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Chromatin as an expansive canvas for chemical biology

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Abstract

Chromatin is extensively chemically modified and thereby acts as a dynamic signaling platform controlling gene function. Chromatin regulation is integral to cell differentiation, lineage commitment and organism development, whereas chromatin dysregulation can lead to age-related and neurodegenerative disorders as well as cancer. Investigating chromatin biology presents a unique challenge, as the issue spans many disciplines, including cell and systems biology, biochemistry and molecular biophysics. In recent years, the application of chemical biology methods for investigating chromatin processes has gained considerable traction. Indeed, chemical biologists now have at their disposal powerful chemical tools that allow chromatin biology to be scrutinized at the level of the cell all the way down to the single chromatin fiber. Here we present recent examples of how this rapidly expanding palette of chemical tools is being used to paint a detailed picture of chromatin function in organism development and disease.

Eukaryotic cells use a multitude of regulatory mechanisms to control the DNA transactions that are involved in replication, repair and transcription. Chemical modification of the DNA template, and the proteins it interacts with, lies at the heart of many of these regulatory processes. In the eukaryotic nucleus, the DNA is tightly packaged in chromatin, a nucleoprotein complex containing canonical histones (histone 2A (H2A), H2B, H3 and H4) that form nucleosomes together with approximately 147 base pairs (bp) of DNA, a linker histone (H1) and numerous nonhistone proteins (Fig. 1a). Chromatin allows for the spatial organization of the genome. Arrays of nucleosomes fold into fibers and higher-order structures, which are stabilized by linker histones and other structural proteins¹. On the basis of chromosomal staining patterns, two basic classes of chromatin can be differentiated: (i) areas of low staining density (euchromatin) that are associated with less compacted chromatin and often (but not always) active gene expression and (ii) regions of high staining density (heterochromatin) that are associated with compacted and generally silent chromatin.

Several chemical mechanisms are used by nature to enact chromatin-mediated gene regulation, including DNA methylation and the installment of post-translational modifications (PTMs, often also referred to as ‘marks’; Box 1 and Fig. 2) on all histone proteins and their many isoforms. Many PTMs are highly dynamic, with specialized enzymes catalyzing their introduction (‘writers’) or removal (‘erasers’). Examples include

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histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases. The large number of modified residues, primarily situated in the unstructured N-terminal domains of histones, allow for the formation of combinatorial patterns of marks within a chromatin fiber or even within a single nucleosome. Indeed, it has been hypothesized that histone PTMs form a code² in which a specific biological output is determined by the local pattern of marks.

Histone PTMs can directly regulate chromatin structure by changing its physicochemical properties, thereby altering the physical accessibility of the DNA template. They can also affect chromatin activity by serving as recruitment platforms for a large variety of protein factors ('effectors') containing specialized protein modules that recognize distinct modifications ('reader' domains). Through such protein-protein interactions, effectors involved in transcriptional activation or silencing are localized on the chromatin. Many effectors are multiprotein complexes that contain several reader domains, thereby allowing the integration of different marks on single or neighboring nucleosomes. Furthermore, coupling of reader domains to writer or eraser domains results in crosstalk between modifications. This allows for the generation of signaling networks, which establish a certain chromatin state that can be stable over cell generations. Conversely, misregulation of these systems has been implicated in many cancers³. Therefore, insight into the molecular basis of the establishment of stable chromatin states is crucial for a better understanding of cell differentiation, homeostasis and malignancy.

In this review, we present developments in applying chemical and biophysical methods to investigate chromatin regulation through histone PTMs. Because of space constraints, we mainly focus on lysine acetylation and methylation, as these marks are the most studied to date. For more information on chemical biology investigations targeting other modifications, we refer the reader to other recent reviews^{4,5}. Following the hierarchical organization of chromatin (Fig. 1a), we begin at the cellular level and discuss methods to globally modulate chromatin effectors using small-molecule compounds. Narrowing our focus to the biochemical details, we discuss chemical biology methods for analyzing the readout of histone marks by effector proteins. Such studies are enabled by the chemist's ability to manufacture chemically defined chromatin (what we refer to as designer chromatin; Box 1). Finally, moving down to the level of the chromatin fiber and the individual nucleosome, we present new findings on the biophysical basis of chromatin regulation by effector proteins and histone PTMs as elucidated in quantitative *in vitro* experiments.

Chemical probes to rewrite histone PTM patterns

Genome-wide profiling approaches, based on DNA adenine methyltransferase identification⁶ and chromatin immunoprecipitation paired with next-generation sequencing^{7,8}, have identified multiple distinct chromatin states that are characterized by combinations of histone modifications and chromatin-associated proteins (Fig. 1b). A substantial portion of current research in the study of chromatin has been geared toward unraveling the mechanisms controlling the establishment and maintenance of these chromatin states. Through the application of genetic and biochemical approaches, many key protein factors have been identified and analyzed in recent years (reviewed elsewhere⁹). As

in many areas of cell biology, small-molecule inhibitor (and activator) compounds are proving to be of great utility for dissecting the precise role of these effectors in choreographing chromatin biology. Some of these compounds have been instrumental in tracking down the enzymes responsible for long-known histone-modifying activities¹⁰, whereas other compounds have already made their way into the clinic for the treatment of cancer¹¹. These successes notwithstanding, many of these first-generation compounds have rather broad specificity profiles, which limits their utility as research tools⁴. Consequently, there is great interest in developing more specific inhibitors of chromatin effector proteins. Further motivating this entire area of research is the potential of such compounds to be developed into next-generation epigenetic drugs tailored toward diseases that are associated with a specific dysregulated effector.

Targeting erasers: histone deacetylases

Modulating the global amount of histone acetylation has a major impact on chromatin states and, ultimately, on cellular phenotype. The amount of histone acetylation is controlled by the action of writers (HATs) and erasers (HDACs). In particular, inhibitors of HDACs have been very successful in altering chromatin regulation, and we will focus on new developments in the discovery of isoform-selective inhibitors of this enzyme family in this section.

