Quantitative Proteomic Analysis of Post-translational Modifications of Human Histones*

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Histone proteins are subject to a range of post-transcriptional modifications in living cells. The combinatorial nature of these modifications constitutes the “histone code” that dictates chromatin structure and function during development, growth, differentiation, and homeostasis of cells. Deciphering of the histone code is hampered by the lack of analytical methods for monitoring the combinatorial complexity of reversible multisite modifications of histones, including acetylation and methylation. To address this problem, we used LC-MSMS technology and Virtual Expert Mass Spectrometrist software for qualitative and quantitative proteomic analysis of histones extracted from human small cell lung cancer cells. A total of 32 acetylations, methylations, and ubiquitinations were located in the human histones H2A, H2B, H3, and H4, including seven novel modifications. An LC-MSMS-based method was applied in a quantitative proteomic study of the dose-response effect of the histone deacetylase inhibitor (HDACi) PXD101 on histone acetylation in human cell cultures. Triplicate LC-MSMS runs at six different HDACi concentrations demonstrated that PXD101 affects acetylation of histones H2A, H2B, H3, and H4 in a site-specific and dose-dependent manner. This unbiased analysis revealed that a relative increase in acetylated peptide from the histone variants H2A, H2B, and H4 was accompanied by a relative decrease of dimethylated Lys from histone H2B. The dose-response results obtained by quantitative proteomics of histones from HDACi-treated cells were consistent with Western blot analysis of histone acetylation, cytotoxicity, and dose-dependent expression profiles of p21 and cyclin A2. This demonstrates that mass spectrometry-based quantitative proteomic analysis of post-translational modifications is a viable approach for functional analysis of candidate drugs, such as HDAC inhibitors. Molecular & Cellular Proteomics 5: 1314–1325, 2006.

Eukaryote DNA is wrapped around nucleosomes that are comprised of octamers of core histones (two heterodimers of histones H2A and H2B and a heterotetramer of histones H3 and H4). Nucleosomes are joined by linker DNA and histone H1 to form chromatin. A central mechanism for regulating chromatin activity is the (reversible) covalent modification of histones by enzymes. A complex interplay between post-translational modifications (PTMs) either represses or activates transcription in a site-specific and cooperative manner (1). The NH2- and COOH-terminal tails of the core histones are subjected to multisite modifications by methylation, acetylation, phosphorylation, ADP-ribosylation, ubiquitination (2, 3), and sumoylation (4). A “histone code” model has been hypothesized where the cell uses a combinatorial system of (clustered) covalent modifications to regulate specific genomic functions (5–7). The current hypothesis is that the pattern and types of modifications modulate protein-protein and protein-DNA interactions, e.g., for recruitment of transcription factors, as a function of the cellular state and environment (8).

Acetylation of lysine residues is a major mediator of the histone code. Hyperacetylation of histone tails is usually a sign of high transcriptional activity, whereas hypoacetylation indicates transcriptional silencing (9). Reversible acetylation/deacetylation of lysine residues is a dynamic balance between the activities of histone acetyltransferases and histone deacetylases (HDACs). Inhibition of HDAC activity results in hyperacetylation of lysine residues in the NH2 tails of histones H3 and H4 (10), and it leads to the expression of genes that induce growth arrest, cell differentiation, and apoptotic cell death in cultured tumor cells (11, 12). Non-toxic levels of several HDACis have shown promising pharmacological properties by inhibiting tumor growth in animal models (13–15). Consequently there is a great interest in developing HDACis as anticancer drugs, and some of these compounds are currently being evaluated in clinical trials (16, 17).

Mass spectrometry is rapidly becoming a key analytical technology in molecular cell biology and proteomics. Mass spectrometry is particularly suited for the examination of protein primary structure and for determining post-translational modifications of proteins as it reveals covalent modifications.
via detection of modification-specific changes of peptide molecular weight (18, 19). Peptide mass fingerprinting is a useful tool for detecting these modification-specific molecular weight changes, whereas tandem mass spectrometry facilitates amino acid sequencing of modified peptides for exact localization and identification of post-translational modifications. For these reasons, mass spectrometry is extensively used to study histones and the histone code (20). For example, a recent proteomic study of histones revealed more than 20 novel covalent modifications (acetylations and methylations) within the calf thymus histones H2A, H2B, H3, and H4, demonstrating that histones are more uniformly modified than previously recognized (21). Many of these modifications were located in positions where they may affect binding of DNA to the nucleosome lateral surface.

Structural and functional analysis of histone modifications is a prerequisite to understanding chromatin function. Thus, determination of the dynamics of post-translational modifications of histones as a function of different cellular states may reveal the underlying principles and biological importance of the histone code. Histone modifications are frequently and efficiently analyzed using immunoassays based on site-specific antibodies (22) (Western blotting). Although very sensitive, these antibody-based methods require prior knowledge of modification type and site, and there is a lack of antibodies with sufficient specificity to be able to study each modification individually. In contrast, mass spectrometry provides an unbiased alternative with high specificity and selectivity that can detect known post-translational modifications in histones as well as identify novel types of modifications and their sites.

The systematic analysis by mass spectrometry of the four core histones isolated from human small cell lung cancer cells exposed to the HDACi PXD101 is described. A total of 32 post-translational modifications at 29 sites were detected, including seven novel ones in histones H2A, H2B, and H4. Comparative analysis of LC-MSMS datasets allowed identification, relative quantification, and general monitoring of the changes in histone acetylation and methylation upon treatment with HDAC inhibitor, providing new insight into the molecular effects of this anticancer drug.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human small cell lung cancer carcinoma OC-NYH cells have been described previously (23). For histone purification, OC-NYH cells were grown in 150-cm² tissue culture flasks (Nunclon) in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 100 units/ml penicillin-streptomycin (Invitrogen). Cells were grown in 150-cm² tissue culture flasks (Nunclon) in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 100 units/ml penicillin-streptomycin (Invitrogen). Cells were grown in a humidified atmosphere containing 5% CO₂ in the dark at 37 °C. Increasing concentrations of the HDACi PXD101 (0, 0.1, 0.3, 1.0, 3.0, and 10 μM) were added to the cell cultures when they approached confluence (corresponding to 10⁷ cells/flask). Cells were incubated with PXD101 for 24 h prior to histone purification.

