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Integrating proteomics and targeted metabolomics to understand global changes in histone modifications

Johayra Simithy, **Simone Sidoli**, and **Benjamin A. Garcia**[*]

Department of Biochemistry and Biophysics, Epigenetics Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Abstract

The chromatin fiber is the control panel of eukaryotic cells. Chromatin is mostly composed of DNA, which contains the genetic instruction for cell phenotype, and histone proteins, which provide the scaffold for chromatin folding and part of the epigenetic inheritance. Histone *writers/erasers* "flag" chromatin regions by catalyzing/removing covalent histone post-translational modifications (PTMs). Histone PTMs chemically contribute to chromatin relaxation or compaction and recruit histone *readers* to modulate DNA readout. The precursors of protein PTMs are mostly small metabolites. For instance, acetyl-CoA is used for acetylation, ATP for phosphorylation, and S-adenosyl methionine for methylation. Interestingly, PTMs such as acetylation can occur at neutral pH also without their respective enzyme when the precursor is sufficiently concentrated. Therefore, it is essential to differentially quantify the contribution of histone *writers/erasers* vs the effect of local concentration of metabolites to understand the primary regulation of histone PTM abundance. Aberrant phenotypes such as cancer cells have misregulated metabolism and thus the composition and the modulation of chromatin is not only driven by enzymatic tuning. In this review, we discuss the latest advances in mass spectrometry (MS) to analyze histone PTMs and the most adopted quantification methods for related metabolites, both necessary to understand PTM relative changes.

Keywords

acetyl-CoA; epigenetics; histone; metabolism; post-translational modifications

Introduction

Epigenetics is the scientific field investigating inheritable changes in gene regulation not coded in the DNA sequence. It is currently one of the fastest growing fields in biology and medical science, specifically because epigenetic alterations play a causal role in many human disorders [1,2]. Uncovering the role of epigenetic changes in human diseases and normal human traits has thus become a priority. DNA is packaged into basic units known as

[*]Corresponding author: Benjamin A. Garcia, Epigenetics Institute, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Room 9-124, 3400 Civic Center Blvd, Bldg 421, Philadelphia, PA 19104, USA. bgarci@mail.med.upenn.edu; Phone: 1-215-573-9423; Fax: 215-573-4764.

Conflicts of interest

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nucleosomes, which form the basic structural elements of chromatin [3,4]. Each nucleosome is composed of 147 base pairs of DNA and two copies each of the four major nucleosomal histones (H2A, H2B, H3, and H4), and is linked by a linker histone called H1 [5]. These nucleosomes are then folded into more complex three-dimensional structures. Histones are small proteins containing a high number of positively charged amino acid residues (lysine and arginine) that facilitate binding to negatively charged DNA. Chromatin structure is tightly modulated by histones and their covalent post-translational modifications (PTMs) [6]. While some of these modifications have been identified at the histone fold domains, most of them occur on the flexible N-terminal tails, which extend outward from the nucleosome core [7]. Histone PTMs can alter chromatin structure, influencing several processes such as gene transcription, DNA replication, DNA damage repair and alternative splicing [8,9]. Every process that requires access to genomics information must overcome this tight packaging of chromatin.

Lysine acetylation (ac) and methylation (me) were the first histone PTMs to be discovered (1960s), and they are the most abundant and well-characterized [10]. Lysine acetylation neutralizes the positive charge on histones, reducing their interactions with negatively charged DNA and allowing transcription activators to access DNA and promote gene expression [11]. Lysine acetylation is therefore commonly associated with transcriptionally active chromatin [12,13]. Histone lysine methylation has been correlated with either activation or repression of chromatin, depending on the localization of the methylated residue [14,15]. Since the discovery of these two marks, an ever-growing number of histone modifications has been identified, including ubiquitination (ub) [16], biotinylation (bio) [17], ADP-ribosylation (Ar) [18], and phosphorylation (ph) [19]. More recently, several less-characterized lysine acylations were included in the already long list of histone PTMs, including malonylation (ma) [20], succinylation (suc) [21], crotonylation (cr) [22], propionylation (pr) [23], butyrylation (bu) [23], glutarylation (glu) [24], 2-hydroxyisobutyrylation (hib) [25], and β-hydroxybutyrylation (bhb) [26]. For an updated review of all histone PTMs identified to date, we refer readers to the "*Comprehensive Catalog Of Currently Documented Histone Modifications*" [27]. Altogether, more than 400 modifications are known to occur on histone proteins to date, many of which have still uncharacterized function.