The human genome encodes 11 different zinc-dependent histone deacetylases, considered 'classical' HDACs, that are grouped into class I (HDAC1, HDAC2, HDAC3 and HDAC8), class IIa (HDAC4, HDAC5, HDAC7 and HDAC9), class IIb (HDAC6 and HDAC10) and class IV (HDAC11) HDACs^{10,12} (some members of the NAD⁺-dependent sirtuin enzyme family constitute another class of HDACs, class III, but are not discussed here because of space constraints). The HDACs share highly homologous active sites and operate within multiprotein complexes that have distinct biological functions. In addition, a given HDAC isoform can exist in more than one complex. The activity of the HDACs is required in many nuclear processes, including transcription, and HDAC substrates also include proteins not associated with chromatin, for example, the HDAC6 deacetylates tubulin and heat shock protein 90 (hsp90) (ref. 12). Small-molecule inhibitors such as trichostatin A, suberoylanilide hydroxamic acid (SAHA), trapoxin B and tubacin (Fig. 3a) have had key roles in the identification of these enzymes and in elucidating their functions⁴. Prolonged treatment of cells with HDAC inhibitors induces globally increased histone acetylation. These changes in chromatin states result in alterations of gene expression profiles¹² and large-scale rearrangements in chromatin compaction^{13,14}. The biological responses to HDAC treatment vary depending on cell type and state. Generally, transformed cells show cell-cycle arrest, differentiation and apoptosis, making HDAC inhibitors promising chemotherapeutics. Indeed, broad-spectrum HDAC inhibitors such as SAHA and romidepsin (Fig. 3a) are used clinically to combat cutaneous T cell lymphoma^{11,15}. Other applications include the use of HDAC inhibitors to increase the efficiency of pluripotent stem cell induction¹⁶.

HDAC inhibitors have common structural features, including a cap, a linker and a metal-binding moiety to chelate the active-site Zn²⁺ ion (Fig. 3a)¹⁷. By modifying the chemical

structure of each of these elements, a multitude of molecules have been produced in recent years with varying degrees of subtype specificity¹⁸. However, a major impediment in the identification of selective inhibitors stems from the difficulties accompanying the development of sensitive screening assays. Modern chemical biology methods have yielded several improvements in characterizing panels of HDACs and probing the selectivity of inhibitor compounds. This concept is exemplified by a study in which the selectivity of HDAC inhibitors was investigated using optimized assays with two fluorogenic substrates containing either acetyllysine or a trifluoroacetyl analog¹⁹. Surprisingly, these experiments revealed subtype specificity for all the tested compounds, even for presumed pan inhibitors such as SAHA. In particular, subtype IIa HDACs were inefficiently inhibited by all the compounds tested. This knowledge ultimately allowed for the development of a pan-active HDAC inhibitor with an overall lower subtype discrimination.

Most screening assays are performed using isolated recombinant HDAC proteins (or the catalytic domains thereof), meaning in the absence of their natural binding partners. This reductionist approach could affect both the activity and specificity of the enzymes. To address this problem, an elegant chemoproteomics profiling approach was developed to probe HDAC inhibition using native complexes²⁰. Using bead-anchored small-molecule inhibitors, native HDAC complexes were isolated from cell lysates and identified by MS. Pretreatment of the cells with an array of HDAC inhibitors resulted in a reduced binding to the affinity matrix that was dependent on the inhibitor concentration. Moreover, by using a mass-labeling approach, inhibition constants for the various compounds were determined. The data revealed unanticipated context sensitivity within multiprotein complexes. HDAC1 and HDAC2, for example, were found to be resistant to aminobenzamide inhibitors such as BML-210 (Fig. 3a) in the context of the Sin3 transcriptional repressor complex but not in the NuRD or CoREST repressor complexes. These findings highlight the value of using sophisticated assays for the development of class- and subtype-selective HDAC inhibitors for use as chemical probes and potential drugs.

Targeting writers: histone lysine methyltransferases

In recent years, there has been rising interest among researchers in the pharmacological modulation of histone lysine methyltransferases for the purpose of the targeted alteration of histone methylation. Histone methyl marks are a core component of all chromatin states (Fig. 1b). Consistently, deregulation of histone lysine methylation is implicated in disease, as many major developmental chromatin regulatory pathways rely on distinct histone-methyl patterns. About 50% of all known HMTs have been linked to disease, either when mutated or on dysregulation of their expression³. Therefore, in addition to being useful as cell biological probes, small-molecule modulators of these writer enzymes could also be used to reprogram cancer cells as a treatment option.

Before the discovery of the first specific HMT inhibitors, most modulators of this enzyme family were close analogs of the general cofactor of the methyltransferases, S-adenosylmethionine (SAM), for example, sinefungin, methylthioadenosine and S-adenosylhomocysteine⁴. This changed in 2005 with the discovery that the complex natural product chaetocin (Fig. 3b) inhibits the H3 Lys9 (H3K9)-specific methyltransferase,

SU(VAR)3-9 (also commonly referred to as lysine methyltransferase 1, KMT1) (ref. 21). H3 dimethyl Lys9 (H3K9me2) and H3 trimethyl Lys9 (H3K9me3) are classical heterochromatin marks that are crucial for maintaining gene repression. H3K9 methylation was specifically reduced in cells treated with chaetocin, as observed by MS and western blot analyses, showing the ability of this compound to modulate chromatin states *in vivo*. At euchromatic sites, monomethylation and dimethylation of H3K9 is carried out by the methyltransferases G9a (KMT1C) and GLP (KMT1D). H3K9me1 and H3K9me2 are the only marks that are lost when tumor-suppressor genes are reactivated by treatment with DNA-demethylating drugs²². Thus, both enzymes are key pharmaceutical targets. Using a high throughput *in vitro* screen, a quinazoline amine derivative, BIX-01294 (Fig. 3b), was identified that specifically inhibits G9a with a half-maximal inhibitory concentration (IC₅₀) of 1.7 μ M and in a noncompetitive manner with SAM²³. A subsequent analysis of the co-crystal structure revealed that the inhibitor binds to the peptide-binding pocket in the presence of bound cofactor²⁴. After treatment with BIX-01294, a significant reduction in H3K9me2 was observed in various cell lines, leading to the upregulation of G9a target genes. Building on this initial success, several analogs of BIX-01294 with enhanced potency and cell penetration have been generated. UNC0638, the most optimized probe reported to date²⁵ (Fig. 3b), inhibits both G9a and GLP with IC₅₀ < 20 nM. Interestingly, BIX-01294 treatment can replace the transcription factor Oct3/4 for reprogramming adult cells into induced pluripotent stem cells²⁶. Changes in the chromatin state induced by inhibitor treatment seem to sensitize the cells to reprogramming, possibly through relieving the repression of genes silenced during differentiation.

