in the cytoplasm [33]. 14-3-3 isoforms (γ and ζ) are known to be expressed in breast cancer cells, and they bind to the tumor suppressor, tuberlin, and negatively regulate AKT signaling [34,35]. Aside from this, very little is known regarding the post-transcriptional modification of 14-3-3 in human cancer cells and our studies show that 14-3-3 is acetylated in SAHA treated cells.

In summary, our data provide for the first time a comprehensive assessment of alterations in the lysine proteomic profile in MDA-MB-231 cancer cells treated with an FDA-approved HDAC inhibitor, SAHA. This analysis shows that SAHA targets proteins that function in multiple histone and non-histone-based cellular pathways. They include previously unrecognized lysine acetylation targets in the transcription factors/regulators, chromatin structure, cytoskeletal proteins, cell cycle regulators and tumor antigen families. Our studies also suggest that prolonged treatment with SAHA can sustain lysine acetylation, an effect that may be critical for the full therapeutic effect of SAHA in cancer therapy. Functional characterization of these acetylated proteins in signal transduction is under way, and may help to elucidate novel mechanisms that underlie the biological effects of HDAC inhibition in cancer therapy.

Glossary

Abbreviations

Ac	acetylation
EF-1α	elongation factor-1 α
HDACs	histone deacetylases
Hsp	heat shock protein
IP	immunoprecipitation
K	lysine
LC-MS/MS	liquid chromatography tandem mass spectrometry
R	arginine
SAHA	suberoylanilide hydroxamic acid
SILAC	stable isotope labeling with amino acids in cell culture

TSA trischostain A
WB western blot

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A **B**

1C

Fig. 1. Effect of SAHA on lysine acetylation

(A) Western blot with anti-DNMT1 antibodies of whole cell lysates from 10 μM SAHA treated MDA-MB-231 (top) and SILAC-labeled MDA-MB-231 cells (bottom) demonstrates no differences in DNMT1 inhibition. (B) Acetylated proteins were detected by using anti-acetyllysine antibodies in SILAC-labeled MDA-MB-231 cells. β -actin was used as a loading control. Three experiments showed similar results. (C) Schematic summary of SILAC-based experimental design.

Fig. 2. Immunoprecipitation of acetylated proteins

Cell lysates from control and SILAC labeled MDA-MB-231 cells treated with SAHA for 24 or 48 h were combined, and lysine-acetylation containing proteins were immunoprecipitated with anti-acetyllysine antibodies followed by colloidal Coomassie staining (left panel) or immunoblotting (right panel).

Enolase -1

Hsp90

EF-1 α 14-3-3 γ

Fig. 3.
Representative MS/MS spectra of peptides derived from enolase-1, Hsp90, EF-1 α and 14-3-3 γ . Sequences identified with * are the SILAC labeled residues.

Fig. 4. Confirmation of acetylated proteins by immunoprecipitation and Western blots
 Immunoprecipitation was performed with antibodies for candidate lysine acetylated proteins using lysates of MDA-MB-231 cells in the presence or absence of SAHA treatment. Western blotting was done with antibody against the specific protein or an anti-lysine acetylation antibody. Three experiments showed similar results.

Table 1

Proteins with 1.5 Fold Increase in Acetyl Lysine Specific Enrichment Upon Treatment of MDA-MB-231 Cells with SAHA