There are at least four mechanisms by which histone modifications are regulated on the chromatin (Figure 1); they divide in (i) regulation of histone writers/erasers by signaling cascade led by external events, (ii) abundance of metabolite precursors of PTMs, (iii) chromatin accessibility due to pre-existing modifications, and (iv) protein-nucleic acid interactions to facilitate enzyme recruitment. Interestingly, these aspects can all be investigated using mass spectrometry (MS)-based proteomics, including protein-nucleic acid interaction [28]. In this review, we mainly focus on (ii) and (iii). The wide chemical variety of histone PTMs rely on central key metabolites that act as substrates for their catalysis in common. Acetyl-CoA, the main donor for histone acetylation, arises mostly from glucose-derived citrate by the intervention of ATP citrate lyase (ACL) or by the enzymatic activation of acetate by acetyl-CoA synthase 1 (AceCS1) (Figure 4, end of the manuscript) [29]. Likewise, there are other acyl-CoAs that act as co-substrates for newly-identified histone acyl-PTMs. This includes propionyl-, butyryl-, crotonyl-, glutaryl-, succinyl- and malonyl-

CoA, all derived from either β-oxidation of fatty acids or from the catabolism of amino acids [30]. ATP, the main source of energy in the cell, is also the donor for protein phosphorylation. Moreover, both histone and DNA methylation require the methionine-derived cofactor S-adenosylmethionine (SAM) as a source of methyl groups. It is therefore intuitive that metabolic changes impacting the availability of these cofactors affect histone PTMs, and thereby influence cell function.

Over the last decade, the implication of metabolism in various aspects of epigenetic control has been revealed in multiple systems. For example, it has been shown that siRNA-mediated silencing of ACL directly affects the transcription of genes regulating the metabolism of glucose [31]. In fact, silencing of ACL results in a decrease of nuclear acetyl-CoA pools and consequently a decrease in the levels of histone acetylation. Although less profound than acetylation, examples of metabolic control have also been seen for histone methylation. Decreasing the levels of SAM through threonine-depleted media in mouse embryonic stem cell (mESCs) was found to significantly decrease H3K4me3 levels, slowing the growth and increasing the differentiation of mESCs [32]. However, the catabolism of threonine is exclusive to mESCs; it is thus unclear whether the influence of metabolism in methylation can affect human stem cells as well.

PTMs are frequently co-localized on histones, which has led to the hypothesis of the “histone code”. This hypothesis proposes that histone PTMs can serve either alone or in combination as a signaling platform to recruit a network of histone-modifying enzymes and nucleosome remodeling complexes to fine-tune gene regulation [33,34]. In fact, dysregulation of combinations of histone PTMs has been implicated in the pathology of many human diseases including cancer [35–39]. For instance, low levels of H3K9ac, H3K9me3 and H4K16ac are positively correlated with tumor recurrence in patients with lung cancer, whereas high levels of H3K18ac and H3K4me2 are associated with an increased risk of prostate cancer progression and metastasis [40]. Nevertheless, the lack of a full understanding of PTM-mediated chromatin processes remains a challenge, as new analytical tools for the comprehensive study of the combinatorial nature of the code are just emerging. This review will give a brief summary of the MS-based approaches that are commonly applied for the analysis of histone PTMs and the possibility of employing MS-based metabolomics to help unravel the role of metabolism in epigenetic control. A detailed discussion of the basic principles of MS for the analysis of proteins is outside the scope of this paper, as such, we refer the interested reader to specialized reviews [41–43].

Overview of all methods for the analysis of histone PTMs

Over the past decade, advances in high-resolution MS have revolutionized the analysis of histone PTMs. The ability to perform high-throughput analysis of single and/or combinatorial patterns of histone PTMs has made MS the most suitable technique for the comprehensive analysis of histone proteoforms, where “proteoform” is defined as all potential isoforms comprising different combinations of PTMs and sequence variations in which a protein can be found [44]. MS provides by far the most accurate quantification and highest throughput in terms of how many PTMs can be identified and quantified in a single analysis. However, histone PTMs can also be investigated by means of site-specific

antibody-based methods such as Western Blot, Immunofluorescence and Chromatin Immunoprecipitation (ChIP). The latter can be coupled to DNA microarrays (ChIP-chip) or highly parallel DNA sequencing (ChIP-seq) for genome-wide analysis [45,46]. Nonetheless, it is important to specify that antibody-based techniques have well-known biases in epitope recognition when co-existing PTMs are present (Figure 2) [47]. For instance, site-specific antibodies for H3S10ph fail to recognize the phosphorylated epitope when the nearby H3K9 or H3K14 residues are acetylated, giving rise to false negative results [48]. Moreover, antibody cross-reactivity is often observed with different modification states (e.g., mono-, di-, or trimethylation), especially when they are placed within the same amino acid context, as is the case for H3K9 and H3K27, which have a common A-R-K-S sequence motif [49]. Cross-reactivity has also been reported for pan-anticrotonyl-lysine and pan anti-butyryl-lysine antibodies with structurally related acyl marks including lysine acetylation, propionylation, crotonylation and butyrylation [50].