SU(VAR)3-9 and G9a, and, indeed, most other known HMTs, contain a conserved SU(VAR)3-9, enhancer of zeste, trithorax (SET) methyltransferase domain. In contrast, the HMT disruptor of telomeric silencing-1 (Dot1, KMT4) has a unique catalytic domain with a fold that is more similar to that seen in the arginine methyltransferases²⁷. Dot1 is specific for the methylation of H3K79, a mark that is associated with active transcription, and is the only known methyltransferase for this site^{28–30}. Because misregulation of Dot1 is involved in hematopoietic malignancies^{31,32}, there has been strong interest in finding an inhibitor compound for it. Recently, an adenosine derivative (EPZ004777; Fig. 3b) was described that acts as a competitive inhibitor of Dot1 and has an inhibitor binding affinity (K_i) of 0.3 nM and a greater than 1,000-fold selectivity over other methyltransferases³³. The activity of this molecule is all the more remarkable when considering the structural similarity of EPZ004777 to SAM, the cofactor of all HMTs. Notably, treatment of leukemia cell lines with this compound selectively reduced global amounts of H3K79 monomethylation and dimethylation. The pharmacological reversal of H3K79 hypermethylation, and the resulting change in chromatin states, led to the repression of genes upregulated in leukemic cell lines while not altering the expression profiles of the housekeeping genes. These changes in gene expression subsequently caused cancer cell differentiation and apoptosis *in vitro* and in mouse xenograft models³³.

The recent successes discussed above show that it is possible to generate highly specific inhibitors of chromatin effector proteins such as HDACs and HMTs. However, as many of the writer and eraser enzymes in a particular class are highly homologous, the problem of

specificity is nontrivial. For protein kinases, one way around this problem has been to use allele-specific inhibitors created using the so-called 'bump-hole' approach³⁴. Similar strategies have been developed to inhibit arginine methyltransferases³⁵. It seems probable that these chemical biology strategies will be used increasingly in the next few years to modulate and study chromatin states on a cellular level.

Readout of histone modification states

It is widely accepted that the interpretation of chromatin states by protein effectors is driven by the localization of these factors to particular chromatin loci³⁶. Effector recruitment must involve interaction with specific DNA elements (either directly or through transcription factors or RNA-mediated processes), distinct histone PTMs or both³⁷. With respect to PTMs, several classes of reader domains (Fig. 4a) have been characterized over the last decade that bind specific histone marks and are widely found in chromatin effectors⁹. Researchers are now beginning to understand, through the use of chemical biology tools, the molecular recognition principles underlying effector recruitment to specific chromatin sites (Fig. 4b).

Multisite binding

The interaction between individual reader domains and histone marks tends to be of modest affinity: for example, acetylated lysine residues are recognized by bromodomains with dissociation constants (K_d) in the high micromolar range⁹. This raises a question: just how specific for a distinct sequence context are the binding interactions between a reader domain and a histone mark? To investigate this issue, an unbiased protein microarray approach was used to gauge the specificity of individual reader domains³⁸. A large selection of readers, including bromodomains, plant homeodomain (PHD) fingers and royal superfamily domains (chromo, tudor and malignant brain tumor (MBT) domains; Fig. 4a), were spotted onto glass slides to form a protein array. Fluorescently labeled histone peptides carrying different methyllysine marks were incubated with the protein array, which was followed by imaging of the bound peptides. These experiments revealed several previously unidentified interactions between reader domains and histone marks. In particular, tudor domains were found to interact with a variety of methyl marks in H3 and H4 tail peptides. For example, the double tudor domain of the DNA damage checkpoint protein p53-binding protein 1 (53BP1) interacts with dimethylated H3K4, H3K9 and H4K20. Similarly, the hybrid tudor domains (HTDs) of the Jmj family demethylase JMJD2A (lysine demethylase 4A, KDM4A) bind both dimethylated and trimethylated H3K4 and H4K20. Plasticity in histone mark recognition is not limited to tudor domains, as was shown in a study investigating the binding specificity of members of the chromodomain Y-chromosome (CDY) protein family³⁹. Proteins in this family contain a chromodomain that is closely related to those found in heterochromatin protein 1 (HP1) (recognizing H3K9me3) and polycomb (binding to H3K27me3)⁴⁰. At both sites, H3K9 and H3K27, the lysine carrying the methyl groups is embedded in a homologous ARKS/T motif. Using a small library of synthetic H3 peptides, it was shown that the CDY chromodomain binds to trimethylated H3K9 and H3K27 peptides. Thus, multisite recognition seems to be a common theme for reader domains and allows for considerable flexibility in the readout of histone PTM patterns.

Combinatorial PTM readout

If the sequence around the modified residue does not in itself impose the specificity required for the proper recruitment of effectors, are there other determinants involved? One such mechanism seems to be the combinatorial readout of two or more neighboring histone marks by a single reader domain (Fig. 4b). Through the action of secondary PTMs, the binding interaction of a reader with its cognate mark can then be completely masked, gradually modulated or even enhanced. The best known example of masking occurs at H3K9me₃, where the association with HP1 was shown to be effectively abolished on phosphorylation of the adjacent Ser10 residue by aurora kinase during the M phase of the cell cycle⁴¹. Another example of combinatorial readout involves the H3K4me₃ mark. Most readers of H3K4me₃ make crucial contacts with flanking residues in the H3 tail, and modification of these residues may alter this binding. This type of sensitivity was found for the double chromodomains of chromodomain helicase DNA-binding protein 1 (CHD1), which binds H3K4me₃ in a noncanonical surface groove that is formed at the interface of both domains⁴². Dimethylation of Arg2 in H3K4me₃-containing peptides reduced the affinity of CHD1 by about twofold, whereas phosphorylation of Thr3 further decreased this binding by a factor of 25. An interesting case is the PHD finger of the V(D)J recombination activator RAG2, which binds the H3 peptide in a unique conformation that is distinct from other readers of the H3K4me₃ mark. In this case, symmetric dimethylation of Arg2 modestly increased binding of this PHD domain to the H3 tail, allowing for a combinatorial readout of these two marks⁴³.

