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REVIEW ARTICLE

Revealing histone variant induced changes via quantitative proteomics

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Abstract

Histone variants are isoforms of linker and core histone proteins that differ in their amino acid sequences. These variants have distinct genomic locations and posttranslational modifications, thus increasing the complexity of the chromatin architecture. Biological studies of histone variants indicate that they play a role in many processes including transcription, DNA damage response, and the cell cycle. The small differences in amino acid sequence and the diverse posttranslational modification states that exist between histone variants make traditional analysis using immunoassay methods challenging. In recent years, a number of mass spectrometric techniques have been developed to identify and quantify histones at the whole protein or peptide levels. In this review, we discuss the biology of histone variants and methods to characterize them using mass spectrometry-based proteomics.

Introduction

The nucleosome, the basic repeating unit of chromatin, consists of DNA wrapped around an octamer of core histone proteins, two copies each of H2A, H2B, H3, and H4. Linker histone H1 may also be present, and contribute to chromatin structure. The presence of covalent histone posttranslational modifications (PTMs) and the incorporation of histone sequence variants alter the composition of the nucleosome (Figure 1). Most PTMs occur on the N-terminal tails of histone proteins and include methylation (mono-, di-, tri-), phosphorylation, acetylation, and ubiquitination. The observation that certain histone modifications are associated with active genes and others with repressed genes lead to the hypothesis that information contained in histone PTMs forms a "histone code," read by numerous effector proteins to influence chromatin structure and downstream events such as transcription (Strahl & Allis, 2000). Histone variants affect a variety of chromatin-related processes, are localized to different areas of the genome, and have unique modification patterns; they are proposed to form an extra layer of the histone code (Hake & Allis, 2006).

Histone PTMs and variants impact a variety of biological processes, including transcription, DNA damage response, cell cycle, viral infection, stem cell pluripotency, and fertility. Chromosome condensation and proper segregation during mitosis are correlated with histone H3 phosphorylation at serine 10 and serine 28, implicating these PTMs in cell cycle regulation (Garcia et al., 2005). Histone deacetylase inhibitors (HDACi), drugs that increase global histone acetylation by blocking deacetylase activity, have been used to induce pluripotent stem cells (Huangfu et al., 2008a, 2008b) and to reactivate latent human immunodeficiency virus (HIV; Van Lint et al., 1996), thus providing evidence for histone acetylation's role in these processes. Additionally, histone H2A variant H2A.X is required for male fertility in mice; in its absence, spermatogenesis halts at the pachytene phase (meosis I), resulting in loss of mature sperm production

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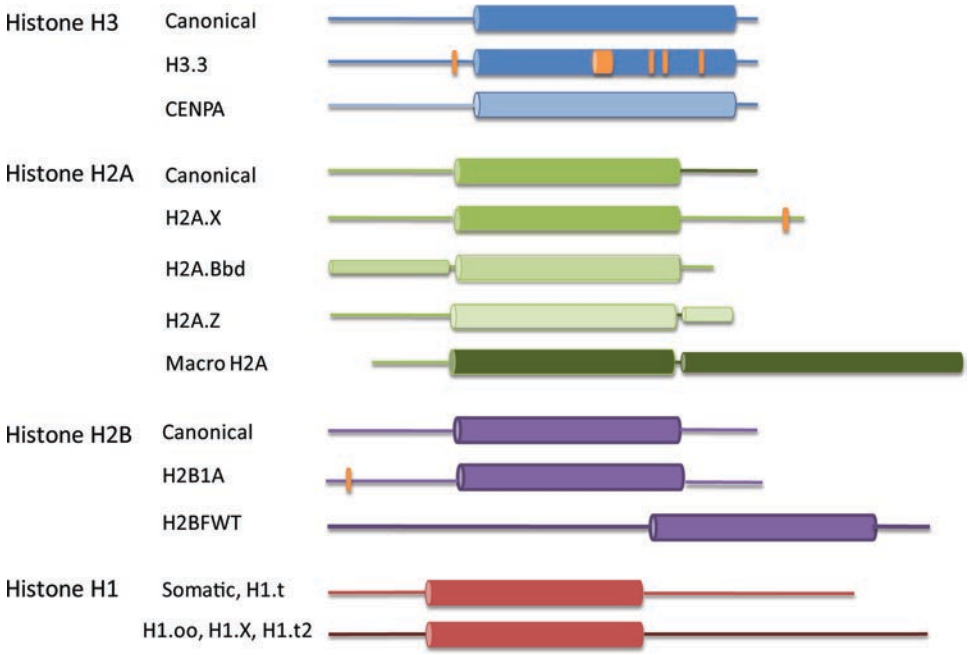

Figure 1. All of the histone variants contain a highly conserved histone fold domain and vary mainly in their C- and N-terminal sequences. Shown above is a schematic comparing histone-variant sequences. Boxes represent the histone fold domain and orange lines represent site-specific sequence variations. Histones that are in different shades of the same color are from the same histone family but have large differences in sequence.

and infertility (Celeste et al., 2002). These examples highlight the biological importance of select histone variants and PTMs. Many of the other variants and PTMs remain to be characterized.

