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Qualitative and quantitative analysis of lysine acetylation and methylation in yeast histone H3

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Abstract

Histone post-translational modifications play important roles in cell functions and the modification patterns vary significantly among different organisms. It is important that histone modification patterns be identified. Flowing our previous work-identification of acetylation and methylation sites of histone H3 in a typical transcription most inactive chromatin isolated from chicken erythrocytes, here, we report using mass spectrometry to qualitatively and quantitatively analyze histone modification pattern of H3 in a typical transcription most active chromatin isolated from *Saccharomyces cerevisiae*. We compared the modification patterns of histone H3 between these two functionally opposite chromatins and observed that acetylation level at K9, K14, K27, K56 and methylation level at K4 and K79 are significantly higher in *S. cerevisiae* than in chicken erythrocytes, methylation at K9 is higher in chicken erythrocytes than in *S. cerevisiae* and methylation level at K36 is unchanged in these two chromatins. Contrary to other sites, acetylation levels at K18 and K23 are higher in chicken erythrocytes than in *S. cerevisiae*. Our data revealed the difference of acetylation and methylation pattern of individual H3 lysine between two distinct chromatins, one with more inactive form versus the other with more active form.

Keywords: Histone; Histone acetylation; Histone methylation; Mass spectrometry

1. Introduction

Histones are the protein wrappers of genes in cell nuclei that bind to DNA or dissociate from it depending on their acetylation states. Histones are the targets of various post-translational modifications (acetylation, methylation, phosphorylation, ubiquitination) that play essential roles in gene regulation [1–3]. Acetylation, which occurs at specific lysine residues predominantly in the amino-terminal tails, is generally associated with transcriptional activity. In contrast, methylation at different sites of histones has been linked to either gene activation or repression. For example, methylation at lysine 4 and lysine 79 of histone H3 is found in euchromatin [4] while lysine 9 and lysine 27 methylations have been linked to repression in heterochromatin or heterochromatin-like regions [5,6].

Although the modification sites of the N-terminus of histones, especially histone H3, are well documented, quantification of global acetylation level has not been available until recently [7,8]. A method to analyze the distribution of acetylation at specific modification site or the distribution among mono-, di- and tri-methylation at a methylated lysine residue in histone H3 has not been well established. In this circumstance, an approach to identifying histone modification sites as well as evaluation of the modification levels by mass spectrometry was illustrated in this report.

Previously, a comprehensive understanding of acetylation and methylation sites in chicken erythrocytes histones was obtained by mass spectrometric method [9]. A non-tail amino acid lysine 79 was found to be methylated in chicken and other organisms [10–12]. Because histones are highly conserved from yeast to animals, *Saccharomyces cerevisiae* (budding yeast) has been widely employed as a model system for chromatin study. It is well known that the majority of the yeast chromatin is in transcriptional active form and most of the chicken erythrocytes chromatin is in an inactive form. Herein, in order to investi-

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gate the variation of modification patterns between two different chromatins, active versus inactive, we analyzed the acetylation and methylation of yeast (*S. cerevisiae*) histone H3 by mass spectrometry. In comparison with our previous data of chicken erythrocytes histones, the result from yeast histone directly supports the existing models of acetylation and methylation at several lysine sites involved specifically in active or inactive chromatin. We also identified a novel acetylation site at lysine 56 in histone H3 from yeast. This site modification is absent in chicken erythrocytes.

2. Experimental

2.1. Isolation of histone H3 from yeast S. cerevisiae

Yeast wild-type strain YDS2 was inoculated in 25 mL YPD medium and grown for 9–10 h at 30 °C before further diluted in 1-L YPD medium and grown overnight to stationary phase. Cells were harvested by centrifugation for 5 min at 5000 × *g* and washed by 200–400 mL of sterile water. The pellets were re-suspended in 50 mL DTT/Tris buffer (0.1 mM Tris, 10 mM DTT, pH 9.4) and shaken for 15 min at 30 °C. After spun at 5000 × *g* for 5 min, cells were washed once by 50 mL of buffer containing 1.2 M sorbitol, 20 mM HEPES (pH 7.4), weighed, and then suspended in 50 mL of the same buffer as above with 2.75 mg zymolyase per gram yeast cell. The zymolyase digestion was carried out at 30 °C for 45 min. Cell pellets were spun down and washed with 100 mL of cold buffer containing 1.2 M sorbitol, 20 mM PIPES, 1.0 mM $MgCl_2$ (pH 6.8). After centrifuged for 5 min at 3500 × *g* and 4 °C, the cell pellets were re-suspended in 50 mL of nuclear isolation buffer (NIB) containing 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM MES (pH 6.6), 0.8% Triton X-100 and freshly added 1 mM PMSF, incubated on ice/water for 20 min, and then spun for 5 min at 4000 × *g* and 4 °C. This process was repeated three times. The nuclei were washed twice by 50 mL of washing buffer A containing 10 mM Tris (pH 8.0), 0.5% NP 40, 75 mM NaCl, 30 mM sodium butyrate and 1 mM freshly added PMSF, each time incubated on ice water for 15 min before centrifugation (4000 × *g* and 4 °C). The pellet was washed again by 50 mL of washing buffer B containing 10 mM Tris (pH 8.0), 0.4 M NaCl, 30 mM sodium butyrate, 7.5N HCl and 1 mM freshly added PMSF, incubated on ice-water for 5 min before centrifugation (4000 × *g* and 4 °C), and then re-suspended in 25 mL of washing buffer B and centrifuged at 4000 × *g* for 5 min. The washed pellet was re-suspended in three volumes of cold water and 5N HCl was added to a final concentration of 0.25N to extract histones, held in ice-water for 30 min, vortexed occasionally and then centrifuged at 30,000 × *g* for 10 min. The supernatant contains histones. In the supernatant, pure trichloroacetic acid (TCA) was added to a final concentration of 20% and incubated in ice-water bath for 30 min before centrifugation (30,000 × *g* for 30 min). The pellet, mainly histones, was washed once with cold acetone with 0.5% HCl and then with pure cold acetone. The purified histones were air-dried and stored at −20 °C.

2.2. HPLC separation of histone H3 from yeast core histones

A capillary HPLC (Agilent 1100, Agilent Technologies) was used to perform the analysis. A phenomenex 2 mm × 150 mm Jupiter C4 column (5-μm particle diameter, 100 Å pore size) with mobile phase of buffer A (0.1% trifluoroacetic acid in water) and buffer B (0.065% trifluoroacetic acid in acetonitrile) was used with a two-step gradients from 38 to 55 of mobile phase B for 50 min, 55% of mobile phase B for 5 min, and then from 65% to 85% of mobile phase B for 13 min at a flow rate of 50 μL/min. Ten microliter of sample with a concentration of about 30 μg/μL H3 in water was injected for each run. All the fractions were manually collected and analyzed by mass spectrometry to assign the peaks in the HPLC chromatogram.

2.3. Enzymatic digestion of histone H3

The HPLC fraction containing about 30 μg of H3 was dried and re-dissolved in 20 μL of 25 mM ammonium bicarbonate buffer solution and digested at 37 °C with 0.15 μg trypsin for 1.5 h or with 1.0 μg Arg-C overnight for quantification assays.

2.4. HPLC purification of peptides from H3 digests

The H3 digestion solution was completely dried by a Speed-vac and then dissolved in 3 μL 0.1% TFA. This solution was loaded onto a HPLC column (Agilent) by three 1-μL injections with 15 s interval. The HPLC was run 6 μL/min in a reverse-phase mode with a gradient increasing from 2% to 65% buffer B over 65 min, staying at 65% B for 10 min, and then increasing from 65% B to 90% B over 10 min. A photo-diode array detector was used to record the chromatogram and each fraction was manually collected in a 0.5 mL silicanized eppendorf tube. Fractions were immediately dried and re-dissolved in 5–50 μL of solvent of 50/50 acetonitrile/0.1% formic acid in water. The amount of solvent was approximately determined by the peak intensity corresponding to each fraction.

