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Comprehensive Mapping of Post-Translational Modifications on Synaptic, Nuclear, and Histone Proteins in the Adult Mouse Brain

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Post-translational modifications (PTMs) of proteins in the adult brain are known to mark activity-dependent processes for complex brain functions such as learning and memory. Multiple PTMs occur in nerve cells, and are able to modulate proteins in different subcellular compartments. In synaptic terminals, protein phosphorylation is the primary PTM that contributes to the control of the activity and localization of synaptic proteins. In the nucleus, it can modulate histones and proteins involved with the transcriptional machinery and, in combination with other PTMs such as acetylation, methylation and ubiquitination, acts to regulate chromatin remodelling and gene expression. The combination of histone PTMs is highly complex and is known to be unique to each gene. The ensemble of PTMs in the adult brain, however, remains unknown. Here, we describe a novel proteomic approach that allows the isolation and identification of PTMs on synaptic and nuclear proteins, in particular on histones. Using subcellular fractionation, we identified 2082 unique phosphopeptides from 1062 phosphoproteins, and 196 unique PTM sites on histones H1, H2A, H2B, H3 and H4. A comparison of phosphorylation sites in synaptic and nuclear compartments, and on histones, suggests that different kinases and kinase motifs are involved. Overall, our data demonstrates the complexity of PTMs in the brain and the prevalence of histone PTMs, and reveals potentially important regulatory sites on proteins involved in synaptic plasticity and brain functions.

Keywords: Histones • Brain • Histone code • Acetylation • Methylation • Ubiquitination • Phosphorylation • Synapse • Nucleus

Introduction

The acquisition and storage of information in memory requires specific long-lasting changes in gene expression. These changes involve multiple neuronal components in distinct subcellular compartments that include synaptic terminals and the nucleus. Among these components, neurotransmitter receptors and associated proteins at synapses, and histone proteins in the nucleus, are important because they allow the coupling of synaptic events to intracellular signal transduction pathways and to activity-dependent gene expression. These signaling pathways rely on reversible and site-specific post-translational modifications (PTMs) of proteins in synaptic and nuclear compartments. Protein phosphorylation, in particular, is a key regulatory PTM that is highly dynamic and reversible. The switch between phosphorylation and dephosphorylation is controlled by the balance of protein kinases and protein phosphatases. This balance regulates multiple synaptic and nuclear proteins, including transcription factors and components of the transcriptional machinery for the control of gene expression. In addition to phosphorylation, other PTMs including acetylation, methylation and ubiquitination are also required. When occurring on histone proteins, they can change chromatin structure and modulate transcriptional activity. Histone PTMs are highly dynamic and are controlled by multiple enzymes that colocalize to form histone-modifying complexes. The best characterized PTM is histone acetylation, which is catalyzed by histone acetyl transferases (HATs) and reversed by histone deacetylases (HDACs). Histone methylation is mediated by methyltransferases (HMTs), and demethylation by histone demethylases, while histone ubiquitination is induced by ubiquitin conjugating enzymes. Recent studies have demonstrated that histone H3 phosphorylation and acetylation are induced during memory formation, and that they occur through signaling pathways involving IκB and MAPKs.[1] Additional evidence has also suggested that the epigenetic regulation of chromatin structure mediates long-lasting changes that strongly influence behavior and cognitive functions.[2] However, these studies have not examined the complete ensemble of PTMs that can occur on brain proteins, and to date, a complete map of protein PTMs in synaptic and nuclear compartments is lacking. Such a map in the brain is, however, a prerequisite to a better understanding of signaling pathways underlying synaptic plasticity and higher-order cognitive functions.

Mass spectrometry (MS) is an ideal tool for generating such a map, and for analyzing and identifying *de novo* protein PTMs. Its application in neuroscience has grown significantly over the

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past years and has allowed novel analyses of biochemical processes in the nervous system.[3] Several recent studies have for instance revealed the existence of thousands of phosphorylation sites on brain proteins.[4−6] However, while these analyses have been performed on brain tissue, to date, histone PTMs have been almost exclusively identified in cultured cells. Since *in vitro* conditions do not necessarily reflect *in vivo* conditions,[7] their identification may be biased and require confirmation in *in vivo* preparations.

To achieve a physiologically relevant method for the identification of protein PTMs *in vivo*, we applied high mass accuracy MS/MS to synaptic, nuclear and histone proteins extracted from the adult mouse brain. This approach enables us to detect and identify with high confidence novel PTM sites on key synaptic, nuclear and histone proteins. These sites were found on proteins belonging to diverse functional groups, and their features, for instance phosphorylation motifs, were revealed to differ between synaptic, nuclear, and histone proteins. Overall, these analyses highlight the existence of an extensive pool of histone PTMs in the brain.

Materials and Methods

C57BL/6 Mice. Brain tissue isolated from adult 6−12 month old C57BL/6 mice was used for all experiments. Mice were maintained in standard conditions under a reversed light cycle (dark phase, 7 a.m. to 7 p.m.). All experiments were carried out in accordance with guidelines and regulations of the Cantonal Veterinary Office, Zürich.

Subcellular Fractionation of Brain Tissue. Synaptosomes and synaptic membranes were isolated as described previously.[4] Briefly, mice were sacrificed by cervical dislocation and the cortex was rapidly removed and washed in ice-cold buffer A (0.32 M sucrose, 4 mM HEPES, pH 7.4, 1× protease cocktail inhibitor and phosphatase inhibitor cocktail I and II (Sigma)). The tissue was homogenized in 10 vol of buffer A by 10 up-and-down strokes of a homogenizer. The resulting homogenate was centrifuged at 1000*g* for 10 min to remove nuclei and cell debris, and the supernatant was collected. The pellet was washed again and both supernatants were combined. The combined supernatants were centrifuged at 17 000*g*; the pellet was washed, layered on a 1.2 M sucrose gradient, and then centrifuged at 15 000*g* for 20 min. The interphase was collected, layered on a 0.8 M sucrose gradient, and spun again at 15 000*g* for 20 min to give a pellet containing synaptosomes. Synaptosomes were lysed by hypotonic shock in 9 vol of H_2O, HEPES was added to give 4 mM, and samples were incubated with shaking for 30 min on ice before centrifugation at 25 000*g* to pellet membranes. The resulting synaptic membranes were mixed with 0.32 M sucrose, layered over a 0.8 M sucrose solution, and centrifuged for 20 min at 230 000*g* to separate synaptic membranes and mitochondria from myelin. The pellet containing synaptic membranes and mitochondria was resuspended in 0.32 M sucrose, layered over a 1.2 M sucrose solution, and centrifuged for 20 min at 230 000*g* to pellet synaptic membranes and then snap frozen. Nuclei were obtained by homogenizing brain tissue in lysis buffer (Sigma Nuclei Pure isolation kit) with the addition of 1× protease cocktail inhibitor and phosphatase inhibitor cocktail I and II (Sigma). Homogenates were mixed with 2 vol of 1.8 M sucrose and layered on top a 1.8 M sucrose gradient. Nuclei were pelleted by centrifugation at 13 000*g* for 45 min and then snap frozen at −80 °C until analyzed.

Histone Isolation and Fractionation. Isolated nuclei were resuspended in 400 μL of 0.4 N H_2SO_4 and incubated for >2 h at 4 °C with end-over-end rotation, then centrifuged at 16 000*g* for 10 min to remove nuclear debris. The histone-containing supernatant was transferred to a new tube and the histones were precipitated by the dropwise addition of 132 μL of trichloroacetic acid (TCA) followed by a 30 min incubation on ice. After centrifugation at 16 000*g* for 10 min, the pellet containing histones was washed twice with ice-cold acetone and centrifuged a second time. The pellet was then dissolved in 100 μL of H_2O, sonicated for 10 min, and spun at 13 000 rpm for 10 min, and the supernatant was loaded on an Agilent C8 column attached to an Agilent HP1100 binary HPLC system. Histone variants were separated and eluted with the following gradient: 0−5 min, 0% solvent B; 5−15 min, 0−35% Buffer B; 15−25 min, 35% Buffer B, 25−75 min, 35−65% Buffer B (see Supplementary Figure 2). Buffer A was 5% acetonitrile (ACN) in 0.1% TFA and Buffer B was 90% ACN in 0.1% TFA.[8]

In-Solution Trypsin Digestion of Synaptic and Nuclear Proteins. For tryptic digestions, synaptic and nuclear protein fractions were desalted by acetone precipitation and then solubilized in 50 mM ammonium bicarbonate, pH 8.0, and 0.1% TX-100. Samples were reduced with 12.5 mM dithiothreitol (DTT) for 30−60 min and alkylated with 40 mM iodoacetamide for 1 h. Samples were digested overnight with trypsin (Promega) at 37 °C (1:30 enzyme/substrate).

In-Solution Semitryptic Digestion of Histone Proteins. For semitryptic digestions, RP-HPLC fractions of histones were collected, lyophilized and redissolved in 50 mM ammonium bicarbonate, pH 8.0. The samples were heated for 15 min at 60 °C prior to digestion for 2 h with trypsin (Promega) at 37 °C (1:200 enzyme/substrate). TFA was added to a final concentration of 0.1% to stop the digest. To prevent potential chemical artifacts,[9] histone samples were not alkylated with iodoacetamide.

SCX-HPLC Enrichment of Phosphopeptides Prior to IMAC/TiO_2. The peptide digests were acidified to pH < 3 with 10% TFA, and ACN was added to a final concentration of 25%. The solution was then centrifuged at 16 000*g* for 10 min to remove insoluble matter. Peptides were loaded onto a 4.6 × 200 mm polySULFOETHYL aspartamide A column (PolyLC) on an Agilent HP1100 binary HPLC system. Phosphopeptide rich fractions were eluted with an increasing KCl gradient (0−105 mM over 30 min and 105 mM to 350 mM over the following 20 min) in 10 mM KH_2PO_4 and 25% ACN, pH 3, as described previously.[4] The early eluting phosphopeptide-rich fractions were lyophilized to remove acetonitrile, desalted with Sep-Pak reversed-phase cartridges (Waters, U.K.) and lyophilized again prior to phosphopeptide enrichment with IMAC or TiO_2.

IMAC Phosphopeptide Enrichment. Iminodiacetic acid-coupled sepharose Fast-Flow Beads (Amersham Biosciences) were washed with 5 vol of H_2O, 5 vol of wash buffer (74:25:1 H_2O/ACN/acetic acid), and 5 vol of 1 mM $FeCl_3$ before equilibration with 5 vol of wash buffer. Peptides were loaded onto 75 μL of a 25% bead-slurry and incubated at room temperature for 30 min. The samples were carefully washed three times with wash buffer and eluted with 100 mM sodium phosphate buffer, pH 8.9, as described previously.[4] Samples were concentrated and desalted with C18 MicroTip Columns (Nest Group), lyophilized and stored at −80 °C prior to MS analysis.

TiO_2 Phosphopeptide Enrichment. Two milligrams of TiO_2 resin (5 μm titanosphere, GL Sciences) was washed consecu-

tively with 200 μL of H_2O and 200 μL of methanol, then equilibrated with 300 μL of saturated phthalic acid solution (80% ACN, 0.2% TFA, phthalic acid). Peptides were resuspended in 300 μL of phthalic acid solution, applied to the TiO_2 resin, then incubated for 15 min with end-over-end rotation. The resin was washed twice with 200 μL of phthalic acid solution, then once with 300 μL followed by 150 μL of 80% ACN and 0.1% TFA solution, then twice with 300 μL of 0.1% TFA. Phosphopeptides were eluted twice with 150 μL of 0.3 M ammonium hydroxide solution, and quickly acidified to pH < 3 using 10% TFA. Samples were concentrated and desalted with C18 MicroTip Columns (Nest Group) before lyophilization and stored at −80 °C prior to MS analysis.

MS Analysis. Samples were analyzed on a 4800 Proteomics Analyzer MALDI TOF/TOF, ESI-LTQ-FT or ESI-LTQ-Orbitrap as described in the Supplementary Methods.

