A Modified “Cross-talk” between Histone H2B Lys-120 Ubiquitination and H3 Lys-79 Methylation*

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Western blot analysis is currently the major method utilized for quantitatively assessing histone global modifications. However, there is a growing need to develop a highly specific, accurate, and multisite quantitative method. Herein, we report a liquid chromatography–tandem mass spectrometry–multiple reaction monitoring method to simultaneously quantify multisite modifications with unmatched specificity, sensitivity, and throughput. With one set of purification of histones by high pressure liquid chromatography or SDS-PAGE, nearly 20 modification sites including acetylation, propionylation, methylation, and ubiquitination were quantified within 2 h for two samples to be compared. Using this method, the relative levels of H2B ubiquitination and H3 Lys-79 methylation were quantified in the U937 human leukemia cell line, U937 derivative cell lines overexpressing anti-secretory factor 10 (AF10) and mutant AF10 with the deletion of the hDot1 binding domain OM-LZ. We found that H2B ubiquitination is inversely correlated with H3 Lys-79 methylation. Therefore, we propose that a catalytic and inhibitory loop mechanism may better describe the cross-talk relationship between H2B ubiquitination and H3 Lys-79 methylation.

Acetylation, methylation, and ubiquitination are widely known modifications of lysine in histones (1). Histone acetylation normally plays a vital role in gene activation with an exception when acetylation is utilized for other functions such as protein–protein interactions (2, 3). The function of histone methylation depends on the site that is modified, i.e. H3 Lys-9, Lys-27 and H4 Lys-20 methylation are associated with heterochromatin where genes are predominantly in a silenced state, whereas H3 Lys-4 and Lys-79 methylation are linked to euchromatin where the majority of genes are in an active format (4). Methylation may also function oppositely, under two different conditions such that both H3 Lys-9 and Lys-36 methylation have a positive effect in the coding region and negative effect in the promoter region (5, 6). Histone H2B ubiquitination has been demonstrated in vitro to be required for histone H3 Lys-4 and Lys-79 di- and tri-methylation, indicating a cross-talk pathway exists between the two types of modifications in two histone subclasses in the process of regulating gene expression (7–9).

Human leukemia cell line U937 typically contains reciprocal CALM-AF102 fusion genes resulting from the rearrangement of CALM and AF10 genes (10). These CALM-AF10 and/or AF10/CALM fusion proteins were identified in many leukemia patients with acute myeloid, acute lymphoblastic, and T cell acute lymphoblastic leukemia (11–13). The AF10 protein associates with the histone H3 methyltransferase hDot1L in the nucleus (14, 15). Literally, the fusion protein, CALM-AF10, might compete with the endogenous AF10 protein for hDot1L binding and bring the bound Dot1 out of the nucleus to the cytoplasm along with the shuttling vehicle of CALM. As a consequence, the net concentration of the hDot1L protein in the nucleus decreases, resulting in H3 Lys-79 hypomethylation (16). Concomitantly, H3 Lys-23 hyperpropionylation was also detected in U937 cells. This alteration was absent in the HL60 cell line, which does not have a CALM-AF10 fusion (17). This discrepancy of histone H3 Lys-79 methylation and H3 Lys-23 propionylation between two leukemia cell lines prompted the need of a highly sensitive, specific, and widely attainable throughput quantitative method for a comprehensive examination of histone modifications, especially for the low-abundance modification such as ubiquitination, in various leukemia cell lines.

Western blot analysis and immunochemistry have been widely employed to analyze histone global modifications. However, from one sample preparation, i.e. an SDS-PAGE gel, only one type of modification at one specific site can be analyzed with a highly specific antibody. Along with the rapid development of mass spectrometers and mass spectrometric methodologies, quantitative analysis of protein expression and protein modifications is now a routine operation in many mass spectrometry facilities or research laboratories with either stable isotope labeling or label-free strategies (18). The mass spectrometry-based quantification method was also applied in anal-

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ysis of histone modification using LC/MS/MS in conjunction with isotope labeling (i.e. deuterated acetyl anhydride) to quant-
ify acetylation (19), heavy methyl SILAC (stable isotope label-
ing with amino acids in cell culture) by incorporation of
[^13]CD₂⁺ methionine and D₂-l-lysine in culture medium to iden-
tify and quantify methylation (20), or label-free LC/MS to quan-
tify histone acetylation and methylation (21). However, chal-
enges in these techniques still remain for a comprehensive quan-
tification of multisite modifications and accurate quanti-
fication of low-abundance modifications.

