Review

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Chemical tools in chromatin research‡

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Eukaryotes organize their DNA in the form of chromatin. This complex of DNA and packaging proteins, the histones, ensures that all genomic information fits into the limited space of the cell nucleus. In addition to compacting DNA, chromatin itself regulates the activity of encoded genes. This regulatory process involves many posttranslational modifications of histone proteins and deciphering the complex crosstalk between histone modifications and gene activity represents a central challenge for biomedical science. This task has often been supported by sophisticated chemical tools, which were crucial for many important discoveries in this field. This review provides an overview of chemical tools for chromatin research, with emphasis on classical and current examples of their applications. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chromatin; histones; protein acetylation; protein methylation; protein phosphorylation; chemical tools; chemical biology

Background

All organisms must deal with the problem of packaging DNA into the limited space of their cellular nuclei. Eukaryotes solve this problem by organizing their DNA into chromatin, a complex of DNA and dedicated packing proteins, so-called histones. Chromatin can condense the DNA up to 10 000-fold, compared to its free form. The basic structural unit of chromatin is the nucleosome, which comprises four pairs of core histones (H2A, H2B, H3 and H4) with about 150 base pairs of DNA wrapped around (Figure 1) [1]. The formation of nucleosomes is the first step in packaging DNA, which reduces the spatial requirement of the genome about sevenfold. The second condensation step is the formation of chromosomal fiber with a diameter of 30 nm, which can be further arranged into even more compact structures of mitotic chromosomes [2]. Different forms of condensed chromatin have been observed by light microscopy as early as 1920. Simple dyes stain highly condensed mitotic chromatin, which can easily be observed side by side to the lightly stained more relaxed forms of chromatin [3]. Interestingly, highly condensed patches of chromatin remain intact during interphase where most chromatin is usually less condensed. These condensed patches were termed heterochromatin in order to distinguish them from decondensed chromosomal regions called euchromatin. It is known today that condensed heterochromatin contains mostly inactive genes, whereas euchromatin harbors active genes [4].

Chromatin exerts an important role in the regulation of gene activity and histones have a dedicated function in this process. In order to compensate for the high negative charge of the DNA phosphodiester backbone, all histones share a high content of positively charged lysine and arginine residues, which represent up to 25% of all histone amino acids. The histone architecture comprises *C*-terminal globular folds that make up the protein scaffold around which the DNA is wrapped [5]. In addition, histones possess unstructured *N*-terminal tails that protrude from the nucleosomal core in an extended conformation. Histone tails are not strictly required for nucleosome formation and some organisms like archaea employ 'tailless' histone variants for packaging their genomes [6,7]. Despite these facts, amino acid sequences of histone tails are highly conserved in eukaryotes, which suggests an important biological function. One likely role of histone tails is in the formation of higher order chromatin, but this structural function is still poorly understood. On the other hand, posttranslational protein modifications of histone tails exert important regulatory roles in modulating gene activity [8]. Well-known histone modifications include acetylation and methylation of lysine residues, as well as phosphorylation of serines and threonines (Figure 2) [9–11]. These three types of modifications have been identified in the early 1960s, but research over the past couple of years has unveiled several additional histone modification states including methyltion of arginine, phosphorylation of histidine, ubiquitylation and SUMOylation of lysine, and poly(ADP-ribosyl)ation of aspartic or glutamic acid residues [12–15]. Investigations of the complex crosstalks between histone modifications and chromatin structure constitute a vibrant field of biomedical science, which is often aided by sophisticated chemical tools [16]. Here, we focus on the three 'classical' histone modifications: acetylation, methylation and phosphorylation. We provide an overview about the roles of these modifications in the regulation of chromatin structure and gene activity and introduce some of the chemical tools that researchers employ to decipher their modes of action.