As a result, several efforts have been made to provide researchers with resources to assess the quality of histone modification-specific antibodies [51–55]. One such resource is the recently published “Histone Antibody Specificity Database,” where authors have evaluated the reactivity of more than 100 commercial PTM-specific antibodies against 250 modified histone peptides and have made the results available on an open-access website [55]. In this report, the evaluated antibodies displayed various degrees of cross-reactivity and influence by neighboring PTMs, highlighting the importance of carefully validating antibodies to avoid misleading conclusions. A recent work employing antibody-based strategies has succesfully identified p300, a histone acetyltransferase (HAT), as able to perform histone crotonylation [56]. The study also showed that p300-catalyzed histone crotonylation is able to stimulate transcription to a greater degree than histone acetylation and that this mechanism is dependent on the metabolic concentrations of the cofactor crotonyl-CoA. The study did not find remarkable differences in the genome-wide localization between acetyl and crotonyl, raising doubts on whether the two marks have different biological function.

In contrast to PTM-specific antibodies, MS represents a more unbiased technique that overcomes many of the discussed challenges. The high resolution offered by MS allows to discriminate between modifications with nearly the same nominal mass shift such as acetylation (ΔM = 42.0106 Da) and trimethylation (ΔM = 42.0470 Da) [57]. MS is also the most suitable technique to identify novel histone PTMs. There are three different strategies for the analysis of histone proteins via MS: “bottom- up”, “top-down” and “middle-down” (reviewed in [58]). These strategies differ by the length of the amino acid sequence being analyzed, type of chromatography and MS acquisition method (Figure 3).

MS strategies for the analysis of individual and combinatorial histone PTMs

“Bottom-up” is the most conventional MS strategy for the study of histone PTMs. Histones are cleaved into short (4–20 aa) peptides by trypsin (cleaves after KR) or ArgC (cleaves after R) digestion. Due to the high frequency of K and R residues on histones, the use of trypsin is combined with derivatization of lysine residues with propionylation or heavy labeled acetylation [59–61]. Chemical derivatization prevents trypsin from digesting after lysine residues, but also leads to longer and more hydrophobic peptides, more suitable for reversed-

phase chromatography. Data-independent acquisition (DIA) is becoming more and more popular for acquiring histone peptides [62]. This is because histone peptides have numerous isobaric forms, where MS/MS fragmentation is required for differential quantification. The main advantage of bottom-up MS is the ease of use and the high sensitivity achieved; i.e. in a recent publication, we proved that the analysis of common PTMs can be performed with as low as 50,000 cells as starting material [63]. Bottom-up MS has been proven successful in identifying and quantifying many novel histone PTMs including crotonylation, malonylation, succinylation and O-GlcN-acylation [22,64,65]. One drawback of the bottom-up MS approach is its limitation in discriminating histone variants, as several PTMs belong to peptides that have the same exact sequence between isotypes. In addition, the physical linkage among PTMs on a given histone is lost, allowing only the analysis of short-range PTM combinations (e.g., H3K27 and H3K36) [66]. However, the high sensitivity can compensate for these limitations when performing MS analysis with specific enrichment methods. For instance, in combination with PTM-specific antibodies for enrichment of distinct histone modifications (H3K27me2/3 and H4K20me1), Voigt *et al.* demostrated the application of bottom-up MS for the study of PTM patterns on single and separate histone copies within a nuclesome [67].

In “top-down” MS no proteolytic digestion is performed prior to MS analysis. Hence, combinatorial patterns of PTMs and the exact histone variant are potentially identified unambiguously due to the unique signal they produce [68]. Core histones are more suitable for this approach due to their low molecular weight (11–15 kDa) and their high abundance. This analysis necessarily requires high resolution MS and MS/MS due to the high charge state of the analytes. Moreover, electron capture dissociation or electron transfer dissociation (ECD/ETD) [69] are more suitable fagmentation methods than typical collision based methods used in bottom-up MS. Despite the intuitive advantages, top-down MS still remains an elitary approach;due to its limitations, it requires highly specialized analysts [70]. The main challenge is the number of potential isobaric forms when considering the intact protein. For example, H4 alone has over 26 billion theoretical proteoforms [71] which hardly separate by chromatography, and many of them end up populating the same MS/MS spectrum. Typically, offline two-dimentional liquid chromatography (2D LC) separation of histones has been carried out. In the first dimension, histones are separated by family members (H4, H2B, H2A and H3) based on their order of hydrophobicity using reversed-phase (RP) chromatography [72]. The second dimension involves separation of modified states (mostly acetylation or methylation) using weak cation exchange-hydrophilic interaction liquid chromatography (WCX-HILIC) [73]. Using this combined approach, Pesavento *et al.* were able to identify 42 unique combinatorial PTMs on histone H4 and their dynamic changes during the cell cycle in HeLa cells [74]. Even though the entire workflow remains challenging, most histones and histone variants have been characterized using top-down MS [75–79]. Top-down MS is also computationally challenging. DiMaggio *et al.* proposed an algorithm based on a mixed integer linear optimization using ETD LC-MS/MS data [80]. Commercial software adopted to the analysis of heavily modified histone proteins are ProSightPC (Thermo), developed from the free version ProSightPTM [81], and BIG Mascot [82], a version of Mascot (MatrixScience) that can analyze polypeptides and intact proteins >16 kDa. More recently, LeDuc *et al.* developed the C-score [83] to enhance the

