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Mapping post-translational modifications of histones H2A, H2B and H4 in *Schizosaccharomyces pombe*

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ABSTRACT

Core histones are known to carry a variety of post-translational modifications (PTMs), including acetylation, phosphorylation, methylation and ubiquitination, which play important roles in the epigenetic control of gene expression. The nature and biological functions of these PTMs in histones from plants, animals and budding yeast have been extensively investigated. In contrast, the corresponding studies for fission yeast were mainly focused on histone H3. In the present study, we applied LC–nano-ESI-MS/MS, coupled with multiple protease digestion, to identify PTMs in histones H2A, H2B and H4 from *Schizosaccharomyces pombe*, the typical model organism of fission yeast. Various protease digestions provided high sequence coverage for PTM mapping, and accurate mass measurement of fragment ions allowed for unambiguous differentiation of acetylation from tri-methylation. Many modification sites conserved in other organisms were identified in *S. pombe*. In addition, some unique modification sites, including N-terminal acetylation in H2A and H2B as well as K123 acetylation in H2A.β, were observed. Our results provide a comprehensive picture of the PTMs of histones H2A, H2B and H4 in *S. pombe*, which serves as a foundation for future investigations on the regulation and functions of histone modifications in this important model organism.

1. Introduction

The eukaryotic nucleosome, which constitutes the fundamental repeating unit of chromatin, plays an important role in packaging and organizing the genetic material [1]. It is comprised of 146 base pairs of DNA wrapped around an octamer of core histone proteins-H2A, H2B, H3, and H4 [1,2]. Core histone proteins are evolutionarily conserved and consist mainly of flexible amino-terminal tails protruding outward from the nucleosome, and globular carboxy-terminal domains making up the nucleosome scaffold [2]. The histone N-terminal tails are involved in the establishment of chromatin structural states, whereas their histone fold domains mediate histone–histone and histone–DNA interactions [3].

Core histones carry a variety of post-translational modifications (PTMs), which include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation. These modifications, occurring mainly on the N-terminal tails [4,5], can affect the interactions of nucleosomes with transacting factors, and are thought to play a role in the assembly and disassembly of chromatin states, ultimately controlling the accessibility of DNA for important cellular processes including transcription, replication, and DNA repair [6–10]. Distinct modifications of the histone tails can recruit specific chromatin-binding proteins, and modifications on the same or different histone tails may be interdependent and generate various combinations on any individual nucleosome [11–13].

Mass spectrometry has been widely used for assessing histone PTMs. It provides direct information about the sites and types of modifications, differentiates isobaric modifications (e.g., acetylation vs. tri-methylation) [14], and allows for quantitative analysis [15]. New histone modifications identified by mass spectrometry facilitated genome-wide chromatin-related functional studies by using chromatin immunoprecipitation [10,16–19].

Schizosaccharomyces pombe, different from budding yeast *Saccharomyces cerevisiae*, is an excellent model eukaryotic organism as fission yeast for studies on epigenetic regulation [20,21]. Previous studies on the PTMs of histones in fission yeast and their biological functions were mainly focused on H3. For example, H3 K4 acetylation was found to mediate chromodomain switch which can regulate heterochromatin assembly in fission yeast [22]. K9-methylated histone H3 can bind strongly to the chromodomain of Chp1, which acts upstream of siRNAs during the establishment of centromeric heterochromatin [23]. H3 K56 acetylation plays an important role in DNA damage response [24]. In these previous studies, identification and characterization of PTMs in *S. pombe* his-

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tones have all relied on the use of modification-specific antibodies, which may bear some potential problems such as cross-reaction, variable specificity, epitope occlusion, and large consumption of time and reagents [25]. Very recently, a few mass spectrometry-based investigations of the PTMs of core histones in *S. pombe* have been reported, which concentrated on core histones H3, H4, and a variant of histone H2A (i.e., H2A.Z) [26,27].

In the present study, we extracted core histones from *S. pombe* and achieved a systematic PTM mapping of histones H2A, H2B and H4, with the combination of digestion with various proteases and analysis with LC–nano-ESI-MS/MS. We report the identification of the conserved PTM sites in *S. pombe* histones that were found previously in other organisms and, more importantly, the unique acetylation sites in H2A and H2B. Our analysis on core histone PTMs sets a stage for examining the regulation of histone modifications and for genome-wide functional studies in fission yeast.