Database Analysis. MS and MS/MS data were searched using Mascot version 2.1.[10] Database searches of MS/MS spectra were performed using a mouse protein database downloaded from the European Bioinformatic Institute (EBI, 42 656 sequences; 20 120 892 residues, release date: 29/04/2008). Modifications used for searches included carbamidomethyl (C, fixed, nonhistone searches), phosphorylation (STY, variable), pyro-Glu (N-term Q, variable), acetyl (N-term protein and K, variable, histone searches), mono-, di-, and trimethylation (R and K, variable, histone searches) and the GlyGly and LRGG motifs for ubiquitination (K, variable, histone searches). Only strictly tryptic peptides[11] with a maximum of 1 (fully tryptic digests) or 3 (semitryptic histone digests) missed cleavage sites were allowed in database searches. For ThermoFinnigan LTQ-FT and Orbitrap measurements, the monoisotopic masses of +1, +2 and +3 charged peptides were searched with a peptide tolerance of 6 ppm and an MS/MS tolerance of 0.5 Da for fragment ions. For MALDI measurements, the monoisotopic masses of +1 charged peptides were searched with a peptide tolerance of 25 ppm and MS/MS tolerance of 0.2 Da for fragment ions. Data was searched against a forward-reverse concatenated mouse database to calculate the false discovery rate.[12,13]

Positive Identification of Modified Residues. Positive identification of phosphorylated, acetylated, methylated or ubiquitinated peptides was performed using a variety of strict criteria including manual inspection of spectra. Only bold-red, rank 1 peptides with Mascot expect values of less than 0.05 were considered. Normalized delta ion-scores were calculated for all phosphopeptides by taking the difference in the ion score for the two top ranking peptides and dividing that difference by the first ranking peptide's ion score.[4,14] All phosphopeptides with normalized delta ion-scores of less than 0.4 were evaluated for precise site assignment, and ambiguous sites were indicated by parentheses. In addition, spectra from all peptides derived from histones were manually validated for site placement and ambiguous sites indicated by parentheses. The confirmation of modification sites was primarily based on the presence of site-specific singly or doubly charged b and y type fragment ions (b and y ions generated by cleavages between two potentially modified residues). Relative intensities of essential diagnostic fragment ions were checked in MS/MS spectra. Rules for increased or decreased peptide cleavage probability were taken into account (enhanced cleavage on the N-terminal side of proline and the C-terminal side of aspartic acid; reduced cleavage on the C-terminal side of proline). All identified peptides with PTMs and their respective spectra are listed in Supplementary Figure 10 and Supplementary Table 1 (Synaptic and Nuclear phosphopeptides), and Supplementary Figure 11 and Supplementary Table 2 (Histone peptides). For many spectra, the dominant peak was not labeled by Mascot but could be manually identified as the precursor ion with neutral loss or loss of water (see also Figure 4A,C). Using commercially available histone PTM antibodies, we could also independently demonstrate the existence of a selection of these histone modifications in our histone samples (see Supplementary Figure 6).

Bioinformatics. WebGestalt[15] was used to determine significantly enriched GO classes. UniProt[16] was used for analysis of biological function, molecular function and cellular location. Heat plots were generated using Genesis.[17] Motif-X[18] was used for extracting PTM motifs and for aligning peptides. WebLogo[19] was used for generating frequency plots of amino acids surrounding the site of modification. Scansite[20] was used to determine likely kinases for detected phosphorylation sites. The human protein reference database (HPRD) was used for analyzing phosphorylation motifs.[21] PESTfind[22] was used to find hydrophilic regions in histones.

Results and Discussion

A Comprehensive Data Set of PTMs in Subcellular Fractions of the Brain. In the nervous system, many proteins are subjected to PTMs in an activity dependent-fashion and in discrete cellular compartments. In synaptic terminals, phosphorylation controls basal synaptic functions,[23,24] and can regulate gene expression in the nucleus. The ensemble of histone PTMs that constitute the 'histone code' is a particularly important mechanism of regulation through which histone phosphorylation together with methylation, acetylation and ubiquitination alters the accessibility of DNA to the transcriptional machinery.[25] To analyze protein PTMs in distinct subcellular fractions in brain tissue and collect a comprehensive data set of PTMs sites on brain proteins, we combined methods for brain tissue fractionation,[3,4] phosphopeptide enrichment[26] and several MS platforms.[27] Subcellular fractionation enabled us to selectively isolate synaptic, nuclear and histone proteins, and conduct highly specific analyses (Supplementary Figure 1). These analyses identified approximately 2000 phosphorylation sites on synaptic and nuclear proteins, and 196 unique sites of acetylation, methylation, phosphorylation and ubiquitination on histone proteins (Tables 1 and 2 and Supplementary Tables 1 and 2). A large number of these PTMs were novel sites located on key regulatory proteins involved in signal transduction from synapses to the nucleus, including NR2B, stargazin, PKC and MAPK (Figure 1D). In total, we identified 142 novel sites of modification on histone proteins (Figure 3C, Table 1 and Supplementary Table 2). These results are notable because they greatly extend the list of currently known PTMs, which to date was only approximately 60 across all histone variants.[7] The depth of coverage of our analyses is evident by the fact that, in addition to novel sites, more than two-thirds of all previously described histone PTMs were identified in our data set (Supplementary Table 3). This was achieved in part by the use of high accuracy MS/MS that allowed accurate site placement, and sequence coverage greater than 95% across most histone variants, which enhanced confidence in peptide and PTM identification in these analyses. High accuracy data also meant that the false discovery rate, as determined by reverse database searches, was only 0.53% at the peptide level. This data overall represents a significant increase in knowledge and should help

Table 1. Unique PTM Sites Found on Histones H1, H2A, H2B, H3, H4 and Variants[a]

Histone	Peptide sequence and modification	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability	Spectral count
Core histone macro-H2A.2	K.AKDSDKEGTpSNSTSEDGPGDGFTILSSK.S	2909.24440	2909.24510	-0.00070	8.90E-07	2
H1 histone family, member X	M.ac-SVELEEALPPTSADGTAR.K	1883.90910	1883.91120	-0.00210	4.80E-16	3
H1 histone family, member X	M.ac-SVELEEALPPTSADGTARK.T	2012.01080	2012.00620	0.00470	2.50E-06	2
H1 histone family, member X	R.RGApSAASSPAPK.A	1178.54470	1178.54450	0.00030	7.10E-03	3
Histone H1.0	-.ac-MTENSTSAPAAKPK.R	1473.71300	1473.71330	-0.00030	2.30E-05	2
Histone H1.0	K.KAKKKub1PAATPK.K	1280.79420	1280.79280	0.00140	4.20E-02	2
Histone H1.0	K.KPAATPKub1K.A	953.56510	953.56580	-0.00070	3.40E-02	2
Histone H1.0	M.ac-TENSTSAPAAKPK.R	1342.67400	1342.67280	0.00120	2.20E-06	21
Histone H1.0	M.ac-TENSTSAPAAKPKR.A	1498.77880	1498.77390	0.00480	7.80E-07	9
Histone H1.0	R.LVTTGVLKQTKub1.G	1300.77320	1300.77150	0.00170	9.10E-03	2
Histone H1.0	K.KPKApTPVKK.A	1075.61680	1075.61540	0.00140	3.70E-02	2
Histone H1.0	M.ac-TENpSTSAPAAKPK.R	1422.64040	1422.63910	0.00120	7.60E-03	2
Histone H1.0	M.TENpSTSAPAAKPK.R	1380.62980	1380.62860	0.00120	9.50E-05	5
Histone H1.1	M.ac-pSETAPVAQAASTATEKPAAAK.K	2121.01020	2120.99900	0.01120	1.00E-05	5
Histone H1.1	M.ac-SETAPVAQAApSTATEKPAAAK.K	2121.00800	2120.99900	0.00900	1.80E-03	2
Histone H1.1	M.ac-SETAPVAQAASTApTEKPAAAKK.T	2249.09720	2249.09400	0.00330	5.80E-03	3
Histone H1.1	M.ac-SETAPVAQAASTATEKacPAAAKK.T	2211.14820	2211.13820	0.01000	9.40E-10	3
Histone H1.1	M.ac-SETAPVAQAASTATEKPAAAK.K	2041.03190	2041.03270	-0.00080	3.20E-13	10
Histone H1.1	M.ac-SETAPVAQAASTATEKPAAAKK.T	2169.13280	2169.12770	0.00520	2.30E-15	7
Histone H1.1	M.ac-SETAPVAQAASTATEKPAAAKKme1.T	2183.14680	2183.14330	0.00350	4.30E-05	3
Histone H1.1	M.ac-SETAPVAQAASTATEKme1PAAAKK.T	2183.15040	2183.14330	0.00710	2.80E-05	2
Histone H1.1	M.ac-pSETAPVAQAASTATEKPAAAKK.T	2249.09920	2249.09400	0.00530	1.10E-07	4
Histone H1.1	M.ac-SETAPVAQAA(pSpT)ATEKPAAAKK.T	2249.09380	2249.09400	-0.00020	2.70E-02	2
Histone H1.1, H1.2, H1.3, H1.4	R.SGVSLAALKub1K.A	1086.63940	1086.63970	-0.00030	8.60E-04	2
Histone H1.1, H1.3, H1.4	R.SGVSLAALKKub1.A	1086.64060	1086.63970	0.00090	7.20E-05	2
Histone H1.2	M.ac-SEAAPAAPAAAPPAEK.A	1489.73670	1489.74120	-0.00450	3.60E-09	4
Histone H1.2	M.ac-SEAAPAAPAAAPPAEKAPAK.K	1856.96300	1856.96320	-0.00010	5.70E-14	14
Histone H1.2	M.ac-SEAAPAAPAAAPPAEKAPAKK.K	1985.05480	1985.05810	-0.00330	1.10E-13	8
Histone H1.2	M.ac-SEAAPAAPAAAPPAEKAPAKKKac.A	2155.17260	2155.16360	0.00900	6.10E-04	2
Histone H1.2	M.ac-SEAAPAAPAAAPPAEKAPAKKK.A	2113.14940	2113.15310	-0.00360	1.90E-12	5
Histone H1.2	M.ac-pSEAAPAAPAAAPPAEKAPAKK.K	2065.02710	2065.02440	0.00270	9.20E-04	3
Histone H1.2 , H1.3	K.KATGAApTPK.K	923.44720	923.44770	-0.00050	5.70E-03	4
Histone H1.2 , H1.3	R.KApSGPPVSELITK.A	1405.72320	1405.72180	0.00140	4.50E-06	5
Histone H1.2 , H1.3	R.Kub1ASGPPVSELITK.A	1439.79800	1439.79840	-0.00030	9.80E-04	2
Histone H1.2 , H1.3	R.Kub1ASGPPVSELITKub1.A	1553.85020	1553.84130	0.00890	3.20E-04	2
Histone H1.2 , H1.3, H1.4	K.AGAAKacAKKPAGAAK.K	1280.75630	1280.75640	-0.00010	2.80E-02	2
Histone H1.2 , H1.3, H1.4	K.AKKPAGAAKacKPK.K	1235.77090	1235.77130	-0.00040	9.80E-05	7
Histone H1.2 , H1.3, H1.4	K.AKKPAGAAKKacPK.K	1235.76950	1235.77130	-0.00180	8.80E-03	3
Histone H1.2, H1.3, H1.4, H1.5	K.GTGApSGSFKLNK.K	1245.57480	1245.57550	-0.00070	1.80E-04	4
Histone H1.2, H1.3, H1.4, H1t	K.ALAAAGYDVEKub1NNSR.I	1691.82870	1691.82270	0.00610	9.30E-05	4
Histone H1.2, H1.3, H1.4, H1t	K.Kub1ALAAAGYDVEK.N	1348.70240	1348.69860	0.00380	3.10E-04	2
Histone H1.2, H1.3, H1.4, H1t	K.Kub1ALAAAGYDVEKNNSR.I	1819.93560	1819.91760	0.01800	1.30E-03	2
Histone H1.3	M.ac-pSETAPAAPAAPAPVEKTPVK.K	2053.01440	2053.01320	0.00120	9.70E-09	4
Histone H1.3	M.ac-pSETAPAAPAAPAPVEKTPVKKK.A	2309.20600	2309.20320	0.00290	2.20E-03	4
Histone H1.3	M.ac-SEpTAPAAPAAPAPVEKTPVKKK.A	2309.20500	2309.20320	0.00190	2.30E-05	3
Histone H1.3	M.ac-SETAPAAPAAPAPVEK.T	1547.77960	1547.78310	-0.00350	2.00E-12	12
Histone H1.3	M.ac-SETAPAAPAAPAPVEKpTPVK.K	2053.02160	2053.01320	0.00840	1.10E-04	3