Because of its inherently unmatched selectivity, LC/MS/MS running in the multiple-reaction-monitoring (MRM) mode is hereto-
fore the highest sensitive mass spectrometry-based methodology for determining the concentration of small mol-
ecules in biological entities. Principally, quantification of pro-
teins can also be implemented by LC/MS/MS-MRM analysis of
tryptic peptides. With well defined transitions and chromato-
graphic profiling, up to 100 transitions of known proteins could
be quantified simultaneously (22). In practice, LC/MS/MS-MRM has been employed to validate biomarkers identified by
other high throughput quantitative proteomics (23, 24). In
the work reported here, we developed an LC/MS/MS-MRM method to relatively quantify multisite modifications of his-
tones simultaneously with high selectivity and up to 5 orders of
magnitude of linearity. This quantification can be either rela-
tive for comparison of histone modifications between two cell
lines or absolute when a known amount of internal standards is
added to the samples. With one set of purification of histones by
high pressure liquid chromatography (HPLC) or SDS-PAGE,
neart 20 modification sites including acetylation, propionyla-
tion, methylation and ubiquitination were quantified within 2 h
for two samples to be compared. We leveraged this novel ana-
lytical method to quantify histone modifications in the U937
cell line and its derivatives to study the cross-talk between his-
tone H2B ubiquitination and histone H3 Lys-79 methylation.
An unexpected inhibitory effect of histone H79 methylation, presumably through the hDot1L-binding protein AF10, on his-
tone H2B ubiquitination, was unraveled.

EXPERIMENTAL PROCEDURES

Cell Cultures—HL60, U937T cells, and their derivatives were
cultured as previously described (17). Briefly, U937T stable cell
lines expressing FLAG-AF10 or FLAG-AF10ΔOM (octapep-
tide motif-leucine zipper (OM-LZ)) were established using the
pUHD10S-1 vector (gift from Dr. Gerard Grosveld). The stable
cell lines were maintained in RPMI 1640 medium supple-
mented with 10% fetal bovine serum, 1 µg/ml tetracycline, 0.5
µg/ml puromycin, and 1 ng/ml G418 (Invitrogen). HeLa and
IMR90 cell lines were purchased from the American Type Cul-
ture Collection (ATCC) and cultured as recommended.

Purification of Histones—Cultured cells were centrifuged in
ice-cold phosphate-buffered saline solution supplemented with
5 mM sodium butyrate. The cell pellets were re-suspended in
ice-cold Triton extraction buffer (phosphate-buffered saline
containing 0.5% Triton X 100 (v/v), 2 mM phenylmethylsulfonyl
fluoride, 0.02% (w/v) NaN₃, and protein inhibitor mixture) at a
cell density of 5 × 10⁶ cells/ml and incubated on a rotator. The
cell nuclei were isolated and lysed in 0.4 N H₂SO₄ by incubation at
4 °C overnight. The supernatant of the centrifugation was added with trichloroacetic acid to reach a final concentration of
33% (v/v) so that histones were precipitated. The core histones
were further fractionated into H2A, H2B, H3, and H4 by
reversed-phase HPLC (17).

Synthesis of Standard Peptides—Stable-isotope labeled pep-
tides were synthesized using solid phase synthesis and fast Fmoc
chemistry on an ABI model 433A peptide synthesizer. Deuterated
valine-d₅ was reacted with N-(9-fluorenylmethoxycarbonyloxy)
succinimide to form the needed Fmoc-protected amino acid pre-
cursor. The branched peptide, AVTK₆₀GTYSSK, that results when
ubiquitinated histone H2B is cleaved with trypsin, was synthesized
using Fmoc-Lys(Mmt)-OH and t-butoxycarbonyl-Ala-OH (25,
26). The Mmt protecting group was selectively cleaved with acetic
acid in trifluoroethanol/dichloromethane (1:2.7) followed by two
more cycles to attach two glycine residues to the lysine side chain.
Incorporation of a propionylated lysine was similarly accom-
plished by reacting Fmoc-Lys(Mmt)-OH with propionic anhy-
dride in N-methylpyrrolidone followed by selective removal of
the lysine side chain-protecting group-Mmt. The protecting groups
and resin support were cleaved from the peptides using a reagent
containing 88% trifluoroacetic acid, 5% phenol, 5% water, and 2%
tri-isopropl silane. After filtering off the resin and concentrating
from the remaining solution, each peptide was precipitated in cold
diethyl ether. After the ether was removed, the peptides were
redissolved in water with a minimum amount of acetonitrile.
Reversed-phase HPLC using a semiprep C18 column with mobile
phase A (0.1% trifluoroacetic acid in water) and mobile phase B
(0.085% trifluoroacetic acid in acetonitrile) was used to obtain the
purified peptide. The identities of the peptides were confirmed by
electrospray ionization-MS analysis on a Thermo-Finnigan LCQ
Deca XP mass spectrometer. The purity of the peptides was veri-
fied to be >95% using a reversed-phase HPLC with a C18 analyti-
cal column.