2.5. MALDI-TOF mass spectrometry

0.5 μL of peptide solution was mixed with 0.5 μL of α-cyano-4-hydroxycinnamic acid (Sigma) matrix solution (5 μg in 1 mL of acetonitrile/0.1% formic acid in water (50/50)) and subjected to MALDI analysis. Mono-isotopic masses of all peptides were measured by MALDI using a Voyager DE-STR Biospectrometry station (ABI Biosystems) with delayed extraction operated in the reflectron mode. High-accuracy mass measurements were done as previously described [9].

2.6. Sequence analysis by QTOF mass spectrometry

Sequence analyses of peptides were done by nano-ESI/MS/MS when a collision energy varying from 20 to 40 eV, which was decided by the precursor ion masses, was applied in the hexapole collision cell with Ar (12 psi) as the collision

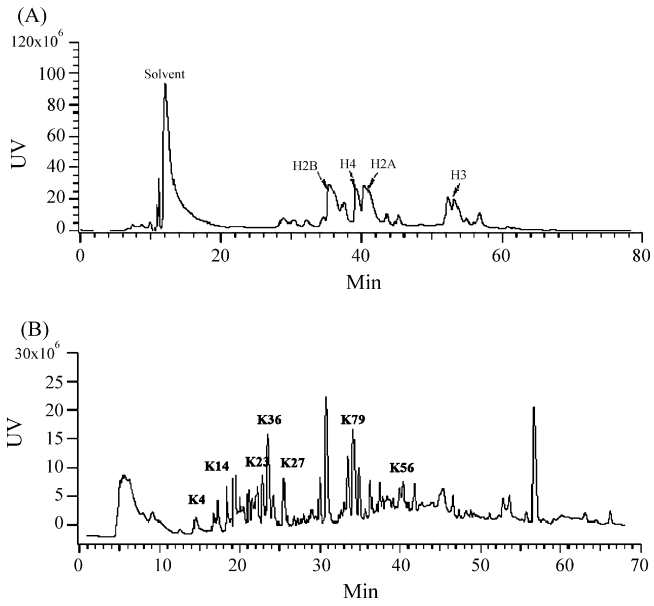

Fig. 1. Isolation of modified peptides from yeast histone H3. (A) Chromatogram of 200–300 μg of yeast core histones applied to a 150 × 2 Phenomenex C4 microbore column in water and eluted at a flow-rate of 50 μL/min using a two-step gradient increasing from 38% B to 55% B within 50 min, staying at 55% B for 5 min and then climbing to 85% B within 13 min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile/water (50/50). Column eluants were monitored by a diode array detector (DAD) and fractions corresponding the peaks in the chromatogram were manually collected into 0.5 mL eppendorf tubes and analyzed by MALDI-TOF mass spectrometry after trypsin digestion. Histones were eluted in the order of H2B (∼36 min), H4 (∼39 min), H2A (∼41 min) and H3 (∼53 min). (B) Chromatogram of trypsin-digests of yeast histone H3 (∼50 μg) injected onto a 150 × 0.5 Agilent Zorbax SB-C18 capillary column and eluted at a flow rate of 6 μL/min using a two-step gradient increasing from 2% B to 65% B within 65 min and then climbing to 90% B for 10 min. The column eluants were monitored by DAD and the fractions corresponding to one peak or a few peaks together were manually collected into 0.5 mL eppondorf tubes. The fractions (1–7) containing modified peptides were labeled with modified lysines.

gas. The QTOF was run at a capillary voltage of 1.0 kV and a cone voltage of 75 V. The source block temperature was 80 °C. Around 4 μL of sample was loaded into the nano-electrospray tip (Protana, Denmark) for each ESI/MS or ESI/MS/MS analysis.

2.7. Quantification of acetylation and methylation levels

Quantification of acetylation or methylation levels of H3 at different modification site was achieved by dividing the amount of modified peptides containing a modification site of interest by the sum of the amount of modified and un-modified peptides sharing the same peptide sequence under the assumption that the difference of ionization efficiencies among those peptides is not significant or the difference becomes less important when the modification levels between two studying samples are compared. This percentage was represented from the intensity percent of the peaks in the mass spectra corresponding to the investigating peptides of modification sites. In order to prevent the peptide with un-modified lysines from digestion by trypsin, two alternate approaches were used: (1) Arg-C digestion method: H3 was digested by Arg-C so that the lysine residues in a peptide, regardless of being modified or un-modified, were not cleaved; (2) chemical acetylation method: acetyl group was chemically introduced into H3 by D6-acetic anhydride treatment. This artificial acetyl group's mass is 45 and herein distinguished from the natural acetyl group. When it is necessary, LC/MS/MS analyses of the trypsin digests of D6-acetic anhydride treated H3 were performed.

3. Results

3.1. Isolation of yeast histone H3 from core histones by HPLC

Core histones isolated from yeast cells were analyzed by SDS-PAGE. An estimated amount of 0.8 mg core histones were dissolved in 30 μL of water and 10 μL was injected onto the HPLC column. HPLC was run as described in the experimental section and the legend of Fig. 1A. The identities of different histones were determined by their trypsin-digests that were analyzed by MALDI-TOF mass spectrometry (the spectra were not shown). Under this HPLC condition, histone H3 was well separated from H2A, H2B and H4. The elution order was H2B (∼36 min), H4 (∼39 min), H2A (∼41 min) and H3 (∼53 min). There were some higher-molecular-weight nuclear proteins co-

Table 1
MALDI-TOF high accuracy measurement of H3 K27 and K36 modification

Measured MH^+	Calculated MH^+	Deviation (ppm)	Sequence	Species
1477.8665	1477.8604	4.1	^{27}KSAPSTGGV^{36}K$_{me2}$KPHR	Yeast
1491.8760	1491.8760	ISTD[a]	^{27}KSAPSTGGV^{36}K$_{me3}$KPHR	Yeast
1505.8584	1505.8552	2.1	^{27}K$_{ac}$SAPSTGGV^{36}K$_{me1}$KPHR	Yeast
1519.8731	1519.8708	1.5	^{27}K$_{ac}$SAPSTGGV^{36}K$_{me2}$KPHR	Yeast
1533.8873	1533.8866	0.4	^{27}K$_{ac}$SAPSTGGV^{36}K$_{me3}$KPHR	Yeast
1447.8548	1447.8497	3.5	^{27}KSAPATGGV^{36}K$_{me1}$KPHR[b]	Chicken
1461.8654	1461.8654	0	^{27}KSAPATGGV^{36}K$_{me2}$KPHR[b]	Chicken
1475.8811	1475.8811	ISTD[a]	^{27}KSAPATGGV^{36}K$_{me3}$KPHR[b]	Chicken
1489.8950	1489.8967	−1.1	^{27}K$_{me1}$SAPATGGV^{36}K$_{me3}$KPHR[b]	Chicken
1503.9051	1503.9124	−4.8	^{27}K$_{me2}$SAPATGGV^{36}K$_{me3}$KPHR[b]	Chicken

Note: Data were retrieved from Fig. 5A and E.

[a] The peptide sequence was confirmed by MS/MS and served as internal standards (ISTD).

[b] The predominant form of sequence was shown.