Table 1. Continued

Histone	Peptide sequence and modification	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability	Spectral count
Histone H1.3	M.ac-SETAPAAPAAPAPVEKTPVK.K	1973.04300	1973.04690	-0.00390	3.30E-14	16
Histone H1.3	M.ac-SETAPAAPAAPAPVEKTPVKK.K	2101.14310	2101.14190	0.00120	1.60E-15	15
Histone H1.3	M.ac-SETAPAAPAAPAPVEKTPVKKK.A	2229.23830	2229.23680	0.00150	8.50E-15	7
Histone H1.3	K.KAAKpSPAKAK.A	1078.59060	1078.58990	0.00060	3.50E-04	2
Histone H1.3	K.KATGAATPKKpTAK.K	1351.72310	1351.72240	0.00060	1.40E-04	2
Histone H1.3	K.KVSKpSPK.K	852.44920	852.44700	0.00220	2.60E-04	2
Histone H1.3	K.KVSKpSPKK.V	980.54160	980.54190	-0.00030	3.50E-03	2
Histone H1.3	M.ac-pSETAPAAPAAPAPVEKTPVKK.K	2181.11100	2181.10820	0.00280	3.50E-05	3
Histone H1.3	M.ac-SETAPAAPAAPAPVEKpTPVKK.K	2181.11160	2181.10820	0.00340	1.20E-02	2
Histone H1.3	M.ac-SETAPAAPAAPAPVEKpTPVKKK.A	2309.20580	2309.20320	0.00270	1.80E-04	3
Histone H1.3	M.pSETAPAAPAAPAPVEK.T	1585.73270	1585.73890	-0.00610	3.60E-02	3
Histone H1.3	M.pSETAPAAPAAPAPVEKTPVKK.K	2139.09870	2139.09760	0.00110	5.20E-03	3
Histone H1.3, H1.4	K.AKKPAAAAGAKacK.V	1152.69590	1152.69780	-0.00190	5.00E-04	4
Histone H1.3, H1.4	K.KacPAAAAGAK.K	825.47020	825.47080	-0.00060	4.00E-02	2
Histone H1.3, H1.4	K.KAKKPAAAAGAKacK.V	1280.79420	1280.79280	0.00150	8.60E-05	6
Histone H1.3, H1.4	K.KPAAAAGAKacK.A	953.56510	953.56580	-0.00070	3.40E-04	2
Histone H1.3, H1.4, H1.5	K.GTLVQTKub1GTGASGSFK.L	1651.85720	1651.85300	0.00420	1.20E-02	3
Histone H1.3, H1.4, H1.5	K.SLVSKacGTLVQTK.G	1301.75580	1301.75550	0.00040	2.10E-04	2
Histone H1.4	K.KAAGTATAKKac.S	987.56970	987.57130	-0.00160	2.30E-03	3
Histone H1.4	K.RAGAAKacAK.K	813.48150	813.48200	-0.00050	8.50E-03	2
Histone H1.4	K.RKpTSGPPVSELITK.A	1591.83920	1591.83350	0.00570	3.20E-03	2
Histone H1.4	K.TSGPPVSELITKub1.A	1341.71660	1341.71400	0.00260	1.50E-06	2
Histone H1.4	K.TSGPPVSELITKub1AVAASK.E	1869.02470	1869.02070	0.00400	3.40E-06	2
Histone H1.4	M.ac-(pSEpT)APAAPAAPAPAEKTPVKK.K	2153.08520	2153.07690	0.00840	9.30E-03	3
Histone H1.4	M.ac-pSETAPAAPAAPAPAEK.T	1599.71600	1599.71810	-0.00210	7.50E-08	2
Histone H1.4	M.ac-pSETAPAAPAAPAPAEKTPV(Kme2Kme2).K	2181.11160	2181.10820	0.00350	6.10E-03	2
Histone H1.4	M.ac-pSETAPAAPAAPAPAEKTPVKme2KK.A	2309.20620	2309.20310	0.00310	7.00E-04	3
Histone H1.4	M.ac-pSETAPAAPAAPAPAEKTPVKKme2.K	2181.11100	2181.10820	0.00280	5.90E-04	4
Histone H1.4	M.ac-pSETAPAAPAAPAPAEKTPVKK.K	2153.08000	2153.07690	0.00320	7.50E-06	5
Histone H1.4	M.ac-pSETAPAAPAAPAPAEKTPVKKK.A	2281.17160	2281.17180	-0.00020	1.30E-05	2
Histone H1.4	M.ac-pSpETAPAAPAAPAPAEKTPVKKme2K.A	2309.20500	2309.20310	0.00190	4.00E-04	2
Histone H1.4	M.ac-SEpTAPAAPAAPAPAEKTPVK.K	2024.98360	2024.98190	0.00170	3.70E-06	3
Histone H1.4	M.ac-SEpTAPAAPAAPAPAEKTPVKK.K	2153.08760	2153.07690	0.01080	8.20E-04	3
Histone H1.4	M.ac-SETAPAAPAAPAPAEKacTPVK.K	1987.02700	1987.02620	0.00090	1.70E-10	3
Histone H1.4	M.ac-SETAPAAPAAPAPAEKacTPVKK.K	2115.12200	2115.12110	0.00090	1.50E-08	2
Histone H1.4	M.ac-SETAPAAPAAPAPAEKacTPVKKK.A	2243.25270	2243.21610	0.03660	9.60E-06	2
Histone H1.4	M.ac-SETAPAAPAAPAPAEKme2TPVKK.K	2101.15150	2101.14190	0.00970	1.20E-03	5
Histone H1.4	M.ac-SETAPAAPAAPAPAEK.T	1519.74500	1519.75180	-0.00680	2.10E-13	11
Histone H1.4	M.ac-SETAPAAPAAPAPAEKpTPVK.K	2024.98900	2024.98190	0.00710	5.20E-05	2
Histone H1.4	M.ac-SETAPAAPAAPAPAEKTPVKacK.K	2115.13140	2115.12110	0.01030	9.40E-09	2
Histone H1.4	M.ac-SETAPAAPAAPAPAEKTPVK.K	1945.01440	1945.01560	-0.00120	1.60E-14	27
Histone H1.4	M.ac-SETAPAAPAAPAPAEKTPVK(Kme2Kme2).K	2101.14250	2101.14190	0.00070	7.30E-03	1
Histone H1.4	M.ac-SETAPAAPAAPAPAEKTPVKK.K	2073.10650	2073.11060	-0.00400	9.50E-16	20
Histone H1.4	M.ac-SETAPAAPAAPAPAEKTPVKKKme2.A	2229.23920	2229.23680	0.00240	3.70E-03	5
Histone H1.4	M.ac-SETAPAAPAAPAPAEKTPVKKK.A	2201.20410	2201.20550	-0.00140	1.30E-14	12
Histone H1.4	M.ac-SETAPAAPAAPAPAEKTPVKKKme1.A	2215.22120	2215.22120	0.00010	2.80E-04	4
Histone H1.4	M.ac-SETAPAAPAAPAPAEKme1pTPVKme1KK.A	2309.20580	2309.20320	0.00270	2.90E-03	5

Table 1. Continued

Histone	Peptide sequence and modification	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability	Spectral count
Histone H1.4	M.pSETAPAAPAAPAPAEKTPV(Kme2Kme2).K	2139.09870	2139.09760	0.00110	1.00E-02	2
Histone H1.4	M.SETAPAAPAAPAPAEKTPVKme2.K	1931.03670	1931.03630	0.00030	1.60E-02	2
Histone H1.4	M.SETAPAAPAAPAPAEKTPVKme2K.K	2059.13550	2059.13130	0.00420	3.20E-02	2
Histone H1.4	R.KAAGGAKacR.K	799.46610	799.46640	-0.00030	1.00E-02	2
Histone H1.4	R.KTpSGPPVSELITK.A	1435.73850	1435.73230	0.00620	3.40E-07	5
Histone H1.4	R.Kub1TSGPPVSELITK.A	1469.80940	1469.80890	0.00050	4.20E-05	3
Histone H1.4	K.APKpSPAKAK.T	976.51040	976.51060	-0.00020	2.00E-03	3
Histone H1.4	K.KAPKpSPAK.A	905.47540	905.47350	0.00190	1.00E-05	4
Histone H1.4	K.KAPKpSPAKAK.T	1104.60560	1104.60560	0.00000	1.30E-05	3
Histone H1.4	K.RKTpSGPPVSELITK.A	1591.83610	1591.83350	0.00270	1.50E-02	2
Histone H1.4	M.ac-pSETAPAAPAAPAPAEKTPVK.K	2024.98500	2024.98190	0.00310	1.20E-03	8
Histone H1.4	M.ac-SEpTAPAAPAAPAPAEKTPVKKK.A	2281.17520	2281.17180	0.00340	5.30E-05	3
Histone H1.4	M.ac-SETAPAAPAAPAPAEKpTPVKK.K	2153.08160	2153.07690	0.00480	2.80E-04	3
Histone H1.4	M.ac-SETAPAAPAAPAPAEKpTPVKKK.A	2281.17600	2281.17180	0.00420	1.50E-04	4
Histone H1.4	M.pSETAPAAPAAPAPAEKTPVKK.K	2111.06710	2111.06630	0.00070	8.90E-04	3
Histone H1.5	K.KAKKPAAAGV(KacKac).V	1237.78750	1237.78700	0.00050	7.30E-04	2
Histone H1.5	M.ac-SEpTAPAETAAPAPVEKpSPAK.K	2152.94080	2152.93300	0.00780	1.10E-04	2
Histone H1.5	M.ac-SETAPAEpTAAPAPVEKSPAKK.K	2201.07000	2201.06160	0.00840	4.80E-03	2
Histone H1.5	M.ac-SETAPAETAAPAPVEKacSPAK.K	2035.02080	2035.01090	0.00990	6.30E-04	4
Histone H1.5	M.ac-SETAPAETAAPAPVEK.S	1609.77930	1609.78350	-0.00420	5.80E-12	10
Histone H1.5	M.ac-SETAPAETAAPAPVEKpSPA(Kme1Kme1Kme1).T	2343.17420	2343.17220	0.00200	7.20E-06	1
Histone H1.5	M.ac-SETAPAETAAPAPVEKpSPAK.K	2072.96560	2072.96670	-0.00110	1.50E-08	8
Histone H1.5	M.ac-SETAPAETAAPAPVEKpSPAKKme2K.T	2357.19040	2357.18790	0.00250	9.00E-04	2
Histone H1.5	M.ac-SETAPAETAAPAPVEKpSPAKK.K	2201.06910	2201.06160	0.00750	9.80E-04	5
Histone H1.5	M.ac-SETAPAETAAPAPVEKpSPAKKK.T	2329.14970	2329.15660	-0.00690	8.70E-06	11
Histone H1.5	M.ac-SETAPAETAAPAPVEKSPAK.K	1992.99760	1993.00040	-0.00270	2.00E-13	9
Histone H1.5	M.ac-SETAPAETAAPAPVEKSPAKK.K	2121.09820	2121.09530	0.00290	2.40E-12	9
Histone H1.5	M.ac-SETAPAETAAPAPVEKSPAKKK.T	2249.18410	2249.19030	-0.00610	3.40E-11	4
Histone H1.5	M.SETAPAETAAPAPVEKpSPAKKK.T	2329.14970	2329.15660	-0.00690	1.50E-06	6
Histone H1.5	R.Kub1ATGPPVSELITK.A	1453.81530	1453.81400	0.00130	4.10E-05	2
Histone H1.5	K.AVpSASKER.G	926.42240	926.42220	0.00020	3.40E-02	3
Histone H1.5	M.ac-SEpTAPAETAAPAPVEKpSPAKKK.T	2409.12810	2409.12290	0.00520	1.50E-05	2
Histone H1.5	M.SETAPAEpTAAPAPVEKSPAK.K	2030.94610	2030.95610	-0.01010	3.60E-02	4
Histone H1.5	M.SETAPAETAAPAPVEKpSPAK.K	2030.96080	2030.95610	0.00470	8.30E-06	6
Histone H1t	K.GTGASGSFKLSKme2K.A	1294.69300	1294.72450	-0.03150	5.40E-05	2
Histone H2A type 1-F	K.Kub1TESHHKPK.G	1204.63380	1204.63120	0.00260	2.30E-02	1
Histone H2A type 1, 1-F, 1-K, 2-A, 2-B, 2-C, 3, H2A.J, H2A.x	R.VTIAQGGVLPNIQAVLLP(Kub1Kub1).T	2172.29960	2172.29940	0.00020	2.30E-11	7
Histone H2A type 1, 1-F, 1-K, 2-A, 2-B, 2-C, 3, H2A.J, H2A.x	R.VTIAQGGVLPNIQAVLLPKme2.K	1958.19530	1958.19280	0.00240	2.30E-05	5
Histone H2A type 1, 1-F, 1-K, 2-A, 2-B, 2-C, 3, H2A.J, H2A.x	R.VTIAQGGVLPNIQAVLLPKme1.K	1944.18040	1944.17720	0.00320	4.00E-05	3
Histone H2A type 1, 1-F, 1-K, 2-A, 2-B, 2-C, 3, H2A.J, H2A.x	R.VTIAQGGVLPNIQAVLLPKub1.K	2044.20560	2044.20450	0.00120	1.30E-06	7
Histone H2A type 1, 1-F, 1-K, 2-A, 2-B, 2-C, 3, H2A.J, H2A.x	R.VTIAQGGVLPNIQAVLLPKub1K.T	2172.30600	2172.29940	0.00660	7.70E-08	4
Histone H2A type 1, 1-F, 1-K, 3, H2A.J	R.NDEELNKub1LLGR.V	1413.72190	1413.72120	0.00080	3.60E-04	2
Histone H2A type 1, 1-F, 2-A, 3	K.VTIAQGGVLPNIQAVLLPKub1KTESHHK.A	2891.67580	2891.63450	0.04130	2.70E-12	1
Histone H2A type 1, 1-F, 2-A, 3	R.VTIAQGGVLPNIQAVLLP(Kub1Kub1)TESHHK.A	2891.63690	2891.63450	0.00240	1.50E-03	2
Histone H2A type 1, 1-F, 2-A, 3	R.VTIAQGGVLPNIQAVLLPKKTESHHKub1.A	2891.63880	2891.63450	0.00430	2.20E-04	2
Histone H2A type 1, 1-F, 2-A, 3	R.VTIAQGGVLPNIQAVLLPKub1KTESHHK.A	2891.63410	2891.63450	-0.00040	6.10E-03	3
Histone H2A type 1/ 1-F/1-K, 2-A, 2-B, 2-C, 3	R.GKacQGGKacAR.A	884.48320	884.48280	0.00040	1.80E-05	5