Histone variants and their PTMs often need to be quantified across different conditions in order to determine their functions in the cell. The reliable identification and quantification of histone PTMs is challenging because histones may be extensively modified and similar in structure and molecular weight. Histone variant analysis is equally difficult because variants can differ in sequence by as little as one amino acid. The two main methods currently used to study histone variants and their PTMs are immunoblotting and mass spectrometry. Immunoblotting is very sensitive but it is not very quantitative and it is a laborious task that provides information only about a single modification or a subset of modifications in a given sample. In addition, many histone modifications and variants are similar in structure and sequence, making the specificity and cross-reactivity of antibodies a problem (Egelhofer et al., 2011; Fuchs et al., 2011). Epitope occlusion is a concern because modifications are also often closely spaced, e.g. Histone H3 Lysine 9 is acetylated and serine 10 is phosphorylated during mitosis (Hirota et al., 2005). In contrast, liquid-chromatography mass spectrometry (LC-MS) can be used to identify large numbers of proteins, their modifications, and the locations of their modifications in just a couple of hours. In addition, there are many MS techniques for the quantification of modified proteins. This review will focus on the progress that has been made in applying quantitative LC-MS techniques to study histone variants and their modifications.

Histone overview

Histone variants differ from canonical histones in their genomic organization and gene expression. Canonical histone genes are intronless and occur in clusters, facilitating joint gene regulation (Albig & Doenecke, 1997). Their mRNAs lack a polyA tail, instead having a dyad sequence that forms a 3′ stem loop structure. Their expression is replication dependent, which means that their expression is linked to the S-phase of the cell cycle. Expression increases as much as 35-fold in S-phase to allow for sufficient histone protein incorporation during DNA synthesis (Harris et al., 1991). The number of canonical genes that code for each histone family member can exceed 10 genes (Marzluff et al., 2002). These canonical genes can produce different protein sequences, drastically increasing variability of the histone population. The only exception is histone H4, whose 12 genes all produce the same sequence. Canonical histone proteins (H3, H4, H2A, and H2B) are small, ranging in size from 11–15 kDa and consist of a lysine-rich N-terminal tail, a globular histone fold domain, and a C-terminal tail. Different combinatorial PTMs to these core proteins add to the complexity.

In contrast to canonical histone genes, histone variants are replication independent with expression occurring throughout the cell cycle. Genes encoding histone variants typically lay outside of clusters, can contain introns, and occur in low copy number. Like standard genes, mRNA produced from variant genes possesses a polyA tail (Doenecke et al., 1997). Histone variants have protein sequences, modifications, structures, and bio-

logical functions distinct from their respective canonical family members.

MS analysis of histones

Most commonly, acid extraction is used to isolate histones from mammalian cell nuclei. This method gives reasonably pure histones for analysis (>90% purity), thus decreasing the sample complexity and diminishing the need for complicated separation strategies seen in whole cell proteomics (Shechter et al., 2007). Several alternatives also exist for isolating histones under nondenaturing conditions, as histones can be extracted from nuclei after micrococcal nuclease digestion using high salt concentrations or by hydroxyapatite chromatography (von Holt et al., 1989; Benson et al., 2006). Different protocols for isolating nuclei and histones are required for plants and some fungi (including *Saccharomyces cerevisiae*) as they have tough-to-lyse cell walls, and, typically, the histone yield and purity are observed to be somewhat lower for these eukaryotic systems. Nevertheless, the histone isolation procedures are essentially the same as for mammalian cells and can be very easily implemented and executed with very little issues (Ide & Saunders, 1981; Waterborg et al., 1987; Edmondson et al., 1996). After extraction, there are different processing steps depending on the type of MS analysis and the method of quantification. There are three main MS analysis techniques used to analyze histones: Bottom Up, Middle Down, and Top Down proteomics. These methods are compared in Figure 2, with a specific emphasis on differences in their utility during histone variant analysis.

Bottom Up Proteomics

Bottom Up proteomics is probably the most widely used form of MS analysis for histones and PTMs. For this method, histones are first subjected to proteolytic digestion and then the resulting peptides are desalted and loaded onto a C8 or C18 column for reverse phase LC-MS analysis. As the peptides elute, they are subjected to MS analysis and fragmentation, known as *MS/MS analysis*. The most common type of fragmentation for Bottom Up analysis is collision-activated dissociation (CAD), which breaks the peptide at the amide bond to create a series of b- and y-type ions. The difference in mass between ions in the series can be used to determine the peptide sequence and the positions of PTMs.

Trypsin, a protease that cleaves after arginine and lysine, is the enzyme most commonly used for digestion during Bottom Up MS analysis. However, histone proteins are arginine and lysine rich, especially in their N-terminal tails where modifications are prevalent; so the standard tryptic digest creates ions that are too small to be retained on a high performance liquid chromatography (HPLC) column and thus cannot be detected using LC-MS analysis. The protease ArgC, which only cleaves after arginines, can be used in the place of trypsin to create longer peptides for LC-MS analysis but ArgC is not as robust as trypsin. Another alternative to a pure tryptic digest is to chemically derivatize lysines using propionic anhydride or acetic anhydride, blocking their cleavage by trypsin (Smith et al., 2002; Bonaldi et al., 2004; Garcia et al., 2007a). The derivatization method is efficient; in addition, propionic or acetic anhydride derivatization decreases the charge on peptides, increasing their retention on reverse phase columns and improving chromatographic resolution.