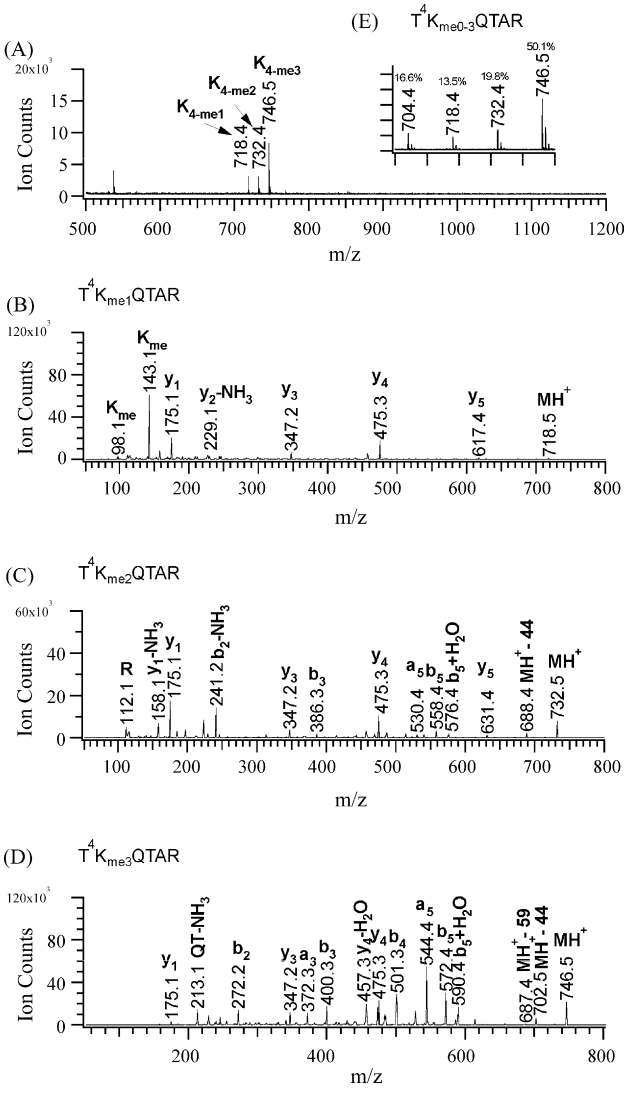

Fig. 2. Analysis of lysine 4 methylation. (A) MALDI-TOF mass spectrum of HPLC fraction (1) (Fig. 1B) containing methylated lysine 4 peptides (trypsin digests of H3). Peaks at 718.4, 732.4 and 746.4 are the mono-isotopic masses of peptides $T^4K_{me1\text{-}3}QTAR$, where $K_{me1\text{-}3}$ is referred to as mono-, di- and tri-methylated lysines. (B) ESI/MS/MS spectrum of precursor ion at *m/z* 718.4 (observed doubly charged ion at *m/z* 459.7 in the ESI mode). The y series ions match the peptide sequence $T^4K_{me1}QTAR$, where lysine 4 is mono-methylated. The mono-methylation of lysine is also confirmed by the observed immonium ion at *m/z* 98.1. (C) ESI/MS/MS spectrum of precursor ion at *m/z* 732.4 (observed doubly charged ion at *m/z* 466.7 in the ESI mode). The y series ions match the peptide sequence $T^4K_{me2}QTAR$, where lysine 4 is di-methylated. (D) ESI/MS/MS spectrum of precursor ion at *m/z* 746.4 (observed doubly charged ion at *m/z* 473.7 in the ESI mode). The y series ions match the peptide sequence $T^4K_{me3}QTAR$, where lysine 4 is tri-methylated. The tri-methylation of lysine is also confirmed by the observed MH^+-59 ion at *m/z* 687.4. (E) Expanded display (insert in A) of MALDI-TOF spectrum of HPLC fraction (1) (Fig. 1B) containing methylated lysine 4 peptides (Arg-C digests of H3). Quantification by intensity percent demonstrates that lysine 4 is 16.6% un-modified, 13.5% mono-methylated, 19.8% di-methylated and 50.1% tri-methylated.

eluted with H3 or eluted at its neighborhood. One identified protein was protein Yp10004cp (NCBinr accession number: 6325253). The H3 fraction was collected and dried for the study of modification-site identification and quantification.

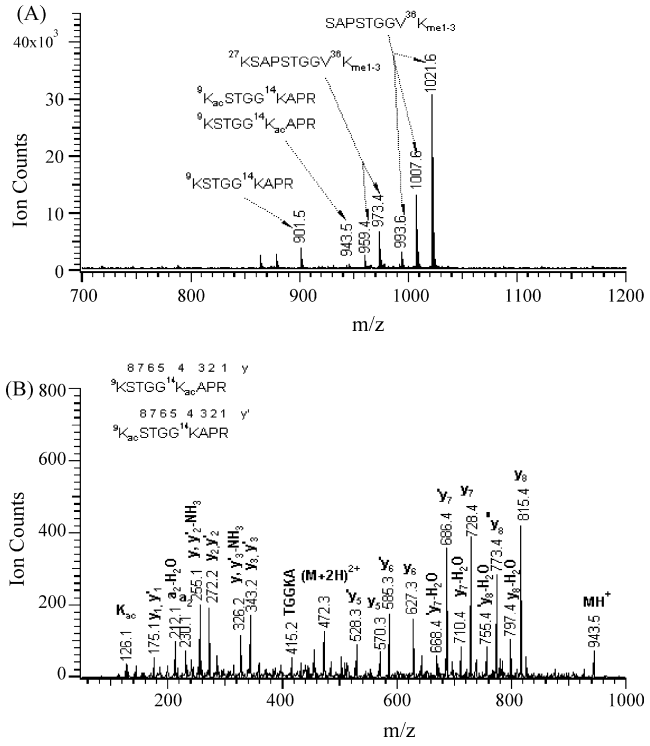

Fig. 3. Analysis of lysine 9 and lysine 14 acetylation. (A) MALDI-TOF mass spectrum of HPLC fraction (2) (Fig. 1B) containing acetylated lysine 9 and lysine 14 peptides (Arg-C digests of H3). Peaks at *m/z* 901.5 and 943.5 are the mono-isotopic masses of peptides $^9K_{ac}STGG^{14}KAPR$ and $^9KSTGG^{14}K_{ac}APR$, where K_{ac} is referred to acetylated lysine. Peaks at *m/z* 959.4, 973.4, 993.6, 1007.6 and 1021.6 are the mono-isotopic masses of peptides (labeled on top of the peaks) containing methylated lysine 36. Quantification by intensity percent demonstrates that lysine 9 and lysine 14 are together 19.6% acetylated. (B) ESI/MS/MS spectrum of precursor ion at *m/z* 943.5 (observed doubly charged ion at *m/z* 472.3 in the ESI mode). Two series ions y and y′ match the peptide sequences $^9KSTGG^{14}K_{ac}APR$ and $^9K_{ac}STGG^{14}KAPR$, respectively. An immonium ion at *m/z* 126.1 was observed to verify acetylation of lysine. The intensities of y ions and y′ ions are approximately equal so that the acetylation levels of lysine 9 and 14 are determined to be 9.8% and 9.8%.

3.2. Identification of modification sites

Histone H3 was dissolved in 50 μL of 25 mM ammonium bi-carbonate and digested by trypsin at 37 °C for 1.5 h. The digests were dried and re-dissolved in 3 μL of 0.1% TFA solution and injected onto HPLC column and separated into fractions as Section 2.4 described. The HPLC chromatogram is shown in Fig. 2C. Fourteen fractions corresponding to single or multiple peaks in HPLC chromatogram were collected, dried, and then dissolved in a solvent containing 50% acetonitrile, 49% water and 1% formic acid. Each fraction was then analyzed by MALDI-TOF mass spectrometry. Seven out of 14 fractions were determined to have modified peptides and labeled using the name of the amino acid. Other fractions containing H3 peptides that do not have modification site were not labeled in the chromatogram and discarded without further analysis. The seven fractions were in the order of methylation of lysine 4 (fraction (1)), acetylation of lysine 9 and 14 (fraction (2)), acetylation of lysine 18 and 23 (fraction (3)), methylation of lysine 36 (fraction (4)), acetylation of lysine 27 (fraction (5)), methylation of lysine 79