Table 1. Continued

Histone	Peptide sequence and modification	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability	Spectral count
Histone H2A type 2-A, 2-C	R.HLQLAIRNDEELNKLLGKme2.V	2131.19000	2131.21130	-0.02120	1.00E-03	5
Histone H2A.J	R.VTIAQGGVLPNIQAVLLP(Kub1Kub1)TESQK.V	2745.61380	2745.57520	0.03860	1.20E-11	5
Histone H2A.V, H2A.Z	M.ac-AGGKAGKacDSGKacAK.A	1299.67620	1299.67820	-0.00200	8.50E-05	3
Histone H2A.x	K.Kub1SSATVGPK.A	1256.71970	1256.72010	-0.00030	2.00E-02	2
Histone H2A.x, H2A type 2-B	K.LLGGVTIAQGGVLPNIQAVLLPKub1K.S	2512.50790	2512.51050	-0.00250	2.80E-07	2
Histone H2A.x, H2A type 2-B	R.NDEELNKLLGGVTIAQGGVLPNIQAVLLPKKub1.S	3354.89420	3354.88750	0.00680	6.40E-06	2
Histone H2A.Z, H2A.V	K.AGKacDSGK.A	703.35260	703.35010	0.00260	7.90E-03	2
Histone H2A.Z, H2A.V	K.SLIGKub1KGQQK.T	1199.69800	1199.69860	-0.00060	1.40E-03	2
Histone H2A.Z, H2A.V	M.ac-AGGKAGKacDSGKacAK.T	1299.68330	1299.67820	0.00500	7.90E-05	2
Histone H2A.Z, H2A.V	M.AGGKacAGKacDSGKacAK.T	1299.67970	1299.67820	0.00140	9.80E-06	4
Histone H2A.Z, H2A.V	M.AGGKacAGKDSGK.A	1016.52430	1016.52510	-0.00070	2.00E-03	3
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B	R.(pSpT)ITSREIQTAVR.L	1540.76290	1540.76100	0.00190	7.70E-04	4
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B	R.STITpSREIQTAVR.L	1540.76260	1540.76100	0.00160	3.30E-04	3
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B, 2-E, 3-A, 3-B	K.HAVpSEGTK.A	907.37950	907.38010	-0.00050	6.40E-06	6
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B, 2-E, 3-A, 3-B	K.HAVpSEGTKAVTK.Y	1306.62890	1306.62820	0.00060	2.60E-05	6
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B, 2-E, 3-A, 3-B	K.HAVSEGpTKAVTK.Y	1306.62810	1306.62820	-0.00010	1.10E-02	3
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B, 2-E, 3-A, 3-B	K.HAVSEGTKub1AVTK.Y	1340.70360	1340.70480	-0.00120	3.10E-02	2
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B, 2-E, 3-A, 3-B	K.VLKub1QVHPDTGISSK.A	1621.88240	1621.87880	0.00360	8.70E-03	5
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B, 2-E, 3-A, 3-B	R.LLLPGELAKacHAVSEGTK.A	1804.01250	1804.00940	0.00310	2.00E-05	3
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 1-P, 2-B, 2-E, 3-A, 3-B	R.LLLPGELAKub1HAVSEGTK.A	1876.04570	1876.04180	0.00390	3.10E-09	4
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-M, 1-P, 2-B, 3-A, 3-B	K.HAVSEGTKAVTKub1YTSSK.-	1906.97770	1906.97480	0.00290	7.90E-04	3
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-M, 2-B, 3-A, 3-B	K.AVTKacYTSSK.-	1025.53720	1025.53930	-0.00210	3.50E-04	2
Histone H2B type 1-A, 1-B, 1-C/E/G, 1-F/J/L,1-H,1-M,1-P, 2-B, 3-A, 3-B	K.AVTKub1YTSSK.-	1366.75510	1366.75690	-0.00180	3.00E-02	3
Histone H2B type 1-A, 1-B, 1-F/J/L, 1-K, 1-P, 3-A, 3-B	R.IApSEASR.L	812.34240	812.34290	-0.00050	2.10E-03	3
Histone H2B type 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 2-B, 1-P, 2-B	R.Kub1ESYSVYVYKVLKQVHPDTGISSK.A	3137.70130	3137.68730	0.01400	4.10E-05	4
Histone H2B type 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 2-B, 2-E, 3-B	K.SAPAP(KacKac)GS(KacKac).A	1181.67590	1181.67680	-0.00080	5.80E-03	5
Histone H2B type 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 2-B, 2-E, 3-B	K.SAPAPKacKacGSKacK.A	1223.68760	1223.68730	0.00030	1.70E-07	5
Histone H2B type 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 2-B, 2-E, 3-B	K.SAPAPKacKacGSK.K	1053.58210	1053.58180	0.00030	9.50E-04	3
Histone H2B type 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 2-B, 2-E, 3-B	K.SAPAPKacKacGSKK.A	1223.69140	1223.68730	0.00410	1.60E-06	4
Histone H2B type 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 2-B, 2-E, 3-B	K.SAPAPKacKGSK.K	1011.56960	1011.57130	-0.00170	2.20E-04	5
Histone H2B type 1-B, 1-C/E/G, 1-F/J/L, 1-H, 1-K, 1-M, 2-B, 2-E, 3-B	K.SAPAPKacGSKK.A	1139.66590	1139.66620	-0.00030	3.70E-02	4
Histone H2B type 1-C/E/G, 1-F/J/L, 1-H, 1-K	M.PEPAKacSAPAPK.K	1133.60670	1133.60800	-0.00130	3.40E-06	5
Histone H2B type 1-C/E/G, 1-F/J/L, 1-H, 1-K	M.PEPAKpSAPAPK.K	1171.56420	1171.56380	0.00040	5.80E-05	3
Histone H2B type 1-C/E/G, 1-F/J/L, 1-H, 1-K	M.PEPAKme1SAPAPK.K	1105.61290	1105.61310	-0.00030	2.50E-03	4
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B	K.KacAVTKacAQK.K	956.56490	956.56540	-0.00060	6.50E-05	8
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B	K.KacAVTKacAQKK.D	1084.65810	1084.66040	-0.00230	3.40E-05	10
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B	K.AVTKacAQK.K	786.45990	786.45990	0.00000	8.00E-04	7
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B	K.AVTKacAQKKDGK.K	1214.69850	1214.69830	0.00020	4.60E-05	3
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B	K.KacAVTKacAQ(KacKac).D	1126.67110	1126.67100	0.00020	3.50E-05	2
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B	K.AVTKacAQKK.D	914.55520	914.55490	0.00030	1.20E-02	2
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B	K.KAVTKacAQKK.D	1042.64830	1042.64980	-0.00150	5.80E-04	7
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B, 2-B	K.KacGSKacKacAVTK.A	1071.63140	1071.62880	0.00260	1.20E-06	8
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B, 2-B	K.KGSKacKacAVTK.A	1029.61960	1029.61820	0.00140	3.40E-02	5
Histone H2B type 1-C/E/G, 1-F/J/L, 1-K, 1-M, 1-P, 2-E, 3-B, 2-B	K.KGSKacKacAVTK.V	1029.61660	1029.61820	-0.00160	3.10E-05	5

Table 1. Continued

Histone	Peptide sequence and modification	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability	Spectral count
Histone H2B type 1-H	K.KacALTKacAQK.K	970.58110	970.58110	0.00000	1.30E-03	3
Histone H2B type 1-H	K.KacALTKacAQKK.D	1098.67750	1098.67600	0.00150	1.60E-03	2
Histone H2B type 1-H	K.KacGSKacKacALTK.A	1085.64450	1085.64440	0.00010	1.20E-06	8
Histone H2B type 1-K, 2-E	K.AVTKub1YTSAK.-	1081.57590	1081.57680	-0.00080	7.40E-04	3
Histone H2B type 1-M	M.PEPTKacSAPAPK.K	1163.61710	1163.61860	-0.00150	8.50E-06	5
Histone H2B type 2-B	K.KacAVTKacVQK.K	984.59560	984.59680	-0.00110	3.30E-05	4
Histone H2B type 2-B	K.KacAVTKacVQKK.D	1112.69170	1112.69170	0.00000	7.30E-05	7
Histone H2B type 2-B	K.AVTKacVQKK.D	942.58490	942.58620	-0.00130	4.90E-04	13
Histone H2B type 2-B	K.AVTKacVQKKDGK.K	1242.73030	1242.72960	0.00080	3.30E-02	3
Histone H2B type 2-B	K.KAVTKacVQKK.D	1070.67990	1070.68120	-0.00130	1.40E-05	8
Histone H2B type 2-B	K.KAVTKVQ(KacKac).D	1070.67900	1070.68120	-0.00210	2.20E-03	2
Histone H2B type 2-B	M.PDPAKacSAPAPK.K	1119.59330	1119.59240	0.00090	1.40E-06	4
Histone H2B type 3-A	M.PEPSRSpTPAPK.K	1245.57610	1245.57540	0.00070	9.60E-04	14
Histone H2B type 3-A, 3-B	R.EVQTAVRme1LLLPGELAKHAVSEGTK.A	2559.44600	2559.43840	0.00750	1.50E-03	2
Histone H2B type 3-A, 3-B	R.STI(pTpS)REVQTAVR.L	1526.74470	1526.74540	-0.00070	2.20E-02	3
Histone H3-like centromeric protein A	R.RRPpSSPAPGPSR.Q	1343.64800	1343.64590	0.00210	1.30E-02	4
Histone H3.1, H3.2	K.AARKme2SAPATGGVKme2KPHR.Y	1787.07080	1787.06420	0.00660	1.30E-03	2
Histone H3.1, H3.2	K.SAPATGGVKme1APAR.Y	1318.74690	1318.74700	0.00000	2.00E-05	1
Histone H3.1, H3.2	R.Kme2SAPATGGVKme2KPHR.Y	1488.88930	1488.88890	0.00050	5.00E-05	18
Histone H3.1, H3.2	R.Kme2SAPATGGVK.K	942.55050	942.54980	0.00070	2.10E-04	13
Histone H3.1, H3.2	R.Kme2SAPATGGVKKPHR.Y	1460.85590	1460.85760	-0.00170	1.50E-04	6
Histone H3.1, H3.2	R.Kme2SAPATGGVKme1.K	956.56400	956.56550	-0.00150	3.90E-02	3
Histone H3.1, H3.2	R.Kme2SAPATGGVKme1KPHR.Y	1474.88170	1474.87320	0.00850	7.40E-08	9
Histone H3.1, H3.2	R.KSAPATGGVKme1KPHR.Y	1446.84530	1446.84190	0.00340	1.20E-04	1
Histone H3.1, H3.2	R.Kme1SAPATGGVK.K	928.53470	928.53420	0.00050	8.70E-03	2
Histone H3.1, H3.2	R.Kme1SAPATGGVKKPHR.Y	1446.84090	1446.84190	-0.00100	7.40E-04	2
Histone H3.1, H3.2	R.Kme3SAPATGGVKme2KPHR.Y	1502.90350	1502.90450	-0.00100	1.30E-02	4
Histone H3.1, H3.2	R.Kme3SAPATGGVK.K	956.56550	956.56550	0.00010	5.50E-04	8
Histone H3.1, H3.2	R.Kme3SAPATGGVKme1KPHR.Y	1488.88660	1488.88890	-0.00220	1.80E-02	1
Histone H3.1, H3.2, H3.3	K.QLATKacAAR.K	899.52220	899.51880	0.00340	1.80E-05	16
Histone H3.1, H3.2, H3.3	K.STGGKacAPR.K	814.42860	814.42970	-0.00110	5.70E-03	7
Histone H3.1, H3.2, H3.3	R.KacpSTGGKme3APR.K	1064.54090	1064.53790	0.00290	1.50E-02	3
Histone H3.1, H3.2, H3.3	R.KacQLATKacAAR.K	1069.62420	1069.62430	-0.00010	5.90E-08	20
Histone H3.1, H3.2, H3.3	R.KacQLATKacAARK.S	1197.71980	1197.71930	0.00050	2.00E-02	7
Histone H3.1, H3.2, H3.3	R.KacQLATK.A	729.43790	729.43850	-0.00050	1.10E-03	18
Histone H3.1, H3.2, H3.3	R.KacSTGGKacAPR.K	984.53510	984.53520	-0.00010	4.40E-06	16
Histone H3.1, H3.2, H3.3	R.KacSTGGKme2APR.K	970.55470	970.55600	-0.00120	2.20E-03	4
Histone H3.1, H3.2, H3.3	R.KacSTGGKAPR.K	942.52570	942.52470	0.00100	3.70E-04	6
Histone H3.1, H3.2, H3.3	R.KacSTGGKme1APR.K	956.54060	956.54030	0.00030	9.50E-05	2
Histone H3.1, H3.2, H3.3	R.KacSTGGKme3APR.K	984.57220	984.57160	0.00060	3.30E-04	9
Histone H3.1, H3.2, H3.3	R.KacSTGGKme3APRK.Q	1112.66700	1112.66660	0.00040	2.10E-04	3
Histone H3.1, H3.2, H3.3	R.Kme2pSTGGKAPR.K	1008.51350	1008.51170	0.00180	1.70E-02	3
Histone H3.1, H3.2, H3.3	R.Kme2STGGKacAPR.K	970.55560	970.55600	-0.00040	5.10E-05	33
Histone H3.1, H3.2, H3.3	R.Kme2STGGKacAPRK.Q	1098.65260	1098.65090	0.00170	4.50E-02	2
Histone H3.1, H3.2, H3.3	R.Kme2STGGKAPR.K	928.54620	928.54540	0.00080	2.10E-04	25
Histone H3.1, H3.2, H3.3	R.EIAQDFKme2TDLR.F	1362.72020	1362.71430	0.00590	3.80E-08	10
Histone H3.1, H3.2, H3.3	R.EIAQDFKpTDLR.F	1414.65480	1414.64930	0.00540	6.30E-04	5

