Review

# Interpreting the language of histone and DNA modifications ☆

Scott B. Rothbart, Brian D. Strahl *

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA
Department of Biochemistry & Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

A B S T R A C T

A major mechanism regulating the accessibility and function of eukaryotic genomes are the covalent modifications to DNA and histone proteins that dependably package our genetic information inside the nucleus of every cell. Formally postulated over a decade ago, it is becoming increasingly clear that post-translational modifications (PTMs) on histones act singly and in combination to form a language or 'code' that is read by specialized proteins to facilitate downstream functions in chromatin. Underappreciated at the time was the level of complexity harbored both within histone PTMs and their combinations, as well as within the proteins that read and interpret the language. In addition to histone PTMs, newly-identified DNA modifications that can recruit specific effector proteins have raised further awareness that histone PTMs operate within a broader language of epigenetic modifications to orchestrate the dynamic functions associated with chromatin. Here, we highlight key recent advances in our understanding of the epigenetic language encompassing histone and DNA modifications and foreshadow challenges that lie ahead as we continue our quest to decipher the fundamental mechanisms of chromatin regulation. This article is part of a Special Issue entitled: Molecular mechanisms of histone modification function.

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## 1. Introduction

### 1.1. Chromatin balances DNA packaging and accessibility

Eukaryotic DNA is tightly packaged within the nucleus of each cell and must be faithfully regulated, copied, and transmitted during cell division. DNA packaging amounts to an amazing feat, often requiring several meters of DNA to be compacted into the confines of a 2–10 micron nucleus. This high level of compaction presents a potential problem, as the underlying DNA must remain accessible to the vast protein machineries that utilize it for critical biological functions. Thus, a fundamental question being addressed by many labs has been how these diverse genomic functions, such as gene transcription, DNA repair, replication, and recombination, occur at the appropriate place and time to promote cellular growth, differentiation, and proper organismal development.

Key contributors to DNA packaging are the highly basic histone proteins, which wrap ~147 base pairs of DNA around an octamer of histones (2 copies each of H3, H4, H2A and H2B) to form the nucleosome core particle [1,2]. This repeating nucleosomal subunit is the fundamental building block of chromatin. Chromatin is organized into distinct domains, such as euchromatin and heterochromatin, which are defined by the level of compaction and associated genomic functions. For example, euchromatin has relatively loose compaction and is typically transcriptionally permissive, whereas heterochromatin (facultative or constitutive) is more condensed and typically transcriptionally repressive [3–5]. The degree to which chromatin is organized and packaged is highly influenced by numerous factors, including the actions of linker histone H1 that regulate the formation of higher-order chromatin states [6], histone variants that can be substituted for canonical histones in the nucleosome core particle [7], chromatin remodelers that use the power of ATP hydrolysis to slide and evict histones [8], histone chaperones that facilitate deposition and eviction of histones [9,10], and small chemical modifications to histones and DNA [11–15], whose interwoven functions are the primary focus of this review.

### 1.2. Histone PTMs and the 'histone code': a rich and vibrant new language has emerged

A major mechanism by which chromatin structure and function is regulated is through the actions of histone post-translational modifications (PTMs). An astonishing number of PTMs, including lysine acetylation, lysine and arginine methylation, arginine citrullination, lysine ubiquitination, lysine sumoylation, ADP-ribosylation, proline isomerization, and serine/threonine/tyrosine phosphorylation occur on histones [11,15] (Table 1). While the majority are found in the flexible N- and C-terminal 'tail' domains that protrude away from the nucleosome

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core particle, a significant number also occur in the histone fold or globular domains that regulate histone–histone and histone–DNA interactions [16,17].

A long-standing question in the field has been how histone PTMs function in chromatin regulation. While it has been half a century since Vincent Allfrey first described the presence of acetylation and

**Table 1**
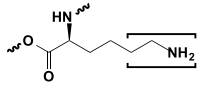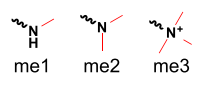Reading and interpreting histone post-translational modifications.
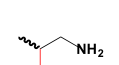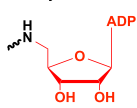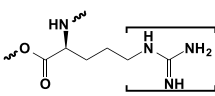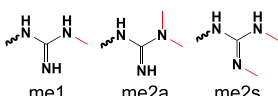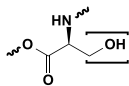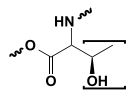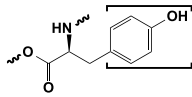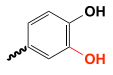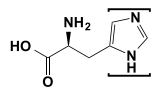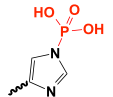
| Modification type | Structure | Associated functions | Reader domains |
|---|---|---|---|
| Lysine |  | Gene regulation | PHD, ADD, WD40 |
| Methylation |  me1 me2 me3 | Gene regulation DNA repair, DNA replication, heterochromatin | Chromo, PHD, Tudor, MBT, ZF-CW, PWWP, ADD Ankyrin Repeats, WD40, BAH |
| Acetylation |  | Gene regulation, chromatin structure | Bromo, PHD |
| Formylation |  | ? | ? |
| Proprionylation |  | ? | ? |
| Butyrylation |  | ? | ? |
| Crotonylation |  | Gene regulation, chromatin structure | ? |
| Malonylation |  | ? | ? |
| Succinylation |  | ? | ? |
| 5-hydroxylation |  | ? | ? |
| Ubiquitination | 8.5 kDa polypeptide, C-term Gly isopeptide bonded with Lys ε-amine | Gene regulation DNA repair, heterochromatin | ? |
| Sumoylation | 11 kDa polypeptide, 2° structure similar to ub, C-term Gly isopeptide bonded with Lys ε-amine | Gene regulation | ? |
| ADP-ribosylation |  | Gene regulation DNA repair, heterochromatin | PAR |
| Arginine |  | Gene regulation | PHD |
| Methylation |  me1 me2a me2s | Gene regulation | Tudor, WD40, ADD |
| Citrullination |  | Gene regulation, chromatin structure | ? |

**Table 1** (*continued*)

| Modification type | Structure | Associated functions | Reader domains |
|---|---|---|---|
| Serine | | | |
| Phosphorylation | | Gene regulation, DNA repair, mitosis | BRCT, 14-3-3 |
| Acetylation | | ? | ? |
| Glycosylation | β-*N*-acetylglucosamine (*O*-GlcNAc) | Gene regulation, | ? |
| Threonine | | | |
| Phosphorylation | | mitosis | BIR |
| Acetylation | | ? | ? |
| Glycosylation | β-*N*-acetylglucosamine (*O*-GlcNAc) | Gene regulation, | ? |
| Tyrosine | | | |
| Phosphorylation | | Gene regulation, | ? |
| Acetylation | | ? | ? |
| Hydroxylation | | Gene regulation, | ? |
| Histidine | | | |
| Phosphorylation | | Gene regulation, | ? |

methylation on histones [18], and Lubomir Hnilica documented histone phosphorylation [19], the functional significance of these modifications remained elusive for many years. Fundamental breakthroughs in our understanding of histone PTM function (many of which occurred in recent years) have been made through the identification of the protein machineries that incorporate (write), remove (erase), and bind (read) histone PTMs. Two landmark discoveries in this regard were the identifications in 1996 of p55/Gcn5 and HDAC1/Rpd3 as transcription-associated histone acetyltransferases [20] and deacetylases [21], respectively — thereby linking dynamic histone modification activity directly to the transcription process. These findings changed the landscape of how the transcription and chromatin fields viewed histone

PTMs and have resulted in a fast-paced and exciting field that shows no signs of slowing down.

In 2000, the concept of a 'histone code' emerged as a hypothesis to stimulate new thinking about how histone PTMs might function [22, 23]. This postulate grew out of the observation that histone H3 serine 10 (H3S10) phosphorylation could be associated with seemingly opposite functions in chromatin (*i.e.*, chromatin decompaction in transcription and chromatin condensation in mitosis) [24–28]. From analysis of this and other PTMs where the associated functions were known, it was possible to infer that PTMs might work singly as well as in combination (on one or more histone tails) to mediate the distinct functions associated with them. It was envisioned that, in addition to histone PTMs having a direct physical effect on chromatin structure (as is the case for lysine acetylation negating the positive charge of this residue) [29], they might also function through the selective recruitment of effector proteins or readers that 'dock' onto histone PTMs to direct specific downstream events in chromatin. One of the major landmarks in deciphering this aspect of the hypothesis was the discovery of the bromodomain module as an acetyl-lysine reader motif [30]. This result paved the way for subsequent characterization of this important family of chromatin effectors [31,32], and further suggested the existence of other, yet-to-be identified protein domains, that read histone PTMs.