**Histone Purification**—After PXD101 treatments, cells were pelleted by centrifugation at 2500 x g for 10 min at 4 °C and washed in lysis buffer followed by wash buffer (10 mM Tris-HCl, 13 mM EDTA, pH 7.4; from Sigma). The pellet was next resuspended in 100 μl of ice-cold 0.4 M H₂SO₄ and incubated for 1 h at 4 °C prior to centrifugation at 2500 x g for 10 min. The supernatant was transferred to a clean tube, and 1 ml of ice-cold acetone was added followed by incubation overnight for histone precipitation. After centrifugation the pellet was air-dried and resuspended in distilled H₂O, and the protein concentration was determined by Bradford protein assay (Bio-Rad).

**Western Blotting**—Five micrograms of purified histones were denatured and reduced by boiling in SDS sample buffer containing dithiothreitol, separated by SDS-polyacrylamide electrophoresis in NuPAGE™ 4–12% bis-Tris gels (Invitrogen), and transferred to nitrocellulose paper. The membranes were blocked with skimmed milk and incubated overnight at 4 °C with the primary antibody and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Upstate Cell Signaling Solutions catalog number 12-348) as secondary antibody for 45 min at room temperature. Detection was performed using ECL Plus Western blotting detection (Amersham Biosciences), and the signal was visualized with the ChemiDoc XRS system (Bio-Rad). Polyclonal antibodies against histones H2B (catalog number 07-371) and H4 (catalog number 07-108) as well as acetylation-specific antibodies against H2B (Lys5) (catalog number 07-382), H2B (Lys12) (catalog number 07-336), H2B (Lys16) (catalog number 07-343), H2B (Lys20) (catalog number 07-347), and H4 (Lys12) (catalog number 06-762) were all obtained from Upstate Cell Signaling Solutions, and polyclonal antibodies directed against histone H4 acetylated at each of Lys5, Lys8, Lys12, and Lys16 were obtained from Serotec (catalog number APH418).

**Protein Digestion**—Separated histones (10 μg loaded, SDS-PAGE, 12% NuPAGE Novex bis-Tris gels, 1.0 mm, MES running buffer; Invitrogen) were in-gel trypsinized as described previously (24). Briefly the separated histones (visualized by Coomassie Blue staining using Coomassie Brilliant Blue G-250 and R-250) were excised and cut into small pieces, washed, shrunk with acetonitrile, swelled with a 10 ng/μl trypsin, 50 mM ammonium carbonate digestion buffer, and incubated overnight at 37 °C. The molecular standard used was Mark12™ standard (Invitrogen). In-solution digestion of 10-μg histone fractions was performed in 50 μl of a 1 ng/μl trypsin, 100 mM ammonium carbonate digestion buffer incubated overnight at 37 °C.

**Quantitative Real Time PCR**—Total RNA was extracted from OC-NYH cells by using TRIzol reagent (Invitrogen) according to the standard protocol. Quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. Total RNA (5 μg) was processed to cDNA by reverse transcription with the TaqMan® reverse transcription system (Applied Biosystems) with random hexamers in a total volume of 50 μl. The reaction proceeded at 25 °C for 10 min followed by 37 °C for 2 h. TaqMan gene expression assays for β-actin (Hs99999903), p21/CIP1/WAF1 (Hs00355782), and cyclin A2 (Hs00153138) labeled with the reporter dye carboxyfluorescein in the 5’-end and the quencher dye tetramethylrhodamine in the 3’-end were purchased from Applied Biosystems. The quantitative PCR was performed in a 20-μl reaction with 9 μl of 50× diluted cDNA, 1 μl of probe, and 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) by the use of the Applied Biosystems 7500 Real-Time PCR System. The thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Each cDNA sample was analyzed in triplicates from three independent experiments and analyzed by calculating the relative number of cDNA molecules by the amplification plot method differential amplifying RT-PCR of Peirson et al. (25). To determine the relative expression of p21/CIP1/WAF1 and cyclin A2, the values were multiplied with the amount of endogenous control β-actin and normalized to the drug-untreated sample.
Quantitative Proteomics of Histone Modifications

Mass Spectrometry—Automated nanoflow liquid chromatography/tandem mass spectrometric analysis was performed using a Q-Tof Ultima or a Q-Tof Micro mass spectrometer (Micromass UK Ltd., Manchester, UK) using automated data-dependent acquisition. A nanoflow HPLC system (Ultimate; Switchos2; Famos; LC Packings, Amsterdam, The Netherlands) was used to deliver a flow rate of 100 nl/min. Chromatographic separation was accomplished by loading peptide samples onto a homemade 2-cm fused silica precolumn (75-μm inner diameter, 360-μm outer diameter packing material; ReproSil-Pur C18-AQ 3 μm; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using an autosampler. Sequential elution of peptides was accomplished using a linear gradient from Solution A (0% acetone in 0.5% acetic acid) to 50% Solution B (80% acetone in 0.5% acetic acid) in 30 min over the precolumn in line with a homemade 8-cm resolving column (50-μm inner diameter; 360-μm outer diameter; ReproSil-pur C18-AQ 3 μm; Dr. Maisch GmbH). The resolving column was connected using a fused silica transfer line (20-μm inner diameter) to a distally coated fused silica emitter (360-μm outer diameter, 20-μm inner diameter, 10-μm tip inner diameter; New Objective, Cambridge, MA).