Western Analysis of Histone H2B Ubiquitination—Approxim-
ately 5 µg of core histones isolated from each cell line were
separated by SDS-PAGE, which ran at a constant voltage of 120
V using a 16% Tris-glycine precast mini-gel (Invitrogen). West-
ern blot analysis was carried out using H2B antibody (Abcam)
to analyze both the H2B protein and the mono-ubiquitinated
H2B protein and ubiquitin antibody (Abcam) to analyze mono-
ubiquitinated H2B protein.

Liquid Chromatography—HPLC was run on an Agilent 1200
system with quaternary pumps. A 25-min gradient (mobile
phase A, 0.1% formic acid and 25 mM ammonium acetate in
water; mobile phase B, 0.1% formic acid in acetonitrile) with a
flow rate of 0.3 ml/min was run through the Waters YMC
ODS-AQ (150 × 2.0 mm, S-5 µm) column to the ion source of
the triple quadrupole mass spectrometer.

Mass Spectrometry—Histones were digested overnight with
trypsin (enzyme/protein ratio was ~50; Roche Applied Sci-
ence). After digestion, the peptides were dried by vacuum cen-
trifugation, redissolved in 0.1% formic acid, and then directly
subjected to LC-MS/MS analysis. The LC-MS/MS experiments
were run on the Agilent 6410 triple quadrupole mass spectrometer.
For the regular electrospray, the ion source was kept at 300 °C; nebulizer gas (N₂) flow was 10 liters/h (42.0 p.s.i. in
pressure); capillary voltage was 4.5 kV; capillary current was 75
Quantification of Histone Modifications by LC-MS/MS-MRM

RESULTS

LC/MS-MS-MRM Transition Selection and Optimization

Core histones were purified from 10 million cells using the acid-precipitation method (17). 200 μg of core histones were obtained from this purification. Each time ~10 μg of core histones were separated into H2A, H2B, H3, and H4 by using reversed phase HPLC or, alternatively, by SDS-PAGE. The fractions from HPLC or gel bands containing each histone were digested by trypsin. The tryptic peptides were split into three aliquots that were analyzed by LC/MS/MS with MRM on the Agilent 6410 triple quadrupole mass spectrometer (Fig. 1). The ion-chromatographies of MRM transitions optimized for the quantification of histone modifications relative to the unmodified histone peptides were demonstrated in Fig. 2. For instance, the intensity of ubiquitinated H2B peptide versus the intensity of H2B peptide that has been proven unmodified was treated as the “percentage” of H2B ubiquitination. HPLC peaks were verified by the overlapped retention time from two transitions with the same precursor ion and two different product ions (y ions) (Fig. 2A). The transitions m/z 549.8 (2+, precursor ion) → m/z 585.3 (y5, product ion) and m/z 408.7 (2+, precursor ion) → m/z 446.3 (y4, product ion) were chosen for the quantification of ubiquitinated H2B peptide, AVTKGGYTSSK, and the quantification of H2B peptide, EIQTAVR, respectively, because they gave higher sensitivity than the other two pairs of transitions. Collision energies were optimized at a fixed fragmentor voltage of 130 volts, which were optimized with preset collision energy, indicating that 22 and 16 eV, respectively, for the H2B ubiquitinated peptide and the H2B peptide gave maximum signal responses. Similar strategies were implemented for choosing MRM transitions and optimizing mass spectrometer parameters for the quantification of H3 Lys-79 methylation as well as other types of modifications listed in Table 1. The optimization of the LC-MS/MS-MRM transition for the quantification of the low-abundance H2B ubiquitination was realized by a synthetic peptide standard, whereas μA, chamber current was 1.66 μA. MRM mode was chosen for qualitative and quantitative analysis of peptides. The collision energies and fragmenter voltages were optimized by a series of changes of parameters using standard peptides or test samples. A 20-μL solution of peptides was injected each time through the HTC PAL autosampler for method development, and a maximum amount of a 40 μL solution of peptides was injected through a Rhodyne manual injector for each LC/MS/MS analysis of samples.

FIGURE 1. An outline of LC-MS/MS-MRM method for the quantification of multisite modifications of histones purified from HPLC or SDS-PAGE on a triple quadruple (QQQ) mass spectrometer.