The Role of Histone Modifications in Gene Regulation

Biochemical and cell biological experiments have produced two theories that interpret the relationships between histone modifications, different chromatin states and gene transcription. The first theory postulates a direct involvement of histone modifications in chromatin structure, which in turn affects gene

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Biography

Dirk Schwarzer, born in 1972 in Schleswig (Germany), studied chemistry at the Philipps-Universität in Marburg and completed his PhD in 2002 with Mohamed A. Marahiel. After postdoctoral research with Philip A. Cole at the Johns-Hopkins University, in 2006 he moved to the group of Henning D. Mootz at the Technische Universität Dortmund. Since 2007 he has been the head of an Emmy-Noether Group at the Leibniz-Insititut für Molekulare Pharmakologie (FMP) in Berlin. His research interests focus on the development and application of chemical tools to study the physiological function of protein modifications.

activity. In essence, it is restricted to providing an explanation to the molecular consequences of histone acetylation events, which are associated with active gene transcription. Histone tails are positively charged due to their high Lys/Arg content. It is conceivable that this positive charge is compensated by the negative charge of the DNA phosphodiester backbone in a tail conformation that might clamp onto the DNA moiety of the nucleosome, thereby inhibiting efficient transcriptional factor (TF) binding to DNA sites. Upon histone tail acetylation, the positive charges are neutralized, the tails can detach from the DNA and TF sites may become accessible [17]. Biochemical investigations of DNA binding of the TFIIA transcriptional factor support this model. TFIIA has a well-defined binding site on the 5S RNA gene. When this site is incorporated into a nucleosomal structure, the binding of TFIIA is largely inhibited unless histone tails are either hyperacetylated or removed all together [18].

Although several observations connected to histone acetylation can be explained by this first theory, adapting a similar rationale to other modifications is not straightforward. Protein phosphorylation introduces a competing negative charge that can potentially reduce the positive net charge of a tail like lysine acetylation. However, the physiological effects of protein phosphorylation are more intricate. Phosphorylation of Ser-10 of histone H3 is associated with active gene transcription during interphase, but also with highly condensed, transcriptionally silent, mitotic chromatin during metaphase [19]. Furthermore, lysine methylation does not alter the net charge of the modified residue and is associated with both gene activation, as well as silencing [20,21,8]. These observations have led to a second theory that interprets patterns of histone modification states as recruitment sites for different regulatory proteins. Those regulators can function as either activators of gene transcription or as silencers [22,23]. This so-called 'histone code theory' is not restricted to a single type of modification but adds a new level of complexity that is based on the functional nature of the respective regulatory proteins. This hypothesis is still a subject of debate because apparently not all modification patterns form a 'code' which triggers a unique alteration of gene activity [24,25]. However, one important prediction from this theory is the existence of regulatory proteins ('readers') that can selectively interact with different histone modification states. Indeed, the existence of specific 'chromatin binders' has been experimentally confirmed and many of them have been well characterized in the meantime. Common features of those chromatin regulators are the presence of dedicated effector domains, structural entities that interact with certain histone modification marks in a site-specific manner and thereby recruit other chromatin-associated proteins [26].

Table 1. Selected histone modifications and dedicated binding domains

Modification mark	Binding domain
Lysine acetylation	Bromodomains
Lysine methyltion	Chromodomains
	MBT repeats
	PHD fingers
Serine/threonine phosphorylation	14-3-3 proteins BRCT domains

Effector Domains: Readers of Histone Modifications

Acetylation marks are recognized by bromodomains. These are approximately 110 amino acids in size and can be found in many chromatin-associated proteins (Table 1) [27,28]. One of the first bromodomains to be characterized is part of the PCAF protein (p300/CBP-associated factor). It recognizes acetylated Lys-8 and Lys-16 on the *N*-terminal tail of histone H4 [29,30]. Several other bromodomain containing proteins have been identified since then, including bromodomains that occur in tandem. In such an arrangement, the two bromodomains are positioned in close proximity as if to interact with two individual acetylation marks on the same histone tail. However, in case of the tandem bromodomain of the TATA-binding protein factor 1 homolog Brdt, bromodomain 2 was found to bind acetlyted Lys-18 on histone H3, whereas the binding pocket of bromodomain 1 accommodated two acetylated lysine residues (Lys-5 and Lys-8) of histone H4 in a cooperative manner [31]. In this regard, the Brdt protein employs its' two bromodomains to read a triplet 'acetylation codon' on two separate histone tails.