2. Experimental

2.1. Extraction of core histones from S. pombe

S. pombe strain 927 was cultured at 30 °C in a medium containing 0.5% yeast extract, 3% glucose and 0.0225% adenine. Cells were harvested when OD_{600} reached 0.8 and collected by centrifugation at 5000 rpm at 4 °C for 5 min. The cell pellets were washed with sterile water, resuspended in buffer 1 (0.1 M Tris–HCl, pH 8.0, 0.1 M EDTA, 0.5% 2-mercaptoethanol) and incubated at 30 °C for 10 min. After centrifugation, cells were washed with buffer 2 [1 M sorbitol, 20 mM K_3PO_4, 0.1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.5] and resuspended in 20 mL of the same buffer containing 10–15 mg zymolyase T20 and incubated at 30 °C for 30 min with gentle shaking to digest the cell wall. The resulting spheroplasts were incubated in an ice-cold nuclei isolation buffer [0.25 M sucrose, 60 mM KCl, 14 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 mM PMSF, and 0.8% Triton X-100] on ice for 30 min and homogenized substantially with a mini glass homogenizer. The cell pellets were collected by centrifugation at 4000 rpm for 8 min, and washed again with nuclei isolation buffer, followed by washing three times with buffer A (10 mM Tris, pH 8.0, 0.5% NP-40, 75 mM NaCl, 30 mM sodium butyrate, 1 mM PMSF) and twice with buffer B (10 mM Tris, pH 8.0, 0.4 M NaCl, 30 mM sodium butyrate, 1 mM PMSF). After centrifugation, the resulting pellets were resuspended, with occasional vortexing, in 1 mL 0.4 N sulfuric acid at 4 °C for 1 h. The core histones in the supernatant were precipitated with cold acetone, centrifuged, dried and redissolved in water.

2.2. HPLC separation and protease digestion

Core histones were isolated by HPLC on an Agilent 1100 system (Agilent Technologies, Santa Clara, CA) as described previously [28]. The wavelength for the UV detector was set at 220 nm. A 4.6 mm × 250 mm C4 column (Grace Vydac, Hesperia, CA) was used. The flow rate was 0.8 mL/min, and a 60-min linear gradient of 30–60% acetonitrile in 0.1% trifluoroacetic acid (TFA) was employed.

In order to obtain high sequence coverage, purified histones were digested separately with several proteases, including trypsin, Glu-C, Asp-N, and chymotrypsin. A protein/enzyme ratio of 50:1 (w/w) was employed for trypsin and 20:1 for other proteases. The different buffers used for the digestions were 100 mM NH_4HCO_3 (pH 8.0) for trypsin or Glu-C; 50 mM sodium phosphate (pH 8.0) for Asp-N; and 100 mM Tris–HCl (pH 7.8) along with 10 mM $CaCl_2$ for chymotrypsin. The digestion was carried out overnight at room temperature for chymotrypsin and at 37 °C for other proteases. For the limited tryptic digestion, the same protein/enzyme ratio was used but the incubation time was decreased to 4 h. The peptide mixture from the digestion of ~0.2 μg of core histone was subjected directly to LC–MS/MS analysis.

2.3. Mass spectrometry

LC–MS/MS experiments were performed on a 6510 QTOF LC/MS system with HPLC-Chip Cube MS interface (Agilent Technologies). The sample enrichment, desalting, and HPLC separation were carried out automatically on the Agilent HPLC-Chip with an integrated trapping column (40 nL) and a separation column (Zorbax 300SB-C18, 75 μm × 150 mm, 5 μm in particle size). The flow rates for sample enrichment and peptide separation were 4 μL/min and 0.3 μL/min, respectively. A 60-min linear gradient of 2–35% acetonitrile in 0.1% formic acid was applied for peptide separation. The Chip spray voltage (VCap) was set at 1950 V and varied depending on chip conditions. MS/MS experiments were carried out in either the data-dependent scan mode or the pre-selected ion mode. The width for precursor ion selection was 4 *m*/*z* units. The temperature and flow rate for the drying gas were 325 °C and 4 L/min, respectively. Nitrogen was used as collision gas, and collision energy followed a linear equation with a slope of 3 V per 100 *m*/*z* units and an offset of 2.5 V. The raw data obtained in the data-dependent scan mode were converted to Mascot generic format files, and submitted to the Mascot database search engine (Matrix Science, Boston, MA) for protein and PTM identification.