A large body of data now supports the notion that histone PTMs function, at least in part, through the recruitment of effector proteins harboring specialized reader domains [33,34]. While the 'histone code' hypothesis provided a retrospectively simplistic explanation of how histone PTMs function, recent advances have revealed that the context in which histone PTMs operate is much more complex than originally envisioned. In addition to combinatorial PTMs that function together both synergistically and antagonistically, there is now an appreciation for PTM asymmetry within individual nucleosomes [35], novel types of PTMs with unique functions [36], nucleosomes bearing histone variants [7], and nuclear compartmentalization events [5] that are all contributing to the final output of chromatin organization and function. Furthermore, fueled in part by the recent discovery of an active DNA demethylation pathway and proteins that read these pathway intermediates [14,37], a new appreciation for the function of histone PTMs in coordination with DNA modifications is emerging. This review will focus on several key findings from the last few years that have helped to expand our current understanding of how histone PTMs and DNA modifications function in the context of a more integrated epigenetic language [22,38]. Posing several key questions to the field, we also foreshadow where studies into chromatin organization and function are headed and highlight several new technologies that are taking us there.

## 2. Increasing complexity of the histone PTM landscape

### 2.1. Identifying and mapping histone PTMs beyond the histone tails

Technological advances in mass spectrometric (MS)-based analyses of histones have greatly facilitated our understanding of the types of PTMs that occur on histones, as well as their abundance and co-occurrence [39–43]. Perhaps one of the most surprising findings from early MS studies using the high resolving power of Fourier transform ion cyclotron resonance MS was the significant number of previously unknown acetylation, methylation and phosphorylation events that were detected on the N- and C-terminal tail domains as well as on the globular regions of histones [44]. From this analysis and additional studies [16,45–47], a vast and mostly unexplored PTM landscape emerged that has greatly expanded the potential complexity of the histone modification language (Table 1). Underappreciated at the time of our original postulate was the potential for a significant number of PTMs to occur along or near the histone–DNA interface where the histone octamer contacts wrapped DNA — thus providing an additional means by which nucleosome stability and/or DNA interactions on the surface can be regulated [16]. Histone–DNA interface PTMs (in contrast

to those located on the histone tail domains) largely act in a physical manner by tweaking or fine-tuning histone–DNA interactions that in turn affect nucleosome stability and/or mobility. Evidence for this effect came from elegant *in vitro* studies employing semi-synthetic or genetically modified nucleosomes to show that histone fold acetylation at H3K56, H3K155 and H3K122, in addition to phosphorylation of H3T118, resulted in weakened histone–DNA interactions, increased nucleosome mobility, and DNA unwrapping [48–53]. Significantly, H3K122 was recently identified as a substrate for the p300/CBP histone acetyltransferase [54]. H3K122ac was found to be enriched at gene promoters and is required for proper transcriptional activation, but how p300/CBP targets H3K122, which is in a largely inaccessible and buried region in the nucleosome core particle, is not yet understood.

While PTMs located at the histone–DNA interface regulate the intrinsic properties of the nucleosome core particle, it is noteworthy that these modifications can also regulate effector protein association similar to those located in the tail domains. For example, JAK2-mediated phosphorylation of H3Y41, a site located at the DNA entry/exit point [55], was found to be refractory to HP1α chromatin association by perturbing an interaction between the HP1α chromoshadow domain and H3 in this region. While H3Y41 was shown to relieve HP1-mediated gene repression, it remains to be determined whether H3Y41 phosphorylation negates HP1α interaction in the context of H3K9me3 [56,57]. Interestingly, neighboring H3R42 was recently identified as a substrate for CARM1 and PRMT6-mediated asymmetric dimethylation (H3R42me2a) [58]. H3R42me2a was linked to active transcription *in vitro*, suggesting this PTM alters chromatin structure by removing a potential hydrogen bond donor contributing to histone–DNA interaction. It is intriguing to also speculate that H3R42me2a may elicit positive transcriptional effects in the cell by negating HP1 binding.

### 2.2. An expanding dictionary of histone PTMs

Along with the identification of new PTM sites on histones, recent MS analyses have also focused on the identification of novel types of histone PTMs. From these studies, a surprising number of newly-identified modifications have been uncovered (Table 1), including lysine crotonylation, lysine butyrylation, lysine propionylation, lysine succinylation, lysine malonylation (all of which are derived from distinct co-enzyme A molecules), lysine 5-hydroxylation, lysine N-formylation, tyrosine hydroxylation, serine/threonine/tyrosine acetylation, and serine/threonine *O*-GlcNAcylation (for an in depth review, see [36]). It is important to note that a number of these above-mentioned PTMs are low in abundance as compared to more well-studied modifications, suggesting that their roles in physical and functional cross-talk may be discreet. While the functions of these newly described PTMs are largely awaiting discovery, exciting recent studies are revealing that they can play key roles in genome function. Several examples are highlighted below.

Lysine crotonylation is a highly conserved PTM that is causally linked to gene activation and nucleosome disruption [59]. Crotonylation is a four-carbon moiety, and (like lysine acetylation) neutralizes the positive charge of this residue. Using pan-lysine crotonylation-specific antibodies that detect each core histone in addition to H1, this novel modification was shown to be enriched at gene promoters, predicted enhancers, and transcriptionally active X/Y sex-linked genes that escape repression in male post-meiotic spermatids [59–61]. While these studies suggest a role for lysine crotonylation in active transcription, it remains unclear which specific crotonylated lysine residues are linked to the observations above. Future work will therefore be needed to identify all of the residues in histones that harbor this mark, and how each mark may contribute to gene regulation or other functions associated with DNA. It is noteworthy that histone acetyltransferases capable of utilizing butyryl-CoA and propionyl-CoA are unable to utilize the crotonyl-CoA cofactor due to its flattened, extended, and more rigid

carbon–carbon π-bond [59]. Thus, it will be exciting to identify the class of histone-modifying enzymes that write and erase this PTM.

Intriguingly, two sites of lysine crotonylation have recently been identified to occur at H3K122 (a site also found to be acetylated, see above) and H4K77 [62]. These lysines lie near the dyad axis on opposite sides of the nucleosome, and thus, are in a position to directly regulate histone–DNA interactions and nucleosome stability. This study found that defective incorporation of the H2B variant TH2B, normally required for the disruption and replacement of histones with protamines during spermatogenesis, resulted in increased lysine crotonylation (in addition to increased arginine methylation) in the histone fold domains of H4 and H2B. This appears to be a compensatory mechanism for not having TH2B present.

Another example where significant insight has been revealed for a novel histone PTM is with the serine/threonine sugar modification *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc). Studies have found that *O*-GlcNAc transferase (OGT) and the hydrolase *O*-GlcNAcase (OGA) are the enzymes that write and erase this PTM, both on histones and non-histone proteins [63–66]. The nucleotide sugar donor used by OGT (UDP-GlcNAc) is highly regulated by nutrient levels and is rate limiting in the cell [67,68]. Studies therefore suggest that *O*-GlcNAc is a sensor of nutrient status, controlling gene expression programs in response to changing nutrient conditions and environmental stress. In *Caenorhabditis elegans*, OGT has been mapped to gene promoters that are connected to metabolism, growth, aging, and the stress response, and is important for the proper transcriptional regulation of these genes [69]. In *Drosophila melanogaster*, OGT has been identified as the protein product of the *super sex combs* (*sxc*) gene, a member of the polycomb group (PcG) proteins required for the PcG-mediated repression of developmentally regulated genes [70,71]. Given *O*-GlcNAcylation is intimately associated with nutrient sensing and metabolism, this finding suggests that there is a nutrient responsive and/or metabolic component to PcG repression and organismal development. Important to note, however, is that *O*-GlcNAcylation is well known to occur on non-histone proteins involved in transcription (*e.g.*, MLL5, polymerase II (RNAPII), and the PcG factor Polyhomeotic) [70,72–74]. Therefore, it will be important to resolve whether *O*-GlcNAcylation that occurs on histones is indeed contributing to the gene regulation programs identified above.