Peptide libraries have been used to obtain a more comprehensive picture of the combinatorial effects of multiple marks on reader interactions. This strategy is exemplified by an investigation into the effects of multiple marks on the binding of the H4 tail by the HTD of JMJD2A⁴⁴. To this end, an 800-member H4-tail peptide library containing all permutations of the then-known modifications (lysine and arginine methylation, citrullination, lysine acetylation and serine phosphorylation) was synthesized on beads. The beads, each carrying a unique peptide, were then incubated with the HTD, and binding was assessed using a colorimetric assay followed by an MS analysis of the hits. These experiments revealed that although methylation of H4K20 had a strong positive influence on JMJD2A binding, all other neighboring modifications decreased the HTD association. In particular, acetylation of Lys5, Lys8, Lys12 and Lys16 resulted in a gradual reduction of the interaction between JMJD2A and the H4 peptides. In a subsequent study, these experiments were expanded to cover combinatorial PTMs on H3 (ref. 45). A 5,000-member library was generated incorporating all modifications of the N-terminal portion of the H3 tail. This library was used to investigate the multisite discrimination of a panel of H3K4-specific PHD fingers, each recognizing different lysine methylation states, as well as for the JMJD2A HTD. Several marks were identified that affected reader binding, including Thr6 phosphorylation (which reduces PHD finger binding but not JMJD2A HTD binding) and Arg2 methylation (which is preferred by the RAG2 PHD finger compared to other readers). Therefore, finely tuned protein-protein interactions allow protein effectors carrying single reader domains to integrate combinatorial patterns of histone marks.

Multivalent PTM binding

Emerging research points to a key role for multivalent interactions (binding to combinations of DNA sequences, histone marks or both) in the proper recruitment of chromatin effectors to target sites (Fig. 4b). Many chromatin effectors contain multiple reader domains⁴⁶, making multivalent engagement of histone modifications a clear strategy for imposing binding selectivity. A pioneering study showed that the double bromodomain module of the TAF1 subunit of the general transcription factor TFIID could simultaneously engage H4 peptides acetylated at both Lys5 and Lys12 (ref. 47). Recently, designer chromatin (Box 1) was used to show that a subunit of the nucleosome remodeling factor (NURF) complex, bromodomain PHD finger transcription factor (BPTF), can simultaneously engage H3K4me3 and H4 that is acetylated at Lys16 (H4K16ac) using a combination of a PHD finger and a bromodomain⁴⁸. These two reader domains in BPTF are separated by a short α -helix, the register of which is crucial for bivalent binding; changes in helix length alter the relative orientation of the PHD and the bromodomains and impair simultaneous recognition of both H3K4me3 and H4K16ac. As the PHD-bromodomain motif is quite common in chromatin effector proteins, this mode of multivalent histone mark recognition could be a recurrent theme in chromatin biology.

Modern proteomics approaches provide an expedient route to the discovery of protein complexes recognizing specific patterns of histone marks. Particularly potent is the use of stable isotope labeling by amino acids in cell culture (SILAC)⁴⁹ in combination with a pull-down strategy and a chemically defined bait (for example, a modified histone peptide). Using this integrated approach, new binders of the H3K4me3 mark were identified, including subunits of the TFIID complex⁵⁰. A second pull-down experiment revealed that acetylation of H3K9 and H3K14 increased TFIID binding considerably because of the presence of two reader domains in different subunits of the complex: a PHD finger in TAF3 and the aforementioned double bromodomain in TAF1 (ref. 47). Having established these proteomic methods, the study was expanded to include other activating (H3K4me3 and H3K36me3) and repressive marks (H3K9me3, H3K27me3 and H4K20me3) in both H3 and H4 tails⁵¹. Among the many interesting findings to come out of this study was the observation that the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex, a histone acetyltransferase that is involved in gene activation, binds to H3K4me3 together with H3K9ac and H3K14ac through a combination of a double tudor domain and a bromodomain on two separate subunits of the complex.

With the development of methods to synthesize modified designer chromatin (Box 1), affinity experiments using more physiological substrates are now possible. In a pilot study, nucleosomes were assembled from semisynthetic histones containing combinations of lysine-methyl marks (H3K4me3, H3K9me3 and H3K27me3), as well as CpG-methylated nucleosomal DNA⁵². These substrates were subsequently applied in SILAC pull-down experiments to identify protein complexes interacting with the modified chromatin. Interestingly, different DNA methylation states of the nucleosomes resulted in differential effects on the association of the effectors. Whereas nucleosome binding of the origin recognition complex was enhanced by DNA methylation, polycomb repressive complex 2 (PRC2) components and KDM2A, an H3K36-specific demethylase, were excluded from the

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methylated DNA. In a similar study, either modified peptides or synthetic arrays of 12 nucleosomes were used to probe the inter-actomes of the H3K4me3 and H3K9me3 marks⁵³. The peptide- and chromatin-based pull-down experiments each produced a comparable number of bound proteins, however, the datasets from the two experiments overlapped only partially. For example, Fanconi anemia complementation group F (FANCF) preferentially bound to the H3K4me3-modified chromatin template over the modified histone peptide. These findings underscore the value of using chromatin for interaction studies, as essential binding determinants might be missed or misrepresented in peptide-based assays alone. In this context, we also note that many chromatin effectors contain DNA-binding domains in addition to histone reader domains⁴⁶.

Modulators of chromatin-binding domains

Although the various approaches discussed in the preceding section are useful for cataloguing interactions between reader domains and histone marks, they do not provide direct information on the functional role of these associations in localizing effector complexes to specific chromatin loci *in vivo*. Targeted mutagenesis (for example, deleting, incapacitating or exchanging reader modules) in combination with chromatin immunoprecipitation approaches provides one option for *in vivo* validation^{54–56}. However, the lack of temporal control associated with this approach, combined with the possibility of generating a lethal phenotype, creates an opening for the application of small-molecule antagonists of interactions between reader domains and histone marks. Beyond their utility as research probes, inhibitors of reader domains could also have widespread therapeutic applications. Genomic translocations that result in hybrid effectors with altered chromatin-binding properties have been observed in many cancers³, providing a rationale for the pharmacological targeting of reader domains.