3. Results

To improve the foundation of information on chromatin structure and function in fission yeast, we initiated a systematic investigation of the PTMs of core histones in *S. pombe*. We first extracted core histones from *S. pombe* cells and fractionated individual core histones by using reverse-phase HPLC. Despite multiple attempts using different histone extraction protocols, we were not able to isolate histone H3 from this organism, which might be due to the selective loss of this histone during the extraction processes. The remaining core histones were eluted in the order of H4, H2B and H2A. It is worth noting that, while we were writing up the results of our study, Sinha et al. [27] reported an extraction protocol for core histones from *S. pombe* with the use of glass beads in a beadbeater. With that protocol, these researchers were able to extract core histones including histone H3 from *S. pombe* [27].

Since the PTMs of histones H3 and H4 have been examined by Sinha et al. [27], we decided to focus on the three types of core histones that we were able to isolate and place emphasis on the PTMs of histones H2A and H2B. To this end, we digested the core histones individually with different proteases and analyzed the peptide mixtures with LC–nano-ESI-MS/MS to obtain high sequence coverage and achieve unambiguous PTM assignment. The identified PTM sites are summarized in Fig. 1 and the sequence coverage is shown in Fig. S1.

3.1. Identification of PTMs in histone H2A

Purified H2A was digested individually with trypsin, Asp-N or chymotrypsin under optimized conditions to obtain peptides in appropriate lengths and with good sequence coverage. The digestion mixtures were subjected subsequently to LC–MS/MS analysis, and the acquired mass spectra were searched with Mascot search engine and the results were manually verified.

In H2A, we identified conserved acetylation on K4 and K8, and the unique N-terminal acetylation. Fig. 2A depicts the MS/MS for the doubly charged H2A N-terminal peptide $_1$SGGKSGGKAAVAK$_{13}$ with

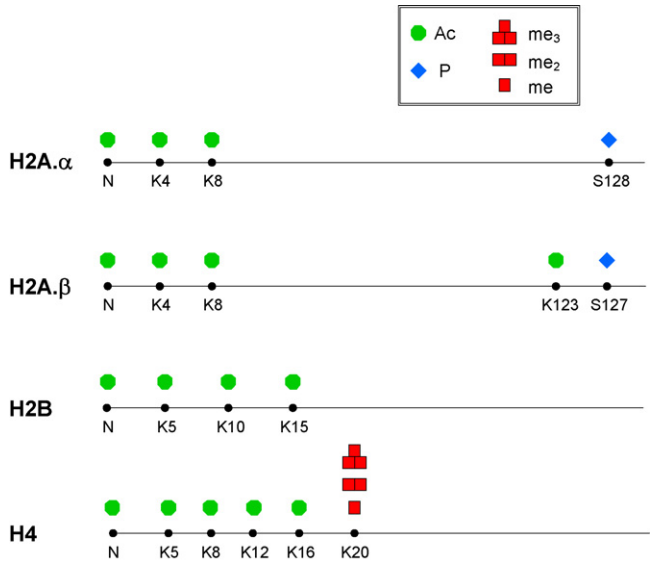

Fig. 1. Summaries of the detected PTMs of *S. pombe* histones H2A, H2B and H4. The modified residues are labeled, and "N" represents N terminus. Acetylation is designated with solid octagon, phosphorylation is shown with solid diamond, and mono-, di-, and tri-methylation are represented by one-, two, and three square boxes, respectively.

three acetyl groups. The presence of the b_2+Ac, b_3+Ac and y_{10}+2Ac, y_{11}+2Ac, y_{12}+2Ac ions supports unambiguously N-terminal acetylation, and this modification has not been found in other organisms. Additionally, the observation of the b_3+Ac and b_4+2Ac as well as y_9+Ac and y_{10}+2Ac ions suggests K_4 acetylation, whereas the presence of y_5, y_6+Ac, y_7+Ac, and y_8+Ac ions demonstrates K8 acetylation.

More interestingly, we found a novel K123 acetylation in the C-terminal region of H2A.β. In the MS/MS of the H2A.β C-terminal peptide $_{120}$QSGKGKPSQEL$_{130}$ (Fig. 2B), we observed an almost complete series of b and y ions; the presence of b_1, b_2, b_3, b_4+Ac and y_5, y_6, y_7, y_8+Ac ions provides solid evidence supporting the K123 acetylation.