In mammals, *O*-GlcNAcylation is a modification that occurs on each of the core histones and is regulated in a cell cycle-dependent manner [75]. Peak levels of *O*-GlcNAc occur during G1 and drop in S-phase, followed by a rise occurring through G2 and M phase. *O*-GlcNAc was found to occur at H3S10, posing an interesting question as to whether *O*-GlcNAcylation is functionally antagonistic with H3S10 phosphorylation. Since *O*-GlcNAc levels are highest in G1, it is plausible that H3S10 *O*-GlcNAcylation acts as a molecular 'switch' to negatively regulate gene expression normally controlled by H3S10 phosphorylation. Intriguingly, *O*-GlcNAc has also been found to crosstalk with other histone PTMs, notably H2BK120 monoubiquitination [76]. Specifically, *O*-GlcNAcylation of H2BS112 in *Drosophila* was found to promote H2BK120Ub1 through the recruitment of the ubiquitination machinery [76]. These studies indicate a positive role for *O*-GlcNAcylation in transcription. In agreement with this notion, it was shown that OGT recruitment by the DNA methylcytosine dioxygenase TET2 (see Section 3.3) facilitates H2BS112 *O*-GlcNAcylation during gene reactivation [77]. As OGT was found to be required with TET2 for gene reactivation, the study suggests that DNA methylcytosine oxidation is coordinated with other histone marks to facilitate reactivation of TET2-regulated genes. This serves as an important example of how our understanding of histone PTM function has evolved over the years to encompass an intimate DNA component (a topic discussed in detail below).

The studies described above have provided important insights into the function of newly discovered PTMs that occur on histones — yet fundamental questions remain about the role of these and other aforementioned PTMs in chromatin regulation. For example, what are the writers,

erasers, and potential readers of these modifications? In the case of lysine butyrylation and propionylation, which use distinct co-enzyme A cofactors (butyryl-CoA and propionyl-CoA), studies have shown that they can be catalyzed and removed by histone acetyltransferases and deacetylases [78,79]. Given that these different Co-A forms are created by distinct metabolic pathways, and thus can be regulated by metabolic flux, it may be that these distinct lysine modification forms play specialized roles in fine-tuning gene expression programs in response to nutrient imbalance. Significantly, this principle of metabolic flux regulating epigenetic signatures is a fundamental aspect of chromatin regulation, recently reviewed in detail elsewhere [80,81].

It is also intriguing to speculate that, like lysine acetylation, distinct Co-A forms are read by bromodomain-containing proteins. Indeed, structures of the first bromodomain of Brd4 solved in complex with proprionylated H3K23 and butyrylated H3K14 demonstrate that these moieties are coordinated similarly to acetyllysine [82]. For these and other newly identified PTMs, it will be important to determine where they occur across the genome, how they show crosstalk with other PTMs, and what functions are mediated downstream of these modifications. Given what has been discovered so far with lysine crotonylation and *O*-GlcNAcylation, it is likely that these other novel modifications will also have important and exciting roles in chromatin function.

### 2.3. PTM combinations and asymmetry add increasing diversity to histone function

In addition to expanded mapping of individual histone PTMs, the co-existence of histone modifications has gained new appreciation based on recent studies employing high resolution tandem MS and improved MS approaches to detect the short and long-range histone PTM combinations that occur. These studies, examining the N-termini of H3$_{1-50}$ and H4$_{1-23}$, have identified a staggering number of diversely modified H3.2 and H4 N-terminal forms (>200 each for H3.2 and H4) in mammalian histones [40,83,84]. The H3 N-terminus was found to be the most highly modified histone N-terminus compared to the other core histones, and can contain up to 7 PTM combinations in a single N-terminal fragment. These studies also confirm several previously observed findings, including the co-occurrence of H3K4 methylation with neighboring acetylation [85, 86]. Given that the C-terminal regions of histones have not been examined to this same extent, along with the inclusion of phosphorylation events that were not readily observed from the asynchronous cells used in the above studies (but would exist during mitosis), there appears be an astonishing number of potential PTM combinations that add diversity to the histone language. Key to our understanding of how histone PTMs function is to elucidate how the readers, writers, and erasers behave in this combinatorial context. Along these lines, a number of high-throughput technologies, including peptide microarray-based screening approaches, have recently been employed to begin characterizing these complex interactions and substrate specificities [87–92].

While much progress has been made in identifying the co-occurrence and mutual exclusivity of histone PTM combinations, it has been technically challenging to determine whether polypeptides within a histone homodimer are modified in the same way. Further challenges have been faced in determining whether identified combinations occur along the same histone polypeptide (symmetrically) or on adjacent polypeptides within a histone homodimer (asymmetrically). Major advances in our understanding of histone PTM patterning within a single nucleosome came from a recent study by Reinberg, Garcia, and colleagues [35]. Histone PTM-specific antibodies were used to enrich for mononucleosomes bearing a PTM of interest, and the abundance of these marks was quantified following tryptic digestion using liquid chromatography-coupled MS (LC/MS). Assuming that the histone PTM antibody was specific and did not enrich for mononucleosomes lacking the mark of interest (a key assumption for accurate interpretation of these data) [93], the relative abundance of unmodified *vs.* modified histone peptide in the MS pool provided insight into the degree of

symmetry of a specific PTM. Applying this approach to H3K27me2 and H3K27me3 (marks of facultative heterochromatin written by the EZH2 subunit of the PRC2 complex [94]) as well as H4K20me1 (written by PR-Set7 and involved in the DNA damage response and mitotic chromatin condensation [95]), populations of symmetrically and asymmetrically modified nucleosomes were identified. These experiments provided the first evidence that histone H3 and H4 homodimers are not necessarily modified identically within a single nucleosome.

A potentially important role for asymmetry has now been realized in the context of development. Genome-wide mapping studies have documented the co-occurrence of H3K4me3 (associated with active transcription) and H3K27me3 (a dominant mark of transcriptional repression) at developmental gene promoters in embryonic stem cells (defined as 'bivalency') [96] — the notion being that these two marks might reside on the same H3 tail to transcriptionally 'poise' these loci for rapid transcriptional activation in response to cellular cues leading to demethylation of H3K27me3. Surprisingly, high-resolution MS studies found that these marks do not co-exist on the same H3 tail [83]. This seemingly opposing result was clarified by the finding that H3K4me3 and H3K27me3 exist within a single nucleosome, but occur on adjacent histones within a homodimer [35]. Consistent with this observation, it has been demonstrated that PRC2 cannot methylate H3K27 when H3K4me3 is present on the same tail [35,97].

The identification of asymmetrically modified nucleosomes greatly increases our awareness for how histone PTMs operate. It will be important to extend these studies to other histone modifications to determine the degree of symmetry and asymmetry across the spectrum of histone PTM combinations. Furthermore, the enzymology and biochemistry directing symmetric and asymmetric PTM combinations will surely be exciting new avenues of discovery. Given our recent appreciation for the ability of histone effector proteins to sense PTMs on adjacent histone tails (see below and Fig. 1), it is intriguing to also speculate that the functional outcomes of symmetric and asymmetric PTM combinations may be distinct.

### 2.4. Multivalency in histone PTM recognition

Much of our understanding of histone PTM function has come from the identification that unique folds or domains within chromatin-associated proteins can 'read' histone PTMs and their combinations to drive distinct functions in chromatin, a central tenet in the original 'histone code' postulate. A structurally diverse family of histone reader domains has been discovered over the last decade, including bromo-domains that bind acetyllysine, chromodomains that bind methyllysine, plant homeodomain (PHD) fingers that bind acetyllysine, methyllysine, and even unmodified lysine, Tudor domains that bind methyllysine and methylarginine, and BRCT domains that bind phosphoserine (Table 1). Recent comprehensive reviews detail the structural properties of these and other identified histone reader domains and highlight the diverse biochemical implications of their interactions [33,34].

It has become increasingly apparent that many chromatin-associated factors harbor multiple known or predicted histone binding domains, both within a single protein, and within distinct subunits of macromolecular complexes. This offers a diverse and exciting potential for multivalent histone engagement [98] (Fig. 1) that adds a previously underappreciated layer of specificity to histone PTM recognition. A number of closely spaced histone binding domains within single proteins have been shown to coordinately engage histone PTM signatures on a single histone tail, termed *cis* interactions [99–106]. A recent example is the tandem Tudor domain (TTD) and PHD finger of UHRF1, which together, coordinate an interaction with H3K9me3 and the N-terminus of an H3 tail, respectively [100,107,108]. Importantly, disrupting the reader function of either domain individually, or perturbing the ability for this dual domain to engage the H3 tail in *cis*, was shown to inhibit the UHRF1 chromatin interaction and prevent UHRF1 from facilitating the epigenetic inheritance of DNA methylation [104] (Fig. 3).