Different domain types have evolved as binders of methylation marks including chromodomains, MBT repeats (malignant brain tumor repeats) and PHD fingers (plant homeodomain) (Table 1). The chromodomain of heterochromatin protein 1 (HP1) is a prototype example [32,33]. HP1 associates with heterochromatin, which is marked by methylated Lys-9 on histone H3. Detailed biochemical investigations discovered that this interaction is mediated by the chromodomain of HP1. An interesting feature of lysine methylation is the existence of three methylation states: mono-, di- or trimethylation, which can be recognized in a differential manner [26]. For example, the chromodomain of HP1 preferentially binds to di- and tri-methylated lysines [34]. This reaction is primarily mediated by cation-π interactions of the methyl-ammonium moieties of the histone lysine side-chains and aromatic residues of the chromodomain binding pocket of HP1 [35]. Binding sites for 'lower' methylation states are less hydrophobic and include an acidic residue that specifically interacts with the remaining proton that is present in mono- and di-methyl ammonium moieties [36].

Finally, effector proteins for phosphorylated histone tails have also been identified. However, despite the many phosphate-binding domains described for non-histone proteins, only two effector modules are known for phosphorylated histones to date [26]. The first example is a 14-3-3 protein binding to phosphorylated Ser-10 on histone H3 (Table 1) [37]. This interaction is

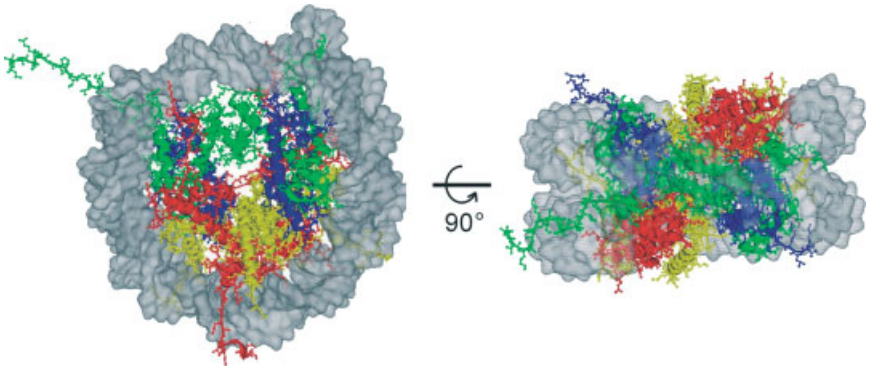

Figure 1. The structure of the nucleosome: H2A, red; H2B, yellow; H3, green; H4, blue; DNA, gray. Prepared from pdb ID: 1AOI [5].

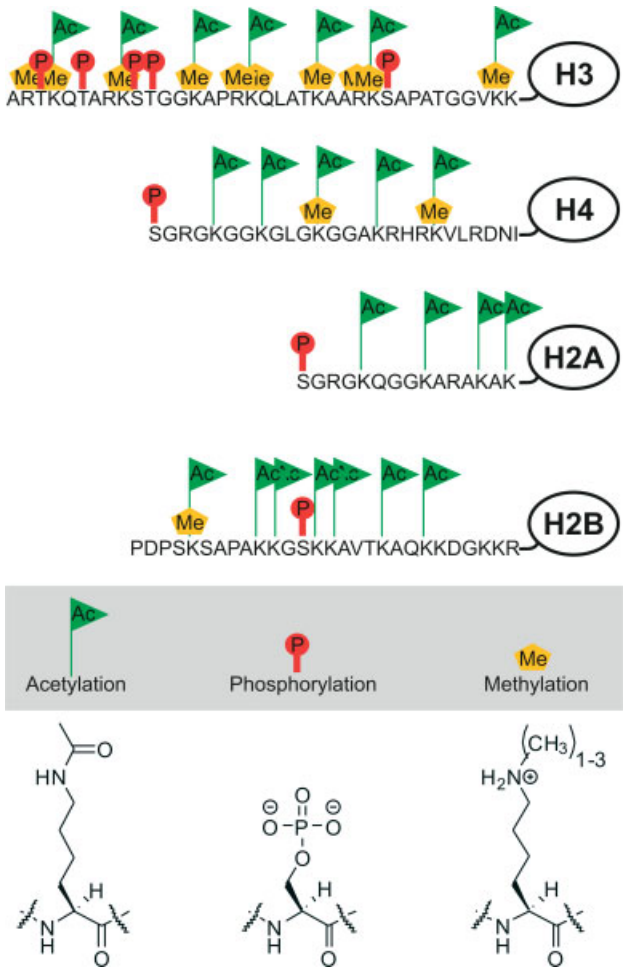

Figure 2. Modification map of histone acetylation, methylation and phosphorylation on the *N*-terminal histone tails.