confidence in the correct mapping of PTMs on the identified intact protein sequences. In fact, the real challenge of identifying a top-down MS/MS spectrum is not the identification of the histone sequence, but the unambiguous mapping of the modified amino acid residues.

The “middle-down” MS strategy is a compromise between top-down and bottom-up. In middle-down MS, proteins are digested into longer peptides (3–9 kDa) with proteolytic enzymes that have less frequent cleavage sites in histones such as AspN or GluC. Digestion by these proteases generates polypeptides that consist of intact histone N-terminal tails (40–50 aa residues). Thus, this approach allows for the analysis of co-existing PTMs on the histone tail, where most PTMs reside, without the burdensome technical challenges of top-down MS. This approach proved its high throughput feasibility in a publication of Young *et al*. [84]. It was then optimized in 2014 [85], and today it can be effectively combined with metabolic labeling of the protein sequence or the PTMs, allowing turnover studies [86]. This last study showed highly comparable results between the bottom-up and the middle-down MS quantification of single histone PTM, further improving previous optimization [87]. Like top-down though, middle-down MS requires different LC separation, MS acquisition and informatics analysis than bottom-up MS. Histone N-terminal tails are commonly separated by “saltless” pH gradient using a WCX-HILIC type of chromatography developed by Young *et al*. [84]. Detection is performed using high resolution MS and MS/MS, and ideally ETD fragmentation. More recently, separation of isobaric histone tails was achieved by using ion mobility [88], paving the way to potential new orthogonal ways to resolve complex mixtures and improve sensitivity. The challenges of data analysis are also mostly based on the accuracy by which a software is able to unambiguously map the localization of PTMs. Frankly, there is no software developed on purpose for middle-down MS. Most of the analyses are performed with abovementioned software tools intended for top-down MS. To date, the only workflow which proved to provide a comparable quantification to bottom-up MS [86,87] is the following: MS/MS ETD spectra deconvolution using Xtract (Thermo), database searching using Mascot (MatrixScience) and data filtering and quantification using isoScale [85].

MS strategies for the analysis of metabolites precursors of histone PTMs

In recent years, metabolomics has made enormous steps towards untargeted identification and more accurate quantification of metabolites using MS. The Human Metabolome Database (HMDB) was released in 2007 [89], and it currently contains >100,000 metabolites. Identifying the entire metabolome of an organism is still very much a challenge, as metabolites have an extremely dynamic regulation, and they span between a wide range of abundances. Specifically, metabolites are considered abundant if > 1 μM, or relatively rare if < 1 nM. Luckily, the list of metabolites precursors of histone PTMs is confined and it can be performed with targeted acquisition. It can also be performed with low resolution MS, as long as MS/MS is performed to increase the confidence in depicting the proper signal [90]. When using high resolution MS, it is possible to profile metabolites even without using necessarily MS/MS [91]. Metabolite extraction is generally a straightforward protocol, especially if the interest is limited to glycolysis, the tricarboxylic acid (TCA) cycle, the pentose-phosphate pathway, and metabolism of amino acids. Differently from proteomics, acquisition is normally performed by switching between positive and negative polarity, as

selected metabolites are more easily ionized in negative mode, e.g. ATP. The type of chromatography may vary depending on the class of metabolites, although a general good starting point is the use of C_{18} reversed phase chromatography with the addition of ion pairing reagents, e.g. tributylamine [91] (Figure 3). Feeding cell cultures using stable isotope labeled metabolites opens up for exciting experiments where the turnover of protein PTMs can be investigated based on the ratio heavy/light of the quantified modification on a peptide. In a recent publication, Kori *et al.* estimated the turnover of acetylations on proteins by labeling either glucose or acetate with heavy isotopes [92]. Results showed a faster acetylation turnover for all proteins when glucose was labeled. However, quantification of heavy labeled acetyl-CoA proved that the cell uses more glucose than acetate to produce acetyl-CoA, implying that the calculated turnover rate was biased by which metabolite was labeled as reference. This, once again, confirmed that monitoring the relative levels of metabolites can reveal much about the regulation of PTMs occurring on the chromatin proteome. Several other publications present excellent quality analysis of targeted metabolites. In our personal experience, we found that C_{18} reversed phase chromatography (with ion pairing reagents) running in the high microliter flow-rate (100–200 μL/min) combined with targeted MS acquisition is the safest approach for combined sensitivity, confidence and reproducibility.