Table 1. Continued

Histone	Peptide sequence and modification	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability	Spectral count
Histone H3.1, H3.2, H3.3	R.EIAQDFKme1.T	863.43600	863.43890	-0.00290	1.70E-04	3
Histone H3.1, H3.2, H3.3	R.EIAQDFKme1TDLR.F	1348.70110	1348.69870	0.00240	4.30E-04	16
Histone H3.1, H3.2, H3.3	R.EIAQDFKub1TDLR.F	1448.72650	1448.72590	0.00060	3.20E-04	3
Histone H3.1, H3.2, H3.3	R.KpSTGGKacAPR.K	1022.49090	1022.49100	-0.00010	5.30E-04	2
Histone H3.1, H3.2, H3.3	R.KQLATKacAAR.K	1027.63550	1027.61380	0.02170	1.10E-06	48
Histone H3.1, H3.2, H3.3	R.KQLATKacAARK.S	1155.70910	1155.70870	0.00030	6.90E-04	6
Histone H3.1, H3.2, H3.3	R.KQLATKme2AAR.F	1730.96300	1730.96790	-0.00490	2.10E-05	2
Histone H3.1, H3.2, H3.3	R.KQLATKme1AAR.K	999.61990	999.61890	0.00100	1.70E-02	2
Histone H3.1, H3.2, H3.3	R.KSTGGKacAPR.K	942.52500	942.52470	0.00030	1.00E-05	24
Histone H3.1, H3.2, H3.3	R.KSTGGKacAPRK.Q	1070.62010	1070.61960	0.00050	2.50E-02	3
Histone H3.1, H3.2, H3.3	R.Kme1QLATKacAAR.K	1041.63060	1041.62940	0.00120	7.30E-05	4
Histone H3.1, H3.2, H3.3	R.Kme1SpTGGKacAPR.K	1036.50480	1036.50660	-0.00180	1.90E-02	2
Histone H3.1, H3.2, H3.3	R.Kme1STGGKacAPR.K	956.54280	956.54030	0.00250	4.70E-05	14
Histone H3.1, H3.2, H3.3	R.Kme1STGGKAPR.K	914.53010	914.52980	0.00040	7.40E-05	7
Histone H3.1, H3.2, H3.3	R.pYQKSTELLIR.K	1329.66970	1329.66930	0.00040	4.40E-04	3
Histone H3.1, H3.2, H3.3	R.TKacQTAR.K	745.40940	745.40820	0.00120	1.60E-02	4
Histone H3.1, H3.2, H3.3	R.Kme3STGGKAPR.K	942.56140	942.56110	0.00040	4.80E-05	9
Histone H3.1, H3.2, H3.3	R.VTIMPKme2DIQLAR.R	1411.78800	1411.82210	-0.03410	7.80E-05	2
Histone H3.1, H3.2, H3.3	R.YQKpSTELLIR.K	1329.66820	1329.66930	-0.00110	2.90E-02	3
Histone H3.1, H3.2, H3.3	R.YQKSpTELLIR.K	1329.67020	1329.66930	0.00080	4.20E-03	3
Histone H3.1, H3.2, H3.3	R.YQKub1STELLIR.K	1363.74620	1363.74590	0.00030	6.70E-03	4
Histone H3.3	K.AARKme2SAPSTGGVKme2KPHR.Y	1803.06660	1803.05910	0.00750	2.30E-03	1
Histone H3.3	K.SAPSTGGVKme2KPHR.Y	1348.75910	1348.75750	0.00160	9.70E-07	3
Histone H3.3	K.SAPSTGGVKKme2PHR.Y	1348.75800	1348.75750	0.00050	3.00E-06	1
Histone H3.3	K.SAPSTGGVKme1KPHR.Y	1334.74160	1334.74190	-0.00030	2.60E-04	3
Histone H3.3	R.KacSAPSTGGVKacKPHR.Y	1532.87910	1532.84230	0.03680	7.40E-04	5
Histone H3.3	R.KacSAPSTGGVKme2Kme1PHR.Y	1532.87910	1532.87870	0.00040	7.20E-04	3
Histone H3.3	R.KacSAPSTGGVK.K	972.52130	972.52400	-0.00270	3.90E-04	4
Histone H3.3	R.KacSAPSTGGVKme3KPHR.Y	1504.84640	1504.84740	-0.00100	1.70E-02	3
Histone H3.3	R.Kme2SAPSTGGVKme2KPHRme1.Y	1518.89810	1518.89940	-0.00130	2.30E-03	2
Histone H3.3	R.Kme2SAPSTGGVKme2KPHR.Y	1504.88450	1504.88380	0.00070	2.10E-04	9
Histone H3.3	R.Kme2SAPSTGGVKme2Kme1PHR.Y	1518.89810	1518.89940	-0.00130	4.80E-05	2
Histone H3.3	R.Kme2SAPSTGGVK.K	958.54170	958.54470	-0.00310	1.50E-03	5
Histone H3.3	R.KSAPSTGGVKacKPHR.Y	1532.84470	1532.84230	0.00240	6.10E-08	2
Histone H3.3	R.KSAPSTGGVKme2KPHR.Y	1476.85410	1476.85250	0.00160	3.10E-02	3
Histone H3.3	R.Kme1SAPSTGGVKme2KPHR.Y	1490.87530	1490.86810	0.00720	3.50E-04	3
Histone H3.3	R.Kme1SAPSTGGVK.K	944.52980	944.52910	0.00070	1.20E-04	2
Histone H3.3	R.Kme1SAPSTGGVKKPHR.Y	1462.83950	1462.83680	0.00270	4.20E-02	2
Histone H4	K.GGKacGLGKacGGAKacR.H	1210.69020	1210.67820	0.01200	3.20E-08	10
Histone H4	K.GGKacGLGKacGGAK.R	1012.56570	1012.56650	-0.00080	1.00E-02	2
Histone H4	K.GGKacGLGKacGGAKR.H	1168.66980	1168.66760	0.00220	2.10E-05	5
Histone H4	K.GGKGLGKacGGAKacR.H	1168.67470	1168.66760	0.00710	2.70E-07	9
Histone H4	K.GGKGLGKacGGAK.R	970.55990	970.55600	0.00390	1.30E-03	4
Histone H4	K.GGKGLGKacGGAKR.H	1126.65560	1126.65710	-0.00140	2.80E-06	6
Histone H4	K.GGKGLGKGGAKacR.H	1126.65520	1126.65710	-0.00190	1.10E-05	9
Histone H4	K.GLGKacGGAKacR.H	926.53370	926.52970	0.00390	5.10E-05	9
Histone H4	K.GLGKacGGAKR.H	884.52220	884.51920	0.00300	9.30E-05	2

Table 1. Continued

Histone	Peptide sequence and modification	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability	Spectral count
Histone H4	K.GLGKacGGAKme3R.H	926.56070	926.56610	-0.00550	3.20E-03	4
Histone H4	K.GLGKGGAKacR.H	884.52200	884.51920	0.00280	7.40E-04	8
Histone H4	K.RIpSGLIYEETR.G	1415.67890	1415.68100	-0.00210	2.10E-04	5
Histone H4	K.RIpSGLIYEETRGVLK.V	1812.95030	1812.94990	0.00040	5.40E-03	2
Histone H4	K.TVTAMDVVpYALKR.Q	1545.76480	1545.76260	0.00220	1.30E-07	6
Histone H4	M.ac-SGRGKGGKacGLGK.G	1240.68760	1240.68880	-0.00110	1.70E-03	1
Histone H4	R.(Kme1VLRme1)DNIQGITKPAIR.R	1835.10760	1835.11050	-0.00290	3.00E-03	6
Histone H4	R.KacVLRDNIQGITKPAIR.R	1863.13820	1863.10540	0.03280	3.60E-02	2
Histone H4	R.KacVLRDNIQGITKPAIRR.L	2019.24900	2019.20650	0.04250	6.70E-03	2
Histone H4	R.DAVTYTEHAKub1.R	1247.57940	1247.57820	0.00120	5.00E-04	3
Histone H4	R.Kme2VLRDNIQGITKPAIRme1.R	1863.13820	1863.14180	-0.00350	2.50E-02	2
Histone H4	R.Kme2VLRDNIQGITKPAIR.R	1849.12490	1849.12610	-0.00130	6.30E-04	9
Histone H4	R.Kme2VLRDNIQGITKPAIRR.L	2005.23340	2005.22720	0.00620	3.40E-06	5
Histone H4	R.DNIQGITKPAIRme1.R	1338.76350	1338.76190	0.00160	3.60E-02	2
Histone H4	R.DNIQGITKub1PAIR.R	1438.79330	1438.78920	0.00410	5.80E-05	5
Histone H4	R.GKacGGKacGLGKacGGAK.R	1239.69550	1239.69350	0.00200	6.00E-08	4
Histone H4	R.GKacGGKacGLGK.G	884.50820	884.50800	0.00030	2.60E-05	5
Histone H4	R.GKacGGKGLGKacGGAK.R	1197.67800	1197.68300	-0.00490	3.60E-06	2
Histone H4	R.GKGGKacGLGKacGGAK.R	1197.68160	1197.68300	-0.00140	8.50E-06	3
Histone H4	R.GKGGKacGLGK.G	842.49640	842.49740	-0.00100	4.00E-03	4
Histone H4	R.HRKme2VLRDNIQGITKPAIR.R	2142.29640	2142.28610	0.01030	2.70E-02	3
Histone H4	R.HRKVLRme2DNIQGITKPAIR.R	2142.28370	2142.28610	-0.00240	1.50E-02	2
Histone H4	R.KVLRme2DNIQGITKPAIR.R	1849.12630	1849.12610	0.00020	1.90E-04	3
Histone H4	R.KVLRme1DNIQGITKPAIR.R	1835.11110	1835.11050	0.00060	6.00E-03	5
Histone H4	R.Kme1VLRDNIQGITKPAIR.R	1835.11150	1835.11050	0.00100	1.10E-03	4
Histone H4	R.Kme3VLRDNIQGITKPAIR.R	1863.14150	1863.14180	-0.00020	9.30E-03	3
Histone H4	R.KTVTAMDVVpYALKR.Q	1673.85800	1673.85760	0.00040	8.90E-07	4
Histone protein Hist1h2aa, Histone H2A type 2-B, 2-C	R.VTIAQGGVLPNIQAVLLPKKTESHKub1.S	2754.58030	2754.57560	0.00470	7.40E-03	1
Histone protein Hist1h2aa, Histone H2A type 2-B, 2-C	R.VTIAQGGVLPNIQAVLLPKub1KTESHK.S	2754.58040	2754.57560	0.00480	4.70E-02	3

[a] A total of 196 unique PTM sites were found on histones H1, H2A, H2B, H3, H4 and variants. In the peptide sequence, the site/s of phosphorylation are designated by "p", acetylation by "ac", N-terminal acetylation by "ac-", mono-/di-/trimethylation by "me1, me2 or me3", respectively, and ubiquitination by "ub1". If the site of modification is ambiguous and cannot be assigned to a single residue, the possible region is in parentheses, e.g., (Kme1Kme1). For each peptide, the frequency at which it was detected across all experiments, which estimates its abundance,[55−57] is indicated in the 'spectral count' column.

better delineate the regulatory mechanisms of gene expression by protein PTMs.

