Part One
General Aspects and Methodologies

Epigenetic Targets in Drug Discovery. Edited by Wolfgang Sippl and Manfred Jung

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New Frontiers in Epigenetic Modifications

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1.1
Introduction

The basic packaging unit of the genome, the nucleosome, consists of ~146 bp of DNA wound around an octamer of histone proteins. The histone octamer is composed of an H3/H4 tetramer and two H2A/H2B dimers. The unstructured N-terminal regions (tails) of histones protrude outward from the nucleosomal core through superhelical gyres of DNA. While genetic information is encoded in the DNA sequence, processes such as transcription, recombination, DNA replication and DNA repair are controlled by the "epigenome" (*epi* is Greek for upon or in addition to). The epigenome is often characterized by heritable or long-term alteration in gene expression patterns that cannot be ascribed to changes in DNA sequence [1]. At the molecular level, epigenetics involves the dynamic regulation of covalent modifications to DNA and the histone proteins. Epigenetics is implicated in processes such as gene expression and silencing, apoptosis, maintenance of stem cell pluripotency, X-chromosome inactivation and genomic imprinting [2]. Therefore, epigenetics can be viewed as the conduit from genotype to phenotype. This chapter provides a framework for our current understanding of molecular epigenetics with particular emphasis on the histone code and it examines the utility of small molecule inhibitors of enzymes that modify DNA and histones.

1.2
DNA Methylation

In multicellular eukaryotes, DNA methylation is associated with transcriptional silencing [3]. In these organisms, DNA methylation has been observed exclusively on the C5 position of the cytosine ring and is frequently found in CpG-rich regions. This process is attributed to the action of DNA methyltransferases (DNMTs), which utilize the cofactor, *S*-adenosyl-L-methionine. Approximately half of all human genes have CpG islands in their promoter regions but these stretches of DNA are typically

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hypomethylated and transcriptionally permissive. Methylation in the proximity of the transcription start site or within a gene is associated with transcriptional repression [1, 4, 5].

DNA methyltransferases can be classified in two categories: (1) *de novo* DNMTs and (2) maintenance DNMTs [3]. *De novo* DNMTs methylate previously unmodified cytosines in CpG islands, while maintenance DNMTs duplicate existing DNA methylation patterns onto newly synthesized DNA strands during replication. DNMT3a and 3b are examples of *de novo* DNMTs and are capable of methylating both unmethylated and hemimethylated (only one strand in the DNA duplex is methylated) sites of DNA. Another protein, DNMT3-like (DNMT3L), acts as a regulatory factor in *de novo* methylation of DNA despite lacking a catalytic domain. This protein is involved in genetic imprinting [6] and methylation (indirectly) of retrotransposons in pre-meiotic spermatogonial stem cells [7]. In the case of genomic imprinting, DNMT3L was found to collaborate with DNMT3a to achieve DNA methylation by localizing the latter to unmodified K4 of histone H3 via a plant homeodomain (PHD)-like domain [8]. DNMT1, which has a catalytic preference for hemimethylated DNA, is an example of a maintenance DNA methyltransferase [3]. DNMT1 is localized to replication foci by interaction with proliferating cell nuclear antigen (PCNA) [9]. Recently, an accessory protein, UHRF1, was shown to target DNMT1 to hemimethylated DNA during S phase [10, 11]. UHRF1 is known to bind methylated DNA in the context of CG, CXG (X = A, T or C) or an asymmetrical sequence using a SET and RING associated (SRA) domain [12]. Furthermore, UHRF1 was found to be required for stable association of DNMT1 with chromatin [10]. Therefore, DNMT1 appears to regulate epigenetic inheritance in a mechanism that involves a complex with UHRF1 and PCNA in regions of replicating heterochromatin (tightly packed, transcriptionally repressive chromatin) that contain hemimethylated DNA [11].

Several other means for targeting DNMTs have been identified. In a sequence-dependent manner, DNMTs bind directly to DNA by virtue of a conserved proline and tryptophan (PWWP) domain [13]. For both *de novo* methyltransferases, DNMT3a and DNMT3b, a PWWP domain is essential for chromatin targeting [14]. Missense mutation of the PWWP domain in the *DNMT3B* gene triggers centromeric heterochromatin instability, pericentromeric instability and facial anomalies (ICF) syndrome [14]. A second mechanism for DNMT targeting is through recruitment by site-specific transcriptional repressors [3]. The oncogenic fusion protein, promyelocytic leukemia–retinoic acid receptor (PML-RAR), localizes methylation to specific genes in cancer cells by recruitment of DNMTs [15]. More recently, the polycomb group protein, enhancer of Zeste homolog 2 (EZH2), was found to target DNMTs to EZH2-repressed genes [16]. Finally, small RNA molecules have been implicated in DNMT targeting [3].