Gene Symbol	gi	Protein Name	Unique Peptides	Manually Validated Peptide(s)	Fold Change		
					Control	24hr	48hr
<i>Heat Shock Proteins</i>							
CCT3	58761484	Chaperonin containing TCP1, subunit 3	1	R.AVAQALEVIPR.T	1	1.4	1.8
HSPA8*	5729877	Heat shock 70kD protein 8	12	R.GTLDPVK.A	1	1.4	2
				R.TTPSYVAFTDTER.L			
HSPA4L	31541941	Heat shock 70kD protein 4L	5	K.ALNDLGKK.I	1	1.2	1.5
HSPA5	16507237	Heat shock 70kD protein 5	6	R.IINEPTAAAIAYGLDKR.E	1	1.4	3.6
HSPA1B	4885431	Heat shock 70kD protein 1B	2	K.NQVALNPQNTVFDAKR.	1	1.3	1.7
HSP90AA1*	40254816	Heat shock protein 90kDa alpha (cytosolic)	11	K.ADLINNLTGIAK.S	1	2.6	8
				R.GVVDSSEDLPLNISR.E			
HSP90AB1*	20149594	Heat shock 90kDa protein 1, beta	12	R.ALLFIPR.R	1	2.2	9.7
				R.NPDDITQEEYGEFYK.S			
<i>Histones</i>							
HIST1H2AD*	10800130	Histone 1, H2ad	3	R.AGLQFPVGR.V	1	1.8	2.1
H2AFZ*	4504255	H2A histone family, member Z	2	R.GDEELDSLJK.A	1	0.9	1.5
H2AFY*	4758496	H2A histone family, member Y isoform 2	5	K.AGVIFPVGR.M	1	1.7	1
LOC347376*	89059701	H3 histone	2	K.STELLIR.K	1	1.5	1.1
HIST1H4A*	4504301	H4 histone family, member A	1	R.DNIQGITKPAIR.R	1	2.1	3
<i>Metabolic Enzymes</i>							
DLST	19923748	Dihydrolipoamide S-succinyltransferase	1	R.GLVVPVIR.N	1	1.2	2.4
ENO1*	4503571	Enolase 1	4	R.IEEELGSK.A	1	4.4	9.7
GAPDH	7669492	Glyceraldehyde-3-phosphate dehydrogenase	4	R.DGRGALQNIIPASTGAAK.A	1	2	2.6
				R.VVDLMAHMASKE			
LDHA	5031857	Lactate dehydrogenase A	1	K.VTLTSEEEAR.L	1	1.7	2
PKM2	33286420	Pyruvate kinase 3 isoform 2	4	R.GDLGIEIPAEEK.V	1	1.4	3
<i>Chromosome Associated</i>							
SMC3	4885399	Structural maintenance of chromosomes 3	3	K.LLEKIEEK.Q	1	1	1.7

Gene Symbol	gi	Protein Name	Unique Peptides	Manually Validated Peptide(s)	Fold Change	
					Control	24hr 48hr
CEP170	109255230	Centrosomal protein 170kDa isoform beta	3	K.QDADRPLSK.M	1	1 18.9
CEP290	109255234	Centrosomal protein 290kDa	4	K.VINELRLR	1	1 34
MCM8	33469924	Minichromosome maintenance protein 8	1	R.KSQRLNSSPITTR.Q	1	1 26.4
<u>Cell Structure</u>						
ACTN4*	12025678	Actinin, alpha 4	11	R.TINEVENQILTR.D	1	2 6.4
ANXA2	4757756	Annexin A2 isoform 2	8	R.RAEDGSVIDYELIDQDAR.D	1	1.7 6.3
ACTB*	4501885	Beta actin	19	R.VAPEEHPVLLTEAPLNPK.A	1	1.3 2.9
R.GYSFTTTAERE						
COL6A1	87196339	Collagen, type VI, alpha 1 precursor	2	K.VKSFTKRFIDNLR.D	1	1 30
CAPZB	4826659	F-actin capping protein beta subunit	2	R.STLNEIFYFGK.T	1	1 6
OBSCN	58331253	Obscurin	2	R.TSAMLTVRALPIK.F	1	1 26.3
SSRP1	4507241	Structure specific recognition protein 1	5	K.IKSDHPGISITDLSK.K	1	1.2 2.4
TUBA1B*	57013276	Tubulin, alpha, ubiquitous	3	K.DVNAAIATIK.T	1	1.3 1.8
R.LDHKFDLMYAK.R						
TTLL7	94536793	Tubulin tyrosine ligase-like family, 7	2	R.RSVSCPR.S	1	1 10.8
<u>Mitochondrial</u>						
MTRFIL	19923495	Mitochondrial translational release factor 1	1	K.DLRIDTK.R	1	1 5.7
<u>Cell Cycle and Differentiation</u>						
CDC2	4502709	Cell division cycle 2 protein isoform 1	3	K.LADFGLAR.A	1	1.4 6.6
R.SPEVLLGSAR.Y						
GDPD2	21361649	Osteoblast differentiation promoting factor		R.RAPGMR.Q	1	1 86.5
<u>Transcription Factors</u>						
EEF1A2*	4503475	Eukaryotic translation elongation factor 1, α 2	4	K.IGGIGTVPVGR.V	1	3.1 8.3
EEF2	4503483	Eukaryotic translation elongation factor 2	2	M.VNFTVDQIR.A	1	1.9 3.1
EIF2B1	4503503	Eukaryotic translation initiation factor 2B	2	R.TLLEFLKR.D	1	3.3 11.5
ESR2	10835013	Estrogen receptor beta isoform 1	1	R.KVSGNR.C	1	1 3.1
PTCHD2	88942356	Dispatched homolog 1	1	R.SRQASRAPR.V	1	1 6
HLTF	21071052	Helicase-like transcription factor	1	R.MSELSSSRPKRRK.T	1	3.2 1
TCEB3C*	21717824	Transcription elongation factor B	1	R.LRVVTTK.I	1	1 15.3
<u>Regulatory</u>						