Large-Scale Phosphorylation Site Mapping in Synaptic, Nuclear and Histone Proteins *in Vivo*. A total of 2082 unique phosphopeptides corresponding to 1062 phosphoproteins were identified in synaptic and nuclear fractions and served as a reference data set for the analyses of histones. Overall, in these two fractions, phosphorylation ratios were 85% pS, 14% pT and 1% pY, consistent with other large-scale analyses.[28] The majority of peptides were monophosphorylated (89%), and a small but significant number were biphosphorylated (10%) or triphosphorylated (0.8%). No significant difference in subcellular ratios of pSTY sites was observed between synaptic and nuclear fractions, except for a slightly lower level of pY in the nuclear fraction, consistent with the fact that this site is primarily associated with cytoplasmic events.[29] When comparing this data set to phosphorylation sites identified on histones, we observed that histones overall had a lower proportion of pS but a higher level of both pT and pY (Figure 2C). The reason for this difference is unclear since the relative proportions of the amino acids S, T and Y is the same in histones and other proteins (Supplementary Figure 3). This difference might be due to the action of different histone-specific kinases and/or phosphatases. As expected, rough protein abundance estimation using emPAI (exponentially modified protein abundance index)[30] showed that phosphorylated proteins were generally among the least abundant proteins identified (Supplementary Figure 8).

Identified Proteins Are from Distinct Categories. Following identification analyses, we investigated the functional significance of collected phosphopeptides and analyzed the annotated molecular functions. Gene ontology (GO) analysis revealed a high functional diversity between these different fractions (Figure 1A,B). Synaptic and nuclear fractions clearly

Table 2. Overview of Phosphorylated Peptide Sequences Identified on Well-Characterized Synaptic and Nuclear Proteins

Protein	Accession	Phosphopeptide	Residue	Measured mass (Da)	Calculated mass (Da)	Mass Accuracy (Da)	Peptide probability
BSN	BSN_MOUSE	R.APpSAGTDGPLALYGWGALPAENISLCR.I	S1623	2836.32894	2836.32550	0.00344	2.62E-06
BSN	BSN_MOUSE	R.IADSpSVQTDDEEGEGR.Y	S2592	1866.65695	1866.65576	0.00119	7.74E-04
BSN	BSN_MOUSE	R.QTpSLADLEQK.V	S3158	1211.54404	1211.54347	0.00057	2.09E-03
BSN	BSN_MOUSE	R.SLSDPKPLSPpTAEESAK.E	T2868	1915.82545	1915.82167	0.00378	8.09E-03
CaMKIIa	KCC2A_MOUSE	K.GAILTpTMLATR.N	T306	1226.61154	1226.60939	0.00215	4.83E-05
CaMKIIa	KCC2A_MOUSE	R.FpTEEYQLFEELGK.G	T10	1711.74130	1711.73821	0.00310	4.97E-05
CaMKIIa	KCC2A_MOUSE	R.SpTVApSCMHR.Q	T276, S279	1207.39121	1207.39163	-0.00042	1.99E-03
CaMKIId	KCC2D_MOUSE	K.ANVVTpSPK.E	S338	894.42545	894.42119	0.00426	3.51E-03
CaMKIIβ	KCC2B_MOUSE	K.NSpSAITSPK.G	S363	1271.63425	1271.63660	-0.00235	3.49E-04
CaMKIIβ	KCC2B_MOUSE	K.NSSAIpTSPK.G	T366	983.43265	983.43247	0.00019	7.95E-03
Centg1	CENG1_MOUSE	K.FIpSGIFTK.S	S343	991.47861	991.47798	0.00063	1.25E-02
DAP-2	DLGP2_MOUSE	K.GLQFGpSSFQR.H	S805	1205.52438	1205.52304	0.00134	1.99E-03
GABBR2	GABR2_MOUSE	K.DLEEVTMQLQDpTPEK.T	T818	1854.80272	1854.79581	0.00692	3.35E-02
GABRA1	GBRA1_MOUSE	K.NNTYAPTATSYpTPNLAR.G	T375	1933.84545	1933.85709	-0.01164	5.61E-05
GLT-1	EAA2_MOUSE	K.pSADCSVEEEPWK.R	S558	1515.55881	1515.55887	-0.00005	6.39E-05
Inpp4a	INP4A_MOUSE	K.YAFNpSLQLK.A	S918	1162.54282	1162.54234	0.00048	1.83E-02
Mecp2	MECP2_MOUSE	K.VGDTSLDPNDFDFTVpTGRGpSPSR.R	S160, T164	2519.10661	2519.09662	0.00999	8.58E-05
Mecp2	MECP2_MOUSE	R.KPGpSVVAAAAAEAK.K	S274	1348.67428	1348.67514	-0.00086	2.15E-05
NR2A	NMDE1_MOUSE	K.SPDFNLTGpSQSNMLK.L	S890	1717.74545	1717.73824	0.00721	3.99E-09
NR2B	NMDE2_MOUSE	K.NMANLSGVNGSPQpSALDFIR.R	S920	2169.99496	2169.98779	0.00717	1.06E-07
NR2B	NMDE2_MOUSE	K.YPQSPpTNSK.A	T1286	1100.44545	1100.45393	-0.00849	3.40E-03
PACSIN	PACN1_MOUSE	K.TEQSVpTPEQQK.K	T181	1353.57980	1353.58133	-0.00153	3.16E-05
PACSIN	PACN1_MOUSE	R.GSVSpSYDR.G	S346	949.35403	949.35423	-0.00020	2.19E-04
Pias1	PIAS1_MOUSE	K.GILSLPHQApSPVSR.T	S503	1540.78097	1540.77628	0.00469	8.10E-04
Palm	PALM_MOUSE	R.pTSTPVRpSPGGSTMMK.A	S157	1695.67465	1695.67625	-0.00160	2.16E-04
PKCγ	KPCG_MOUSE	K.QPpTFCSHCTDFIWGIGK.Q	T47	2115.84262	2115.85843	-0.01581	1.26E-03
Piccolo	PCLO_MOUSE	R.ADPTVQLAPSPPKpSPK.V	S3519	1711.85715	1711.85458	0.00257	4.89E-02
Piccolo	PCLO_MOUSE	R.pSMSDPKPLpSPTADESSR.A	S3578, S3586	1963.76852	1963.76352	0.00501	4.03E-04
Piccolo	PCLO_MOUSE	R.SPSTIpSLK.E	S131	911.44545	911.43649	0.00896	6.81E-03
PKCγ	KPCG_MOUSE	R.SPpTSPVPVPVM.-	T689	1269.51132	1269.51175	-0.00043	1.60E-03
PKCγ	KPCG_MOUSE	R.SPTpSPVPVPVM.-	S690	1189.54599	1189.54543	0.00057	1.22E-03
PSD-95	DLG4_MOUSE	K.DWGSSSGpSQGR.E	S512	1202.43417	1202.43535	-0.00118	5.21E-04
PSD-95	DLG4_MOUSE	R.EQLMNSSLGSGTApSLR.S	S422	1729.76545	1729.77058	-0.00514	1.93E-08
Shank2	SHAN2_MOUSE	R.NpSPAFLSTDLGDEDVGLGPPAPR.M	S724	2404.08545	2404.09479	-0.00934	1.33E-07
Shank2	SHAN2_MOUSE	R.NSPAFLpSTDLGDEDVGLGPPAPR.M	S729	2404.10654	2404.09479	0.01176	2.60E-09
Shank3	SHAN3_MOUSE	R.LFSpSLGELSTISAQR.S	S1614	1687.82431	1687.81819	0.00612	2.67E-07
Stargazin	CCG2_MOUSE	R.IPpSYR.Y	S228	714.31015	714.31017	-0.00001	4.44E-02
Stx1a	STX1A_MOUSE	K.HSAILApSPNPDEK.T	S64	1457.65414	1457.65514	-0.00099	3.27E-05
Syn1	SYN1_MOUSE	K.TNTGpSAMLEQIAMSDR.Y	S341	1803.75668	1803.75322	0.00346	1.36E-05
Syn1	SYN1_MOUSE	R.GSHSQSpSPGALTLGR.Q	S437	1620.72458	1620.72571	-0.00112	5.02E-05
Syn1	SYN1_MOUSE	R.KpSFASLFSD.-	S699	1080.45432	1080.45288	0.00144	1.19E-02
Srrm2	SRRM2_MOUSE	R.DKFSPpTQDRPESSTVLK.V	T1153	2013.94585	2013.94084	0.00501	5.00E-06
Srrm2	SRRM2_MOUSE	R.ERAPpSPASR.M	S2381	1049.46777	1049.46549	0.00228	1.08E-03
Srrm2	SRRM2_MOUSE	K.SEQPLSQVLPpSLSPEHK.E	S1214	1954.94713	1954.94009	0.00704	9.46E-04
U2af	U2AF2_MOUSE	K.EEHGGLIRpSPR.H	S79	1474.72560	1473.71830	-0.00280	5.80E-03
Znf521	ZN521_MOUSE	R.ALpSPLSPVAIEQTTLK.M	S605	1746.92521	1746.91684	0.00837	2.68E-06

differed in their composition, particularly in areas associated with synaptic functions and translational/transcriptional regulation. Nuclear fractions, for instance, contained a significantly higher representation of proteins belonging to the GO categories 'DNA/RNA binding' and 'transcription'. In comparison, synaptosome and synaptic membrane fractions were significantly enriched for GO categories including 'enzyme/G protein' and 'receptor'. As expected, identified histones were categorized into the GO categories representing their major functions of 'DNA binding' and 'chromatin' (Figure 1A,B, Supplementary