Histone modifications and metabolism: co-dependency in health and disease

As most chromatin-modifying enzymes demand the use of metabolic intermediates, it is expected that the proper functioning of chromatin to regulate physiological processes and to maintain homeostasis requires an intimate coordination with cell metabolism. The relationship between histone modifications and the cellular metabolic state is a theme that has sparked the interest of many researchers over the last few years [93–96]. For instance, by employing MS and [^{13}C] glucose-labeling in cell culture, Evertts *et al.*, monitored the rate of the incorporation of histone acetylations by regulation of glucose flux [97]. This study showed once again that glucose is the predominant source for nuclear acetyl-CoA, as near complete acetylation turnovers were observed when using isotopically labeled glucose, followed by glutamine and acetate in cultured HEK293 cells. Similarly, other studies have investigated protein PTM turnover, including O-GlcNAc [98] (Figure 4), providing an important timescale perspective on how frequently modifications are exchanged on histones and other proteins.

Sabari *et al.* showed that manipulations of the levels of crotonyl-CoA through the addition of crotonate to cell culture can shift the levels of histone lysine crotonylation (Kcr) [56]. Similarly, starvation-induced ketogenesis marked by an increase in the concentration of β-hydroxybutyrate in the liver and kidneys was found to dramatically induce the levels of histone lysine β-hydroxybutyrylation (Kbhb) [99]. Further evidence suggesting that the levels of precursor metabolites directly affect the levels of histone PTMs has also been shown *in vitro*. Through *in nucleo* experiments, we were able to demonstrate that the availability of acyl-CoA co-substrates has a direct impact on the levels of nuclear histone acylations [50]. Strikingly, this study also showed a strong positive correlation between the levels of various acyl-CoA donors and their corresponding histone acylations in proliferative and differentiated myogenic cells *in vivo*. Although the mechanisms for the catalysis of

histone marks, in particular histone acylations, has proven to be both enzymatic and non-enzymatic in nature [50,100,101], the existence of a quantitative link between intracellular metabolite concentrations and chromatin modifications, and possibly epigenetic regulation, has become more concrete.

Metabolic regulation of chromatin has already been recognized in both healthy and diseased conditions. For example, multiple studies have investigated the implication of nutritional challenge through a high-fat diet in the reprogramming of genes regulating the circadian clock in the liver [93,102,103]. This mechanism involves large oscillation of transcripts mediated by chromatin remodeling via acetylation of histone H3 at positions K9 and K14 [93,104]. Similarly, the implication of epigenetic regulation in cell senescence and aging has been extensively discussed [93]. While further investigation is required to ascertain the involvement of cell metabolism in normal epigenetic traits, most research has focused on elucidating the implications of altered metabolism in the development of disease, specifically cancer. The proposed mechanisms through which dysfunctional metabolic states can elicit epigenetic changes contributing to the formation of cancer could be summarized as follows: first, extensive metabolic rewiring has been recognized as a hallmark of cancer cells [105,106]. As such, tumorigenesis-associated metabolic alterations can influence the availability of cofactors required for chromatin-modifying enzymes, directly affecting the epigenome and in turn, the transcriptome [1,94,107,108]. Secondly, the altered epigenetic landscape in cancer cells can affect the expression of genes involved in cell metabolism, mostly through aberrant DNA methylations and histone modifications, and deregulation of metabolic signaling pathways by microRNAs (miRNAs) [108–110]. While the mechanisms driving tumorigenesis are still not fully understood, it is intuitive that integrating metabolomics and proteomics approaches could significantly increase our understanding of the bigger picture and, in particular, the interplay between epigenetics and metabolism.