Transcriptional repression by DNA methylation is achieved by various modes of action. In one such mechanism, DNA methylation simply inhibits the binding of a transcription factor [17]. By a more complex means of action, a number of DNA methyl-binding proteins potentiate transcriptional silencing. In some cases, binding is accompanied by the action of an associated histone-modifying enzyme.

For example, one DNA methyl-binding protein, MBD1, associates with SET domain bifurcated 1 (SETDB1), a histone methyltransferase (HMT). During DNA replication, SETDB1 associates with MBD1 in addition to chromatin assembly factor-1 and catalyzes methylation of histone H3 at lysine 9. Trimethylation at H3K9 is associated with heterochromatin [18]. A second example that links DNA methylation to a histone modification state involves PML-RARα [19]. MBD1 can form a repressor complex with N-CoR, DNMTs, HDAC3 and PML-RARα to silence PML-RARα-dependent promoters. This aberrant gene silencing is manifested by HDAC-mediated histone deacetylation, DNA methylation, as well as time-dependent spreading of MBD1 outside of the promoter region. The spreading of MBD1 along regions of methylated DNA is thought to recruit additional repressor enzymes [19].

1.3
Histone Modifications and the Histone Code Hypothesis

A vibrant area in epigenetic research involves covalent modifications of histones [20]. The most commonly observed histone modifications include acetylation, methylation and phosphorylation. However, other modifications such as citrullination, ubiquitination, SUMOlyation, prolyl isomerization, ADP-ribosylation and biotinylation are being increasingly recognized. These modifications are primarily located on the unstructured N-terminal tails of histones (Figure 1.1), yet an increasing number of modifications in the α-helical histone fold domains have been documented. In this chapter, we limit our detailed discussion to histone acetylation and methylation. One interpretation of the epigenetic consequences of histone modification is the histone code hypothesis, which predicts that combinatorial histone modification states result in unique biological outcomes [21]. This molecular "code" is regulated by alteration of histone–histone interactions, histone–DNA interactions and histone–nonhistone protein interactions. While there is some dispute whether each histone modification state results in a unique downstream function, it is generally agreed that histone modifications can lead to differences in binding and selectivity and that these differences can propagate a variety of epigenetic outcomes.

Figure 1.1 PTMs found on the first 30 residues of the human histone proteins. Only acetylation, methylation, deimination (formation of citrulline) and phosphorylation are shown.

A number of strategies have emerged for characterizing histone modifications and understanding their significance. The workhorses of histone modification mapping have been modification-specific antibodies and mass spectrometry [20] (see the requisite chapters in this book for more details). In the ChIP on chip approach, DNA is crosslinked to DNA-binding proteins, digested and immunoprecipitated with an antibody for the histone modification of interest. Following PCR amplification of the associated DNA, the histone modification can be linked to particular gene regions using microarray technology. This methodology was used extensively in a large-scale human and mouse epigenome study [22]. While this method was extremely powerful for mapping locations of histone modification on genes, it is limited by the specificity of available antibodies and cannot be used to determine the modification status of histones within the same nucleosome. Mass spectrometry, in contrast, is capable of determining the modification status of individual histone proteins. A recent study identified 74 unique histone H4 modification patterns in differentiating human embryonic stem cells [23]. Several chemically driven strategies have found utility in studying the effects of histone posttranslational modifications. For example, native chemical ligation/expressed protein ligation [24–26], chemical incorporation of methyl-lysine mimetics [27] and genetic incorporation of modified amino acids (such as acetyl lysine) [28] have enabled the generation of site-specifically modified histones. In one study employing semisynthetic histones, acetylation of H4K16 was shown to modulate chromatin compaction and its ability to form cross-fiber interactions [25]. Recently, we developed a method for assaying the specificity of enzymes and proteins that read the histone modification patterns using combinatorial peptide libraries based on the modification patterns of N-terminal histone tails [29].