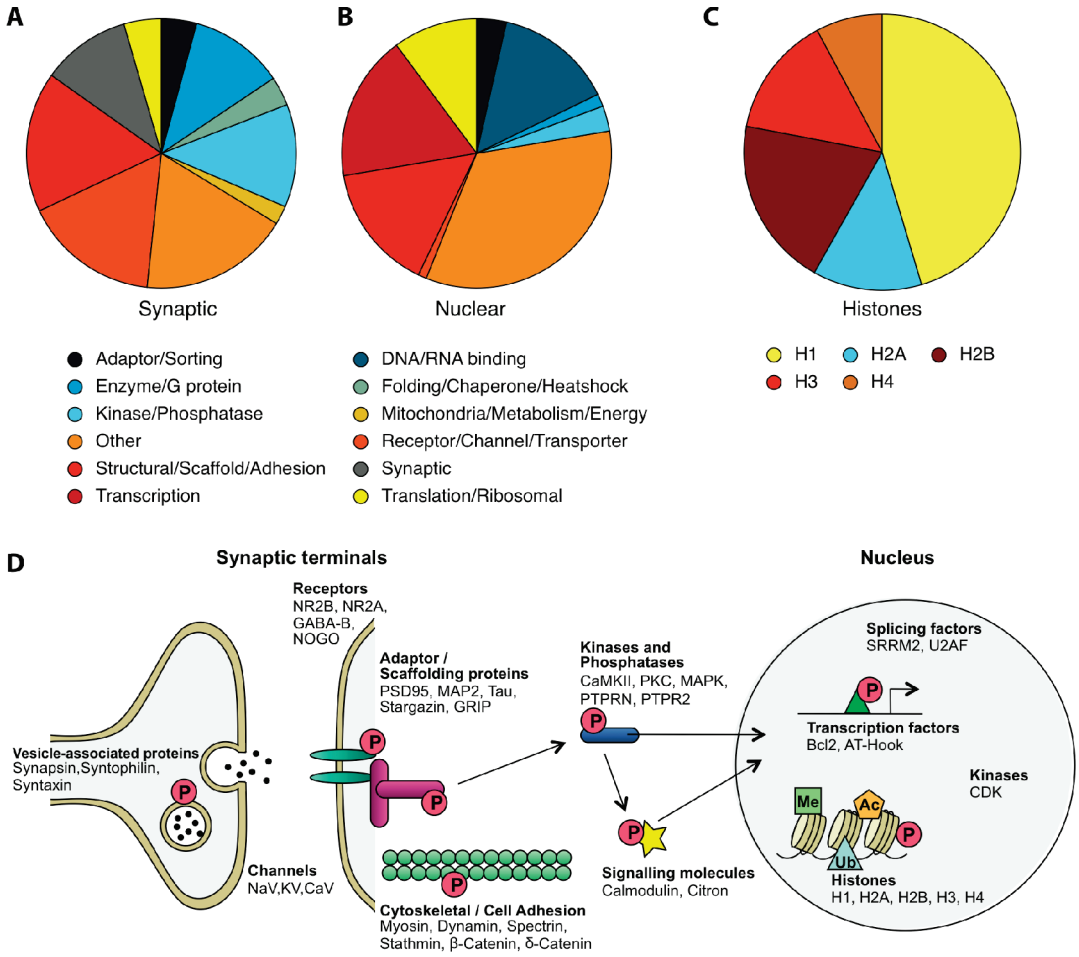

Figure 1. (A and B) Gene ontology analysis showing that phosphoproteins identified in synaptic fractions (A) belong to functional groups distinct from those identified in the nucleus (B), in particular, in areas associated with synaptic functions and translational/transcriptional regulation. (C) Novel histone PTMs were found on all 5 major histone subtypes, with about half found on H1. (D) Novel PTMs sites described in these analyses were found on proteins involved in key signaling pathways in the synapse, the nucleus and on chromatin.

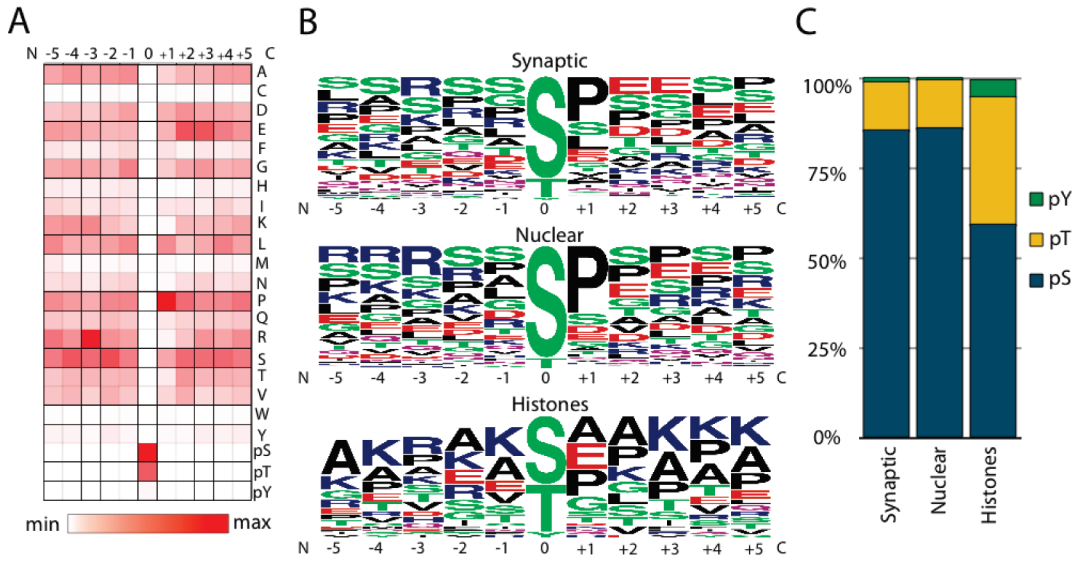

Figure 2. (A) Heat map of amino acid frequency surrounding all synaptic and nuclear phosphosites showing that certain amino acids such as proline (P) in the +1 position are clearly favored. (B) Amino acids surrounding synaptic (top) and nuclear (middle) phosphorylation sites were different to those found surrounding phosphorylation sites on histones (bottom). (C) Phosphorylated histones have a lower proportion of pS and a higher level of both pT and pY relative to synaptic and nuclear phosphoproteins.

Table 3. Determination of Overrepresented Motifs Surrounding Phosphorylation Sites Using Motif-X[18a]

Modification	Motif	Kinase Motif in literature – HPRD Phosphomotif finder	Binding Motif in literature - HPRD Phosphomotif finder
Synaptic pS	.....pSPT...	Unknown	WW domain binding motif
Synaptic pS	.....pSP....	GSK-3, ERK1, ERK2, CDK5 substrate motif	WW domain binding motif
Synaptic pS	..R..sL....	Unknown	14-3-3 domain binding motif
Synaptic pS	.....pS.E...	Casein Kinase II substrate motif	Unknown
Synaptic pS	..R..pS.....	PKA, PKC, CamKII substrate motif	14-3-3 domain binding motif
Synaptic pS	..... pS.D...	Unknown	Unknown
Synaptic pS	.....pS..E..	Casein kinase II substrate motif	Unknown
Synaptic pS	..K..pS.....	PKA, PKC kinase substrate motif	Unknown
Synaptic pS	..S..pS.....	Unknown	Unknown
Synaptic pT	.....pTP....	GSK-3, ERK1, ERK2, CDK5 substrate motif	WW domain binding motif
Synaptic pT	.....pTSP...	Unknown	WW domain binding motif
Synaptic pT	....SpT.....	Unknown	MDC1, BRCT, Plk1, PBD domain binding motif
Synaptic pT	.....pT.S...	Unknown	Unknown
Synaptic pY	....G.pY.....	Unknown	Unknown
Synaptic pY	....K.pY.....	Unknown	Unknown
Synaptic pY	.....VpY.....	Unknown	Unknown
Nuclear pS	..R..pSP....	DYRK1A	WW, 14-3-3 domain binding motif
Nuclear pS	.....pSP.R..	CDK-1, -2,- 4, -6, Growth associated histone HI kinase, Cdc2, CDK kinase substrate motif	WW domain binding motif
Nuclear pS	.R...pSP....	Unknown	WW domain binding motif
Nuclear pS	.....pSP...K	Unknown	WW domain binding motif
Nuclear pS	.....pSP....	GSK-3, ERK1, ERK2, CDK5 substrate motif	WW domain binding motif
Nuclear pS	..R..pS.....	PKA, PKC, CamKII substrate motif	14-3-3 domain binding motif
Nuclear pS	.....pS..E..	Casein kinase II substrate motif	Unknown
Nuclear pS	.....pS SP...	Unknown	Unknown
Nuclear pT	.....pTP....	GSK-3, ERK1, ERK2, CDK5 substrate motif	WW domain binding motif
Histones pS	.K...pS.....	PKA kinase substrate motif	Unknown
Histones pS	.....pS..AP.	Unknown	Unknown
Histones pT	A....pT.....	Unknown	Unknown

[a] Differences in motifs between sites on histones and sites on nuclear and synaptic proteins were revealed. One-third of the identified motifs could not be found in the HPRD[21] and may represent novel phosphorylation motifs.

Figure 5). These GO analyses, therefore, demonstrate the ability to target phosphoproteins in specific neuronal compartments through subcellular fractionation.

Analysis of Phosphorylation Site Motifs and Kinase Specificity. Kinase specificity typically depends on the primary amino acid sequence surrounding a given phosphorylation site, and major kinase classes include the proline-directed basophilic kinases and acidophilic kinases.[31] To detect differences in phosphorylation site motifs in distinct fractions, we used motif-X.[18] This allowed us to extract overrepresented patterns/motifs surrounding pS, pT and pY residues from each of the synaptic, nuclear and histone phosphorylation data sets (Table 3 and Supplementary Figure 4) and isolate the amino acids surrounding each phosphorylation site identified. The amino acid composition surrounding nuclear and synaptic phosphorylation sites (Figure 2B, top and middle panels) was similar, and certain amino acids surrounding the site of phosphorylation were clearly favored (Figure 2A). However, in comparison, amino acids surrounding histone phosphorylation sites (Figure 2B, bottom panel), and overrepresented histone phosphorylation motifs found with Motif-X were distinct from those found in the synapse and nucleus. We additionally compared the putative kinase motifs detected with motif-X, with motifs in the literature using HPRD.[21] We found that the majority of the motifs (16/24) mapped to known kinases and/or binding motifs, but a third (8/24) were unknown and may therefore represent novel phosphorylation motifs (Table 3 and Supplementary Figure 4). Furthermore, we predicted kinase/substrate relationships for all three fractions in our data set using ScanSite.[20] In agreement with the detected motifs, ScanSite predicted that a large proportion of phosphorylation sites were targets of proline-directed kinases (CK2, MAPK14, etc.), along

with the other major classes of S/T kinases, including the basophilic kinases (PKA, PKC alpha/beta/gamma, Akt), acidophilic kinases (GSK3, CK2, ATM) and pY kinases (Lck, Abl).[32] Phosphorylation sites on histone proteins were mainly predicted to result from the activity of cyclin dependent kinases (CDKs), which phosphorylate sites with the (S/T)-P-X-K consensus motif[33] (Supplementary Table 5). In agreement with this analysis, currently known kinases that phosphorylate histones (MSK1, RSK1, CDKs and IPL1) are distinct from kinases in other subcellular compartments, and are often purely nuclear in localization and in some cases, bound to chromatin.[34] However, the ScanSite analysis also revealed that for all 3 cellular fractions, the majority of phosphorylation sites (75%) could not be assigned to a specific kinase, indicating that kinase motif information is either incomplete and/or not yet fully understood.

Phosphorylation Is Abundant on All Histones. Phosphorylation is perhaps the most interesting histone PTM because it involves protein kinases and phosphatases that are present in the cytoplasm and the nucleus, and therefore provides a direct functional link between intracellular signaling pathways and chromatin remodelling.[34] Surprisingly, nearly half of all identified phosphorylation sites were found on the linker histone H1 and subtypes (Figure 1C, Figure 5 and Table 1). It is thought that the cyclin-dependent kinases (CDKs) are primarily responsible for H1 linker histone phosphorylation.[33,35] While most H1 phosphorylation sites identified in these experiments are within CDK-consensus sequences, novel sites were found within both, CDK consensus sequences such as Ser 18 (H1.3, H1.4), and outside CDK-consensus sequences, Ser 1 (H1.1, H1.2, H1.3, H1.4), Ser 5 (H1.0), Thr 8 (H1.5), Ser 12 (H1.0) and Ser15 (H1.0). Analyses with ScanSite identified CDK5 as the likely kinase for Thr8 phosphorylation on H2B3A, but it did not identify any likely kinase(s) for other novel sites on H1, H2A, H2B, H3 or H4, none of which were within known kinase motifs (Table 1). Our data, therefore, confirms the role of CDKs in histone phosphorylation, but further strengthens the notion that histone phosphorylation is mediated by additional kinases targeting currently unknown phosphorylation motifs.[33,36]

Histone Acetylation and Methylation. Acetylation and methylation of the ε-amino group of lysine and guanidinyl group of arginine residues are the most frequent PTMs on histone proteins[37] (Figure 5). Acetylation of histones is usually linked to transcriptional activation, whereas methylation can result in either activation or repression, depending on the modified residue and the surrounding PTMs on the histone.[2] Unlike phosphorylation, little is known about the specificity of the enzymes that generate these modifications. Acetylated and methylated lysine and arginine residues were extremely common on identified histone peptides in our samples. However, despite the number of previously identified sites, novel PTMs were detected on all core and linker histones (Figure 3 and Table 1), particularly on N- and C-terminal tails (Supplementary Figure 7). Several novel sites identified here (in the mouse) have been demonstrated to play specific roles in yeast, such as N-terminal H4 acetylation in chromatin binding,[38] and H3K36 methylation in transcriptional activation.[39] These sites may have similar roles in the mouse but will need further analysis. However, most novel sites presented here have not yet been studied or identified in other species. In general, we observed that dimethylated lysine residues were the most frequent (67%), and trimethylated the least frequent (5%). This is an interesting finding because each PTM is proposed to have