FIGURE 2. Ion chromatographies of histone modifications in U937 cells analyzed by LC-MS/MS-MRM using the transitions listed in Table 1. A, shown is measurement of H2A di-acetylation on Lys-5/Lys-9 relative to an unmodified H2A peptide. B, shown is measurement of H2B Lys-120 ubiquitination (Ub) relative to an unmodified H2B peptide. C, shown is measurement of H3 Lys-9 methylation, Lys-9/Lys-14 di-acetylation, Lys-18/Lys-23 di-acetylation, Lys-23 propionylation, and Lys-79 methylation relative to an unmodified H3 peptide. D, shown is measurement of H4 Lys-5/Lys-8/Lys-12/Lys-16 di-, tri-, tetra-acetylation and Lys-20 methylation relative to an unmodified H4 peptide.
other higher-abundance modifications, such as H3 Lys-79 mono- and di-methylation, were implemented by direct injection of histone tryptic digests.

**Linearity and Dynamic Range of Quantification**

Standard ubiquitinated peptide AVTKGGYTSSK and H2B peptide EIQTAVR and their stable-isotope-labeled counterparts A*VTGGGGYTSSK and EIQTAVR* (V, valine is replaced by d8-valine) were synthesized by solid-phase peptide synthesis. The concentration of the ubiquitinated peptide was determined using the UV absorbance at the 274 nm and a tyrosine coefficient of 1368 \( m^{-1}cm^{-1} \), and the concentration of the H2B peptide was estimated by comparison of the UV absorbance with that of the ubiquitinated H2B peptide at the wavelength of 210 nm. A series of 10-fold dilutions from the highest concentration of 0.73 mg/ml to the lowest concentration of 7.3 ng/ml was prepared to establish the quantification curve of ubiquitinated H2B peptide. As shown in Fig. 3B, a linear response (regression coefficient \( R^2 = 0.993 \)) of MRM signals to peptide concentrations was established within the six data points spanning 5 orders of magnitude. The lowest quantification of H2B ubiquitinated peptide (\( M_j \) 1098) was 7.3 ng/ml or 100 fmol on the column when 15 \( \mu \)l of sample was injected. A similar result was obtained for the quantification of H3 Lys-23 propionylation (supplemental Fig. S1).

**Reproducibility and Accuracy**

We have constructed four mixtures from four synthesized and HPLC-purified H2B peptides to mimic \( \sim 1 \) and \( \sim 10\% \) ubiquitination of H2B tryptic peptide solutions with two concentrations, \( \sim 100 \) fmol and \( \sim 1 \) pmol. First, 1 pmol each of H2B ubiquitination peptides, AVTKGGYTSSK and internal standard A*VTGGGGYTSSK (V, d8-valine), was mixed with 10 pmol each of H2B peptides EIQTAVR and internal standard EIQTAVR* from their stock solutions to form solution A; solution A was diluted 10 times with 0.1% formic acid to form solution B. Second, 1 pmol each of H2B ubiquitination peptides AVTKGGYTSSK and A*VTGGGGYTSSK was mixed with 100 pmol each of H2B peptides EIQTAVR and EIQTAVR* from their stock solutions to form solution C; solution C was diluted 10 times with 0.1% formic acid to form solution D. Each solution was analyzed by LC-MS/MS-MRM 20 consecutive times, and therefore, a total of 80 acquisitions were made from the aforementioned prepared four solutions. H2B ubiquitination was calculated by the ratio of the area of the peak at a retention time of 13.2 min corresponding to the H2B ubiquitinated peptide over the area of the peak at a retention time of 13.9 min corresponding to the H2B unmodified peptide. H2B ubiquitination was calculated in two ways; one way with the consideration of the internal standards, and the other was without. The result of the data analysis was summarized as shown in Fig. 4, demonstrating that the variations of measurements without internal standards were less than 20%, and the variations of measurements with internal standards were less than 15%, the variations at low concentration were slightly higher than those at higher concentration, and the averages of measurements were nearly constant between the two concentrations (~100 fmol and ~1 pmol) for both percentages of ubiquitination (~1 and ~10%); however, the average percentages of ubiquitination calculated without internal standards were significantly different from the average percentages calculated with internal standards that were close to the absolute percentages of ubiquitination in samples. This difference arose from the unequal ionization efficiencies of the two peptides, which were compensated by the internal standards. This set of data demonstrated that the LC/MS/MS-MRM method is highly capable for the quantification of histone modifications; with internal standards, quantification is more accurate and absolute, and without internal standards, quantification is still satisfactory for comparative analysis between two samples as long as the peptide concentrations are within the dynamic range shown by the quantitative curve (Fig. 3B).