Antagonizing protein-protein interactions with small molecules is very challenging⁵⁷. Nonetheless, there have been several notable successes in the study of histone reader domains in the past few years. A particular focus has been to find small-molecule compounds that inhibit the bromodomain-acetyllysine interaction because of the widespread functions of this reader in transcription regulation. In early examples, NMR-based screening approaches were used to identify low-micromolar binders to the bromodomains of the HATs PCAF (lysine acetyltransferases 2B, KAT2B)⁵⁸ and CBP (KAT3A)⁵⁹. In both cases, the respective bromodomains are able to bind nonhistone acetyllysine substrates. A nitroaniline derivative (Fig. 5a) showed promise as an inhibitor of the interaction between PCAF and the human immunodeficiency virus (HIV) protein Tat to block HIV transcription and replication⁵⁸. Conversely, a substituted tetrahydrocarbazole (MS7972; Fig. 5a) was found to inhibit p53 activation by disruption of the p53-CBP interaction⁵⁹. These studies were extended with the development of ischemin, a compound based on a diazobenzene scaffold that antagonizes the p53-CBP interaction with a micromolar affinity⁶⁰ (Fig. 5a).

The first highly specific inhibitors of a bromodomain were described only recently. Two independent studies converged on related compounds that are based on a diazepine core and have a nanomolar affinity for bromodomain and extraterminal (BET) family bromodomains^{61,62}. BET bromodomains occur in the transcription-associated proteins

bromodomain-containing 2 (BRD2), BRD3 and BRD4, as well as in the testes-specific bromodomain (BRDT) protein. These domains are notable for their distinct binding surfaces and their ability to simultaneously bind to two acetyllysine marks in the same histone tail⁶³. In the first study, a thienodiazepine-based BET bromodomain inhibitor, JQ1 (Fig. 5a), was designed based on an earlier discovery of a small-molecule ligand of the bromodomains of BRD4 (ref. 64). JQ1 was found to be very selective for the BET bromodomains; the compound bound with a K_d of 50 nM and a K_d of 90 nM to bromodomains 1 and 2 of BRD4, respectively⁶¹. A crystal structure of one of the complexes showed that the triazole ring of JQ1 inserts into the acetyllysine recognition channel and forms a hydrogen bond to the conserved asparagine residue that is normally involved in the recognition of the acetamide moiety. The BRD4 bromodomains have been implicated in a rare cancer, NUT midline carcinoma, in which a genetic translocation results in the fusion of these bromodomains to an unrelated protein, nuclear protein in testes (NUT). The BRD4-NUT fusion protein is mislocalized in the nucleus in this cancer, leading to the hyperacetylation of inactive chromatin domains and p53 inactivation⁶⁵. As JQ1 was shown to prevent the association of BRD4-NUT fusion protein with acetylated chromatin, the compound has the potential to block the pathogenic association of the oncogenic fusion. Indeed, tumor cell lines that are dependent on the activity of the BRD4-NUT oncogene entered apoptosis after JQ1 treatment, and JQ1 was also active in the context of mouse xenograft tumor models.

The therapeutic potential of BET bromodomain inhibitors is not restricted to cancer. BET transcription factors are known to regulate the expression of inflammation-related genes⁶⁶. Thus, compounds that interfere with these interactions might be effective at modulating inflammation. In a remarkable example of pharmacological convergence, a benzodiazepine compound termed I-BET (Fig. 5a) was identified from a medicinal chemistry program and was subsequently shown to bind to BET bromodomains with a K_d of 50–60 nM and with a similar binding orientation as JQ1 (ref. 62). Treatment of lipopolysaccharide-stimulated macrophages with I-BET resulted in suppression of the expression of inflammation-related genes by altering the acetylation patterns at their promoters. Moreover, I-BET treatment led to protection against endotoxic shock arising from bacterial sepsis in mouse models.

In contrast to bromodomains, the search for selective inhibitors of methyllysine-binding domains, such as PHD fingers and royal family members, has somewhat lagged. Some success has been reported in developing small-molecule inhibitors of the MBT domains in the MBT-family protein L3MBTL1 (ref. 67), which recognize lower methylation states of lysine residues in histone tails (Fig. 5b). Although there is considerable work left to do in this area, these initial studies show that selective targeting of methyllysine-binding proteins is achievable. As the recognition of distinct methyl marks is a cornerstone of chromatin regulation, selective targeting of methyllysine reader domains may have great utility in influencing chromatin readout and gene expression to modulate cell development and combat cancer.

Molecular analysis using systems designed *in vitro*

To gain an in-depth molecular understanding of how histone modifications regulate chromatin function, detailed *in vitro* biochemical, structural and biophysical studies are

required. The development of synthetic methods to produce in the test tube chromatin that is modified at specific sites has greatly extended the possibilities in this area. In this section, we describe bottom-up approaches using chemically defined chromatin to elucidate the interplay between histone modifications and chromatin structure, dynamics and function.

Crosstalk between histone marks

Preexisting marks on histones can influence the activity of writer and eraser enzymes. Differential recruitment of such effectors carrying reader domains alters their ability to modify chromatin through positioning, as well as through local concentration effects. Alternatively, writer and eraser enzymes may be allosterically modulated by preexisting marks. These mechanisms can be discriminated in carefully conducted experiments using chemically defined histones and reconstituted nucleosomes.

An example of effector recruitment is provided by the results from a study investigating the kinetics of H3 tail acetylation by the SAGA complex⁶⁸. In contrast to H3 peptides, SAGA acetylation activity was found to be highly cooperative in reconstituted nucleosomal arrays. Extensive kinetic analyses showed that this cooperativity depends on the availability of two H3 tails within the same nucleosome. Further investigation identified the bromodomain of GCN5 (KAT2A), a SAGA complex member, to be responsible for this effect⁶⁹. Interestingly, in nucleosomes containing one acetylated H3 molecule, synthesized using EPL (Box 1 and Fig. 2a), SAGA activity was markedly stimulated in a manner that was dependent on an intact bromodomain. Thus, SAGA is recruited to preacetylated chromatin sites and, through recognition of the product of its catalytic activity, may propagate an acetylated chromatin state (Fig. 6a). Similar mechanisms have been proposed for spreading repressive histone marks, in particular, H3K9me3 in heterochromatin and H3K27me3 in polycomb-repressed genes. H3K9me3 is bound by HP1, which interacts with the methyltransferase that is responsible for H3K9 methylation, SUV39H1. The combined action of these proteins could potentially allow for directional spreading of the silencing H3K9me3 mark. Along the same lines, H3K27me3 is recognized by a member of PRC2, the WD40 repeat protein embryonic ectoderm development (EED)⁷⁰. PRC2 also contains an H3K27-specific methyltransferase, enhancer of zeste homolog 2 (EZH2, KMT6), potentially enabling the propagation of polycomb silencing. For both HP1- and polycomb-mediated repression, the mechanistic details of the chromatin-state spreading are lacking. It will be a challenge in the future to dissect these processes kinetically using defined designer chromatin templates.