Bottom Up proteomics has limitations when it comes to analyzing histone variants and combinations of histone modifications. During ArgC or tryptic digests, information about the modifications that occur in combination is lost because of the small size of the resultant peptides (5–20 amino acids in length). In addition, Bottom Up proteomics cannot be used to distinguish between many of the histone variants and their modified states because of small differences in their sequences. For example, there is no unique peptide that distinguishes all of the H2B variants from each other, so they cannot be quantified using Bottom Up MS (see Figure 2). Offline separation techniques can be used to purify histone variants prior to Bottom Up analysis. For example, histones H3.1, H3.2, and H3.3 have many tryptic peptides in common and cannot be distinguished from a mixture of digested histones, but they can be separated prior to digestion and LC-MS analysis using reversed-phase HPLC (RP-HPLC). Alternatively, whole protein MS (Top Down MS) or MS of larger histone peptides (Middle Down MS) can be used to gain more information about histone variants and combinatorial modifications.

Top Down proteomics

Top Down MS is the only method that can distinguish between all of the histone isoforms and their PTMs. Prefractionated whole histones are identified using Top Down MS by their intact mass and fragmentation spectra (Kelleher, 2004). Using this method, even isobaric histones, such as H2B1H and H2B1K, can be identified based on their fragment ions. Analysis of H2B variants from bulk H2B is presented in Figure 2. Histones are very basic proteins and are therefore highly charged and generate multiply charged fragment ions. As a consequence, non-ergodic fragmentation methods such as electron transfer dissociation (ETD) or electron coupled dissociation (ECD) and high-resolution instruments such as the LTQ-Orbitrap or FT-ICR (Zubarev et al., 1998; Syka et al., 2004) are required for Top Down histone analysis.

Despite the advantages of Top Down MS, it is not utilized as often as Bottom Up MS for histone analysis because it is not as sensitive: many of the low-level histone variants and modifications that can be identified and quantified using Bottom Up methods are not readily detected in Top Down spectra. Another disadvantage of Top Down analysis is that it does not lend itself well to high-throughput analysis because it is not easily coupled to online separations. Some advances have been made in combining online separations with Top Down MS for the analysis of histone mixtures (Eliuk et al., 2010; Tian