Reversible lysine acetylation of histones is regulated by the opposing activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of the lysine ε-amino group results in neutralization of a positive charge, while deacetylation reestablishes the presence of a primary amine. Hypoacetylation typically facilitates formation of highly condensed chromatin (heterochromatin) and transcriptional repression, while hyperacetylation tracks with chromatin that is more "loosely" associated with DNA (euchromatin) and transcriptional activity. HATs employ an acetyl-CoA cofactor, operate in large multiprotein complexes and can be classified as members of the GNAT, MYST or CBP/p300 families of enzymes [30]. However, a recently identified HAT in yeast, Rtt109, appears to belong to an entirely different family due to lack of homology with other HATs [31]. HDACs are responsible for the removal of acetyl groups from histone lysines. These protein deacetylases are categorized as class I, II, III and IV HDACs [32]. Class I, II and IV HDACs are metal-dependent acetyl hydrolases that yield acetate as a product. Class III HDACs, or sirtuins, utilize an NAD^+ cofactor and couple deacetylation with the formation of *O*-acetyl-ADP-ribose (*O*AADPr) and nicotinamide. Sirtuins have been postulated to link epigenetics to metabolic processes and act on a number of nonhistone proteins [33].

Reversible histone methylation is a highly specific process that is catalyzed by the action of histone methyltransferases (HMTs) and histone demethylases on lysine and arginine residues. Like DNMTs, HMTs employ a SAM cofactor. Lysine can be

mono-, di- or trimethylated and arginine can be monomethylated and symmetrically or asymmetrically dimethylated. The consequences of histone methylation appear to be largely context-dependent. For example, trimethylation of H3K4 is a mark of transcriptionally active chromatin, while trimethylation of H3K9 tracks with transcriptionally repressive chromatin. Lysine HMTs are divided into the SET and Dot1 families [30]. Members of the SET family contain a SET domain and representative examples include mixed-lineage leukemia (MLL) and EZH2 proteins, which are specific for methylation of H3K4 and H3K27, respectively. Dot1 lacks a SET domain and is responsible for mono- and dimethylation of H3K79 [30]. Protein arginine methyltransferases (PRMTs) usually act on glycine-arginine-rich regions within their substrates. Following N^{ω}-monomethylation, type I PRMTs can catalyze a subsequent methylation on the same atom (asymmetric dimethylation), while type II PRMTs methylate the other ω-nitrogen (symmetric dimethylation) [30].

The discovery of LSD1, the first histone demethylase to be characterized, invalidated the notion that histone methylation was a permanent mark [34]. LSD1 is an FAD-dependent amine oxidase that catalyzes mono- and di- demethylation of H3K4. Interestingly, a byproduct of LSD1 catalyzed demethylation, hydrogen peroxide, is thought to link local DNA oxidation to estrogen-induced gene expression [35]. The Jumonji C (JmjC) hydroxylases, another class of histone demethylases, require iron and α-ketoglutarate and produce formaldehyde as a byproduct. While LSD1 only demethylates mono- and dimethyl lysine residues in proteins, members of the JmjC family such as JMJD2A and JARID1A are specific for di- and trimethyl lysines. JMJD6 demethylates both symmetric and asymmetric arginine to their monomethylated counterparts at H3R2 and H4R3 [36]. Protein arginine deiminases (PADs) have been suggested to catalyze methylated arginines demethylimination to furnish citrulline [37], yet *in vitro* evidence for this transformation suggests that methylarginine is a very poor substrate, if at all [38]. Further studies are needed to resolve these issues. Deimination of arginine to citrulline, however, has been widely characterized [39]. Currently, there is no evidence that protein deimination is a reversible modification. See the relevant chapters of this book for more information on reversible histone aceylation and methylation.

1.4 Origins of Specificity in Histone Binding Proteins/Modifying Enzymes

The primary readers of the histone code are histone binding domains (HBDs). HBDs are often found in histone-modifying enzymes, ATP-dependent chromatin remodeling factors and transcription factors. In this chapter, we focus on domains with the ability to recognize acetylation and methylation. The observation that bromodomains function as histone acetyl-lysine binding modules [40] set the stage for discovery of a number of specialized HBDs. The specificity of the these binding modules appear to be dictated by both the type of modification and the context of the modified amino acid [41]. For example, although both heterochromatin protein 1 (HP1) and polycomb protein PC2 both contain chromodomains (methyl-lysine binding modules), the

former binds trimethylated H3K9 while the latter binds trimethylated H3K27 despite an identical sequence surrounding each lysine (ARKS). The binding specificity is achieved by means of an extended recognition groove in polycomb that recognizes residues 20–24 [42]. Another mechanism for discriminating among potential binding partners is by the extent of a particular modification state (e.g. mono-, di- or trimethylation).