Many effectors contain binding domains that recognize marks that are distinct from the result of their catalytic activity, allowing crosstalk between different marks. Rpd3S, a yeast HDAC complex, contains a chromodomain within its Eaf3 subunit that is selective for trimethylated H3K36. In combination with a PHD finger that binds to nucleosomes, Rpd3S is recruited to chromatin containing methylated H3K36 through multivalent interactions⁵⁵ (Fig. 6b). Further investigations using a cysteine-alkylation approach (Box 1 and Fig. 2b) and reconstituted nucleosomes revealed that the Eaf3 chromodomain is able to discriminate between different methylation states at H3K36 and that dimethylation results in stable nucleosome binding by Rpd3S⁷¹. This in turn recruits the HDAC complex to specific

chromatin regions, where it deacetylates histones to suppress the spurious transcription of silent genes.

A completely different mechanism of crosstalk between histone marks has been observed for Dot1, which has been reported to sense histone ubiquitylation, as inferred from yeast experiments⁷². To explore this crosstalk more carefully, nucleosomes were generated containing H2B that had been site-specifically ubiquitylated at Lys120 (ref. 73). This required the development of a three-piece protein ligation strategy for the preparation of the requisite ubiquitylated H2B (uH2B) (Box 1 and Fig. 2a). The nucleosomes containing uH2B directly stimulated Dot1 activity. Subsequent kinetic investigations revealed that the catalytic rate constant of Dot1 was greatly increased on ubiquitylated nucleosomes as compared to unmodified substrate⁷⁴. Furthermore, this effect was found to be specific for ubiquitin, as ubiquitin-like modifiers such as small ubiquitin-like modifier (SUMO) could not elicit a similar response. In addition, structure-activity studies on ubiquitylated nucleosomes explored the effects of ubiquitin positioning on the nucleosome on Dot1 stimulation⁷⁵. By using a disulfide-directed strategy to attach ubiquitin to single cysteine mutants on H2A and H2B, several attachment sites were screened. Only attachment to sites that were confined to a region around Lys120 resulted in full Dot1 activity. Taken together, the available experimental data point to an allosteric mechanism of Dot1 stimulation that involves a key surface patch on ubiquitin that is positioned on the nucleosome (Fig. 6c). Interestingly, a second methyltransferase, the H3K4-specific multiprotein enzyme hSET1 (KMT2F), is also directly stimulated by H2B ubiquitylation⁷⁶. In methyltransferase assays using the purified hSet1 complex and unmodified reconstituted nucleosomes, only H3K4 monomethylation was detected. However, when using nucleosomes containing uH2B, hSet1 activity was strongly activated and proceeded to form dimethylated and trimethylated H3K4 (ref. 76). This finding is also in agreement with yeast studies showing a correlation between H3K4 methylation and H2B ubiquitylation⁷⁷.

Structure and function of modified chromatin fibers

Beyond serving as a signaling platform for various chromatin regulatory pathways, histone modifications can alter the structural and dynamic properties of chromatin fibers and single nucleosomes. At the fiber level, histone modifications can act by recruiting structural effectors that stabilize a defined chromatin conformation. Alternatively, they can directly influence the long- and short-range properties of the chromatin template, thereby altering downstream biochemical processes.

Recently, designer chromatin was used to investigate the binding mechanism of the heterochromatin-stabilizing protein HP1 (ref. 78). HP1 contains a chromodomain that binds to H3K9me3 and a chromoshadow domain that mediates its dimerization, potentially allowing bivalent binding of H3K9me3 marks in neighboring nucleosomes. Chromatin methylated at H3K9 (prepared by cysteine alkylation; Box 1 and Fig. 2b) was shown to increase the cooperative binding of Swi6 (the *Schizosaccharomyces pombe* homolog of HP1). This effect is dependent on the internucleosomal distance, as increasing the length of the linker DNA connecting the neighboring nucleosomes decreased the ability of Swi6 to discriminate between methylated and nonmethylated nucleosomes. This study provides a

model of HP1-mediated heterochromatin formation in which the binding of HP1 to H3K9me3 marks leads to its multimerization, the cross-bridging of nucleosomes and the stabilization of a compact chromatin state.

The first direct effect of a histone PTM on chromatin fiber structure was shown for a transcription-associated mark, H4K16 acetylation⁷⁹. Using peptide ligation (Box 1 and Fig. 2a), H4 site-specifically acetylated at Lys16 was synthesized and introduced into 12-mer nucleosomal arrays. Sedimentation velocity experiments showed a strong impairment of fiber compaction as a result of the acetylation of this single lysine residue in H4, possibly caused by a disruption of internucleosomal interactions between H4K16 and an acidic patch on H2A in the neighboring nucleosome. Furthermore, the activity of a chromatin-remodeling complex, ATP-utilizing chromatin assembly and remodeling factor (ACF), was inhibited by H4K16 acetylation, independent of the mark's effect on higher-order chromatin structure. These findings show that a single histone mark can function as a chemical switch that controls both chromatin structure and effector protein function.

Additional histone marks have also been shown to modulate higher-order chromatin folding (Fig. 6d). Cysteine alkylation methods (Box 1 and Fig. 2b) were used to investigate the effect of a series of histone-methyl marks on chromatin structure⁸⁰. Interestingly, H4K20me3 was found to increase the propensity of chromatin arrays to compact into fibers. This finding is consistent with the role of H4K20me3 as a silencing mark that is associated with heterochromatin. H4K20me3 is, to date, the only histone mark that has been found to increase chromatin compaction. Ubiquitylation of H2B has long been suspected to influence chromatin compaction by prying open chromatin fibers because of the steric bulk of the PTM⁷⁷. A direct test of this hypothesis was recently reported⁸¹ that relied on a disulfide-directed strategy⁷⁵ (Box 1 and Fig. 2b) to produce homogeneously ubiquitylated chromatin fibers. A fluorescence anisotropy-based assay reporting on chromatin fiber folding revealed a marked decrease in chromatin compaction caused by uH2B incorporation. Interestingly, a different ubiquitin-like protein with low sequence homology to ubiquitin could not elicit this effect, pointing toward a specific role for ubiquitin in disrupting chromatin compaction.