**TABLE 1**

| Histones      | MRM pairs           | HPLC retention time | Quantification peptide sequences |
|---------------|---------------------|---------------------|----------------------------------|
|               | Precursor ions      | Product ions        | min                              |
| H2A           | 472.8               | 428.3 (y2)          | 17.1 AGLOFPVGR                   |
| Lys-4/-9 di-acetylation | 443.3          | 246.2 (y2)          | 12.9 ‘GK_QGGK_A12               |
| H2B           | 408.7               | 446.3 (y2)          | 13.4 EIQTA*VR                    |
| Lys-120 ubiquitination | 549.8          | 585.3 (y2)          | 14.8 YRPGTVLR                    |
| H3            | 516.8               | 303.1 (b2-NH2)      | 14.8 ‘Kme2STGGK_APR              |
| Lys-9 mono-methylation | 479.3          | 272.1 (y2)          | 12.6 ‘Kme1STGGK_APR              |
| Lys-9 di-methylation | 486.3          | 272.21 (y2)         | 12.6 ‘Kme2STGGK_APR              |
| H3_Lys-9 trimethylation | 493.3          | 463.8 (M+–H–)       | 12.6 ‘Kme3STGGK_APR              |
| Lys-9 acetylation | 493.3            | 258.1 (b2)          | 13.3 ‘Kme3STGGK_APR              |
| Lys-18/-23 di-acetylation | 535.8      | 659.4 (y6)          | 14.3 ‘Kme3STGGK_APR              |
| Lys-23 propionylation | 521.8          | 673.4 (y6)          | 14.0 QLALTAK_AAR                 |
| Lys-79 mono-methylation | 675.8          | 288.2 (y2)          | 15.7 ELAQDFKme3TDLR              |
| Lys-79 di-methylation | 682.8          | 288.2 (y2)          | 15.7 ELAQDFKme3TDLR              |
| Lys-79 tri-methylation | 689.8          | 288.2 (y2)          | 15.6 ELAQDFKme3TDLR              |
| H4            | 495.3               | 219.1 (a2)          | 17.8 VFLENVR                     |
| Lys-12/-16 di-acetylation | 464.3          | 473.3 (y2)          | 14.6 GLKG_GGAK_R                 |
| Lys-8/-12/-16 tri-methylation | 606.4          | 530.3 (y2)          | 15.1 GK_GGLK_GGAKme3R            |
| Lys-5/-8/-12/-16 tetra-acetylation | 719.9       | 530.3 (y2)          | 15.3 GK_GGLK_GGLK_GGAKme3R       |
| Lys-20 mono-methylation | 265.2          | 288.2 (y2)          | 12.9 ‘Kme1VLR                    |
| Lys-20 dimethylation | 272.2            | 288.2 (y2)          | 12.9 ‘Kme1VLR                    |
| Lys-20 tri-methylation | 279.2          | 288.2 (y2)          | 12.9 ‘Kme1VLR                    |

MRM transitions optimized for the quantification of histone modifications.
Next, we tested if the accuracy and reproducibility remained for the tryptic digests of histones. Shown as an example, we chose H3 Lys-79 mono- and di-methylation to test the accuracy and reproducibility because they are of high-abundance modification, enabling us to make a series of 10-fold dilutions. Principally, the digest from 1/11011 g of HPLC-purified histone H3 was dissolved in 100/11011 l of 0.1% formic acid, from which 10/11011 l was diluted consecutively by 0.1% formic acid in 3 consecutive orders of magnitude. Each solution (total of four) was split in three aliquots that were injected to HPLC for LC-MS/MS-MRM analyses in an order from the lowest concentration to the highest concentration. The intensities, in triplicate, of H3 tryptic peptide YRPGTVALR and H3 tryptic Lys-79 methylated peptides EIAQDFKme1/me2TDLR were plotted as a function of dilution -fold as shown in Fig. 5A. H3 Lys-79 di-methylation in the solution with a 1000-fold dilution was below the quantification limit, and thus, no data were available. Lys-79 tri-methylation was detected as low abundance in the solution of the highest concentration (before dilution) but not detected and quantified in the following diluted solutions and, thus, not included for validation in this study. The maximum deviation of measurement among three injections related to a specific peptide concentration including before dilution and after dilution was 12.9% for Lys-79 mono-methylation, corresponding to the data point at 1/11011 dilution on Fig. 5, A and B, and 20% for Lys-79 di-methylation, corresponding to the data point at 1 dilution (before dilution) on Fig. 5, A and B. The ratios of H3 Lys-79-methylated peptides over H3 peptides were calculated as the methylation percentages as shown in Fig. 5B, giving consistent percentages of Lys-79 mono-methylation (14.1 ± 0.4%, relative standard deviation 3%) and di-methylation (2.0 ± 0.06%, relative standard deviation 3%) in all the concentrations, demonstrating that both the reproducibility and accuracy of LC-MS/MS-MRM for the quantification of H3 Lys-79 methylation were excellent and irrelevant to the peptide concentrations (because we measure the ratio of the peak areas of two peptides) as long as they are within the dynamic range of quantification.