strengthened by concomitant acetylation states of Lys-9 and Lys-14 on the same histone tail and is important for efficient transcription of the gene encoding histone deacetylase 1 (HDAC 1) [37,38]. The second example is the tandem BRCT repeat (breast cancer gene 1 carboxyl-terminal domain) of the MDC1 protein (mediator of DNA damage checkpoint) which binds to phosphorylated Ser-139 on the histone H2A variant γH2AX in response to DNA double-strand breaks [39].

Histone-Modifying Enzymes

Histone modifications can be either short lived, long lived, or transmitted from one generation to the next in a quasi-permanent manner. Individual lifetimes of histone modification states are determined by the balanced action of enzymes that specifically add or remove these modifications [40]. Acetylation of lysine residues is catalyzed by histone acetyl transferases (HATs) [41]. These enzymes require the ubiquitous metabolite acetyl-coenzymeA (Acetyl-CoA) as a cofactor (Figure 3(A)). HATs are typically part of large multi-protein complexes that can comprise up to 20 different proteins. The *Tetrahymena* Gcn5 protein was one of the first HATs to be identified and has functioned as the founding member of the GNAT HAT family [42]. In yeast, the Gcn5 homologous gene has long been known to encode a transcriptional regulator [43]. Besides the GNAT family of HATs, two other groups of HATs, the MYST and the CBP/p300 family, have been identified [40].

HDACs catalyze the removal of acetyl groups from lysine residues (Figure 3(A)). Together with HATs, they are responsible for maintaining the balance between acetylated and deacetylated histone states. The family of HDACs has been divided into three classes, based on phylogenetic analyses and sequence comparisons. HDACs of the classes I and II remove the acetyl moiety of lysines in a hydrolytic type of reaction that is Zn^{2+}-dependent (Figure 3(A)). Class III HDACs, also known as Sir2 proteins (silent information regulator 2) or sirtuins, share neither evolutionary nor mechanistic relations with HDACs of class I and II [44]. They depend on NAD^{+} as co-substrate and release the acetyl moiety as *o*-acetyl-ADP-ribose (Figure 3(A)) [45]. Eleven HDACs of classes I and II are known in mammals, termed HDAC 1–11. At the same time, there are seven homologs of sirtuines, SIRT 1–7. Like HATs, functional HDACs are often part of large multifunctional protein complexes that contain many other chromatin modifying enzymes and regulatory proteins [46].

Lysine methylation has been known since the 1960s; however, the first histone methyl transferases (HMTase), -Su(var)3-9-, were identified 40 years later [47]. The corresponding gene belongs to the Su(var) group (suppressor of variegation) of transcriptional inactivators that cause gene silencing in regions close to heterochromatin [48]. Detailed experiments uncovered that a conserved protein domain of Su(var)3–9, the SET-domain (Su(var)3–9, Enhancer of zeste and Trithorax), is one of the long sought-after HMTase. The SET-domain of Su(var)3–9 methylates Lys-9 of histone H3, which serves as a hallmark of heterochromatin. Methylation requires *S*-adenosyl-metheonine (SAM) as a cofactor and methyl source (Figure 3(B)). Today, more than 150 SET-domain proteins have been annotated in the human genome [49]. In

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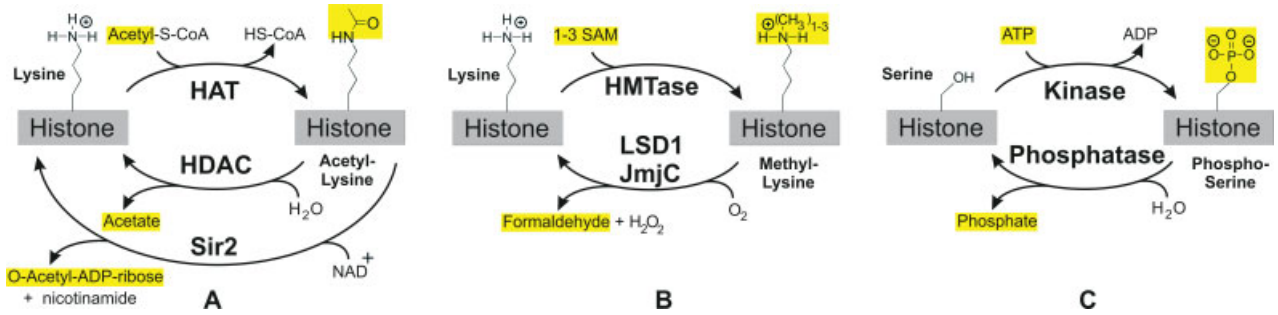