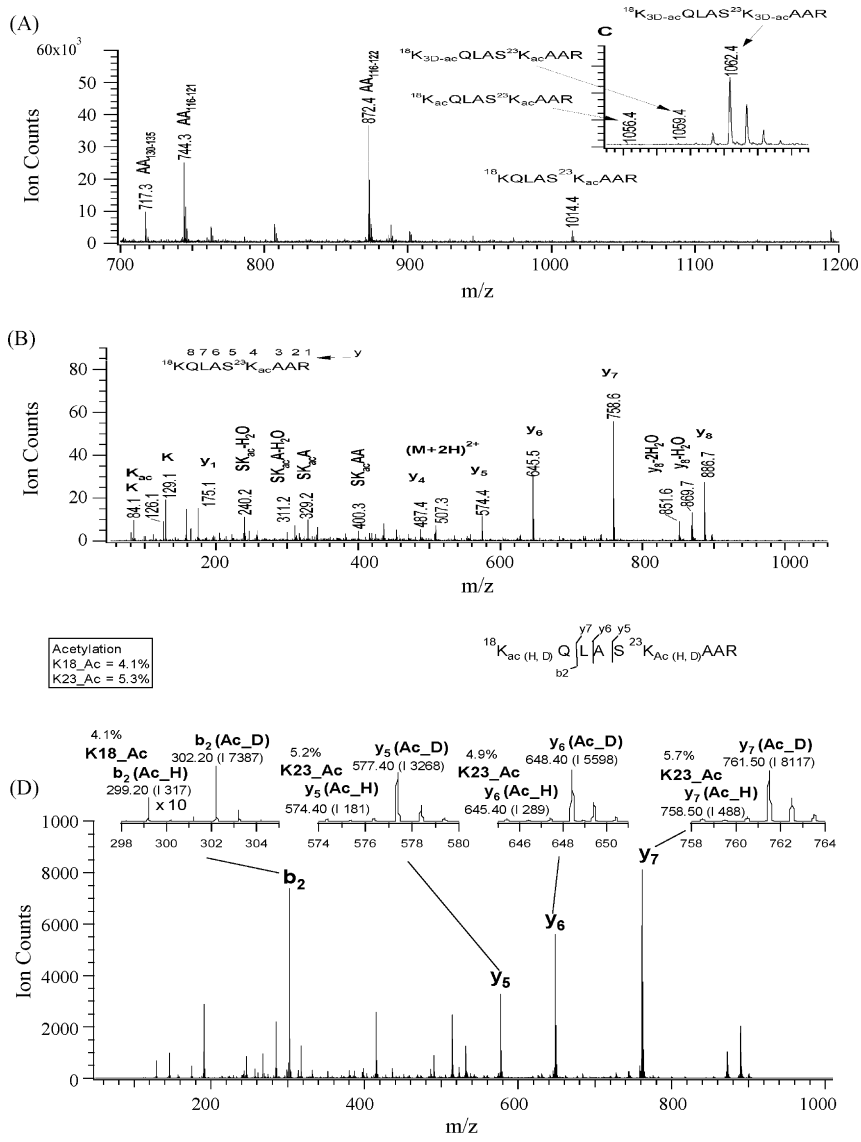

Fig. 4. Analysis of lysine 23 acetylation. (A) MALDI-TOF mass spectrum of HPLC fraction (3) (Fig. 1B) containing acetylated lysine 23 peptides (Arg-C digests of H3). The peak at *m/z* 1014.4 is the mono-isotopic mass of the peptide ^{18}KQLAS^{23}K$_{ac}$AAR, where lysine 23 is acetylated. Other peaks correspond to other trypsin digested peptides of H3 and labeled by AA amino acid sequences in the spectrum. (B) ESI/MS/MS spectrum of precursor ion at *m/z* 1014.4 (observed doubly charged ion at *m/z* 507.3 in the ESI mode). The fragmentation ions, mainly the y series ions, matches the peptide sequence ^{18}KQLAS^{23}K$_{ac}$AAR, where lysine 23 is acetylated. An immonium ion at *m/z* 126.1 was observed to verify acetylation of lysine. (C) (The insert spectrum in A) Expanded-region display of the MALDI-TOF mass spectrum of HPLC fraction (3) (Fig. 1B) containing acetylated lysine 23 peptides (In a separate experiment, H3 was treated with 3D-acetyl and digested by trypsin). (D) MS/MS spectrum of precursor ions showed in C. Acetylation level at lysine 18 (4.1%) was calculated from abundances of b2 ions at *m/z* 299. 2 and 302.2. Acetylation level at lysine 23 (average 5.3%) was calculated from the intensities of y5 ions at *m/z* 574.4 and 577.4, y6 ions at *m/z* 645.4 and 648.4 and y7 ions at *m/z* 758.5 and 761.5.

(fraction (6)) and acetylation of lysine 56 (fraction (7)). Determination of acetylation or methylation at specific modification site in individual fraction was made by high-accuracy MALDI-TOF measurement and ESI/MS/MS analysis and in-detail described in following sections.

3.3. Lysine 4 is mono-, di- and tri-methylated

In fraction (1), MALDI-TOF detected three monoisotopic peaks (Fig. 2A), spaced by 14 Da, corresponding to mono-methylated (mono-isotopic mass is 718.4 Da and labeled as $K_{4\text{-mel}}$), di-methylated (mono-isotopic mass is 732.4 Da and labeled as $K_{4\text{-me2}}$) and tri-methylated (mono-isotopic mass is 746.5 Da and labeled as $K_{4\text{-me3}}$). The peptides corresponding to the three peaks were sequence-analyzed by nano-ESI/MS/MS of their corresponding doubly charged ions in the ESI mode, at *m/z* 359.8, 366.8 and 373.8, respectively. As shown in Fig. 2B, the MS/MS spectrum of the peak at *m/z* 359.8, the y series ion y_1–y_5 confirmed the peptide sequence of K_{me1}QTAR in which the lysine residue is mono-methylated, because y_5 ion at *m/z* 617.4 is 14 Da higher than a 'peak' at *m/z* 603.4 which would be observed if the position 5 (counted from the C-terminus) were replaced by a regular lysine amino-acid. Furthermore, an immonium ion at *m/z* 98.1 was observed, indicating of a mono-methylated lysine

residue in the peptide (note: an immonium ion of regular lysine is 84). Therefore, mono-methylation of lysine 4 in the peptide $T^4K_{me1}QTAR$ was confirmed. Similarly, peaks at *m/z* 732.4 and 746.5 in the MALDI-TOF spectrum were determined by nano-ESI/MS/MS sequence-analysis (Fig. 2C and D) to correspond to $T^4K_{me2}QTAR$ and $T^4K_{me3}QTAR$, where lysine 4 is di- and tri-methylated, respectively. After determination of lysine 4 methylation, a separate digestion of H3 using Arg-C as the protease was done to quantify the methyaltion level. Because Arg-C cleaves H3 mainly at arginine residues, cleavages at R2 and R8 of H3 produce a contact peptide with the sequence of amino acid 3–8, $T^4K_{me0-3}QTAR$, where lysine 4 is either un-methylated or modified with up to three methyl groups. The occupancies for un-, mono-, di- and tri-methylation of lysine 4 were calculated