Bromodomains, which are composed of approximately 110 amino acids, fold into a left-handed antiparallel four-helix bundle. They contain a hydrophobic tunnel, which accommodates binding of acetylated lysine. Relative to other histone binding domains, bromodomains are promiscuous with regard to the sequence to which they bind and typically have dissociation constants in the ~50–350 μM range [2]. These targeting modules are frequently found in HATs. It has been suggested that bromodomains enable a mechanism whereby HATs can propagate acetylation along a histone or a nucleosome. Other proteins that harbor bromodomains include members of the HMT family and ATP-dependent remodeling enzymes. One such example, the SWI/SNF ATP-dependent remodeling complex employs a bromodomain to associate with acetylated promoter nucleosomes [43]. Using the energy liberated from ATP hydrolysis, the SWI/SNF complex mobilizes nucleosomes. The bromodomain binding activity of SWI/SNF is essential for displacing the SAGA HAT complex, as well as facilitating octamer transfer on H3-acetylated nucleosomes [44].

The tudor domain royal superfamily includes the chromodomain, double chromodomain, chromo barrel, tudor, double/tandem tudor and MBT domains [45, 46]. Tudor and chromo domains bind to di- and trimethylated lysine while MBT domains prefer mono- and dimethylated lysine. Tudor domains are also capable of recognizing symmetrically dimethylated arginine. Increasing lysine methylation results in reduced hydrogen bonding potential and solvation properties, as well as greater hydrophobicity and a larger cation radius. Because many methyl binding modules engage in cation-π interactions [46], the degree of methylation is critical in conferring specificity. Domains that preferentially bind to higher lysine methylation states can accommodate a diffuse and hydrophobic cation. In contrast, hydrogen bonding and steric exclusion are dominant factors in regulating specificity for lower methylation states (or unmethylated lysine) [46]. It was noted that the chromodomain of HP1 binds specifically to di- and trimethylated H3K9. HP1 recognizes methylation catalyzed by the action of the suppressor of variegation 3–9 homologue 1 (SUV39H1) methyltransferase [30]. Structural analysis revealed that a H3K9 trimethylated histone peptide was immobilized in an induced β-strand sandwich with the protein [46]. Since the original HP1 structures, a similar induced β-strand fit was observed for the interaction between other methyl-recognizing proteins and their methylated peptide binding partners. An example of a double tudor domain is that of the histone demethylase and transcriptional repressor, JMJD2A. The JMJD2A double tudor domain preferentially binds to di- and trimethylated H3K4 and H4K20 [47–49].

With regard to specificity, plant homeodomain (PHD) fingers constitute the most diverse class of histone binding modules. These modules recognize lysine in unmodified, mono-, di- and trimethylated states. Although not structurally related,

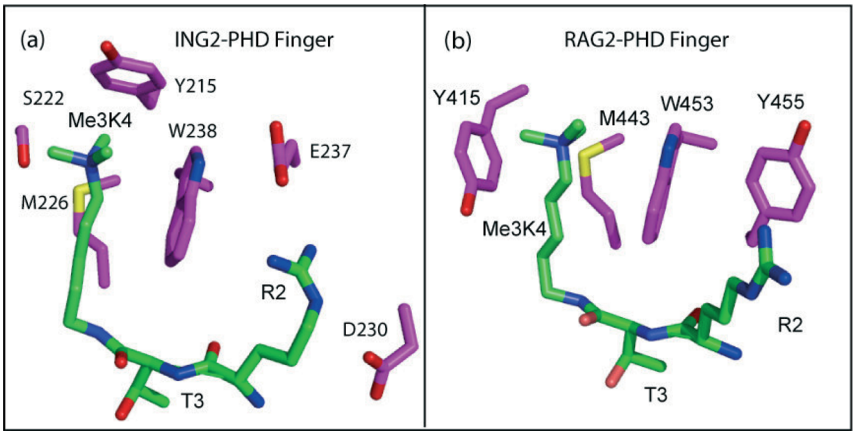

Figure 1.2 Structural comparison of (a) the ING2 [50] (PDB entry 2G6Q) and (b) the RAG2 [52] (PDB entry 2V89) PHD fingers bound to H3 peptides. The ING2-PHD finger binds Me_3K4 in an "aromatic cage," which is adjacent to an R2 binding pocket comprised of W238, E237 and D230. In the RAG2-PHD finger structure, Me_3K4 is enclosed in an "aromatic tunnel," while R2 is splayed outward due to the presence of Y455 and lack of a hydrogen bonding partner.