Differentiation of tri-methylation from acetylation is essential in PTM studies of histones [14]. Besides the typical method based on the immonium ion with *m/z* 126.1 from acetylated lysine and the neutral loss of a trimethylamine (59 Da) from trimethyl lysine-containing precursor and fragment ions [14], we were able to differentiate, by taking advantage of the high mass accuracy of the QTOF mass spectrometer [29,30], these modifications based on subtle difference in mass increase of the lysine residue introduced by acetylation and tri-methylation. Taking Fig. 2B as an example, the measured mass difference between the y_7 and y_9 ions from the peptide segment housing residues 120–130 was 985.5314 – 758.4048 = 227.1266 Da. This mass difference is in much better agreement with the calculated mass difference with the consideration of K123 acetylation (227.1270 Da, with a mass deviation of 1.8 ppm) than the corresponding mass difference with the consideration of K123 tri-methylation (227.1635 Da, with a mass deviation of 162 ppm). Thus, K123 is acetylated. All the acetylation and tri-methylation sites of *S. pombe* core histones were unambiguously established with this method, as summarized in Table 1.

In addition to acetylation, we observed the phosphorylation of S128 in H2A.α and S127 in H2A.β. The modification sites could again be determined from fragment ions, with the consideration of a mass shift of +80 Da introduced by phosphorylation and −98 Da from the loss of an H_3PO_4. In the MS/MS of the Asp-N-produced H2A.α peptide $_{91}$DEELNKLLGHVTIAQGGVVPNINAHLLPKTSGRTGKPSQEL$_{131}$ (Fig. 2C), the existence of y_1, y_2, y_3, $y_5{}^*$, $y_9{}^*$ ions suggests the S128 phosphorylation ("*" designates those fragment ions carrying a phosphorylated residue), while the similar small y ions formed from the corresponding C-terminal peptide of H2A.β with residues 91–130 support the S127 phosphorylation (Fig. S2A). It is worth noting that, although we observed the peptide carrying either K123 acetylation or S127 phosphorylation, the same peptide carrying simultaneously K123 acetylation and S127 phosphorylation could not be found.

3.2. Identification of PTMs in histone H2B

The isolated histone H2B was digested with trypsin and Glu-C separately and subjected subsequently to LC–MS/MS analysis. A sequence coverage of 100% was reached and multiple conserved acetylation sites including the N-terminus, K5, K10, and K15 were identified. In the MS/MS of the tetra-acetylated peptide $_1$SAAEKKPASKAPAGKAPR$_{18}$ (Fig. 3), N-terminus was determined to be acetylated based on the observation of b_2+Ac and y_{17}+3Ac ions. Mass difference between b_4+Ac and b_5+2Ac, together with that between y_{13}+2Ac and y_{14}+3Ac, supports K5 acetylation. Additionally, the presence of b_9+2Ac and b_{10}+3Ac, along with y_8+Ac and y_9+2Ac ions, demonstrates K10 acetylation. Moreover, K15 acetylation is determined by the formation of y_3 and y_4+Ac ions.

Table 1
The fragment ion mass comparison of histone peptides to differentiate tri-methylation from acetylation based on MS/MS data acquired on the Agilent 6510 Q-TOF mass spectrometer. For each modification site, two b or y ions flanking the modified lysine were chosen. The experimental mass difference (Measured ΔMass) of these two flanking b or y ions was calculated, so were the corresponding theoretical mass differences with the lysine being tri-methylated or acetylated (Calcd. ΔMass, ac/me3). The two mass deviations (M.D.) between the Measured ΔMass and Calcd. ΔMass were further calculated, with one being markedly smaller than the other. The modification type at the target lysine could be determined as the one with smaller deviation.