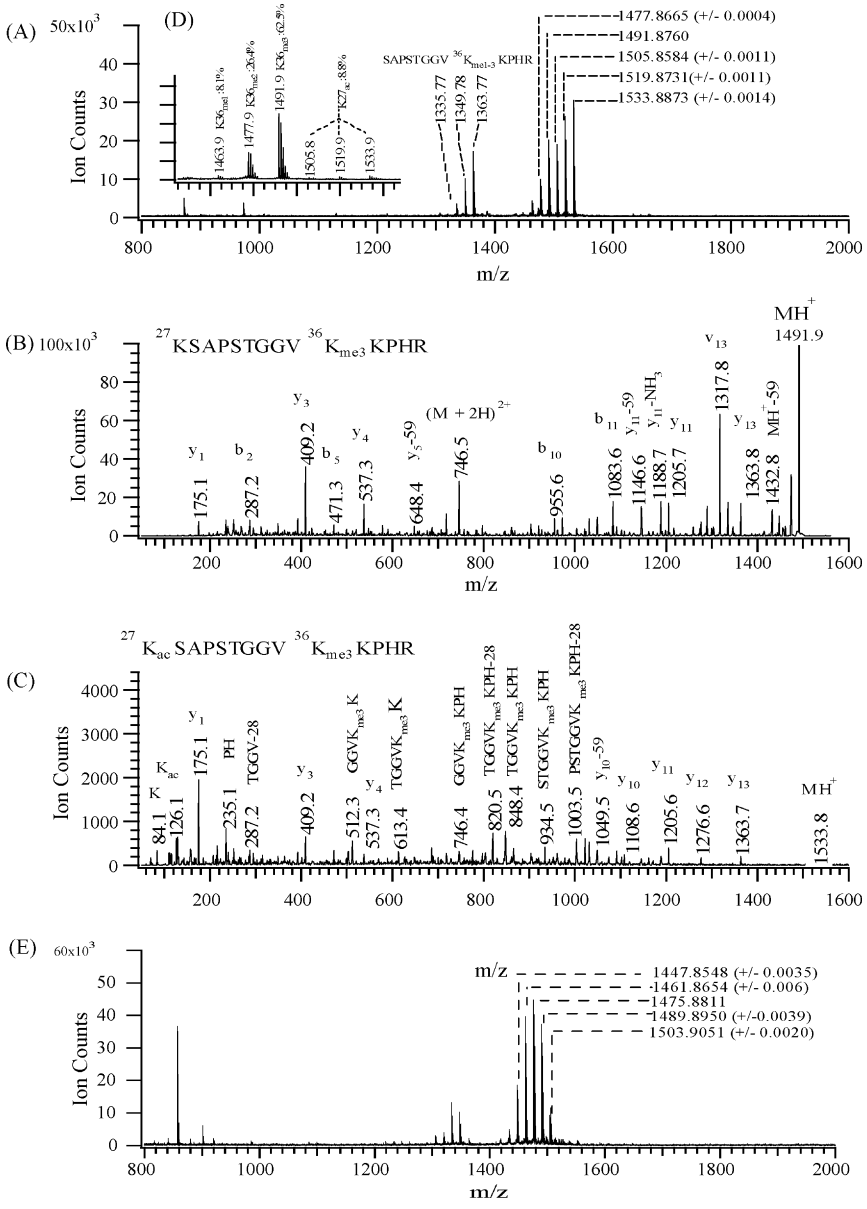

Fig. 5. Analysis of lysine 27 acetylation and lysine 36 methylation. (A) MALDI-TOF mass spectrum of HPLC fractions (4) and (5) (Fig. 1B) containing acetylated lysine 27 and methylated lysine 36 peptides (trypsin digests of H3). Peaks at *m/z* from 1464.9 to 1533.9 spacing 14 are the mono-isotopic masses of the peptide $^{27}KSAPSTGGV^{36}K_{me1-3}KPHR$, where lysine 36 is mono-, di- and tri-methylated. High-accuracy mass measurement data are shown by "- - -" linking to the peaks, which determine methylation of lysine 36 and acetylation of lysine 27. Peaks at *m/z* 1335.8, 1349.8 and 1363.8 correspond to the peptides SAPSTGGV$^{36}K_{me1-3}$KPHR. (B) ESI/MS/MS spectrum of precursor ion at *m/z* 1491.9 (observed doubly charged ion at *m/z* 746.5 in the ESI mode). The fragmentation ions, mainly the y series ions, matches the peptide sequences $^{27}KSAPSTGGV^{36}K_{me1-3}KPHR$, where lysine 36 is tri-methylated. An observed MH$^+$-59 ion at *m/z* 1482.8 verifies the tri-methylation of a lysine residue. (C) ESI/MS/MS spectrum of precursor ion at *m/z* 1533.8 (observed doubly charged ion at *m/z* 766.9 in the ESI mode). The fragmentation ions, mainly the y series ions, matches the peptide sequences $^{27}K_{ac}SAPSTGGV^{36}K_{me1-3}KPHR$, where lysine 36 is tri-methylated and lysine 27 is acetylaetd. An observed immonium ion of *m/z*126.1 verifies the acetylation at lysine residue and the acetylation occurs at lysine 27. (D) (The insert spectrum in A) MALDI-TOF mass spectrum of HPLC fractions (4) and (5) (Fig. 1B) containing acetylated lysine 27 and methylated lysine 36 peptides (Arg-C digests of H3), from which the methylation level of lysine 36 and acetylation level of lysine 27 are calculated: lysine 36 is 8.1% mono-methylated, 26.4% di-methylated and 62.5% tri-methylated; lysine 27 is 10.7% acetylated.

by intensity percent of the four peaks in the MALDI-TOF spectrum (Fig. 2E). The calculation showed that lysine 4 in yeast H3 is 16.6% un-methylated, 13.5% mono-methylated, 19.8% di-methylated and 50.1% tri-methylated. Overall 83.4% of lysine 4 is methylated and tri-methylation is the dominant form. For comparison, a similar work has been done to determine the methylation level of lysine 4 in chicken H3 and the results demonstrated that 82% of lysine 4 is un-methylated, 18.0% of lysine 4 is mono-methylated and no di- and tri-methylation was detected (Table 2).

3.4. Lysine 9 and lysine 14 are acetylated

The MALDI-TOF mass spectrum of the HPLC fraction (2) (H3 was digested by Arg-C) is shown in Fig. 3A. The peak at *m/z* 901.5 is the mono-isotopic mass of H3 N-terminal peptide AA 9–18, ^{9}KSTGG^{14}KAPR, and the peak at *m/z* 943.5 is likely the mono-isotopic mass of peptide AA 9–18 modified with an acetyl group at one of the two lysine residues or three methyl groups at one lysine residue or randomly distributed at two lysine residues. High-accuracy mass measurement of peak at *m/z* 943.5 by MALDI-TOF gave an exact mass of 943.5446, matching the mass of the peptide AA 9–18 modified with one acetyl group with a 1.6 ppm derivation. Nano-ESI/MS/MS analysis of the peak at *m/z* 943.5 (doubly charged ion at *m/z* 472.8 was observed in the ESI mode) demonstrated that the peak at *m/z* 943.5 arose from a mixture of two peptides, in one of which lysine 9 is acetylated and in the other lysine 14 is acetylated. Noted in Fig. 3B, the C-terminal fragmentation ions contain two ion series y_5–y_8 versus y'_5–y'_8, with a mass difference of 42. The y_5–y_8 ions construct the peptide sequence ^{9}KSTGG^{14}K$_{ac}$APR, where lysine 14 is acetylated; and the y'_5–y'_8 ions construct the peptide sequence ^{9}K$_{ac}$STGG^{14}KAPR, where lysine 9 is acetylated. The ratio of y over y′ is approximately 1:1, demonstrating that lysine 9 and lysine 14 are equally acetylated if the ionization efficiencies of the two types of fragmentation ions, y versus y′, are the same. A signature ion of acetylated lysine at *m/z* 126.1 was also observed to further confirm the acetylation of the peptide. Intensity percents for peaks at *m/z* 901.5 and 943.5 are 80.4% and 19.6%, respectively, demonstrating that the acetylation levels of lysine 9 and lysine 14 are 9.8% individually. No methylation at either lysine 9 or lysine 14 in yeast histone H3 was observed. By contrast, in chicken erythrocytes, 84% of lysine 9 is methylated (K9:K9$_{me1}$:K9$_{me2}$:K9$_{me3}$ = 16.0%:11.3%:22.8%:49.8%), 27.5% of lysine 14 is acetylated and 8.3% of lysine 14 is methylated (Table 2).

3.5. Lysine 18 and 23 are acetylated

Peak at *m/z* 1014.4 in the MALDI-TOF mass spectrum (Fig. 4A) of HPLC fraction (3) matches the mono-isotopic mass of peptide AA 18–23 of H3 with one site acetylation. Nano-ESI/MS/MS analysis of the peak at *m/z* 1014.4 (doubly charged ion at *m/z* 507.3 in the ESI mode) determined the peptide sequence to be AA 18–23, where lysine 23 is acetylated. As shown in Fig. 4B, the C-terminal fragmentation ions y_4–y_8 cover the sequence QITS^{23}K$_{ac}$, which has an acetylation at lysine 23. The observed internal fragmentation ions at *m/z* 240.2, 311.2, 329.2 and 400.3 corresponding to the sequences S^{23}K$_{ac}$-H$_2$O, S^{23}K$_{ac}$A-H$_2$O, S^{23}K$_{ac}$A and S^{23}K$_{ac}$AA as well as the signature ion of acetyl lysine at *m/z* 126.1 further confirmed the acetylation of lysine 23. The peak at *m/z* 1056.4 in the MALDI-TOF mass spectrum (spectrum not shown) of HPLC fraction following fraction (3) matches the mono-isotopic mass of peptide AA 18–23 of H3 with two acetylation sites. Because the intensity of ion at *m/z* 1056.4 was very low, we were unable to obtain an MS/MS spectrum by nano-ESI analysis. It has been widely accepted that lysine 18 is acetylated in various species including yeast. Therefore, the peak at *m/z* 1056.4 is very likely from the peptide AA 18–23 where lysine 18 and lysine 23 are both acetylated. In a separate experiment, H3 was treated with deuterated acetic anhydride before trypsin digestion. MALDI-TOF mass spectrum (Fig. 4C) of the peptide containing naturally and chemically acetylated lysine residues 18 and 23 was used to quantify the acetylation levels of lysines 18 and 23. The three peaks at *m/z* 1056.4, 1059.4 and 1062.4 correspond to the peptide AA 18–23, where both lysines 18 and 23 are naturally acetylated, lysine 23 is naturally acetylated and lysine 18 is chemically acetylated (equivalent to un-acetylated lysine 18 in the natural form), and both lysine 18 and lysine 23 are chemically acetylated (equivalent to un-acetylated lysine 18 and un-acetylated lysine 23 in the natural form). MS/MS of the