Stability and dynamics of mononucleosomes

In this section, we narrow our focus to nucleosomes, whose biophysical properties and interactions underpin the formation of higher-order chromatin structure. Nucleosomes must be dynamically remodeled to allow processes such as transcription and DNA replication and repair to proceed. Histone PTMs can alter histone-DNA interactions and thereby modulate the thermodynamic and kinetic properties of nucleosomes. To understand the contribution of histone marks to the regulation of chromatin function, detailed studies of the forces governing nucleosome stability, structure and dynamics are required.

In pioneering studies, restriction enzymes were used to investigate the digestion rates of nucleosomal DNA⁸². These experiments uncovered that nucleosomes are dynamic complexes, continually exposing parts of the wound DNA through local unwrapping events. Such breathing behavior also allows for access to transcription factor binding sites within nucleosomes, as was shown in a subsequent biophysical analysis of nucleosome invasion by DNA-binding proteins⁸³. A fluorescence resonance energy transfer (FRET) system reporting

on the nucleosomal DNA conformation was used to show that LexA, a bacterial transcription factor, could bind its target site within the nucleosomal DNA by stabilizing a transiently unwrapped conformation. Kinetic experiments using stopped-flow and fluorescence-correlation spectroscopy assigned a time constant of ~250 ms to these unwrapping events.

Insight into the mechanism of this site exposure was provided in two recent single-particle spectroscopy studies^{84,85}. In the first study, conformational subensembles in freely diffusing nucleosomes were characterized as a function of salt concentration by applying a multiparameter FRET analysis in combination with fluorescently labeled nucleosomes⁸⁴. A geometric model of nucleosome unfolding was applied for the analysis of the data, indicating that DNA unwrapping occurs as a series of 10-bp steps separated by major energy barriers. In the second study, optical tweezers were used to study DNA unwrapping, indicating that the process occurs in 5-bp steps⁸⁵. Three major barriers were identified in this sequential unzipping process: one barrier at the region of the nucleosomal dyad axis where nucleosome-DNA contacts are particularly strong⁸⁶ and two barriers approximately ± 40 bp from the dyad (Fig. 6e). Crossing these lower barriers resulted in reversible DNA unwrapping, allowing for site exposure of nucleosomal DNA, whereas crossing the major barrier at the dyad axis resulted in irreversible disassembly of the nucleosome. Collectively, these studies provide interaction maps of DNA with the histone core and offer insights into how chromatin remodelers might invade nucleosomal DNA to disassemble nucleosomes.

Lysine acetylation removes a positive charge and thereby disrupts ionic DNA contacts in addition to imposing steric hindrance. Several lysine residues in the histone globular domains make crucial contacts with the nucleosomal DNA. H3K56, the most prominent example, contacts the DNA at its entry-exit site (Fig. 6e), and acetylation of this residue is associated with DNA repair, transcription regulation and chromatin assembly¹². To investigate the effect of this mark on nucleosomal stability, acetylated Lys56 was incorporated into recombinant H3 using an amber-suppression protocol (Box 1 and Fig. 2c)⁸⁷. A single-particle FRET analysis revealed that H3K56 acetylation greatly increased the population of partially unwrapped nucleosomes, whereas the overall nucleosomal stability was not altered. These findings are consistent with a role for H3K56 as a gatekeeper residue, anchoring the DNA at its entry into the nucleosome structure. Such effects are not unique to H3K56, as was shown in experiments investigating two further acetylation patterns, H4K77 and H4K79 and H3K122 and H3K125, which contact the nucleosomal DNA at two crucial sites (Fig. 6e)⁸⁸. Using a combination of force and FRET spectroscopy, acetylation of H3K56 or H4K77 and H4K79 was found to lead to an increase in DNA unwrapping. In contrast, acetylation of H3K122 or H3K125 did not alter DNA unwrapping but decreased octamer binding to the DNA.

As is evident from the results discussed above, the combination of modern protein chemistry methods and high-resolution biophysical approaches is revealing chromatin fibers, as well as single nucleosomes, to be very dynamic complexes with finely tuned stabilities. Nucleosomes must be stable enough to organize chromatin over long periods of time while simultaneously allowing biochemical access to the genomic material. Histone PTMs are strategically used by nature to modulate chromatin structure and nucleosomal stability to

control DNA access. Thus, one can imagine that the activity of the writers and erasers of these ‘gatekeeper’ marks are heavily regulated at all stages of life.

Outlook

The goal of this review has been to illustrate the various ways in which chemical biology is affecting the study of chromatin. Although this is indeed an expansive canvas on which to work, much of this creative space remains untouched, and there are a great many opportunities for the chemical biologist going forward in this field. In particular, there is a rapidly growing mismatch between the volume of information being generated by top-down epigenomic and proteomic approaches and our ability to systematically fill in the molecular details of the associated chromatin biochemistry. Such an understanding would seem to be a prerequisite for the rational design of next-generation epigenetic drugs.

How, then, can the chemical biologist ramp up chromatin biochemistry so that he or she can begin to make a dent in the ever-expanding body of information being accumulated from these large-scale ‘-omics’ initiatives? This is an enormously challenging problem, and it is one that will require new technologies that elevate chromatin biochemistry from an essentially artisanal pursuit, as it generally is now, to something that might be better described as high-throughput quantitative biochemistry. One approach to this will involve the integration of designer chromatin and so-called ‘lab-on-a-chip’ microfabrication methods. For example, one could imagine using microfluidic devices to reconstitute libraries of designer chromatin on the nanoscale and then using them to study, in a high-throughput fashion, the activity of chromatin effectors using microreactors coupled with sophisticated imaging modalities. There are many variations on this theme, but they all share the core idea of doing things in parallel without sacrificing quantitative information, and they all seem ripe for the creative sensibilities that the chemical biologist brings to the table.