Peptide	Modification	Chosen ions	Measured Mass 1	Measured Mass 2	Measured ΔMass	Calcd. ΔMass,ac	M.D./ppm	Calcd. ΔMass,me3	M.D./ppm
H2A: 1–13	N. Ac	b2	0	187.0698	187.0698	187.0714	**8.6**	187.1079	203.6
	K4 Ac	y9, y10	830.4696	1000.5679	170.1053	170.1055	**1.2**	170.1420	215.7
	K8 Ac	y5, y6	459.2917	629.3988	170.1071	170.1055	**−9.4**	170.1420	205.1
120–130	K123 Ac	y7, y9	758.4048	985.5314	227.1266	227.1270	**1.8**	227.1635	162.4
H2B: 1–18	N. Ac	b2	0	201.0823	201.0823	201.0870	**23.4**	201.1235	204.8
	K5 Ac	y13, y14	1362.7919	1532.8941	170.1022	170.1055	**19.4**	170.1422	235.1
	K10 Ac	b9, b10	954.5077	1124.6095	170.1018	170.1055	**21.8**	170.1422	237.4
	K15 Ac	y3, y5	343.2083	570.3375	227.1292	227.1270	**−9.7**	227.1635	151.0
H4: 4–17	K5 Ac	y12, y13	1211.6855	1381.7927	170.1072	170.1055	**10.0**	170.1422	205.7
	K8 Ac	y9, y10	927.5331	1097.6359	170.1028	170.1055	**15.9**	170.1422	231.6
	K12 Ac	y5, y7	530.2975	757.4286	227.1311	227.1270	**−18.1**	227.1635	142.6
	K16 Ac	y1, y2	175.1171	345.2194	170.1023	170.1055	**18.8**	170.1422	234.5
1–17	N. Ac	b2	0	187.0702	187.0702	187.0714	**6.4**	187.1079	201.5
20–35	K20 3Me	b2	0	284.2308	284.2308	284.1969	−119.3	284.2334	**9.1**

Smaller M.D.s are highlighted in bold, which suggested modification types.