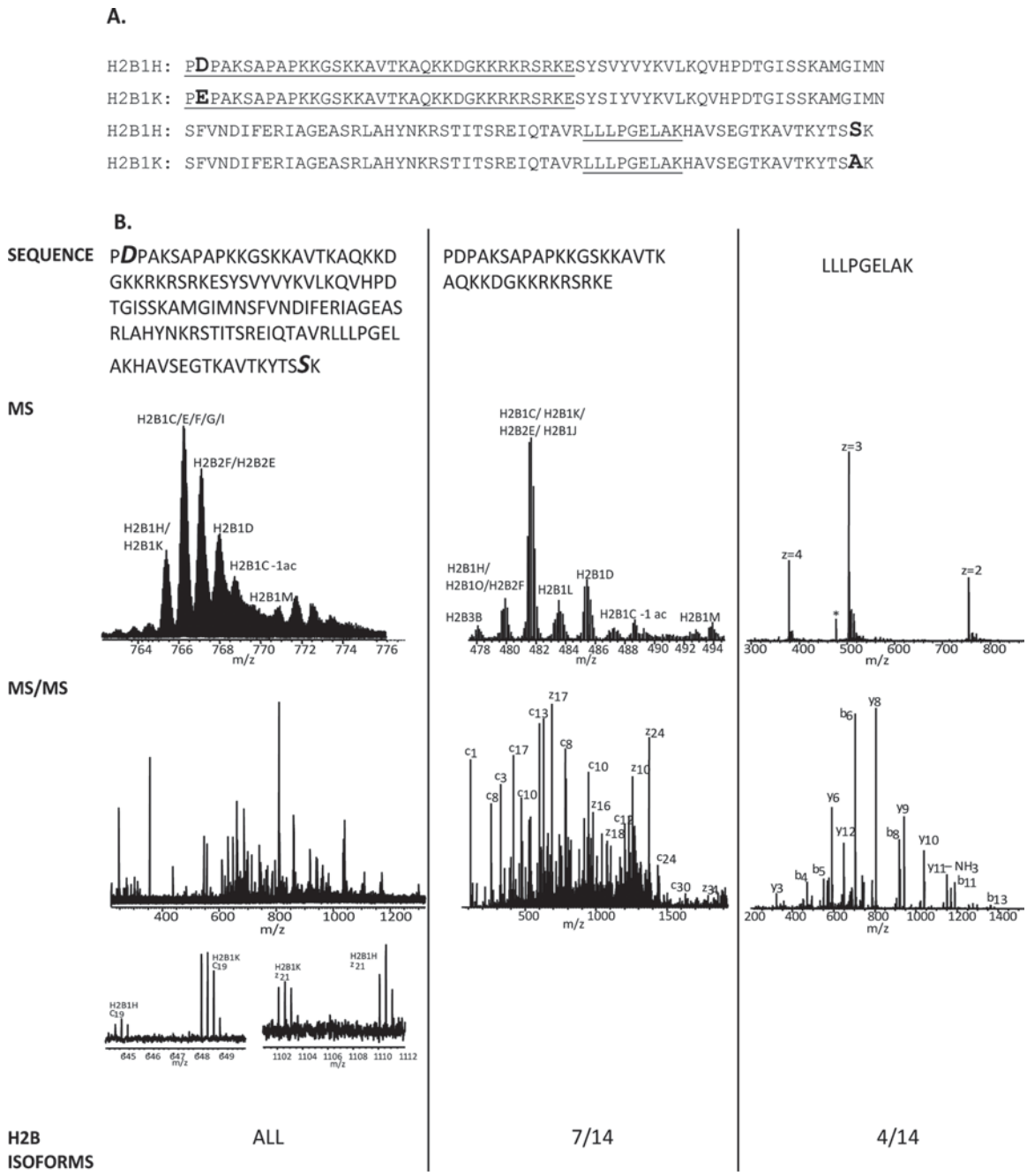

Figure 2. (A) A sequence alignment H2B1K and H2B1H demonstrate the extent of sequence similarity between H2B isoforms. (B) Side-by-side comparison of a mixture of H2B sequences analyzed using Bottom Up, Middle Down, and Top Down mass spectrometry. Top Down: The Top Down mass spectra were collected using direct infusion of purified H2B and fragmented using electron transfer dissociation (ETD) on an LTQ-Orbitrap. Isobaric H2B1K and H2B1H species could be differentiated after MS/MS analysis. Middle Down: Middle Down spectra were collected after online reversed-phase high performance liquid chromatography (RP-HPLC) separation of Glu-C digested H2B and fragmented using ETD. A peak corresponding to the N-terminal tail of H2B1H was chosen for MS/MS analysis, however, the N-terminal tail of H2B1H is identical to that of H2B1O and H2B2F. Seven out of 14 somatic H2B variants can be uniquely identified using Middle Down MS. Bottom Up: Bottom Up spectra were collected after separation of peptides generated from a tryptic digest of H2B and analyzed using collision-activated dissociation (CAD) fragmentation. Four out of 14 H2B variants can be uniquely identified using this method.

et al., 2010), but further improvements need to be made before Top Down methods are used to routinely identify and quantify histone variants and their PTMs.

Middle Down proteomics

Middle Down proteomics combines the advantages of Top Down and Bottom Up methods. Middle Down MS cannot distinguish between as many histone variants as Top Down MS; however it is much more sensitive and can be used with online chromatography (see Figure 2 for a comparison). Large histone peptides (greater than 3000 Da) are generated using Glu-C or Asp-N digests, separated using online or offline HPLC, and then fragmented using ETD or ECD for identification. Middle Down proteomics is used to

profile coexisting histone modifications and can distinguish between more histone variants than Bottom Up MS. So far, Middle Down MS has been applied to H4 as well as H3, H2A, and H2B variants and their modifications (Bonenfant et al., 2006; Garcia et al., 2007b; Young et al., 2009).

Quantification strategies

Multiple proteomic approaches have been developed to allow for protein quantification ranging in scale from the whole cell proteome to single protein analyses. These approaches can be broken down into four general categories—"label-free" quantification, chemical labeling, metabolic labeling, and quantification by standard peptide (Bantscheff et al., 2007). Variations on all of these techniques have been utilized in studying histone posttranslational modifications, histone variants, and histone turnover as shown in Figure 3, and discussed in more detail below.

Label-free quantification

Label-free quantification methods establish relative protein quantities without incorporation of any labels or use of standard peptides, thus abrogating the cost and sample preparation required for labeling or peptide-based strategies and allowing quantification across multiple samples or experimental conditions. A common label-free technique is spectral counting, which bases quantification on the number of MS/MS spectra acquired for each protein (Liu et al., 2004). In spectral counting, the total number of MS/MS spectra corresponding to each peptide is compared across experimental conditions. The quality and reproducibility of the data can be affected by many factors, such as peptide ionization efficiency, settings for data-dependent acquisition, and chromatographic separation (Washburn et al., 2003; Liu et al., 2004; Ott et al., 2007). The large scale nature of some spectral counting experiments utilize multidimensional separation strategies (e.g. strong cation exchange coupled to reverse phase) to better resolve peptide mixtures and allow for analysis of thousands of proteins- including histones (Washburn et al., 2003). Spectral counting of human HEK 293 cells, for example, revealed that treatment with cytokine tumor necrosis factor-α (TNF-α) causes an increase in expression of H2A variants, H2A.Z and macroH2A. This increase in H2A variants coupled with a decrease in DNA replication machinery implicates TNF-α in a cell cycle arrest event as part of the innate immune response (Ott et al., 2007). Such studies indicate that spectral counting may be a way to assess changes in histone variants across whole cells or tissue samples.

An alternative label-free strategy to spectral counting utilizes peak area from an extracted ion chromatogram to estimate peptide abundance. These studies typically rely on the Bottom Up strategies discussed above. The relative abundance of a covalently modified peptide is established by comparing the peak area of that modified form to the total peak area for all forms of the peptide. In Drosophila, it was found by Bottom Up LC-MS quantification of offline RP-HPLC purified H3 variants that histone H3.3 is enriched for acetylation and K4 methylation, suggesting a role for H3.3 in gene activation (McKittrick et al., 2004). A more streamlined analysis of histone PTMs can be done by combining propionylation with trypsin digestion of unfractionated histones (Plazas-Mayorca et al., 2009).