Interestingly, crystal structures of the UHRF1 TTD-PHD bound to $H3_{1-11}$ K9me3 revealed that H3 residues lysine 4 through alanine 7 adopt a single turn of an α-helix [108]. Since the H3 tail peptide in complex with the isolated PHD finger is in an extended conformation, it is assumed that multivalent TTD-PHD engagement induces conformational change in the H3 tail. It is notable that tail domains of histone proteins are rich in positively charged lysines and arginines and lack a detectable secondary structure when observed as nucleosomes, free histones, and/or peptides. These findings have led to the perception that tail domains are intrinsically disordered. However, classic studies from Grunstein and colleagues predicted that regions within the H3 and H4 tails have the propensity to adopt amphipathic α-helices, β-sheets, and β-turns [109]. More recent studies using advanced molecular modeling algorithms have reached similar conclusions, suggesting that the H4 tail
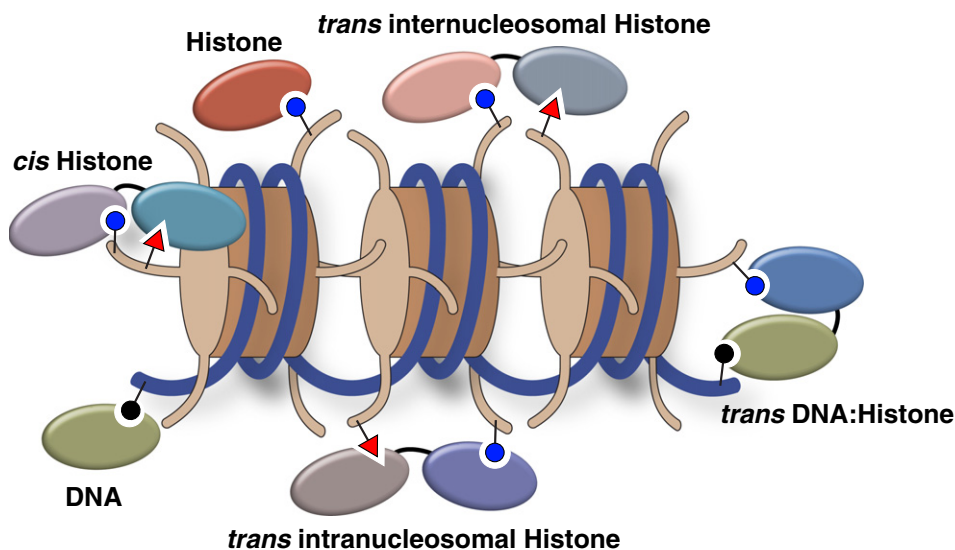


**Fig. 1.** Writing, erasing, and reading the histone and DNA modification landscape. Isolated and linked protein domains coordinate the addition (writing), removal (erasing), and association (reading) of DNA modifications (black circles) and histone PTMs (blue circles and red triangles), creating a dynamic and variable chromatin environment. *Cis* refers to multivalent events occurring on the same histone. *Trans* refers to multivalent events occurring on adjacent histones or spanning histones and DNA, either within the same nucleosome or on neighboring nucleosomes. *Trans* interactions with distant nucleosomes (which may be in 3-dimensional proximity) are not depicted. Multivalent interactions facilitated by membership in macromolecular complexes are also not depicted.

can form β-hairpins, and the H3 and H2B tails may adopt α-helices [110, 111]. Interestingly, it appears that PTMs may influence the departure from extended tail conformations, either directly by stabilizing secondary structures [111] or indirectly (as described above with UHRF1) through the recruitment of effector proteins that appear to induce conformational change in histone tails [57,100,101]. Indeed, $H3_{1-13}$ in complex with the double PHD finger of the MOZ lysine acetyltransferase adopts an extensive α-helix, spanning H3 residues lysine 4 through threonine 11, and, unlike the helix adopted by UHRF1, several residues spanning the helix make hydrogen bonds with MOZ [101]. This helical conformation, and effector protein interaction, is maintained when lysines 9 or 14 are acetylated. Importantly, the acetyl group on lysine 14 forms a hydrogen bond with the main chain amine of isoleucine 260 on MOZ, and the proximity of this interaction appears to be facilitated by the helical turn.

The potential for histone PTMs to either induce or stabilize secondary structures suggests that histone PTMs distant in sequence may in fact be in proximity for combinatorial influence. This possibility adds an exciting new level of regulation that may underlie how histone PTMs function. Future structural work will reveal the dynamics of induced secondary structures upon effector protein binding, the propensity for other effector protein interactions to induce conformational change in histone tails, and the direct effects (or lack-there-of) on PTM-induced conformational change. It is important to note that these recent instances of histone tails with stabilized secondary conformations were observed with peptides. The propensity for histone tail structure in the physiological context of chromatin may be more challenging, both energetically and physically. Furthermore, it will be interesting to question whether effector proteins are able to recognize histone tails that have already adopted a secondary structure, or whether the ground state of histone tails is indeed unstructured.

Multivalent interactions spanning adjacent histone tails within a single nucleosome or on adjacent nucleosomes (Fig. 1), termed *trans* interactions, have been more difficult to study, largely due to technological challenges in generating optimal templates for biophysical assays. However, a number of recent studies provide compelling evidence for *trans* interactions in chromatin [112–114]. The BPTF subunit of the NURF chromatin-remodeling complex harbors a PHD finger adjacent to a bromodomain, a common reader domain combination found in chromatin-associated proteins [98]. Biophysical studies using modified histone peptides connected by rigid DNA linkers, as well as semi-synthetic mono- and di-nucleosomes, demonstrated that PHD engagement of H3K4me3 and bromodomain engagement of H4K16ac within a single nucleosome provided an optimal scaffold for BPTF recruitment [114]. This important finding was supported by structural analysis, which suggested that in nature, a rigid, helical linker connecting the domains provides the optimal spacing to facilitate this *trans* interaction. While disruption of either reader domain in BPTF was demonstrated by chromatin immunoprecipitation (ChIP) to affect HOX gene targeting of the NURF complex, in what way this multivalent *trans* interaction impacts NURF chromatin remodeling function at these loci and others remains to be determined.

These and other recent studies have conveyed awareness that histone binding domains within proteins and protein complexes are often functioning in a coordinate manner. Notably, a number of paired histone binding domains characterized to engage the chromatin template in a multivalent manner harbor single domain reader functions that are broadly shared among chromatin-associated factors. For instance, the PHD finger of BPTF shares the ability to read H3K4me3 with a growing list of chromatin-associated factors [33,115–117], and the TTD of UHRF1 is one of a host of identified H3K9me3 readers [57, 102,118–121]. Importantly, *in vitro* affinity measurements for these interactions are often similar, suggesting the possibility that competition for histone PTMs exists at defined regions of chromatin. It is intriguing to speculate that multivalency may in fact provide a mechanism to circumvent this potential competition problem, as these dual interactions

are often more energetically favorable than single domain interactions [122]. Decoding the energetics and interaction networks of paired or multidomain complexes will certainly be of fundamental importance moving forward. Importantly, these studies indicate that it may be insufficient to characterize isolated histone binding domains without taking into account the potential function or influence of adjacent regions. In addition, the discovery of asymmetry within histone tails in a single nucleosome [35] suggests that *trans*-tail interactions may be more prevalent than initially thought. Technological advances, such as more straightforward routes to facilitate the construction of defined semi-synthetic nucleosomes [123], will also be needed to aid future discoveries of *trans*-tail histone interactions.

## 3. Emergence of a DNA methylation language

Classic studies have unquestionably shown that DNA sequence is a major driving force in genome function through the direct recruitment of sequence-specific binding factors to cognate DNA elements (Fig. 2). Perhaps the clearest examples lie in the control of gene expression, where many landmark studies have identified an expanding list of activators and repressors that target DNA elements to drive transcriptional states [124]. These studies have also shaped our view of how the transcription regulatory machinery, *via* DNA binding, regulates histone modification states and histone variant patterns [125]. In addition to DNA sequence, it has become increasingly apparent that DNA modifications, much like histone PTMs, can regulate the association and downstream functions of factors that directly bind DNA (Fig. 2). High-resolution mapping of DNA modification patterns and systematic characterization of factors that interact specifically with these marks are revealing important new insights into how DNA modifications play an active role in chromatin regulation. Below, we highlight recent advances in our understanding of DNA modifications and how these marks integrate with histone modifications to create a diverse epigenetic landscape that functions coordinately with the underlying DNA sequence to dynamically regulate chromatin function.