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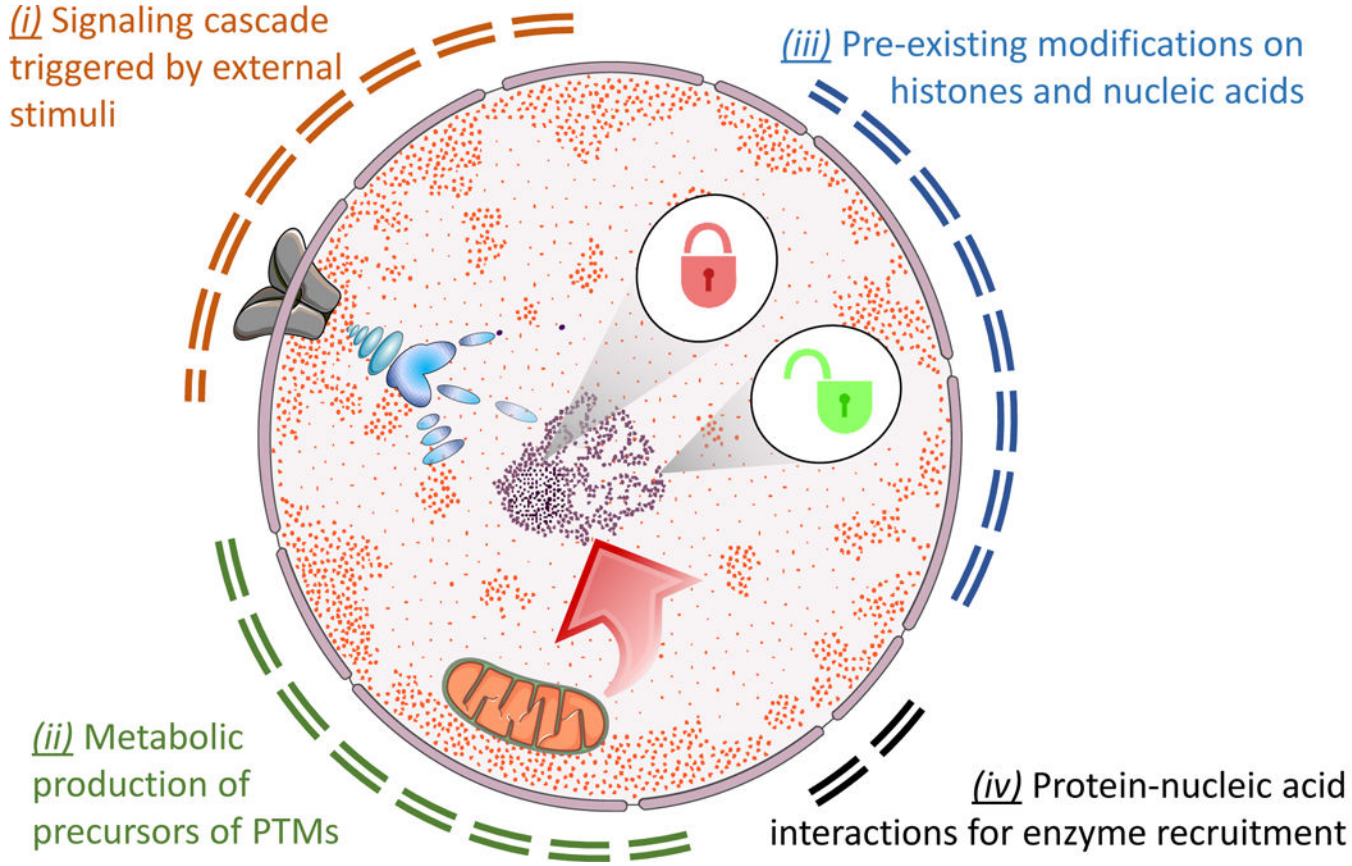

Figure 1: Pathways leading to histone modification catalysis.

(A) Cartoon representation on the mechanisms leading to chromatin regulation. (i) External stimuli to the cell generate signaling cascade through pathways that eventually modify and modulate the activity of histone writers and erasers. Phosphorylation is the most common PTM used as messenger for signaling cascade via kinase/phosphatase catalysis and removal. (ii) The production of metabolites influences the abundance of histone and other protein PTMs. This phenomenon is frequently not considered in studies investigating global changes of protein PTMs. (iii) Chromatin can be modified depending on its accessibility, and by modifications already present that recruit transcription factors and enzymes. (iv) Other mechanisms for chromatin modification are possible, including regulation through protein-RNA binding and chromatin localization within the nucleus. This last has, as typical example, sequestration of chromatin on the nuclear periphery, leading to local inhibition of gene expression.