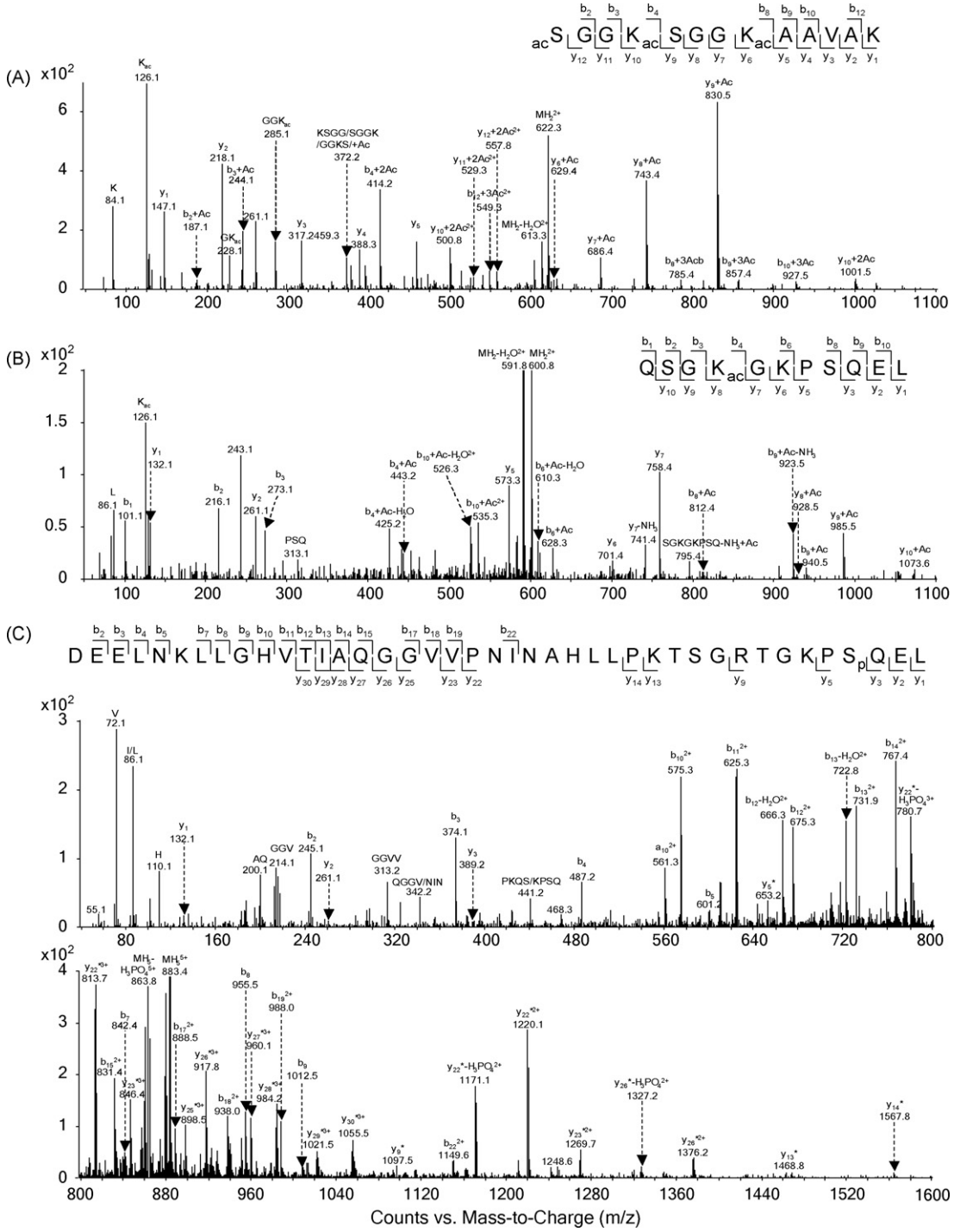

Fig. 2. ESI-MS/MS of tri-acetylated N-terminal tryptic peptide $_{1}$SGGKSGGKAAVAK$_{13}$ (A), mono-acetylated C-terminal tryptic peptide $_{120}$QSGKGKPSQEL$_{130}$ (B) of histone H2A, and Asp-N-produced phosphorylated peptide $_{91}$DEELNKLLGHVTIAQGGVVPNINAHLLPKTSGRTGKPSQEL$_{131}$ (C) of H2A.α isolated from *S. pombe*, in which b_n* and y_n* designate those fragment ions carrying a phosphorylated residue.

3.3. Identification of PTMs in histone H4

Purified histone H4 was digested by trypsin, Asp-N and chymotrypsin separately and subjected to LC–MS/MS analysis. All the modifications were located on the N-terminal segment, similar to the situation observed for other organisms. Upon limited tryptic digestion (i.e., with short incubation time), the miss-cleaved N-terminal peptide $_{1}$SGRGKGGKGLGKGGAKR$_{17}$ was obtained. Fig. 4A shows the MS/MS of the di-acetylated peptide with residues 1–17. The formation of b_n+Ac (n = 2, 3, 5–7, 11–13) and y_{16}+Ac ions, but not y_{16}+2Ac ion, supports the N-terminal acetylation, whereas the observation of y_1 and y_2+Ac ions underscores the K16 acetylation. In addition, K5, K8, K12 were all found to be acetylated, as exemplified by the observations of the complete series of b and y ions in the MS/MS of the tetra-acetylated peptide with residues 4–17 (Fig. 4B).

As conserved in other organisms, K20 in *S. pombe* could be mono-, di- and tri-methylated. In this regard, the positive-ion ESI-MS (Fig. S3A) reveals the presence of 0, 1, 2 and 3 methyl groups in the peptide $_{20}$KILRDNIQGITKPAIR$_{35}$. The MS/MS of this group of