### 3.1. Reading DNA methylation

DNA methylation in eukaryotes, which occurs primarily at the 5-position of cytosine residues (5mC) in the context of CpG dinucleotides (Table 2), is a heritable chromatin modification long-studied for its key role in repressive chromatin regulatory processes like X-chromosome inactivation, genomic imprinting, and retrotransposon silencing [13]. It is estimated that 80% of all CpG sites in the genome are methylated, accounting for roughly 1% of the total nucleotide pool [126]. DNA methylation has classically been shown to function in gene silencing through direct occlusion of the interaction of transcription factors with DNA [127] (Fig. 2) and by serving as a binding site for methyl-binding domain (MBD) proteins often associated with chromatin remodelers and transcriptional co-repressor complexes [13]. Methyl-CpG binding protein 2 (MeCP2) was the first MBD identified to specifically read a symmetrically methylated CpG dinucleotide [128], and mutations in this domain are causally linked to the X-linked neurodevelopmental disorder Rett syndrome [129,130]. More recently, a second mutational cluster identified outside of the MBD domain of MeCP2 [131] was shown to disrupt the interaction with the NCoR/SMRT co-repressor complexes [132], suggesting a key role for MeCP2 as a bridging molecule between 5mC and NCoR/SMRT. Subsequent members of the MBD family (MBD1–MBD4) have been classified based on primary sequence similarity to the minimal region of MeCP2 required to bind symmetrically methylated DNA [128,133]. Potential functional redundancy of MBD1, MBD2, and MBD4, but not MBD3, is suggested from mouse knockout studies [134–137]. Interestingly, MBD3 is unable to read methylated DNA due to two amino acid substitutions in its MBD domain [138]. A recent study suggests that this protein may in fact read 5-hydroxymethylcytosine (5hmC) [139], a proposed intermediate
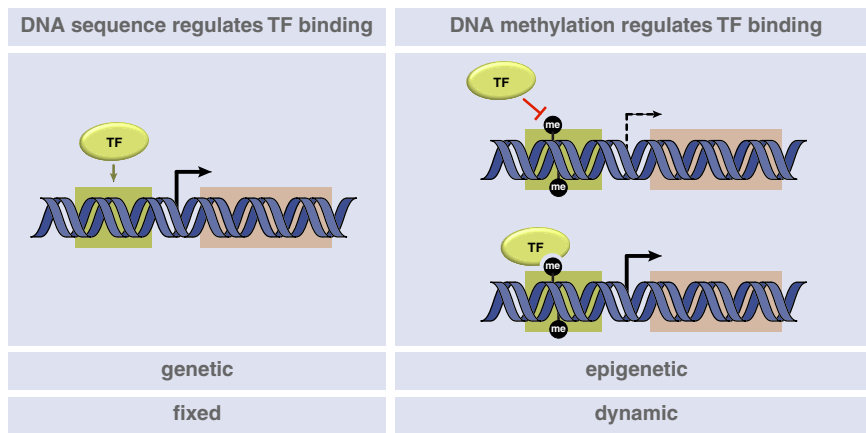
**Fig. 2.** Coordinate genetic and epigenetic mechanisms regulate transcription factor binding. (Left) Transcription factor (TF) binding to cognate DNA sequence motifs (yellow box) regulates transcriptional output (orange box). (Right) TF binding to methylated DNA sequence motifs (black circles) both positively and negatively regulates transcriptional output.

of oxidative DNA demethylation [14] (see below). Genome-wide analysis demonstrated that MBD1, MBD2, MBD4, and MeCP2 occupancy is directly proportional to CpG density [140]. However, *in vitro* affinities for methylated CpG sites vary considerably among this protein family [141], suggesting that other factors, such as additional known or putative chromatin-binding domains within these proteins or unique interacting partners, may also contribute to their cellular specificity.

Several other 5mC reader families have been recently identified, including a family of zinc finger-containing proteins and SET and RING-associated (SRA) domain-containing proteins. Kaiso, the flagship member of the zinc finger family, reads symmetrically modified 5mC (occurring on both DNA strands) in the context of tandem CpG dinucleotides [142]. Similar to MeCP2, Kaiso may be involved in the recruitment of the NCoR co-repressor complex to mediate silencing of target loci [143]. Interestingly, Kaiso also reads with high affinity a specific 5 base-pair unmethylated DNA sequence found at promoters of target

genes regulated by the Wnt signaling pathway [144,145]. As structural analysis of Kaiso binding to methylated and unmethylated DNA templates reveals similar molecular interactions [146], a challenge for future study will be to determine the extent to which this unique bimodal DNA recognition contributes to Kaiso-mediated gene regulation.

The SRA domain was first described as a bona-fide 5mC-binding motif in *Arabidopsis thaliana* [147] and is restricted to the UHRF family of chromatin-interacting proteins in mammals [148]. Evidence for an interaction of the UHRF1 SRA domain with hemi-methylated DNA was initially shown in crude nuclear extracts [149]. Structural studies determined that the UHRF1 SRA domain binds hemi-methylated CpG sites [150–153], similar to nucleotide-modifying enzymes [154,155], by flipping the methyl-cytosine nucleotide out of the DNA double helix into a cage-like hydrophobic pocket [150–153]. Importantly, this is the first example of a sequence-specific non-enzymatic DNA binding domain to harbor base-flipping activity. Hemi-methylated CpG dinucleotides

**Table 2**
Reading and interpreting DNA modifications.

| Modification type | Structure | Associated functions | Reader domains |
|---|---|---|---|
| Cytosine (CpG) | | Gene regulation | CxxC |
| Methylation (5mC) | | X-inactivation, imprinting, long-term silencing, development, gene regulation | MBD, ZF, SRA (others?) |
| Hydroxymethylation (5hmC) | | ? | MBD, SRA (others?) |
| Formylation (5fC) | | ? | ? |
| Carboxylation (5caC) | | ? | SRA (others?) |

are a DNA synthesis intermediate found at replication forks, and the faithful copying of the parental DNA methylation pattern on the daughter strand by DNA methyltransferase 1 (DNMT1) is central to the epigenetic inheritance model of DNA methylation [156,157] (Fig. 3). The interaction of UHRF1 with both DNMT1 and hemi-methylated DNA suggests that UHRF1 is involved in this process [149,158–160]. Indeed, deletion of *Np95* (mouse UHRF1) results in a pronounced reduction in cellular DNA methylation levels [159,160], and the SRA interaction with DNA is required for DNMT1 chromatin targeting and subsequent DNA methylation maintenance [161].

### 3.2. Breaking the dogma of DNA methylation silencing

Large-scale mass spectrometry-based screening approaches are revealing what appears to be an expanded repertoire of proteins that read 5mC either directly or indirectly through membership in macromolecular complexes [37,162–164]. Paradoxical to the silencing function of DNA methylation, a number of DNA-binding transcription factors, notably, three Kruppel-like zinc fingers (KLF2, KLF4, and KLF5), bind specific DNA sequences in a methylation-dependent manner [37,165] (Fig. 2). Interestingly, the Yamanaka reprogramming factor KLF4 associates with both methylated and unmethylated DNA binding motifs in cells, and biochemical studies suggest that these non-competitive reading activities are harbored in distinct domains of the protein [165]. Importantly, 5mC recognition by KLF4 plays a stimulatory role in KLF4-mediated transcription. However, the precise role of 5mC recognition in the stem-cell reprogramming function of KLF4 remains to be determined.

Taken together, these intriguing findings expand the functional role of 5mC in gene regulation, thus underscoring the potential for a DNA methylation language to regulate active and repressive gene states in a site-specific manner. One of the most exciting concepts to emerge from these studies is the realization that a methylated DNA sequence motif can take on a new function by creating a novel DNA binding site for transcriptional activators that could not be predicted from sequence information alone (Fig. 2). Thus, the complexity of DNA modifications regulating genomic function is perhaps as complex now as understanding the dynamics of histone PTM regulation. This complexity is further typified by the recent findings that factors read the newly discovered oxidized derivatives of 5mC, described further below.

### 3.3. Reading DNA hydroxymethylation and oxidized derivatives

Mechanisms facilitating the active removal of DNA methylation have been the subject of much debate [14]. The recent discovery that the ten-eleven translocation (TET) family of enzymes catalyze iterative 5mC oxidation to 5-hmC [166–168], 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (the latter two being substrates for thymine DNA glycosylase-mediated base excision repair) [169,170], significantly advanced our understanding of the active DNA demethylation process. Importantly, recent identification of proteins that read these oxidized derivatives of 5mC [37,139,171,172] suggests that DNA-demethylation independent functions of these DNA modifications may exist.