Top Down MS is not commonly used in conjunction with online LC analysis, so an alternative label-free method has been developed for whole protein analysis. The relative amounts of different modified histone forms are measured by comparing the intensities of abundant fragment ions. The Kelleher lab found that the relative fragment ion intensity of modified histone H4 peptides was the same as the relative concentration of the peptides in solution, within 5% error margins (Pesavento et al., 2006). This technique has been applied to measure the relative abundances of PTMs across H3 variants in rat brains. A slight enrichment in the activating mark K4 methylation in H3.3 and a slight enrichment of the repressive mark K27 trimethylation in H3.2 were found (Garcia et al., 2007c).

Chemical derivatization-based quantification

The postharvest derivatization of histones for Bottom Up using anhydrides or methyl and ethyl esters facilitates relative quantification between experimental conditions. An initial round of anhydride or ester derivatization blocks monomethylated and unmodified lysines. Then, following tryptic digestion, a second round of derivatization with esters or anhydrides block the C-terminus or N-terminus of the peptides, respectively. Heavy isotope-labeled esters or anhydrides, such as D10-propionic anhydride, can be used in the second round of derivatization to add one label per peptide. Mixing equal amounts of labeled and unlabeled propionylated histone samples from two different conditions facilitates relative quantification. A disadvantage of chemical derivatization is that samples are mixed at the peptide level, and results can be skewed if sample loss occurs early on in sample preparation (see Figure 3). Ester derivatization requires peptides to be dried, increasing sample loss and making it a less desirable method compared to propionic anhydride derivatization.

A number of studies have utilized differential chemical derivatization to quantify changes in PTMs or variants (DiMaggio & Garcia, 2010). Most recently, propionic anhydride labeling was used to observe the effect of G9a/Glp1 methyltransferase knockdown on global histone modifications. G9a/Glp1 methyltransferases are responsible for dimethylation of H3K9 and their knockdown resulted in a decrease of this modification. Additionally, H3K14 acetylation, H3K79 and H3K36 methylation levels increased in a H3 variant-specific manner, suggesting cross-talk between these modifications (Plazas-Mayorca et al., 2010).

Metabolic stable isotope labeling

Metabolic labeling is a relative quantification technique that relies on the replacement of a standard light isotope

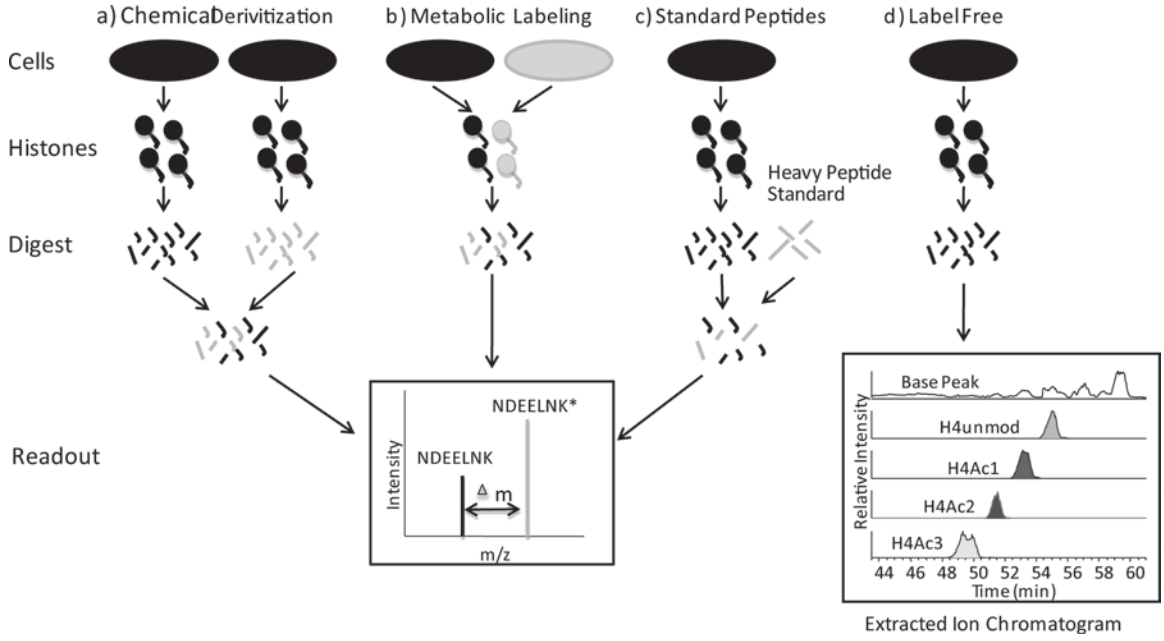

Figure 3. Histone quantification methods. Depicted above are (A) chemical derivatization, (B) metabolic labeling, (C) standard peptides, and (D) label-free quantification. For the labeling-based strategies (A, B, C), the processing step where label incorporation occurs is shown.