Relative Quantification of Histone Modifications among Four Cell Lines

**Evaluation of the Cross-talk Relationship between H3 Lys-79 Methylation and H2B Lys-120 Ubiquitination—**Leukemia cell lines HL60, U937, U937 with ectopically overexpressed AF10, and U937 with ectopically overexpressed OM-LZ domain-deleted AF10 were chosen for this study. In previous studies it was demonstrated that histone H3 Lys-79 global methylation in U937 cells was lower than that in HL60 cells. An overexpression of AF10 in U937 cells resulted in the increase of H3 Lys-79 methylation to the level detected in HL60 cells, whereas an...
overexpression of truncated AF10 protein, in which the H3 Lys-79 methyltransferase binding domain (OM-LZ) was deleted, abolished the increase of methylation. Because there would be cross-talk between histone H2B ubiquitination and histone H3 Lys-79 methylation (and Lys-4 methylation), indicating that histone H2B ubiquitination might be required for histone H3 Lys-79 (and Lys-4) methylation, we were prompted to analyze histone H2B ubiquitination in these four cell lines.

Core histones isolated from the four cell lines were separated into H2A, H2B, H3, and H4 by HPLC sequentially. In parallel, a portion of the core histones was analyzed by SDS-PAGE with Coomassie Blue staining and Western blotting. Each histone fraction was split into three aliquots that were digested by trypsin overnight, respectively. After digestion, the peptides were subjected for LC/MS/MS-MRM analysis for the quantification of histone H2B ubiquitination as well as other modifications, such as H3 Lys-79 methylation. The whole process, from histone purification to LC/MS/MS analysis, was repeated at least once. Therefore, a minimum six sets of data were obtained. Fig. 6A shows the average percentage of histone H2B ubiquitination from a minimum six runs of LC/MS/MS analyses, and the error bars represent the S.D. of measurements, demonstrating that the H2B ubiquitination level in both the U937 cell line and its derivative cell line overexpressing AF10 whose OM-LZ domain was deleted is ~2 times higher than in both the HL60 cell line and the U937 cell line overexpressing the full-length AF10 protein. In parallel, Western blot analyses with an antibody specific to ubiquitin and an antibody specific to H2B (Fig. 6B) revealed the same trend of H2B ubiquitination as observed by mass spectrometry. Fig. 6C was the Coomassie Blue staining of the SDS-PAGE of core histones, confirming that an approximately equal amount of histone H2B from four cell lines was loaded for Western blot analysis. The consistency between LC/MS/MS-MRM quantification and Western blot analysis further validated the accuracy of the former method for the quantification of histone modifications.

Relative Quantification of Histone Methylation and Acetylation on Histones H2A, H3, and H4—Similarly to the work carried out for the quantification of histone H2B ubiquitination, the LC-MS/MS-MRM method was also successfully applied for the quantification of histone acetylation and methylation on histone H2A, H3, and H4. At the initial stage of method development, we focused on the quantification on a few predominant and well known modification sites, such as di-acetylation on H2A Lys-5 and Lys-9, acetylation on H3 Lys-9, di-acetylation of H3 Lys-18 and Lys-23, di-acetylation on H4 Lys-12 and Lys-16, tri-acetylation on Lys-4 Lys-8, Lys-12, and Lys-16, and tetra-acetylation on H4 Lys-5, Lys-8, Lys-12, and Lys-16, mono-, di-, and tri-methylation on H3 Lys-9, mono-, di-, and tri-methylation on H3 Lys-79, and di- and tri-methylation of H4 Lys-20. The MRM transitions and the mass spectrometric parameters to quantify those peptides were listed on Table 1. From this set of data (Fig. 7), we observed that acetylation of H3 and H4 did not fluctuate significantly among the four aforementioned cell lines, which is consistent with our previous data acquired by Western blot (16). However, we did see the reduction of H4 Lys-20 di-methylation in U937 cell line overexpressed with AF10 protein, and this reduction could be forfeited by the deletion of the hDOT1 binding domain OM-LZ, with an opposite effect on Lys-79 methylation, demonstrating that H3 Lys-79 methyltransferase hDOT1 might be agonistic to the H4 Lys-20 methyltransferase SET8. Di-acetylation on H2A Lys-5 and Lys-9 was detected and quantified in the AF10-overexpressed U937 cell line and in HL60 cell line, whereas this modification was barely detectable in U937 cell line and U937 cell line with overexpressed OM-LZ domain-deleted AF10, demonstrating that acetylation of H2A occurs synergistically with H3 Lys-79 methylation. Meanwhile, we observed again that H3 Lys-23 propionylation was dramatically increased with respect to this modification in HL60 cell line, well consistent with our previous data garnered by mass spectrometric analyses on a quadrupole-time of flight instrument (17). We would like to remind the readers that at this moment the LC-MS/MS-MRM method demonstrated in this manuscript, like other LC-MS/MS methods, could not successfully quantify histone H3 Lys-4 methylation, because the Lys-4Me peptides are too small and hydrophilic to be retained on a reverse-phase HPLC column using electrospray-favored solvents as the mobile phases.