Figure 3. Histone-modifying enzymes for: A, acetylation; B, methylation; C, phosphorylation. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

addition, a further class of HMTases that do not contain SET-domains, the Dot1 family of proteins, has recently been identified [50].

Histone methylation was long considered a permanent histone mark until the first histone demethylase, LSD1 (lysine-specific demethylase 1) has been discovered. LSD1 is part of the CoREST complex, which represses neuronal genes in non-neuronal cells [51–53]. The enzyme shares homology with flavin-dependent mono-amine oxigenases (MAO) and utilizes molecular oxygen for catalysis (Figure 3(B)). The reaction mechanism of LSD1 is restricted to mono- and di-methylated lysines, whereas the recently identified JumonjiC family of demethylases catalyzes the removal of methyl groups from tri-methylated lysines [54,55].

Reversible histone phosphorylation is controlled by kinases and phosphatases (Figure 3(C)). Compared to the intensively studied lysine modifying enzymes information about histone kinases and phosphatases is rather scarce. The best-studied histone phosphorylation event is the modification of Ser-10 of histone H3. This site is globally phosphorylated during mitosis and the cellular enzymes that carry out these reactions are aurora B and protein phosphatase 1 (PP1) [56]. H3 Ser-10 phosphorylation has also been observed in interphase cells where it is catalyzed by a multitude of kinases and leads to elevated levels of gene transcription [57].

Chemical Tools for Chromatin Research

Ever since the realization that histone modifications play important roles in chromatin dynamics and gene activity, scientists have aimed at developing appropriate techniques to analyze the resulting landscapes of differential modification states. Here, sophisticated chemical tools have often paved the way for critical discoveries in the field [58].

Synthetic Peptides and Peptide-Based Probes

Unstructured histone tails constitute the programming platforms onto which the differential modification code is 'written' by modifying enzymes, to then be interpreted, or 'read', by various effector proteins, or protein complexes. This concept has led researchers to employ isolated histone tails as powerful probes for chromatin research. With a length of 15 to 38 amino acids, histone tails are well suited for solid-phase peptide synthesis and the availability of modified building blocks has rendered the generation of differentially modified histone tails straightforward. Most histone-modifying enzymes and effector proteins specifically interact with synthetic histone tail peptides in certain modification states, or recognize combinations of histone modification marks,

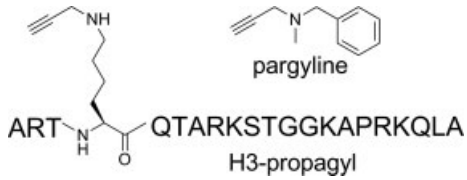

Figure 4. Inhibitors of MAO B and LSD1.

so that tail peptides have become indispensable tools to study their specificity. In addition, scientists have begun to explore peptide-based inhibitors of histone-modifying enzymes. In such constructs, the peptide mediates substrate recognition by the enzyme and a covalently attached chemical moiety interacts with the active site of the enzyme upon recognition.

One such example is the development of a mechanism-based inhibitor for the histone demethylase LSD1 [59]. As stated above, this flavin-dependent enzyme shares homology with mono-amine oxidases (MAO). Human MAO B, like most other MAOs, is irreversibly inhibited by pargyline, a small propargylamine-containing compound that forms a covalent adduct with the cofactor of the enzyme (Figure 4) [60]. Despite its homology to MAOs, LSD1 is not efficiently inactivated by pargyline *in vitro* [61]. This notion led to the development of a peptide-based inhibitor of LSD1, in which a propargylamine moiety is linked to the primary site of LSD1 action, Lys-4 on histone H3. The resulting inhibitor, 'H3-propagyl' (Figure 4), efficiently inhibited the demethylase activity of LSD1 in contrast to pargyline. A detailed biochemical investigation confirmed that the expected H3-propagyl and cofactor adduct were formed and caused the inactivation of LSD1 [62]. Importantly, this compound did not inhibit MAO B and thus demonstrated that the LSD1 specificity is exclusively mediated by the peptide moiety of the H3-propagyl compound.