PHD fingers do share a few salient features with members of the tudor domain royal superfamily: most engage methyllysine through cation-π interactions and induce their peptide binding partners to adopt a complementary β-strand [46]. One of the first PHD fingers to be biochemically and structurally enumerated was that of inhibitor of growth 2 (ING2) [50]. ING2 is a member of the mSin3a-HDAC1 complex and, in response to DNA damage, stabilizes this complex at promoters of proliferation genes via interaction with trimethylated H3K4 [51]. The ING2-PHD finger binds trimethylated lysine by virtue of a two-residue aromatic cage. One of these residues, W238, forms the boundary for an adjacent groove to the H3K4 binding site that accommodates H3R2, which interacts with D230 and E237 (Figure 1.2). A homologous tryptophan has been observed in almost all methyl-binding PHD fingers characterized to date. Unlike the ING2-PHD finger, the RAG2-PHD finger (another trimethylated H3K4 binding module) binds in a conformation in which R2 is twisted out of plane with K4 [52, 53]. Interestingly, this difference in orientation and lack of interaction with a carboxylate permits methylation of R2 without a defect in binding. In fact, it has been suggested that symmetric dimethylation of R2 yields a very modest increase in affinity for peptides trimethylated at H3K4 – an added level of sophistication for the histone code reading capacity of the RAG2-PHD finger [53]. Furthermore, unlike the ING2-PHD finger, the RAG2-PHD finger does not engage trimethylated H3K4 in an aromatic cage (aromatic residues above and adjacent to the quaternary amine) but rather in what is dubbed an "aromatic tunnel" (aromatic residues are only adjacent to the quaternary amine; Figure 1.2). Functional evidence demonstrates that binding at trimethylated H3K4 by the RAG2-PHD finger is necessary for V(D)J recombination *in vivo* [52]. This finding provided the first

evidence for involvement of H3K4 trimethylation in a process other than regulation of gene expression. Interestingly, a W453R mutation in the RAG2-PHD finger disrupts this interaction [52] and results in Omenn's syndrome, a rare human immunodeficiency. In contrast to both the ING2 and RAG2-PHD fingers, the PHD finger of BHC80, a member of the LSD1 co-repressor complex, shows a preference for unmethylated H3K4 [54]. Binding of unmethylated H3K4 by the BHC80-PHD finger is characterized by an electrostatic interaction and hydrogen bonding in addition to steric exclusion of methyl groups. Also, unlike the RAG2 and ING2 PHD fingers, the BHC80-PHD finger-induced β-strand extends to R8 of a histone H3 peptide. Binding activity of the BHC80-PHD finger is essential for LSD1-mediated gene repression and appears to function downstream of H3K4 demethylation by retaining LSD1 at target promoters [54]. A recent study revealed that the autoimmune regulator (AIRE)-PHD finger also recognizes unmethylated lysine and links this process to transcription of tissue-restricted antigens [55].

Perhaps most intriguing are proteins capable of reading a histone code with multiple histone binding domains. Multivalent engagement results in a more favorable Gibbs free energy (ΔG) of binding relative to monovalent association. This is because multivalent binding increases the heat liberated upon association (enthalpy) with minimal entropic penalty due to pre-organization of the domains [2]. An example of a protein that utilizes multiple domains for histone binding is TAF1 (formerly $TAF_{II}250$), the largest subunit of the TFIID complex, which is responsible for initiation of transcription. In this protein, the binding pockets of two bromodomains are separated by ∼25 Å, making them ideally situated to simultaneously recognize and bind acetylated lysines 5 and 12 or 8 and 16 of histone H4 [56] (Figure 1.3). H4 peptides diacetylated at these positions bind ∼28 and ∼7 times more