with a "heavy" isotope (e.g. ^{15}N, ^{13}C, D, or ^{18}O) in cellular proteins. The heavy isotope is incorporated into the protein using a heavy-labeled metabolite that is added to media during cell culture. In a typical metabolic labeling experiment comparing cells grown under two biological conditions, cells under one condition receive heavy-isotope-labeled media, whereas cells grown under the other condition receive normal media. Equal numbers of cells from each condition are mixed and processed for MS analysis. Proteins from different conditions are distinguished by a known mass shift in their spectra. Relative protein amounts can be quantified using the area under the curve in the extracted ion chromatograms for heavy- and light-labeled peptides or by relative fragment ion intensities. The main advantage of metabolic labeling is that the labeled cells and unlabeled cells are mixed as a first step, so proteins have identical losses for all of the proceeding processing steps (Figure 3). The main disadvantage of metabolic labeling is that it has to be done in tissue culture. Two types of metabolic labeling are used for histone quantification: stable isotope labeling of amino acids in cell culture (SILAC) and labeling of metabolites that become incorporated into the histone modifications.

SILAC was developed by the Mann group in 2002 and since then has been used in numerous histone studies (Ong et al., 2002). It was used recently to investigate partitioning of H3 variants during DNA replication (Xu et al., 2010). In this study, FLAG epitope-tagged H3.1 and H3.3 variants were grown in HeLa cells. The cells were arrested and switched into media containing $^{13}C_6{}^{15}N_2$-lysine so that all new histones after cell cycle arrest would become labeled. After immunopurification of the tagged variants, and Bottom Up analysis of the immunopurified histones, they discovered that H3.1–H4 tetramers do not split into dimers during replication-dependent nucleosome assembly, but H3.3–H4 dimers do split during replication-dependent assembly. They present this as support for the hypothesis that histone modifications in newly formed nucleosomes are copied from histones in preexisting nucleosomes.

SILAC can also be used in combination with Top Down MS. It was used to study changes in levels of different canonical HeLa cell H2A isoforms across the cell cycle by following the level of incorporation of ^{13}C, ^{15}N labeled valine and arginine into the H2A isoforms after cell synchronization (Boyne et al., 2006). This study confirmed that the majority of canonical H2A isoforms are produced during S-phase and showed that levels of different H2A isoforms did not vary much across the cell cycle.

Metabolites can also be used to label specific modifications. Jenuwein and coworkers used $^{13}CD_3$-methionine to measure levels of H3K9 methylation after inducing the expression Jmjd2b H3K9 de-methylase in murine cells (Fodor et al., 2006). Later, the Garcia Lab used heavy-methionine labeling in addition to $^{13}C_6{}^{15}N_2$-lysine labeling to study methylation turnover in site-specific manner and to compare methylation turnover to histone turnover (Zee et al., 2010a). They found that activating methylation marks such as H3K4 generally turned over faster than repressive marks such as H3K9. They also found that methyl marks had faster turnover than the histones themselves, demonstrating that histones are dynamically modified. In a later paper, they demonstrated that PTM turnover between variants was fairly constant and that

all histone variants, with the exception of histone H2A variants, turned over at approximately the same rate (Zee et al., 2010b).

Absolute quantification by use of peptide standards

Both relative and absolute quantification of histones and their PTMs are possible through the use of synthetic, isotopically labeled standard peptides. Peptides are synthesized to have the same sequence and modifications as the digested form of the histones of interest. A known concentration of the standard peptide is spiked into samples, then the intensity of the analyte to the standard is compared. Alternatively, the absolute quantity of the analyte can be determined by making a calibration curve of peptide standard concentration versus ion intensity. This method is not entirely accurate because analyte losses occur in processing steps before the standard peptide is added; however, it can be used in combination with reaction monitoring MS techniques to monitor very low levels of analyte. Standard peptide quantification is not commonly used for analysis of histones because standard peptides are expensive and only one PTM or variant can be quantified at one time.

There are a few examples of standard peptides being used to quantify histone PTMs. Darwanto and coworkers used peptide standards to quantify relative levels of H3K79 methylation and H2B ubiquitination in wild-type U937 lymphoma cells and in U937 cells expressing the methyltransferase hDot1 with a deleted methyl-binding domain. They found that a decrease in H3K79 methylation led to an increase in H2B ubiquitination suggesting cross-talk between the modifications (Darwanto et al., 2010). They used standard peptides for this study because ubiquitinated H2B is not very abundant and would not be easily detected using less sensitive techniques.

Function of histone variants

Histone H3 family

Histone H3 has three major variants as well as a testis-specific variant (H3t) and a variant that is localized to centromeres (CENP-A). The three main variants of H3 are surprisingly similar in sequence with H3.1 only differing from H3.2 by a change in Cysteine 96 to serine and H3.3 differing from H3.1 by only five residues. Despite the small differences in sequence homology, the variants have large differences in their expression, localization in chromatin, and modification state. H3.3 is expressed in a replication-independent manner, whereas the canonical variants (H3.1 and H3.2) are only expressed during S-phase (Stein et al., 1984). In addition, different chaperones recognize and assemble H3.1 and H3.3 into nucleosomes in a replication-dependent and independent manner, respectively (Tagami et al., 2004). Compared to H3.1 and H3.2, H3.3 is usually localized to heterochromatin and enriched for histone modifications that are associated with gene activation (Ahmad & Henikoff, 2002; McKittrick et al., 2004). H3.1 and H3.2 do not show specific areas of localization in the genome and are generally considered to be identical; however, one study suggests that H3.2 is modified with marks associated with gene repression, whereas H3.1 shows both activating and repressive marks (Ahmad & Henikoff, 2002; Hake et al., 2006).