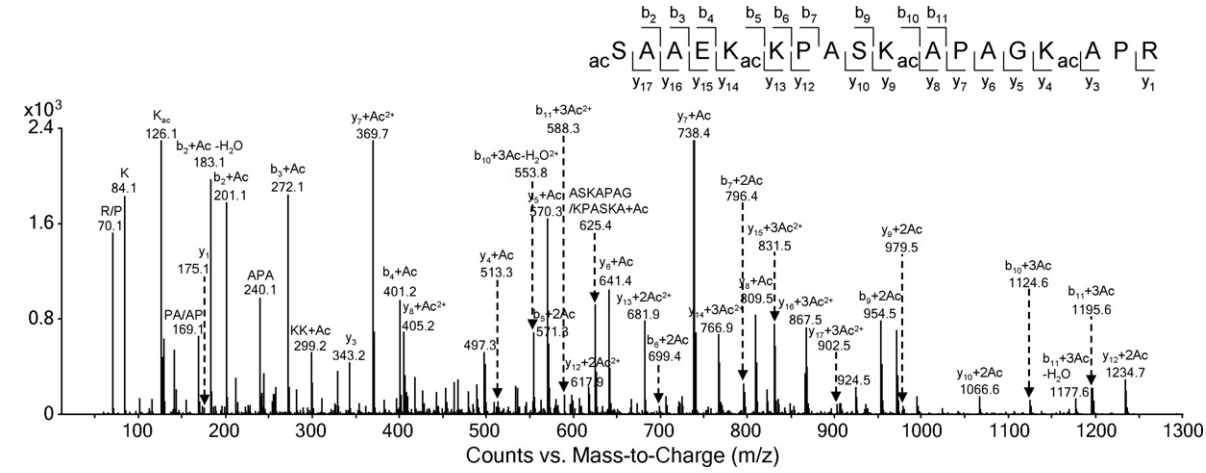

Fig. 3. The ESI-MS/MS of the *S. pombe* H2B N-terminal peptide $_1$SAAEKKPASKAPAGKAPR$_{18}$ with the N-terminus, K5, K10, and K15 being acetylated.

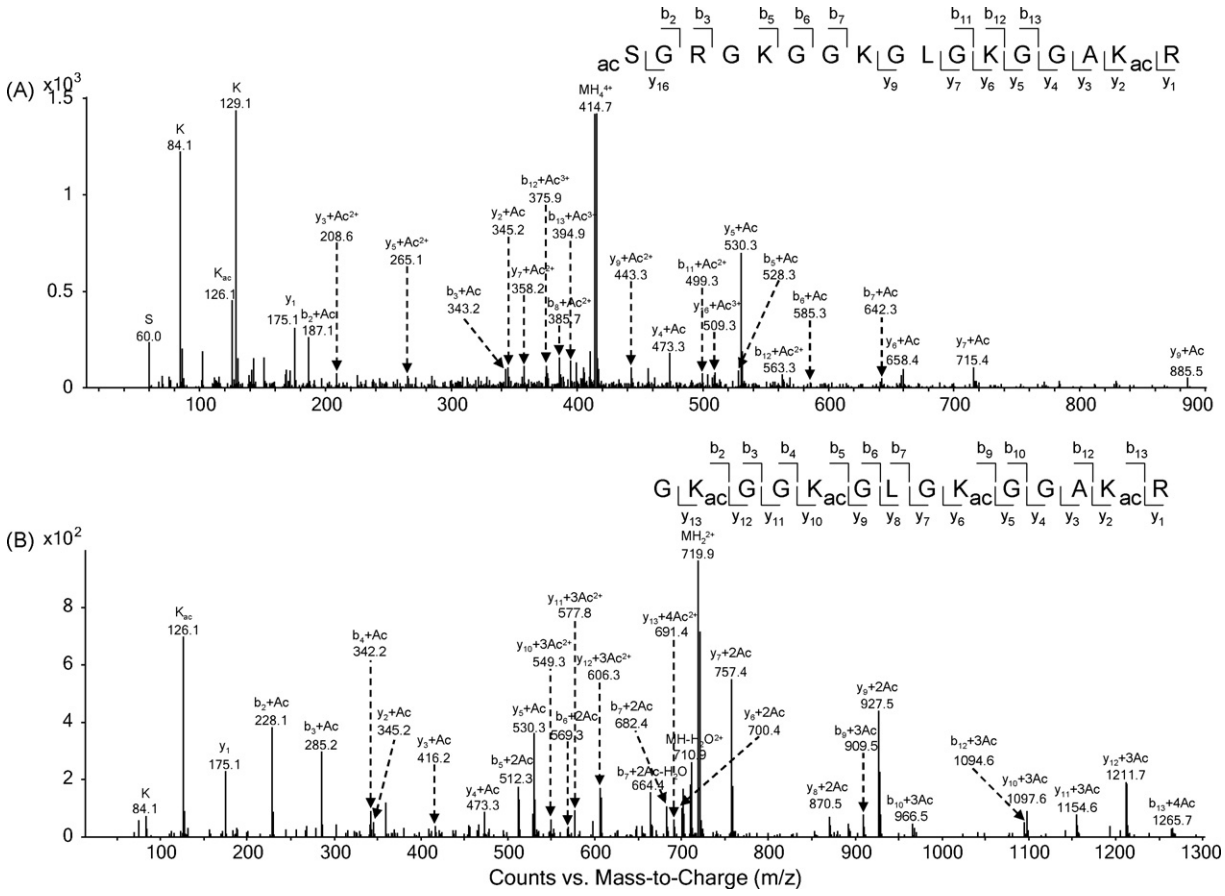

Fig. 4. The ESI-MS/MS of the *S. pombe* H4 N-terminal peptides, $_1$SGRGKGGKGLGKGGAKR$_{17}$ with the N-terminus and K16 being acetylated (A), and $_4$GKGGKGLGKGGAKR$_{17}$ with K5, K8, K12 and K16 being acetylated (B).