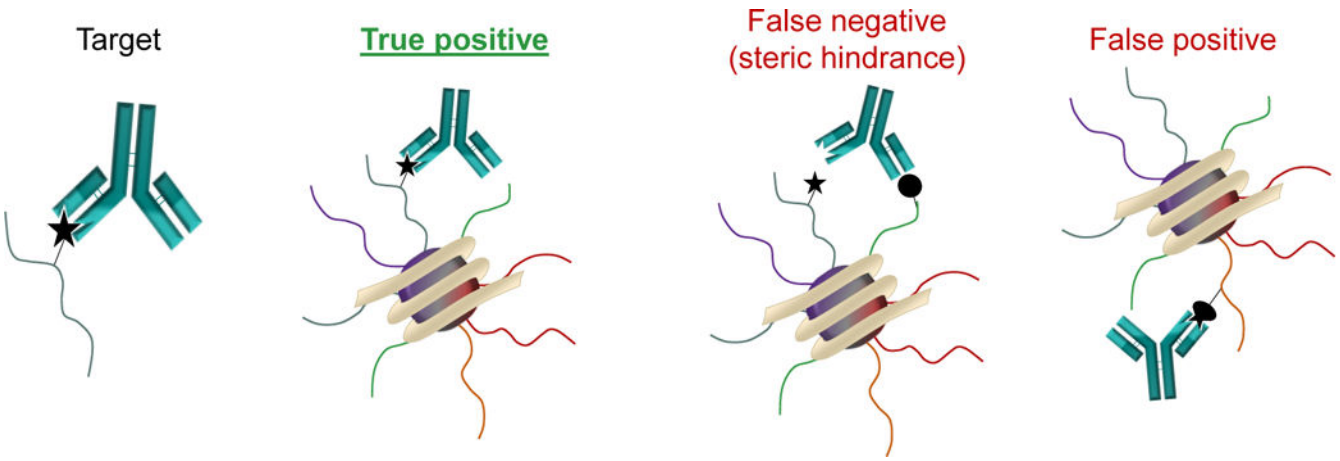

Figure 2: Potential biases of antibody-based strategies for the analysis of histone PTMs.

Recognition of a specific histone modification by an antibody can be hindered by the presence of nearby PTMs, generating a false negative. Generating highly specific antibodies for small modifications is also a challenging task, potentially leading to false positive identifications. For instance, it is difficult to generate antibodies that specifically recognize one type of acyl-PTM and not bind another (e.g. acetyl, propionyl, crotonyl). Moreover, antibodies for these “exotic” modifications are rarely site specific.

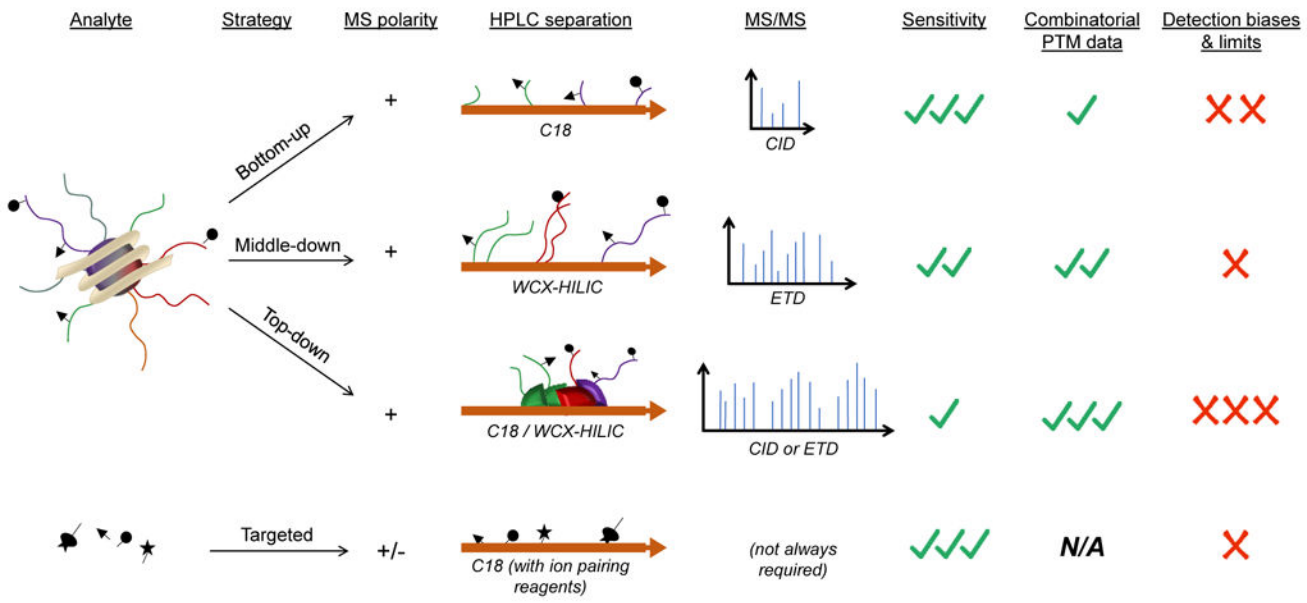

Figure 3: MS methods for the analysis of histone PTMs and their respective precursor metabolites.

Histone analysis can be performed with three different strategies: bottom-up (short peptides), middle-down (long intact tails) and top-down (no digestion, full protein). Histone sequences always ionize preferentially in positive mode. Separation and MS detection varies depending on the strategy; in particular, for middle-down and top-down, chromatography has a critical role due to the large variety of isobaric forms that must be at least partially separated. Because of the number of isobaric forms, and the charge state distribution (wider for larger analytes), the sensitivity is lower for middle-down and top-down as compared to bottom-up. However, middle-down and top-down have fewer biases in ionization efficiency, as the presence of a PTM does not affect the ionization of a long polypeptide as much as compared to short peptides. Despite lower biases, the enormous variety of isobaric forms makes top-down MS still a semi-quantitative strategy. Metabolites can be analyzed in a targeted mode, mostly accompanied by synthetic and isotopically labeled standards to enhance confidence. Because of this, MS/MS is not always required. Separation is commonly performed with C_{18} chromatography aided by ion pairing reagents to improve LC binding and resolution.