Table 2
Acetylation and methylation levels in histone H3 from yeast and chicken erythrocytes

Modification site (%)	Yeast					Chicken erythrocytes				
	K	K$_{me1}$	K$_{me2}$	K$_{me3}$	K$_{ac}$	K	K$_{me1}$	K$_{me2}$	K$_{me3}$	K$_{ac}$
K4	16.6	13.5	19.8	50.1		82.0	18.0			
K9	91.2				9.8	16.0	11.3	22.8	49.8	
K14	91.2				9.8	64.2		8.3		27.5
K18	95.9				4.1	74.8				25.2
K23	94.7				5.3	0				100
K27	90.3				10.7		31.8	8.3	2.5	
K36	3.1	8.1	26.4	62.5			5.9	27.6	66.5	
K56	72.4				27.6	100				
K79	2.6	15.5	23.9	58.0		59.6	23.9	9.5	6.9	
	Total acetylation level				71.1	Total acetylation level				152.7

precursor ions bracketing these three ions (doubly charged ions of *m/z* 1056.4, 1059.4 and 1062.4) resulted in fragmentation ions b2, y5, y6 and y7 ions each containing a pair of ions separated by 3 Da. Acetylation level at lysine18 was calculated from the intensities of b2 ions at *m/z* 299.2 and 302.2 which correspond to naturally acetylated and chemically d3-acetylated forms. Similarly, acetylation at lysine 23 was calculated from the average of the intensities of y5 ions at *m/z* 574.4 and 577.4, the intensities of y6 ions at *m/z* 645.4 and 648.4 and the intensities of y7 ions at *m/z* 758.5 and 761.5. It turned out that 4.1% of lysine 18 and 5.3% of lysine 23 are acetylated. A similar work was done for chicken H3 that was digested by Arg-C. It was found that 25% of lysine 18 is acetylated and almost all of lysine 23 is acetylated in chicken erythrocytes (Table 2).

3.6. Lysine 36 is mono-, di- and tri-methylated and lysine 27 is acetylated in the yeast

As shown in the MALDI-TOF mass spectrum (Fig. 5A) of HPLC fractions (4) and (5), a group of peaks spacing 14 Da encompasses the mass range 1430–1540. Tandem mass analyses of the first three peaks reveal that they are the ions of mono-, di- and tri-methylated peptide AA 27–38 with lysine 36 modified. Only the tandem mass spectrum of the peptide at *m/z* 1491.9 (doubly charged ion at *m/z* 746.5 in the ESI mode) is presented in Fig. 5B. From this spectrum, we noticed that the y series ions starting from ion y5 gain 42 data as compared with calculated y series ions of the unmodified peptide, indicating that the modification is either an acetylation or a tri-methylation at the position 5 counted from the C-terminus, which is lysine 36. Observation of the MH^+-59 ion (unique to a tri-methylated peptide) but no ions at *m/z* 126 (specific for an acetylated lysine) demonstrated that the peptide is tri-methylated at lysine 36 [9]. Tandem mass spectra of peaks at *m/z* 1463.9 and 1477.9 also confirmed that lysine 36 is mono- and di-methylated (spectra not shown). Using the calculated mono-isotopic mass 1491.8760 of the tri-methylated peptide and the mono-isotopic mass at *m/z* 1570.8426 of an added peptide Glu-fib as the internal standards to calibrate the MALDI spectrum, we obtained the exact masses for peaks adjacent to the internal standards. The exact mass measurement showed that the peak at *m/z* 1477.8665 (Fig. 5A), with a standard deviation of 0.0004 Da, matched the peptide with amino acid sequence 27–38 modified with two methyl groups. This measurement reconfirmed the methylation of lysine 36 and the rationality of choosing the peak at *m/z* 1491.8760 as an internal standard peak. Exact masses of other three peaks, at *m/z* 1505.8584 with a standard deviation of 0.0011 Da, at *m/z* 1519.8730 with a standard deviation 0.0011 Da and at *m/z* 1533.8873 with a deviation of 0.0014 Da, matched the peptide with amino acid sequence 27–38 modified at two sites, one with an acetyl group and the other with mono-, di- and tri-methyl groups, respectively (Table 1). Given the fact that lysine 36 is the methylation site, then either lysine 27 or lysine 37 should be the acetylation site. A tandem mass spectrometric experiment was done for peak at *m/z* 1533.8873 to determine the acetylation site of this peptide. As shown in Fig. 5C, the MS/MS spectrum of peak at *m/z* 1533.8873 (observed doubly