In summary, the chromatin field sits at a crossroads between molecular biology, bioorganic chemistry and biophysics. This provides innumerate opportunities for chemical biologists armed with their palette of small-molecule probes, analytical tools and custom-made proteins. Thus, there is plenty of room for the aspiring van Gogh or Picasso to leave his or her ‘mark’ on this expansive canvas.

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box 1**Synthesis of designer chromatin**

Here we illustrate several state of the art methods to generate modified histone proteins and incorporate them into nucleosomes and chromatin fibers.

Synthesis of designer histones

Native chemical ligation (NCL)⁸⁹ and expressed protein ligation (EPL)⁹⁰ allow for the traceless linking of two synthetic peptides or a peptide and an expressed protein, respectively. Many types of PTMs have been incorporated into histones using EPL or NCL in two to three ligation steps (Fig. 2a). For complex modifications, analogs have been developed, such as phosphoryltriazolylalanine for phosphorylated histidine⁹¹, that use an aminooxy linkage to generate neo-ADP-ribosylated peptides⁹² and several different methods to produce ubiquitylated lysines^{74,75,93,94}. However, the synthetic complexity for the internal marks (>50 residues from either terminus) can be substantial because of increasing difficulty in peptide synthesis.

Alkylation of cysteine residues using electrophilic monoethylamines, diethylamines and triethylamines readily produces analogs of methyllysine residues at any site in proteins⁹⁵ (Fig. 2b). More recently, this approach has been extended to acetyllysine by using a radical-initiated thiolene reaction⁹⁶. The ease of these reactions makes this approach attractive, however, regiocontrol is difficult if multiple modifications are required.

A third possibility involves using a nonsense-suppression mutagenesis strategy (Fig. 2c). An evolved orthogonal transfer RNA (tRNA) synthetase and tRNA_{CUA} pair (where CUA is the anticodon of the tRNA) is used to selectively incorporate non-natural amino acids (for example, acetyllysine) during expression in bacteria⁹⁷.

Designer chromatin

Recombinant histones, potentially modified by the above methods, are readily refolded into histone octamers (Fig. 2d). Purified octamers in combination with DNA sequences that contain nucleosome positioning sequences can subsequently be used to reconstitute nucleosomes or defined nucleosomal arrays using either a histone chaperone and remodeler system⁹⁸ or salt dialysis⁹⁹. The stability of the nucleosomes allows for the construction of chromatin fibers modified at specific sites. Using DNA ligation methods¹⁰⁰, reconstituted modified nucleosomes can be connected to form true, heterogeneous, designer chromatin in which the modification state of each nucleosome is precisely controlled.

Figure 1. Organization and analysis of chromatin

(a) Schematic overview of the structural organization of chromatin highlighting the type of chemical biology tools that have been successfully used to explore each level of complexity. For the structure of the nucleosome, see Protein Data Bank (PDB) code 1kx3. MN, mononucleosome. (b) Summary of the major chromatin states with their associated histone marks indicated. me, methylation; ub, ubiquitylation; ac, acetylation.

Figure 2. Chemical synthesis of designer chromatin

(a–c) PTMs and their analogs introduced into histone proteins by EPL or NCL methods (a), modification of single cysteine residues (b) and nonsense suppression methods (c, for details see text). Kme1, monomethylated lysine; Kme2, dimethylated lysine; Kme3, trimethylated lysine; KAc, acetylated lysine; Kub, lysine ubiquitylation; o-ADPR, o-ADP ribosylation; Sph, phosphorylated serine; pHis, phosphorylated histidine; pTza, phosphoryltriethylalanine. (d) Methods to reconstitute designer chromatin of different lengths and compositions. NPS, nucleosome positioning sequence; Nap-1, nucleosome assembly protein

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Figure 3. Small-molecule inhibitors of writers and erasers of histone marks

(a) Structures of HDAC inhibitors with their known targets indicated. (b) Structures of HMT inhibitors and their targets.

Figure 4. Readout of histone marks

(a) Structures of representative acetyllysine and methyllysine reader domains. Each domain is shown in complex with a histone-derived modified peptide. Shown are the GCN5 bromodomain (PDB code 1e6i), the PHD finger from BPTF (PDB code 2f6j), the HP1 chromodomain (PDB code 1kne), the MBT domain of I3MBT11 (PDB code 2pqw) and the HTD of JMJD2A (PDB code 2gfa). (b) Schematic representation of the various ways reader domains can be used to readout chromatin states. As discussed in the text, many domains can bind to multiple sites on histones (each carrying the same modification, left). Specificity can be imposed through the presence of nearby heterotypic marks that impede binding (middle) or through multivalent interactions involving homotypic or heterotypic marks on either the same histone or different histones in a nucleosome (right).

Figure 5. Small-molecule inhibitors of histone-binding modules

Chemical structures of the small molecules that target the peptide-binding pockets of various histone reader domains. See the text for details. BET-bromo, BET bromodomain; PCAF-bromo, PCAF bromodomain; CBP-bromo, CBP bromodomain; 13MBTL1-MBT, 13MBTL1 MBT domain.

Figure 6. Control of chromatin function by histone marks through *cis*- and *trans*-acting mechanisms

The combination of designer chromatin and various biochemical and biophysical methods allows for a deeper understanding of the ways in which histone marks can locally regulate chromatin structure and function. **(a–c)** Examples of histone mark crosstalk. Such crosstalk can occur when a chromatin effector is recruited to chromatin by a specific mark and then proceeds to install additional copies of the same mark, as in the cooperative acetylation of chromatin by the SAGA complex. Alternatively, an effector is recruited by a mark that is distinct from the one it then acts on, as observed in the Rpd3S HDAC complex.

Allosteric crosstalk between marks was determined for the H3K79 methyltransferase Dot1, which is activated by H2B ubiquitylation. **(d)** Certain histone marks intrinsically alter the structural properties of chromatin fibers: H4K20me3 leads to the stabilization of a compact fiber structure, whereas H4K16ac and uH2B impair fiber compaction. **(e)** Histone marks can alter the stability of a single nucleosome: specific acetylation sites in the globular domain of H3 and H4 modulate nucleosomal stability and lead to increased DNA access to downstream effectors.