In addition to its interaction with 5mC, it has been demonstrated that UHRF1 can also read 5hmC [37,171,173]. While the significance of this interaction remains to be determined, it was recently shown that the structurally homologous protein UHRF2 reads 5hmC and 5caC, but not 5mC or 5fC [37]. Importantly, UHRF2 is unable to restore DNA methylation in cells devoid of UHRF1 [174,175], suggesting that while these two proteins share structural identity, they may function in distinct biological pathways. Indeed, oxidative demethylation by
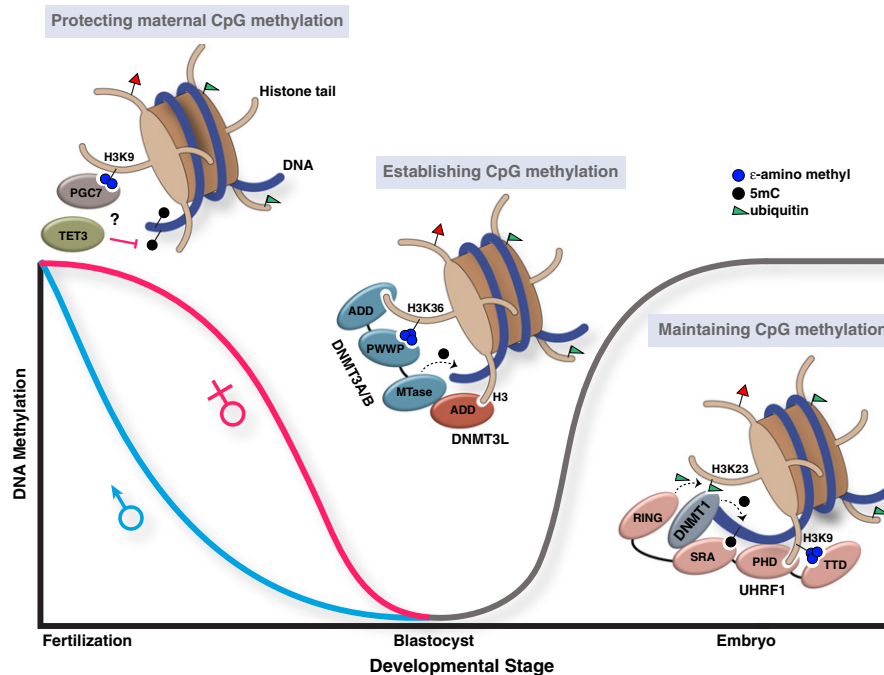


**Fig. 3.** Distinct combinatorial epigenetic modifications play key roles in the erasure, establishment, and maintenance of DNA methylation patterns through organismal development. (Left) PGC7 protects the maternal genome from active DNA demethylation. PGC7 binding to H3K9me2 (blue circles) protects maternal DNA methylation (black circles) from TET3-dependent oxidative demethylation by an unknown mechanism. The paternal genome lacks histones, and TET3 demethylation is therefore active in these cells. (Center) DNMT3A/B and DNMT3L re-establish DNA methylation patterns in early development. DNMT3A/B interacts with the H3 N-terminus through an ADD domain and H3K36me3 (blue circles) through a PWWP domain. DNMT3L also interacts with the H3 N-terminus through an ADD domain and physically associates with the methyltransferase domain of DNMT3A/B to allosterically stimulate *de novo* DNA methyltransferase activity. (Right) UHRF1 facilitates the DNMT1-mediated maintenance of established DNA methylation patterns through embryonic and somatic cell divisions. UHRF1 physically associates with chromatin in a trivalent manner through its TTD and PHD domains that engage a single histone H3 tail (*cis* interaction) that is tri-methylated at lysine 9 (H3K9me3; blue circles) and unmodified at the N-terminus, respectively, and through its SRA domain that engages hemi-methylated DNA (black circles), a DNA replication intermediate. The UHRF1 RING domain aids in the catalysis of H3K23 ubiquitination (H3K23ub; green triangle), which serves as a binding platform for DNMT1. DNMT1 also physically interacts with DNA (see text) and with the SRA domain of UHRF1. Cartoon representations of nucleosomal interactions are depicted based on biochemical and structural studies, but may not be accurate in regards to orientation within the nucleosome.

TET1 in HEK293T cells was enhanced in the presence of ectopically expressed UHRF2 [37]. Clearly, a relationship is emerging between the DNA demethylation pathway and UHRF1/UHRF2, and future biochemical and biological studies are warranted to gain mechanistic insight into how these discreet modifications to the cytosine ring are helping orchestrate the complex recruitment and function of these factors.

Recent studies have also suggested that MBD proteins are capable of reading 5hmC [139,172]. Interestingly, MBD3 localizes to hydroxymethylated gene promoters in a TET1-dependent manner, and an identified physical interaction of MBD3 (unable to read 5mC; see above) with 5hmC suggests that TET1-mediated 5hmC may be a recruitment mechanism for MBD3/NuRD-dependent gene regulation [139]. Additionally, MeCP2 was identified as a reader of 5hmC [172], and a common mutation of MeCP2 found in Rett syndrome [176] disrupts the interaction with 5hmC while maintaining the ability of MeCP2 to read 5mC [172]. The strong correlation between 5hmC and gene activation provides yet another paradigm whereby a dual modification reader like MeCP2 may function as both a co-activator and a co-repressor of transcription depending on the chromatin mark it reads.

Perhaps the most intriguing findings from the above studies are the wide range of potential reader domains capable of interacting with the distinct methylation states on CpG dinucleotides. This finding creates a new hierarchy of regulation and will lead to many important studies into the functional significance of these interactions.

### 3.4. Reading unmodified CpG dinucleotides

While most CpG dinucleotides across the genome are methylated, repetitive clusters known as CpG islands are found at more than half of transcriptionally active mammalian gene promoters, and these islands are largely hypomethylated [177]. The identification of cysteine-rich zinc-finger CxxC-containing proteins as readers of unmethylated CpG dinucleotides suggests that CpG islands can play an active role in transcription [178]. Indeed, CxxC-finger protein 1 (CFP1), a subunit of the SET domain 1 (SETD1) H3K4 methyltransferase complex [179], associates with a large fraction of non-methylated CpG sites in the mouse brain, most of which are also enriched for H3K4me3 [180]. Importantly, integration of artificial CpG islands into promoter-free regions of the mouse genome is sufficient to establish new H3K4me3 domains, suggesting a key role for CFP1 in directing H3K4me3-dependent transcription.

The presence of CxxC domains in TET1 and TET3, but not TET2, suggests that unmethylated CpG sites may constitute, at least in part, a targeting mechanism and nucleation point for subsequent DNA demethylation. However, efforts to characterize the CxxC domains of TET1 and TET3 as bona-fide CpG readers *in vitro* have been difficult, presumably due to the positive charge of these domains promoting non-specific DNA interactions [181–184]. Interestingly, the CxxC domain of IDAX, an ancestral gene product of *TET2*, now transcribed from a divergent promoter [185], is necessary to promote caspase-dependent TET2 degradation [186]. TET3, through the action of its own CxxC domain, is also regulated in a similar manner. These unexpected consequences of TET2 and TET3 chromatin recruitment are suggestive of interesting auto-regulatory loops to ensure the maintenance of hypomethylated genomic states.

## 4. Coordinate function of histone and DNA modifications in chromatin regulation

### 4.1. Multivalency in DNA and histone recognition

Significant insight into chromatin regulation has come from recent studies revealing that DNA and histone modifications are functioning in a cooperative manner to re-shape chromatin organization and facilitate the recruitment of effector proteins and their macromolecular complexes to discreet locations throughout the genome. Significantly,

pioneering work of Kouzarides, Mann, and colleagues using a semi-synthetic mononucleosome assembly strategy coupled with quantitative MS has begun defining an interaction network of proteins and protein complexes that read both histone and DNA modification states [163]. CpG methylated or unmethylated DNAs wrapped around recombinant histone octamers containing uniformly modified H3K4me3, H3K9me3, or H3K27me3 were used as baits to enrich for interacting proteins from HeLa nuclear extracts. From these proteomic experiments, a rich list of factors whose interaction with chromatin may be directed by modifications on both histones and DNA has emerged [163]. For example, UHRF1 binding to H3K9me3-containing nucleosomes was enhanced by CpG methylation, consistent with the identified functions of its TTD-PHD histone binding module [104] and SRA domain [150–153]. In contrast, KDM2A binding to H3K9me3-containing nucleosomes was perturbed by CpG methylation, consistent with the identified function of its CxxC domain as a reader of unmethylated CpG dinucleotides [187]. Importantly, several protein complexes, including ORC and PRC2, were identified that appear to read simultaneous modification states on both histones and DNA, likely through distinct subunits. Indeed, the Mi-2/NuRD complex can sense methylated DNA through its MBD2 subunit and unmodified H3 N-terminal tails through its CHD4 [112] and CHD5 subunits [113], and the repressive function of the MBD2/CHD4-containing NuRD complex appears to be dependent on the coordinate function of these two interactions [188].

Another example of DNA and histone multivalency is the pre-initiation complex for RNAPII transcription. Here, the largest multi-subunit complex, TFIID, harbors the TATA-box binding protein (TBP) in addition to the TAF1 and TAF3 subunits that contain paired bromo-domains and a PHD finger, respectively. A landmark study by Tjian and colleagues in 2000 identified the bromodomains of TAF1 to be dual histone acetyllysine readers [32] — thus providing an expanded mechanism by which TFIID may be recruited and/or stabilized at gene promoters. This mechanism may be particularly important in proper positioning of TFIID at TATA-less promoters that do not utilize TBP. In addition to the TAF1 interaction with histone acetylation, recent SILAC coupled with MS analysis revealed that the PHD finger of TAF3 is a specific reader of H3K4me3 [189] — a mark found at the +1 nucleosome along with H3/H4 acetylation. Significantly, recent studies have shown that TAF3 interaction with H3K4me3 is required for proper gene activation [190]. Taken together, these data underscore the role of multivalent histone and DNA interactions in creating a precise epigenetic signature that is read by the RNAPII transcription machinery.