Gene Symbol	gi	Protein Name	Unique Peptides	Manually Validated Peptide(s)	Fold Change		
					Control	24hr	48hr
ANKRD26	7662474	Ankyrin repeat domain	2	K.KQMDK.I	1	11.3	1
CDC14B	15451936	CDC14 homolog B isoform 2	1	K.SLSISR.T	1	1	27
KIAA1542	55741657	CTD-binding SR-like protein rA9	1	R.NRISTAR.R	1	1	98.6
DDX27	19743937	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	2	R.KMLKEIVK.A	1	1.3	1.6
FBXO32	17158037	F-box only protein 32 isoform 1	1	K.KDMLNSK.T	1	1	2.7
				R.LTPEEIER.M			
HKR1	32401441	GLI-Kruppel family member HKR1	1	R.VSKNGTSK.A	1	1.3	21
C15orf29	13376013	Hypothetical protein LOC79768	1	K.ISNFTNKNMKE	1	3	1
JMJD2C	109255247	Jumonji domain containing 2C	4	R.QRVLSRR.F	1	1.7	17.8
NTSE	4505467	5'nucleotidase	11	K.VIYPAVEGR.I	1	1.6	1.1
YWHAZ	4507953	14-3-3 ζ	2	R.NLLSVAYKN	1	1.5	4.6
				K.SVTEQGAELSNEER.N			
YWHAG	21464101	14-3-3 γ	2	R.LAEQAERYDDMAAAMK.N	1	1.5	3
CCDC19	81295816	Nasopharyngeal epithelium specific protein 1	2	R.AQNLLQRANKLR.M	1	2	4
PMS2	4505913	PMS2 postmeiotic segregation increased 2	1	K.KDVSISR.L	1	1	4.6
RBP2	40354214	Retinol binding protein 2, cellular	1	R.KIAVRLTQTK.V	1	1	5.3
RPL5	14591909	Ribosomal protein L5	7	R.YLMEDEDA YKK.Q	1	1	2.3
SQSTM1	4505571	Sequestosome 1	6	K.NYDIGAALDTIQYSK.H	1	6.4	9.6
				R.AGEARPGPTAESASGPSDDPSVNFLK.N			
				K.LAFPSPFGHLSEGFHSR.W			
TTN	110349713	Titin isoform novex-1	6	K.LKKNVNIKE	1	1	13.6
TPI1	4507645	Triosephosphate isomerase 1	2	K.SNVSDAVAQSTR.I	1	1.4	4.2
ZSCAN2	63176661	Zinc finger protein 29 isoform 1	1	R.SPNLIAHQ.R.T	1	1.8	4.4
LOC153222	23957698	Hypothetical protein LOC153222	1	K.LASRACRL	1	1	29
		<i>Antigen</i>					
HSP90B1	4507677	Tumor rejection antigen (gp96) 1	6	K.SGTSEFLNK.M	1	1.5	3.9
				R.GLFEDEYGSK.K			

* Previously determined to have acetylated lysine residues