The mass spectrometer was operated in the positive ion mode with a resolution of 5000–7000 at full-width half-maximum for Q-Tof Micro or 8000–10,000 Da for the Q-Tof Ultima using a source temperature of 80°C and a counter current nitrogen flow rate of 60 liters/h. Data-dependent analysis was used (three most abundant ions in each cycle): 1-s MS (m/z 350–1500) and maximum 2-s MSMS (m/z 50–2000), 30-s dynamic exclusion. Processing of raw data was done by external mass calibration using NaI and resulted in mass errors of less than 50 ppm, typically 5–15 ppm in the m/z range 50–2000. Raw data were processed using Protein Lynx Global Server 2.05 (smooth 9/3 Savitzy Golay and center four channels/80% centroid), and the resulting MSMS dataset was exported in Micromass pkf format for automated peptide identification using an in-house Mascot server (Version 2.0) (Matrix Sciences, London, UK) or the Virtual Expert Mass Spectrometrist (VEMS Version 3.0) software (26). The search was done against a database containing all known human histone variants and using the following constraints: only trypptic peptides and up to seven missed cleavage sites allowed; ±0.3–0.6-Da tolerance for MS and MSMS fragment ions; carbamidomethyl cysteine was specified as a fixed modification; methionine oxidation, NH2-terminal (protein) acetylation, lysine acetylation, lysine and arginine mono- and dimethylation, lysine trimethylation, serine and threonine phosphorylation, and lysine ubiquitination were specified as variable modifications.

Quantitative Analysis of Protein Modifications—In-solution trypptic digests of histone proteins from cells exposed to HDACi were analyzed by LC-MSMS as described above. Datasets consisting of six samples corresponding to the HDACi concentration used (0, 0.1, 0.3, 1, 3, and 10 μM PXD101) were analyzed in triplicate. Quantification of peptides carrying modified arginine and/or lysine residues was performed by using the features of the VEAMS Version 3.0 software (26, 27). These features included protein identification, PTM assignment, and alignment and calibration of LC-MSMS data with respect to peptide retention times and peptide masses. Isotopically resolved ion intensities of all identified peptides were extracted from the LC-MS part of the datasets (six samples analyzed in triplicates) using the observed precursor peptide ion masses and aligned retention times. Peptides used for normalization of datasets were selected based on their coefficient of variation (CV), <30% (28). CV for a given peptide is defined as standard deviation divided by the mean of the ion intensities of this peptide from the LC-MSMS analysis of the six triplicate samples.

Scaling factors to be used for data normalization were calculated by averaging the intensities of the four internal standard peptides from each LC-MSMS run (6 × 3 runs) and then dividing the highest mean by the mean from the 17 remaining means. Datasets from each LC-MSMS run were then normalized using the corresponding scaling factors.

A Tukey multiple comparison test was performed to determine significant differences in peptide ion intensity between peptides in the different groups (29). Tukey multiple comparison tests were used previously to analyze quantitative RT-PCR results (30). First a single factor analysis of variance (ANOVA) was used to accept or reject the null hypothesis $H_0$: $\mu_A = \mu_B = \mu_3 = \mu_4 = \mu_5 = \mu_6$, which states that the mean is the same in all six groups. Rejection of $H_0$ for the single factor ANOVA means that at least one sample is significantly different from the others. The internal standard peptides were included in the six triplicate groups analyzed by ANOVA. Then a variation of the Tukey multiple comparison tests was used to test each of the pairwise comparisons $H_{\alpha, \beta}$: $\mu_{\alpha, \beta} = \mu_A$, where $A$ and $B$ are integers in the interval 2–6 corresponding to the six different groups. For each modified peptide identified, the Tukey test generates a q-value for each of the six groups of triplicate experiments. The Tukey test used here was slightly modified to compare intensity from different concentrations of HDACi to the peak intensity obtained from the untreated cells. The q-value is defined as the difference between the mean of the group at 0 μM HDACi and the mean of one of the other five groups divided by the standard error. If the q-value is above a critical value, determined to be 4.75 ($q_{0.05}$, $12, 6$ where $\alpha = 0.05$ is the significance level, 12 is the number of degrees of freedom, and 6 is the number of groups) then the sampled group is significantly different from the group corresponding to 0 μM HDACi.

Significance analysis of microarrays (SAM) analysis was performed to identify peptides that showed significant abundance changes over the different concentrations of HDACi. The SAM analysis tests the overall significance of difference in peptide peak intensity in the different runs by compensating for multiple testing (31).

RESULTS

Analysis of Human Histones by LC-MSMS for Annotation of Post-translational Modifications—Histone protein fractions from human small lung cancer carcinoma OC-NYH cells were analyzed. To obtain a highly diverse set of post-translationally modified histones, protein samples from untreated, control cells and from cells exposed to the HDACi PXD101 were analyzed. SDS-PAGE was used to separate histones prior to LC-MSMS analysis as a means to increase amino acid sequence coverage thereby enhancing the likelihood of detecting low abundance modifications. Fig. 1a shows the SDS-PAGE analysis of a histone protein fraction purified from unexposed small lung cancer cells, representative for all histone fractions analyzed. Distinct protein bands were trypsinized and analyzed by capillary LC-MSMS followed by protein sequence database searching. The histones H2A, H2B, H3, and H4 were readily identified (Fig. 1a). Distinct bands on the gel corresponded to histone H1 (36 kDa), mixtures of histones H3 and H2B (18 kDa) and H2A and H2B (16 kDa), and histone H4 (12 kDa), respectively. Another band appeared at 24 kDa and was identified as a histone H2A-ubiquitin conjugate.

Having identified the individual core histones, the datasets were next investigated with the aim to identify post-translationally modified peptides. Trypsin does not cleave efficiently at acetylated lysine residues, and in the case of lysine-rich
histone tails this leads to the accumulation of modified peptides that contain acetylated lysine residues, which in turn facilitates their detection and identification by mass spectrometry.

MSMS spectra of peptides that contain acetylated and/or methylated lysine residues frequently produce distinct ion signals indicative for these modifications facilitating their identification. For example, acetylated lysine residues generate fragment ions at \( m/z \) 143 and 126, whereas monomethylated lysine residues produce diagnostic fragments at \( m/z \) 98 and 115, and dimethylated lysine residues generate an ion at \( m/z \) 129 (32). These low molecular weight modification-specific ion signals in combination with the high mass accuracy of precursor and fragment ions are important for assignment of acetylated and methylated peptides via MSMS (26). A total of 29 modified amino acid residues, including 18 acetylation sites, 10 methylation sites, and one ubiquitination site in the histones H2A, H2B, H3, and H4 (Table I) were mapped. Seven of these PTMs were not previously reported, including three acetylation sites and three methylation sites with one site being either mono- or dimethylated, respectively.