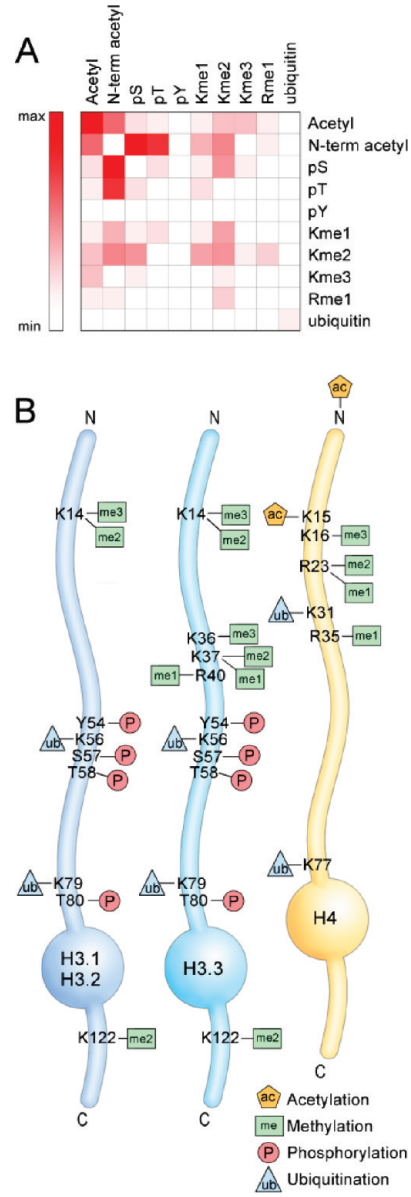

Figure 3. (A) PTMs co-occurring on the same peptide were plotted on a heat-chart to visualize the frequency of co-occurrence of different histone PTMs. Certain combinations were clearly more frequent than others, reinforcing the idea that the histone code has specific combinations of PTMs. (B) A total of 65 sites were identified on H3.1, H3.2, H3.3 and H4 in these analyses. PTMs depicted above were novel at the time of writing. Several of these sites are in close proximity to, or on the same residue as, those known to be involved in memory formation such as H3S10 and H3K14.

a different impact on gene expression, and trimethylation is thought to be the most stable mark.[40]

Histone Ubiquitination Is a Common Histone PTM. Ubiquitination is a PTM classically associated with protein degradation in the cytosol,[41] that has only recently been shown to occur on histones. Ubiquitination involves the addition of a large molecule (ubiquitin, 76 amino acids) that can be chained together to polyubiquinate proteins. While polyubiquitination marks proteins for degradation by the 26S proteasome, monoubiquitination has signaling purposes. It can be reversed by ubiquitin-specific proteases (USPs),[42] and when occurring on H2B and H2A, it is thought to function as a master switch

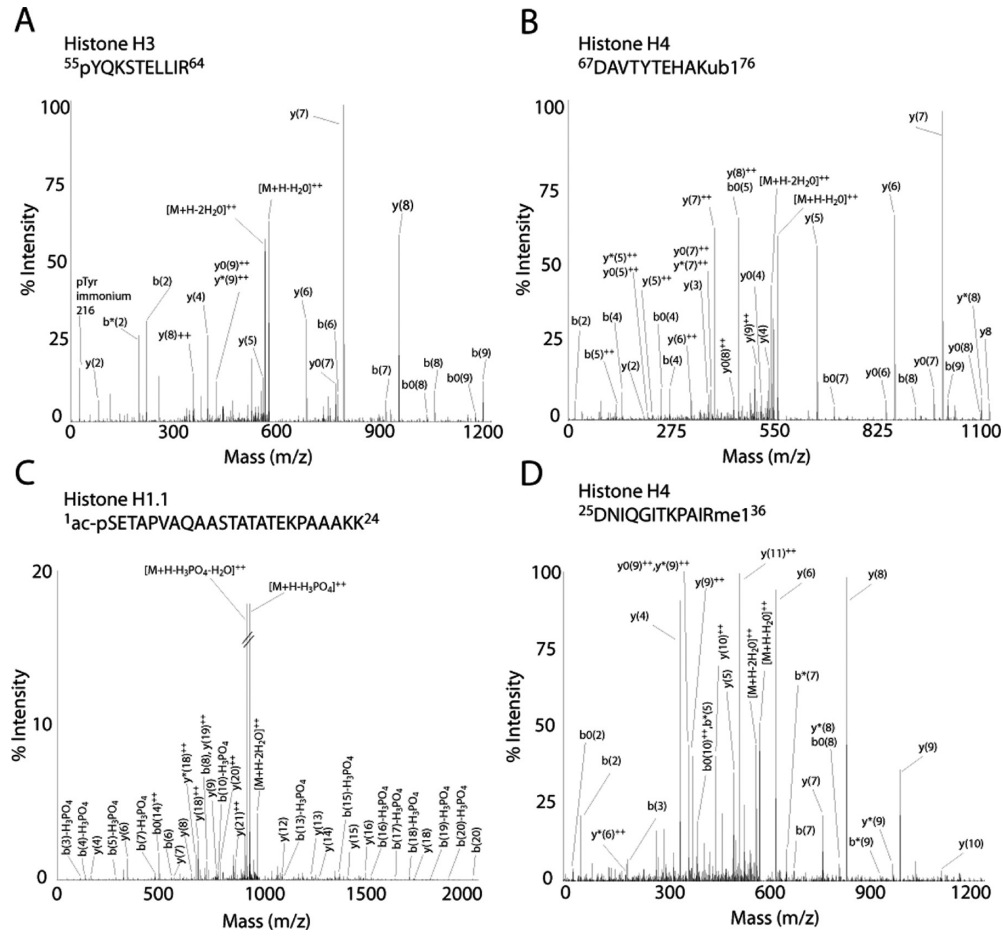

Figure 4. Mass spectra from selected novel histone PTMs. (A) H3pY55 tyrosine phosphorylation, (B) H4K78ub1 lysine ubiquitination, (C) H1.1ac-pS1 N-terminal acetylation with serine phosphorylation, and (D) H4R36me1 arginine methylation.

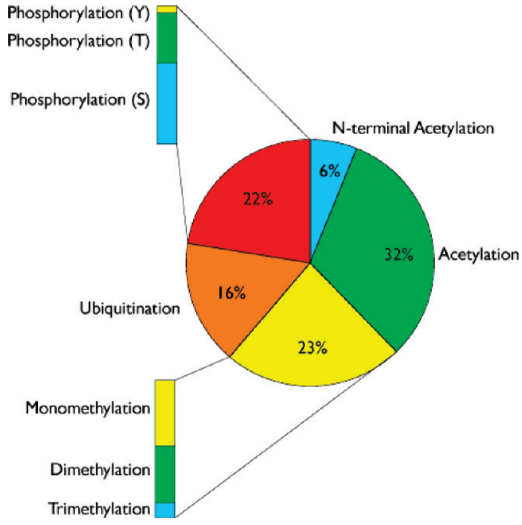

Figure 5. The majority of the sites identified were either acetylation or methylation sites, about 25% were phosphorylation sites and 16% ubiquitination sites. The large proportion of ubiquitination indicates that this PTM is a lot more prominent on histones than anticipated, which suggests that it may have a much larger role in the histone code than previously thought.

regulating multiple modifications on other histones.[43] Actively transcribed genes are known to be enriched in H2B ubiquitination, while inactive genes are enriched in H2A ubiquitination.[44,45] The mechanisms by which ubiquitin increases gene expression are not known but may involve the physical opening of chromatin through a 'wedging process',[37] and may perhaps also favor histone proteolysis.[46] To date, ubiquitination in the mouse has only been found on histones H2A (monoub K119 mediated by PRC1L), H2B (monoub K120 mediated by Rad6/Bre1)[40] and H1 (K46 and K116).[33] Here, we identified 30 additional sites on histone H2A (8 sites), H2B (5 sites), H1 (12 sites), and H4 (2 sites) (Figure 3B and Table 1), in addition to the currently known sites on H2A and H2B. Analyses with PESTfind, a tool that locates regions rich in P, E, S, and Ts, which are associated with ubiquitination and are common on short-lived proteins,[47] did not show any such regions on histone proteins, except on the N-terminal region (residues 1−28) of H1.5. This is consistent with histones being long-lived molecules and with histone ubiquitination being more likely associated with chromatin remodelling than with targeting the protein for degradation. The frequency of these PTM sites in our data set suggests that it potentially has a much larger role in chromatin remodelling than currently thought, but further experiments are needed to determine if these residues are mono- or polyubiquitinated.

Histone H1 and Histone Cores Are Extensively Modified. The role of the four core histones in the 'histone code' has been extensively studied, but little is known about the linker histone H1 and its variants. H1 is subjected to all four types of PTMs, but its peptides are also frequently found nonmodified, indicating that PTMs on H1 are dynamic and unstable. Recent studies have shown that many PTM sites are in regions thought

to be involved in binding to nucleosomal DNA[33] and potentially in gene repression.[48] To date, most known histone PTMs are located in the flexible tail protruding from the nucleosome. However, recent MS analyses have shown that they can also occur in the central histone-fold domains and C-terminal regions.[33,49] Accordingly, we found PTMs not only on C- or N-terminal tails, but also in the histone core. For H1, 14% of PTMs were found in the core (86% in tail), 4% for H2A, 5% for H2B, and 6% for H3 and H4 (see Supplementary Figure 7). In the case of H1, the fact that many PTMs were found in regions not involved with DNA binding suggests a potential role for H1 in 'cross-talk' between core histone PTMs and gene expression.

Close-Range Combinatorial Patterns of Histone PTMs. The histone code hypothesis proposes that PTMs co-occur in specific combinations and patterns, and can regulate each other through controlled 'cross-talk'.[49,50] Some PTMs can influence the occurrence of other subsequent PTMs through steric hindrance or by enhancing/impeding the binding of histone modifying enzymes.[49] Because of the global approach used in our analyses, we were able to not only examine individual PTMs, but also their combinations on identified peptides. Since the average peptide length obtained from histones was 14 amino acids, the observed PTMs were close together, which may increase their propensity for crosstalk. Particular combinations of PTMs such as N-terminal acetylation together with phosphorylation at S2 and T3, and/or acetylation/methylation at K17 and K22, were frequently observed. Ubiquitination and pY in comparison were typically observed alone (Figure 3A). We also determined whether published 'rules' for the histone code[2] were observed in our data set, in particular, whether modifications known to be mutually exclusive were found together or not. Consistent with the literature, sites such as H3K4me1 and H3K9ac, thought to be mutually exclusive,[49] were never seen together on any identified peptides. Furthermore, H3K9me1 was seen both in isolation and in combination with H3K14ac, a PTM thought to be inhibitory but not mutually exclusive with H3K9me1.[49,51] Additional interesting sites of potential crosstalk are novel di- and trimethylation (H3K14) found in close proximity to H3S10, a key phosphosite for long-term memory.[34,52] H3K14me1, but not mono- or dimethylation, was ever observed in combination with H3pS10. Recent studies have also demonstrated that H3 variants have distinct PTM patterns, leading to the H3 'barcode hypothesis'.[53,54] Consistent with this, we detected differences in PTMs on N-terminal tails of H3.1/3.2 and H3.3 (Figure 3B). Overall, this data points to the multiplicity of the histone code and the need for combinatorial analyses of histones PTMs in future studies of synaptic functions.[34,43]

Conclusions

The findings presented here provide an extensive mapping of PTMs on histone proteins in the brain, and on other major synaptic and nuclear proteins. The data demonstrates that histones PTMs are much more prevalent than previously thought, which further strengthens their importance for regulatory mechanisms in the brain. We show that ubiquitination frequently occurs on all histones and, in particular, on the linker histone H1. All PTMs were found to occur on both the core and tail regions of histones demonstrating the complexity of the regulatory mechanisms in which they are involved. Future studies are required to determine the biological significance of these PTMs and their various combinations, and are expected to provide important new insight into the regulation of gene expression and chromatin remodelling in the brain. In addition, the data further strengthens the need for systems approaches to study the combinatorial patterns of histone PTMs using technologies such as ETD and top-down proteomics.

Abbreviations: ac, acetylation; ac-, N-terminal acetylation; CNS, central nervous system; Da, dalton; GO, gene ontology; HPLC, high performance liquid chromatography; HDAC, histone deacetylase, IMAC, immobilized metal affinity chromatography; me1/me2/me3, mono/di/trimethylation; MS, mass spectrometry; p, phosphorylation; PSD, postsynaptic density; PTM, post-translational modification; RP, reversed phase; SCX, strong cationic exchange; Ser(S)/Thr(T)/Tyr(Y), serine/threonine/tyrosine; TiO_2, titanium dioxide; ub1, ubiquitination.

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Supporting Information Available: Supplementary figures, tables and methods are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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