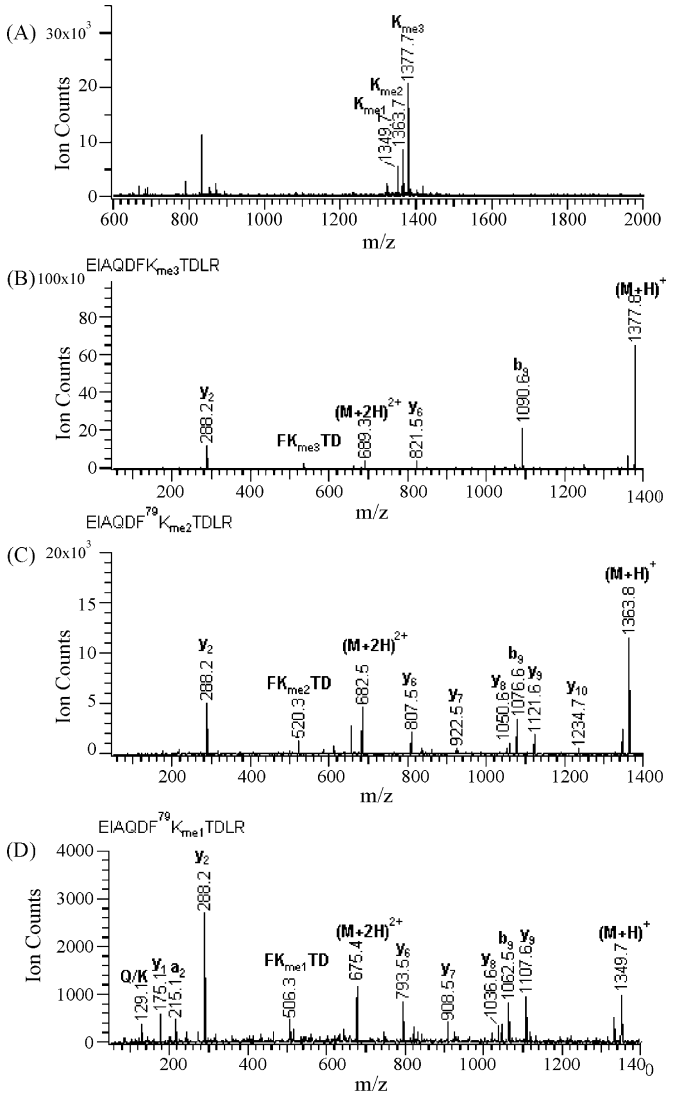

Fig. 6. Analysis of lysine 79 methylation. (A) MALDI-TOF mass spectrum of HPLC fraction (6) (Fig. 1B) containing methylated lysine 79 peptides (trypsin digests of H3). Peaks at *m/z* 1349.7, 1363.7 and 1377.7 are the mono-isotopic masses of the peptide EIAQDF^{79}K$_{me1\text{-}3}$TDLR, where lysine 79 is mono-, di- and tri-methylated. (B) ESI/MS/MS spectrum of precursor ion at *m/z* 1349.7 (observed doubly charged ion at *m/z* 675.4 in the ESI mode). The fragmentation ions, mainly b and y series ions, match the peptide sequence EIAQDF^{79}K$_{me}$TDLR, where lysine 79 is mono-methylated. (C) ESI/MS/MS spectrum of precursor ion at *m/z* 1363.7 (observed doubly charged ion at *m/z* 672.4 in the ESI mode). The fragmentation ions, mainly b and y series ions, match the peptide sequence EIAQDF^{79}K$_{me2}$TDLR, where lysine 79 is di-methylated. (D) ESI/MS/MS spectrum of precursor ion at *m/z* 1377.7 (observed doubly charged ion at *m/z* 679.4 in the ESI mode). The fragmentation ions, mainly b and y series ions, match the peptide sequence EIAQDF^{79}K$_{me3}$TDLR, where lysine 79 is tri-methylated.

charged ion at *m/z* 767.9) shares the same y series ions y1–y13 as the MS/MS spectrum of peaks at *m/z* 1491.9 (observed doubly charged ion at *m/z* 746.5, indicating that lysine 36 is the methylation site, lysine 27 is the acetylation site and lysine 37 is not modified. Lysine 27 acetylation was further confirmed by the evidence of the immonium ion at *m/z* 126. By calculating the intensity percent of each peak in the MALDI-TOF spectrum (Fig. 5D) of the peptide AA 27–38 and its modified

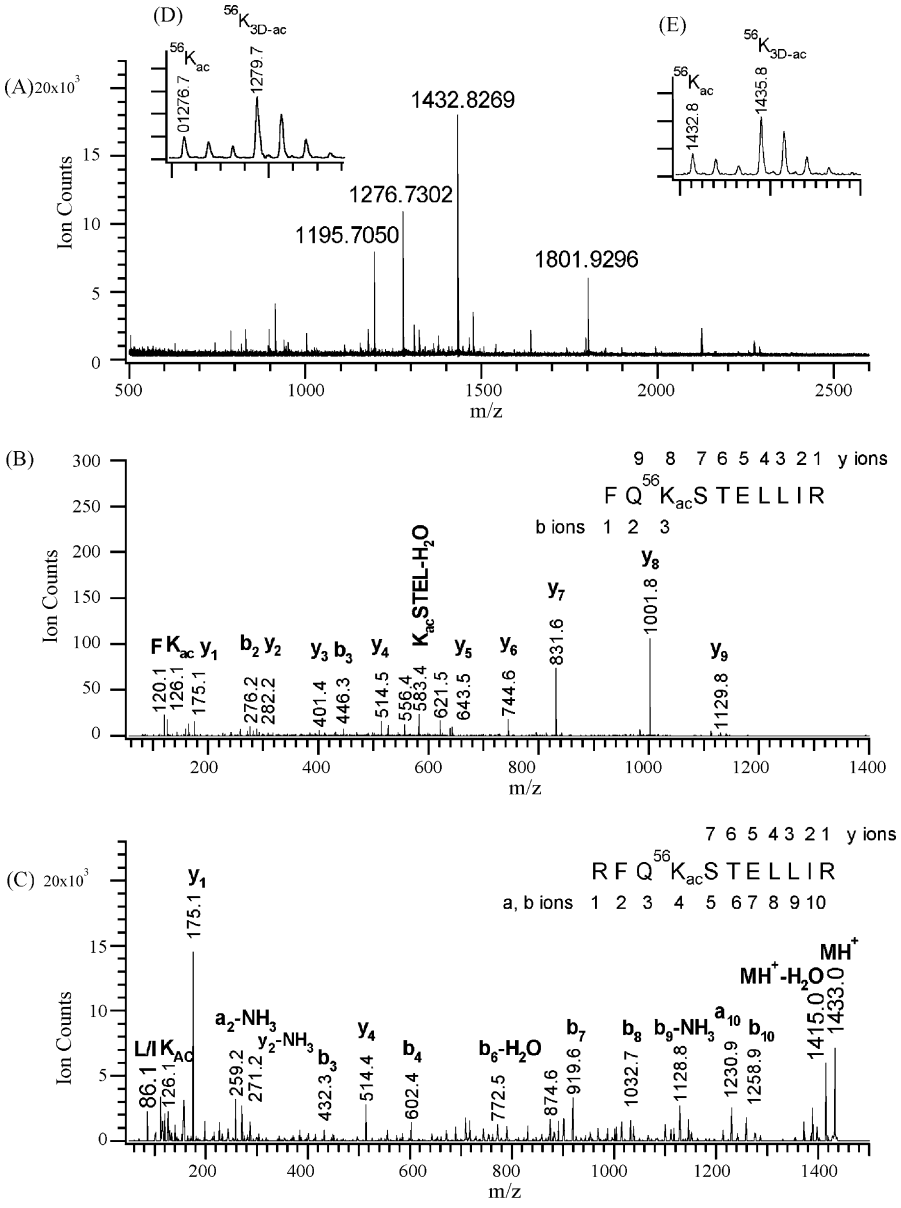

Fig. 7. Analysis of lysine 56 acetylation. (A) MALDI-TOF mass spectrum of HPLC fraction (7) (Fig. 1B) containing acetylated lysine 56 peptide (trypsin digests of H3). Peaks at *m/z* 1276.7 and 1432.8 are the mono-isotopic masses of the peptide FQ^{56}K$_{ac}$STELLIR and RFQ^{56}K$_{ac}$STELLIR, respectively. Peaks at *m/z* 1195.7050 and 1801.9296 (they are sequence-analyzed and data not shown) are the trysin digested peptides IPVLEQELVR and AEAESLVAEAQLSNITR from protein Yp10004cp, which were used as the internal standards to calibrate the spectrum so that an accurate masses (labeled on the top of the peaks) for peaks at *m/z* 1276.7 and 1432.8 were obtained. (B) ESI/MS/MS spectrum of precursor ion at *m/z* 1276.7 (observed doubly charged ion at *m/z* 675.4 in the ESI mode). The fragmentation ions, mainly b and y series ions, match the peptide sequence FQ^{56}K$_{ac}$STELLIR, where lysine 56 is acetylated. An observed immonium ion at *m/z* 126.1 verified the acetylation of a lysine residue. (C) ESI/MS/MS spectrum of precursor ion at *m/z* 1432.8 (observed doubly charged ion at *m/z* 716.9 in the ESI mode). The fragmentation ions, mainly a, b and y series ions, match the peptide sequence RFQ^{56}K$_{ac}$STELLIR, where lysine 56 is acetylated. An observed immonium ion at *m/z* 126.1 verifies the acetylation of a lysine residue. (D) (The insert spectrum in A) Expanded-region display of the MALDI-TOF mass spectra of HPLC fraction (7) (Fig. 1B) containing acetylated lysine 56 peptides (H3 was treated with 3D-acetyl and digested by trypsin), from which the acetylation level for lysine 56 was calculated by intensity-percent to be 27.6%.