Most of the post-translational modifications identified in this study were located on peptides that carried multiple modifications. A majority of these modifications were detected in different peptides that shared parts of the amino acid sequences, resulting in redundant sequence information and a more solid foundation for assignment of the sites and types of modifications. A whole range of known acetylation sites in histones H3 and H4 (Table I) were identified, but no new acetylation sites were mapped onto these proteins.

Novel acetylation sites in H2B were identified at Lys16 and Lys11 as established by MSMS analysis of doubly charged precursor ions at \( m/z \) 721.39 (acKGSacK11acK12acK20) and 527.79 (SAPAPacK11acKGSK), respectively (Fig. 1, b and c). These peptides carry acetylations at Lys12, Lys15, Lys16 and Lys20, and at Lys11 and Lys12, respectively. The product ion spectra exhibited complete \( y \)-ion series for unambiguous sequence assignment. Furthermore an ion at \( m/z \) 126 indicative of lysine acetylation was observed in both MSMS spectra. The novel acetylated lysine residues were identified based on the mass difference of 170 Da between the \( y_4 \) and \( y_5 \) ions for H2B-(6–15)acK11acK12 and between the \( y_2 \) and \( y_3 \) ions for H2B-(12–23)acK12acK15acK16acK20 (Fig. 1, b and c). Trimethylated lysine was also considered, but a neutral loss (\( y_i - 59 \) ion) indicative of lysine trimethylation was absent, and so we
concluded that Lys\textsubscript{11} and Lys\textsubscript{16} of H2B were acetylated. Similarly a novel acetylation site at H2A Lys\textsubscript{11} was found (Table I).

Modification of H4 was not restricted to the NH\textsubscript{2} terminus as demonstrated by assignment of four novel modifications in the core region of this histone: dimethylation of Lys\textsubscript{31} and Arg\textsuperscript{55} as well as monomethylation of Arg\textsuperscript{55}. In addition, MSMS analysis of the doubly charged peptide DAVTYEHAKR (m/z 652.83) indicated methylation of Lys\textsuperscript{77}.

In summary, mass spectrometry analysis of peptides derived from human histones revealed a variety of post-translational modifications in the H2A, H2B, H3, and H4 molecules. Most of the post-translational modifications were assigned for histones H2B, H3, and H4, whereas one novel and two known acetylated lysine residues were mapped onto histone H2A. Acetylated lysine residues were observed most frequently, and they typically occurred as multisite modifications in individual tryptic peptides due to closely spaced lysine residues. As expected, acetylated lysine was not a good substrate for trypsin, leading to the generation of larger tryptic peptides with internal acetylated lysine residues. All the acetylated peptides generated the m/z 126 ion during MSMS analysis. Methylation sites were found as mono- and dimethylated lysine and arginine residues. No trimethylated lysine residues