Quantitative studies of H3 variant modifications have been accomplished using Bottom Up, Top Down, and Middle Down techniques (Hake et al., 2006; Thomas et al., 2006; Garcia et al., 2007c). Fractionation of H3 variants is necessary for complete analysis by MS. The H3 variants are easily resolved using reverse-phase HPLC and different modified forms of each variant can be further separated using cation-exchange chromatography or hydrophilic interaction chromatography (HILIC). The H3 variants need to be separated before digestion for Bottom Up analysis because they have nearly identical N-terminal tails. Top and Middle Down analysis can also be improved by separating H3 variants and their modifications because there are thousands of combinations of modifications associated with each H3 variant. The most successful separation method used in combination with Top and Middle Down MS is weak cation-exchange HILIC, which successfully separates isobaric species based on the number and position of modifications. This technique was used to identify over 200 modified forms of H3.2 (Young et al., 2009).

Histone H2A family

The H2A family of histones is one of the most sequence-divergent families, with 20 unique sequences in humans. Canonical human histone H2A is encoded for by 16 genes within gene clusters and has 12 unique sequences. Sequence diversity comes mainly from divergent C-terminal tails, but the biological relevance of this diversity remains unknown. Phosphorylation of S1 and acetylation sites within the N-terminal have been reported for canonical H2A (Bonenfant et al., 2006; Boyne et al., 2006). Monoubiquitination of H2A at C-terminal residue K119 has been implicated in gene silencing (Wang et al., 2004). The H2A family variants include H2A.X, H2A.Bbd, H2A.Z, and macroH2A; these variants differ in sequence and have diverse functions.

Variant H2A.X is most similar in sequence to canonical but has a divergent C-terminal tail. Unlike canonical genes, the mRNA for H2A.X can have either a polyA tail or the stem loop dyad structure, indicating that it can undergo both replication-dependent and independent transcription (Mannironi et al., 1989). H2A.X can be acetylated, ubiquitinated, and phosphorylated. In response to DNA damage, H2A.X is phosphorylated at serine 139 and localizes to DNA double-strand breaks, helping to recruit proteins to carry out DNA repair (Rogakou et al., 1998, Ikura et al., 2007). Acetylation and ubiquitination of H2A.X also function in this process with acetylation at lysine 5 serving as a prerequisite for ubiquitination and subsequent release of H2A.X from DNA damage sites (Ikura et al., 2007).

A second variant of the H2A family is H2A.Bbd, whose name is derived from the fact that this variant is excluded from the inactive X and therefore Barr body deficient. This along with the finding that it associates with acetylated histone H4 has implicated H2A.Bbd in transcriptional activation (Chadwick & Willard, 2001). Recent reports also suggest a role for H2A.Bbd in spermatogenesis (Ishibashi et al., 2010). The H2A.Bbd protein sequence is short and arginine rich in comparison to canonical. *In vitro* work demonstrates that these sequence differences alter the nucleosome core particle. Nucleosomes containing H2A.Bbd contain 128 bp of DNA as opposed to the traditional 146, indicating that H2A.Bbd incorporation impacts chromatin structure (Doyen et al., 2006).

Histone H2A.Z is highly conserved and is essential for development in higher eukaryotes (Draker & Cheung, 2009). Chromatin immunoprecipitation experiments show that H2A.Z localizes to the promoters of genes and in mammals, localization to the transcription start site correlates with gene activation (Barski et al., 2007). In yeast, it was found that H2A.Z is present in transcriptionally active areas near the telomeres and silent mating locus and prevents spreading of heterochromatin from the silent mating locus. These findings suggest that H2A.Z is important for maintaining active chromatin in regions near silent chromatin (Meneghini et al., 2003). Acetylation of H2A.Z is thought to be linked to its role in gene activation. These facts implicate H2A.Z in gene activation, however, there is evidence to indicate H2A.Z can also act in gene repression (Millar et al., 2006; Draker & Cheung, 2009; Mehta et al., 2010).

MacroH2A is the largest H2A variant containing an approximately 30 kDa macro domain at the C-terminus. Human macroH2A is encoded for by two genes, macroH2A2 and macroH2A1, which has two splice variants, macroH2A1.1 and macroH2A1.2. The ability of macroH2A1.1 to bind metabolites of NAD+ gives this splice variant potential for unique functions. MacroH2A is generally enriched at transcriptionally silent areas both on autosomes and on the inactive X. However, recent ChIP-Chip experiments show that macroH2A1 can be found at a subset of active genes (Gamble et al., 2010). MS analysis reveals that macroH2A can be ubiquitinated, phosphorylated, and acetylated. Ubiquitination at K115 has been implicated in X-inactivation, while phosphorylation of S137 is enriched during mitosis (Chu et al., 2006; Bernstein et al., 2008).