**Quantification of Histone Modifications by LC-MS/MS-MRM**
Quantification of Histone Modifications by LC-MS/MS-MRM

In this manuscript, we developed a LC/MS/MS-MRM method to relatively and absolutely quantify histone modifications. We have tested this method for specificity, speed, dynamic range, reproducibility, and accuracy. We successfully used this method to quantify histone acetylation, methylation, propionylation, and ubiquitination in four chosen cell lines. Specifically, we showed the feasibility of using this method to relatively quantify histone H2B ubiquitination, whereas other mass spectrometry-based methods could have difficulties. This approach allowed us to study the correlation between histone H2B Lys-120 ubiquitination and histone H3 Lys-79 methylation.

The novel LC/MS/MS-MRM method is highly promising for the quantification of histone modifications. Although ubiquitination at Lys-120 was known for human histone H2B, previous product ion scan LC-MS/MS methods were hardly able to quantify ubiquitination at this site in human samples. The current LC-MS/MS-MRM method described in this manuscript demonstrated that it is a highly promising method for the detection and quantification of this low abundance modification in human histones with unmatched specificity, accuracy, wide dynamic range, and high sensitivity and throughput. With current settings, this quantification method was capable of quantifying ~20 modification sites simultaneously, including acetylation, propionylation, and methylation. The limit of quantification on a narrow bore column with a 200 μl/min flow rate and regular electrospay was 100 fmol for the H2B ubiquitinated peptide. The limit of quantification is expected to be easily decreased by 10-fold to 10 fmol with a jet-stream electrospay ion source and further reduced to the low amol with a nano-electrospray ionization source as well as a nano-mobile phase delivery system. Therefore, it will have the potential of quantifying all the known modification sites shown in the histone modification map (Abcam Web site) simultaneously. Furthermore, the LC-MS/MS-MRM quantification method, especially after full development, will be extremely important for the quantification of low abundance modifications, such as phosphorylation in addition to ubiquitination described in this manuscript, with well defined MRM reaction pairs, liquid chromatography, and electrospray conditions.

The LC-MS/MS-MRM method was validated for the quantification of H2B Lys-120 ubiquitination and H3 Lys-79 methylation. This method has demonstrated that it has comparable or better quantification capability than other quantitative LC/MS/MS methods, such as iTRAQ (isobaric tag for relative and absolute quantification). The maximum deviation of quantification was 20% without internal standards. In most cases this accuracy would be sufficient for the quantitative comparison of histone modifications between two cell lines or tissues. With internal standards, the maximum deviation was down to 12.6%, and an absolute quantification would be practical, although a one-time investment for the costly stable isotope-labeled peptides is required as the internal standards. Collectively, judged by all the aspects of validation of the tested method, including specificity, accuracy, speed, and dynamic range as well as simultaneous multisite quantification, the LC/MS/MS-MRM method is much superior to immunochemical methods and other types of mass spectrometric methods. The successful case demonstrated here for the relative quantification of histone H2B ubiquitination and H3 Lys-79 methylation among four leukemia cell lines will lead to the development and validation of the LC-MS/MS-MRM methods for the quantification of multisite modifications on histones (for example, ~100 sites), promising an extensive application in the studies of human diseases and cancer.