| Structure | Detected PTMa | MW\textsubscript{calculated}b | m/z | Mascot score |
|-----------|---------------|-------------------------------|-----|--------------|
| H2B       |               |                               |     |              |
| 1PDPAKASAPAKP\textsubscript{11} | Lys\textsuperscript{5ac} | 1119.59 | 560.79 (2+) | 63 |
| 1PEPAKASAPAKP\textsubscript{11} | Lys\textsuperscript{5ac} | 1133.61 | 567.77 (2+) | 53 |
| 6APAPKKGSK\textsubscript{15} | Lys\textsuperscript{11ac}, Lys\textsuperscript{12ac} | 1053.58 | 527.78 (2+) | 47 |
| 6APAPKKGSK\textsubscript{16} | Lys\textsuperscript{11ac}, Lys\textsuperscript{12ac}, Lys\textsuperscript{15ac} | 1223.69 | 612.83 (2+) | 52 |
| 6APAPKKGSKAVTKAQK\textsubscript{23} | Lys\textsuperscript{12ac}, Lys\textsuperscript{15ac}, Lys\textsuperscript{16ac}, Lys\textsuperscript{20ac} | 2062.13 | 688.38 (3+) | 20 |
| KGSKAVTK\textsubscript{20} | Lys\textsuperscript{12ac}, Lys\textsuperscript{15ac}, Lys\textsuperscript{16ac}, Lys\textsuperscript{20ac} | 1071.63 | 536.78 (2+) | 78 |
| KGSKAVTKAQK\textsubscript{23} | Lys\textsuperscript{12ac}, Lys\textsuperscript{15ac}, Lys\textsuperscript{16ac}, Lys\textsuperscript{20ac} | 1440.83 | 721.39 (2+) | 74 |
| KGSKAVTKVQK\textsubscript{25} | Lys\textsuperscript{12ac}, Lys\textsuperscript{15ac}, Lys\textsuperscript{16ac}, Lys\textsuperscript{20ac} | 1468.83 | 735.39 (2+) | 59 |
| KGSKAVTKAQK\textsubscript{24} | Lys\textsuperscript{12ac}, Lys\textsuperscript{15ac}, Lys\textsuperscript{16ac}, Lys\textsuperscript{20ac} | 1398.81 | 647.28 (3+) | 29 |
| KGSKAVTKAQK\textsubscript{23} | Lys\textsuperscript{12ac}, Lys\textsuperscript{15ac}, Lys\textsuperscript{16ac}, Lys\textsuperscript{20ac} | 1568.92 | 524.00 (3+) | 34 |
| KAVTKAQK\textsubscript{23} | Lys\textsuperscript{16ac}, Lys\textsuperscript{20ac} | 956.56 | 479.28 (2+) | 19 |
| VLKVQHPDGTGIS\textsubscript{S7} | Lys\textsuperscript{77me} | 1303.65 | 652.83 (2+) | 37 |
| 1AGGKAQKDSGKAK\textsubscript{13} | Lys\textsuperscript{4ac}, Lys\textsuperscript{7ac}, Lys\textsuperscript{11ac} | 1299.68 | 650.76 (2+) | 73 |
| H3        |               |                               |     |              |
| 9KSTGKAPR\textsubscript{17} | Lys\textsuperscript{9ac}, Lys\textsuperscript{14ac} | 984.54 | 493.28 (2+) | 61 |
| 16KQLATKVAR\textsubscript{26} | Lys\textsuperscript{23ac} | 1027.65 | 514.81 (2+) | 50 |
| 16KQLATKVAR\textsubscript{26} | Lys\textsuperscript{18ac}, Lys\textsuperscript{23ac} | 1069.62 | 535.82 (2+) | 61 |
| 19QLATKVAR\textsubscript{26} | Lys\textsuperscript{23ac} | 899.52 | 450.77 (2+) | 45 |
| 27KSAPSTGGVK\textsubscript{36} | Lys\textsuperscript{27ac} | 972.52 | 487.25 (2+) | 46 |
| 27KSAPSTGGVKPKHR\textsubscript{40} | Lys\textsuperscript{27ac}, Lys\textsuperscript{36me2} | 1502.87 | 736.72 (4+) | 44 |
| 27KSAPSTGGVKPKHR\textsubscript{40} | Lys\textsuperscript{27me2}, Lys\textsuperscript{36me} | 1474.87 | 736.90 (4+) | 55 |
| 27KSAPSTGGVKPKHR\textsubscript{40} | Lys\textsuperscript{27me2}, Lys\textsuperscript{36me} | 1488.89 | 737.21 (4+) | 74 |
| 73EIAQDFKTDLR\textsubscript{83} | Lys\textsuperscript{7me} | 1348.70 | 674.35 (3+) | 57 |
| 73EIAQDFKTDLR\textsubscript{83} | Lys\textsuperscript{7me} | 1362.71 | 682.36 (2+) | 56 |
| H4        |               |                               |     |              |
| 4GKGKGLGK\textsubscript{12} | Lys\textsuperscript{5ac}, Lys\textsuperscript{8ac} | 884.51 | 443.27 (2+) | 23 |
| 4GKGKGLGKGGAK\textsubscript{16} | Lys\textsuperscript{5ac}, Lys\textsuperscript{8ac}, Lys\textsuperscript{12ac} | 1239.69 | 620.86 (2+) | 80 |
| 4GKGKGLGKGGAKR\textsubscript{17} | Lys\textsuperscript{5ac}, Lys\textsuperscript{8ac}, Lys\textsuperscript{12ac}, Lys\textsuperscript{16ac} | 1395.80 | 646.27 (3+) | 32 |
| 4GKGKGLGKGGAKR\textsubscript{17} | Lys\textsuperscript{5ac}, Lys\textsuperscript{8ac}, Lys\textsuperscript{12ac}, Lys\textsuperscript{16ac} | 1437.81 | 719.92 (2+) | 116 |
| 4GKGKGLGKGGAKR\textsubscript{17} | Lys\textsuperscript{5ac}, Lys\textsuperscript{8ac}, Lys\textsuperscript{12ac}, Lys\textsuperscript{16ac} | 1210.68 | 606.34 (2+) | 70 |
| 9GLGKGA\textsubscript{KR} | Lys\textsuperscript{12ac}, Lys\textsuperscript{16ac} | 926.53 | 464.24 (2+) | 36 |
| 20KLVRDNIQGITKPAIR\textsubscript{35} | Lys\textsuperscript{20me2} | 1849.13 | 463.29 (2+) | 33 |
| 24DNIQGITKPAIR\textsubscript{35} | Lys\textsuperscript{31me2} | 1352.78 | 677.40 (2+) | 39 |
| ISGLIYEETR\textsubscript{55} | Arg\textsuperscript{55me} | 1193.63 | 597.85 (2+) | 50 |
| ISGLIYEETR\textsubscript{55} | Arg\textsuperscript{55me} | 1207.64 | 604.82 (2+) | 40 |
| DAVTYEHAKR\textsubscript{78} | Lys\textsuperscript{77me} | 1303.65 | 652.83 (2+) | 34 |

\(a\) Superscript letters following the position numbers represent the following: ac, acetylation; me, monomethylation; me2, dimethylation; ub1, monoubiquitination.

\(b\) Calculated molecular weight.
Quantitative Proteomics of Histone Modifications

Quantitative Proteomics of Histone Modifications—Histones undergo dynamic post-translational modification via the actions of histone acetyltransferase and histone deacetylase enzymes. Thus, functional investigations of histone acetylation require quantitative analytical methods that allow molecular dissection and structural analysis of modified proteins. Having demonstrated that LC-MSMS-based technology and bioinformatics allowed qualitative analysis of the core histones we next investigated whether LC-MSMS could be adapted for unbiased quantitative analysis of histone acetylation. In particular, we were interested in monitoring changes in acetylation at the novel sites discovered during our analysis of histones. PXD101 is a HDACi that induces hyperacetylation of histones, apoptosis, and cell death in cancer cells (33). We used PXD101 to generate a dose response of histone acetylation in human cell culture and measured the effect by LC-MSMS analysis of tryptic peptides and by Western blotting using anti-acetylysine antibodies.

First the purified histone fraction was separated by SDS-PAGE, and the degree of acetylation was measured using polyclonal antibody raised against histone H4 acetylated at Lys5, Lys8, Lys12, and Lys16. Histone H4 extracted from untreated cells (0 μM PXD101) contained only low levels of acetylation (see Fig. 3a). Addition of PXD101 up to 10 μM resulted in increased acetylation as judged by Western blotting (see Fig. 3a).

The mass spectrometry-based analytical and computational strategy is outlined in Fig. 2. Acid-extracted histones from PXD101-untreated and -treated human small cell lung carcinoma nuclei preparations were digested by trypsin protease and analyzed by capillary LC-MSMS. In this way, the relative peptide abundance in each of the six PXD101-treated samples was determined in triplicate by mass spectrometry. All the LC-MSMS runs were compared by using peptide retention times and peptide masses as well as MSMS-based amino acid sequence information for peptide alignment. Peptide abundance changes were measured via peptide ion intensities determined by mass spectrometry. Hybrid Q-TOF tandem mass spectrometers with relatively high mass accuracy and mass resolution were used to obtain high quality data for computational data analysis, resulting in unambiguous protein identification and confident assignment of post-translational modifications as well as robust peptide quantification. Polyacrylamide gels were avoided to eliminate ambiguities related to separation of histone isoforms, including post-translationally modified forms, as this would bias the relative quantification of modifications.