Beyond Peptides: Semisynthetic Histones

Although peptides are very useful probes for analyzing the function of histone modifications, there are several limits with respect to biological questions that require larger chromatin structures. For example, the involvement of histone modifications in the formation of higher order chromatin, or the synergy between modifications located on two different histones, can only be addressed with whole nucleosomes, or arrays of nucleosomes. This problem can be solved with chemoselective ligation techniques like native chemical ligation (NCL) or expressed protein ligations (EPL). [63,64]. Both methods combine the advantages of solid-phase peptide synthesis and recombinant protein production

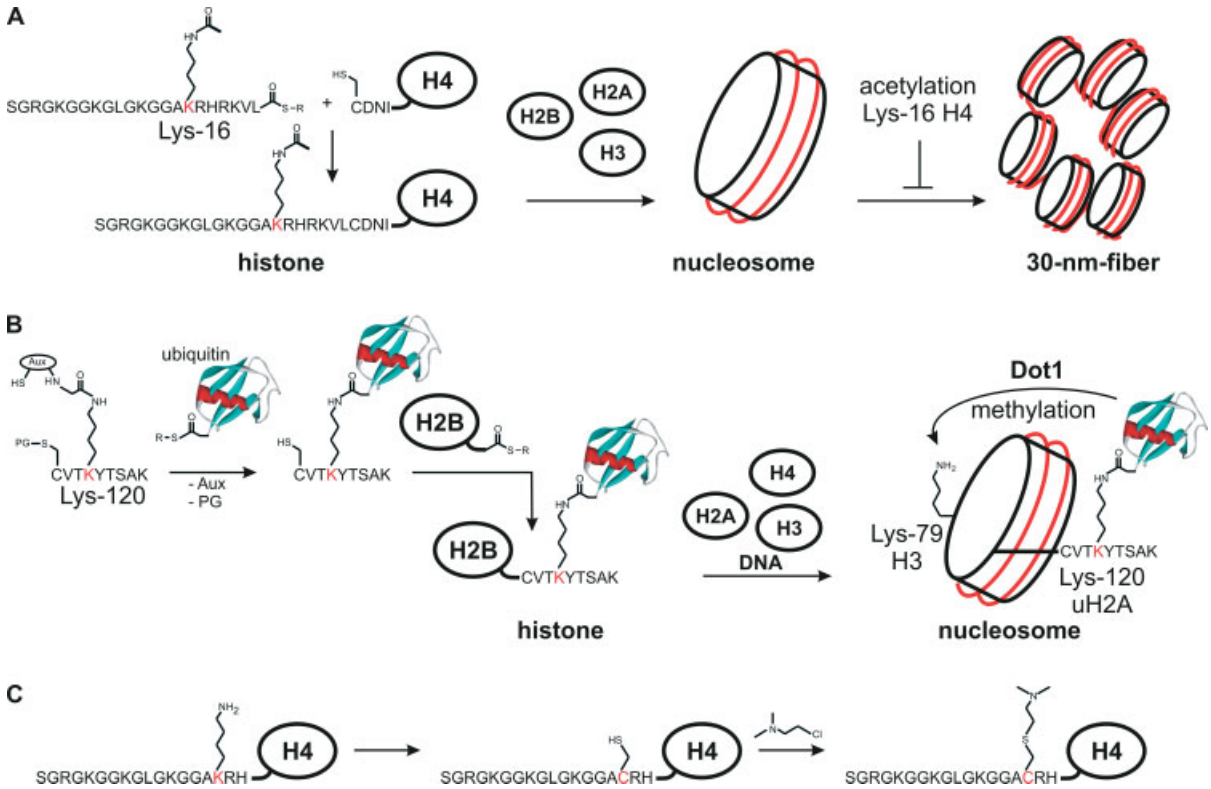

Figure 5. Application of semisynthetic histones in chromatin research. (A) H4 Lys-16 acetylation in the formation of higher order chromatin structures. (B) Crosstalk of histone ubiquitylation and methylation mediated by the HMTase Dot1; Aux: auxiliary group; PG: protecting group (C) Aminoethylation of histones. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

techniques [65]. Modified histone tails can be ligated to the globular core histone domains in order to provide homogenously modified full-length histones, which can then be incorporated into nucleosomes or nucleosomal arrays.