Histone H2B family

Human H2B has 17 isoforms (Figure 1), which are encoded by 25 genes. Most H2B sequences vary by just a few amino acids and are encoded by genes located in the main histone gene clusters. The two H2B proteins that differ significantly from other H2B sequences are the testis-specific H2B proteins, H2B1A and H2BFWT (Zalensky et al., 2002; Churikov et al., 2004). H2B variants have not been studied in as much depth as other histone variants, possibly, because of their sequence similarity, so their biological significance is not well understood. H2B variants are not as extensively modified as H3 or H4, and aside from a few lysine acetylation sites, other modified forms are very low in abundance. H2B isoforms have been identified using Bottom Up, Middle Down, and Top Down MS (Bonenfant et al., 2006; Siuti et al., 2006). No technique gives a complete picture of all H2B isoforms because H2B isoforms have too much sequence homology to be identified from digest peptides, and Top Down MS is not sensitive enough to detect lower level variants. MS has also been used to detect H2B modifications, including some modifications specific to the testis-specific variants (Bonenfant et al., 2006; Lu et al., 2009; Wyrick & Parra, 2009).

Histone H1 family

Histone H1 is commonly referred to as the *linker histone*. A single copy of this histone has been proposed to bind near the entry/exit site of DNA on the nucleosome stabilizing the 30 nM fiber and impacting chromatin structure and DNA linker length (Bustin et al., 2005; Woodcock et al., 2006). The amount of histone H1 present for each core nucleosome has found to differ between cell types. In higher eukaryotes, histone H1 contains a globular domain, a short N-terminal tail, and a longer lysine-rich C-terminal tail. Sequence divergence between histone H1 isoforms occurs mainly in the N- and C-terminal regions of the protein and is high, with mammals having as many as 11 isoforms. These isoforms are generally categorized based on the timing of expression or tissue specificity (Izzo et al., 2008). The replication-dependent isoforms include histone H1.1–H1.5 and H1.t, whereas replication-independent variants include H1.X, H1.t2, HILS1, H1oo, and H1.0 (which is sometimes referred to as H1degrees). Histones H1.0–H1.5 are expressed in somatic cells, with H1.1 restricted to certain tissue types (Daujat et al., 2005; Godde & Ura, 2008). Histone H1.t, H1.t2, and HILS1 are expressed in testes-specific tissues and histone H1oo is oocyte specific (Godde & Ura, 2008). Histone H1.X has mainly been reported in tissue culture cells (Wisniewski et al., 2007). In addition to sequence diversity, MS analysis and other studies have revealed a high number of posttranslational modifications present on different linker histone variants including lysine methylation, phosphorylation, acetylation, ubiquitination, formylation, and ADP ribosylation (Poirier & Savard, 1980; Talasz et al., 1996; Garcia et al., 2004; Wisniewski et al., 2007; Talasz et al., 2009; Weiss et al., 2010). The high number of isoforms and their unique modification patterns cause some to suggest that in combination this diversity results in a linker code, akin to the histone code.

Multiple studies of these isoforms reveal some interesting insights into function and potential for

functional diversity and redundancy (Godde & Ura, 2008; Izzo et al., 2008). Deletion of testes-specific histone H1.t from mice resulted in deposition of other isoforms and normal spermatogenesis (Lin et al., 2000). Mice can also survive lacking copies of the somatic isoforms H1.0, H1.2, H1.3, or H1.4, but deletion of histone H1.2, H1.3, and H1.4 resulted in embryonic lethality (Fan et al., 2005). These results suggest that there is some functional redundancy, but there are cases where individual isoforms have been assigned unique functions. For example, histone H1.4 has been implicated in transcriptional repression (Kuzmichev et al., 2004). Reverse-phase separation of histone H1 from bulk Hela histone followed by digestion, propionylation, and tandem MS revealed methylation at K26 and phosphorylation at S27 of histone H1.4 (Garcia et al., 2004). Methylation at K26 allows for binding of heterochromatin protein 1 (HP1), which functions in heterochromatin formation and gene silencing, while pS27 prevents binding (Daujat et al., 2005). Histone H1.4 is enriched in heterochromatic regions as compared to euchromatic regions, a unique localization pattern in comparison to some other H1 variants (Izzo et al., 2008). Overall, these findings suggest that histone H1.4K26me facilitates HP1 binding, chromatin condensation, and gene silencing, while phosphorylation of neighboring S27 can ameliorate these effects.

Phosphorylation is the most well-characterized modification on histone H1 isoforms. In general, phosphorylation levels on H1 change over the course of the cell cycle with a maximum amount occurring during mitosis. Interestingly, the level of phosphorylation differs on specific variants in mice and rats (Talasz et al., 1996). Hydrophobic interaction chromatography followed by Top Down analysis confirm these results in human cells showing that H1.2 and H1.4 from synchronized Hela cells have a single and double phosphorylation site, respectively, during interphase, while these numbers increase to four and six, respectively, during mitosis (Zheng et al., 2010). Phosphorylation in the C-terminal domain is thought to ease binding of H1 to DNA, facilitating dynamic changes to chromatin organization (Lever et al., 2000). Distinct phosphorylation events on H1 isoforms have been proposed to have various functions including stabilizing condensed chromatin during mitosis (Talasz et al., 2009) and promoting transcription and DNA replication (Talasz et al., 2009; Zheng et al., 2010).

Declaration of interest

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