The acquired LC-MSMS datasets of tryptic peptides were used for protein identification and PTM assignment using the Mascot and VEMS programs for protein database searching. Peptide retention times, precursor ion masses, and intensities were extracted. The experimental peptide m/z values were recalibrated using the theoretical masses obtained from the database-dependent search. The CV of the ion intensity of all detected and sequenced peptides was calculated. Unmodified tryptic peptides to be used for normalization of datasets were assigned based on the criteria that their CVs should be less than 30% (28). The peptides HLQLAIR from H2A, STELLIR from H3.3, and VFLENVIR and ISGLIYETR from H4 fulfilled these conditions having CVs of 22, 21, 15, and 24%, respectively. The peptide traces of these automatically assigned “internal standard peptides” for data normalization are shown in Fig. 3b. The peptide ion abundance is similar across the dose-response experiment, indicating that the ratio between these unmodified histone peptides does not change significantly upon drug treatment. Scaling factors for normalization were calculated by averaging the intensities of the four peptides in each LC-MS run, yielding scaling factors between 0 and 1. Normalization of data was achieved by multiplying each individual dataset by the corresponding scaling factors. After alignment and normalization of 18 LC-MSMS experiments (six samples analyzed in triplicate), the relative changes in intensity of each of the identified (modified) peptides were calculated.

Statistically significant changes of peptide abundance were determined by a Tukey comparison test and a SAM analysis. These two statistical methods complement each other. The Tukey test evaluates single peptides for the significance of peak intensity changes upon HDACi exposure compared with the intensity that is derived from unexposed OC-NYH cells. This approach identifies significant abundance changes for a specific peptide at given HDACi concentrations. The SAM analysis identifies peptides with significant changes in peak
Fig. 3. Outline of the quantification by the internal standard method of the quadruply acetylated peptide \( m/z \) 719.92 (\([\text{MH}^2]^+\)) from histone H4 acetylated at Lys\(^5\), Lys\(^8\), Lys\(^12\), and Lys\(^16\) (acKGGacKGLGacKGGAacKR) from histone fractions isolated from OC-NYH cells exposed to 0, 0.1, 0.3, 1, 3, and 10 \( \mu \)M HDACi PXD101. 

a, Western blot analysis using polyclonal antibody directed against the quadruply acetylated peptide from histone H4. 

b, single ion chromatograms extracted from the MSMS datasets of the internal standard peptides prior to RT correction, HLQLAIR from H2A (A), STELLIR from H3.3 (B), ISGLIYEEETR (C), and VFLENVIR (D) from H4, selected for data normalization and of the quadruply acetylated peptide from histone H4 acetylated at Lys\(^5\), Lys\(^8\), Lys\(^12\), and Lys\(^16\). 

c, increase in the relative abundance of the quadruply acetylated peptide from histone H4. Error bars indicate S.D.; \( n = 3 \).
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intensity over all HDACi concentrations but does not identify the specific HDACi concentrations at which the significant intensity changes have occurred.

For a given peptide the Tukey test compared the variance within a group of triplicate replicate Western blots with the variance between the six groups (0, 0.1, 0.3, 1.0, 3.0, and 10 μM PXD101). This comparison revealed the peptides that differed from the average intensity/abundance values. SAM analysis showed that 13 of the modified histone peptides had an intensity variation over all the groups that were significant. This was, for example, demonstrated by monitoring the changes in the level of the histone H4 acetylated tryptic peptide GacKGacKGLGacKGGAacKR (m/z 719.92, [MH$_2$]$_2$) as a function of cell exposure to 0, 0.1, 0.3, 1, 3, and 10 μM HDACi PXD101. The LC-MSMS ion trace of this quadruply acetylated peptide is also shown in Fig. 3, b and c, and an increase in the level of the acetylated tryptic peptide relative to the levels of the four unmodified peptides used for normalization was observed. This is in accordance with the Western blotting results (Fig. 3a) and demonstrates that the quantitative LC-MSMS method can be used to identify posttranslationally modified peptides that exhibit dose-response behavior upon drug treatment of cells.

Using this approach we measured by LC-MSMS a PXD101 dose response from a series of acetylated peptides and methylated peptides. These results are presented and discussed in more detail in the following sections.

Quantitative Analysis of Post-translational Modifications of Histones—Acetylation of H2B was analyzed by Western blotting using polyclonal antibodies against H2B and acetylated H2B Lys$^5$, Lys$^{12}$, Lys$^{15}$, and Lys$^{20}$ (Fig. 4a). The Western blots of H2B confirmed identical protein loads and dose-dependent increased acetylation in response to PXD101 treatment. Proteomic analysis by LC-MSMS revealed the tryptic peptides H2B-(12–20) (m/z 536.78) acetylated on Lys$^{12}$, Lys$^{15}$, and Lys$^{16}$; H2B-(6–16) (m/z 612.83) acetylated on Lys$^{11}$, Lys$^{12}$, and Lys$^{15}$; H2B-(11–11) (m/z 560.79) acetylated on Lys$^{6}$; and H2B-(12–23) (m/z 721.39) acetylated on Lys$^{12}$, Lys$^{15}$, Lys$^{16}$, and Lys$^{20}$ (Fig. 4b). All these peptides exhibited a dose-dependent increase in levels relative to the internal standard peptides (Fig. 4b). The greatest increase in relative abundance was observed for the peptide that was acetylated on Lys$^{12}$, Lys$^{15}$, and Lys$^{16}$. The intensity increase was detectable from cell exposure at an HDACi concentration of 0.1 μM. A higher HDACi concentration (0.3–1 μM) was required to induce detectable levels of all other acetylated peptides analyzed. These data corresponded well with the data obtained by Western blotting.