The method described here can also be adapted for the quantification of multisite modifications in other non-histone proteins, for instance, p53, tubulin, RNA polymerase II, Cdc25C, and high mobility group proteins (27). Meanwhile, quantification of protein phosphorylation, which was not demonstrated in this paper, can also utilize this strategy. Specifically, the concentrations of phosphorylated peptides/proteins can be measured by LC-MS/MS using MRM transitions corresponding to the tryptic phosphorylated peptides and the MRM transitions corresponding to synthetic stable-isotope labeled phosphorylated peptides, which have identical sequence to the unlabeled peptide, to be served as internal standards. The concentrations of proteins can be measured using the same strategy as for the measurement of the phosphorylated peptides/proteins by choosing the MRM transitions of particular tryptic peptides of the proteins that do not have modifications. Then, the percentage of phosphorylation of a protein at a specific site will be...
calculated by the ratio of the concentration of a phosphorylated peptide over the concentration of non-modified peptide of the protein. The underlined mechanism of this LC-MS/MS-MRM method is similar to the previously reported absolute quantification of abundance methodology by LC-MS/MS selective ion monitoring (28, 29). However, the LC-MS/MS-MRM method, as demonstrated here for the quantification of histone modifications, is more specific, more sensitive, and has a faster throughput.

In mammalian cells, histone H2B ubiquitination inversely correlates with histone H3 Lys-79 methylation. In mammalian cells and yeast, histone H2B ubiquitination, which is mediated by RAD6 through the COMPASS pathway, is required for H3 Lys-4 and Lys-79 methylation (7, 30–33). H3 Lys-79 is methylated by yeast DOT1 whose human homolog is hDOT1L (8, 34, 35). hDOT1L interacts with AF10, a mix lineage leukemia and CALM fusion partner through the OM-LZ region of AF10 required for leukemogenesis (14). The translocation gene t(10;11) (p13;q14) in U937 cell line produces the reciprocal fusion proteins AF10-CALM or CALM-AF10, resulting in an uneven distribution of AF10 protein and its association with H3 Lys-79 methyltransferase hDOT1L with chromatin and subsequent global hypomethylation of H3 Lys-79 (10, 16). This reduction of methylation can be compensated by overexpression of AF10 protein. This compensation can be forfeited by the deletion of the OM-LZ domain in the AF10 gene. As expected from the quantification of H3 Lys-79 mono- and di-methylation by LC-MS/MS-MRM method (Fig. 7A), 0.58% of H3 Lys-79 was di-methylated in U937 cells as opposed to 1.6% in HL60 cells, a decline of 60%. Additionally, the H3 Lys-79 mono-methylation was not as severe as di-methylation but still had 24% from 13.8% in HL60 cells to 8.5% in U937 cells (Fig. 7B). H3 Lys-79 mono- and di-methylation in U937 cell lines ectopically expressed with OM-LZ domain-deleted AF10 were 9.0 and 0.54%, respectively. These values are nearly unchanged from the mock U937 cell lines. This set of LC-MS/MS-MRM data is consistent with previous experimental data determined by quantitative Western blot and matrix-assisted laser desorption ionization time-of-flight analyses (16).
Histone H2B ubiquitination is required for H3 Lys-4 and Lys-79 di- and tri-methylation, whereas mono-methylation is dispensable (36, 37). The H2B mono-ubiquitination-dependent association of Csp35 with chromatin can facilitate the interaction between polymerase II-associated COMPASS (Complex proteins associated with Set1) and Csp35, resulting in H3 Lys-4 methylation-competent COMPASS on chromatin, and hence, H3 Lys-4 di- and tri-methylation (9). Furthermore, Lee et al. (9) proposed a model that mono-ubiquitination could also facilitate the interaction between Dot1 and Csp35 and, therefore, regulate mono-ubiquitination-dependent H3 Lys-79 methylation. Here we observed histone H2B ubiquitination (Fig. 6) and H3 Lys-79 methylation (Fig. 8) in the reverse direction; an increase in H2B ubiquitination resulted from the decrease in H3 Lys-79 methylation and vice versa. These data are consistent with the observation made in previous mass spectrometric analyses that H2B ubiquitination was hardly detectable by mass spectrometry in yeast in which histone H3 Lys-79 is highly methylated (38, 39). On the contrary, Arabidopsis contained spectrometry-detectable amounts of H2B ubiquitination but no H3 Lys-79 methylation (40). Taken together, we hypothesize that H2B ubiquitination (through RAD6) may be required to initiate the methylation of H3 Lys-79, whereas H3 Lys-79 methylation or its modification enzyme Dot1 inhibits histone H2B ubiquitination or accelerates the process of H2B de-ubiquitination (Fig. 9). This positive and negative feedback mechanism still requires a carefully defined study in the future. However, our quantitative LC-MS/MS-MRM method provides very useful information toward this direction.

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FIGURE 9. A hypothetic model to describe the relationship between histone H2B Lys-120 ubiquitination (Ub) and H3 Lys-79 methylation (Me). (Note that Lys-123 in yeast is equivalent to Lys-120 in human.)

Quantification of Histone Modifications by LC-MS/MS-MRM