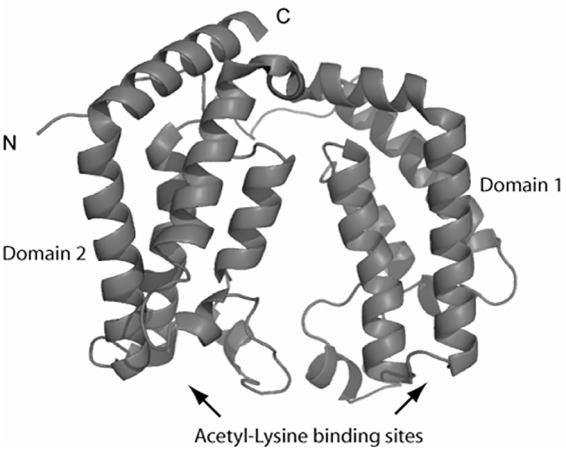

Figure 1.3 Structure of double bromodomain of TAF1 [56] (PDB entry 1EQF). Because the TAF1 bromodomains are separated by ∼25 Å, they are proposed to simultaneously bind acetylated lysines 5 and 12 or 8 and 16 of histone H4.

tightly (respectively) than a peptide monoacetylated at lysine 16. A second example of a histone binding protein with the potential for combinatorial histone code reading is the bromodomain and PHD domain transcription factor (BPTF), the largest subunit of the ATP-dependent chromatin remodeling complex, nucleosome remodeling factor (NURF) [57]. BPTF is of particular interest because it bears two different histone-reading modules: a bromodomain and a PHD finger domain, which are appended by an α-helical linker. The PHD finger binds di- and trimethylated H3K4 but the role of the bromodomain is unclear. Recognition of H3K4 by the BPTF subunit may be involved in NURF-mediated ATP-dependent chromatin remodeling and *Hox* gene expression during development [58]. While TAF1 and BPTF are individual proteins with the capacity to recognize dual covalent histone modifications, the yeast Rpd3S HDAC complex contains two proteins with domains that collaborate to recognize methylated H3K36 [59]. The Rpd3S HDAC complex is responsible for suppressing cryptic initiation of transcription within coding regions of a gene by histone deacetylation. This is achieved when the Rpd3S complex is targeted to active sites of transcription by association of the chromodomain of the Eaf3 subunit and the PHD finger of the Rco1 subunit with methylated H3K36. In this cooperative binding event, the Rco1-PHD finger is primarily responsible for providing affinity for nucleosomes, while the Eaf3 chromodomain contributes specificity for the methylated H3K36 mark [59, 60]. These examples demonstrate how multiple domains can cooperate to read a histone code with high fidelity and enhanced affinity.

1.5
Histone Modification Cross-talk

The crux of the histone code is manifested by a variety of cross-regulation or "cross-talk" mechanisms, which serve to regulate the activities of histone binding proteins/modifying enzymes [61]. The most obvious form of regulation is the mutual exclusivity of some modifications. For example, a di- or trimethylated lysine residue cannot be acetylated by a HAT. The occurrence of regularly spaced modifiable amino acids on histones can facilitate antagonistic or agonistic effects on the activities of histone binding proteins/modifying enzymes. An intriguing example involves the interplay between asymmetric trimethylation of H3R2 and trimethylation of H3K4 [62–64] (Figure 1.4). A quantitative chromatin immunoprecipitation study revealed that while H3K4 trimethylation occurs at the 5′ region of actively transcribed genes, asymmetric dimethylation of H3R2 is found within the body of genes or at inactive promoters in a mutually exclusive manner [62]. After identifying PRMT6 as the methyltransferase responsible for asymmetric methylation of R2 *in vivo*, Guccione *et al.* demonstrated that immunoprecipitated PRMT6 can methylate an unmodified H3 peptide but not an H3K4 trimethylated version [63]. An ASH2/WDR5/MLL family methyltransferase complex, which is responsible for H3K4 trimethylation *in vivo*, can methylate an unmodified H3 peptide, but does not tolerate asymmetric dimethylation at R2. This finding was ascribed to

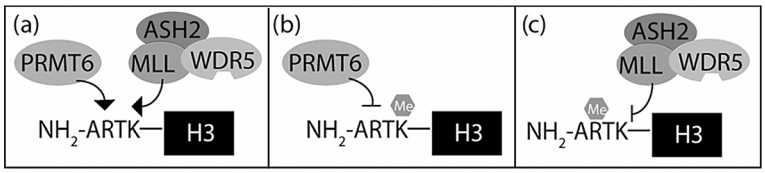

Figure 1.4 Example of histone cross-talk on histone H3. PRMT6 and the ASH2/WDR5/MLL complex are capable of methylation of H3R2 and H3K4, respectively (a). PRMT6 cannot methylate R2 in the presence of H3K4 trimethylation (b). Asymmetric dimethylation of R2 prevents interaction of WDR5 with the H3 tail and subsequent methylation by the MLL complex (c). Trimethylation of H3K4 is a hallmark of active promoters, while asymmetric dimethylation of H3R2 is typically associated with inactive promoters.