The quantitative analysis of modified tryptic peptides from the histone variants H2A, H3, and H4 is summarized in Fig. 4b. Dose-dependent increases in the relative levels of the acetylated peptides H2A-(1–14) acetylated on Lys$^{4}$, Lys$^{7}$, and Lys$^{11}$; H3-(18–26) acetylated on Lys$^{18}$ and Lys$^{23}$; H3-(27–36) and H3.a-(27–36) acetylated on Lys$^{27}$; and H4-(4–17) acetylated on Lys$^{5}$, Lys$^{8}$, Lys$^{12}$, and Lys$^{16}$; and H4-(6–17) acetylated on Lys$^{8}$, Lys$^{12}$, and Lys$^{16}$ were observed. The relative abundances of those peptides increased with increasing PXD101 concentrations. In contrast, three acetylated tryptic peptides from H4 did not follow this trend, the triply acetylated peptide H4-(4–17) acetylated on Lys$^{5}$, Lys$^{12}$, and Lys$^{16}$ and the doubly acetylated peptide H4-(4–12) acetylated on Lys$^{5}$ and Lys$^{8}$ peaked at a PXD101 concentration of 3 μM, whereas the doubly acetylated peptide H4-(9–17) acetylated on Lys$^{12}$ and Lys$^{15}$ showed maximum relative abundance when OC-NYH cells were exposed to 0.3 μM PXD101.

Interestingly we found that the H2B peptide detected at m/z 399.53 (3+), QVHPDTGISSK, that was dimethylated at Lys$^{57}$ decreased in abundance with increasing PXD101 concentrations (Fig. 4b), whereas the H3 peptide carrying the Lys$^{79}$ methylation was unaffected upon PXD101 treatment.

Analysis of Expression of p21 and cyclin A2—To establish a link between a specific set of PXD101-induced modifications and apoptosis in induced OC-NYH cells we analyzed the expression of p21 and cyclin A2 genes by real time PCR (Fig. 5, a and b). Maximum expression of p21 was observed at 1 μM PXD101, whereas a significant decrease in expression of cyclin A2 was observed in NYH cells exposed to 0.3–1 μM PXD101. These data correlated well with the PXD101 concentrations leading to cell death of more than 95% of the cells (Fig. 5c).

DISCUSSION

Distinct sets of histone modifications (primarily acetylation and methylations) form epigenetic codes that control chromatin structure and function. These epigenetic codes can be perturbed upon treatment with HDACis that selectively induce growth arrest and apoptosis in cancer cells. The MS-based qualitative and quantitative approach presented here provides a method for the identification and determination of multisite histone modifications.

Mass accuracy, sensitivity, and resolution are crucial parameters for protein identification and assignment of posttranslational modifications in proteomic studies. In the present study, we exploited the mass accuracy, sensitivity, and resolution of an ESI Q-TOF instrument equipped with a capillary LC system to meet these analytical requirements. The mass accuracy of MS and MSMS experiments and the observation of modification-specific fragment ions allowed us to annotate acetylated and methylated peptides.

Seven novel modifications were detected, including histone acetylation sites and methylation sites. Novel modifications on histone H2B includes acetylation at Lys$^{11}$ and Lys$^{16}$, and histone H2A was found to be acetylated at Lys$^{11}$. Histone H4 contained mono- and dimethylated Arg$^{55}$, dimethylated Lys$^{31}$, and methylated Lys$^{77}$. The modified amino acid residues Lys$^{31}$, Arg$^{55}$, and Lys$^{77}$ of H4 were all located on the outer surface of the nucleosome (Fig. 6), therefore readily accessible to modifying enzymes such as histone acetyltransferases.
and methyltransferases. Thus, these post-translational modifications may modulate the interaction between the proteins and DNA and thereby influence the transcriptional activity of chromatin. Interestingly Lys77 on human histone H4 is involved in the binding of DNA to the nucleosome (34). This site is located in the same region as Lys79 on histone H3 from yeast. Hypomethylation of H3 Lys79, which remained unaffected upon PXD101 treatment, is important for transcriptional silencing and for association of Sir (silent information regulator) proteins (35, 36). Thus, methylation of Lys77 on histone H4 may also influence chromatin structure.

**FIG. 4.** a, Western blot analysis of histone H2B using polyclonal antibodies against H2B protein and acetylation-specific antibodies against acetylated lysine residues Lys5 (Ac-K5), Lys12 (Ac-K12), Lys15 (Ac-K15), and Lys20 (Ac-K20), respectively. b, diagrams showing the intensity changes as a function of HDACi (PXD101) concentrations. The upper value in a box is the average intensity of the three peak intensities in each triplicate set. The lower value in a box describes q-values calculated as described under “Experimental Procedures.” q-values < q_critical are graduated from light blue to dark blue (global q-value minimum), and q-values > q_critical are graduated from light red to dark red (global q-value maximum). Peptides annotated with * showed significant abundance changes over the different concentrations of HDACi as identified by SAM analysis.

**FIG. 5.** Real time PCR analysis of the relative (Rel.) expression of p21 (a) and cyclin (b) upon cell exposure to increased concentrations of PXD101. c, cell death in small lung cancer cells.

**FIG. 6.** Localization of the novel modification sites identified by LC-MSMS on nucleosome crystal structure. The structure was generated using the Deepview/Swiss PDB-viewer 3.7 with atomic coordinates from Protein Data Bank code 2CV5 (46).
methylation of Lys59 on histone H4 is important for silent chromatin structure. The proximity of this site to Lys31 and Arg55 on histone H4 suggests related roles for these modifications.

In an effort to test and validate the method for quantitative analysis of post-translational modifications, we investigated the dose-dependent effect of the HDACi PXD101 on modification of histone proteins from human small lung cancer cells. In previous studies, stable isotope incorporation was applied to monitor the change in acetylation patterns of individual histone tails, including H4 (37) and H3 (32). These studies only characterized a small subset of acetylation sites. In another study, LC-MS was used to characterize global modification levels of the core histones H2A, H3, and H4 from different cell lines treated with HDACi valproic acid and from clinical samples from patients treated with the HDACi depsipeptide (38). In that study, a dose-dependent change in the modification in the distribution of modified core histones from these cell lines was found. Furthermore, an increase in the relative abundance of specific acetylated H4 forms upon HDACi treatment was found in the clinical trial samples. However, what these studies have in common is that they were restricted to the predefined modified proteolytic peptides such as H4-(4–17) (37), required the combination of different mass spectrometric techniques (39), or measured only the global modification levels of the core thus lacking information on the specific amino acid(s) being modified (38).