### 4.2. Multivalency in the generation of defined epigenetic landscapes

Combinatorial DNA and histone modifications also contribute to the diversity of epigenetic landscapes and chromatin states by influencing the generation and removal of distant marks. Several recent examples of multivalent mechanisms underlying the creation of defined chromatin signatures on histones and DNA are discussed below.

The Jumonji C (JmjC) domain-containing histone lysine demethylase KDM2A catalyzes the removal of H3K36me2 [191]. The identified function of its CxxC domain as a reader of unmethylated CpG dinucleotides [187] suggests that DNA recognition may be important for cellular KDM2A catalytic activity. Indeed, this was shown to be the case [187], providing a likely explanation for why CpG island promoters are devoid of H3K36me2. Likewise, CxxC-mediated recognition of unmethylated CpG islands by SET1-associated CFP1 is necessary to prevent the spread of H3K4me3 to distal regulatory elements [192].

Recent studies also demonstrate that the establishment, maintenance, and removal of DNA methylation is intricately linked to the recognition of histone PTMs. Parental DNA methylation patterns, largely erased between fertilization and the blastocyst stage, are re-established during early embryogenesis by the *de novo* DNA methyltransferases DNMT3A and DNMT3B (with the associated, but non-catalytic DNMT3L) [193], and are stably maintained through somatic cell divisions

[194] primarily by the maintenance methyltransferase DNMT1 [195] (Fig. 3). Waves of DNA methylation reprogramming also occur through germ cell development and carcinogenesis, recently reviewed elsewhere [196,197]. DNMT3A, DNMT3B and DNMT3L share a cysteine-rich ADD domain, aptly named for the four proteins identified to harbor this motif (ATRX-DNMT3A/B-DNMT3L), that binds the unmodified N-terminus of histone H3 [198–200]. Furthermore, *in vitro* catalysis of DNA methylation by DNMT3A/DNMT3L on linker DNA of semi-synthetic nucleosomal arrays was disrupted when nucleosomes harbored H3K4me2 or H3K4me3, suggesting a role for the ADD domain in DNMT3A/DNMT3L recruitment [201] (Fig. 3). While these data are consistent with the anti-correlative genome-wide distribution of DNA methylation and H3K4me3, the biological significance of these putative recruitment mechanisms remains to be determined. Interestingly, DNMT3A and DNMT3B also harbor PWWP domains essential for chromatin targeting in cells [202–204], and biochemical studies suggest that the PWWP domain of DNMT3A reads H3K36me3 [205]. Given that H3K36me3 is a histone PTM associated with transcription elongation and is found within the bodies of RNAPII-regulated genes, it has been speculated that DNMT3A/B contributes to gene-body DNA methylation that widely occurs but is poorly understood [206]. Indeed, it was recently shown in mouse ESCs that DNMT3L is a positive regulator of gene body DNA methylation within active genes [207]. Interestingly, this same study showed that a physical interaction between DNMT3L and the EZH2 subunit of the PRC2 complex ensures that poised bivalent promoters remain hypomethylated.

Unlike the *de novo* methyltransferases, mechanisms facilitating the recruitment of the maintenance methyltransferase DNMT1 to chromatin have been somewhat elusive. A replication foci targeting sequence (RFTS) facilitates the interaction of DNMT1 with proliferating cell nuclear antigen (PCNA) at replication forks [208]. However, disruption of this interaction has a minimal effect on cellular DNA methylation [209]. The identification of a CxxC domain in DNMT1 that binds unmethylated CpG dinucleotides and is necessary in cells for enzymatic function [210] suggests an interesting interplay between the ability of DNMT1 to sense both unmethylated and hemi-methylated DNA, the latter of which appears to be an inherent property of its catalytic domain [208,211,212]. Interestingly, recent structural studies suggest that these unbound domains function as auto-regulators of DNMT1 activity, as both an acidic loop C-terminal to the CxxC domain and the RFTS domain limit catalytic function of the enzyme by inserting into the active site [213,214]. Recent studies linking UHRF1 genetically and physically to DNMT1 chromatin targeting and activity [150–153,159,160] strongly suggest that UHRF1 modulates DNMT1 chromatin function. Excitingly, it was recently shown that the UHRF1 RING domain has E3 ubiquitin ligase activity towards H3K23 [161]. H3K23 monoubiquitination was identified as being read by DNMT1 in an RFTS-dependent manner, providing an unexpected mechanism by which a histone modification facilitates DNA methylation maintenance (Fig. 3). This is one of the few examples of how ubiquitination on a histone may be driving recruitment of a chromatin modifier, and further, underscores the more general potential for how histone monoubiquitination may serve as a binding platform, as is found for monoubiquitinated proteins in cell signaling [215].

Aided by important technological advances in both sample collection and genome-wide mapping capabilities, the dynamics of epigenetic reprogramming in the early stages of development have been re-examined extensively over the past several years (for recent in-depth reviews, see [193,197]). Intriguingly, chromatin-templated mechanisms regulating these impressive dynamics of DNA methylation during embryogenesis are being uncovered. The time-scale in which DNA methylation is lost in the paternal and maternal nuclei suggests that both active and passive removal mechanisms, respectively, are in place (Fig. 3). Indeed, recognition of H3K9me2 by the maternal factor PGC7 is necessary to protect maternal 5mC from TET3-mediated oxidative demethylation [216,217] (Fig. 3). Although paternal nuclei also express PGC7, sperm DNA is packaged with protamines rather than

histones, and therefore lack the ability to recruit PGC7, resulting in active TET3. In addition, Dnmt1 and Np95 are excluded from maternal nuclei and primordial germ cells, accounting for the passive loss of DNA methylation in these cells [197,218]. Understanding the relationship between PGC7 and TET3, the structural basis for PGC7 recognition of H3K9me2, and the signals controlling the subcellular localization of Dnmt1 and Np95 at specific cell stages will be required to fully appreciate these key developmental regulatory mechanisms.

## 5. Future challenges towards understanding chromatin regulation

Similar to histone PTMs, we are still just scratching the surface of understanding how key players in the generation, removal, and interpretation of DNA modifications are regulated in the context of the epigenetic landscape. To fully grasp the depth and breadth of complexity underlying how histone and DNA modifications function together, many remaining unanswered questions must be tested experimentally. A number of immediate future challenges towards interpreting the complex epigenetic language are discussed below.

### 5.1. Determine the influence of sequence context on the recognition of DNA modifications

Compared to histone residues, the sequence context surrounding CpG dinucleotides throughout the genome is enormous. This diversity alone suggests that the rules governing DNA modification interactions will be more challenging to decipher than histone PTM interactions. Indeed, several recent studies suggest that, similar to histone PTM recognition and transcription factor binding, DNA readers have the ability to differentially sense the sequence surrounding a modified CpG. For example, in the above-described semi-synthetic nucleosome strategy for identifying dual readers of histone and DNA modifications, two DNA sequences with similar nucleosome forming properties but differing sequences [219] were used to assemble nucleosomes [163] for MS enrichment. Interestingly, factors were identified that bound in a sequence-dependent and independent manner, suggesting that some, but not all CpG readers are influenced by sequence context. Moreover, recent analysis of transcription factor binding to 154 unique CpG methylated DNA sequences demonstrated that most of these newly-identified CpG readers bound methylated DNA in a sequence-dependent fashion [165]. Differing sequence preferences have also been documented for several MBD proteins [220,221], a property to consider when using these domains as methylated CpG affinity enrichment tools. This poses intriguing questions regarding the rules that dictate whether specific neighboring nucleotides (or combinations thereof) attract or repel CpG readers. The already realized level of apparent sequence preference suggests that there is minimal redundancy in the DNA modification language. Rather, in parallel to the original postulate of the 'histone code,' CpG modifications at defined genomic locations may also direct distinct biological outcomes.

### 5.2. Define the structural families of DNA modification readers

Our understanding of functions associated with histone PTMs has been facilitated, in part, by detailed structural and biochemical characterization of the protein domains that bind to histone modifications [33,34]. Undoubtedly, atomic resolution structural mapping of binding interactions for newly identified DNA modification readers will facilitate our understanding of how DNA modifications are read and interpreted. While a number of these proteins harbor DNA binding domains commonly found in transcription factors, including zinc fingers, homeoboxes, and helix–loop–helix domains [37,165], it is likely that many newly discovered DNA modification readers will engage their mark through novel motifs. Excitingly, a number of identified DNA modification readers also harbor known or putative histone binding domains. For example, death inducer obliterator (DIDO) isoforms contain

PHD fingers shown to read H3K4me3 [116,117,163]. The recent identification of DIDO1 as a sequence-specific reader of CpG methylation [165] poses an interesting question regarding the contributions of these DNA and histone reading activities to DIDO functions. Indeed, determining how proteins interact with CpG modifications, as well as cytosine modifications in other, lesser-studied, sequence contexts [222], will ultimately lead to a deeper understanding of known and novel biological functions of these marks.