peptides were all acquired with the selected-ion monitoring (SIM) mode of analysis (Fig. S3). In the MS/MS of the tri-methylated peptide (Fig. S3E), the presence of b_2+3Me and y_{14} ions and the abundant fragment ions with the loss of a trimethylamine reveals the tri-methylation on K20. The observation of similar modified small b ions and unmodified large y ions in the MS/MS of the mono- and di-methylated peptides provides solid evidence for K20 mono- and di-methylation, respectively (Fig. S3C and D). Our above findings with the PTMs of histone H4 are consistent with what was recently reported by Sinha et al. [27].

4. Discussion

We provided a thorough mapping of PTMs for core histones H2A, H2B and H4 in *S. pombe*, by using LC–nano-ESI-MS/MS coupled with digestion using different proteases. Our work complements the results published very recently by Sinha et al. [27], where the post-translational modifications of core histones H3 and H4 were investigated, and provides a complete picture about the PTMs on core histones in *S. pombe*. The various protease digestions provided high sequence coverage and the accurate mass measurement of

fragment ions allowed for unambiguous differentiation of acetylation from tri-methylation. Our results showed that most N-terminal acetylation and methylation sites and H2A C-terminal phosphorylation site are conserved among different organisms including mammals, budding yeast and plants [15,31–34]. In addition, some novel modifications were detected for the first time.

H2A was found to bear acetylation on N-terminus, K4 and K8, and H2A.β carries an additional acetylation site on K123. In addition, phosphorylation occurs on S128 in H2A.α and S127 in H2A.β. The acetylation sites found in H2B include the N-terminus, K5, K10 and K15. Moreover, H4 was found to carry methylation on K20 and acetylation on the N-terminus, K5, K8, K12, and K16 (Fig. 1). Nevertheless, it is worth noting that some low levels of modification in *S. pombe* might escape the detection even with the combination of digestion with multiple proteases and the use of a sensitive Q-TOF mass spectrometer with nanospray ionization interface.

Two *S. pombe* H2A isoforms H2A.α and H2A.β are acetylated at K4 and K8, and the similar acetylation was also found in *S. cerevisiae* at K4 and K7. In *S. cerevisiae*, K4 acetylation has been shown to be essential for efficient silencing [35], and H2A K7 acetylation together with H4 N-terminal acetylation was required to maintain chromosome stability [36]. The functions of these acetylations in H2A of *S. pombe* would require further investigation. More interestingly, H2A N-terminal acetylation and H2A.β K123 acetylation were observed here for the first time. It is important to study their functions and crosstalk with other histone modifications.

Aside from acetylation, we also observed C-terminal phosphorylation in H2A, which was located at S128 in H2A.α and S127 in H2A.β. These phosphorylations were reported to control Crb2 recruitment at DNA breaks, maintain checkpoint arrest, and influence DNA repair in fission yeast [37].

In *S. pombe* H2B, we found conserved acetylation at K5, K10 and K15. It has been reported that the acetylation of these lysine residues in budding yeast H2B activates the transcription of genes involved in NAD biosynthesis and vitamin metabolism [38]. Thus, it will be interesting to assess the role of H2B acetylation in transcription activation in fission yeast. Moreover, we observed acetylation on the N-terminus of H2B.

Histone H4 acetylation sites, including the N-terminus, and residues K5, K8, K12 and K16, are conserved in almost all organisms, including *S. pombe*. They play important roles in many processes including transcriptional activation, DNA double strand break repair and cellular lifespan regulation [39,40]. H4 K20 methylation, which is absent in budding yeast, was found in *S. pombe*, in the form of mono-, di- and mainly tri-methylation [41]. It has been reported that this methylation plays important roles in heterochromatin silencing and DNA damage response, similar as H4 in humans and *Drosophila melanogaster* [41–44].

In summary, a systematic PTM mapping of histones H2A, H2B and H4 in *S. pombe* was obtained by mass spectrometric analyses. The rigorous identification of modification sites provides a foundation for further studies on the function of histone PTMs in fission yeast.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.08.015.

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