forms isolated from the Arg-C digests of H3, we found lysine 36 is 8.1% mono-methylated, 26.4% di-methylated and 62.5% tri-methylated, and lysine 27 is 8.8% acetylated (see Table 2). Using the same strategy, a high accuracy mass measurement was done for the fraction containing peptides with modification at lysine 27 and lysine 36 in chicken histone H3 using the mass (1475.8811 Da) of the peptide ^{27}KSAPATGGV^{36}K$_{me3}$KPHR with tri-methylation at lysine 36 and the mass (1570.6774) of an added peptide Glu-Fib as the internal standards. As shown in Fig. 5E, the exact mass of peak at *m/z* 1447.8 is 1447.8548 Da, matching the peptide ^{27}KSAPATGGV^{36}K$_{me}$KPHR with mono-methylation with a standard deviation of 0.0035 Da; the exact mass of peak at *m/z* 1461.9 is 1461.8654, matching the peptide ^{27}KSAPATGGV^{36}K$_{me2}$KPHR with di-methylation with a standard deviation of 0.0006 Da; the exact mass of peak at *m/z* 1489.9 is 1489.8950, matching the pep-

tide $^{27}K_{me}SAPATGGV^{36}K_{me3}KPHR$ with tri-methylation at lysine 36 and mono-methylation at lysine 27 with a standard deviation of 0.0039 Da; and the exact mass of peak at *m/z* 1503.9 is 1503.9051 Da, matching the peptide $^{27}K_{me2}SAPATGGV^{36}K_{me3}KPHR$ with tri-methylation at lysine 36 and di-methylation at lysine 27 with a standard deviation of 0.0020 Da (Table 1). Modifications at these two sites were also confirmed previously by MALDI-PSD mass spectrometry [9]. Using the intensity-percent quantification method (from a separated Arg-C digestion experiments), we found, in chicken erythrocytes, lysine 36 is roughly 5.9% mono-methylated, 27.6% di-methylated and 66.5% tri-methylated, and lysine 27 is 31.8% mono-methylated, 8.3% di-methylated and 2.5% tri-methylated (Table 2).

3.7. Lysine 79 is hypermethylated

As shown in the MALDI-TOF mass spectrum (Fig. 6A) of HPLC fraction (5), there were three peaks at *m/z* 1349.8, 1363.8 and 1377.8 spacing by 14 Da. They were confirmed by nano-ESI/MS/MS analyses to be the mono-isotopic masses of peptide EIAQDF^{79}KTDL modified with mono-, di- and tri-methyl groups at lysine 79. Intensity-percent calculation showed that lysine 79 in yeast is 15.5% mono-methylated, 23.9% di-methylated and 58.0% tri-methylated (data obtained from Arg-C digestion). The dominant form of lysine 79 methylation is tri-methylation. By contrast, in chicken erythrocytes histone H3 lysine 79 is 59.6 un-modified, 23.9% mono-methylated, 9.5% di-methylated and 6.9% tri-methylated (Table 2). Lysine 79 in chicken erythrocytes is predominately unmodified.

3.8. A novel acetylation site at lysine 56 was observed

The MALDI-TOF mass spectrum of HPLC fraction (5) detected two peaks at *m/z* 1276.7 and 1432.8, which match the mono-isotopic masses of peptides FQKSTELLIR and RFQKSTELLIR, respectively, with a mass gain of 42 Da. This observation indicated that there might be an acetylation or tri-methylation site in these two peptides. Beside peaks 1276.7 and 1432.8, there are other two peaks at *m/z* 1195.7 and 1801.9. Sequence analysis by nano-ESI/MS/MS (spectra not shown) for these two peaks demonstrated that they belong to the mono-isotopic masses of peptide IPVLEQELVR and peptide AEAESLVAEAQLSNITR from protein Yp10004cp which was accompanying with core histones isolated from the yeast nuclei and co-eluted with histone H3 on the HPLC column. Using the known mono-isotopic masses (1195.7050 and 1801.9296) of these two peptides as internal standards to calibrate the MALDI-TOF mass spectrum, the accurate masses for peak 1276.7 and 1432.8 were obtained, which were 1276.7302 and 1432.8269, matching the mono-isotopic mass 1276.7265 of the acetylated peptide FQ$^{56}K_{ac}$STELLIR with an error of 0.0037 Da or 2.9 ppm, and the mono-isotopic mass 1432.8276 of the acetylated peptide RFQ$^{56}K_{ac}$STELLIR with an error of −0.0007 Da or 0.5 ppm, respectively. Sequence analyses were done for these two peptides to confirm their sequence and acetylation sites. Fig. 7B and C showed the MS/MS spectra of ions at *m/z* 1276.7 Da (doubly charged ion at *m/z* 638.9 Da in the ESI mode) and 1432.8 Da (doubly charged ion at *m/z* 716.9 in the ESI mode). The peptide sequences FQKSTELLIR and RFQKSTELLIR were constructed from the fragmentation ions labeled as a, b and y series. The mass difference between y7 and y8, between b2 and b3 in the MS/MS spectrum of the ion at *m/z* 1276.7 Da (Fig. 7B) and the mass difference between b3 and b4 in the MS/MS spectrum of the ion at *m/z* 1432.8 (Fig. 7C) is 170 Da, 42 Da higher than the mass 128 Da of an un-modified lysine, demonstrating that the lysine residues in the two peptides are modified with an acetyl group whose mass is 42 Da. The observed ion at *m/z* 126.1, a signature ion for an acetylated lysine, provided further evidence that this modification is acetylation. Moreover, there was no observation of MH^+-59 ion or y/b-59 ion, indicating that a tri-methylation is not the case for this lysine residue [13]. Therefore, both MALDI-TOF high-accuracy mass measurement and ESI–MS/MS sequence analysis revealed that lysine 56 is acetylated in yeast. Using the chemical acetylation method, 27.6% of lysine 56 was determined to be acetylated (Table 2). We also analyzed chicken H3 and did not observe either acetylation or methylation at lysine 56.

4. Conclusion

We analyzed the lysine acetylation and methylation sites in yeast histone H3 by the combination of mass spectrometry (MALDI-TOF and ESI) and HPLC purification. The relative acetylation/methylation level of each modified site was also quantified by mass spectrometric approaches.

Our dada showed: lysine 4 and 79 are primarily methylated in yeast histone H3 and tri-methylation is the dominant form; by contrast, in chicken erythrocytes histone H3, they are almost un-methylated. Histone H3 lysine 9 is acetylated in yeast, while methylated in chicken erythrocytes. These data support the rationale that methylation of lysine 4, lysine 79 and acetylation of lysine 9 are associated with transcription activation while methylation of lysine 9 is linked to gene silencing [14,15]. Lysine 4 methylation, particularly tri-methyltion, occurs in coding regions of active genes [12]. Lysine 4 and 79 are hypomethylated at silenced loci in yeast, mammalian cells, and chicken erythrocytes [16,17]. It has been well documented that lysine 9 acetylation and methylation are associated with active and inactive chromatin, which is reflected in yeast and chicken erythrocytes [18]. Lysine 14 is acetylated in the yeast, while partially acetylated and partially methylated in chicken erythrocytes. Acetylation of lysine 14 was suspected to initiate phosphorylation of serine 10 [19]. We do not know yet what is the function of lysine 14 methylation. Lysine 18 and 23 are acetylated in yeast, although their acetylation levels are very low compared with chicken erythrocytes in which lysine 18 and 23 are hyper-acetylated. Low acetylation levels of lysine 18 and 23 in yeast probably arise from the removal of acetylation by deacetylases when the cells grow into the stationary phase. However, the reason of hyper-acetylation of lysine 18 and

23 in chicken erythrocytes is still not clear. Mass spectrometric results reported here confirmed previous finding that lysine 27 in yeast is acetylated by an antibody raised against the acetylated lysine 27 [20]. Lysine 27 was also found in hyper-acetylated form in Hela cells [21] in which lysine 4 and 79 are hyper-methylated [10] and all the other N-terminal acetylation target sites (lysine 9, 14, 18 and 23) are acetylated. An association of lysine 27 acetylation with gene activation is logical. By contrast, lysine 27 is methylated in chicken erythrocytes, supporting the perception that methylation of lysine 27 is involved in transcriptional repression [6,22,23]. Interestingly, in both yeast and chicken erythrocytes, lysine 36 is highly methylated. Lysine 36 methylation has been observed to play a role in down-regulating gene expression in yeast [24], and it was also reported to be involved in transcription activation in Tetrahymena [25]. Further work will be necessary to determine the exact biological function(s) of this modification. In addition, a novel acetylation site at lysine 56 in yeast was determined by mass spectrometry. Acetylation at lysine 56 correlates with DNA replication while deacetylation at this site correlates with telomere gene silencing [26,27].

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