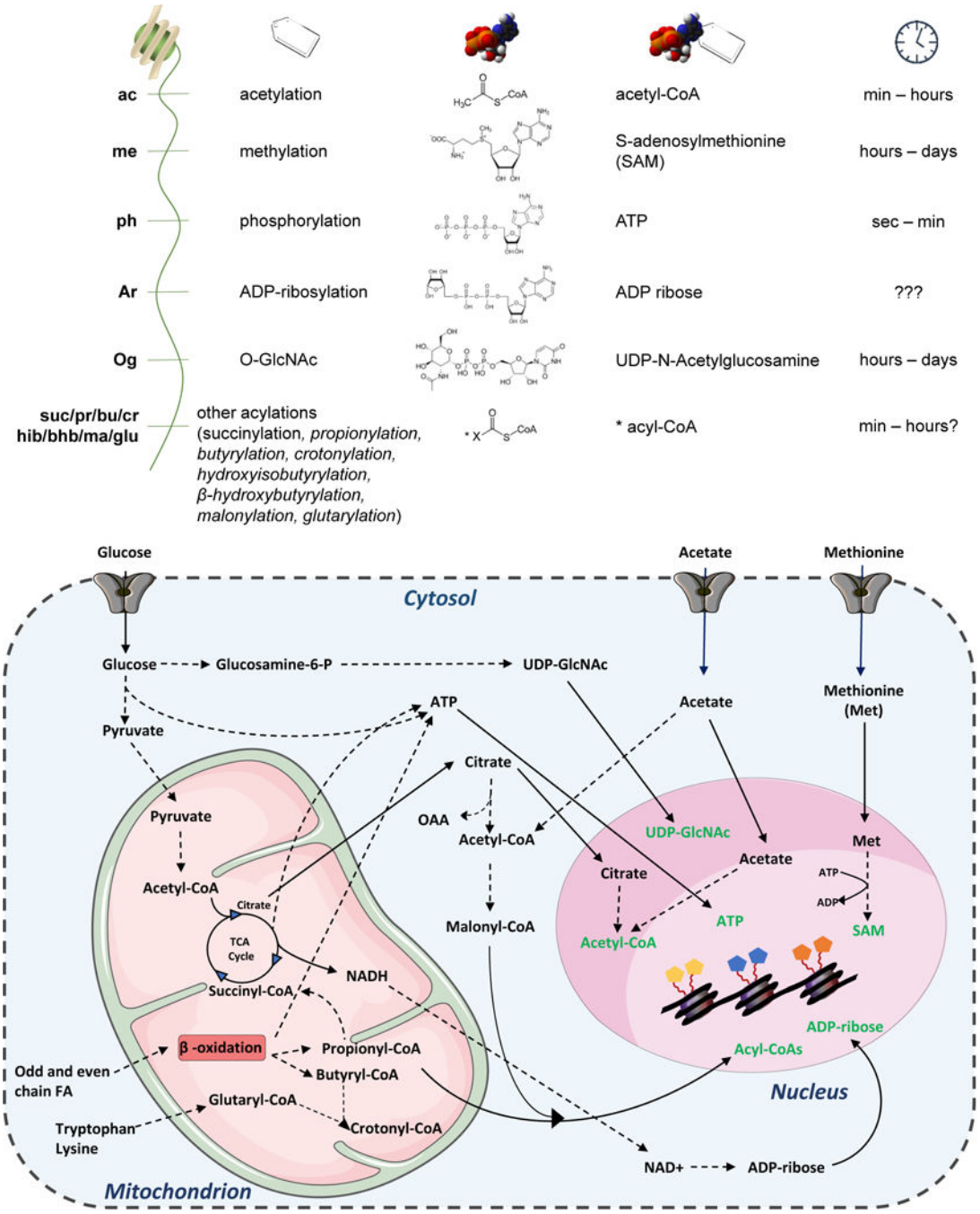

Figure 4: List of common histone PTMs, their immediate metabolic precursor and their estimated turnover rate.

The most common histone modifications have as precursors small molecules derived from metabolism, with the exception for ubiquitinylation. In the figure, histone PTMs are listed as common symbol, full name, structure of immediate precursor metabolite, name of precursor metabolite, and estimated turnover rate of catalysis. Question marks indicate that no study was yet performed to estimate the turnover rate of the given PTMs. Below, major pathways leading to the synthesis of the described biomolecules.