### 5.3. Complete the dictionary of chromatin modifications

Drawing the analogy of DNA and histone modifications being akin to letters of the alphabet, we would be unable to form words and sentences like transcriptional activation or DNA repair without first learning the identity of all of the letters in the alphabet as well as the order in which to place them. Therefore, crucial to our future understanding of chromatin function will be the identification of the full complement of DNA and histone modifications and their combinations, as well as their precise locations throughout the genome.

While much of the PTM landscape on the N-termini of histones have been defined, we surprisingly still know very little about the identity and combinations of PTMs present on histone C-termini, globular domains, and variant histones. Indeed, important functions associated with modifications to these less-studied regions of histones are continually emerging. For example, Zhang and colleagues recently used an elegant combination of yeast genetics and biochemistry to identify a conserved mechanism by which H3K56ac promotes Rtt101-dependent ubiquitination of three lysines in the H3 C-terminal tail (K121, K122, and K125) [223]. This ubiquitination promotes the necessary transfer of H3–H4 dimers between the histone chaperones Asf1 and RTT106/CAF-1 for nucleosome assembly and reveals novel crosstalk between the histone ubiquitination and acetylation machinery beyond competition for the same residue.

Proteomic technologies are also advancing to the point where comprehensive analysis of all core histones and their variants is now possible [36,39,42]. As new types of histone PTMs will likely continue to be identified, a full understanding of epigenetic regulation will require knowledge of how these PTMs function, and how they contribute antagonistically or synergistically with known PTMs to regulate the distinct functions associated with them. Likewise, the recent discovery of oxidized derivatives of CpG methylation suggests that the dictionary of DNA modifications is also incomplete. For example, the existence of CpA, CpT, and CpC methylation has been known for some time now [222], but it is unclear whether these modifications are also susceptible to oxidative demethylation.

### 5.4. Define the modification landscape at individual genomic loci

Another significant set of challenges we face is to develop new technologies that enable characterization of the PTM landscape at defined genomic loci and to determine how this landscape contributes to the architecture and function at these regions, through specific cell stages, cycles, and developmental contexts. From a practical standpoint, knowing this information will be key to understanding how deregulation of the epigenetic machinery contributes to the re-wiring of the epigenetic landscape in human disease.

Several recent studies have indeed begun addressing this challenge using distinct approaches. Notably, Tackett and colleagues recently developed a technique they call Chromatin Affinity Purification with Mass Spectrometry (ChAP–MS) that allows for enrichment of native ~1 kilobase sections of chromatin for site-specific identification of chromatin-associated proteins and histone PTMs [224]. While powerful, this technique requires insertion of a LexA DNA binding site into the genomic region of interest for site-specific targeting of the LexA protein, and is therefore limited to routine use in genetically tractable systems. An important modification to this technique circumvents this problem by using transcription activator-like effector (TALE) proteins as targeting molecules [225]. TALs can easily be designed to target any ~20-nucleotide genomic locus of interest without genetic manipulation, and are therefore a powerful tool for site-specific high-resolution genomic targeting. This modified technique was used to define the chromatin architecture of the *GAL1* gene in *Sacchromyces cerevisae*, which was found to be enriched for a number of single histone PTMs, including H3K14ac, H3K56ac, all three states of H3K79 methylation, H2BK17ac, and H2AK7ac [225]. A number of di-acetyl histone PTM combinations were also found at this locus. Furthermore, over 50 unique proteins were identified to reside at the *GAL1* gene, including a number previously identified transcriptional regulators like the Spt16 subunit of the FACT transcription elongation complex and Gal3.

Recent studies have also begun defining with high-resolution the nucleosomal landscapes occupied by chromatin-associated proteins in an unbiased manner [226,227]. One notable study coupled the powerful approaches of mononucleosome-resolution ChIP coupled with next-generation DNA sequencing (Mnase-ChIP-seq), RNA sequencing, and MS to simultaneously reveal 1) the combinations of histone PTMs associated with HP1 isoforms and several members of the bromodomain and extraterminal family (BET), 2) the genomic loci harboring these nucleosomes, and 3) the transcriptional state of these nucleosomes [226]. BET family proteins (Brd2, Brd3, and Brd4) were enriched at active gene promoters predominantly harboring H3K4me3 in combination with K9ac, K14ac, and K23ac, as well as H4K5ac in combination with K12ac and K16ac. Gene bodies were also enriched for BET family proteins, and histones in these regions were predominantly marked with H3K4me1 in combination with K14ac, K23ac, and K79me1 as well as H4K8ac in combination with K12ac, K16ac, and K20me1. Interestingly, a large number of gene promoters occupied by BET family proteins were the key developmental HOX genes, although the functional significance of this intriguing finding is not understood. Approaches like this are an important step towards understanding how chromatin-associated proteins read and translate the epigenetic language, and future studies incorporating DNA modification analyses will give an even more complete picture of how histone and DNA modification states coordinate functional outcomes in defined regions of chromatin.

### 5.5. Assign function to chromatin modifications

As we continue to discover new chromatin modifications, their combinations, genomic locations, and protein readers, a key challenge moving forward is to define how any particular modification, on DNA or histones, tips the balance towards distinct biological outcomes. Our ability to now site-specifically modify the chromatin landscape in complex eukaryotic systems using re-purposed genome editing tools like zinc-finger (ZF) nuclease, TALE nuclease, and clustered regulatory interspaced short palindromic repeat (CRISPR) technologies [228–231] is an important step towards tackling this central question. In addition, chemical-induced [56] and light-induced [232] chromatin targeting approaches also allow the direct examination of the functional consequences of DNA and histone modifications. For example, Crabtree and colleagues elegantly adapted a chemical-induced proximity system for precision targeting of chromatin modifiers [56], whereby stimulation by a bi-functional small molecule causes the rapid, reversible association of two different peptide tags fused to proteins of interest in the cell. They then engineered the mouse *Oct4* promoter to harbor ZFHD1 and GAL4 DNA binding sites in-frame with a GFP reporter. By tethering ZFHD1 to FKBP, and the chromoshadow domain of HP1α (which directly interacts with the H3K9 methyltransferases SUV39h1/2 and SETDB1) to Frb, they were able to use rapamycin to promote an interaction between FKBP and Frb, which resulted in the recruitment of HP1α and H3K9 methylation to the engineered ZFHD1 binding site of *Oct4*. Excitingly, this resulted in a cascade of events, including the spread of H3K9me3, the loss of H3K4me3, and the gradual increase in DNA

methylation that collectively created a new, stable domain of transcriptionally repressed heterochromatin even in the absence of continued HP1α targeting. Importantly, they showed that the kinetics of DNA methylation at this locus trailed the kinetics of H3K9 methylation and discovered that the maintenance of the repressed domain in the absence of further HP1α targeting was dependent on DNA methylation at this locus. Interestingly, by tethering the transcriptional activator VP16 to PYL1, and GAL4 to ABI1, they were similarly able to recruit VP16 to the GAL4 DNA binding site at this repressed *Oct4* locus by addition of abscisic acid, which resulted in reactivation of silenced GFP. This result suggests that robust transcriptional activation can outcompete the signals of heterochromatin, and the authors propose that DNA methylation at the *Oct4* promoter may function to maintain this heterochromatin domain by directly preventing the binding of transcription factors.

## 6. Concluding remarks

Progress over the past several decades has ushered in a new wave of appreciation for the role of histone modifications in diverse chromatin functions. The complexity of histone modifications in regards to the sheer number of identified PTMs, alone and in combination, is astonishing in itself. The discovery of proteins harboring multiple reader domains that interpret these combinatorial PTMs on histones to perform their chromatin functions suggests that our original one modification–one domain model underestimates the real situation and complexity of histone function. The discovery of new protein families that read DNA modification states and patterns of histone and DNA modification embedded in nucleosomes places the 'histone code' within the framework of a much broader language of epigenetic modifications. Taking into account known single PTMs on the four core histone tail domains, occurring symmetrically or asymmetrically, in conjunction with the known single modification states of CpG dinucleotides, regardless of DNA sequence context, would imply the potential for an enormous number of unique chromatin signatures throughout the genome. Even if only a fraction of these combinations contribute to biological functions of chromatin, we will surely stay busy decoding the astonishing language of histone and DNA modifications for years to come.

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