This strategy was applied in the synthesis of phosphorylated, acetylated and methylated histones for various purposes [66–69]. Linking an individual histone acetylation to the formation of higher order chromatin structures is a prominent example of this approach [70]. An array of 12 nucleosomes can form a structure that resembles the biophysical properties of the elusive 30 nm chromatin fiber [71]. Previous investigations have shown that amino acids 14–23 of the tail of histone H4 are crucial for such a structural arrangement [71]. As this region contains one of the four known acetylation sites of H4, Lys-16, a role of this single modification mark in 30 nm fiber-formation was tested with a semisynthetic version of histone H4, homogeneously acetylated at Lys-16 [71]. In order to produce this protein, a synthetic H4 peptide thioester (residues 1–22) acetylated at Lys-16 was generated and ligated to recombinant H4 (residues 23–102, containing an *N*-terminal Cys) by NCL (Figure 5(A)). This modified, semisynthetic version of histone H4 was incorporated into a nucleosomal array and subsequently employed for biochemical investigations. Strikingly, nucleosomal arrays assembled in this manner did not form 30 nm fibers. This finding clearly demonstrated a direct role of the singly acetylated Lys-16 residue in the formation of higher order chromatin.

Finally, an elaborated semisynthesis approach has been developed and applied to study the functional crosstalk of histone ubiquitylation and lysine methylation, catalyzed by the Dot1 HMTase (Figure 5(B)) [72,73]. Dot1 methylates Lys-79 of histone H3, which serves as an important mark for gene silencing. Lys-79 methylation is strongly affected by ubiquitylation of Lys-120 of histone H2B, by either affecting Dot1 recruitment or by modulating its activity (Figure 5(B)) [72]. In order to resolve this ambiguity, ubiquitylated H2B was generated in a two-step ligation process. At first, an ubiquitin thioester was ligated to Lys-120 of a synthetic H2B peptide (residues 117–125) by an auxiliary-mediated NCL reaction. In a second step, the auxiliary and a protecting group masking the *N*-terminal Cys were removed and the ubiquitylated H2B peptide was ligated to a recombinant H2B thioester (residues 1–116) generated by EPL. The resulting uH2B construct (Figure 5(B)) was assembled into nucleosomes and tested for Dot1 activity. Nucleosomes reconstituted with uH2B strongly stimulated the activity of Dot1 [72]. In contrast, nuclosomes assembled with non-modified H2B did not. Importantly, this stimulation was restricted to the nucleosome containing uH2B, which led to the conclusion that each nucleosome methylated at Lys-79 of H3 must have been ubiquitylated at Lys-120 of histone H2B at some point of time.

Alternative Strategies for Modified Histones

A prominent alternative method for generating homogeneously methylated histones is the aminoethylation of Cys residues. With exception of Cys-110 in histone H3 cysteines are absent in the core histones and can be introduced artificially by site-directed mutagenesis at any site. Recombinant Cys-mutated proteins are subsequently treated with an alkylation reagent like 2-chloro-*N*,*N*-dimethylethylamine hydrochloride, which converts the single Cys residue into a sulfur-containing analog of di-methyl-Lys (Figure 5(C)). Under optimized conditions, this alkylation reaction is specific for thiols thus allowing the generation of homogeneously