Our new mass spectrometry-based method for peptide quantification relies on automated assignment of a set of “internal standard” peptides across LC-MSMS experiments. This was achieved by identification, alignment, and normalization followed by comparison and statistical analysis of peptide ion intensities across all experiments. Previous work has shown that, although some quantification of peptide abundances based on ion signals can be provided by ESI MS methods (40–42), normalization procedures due to randomly occurring effects such as uneven ionization efficiencies and sample loading are critical for the quantitative analysis of proteins without the use of stable isotopes. Therefore, such procedures are included in our approach for quantitative analysis of histone modifications. This method revealed a series of post-translationally modified peptides from all four core histones, which exhibited a dose-response upon HDACi treatment of cells. As expected, the relative abundance of nonmodified peptides did not change significantly.

Our method can be applied to any set of protein samples as it does not rely on metabolic or chemical stable isotope incorporation. However, reproducibility of sample preparation and mass spectrometry analysis is a key issue. Crude histone extracts were directly analyzed by mass spectrometry to minimize sample handling and to detect as many isoforms and modifications as possible. Samples were analyzed in triplicate to minimize the analytical variation. A major advantage of the method is that it allows identification and quantification of multisite modifications and the discovery of novel modifications by MS and MSMS analysis.

The influence of HDACi on acetylation of H4 has been reported (43, 44). A “zip” model for histone acetylation and deacetylation was proposed based on a study of histone H4 in HeLa S3 cells treated with sodium butyrate. It was suggested that acetylation first occurred at Lys16 and then at Lys12, Lys8, and Lys5 in that order, and deacetylation occurred in the opposite direction starting at Lys5. The order of acetylation of Lys16 and Lys15 was confirmed in a study of mouse lymphosarcoma cells treated with the HDACis trichostatin A or depsipeptide (44). However, it was observed that both Lys8 and/or Lys5 were acetylated next irrespective of the HDACi used. We observed a decrease in the relative intensity of the doubly acetylated peptide H4-(9–17) acetylated at Lys12,16 from 0.3 to 10 μM PXD101 and an increase in intensity of the peptides H4-(4–17) acetylated on Lys5,12,16 and H4-(6–17) acetylated on Lys8,12,16 from 0.3 to 3 μM PXD101 and H4-(4–17) acetylated at Lys5,8,12,16 from 0.3 to 10 μM PXD101. These findings support the previous findings (44) that acetylation first occurs at Lys16 followed by acetylation of Lys15, whereas the concomitant acetylation of Lys5 and Lys8 does not occur in a strict order. A missed cleavage at Lys5 in the tryptic peptide H4-(4–17) acetylated on Lys5,12,16 occurred with high reproducibility in all samples analyzed. This peptide was also detected by Ren et al. (44) who also detected a missed cleavage at Lys5 in the peptide H4-(4–17) acetylated on Lys8,12,16. These peptides did not significantly change in abundance upon prolonged trypsin treatment. The resistance of unmodified Lys5 and Lys8 to trypsin cleavage might be explained by the presence of several glycine residues that makes the NH2 terminus of H4 very flexible, and the introduction of an acetylation of Lys5 or Lys8 therefore introduces a conformational change of the peptide that prohibits trypsin cleavage at unmodified Lys5 or Lys8. In contrast to the acetylated histone peptides analyzed we found that the methylation of Lys79 in histone H3 was unaffected upon PXD101, whereas there was a significant decrease in the relative level of the peptide from histone H2B carrying the dimethylation at Lys75 upon PXD101 treatment.

The histone code hypothesis holds that specific combinations of histone modifications control transcriptional output. Also, HDACi treatment of cancer cells leads to growth arrest and cell death. To establish a link between the histone code, gene expression, and apoptosis, we analyzed the expression of p21 and cyclin A2 genes by real time PCR. The protein p21 is a cyclin-dependent kinase inhibitor that associates with cyclin-dependent kinases such as cyclin A2 and inhibits their kinase activities leading to cell cycle arrest (45). Upon treatment with HDACis the expression of p21 is induced with a concomitant repression of the cyclin A2 gene leading to growth arrest and apoptosis. We found that maximum and minimum expression of these genes encoding the proteins p21 and cyclin A2, respectively, was observed in OC-NYH...
cells exposed to 0.3–1 μM PXD101 concentrations (Fig. 5, a and b). Similar PXD101 concentrations led to cell death of more than 95% of the cells (Fig. 5c). At exposure of 1 μM PXD101 a significant increase in acetylated sites was observed for the lysine residues Lys5, Lys8, Lys12, and Lys16 in histone H4 and the acetylation of Lys18 and Lys23 in histone H3 as compared with untreated cells. Furthermore a significant decrease in the relative level of the tryptic peptide from H2B carrying a dimethylation at Lys47 at this PXD101 concentration was found, in all indicating these modifications as important for the induction of apoptosis in PXD101-treated human small lung cancer cells.

In conclusion, mass spectrometry applied to the systematic qualitative and quantitative analysis of histones revealed known and novel post-translational modifications and revealed dose-dependent acetylation and methylation of core histone proteins from human small lung cancer cells exposed to the HDACi PXD101. The qualitative analysis of gel-separated histone fractions revealed seven novel modifications of which four were located on the nucleosome surface where they may interfere with histone-DNA interactions, thereby affecting chromatin structure. A novel internal standard-based LC-MSMS method was successfully applied to the quantitative analysis of known and novel histone modifications using crude histone protein preparations. This analysis revealed a dose-dependent acetylation of the histone NH2 termini and also identified a distinct set of modifications that correlated with expression of genes involved in apoptotic pathways upon PXD101 exposure.

The presented internal standard-based LC-MSMS method does not rely on the incorporation of stable isotopes and is not restricted to quantification of specific modifications, enabling the direct quantification of novel post-transcriptional modifications in proteins from a variety of sources such as tissues and blood samples from clinical trials. We therefore envision that this technology will find widespread use in studies of chromatin dynamics and in other fields of cell biology, molecular medicine, and pharmacology to study the effects of mitogens and drugs on post-translational modifications.

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