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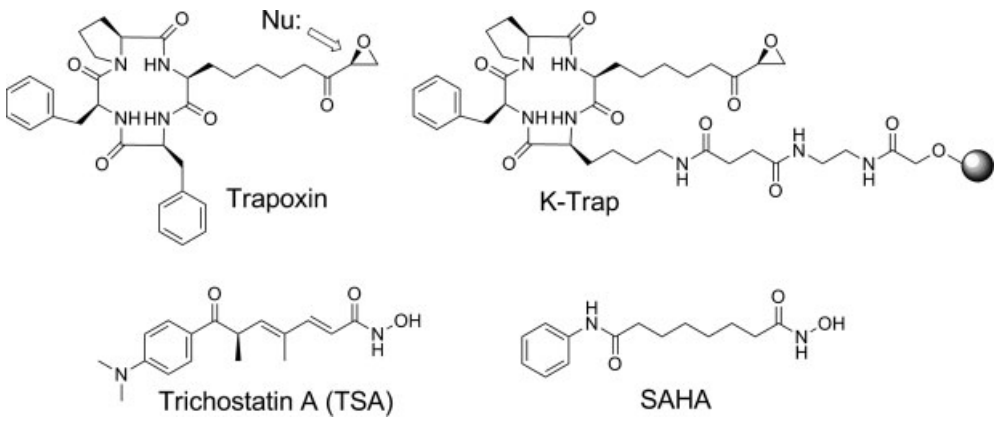

Figure 6. Natural products that inhibit HDACs.

methylated histones in large quantities [74]. This method has been successfully applied to probe the effects of histone methylation on nucleosome structure [75]. Another promising approach entails genetic encoding of modified lysines with evolved orthogonal tRNA synthetase/tRNA pairs. Acetylated and methylated lysine residues have been successfully produced in *E. coli* in this manner [76–78].

Natural Products and Small Molecules

Nature has evolved many chemical modulators of chromatin activity. These natural products are often inhibitors of histone-modifying enzymes. They naturally occur in bacteria and fungi and represent important tools in chromatin research and drug development. Indeed, one key discovery in the chromatin field is directly linked to their utilization. Trapoxin a natural histone deacetylase inhibitor (HDACi) was instrumental in the discoveries of the first HDAC [79]. Trapoxin functions as an irreversible HDACi and causes a dramatic increase in histone acetylation levels in cells treated with this compound [80–82]. The structure of this cyclic peptide enabled the delineation about its mode of action (Figure 6). Trapoxin contains an electrophilic epoxyketone that closely resembles acetylated lysine residues, and it had been hypothesized that this moiety could alkylate a nucleophilic residue of HDACs [79]. This assumption was confirmed by a synthetic Trapoxin derivative, K-trap, which was immobilized on a solid support to isolate the first HDAC from nuclear extracts of mammalian cells (Figure 6). Microsequencing identified the HDAC protein which subsequently initiated a period of intensive HDAC research in the following years.

Apart from academic research endeavors, natural products and synthetic small molecule inhibitors of histone-modifying enzymes are also investigated for their suitability as potential drugs [83]. The dynamic balance of acetylated and deacetylated histone states is important for genome stability and the regulation of gene activity. Alterations in HDAC and HAT activities, including overexpression or aberrant recruitment of HDACs by oncoproteins, or mutations in HATs can be found in several forms of cancer [84]. This notion has challenged scientists to search for, and explore small molecules and natural products that are capable of modulating HDAC/HAT activities for therapeutic intervention. Today, several HDACis are tested in clinical trials and their projected efficaciousness render them most promising drug candidates. The first known HDACis to be clinically tested were short-chain fatty acids like butyric acid, which were shown to exhibit broad HDAC inhibitory potential both *in vivo* and *in vitro*. These compounds, however, suffered from short *in vivo* half-lives, which resulted in low plasma levels [85]. One of the most intensively studied HDACi is trichostatin A (TSA) (Figure 6), which is known to inhibit class 1 and 2 HDACs in the low nanomolare range. Cells treated with TSA display a significant increase in histone acetylation levels, but poor pharmacokinetics prohibit a clinical application of this compound [85]. TSA is a hydroxamic acid-based HDACi that coordinates the essential Zn^{2+}-ion of the active site. With suberoylanilide hydroxamic acid (SAHA), also known as Vorinostat and marked under the trademark name Zolinza, the first HDACi was approved for cancer treatment in 2006 [85]. It is likely that more compounds that target histone-modifying enzymes will be added to this collection in the near future.

Perspective

Deciphering the complex regulation of chromatin structure with respect to differential gene activity remains one of the major challenges in the biomedical sciences today. Detailed knowledge about these processes will be necessary for a general understanding of eukaryotic genomes, as well as for the treatment of several human diseases. Chemical tools are inextricably linked to key discoveries in chromatin research and our current understanding of epigenetics. It is certain that chemical methods will continue to represent indispensible tools in chromatin research and that future challenges will likely inspire new generations of such tools.

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