disruption of binding of the WDR5-WD40 repeats with H3K4 when H3R2 was dimethylated, a previously observed interaction [46]. A similar study in yeast found that trimethylation of H3K4 by the Set1 methyltransferase complex was abrogated in the presence of asymmetrically dimethylated H3R2. Ablation of enzymatic activity was attributed to disruption of the interaction of the Spp1-PHD finger with H3K4, a component of the Set1 complex that is necessary for trimethylation [64]. These studies reveal how modification cross-talk can be leveraged to achieve transcriptional regulation.

A recent study illustrates the complex interplay of histone modifications in regulation of the interaction with the transcription factor, TFIID [65]. The TFIID complex, which consists of several subunits, is involved in initiation of transcription. Genome-wide surveys [22, 66] revealed that TAF1, the largest TFIID subunit, was frequently found in regions with high distributions of H3K4 trimethylation as well as H3K9 and H3K14 acetylation. Using a quantitative mass spectrometry approach, Vermeulen *et al*. discovered that selective anchoring of TFIID to the nucleosome was facilitated in a trimethylated H3K4-dependent manner [65]. This was ascribed to interaction of the TAF3-PHD finger with trimethylated H3K4. *In vitro* binding assays with H3 peptides revealed that the TAF3-PHD finger has ~10-fold increased affinity for trimethylated H3K4 relative to dimethylated H3K4. Furthermore, asymmetric dimethylation of H3R2 resulted in a >25-fold loss in binding affinity for peptides that were trimethylated at H3K4. Interestingly, acetylation of H3K9 and H3K14 enhanced the affinity of TFIID for H3K4 trimethylated peptides in a pull-down assay. Vermeulen *et al*. suggest that the stronger interaction is due to the combined binding of the TAF3-PHD finger and the TAF1 tandem bromodomain. However, the superior affinity of the TAF3-PHD finger for trimethylated H3K4 is thought to be the dominant driving force behind the interaction. In support of this hypothesis, ablation of the PHD finger interaction reduced the mRNA levels of a variety of genes [65].

The examples of cross-talk in the histone code noted thus far involve events occurring on a single histone, or *in cis*. Cross-talk also occurs *in trans*, that is, between histone molecules. A good example of histone cross-talk *in trans* involves the link between H2B monoubiquitination at H2BK120 (H2BK123 in *Saccharomyces cerevisiae*) and methylation at H3K4 and H3K79, both of which are hallmarks of

transcriptionally poised chromatin. During transcription in budding yeast, monoubiquitination of H2B precedes di- and trimethylation of H3K4 and trimethylation of H3K79 [61]. Mechanistic insight for the requirement of H2B ubiquitination came with the recent discovery that ubiquitination of H2BK123 in yeast mediates interaction with Cps35, a subunit necessary for the H3K4 methyltransferase activity of COMPASS, the yeast homology of the MLL complex [67]. Interestingly, monomethylation of these residues is not linked to ubiquitination of H2B [68, 69]. Another instance that highlights cross-talk *in trans* involves the activity of UTX, a JmjC-domain containing protein. UTX mediates H3K27 demethylation, which is linked to H3K4 trimethylation and downregulation of H2A ubiquitination [70, 71]. During retinoic acid signaling, a UTX/MLL complex is recruited to *HOX* genes and facilitates transcription by methylating H3K4 and demethylating H3K27. Demethylation of H3K27 blocks chromodomain-mediated interaction with polycomb repressive complexes (PRCs) and subsequent ubiquitination of H2A. The combined action enables transcription of *HOX* genes.

1.6 Inhibitors of DNMTs and HDACs

Because proper epigenetic regulation is essential for normal functioning of the genome, there is burgeoning interest in the development of drugs that target epigenetic misregulation. A dynamic epigenetic model for complex disease suggests that a pre-epimutation (epigenetic occurrence that predisposes one to disease) is compounded by a variety of factors that occur over time [72]. These factors may include the effects of tissue differentiation, hormones and environment. The model predicts that a critical threshold is reached at some point and this results in a disease phenotype. Most studies of epigenetic misregulation involve its role in carcinogenesis and tumor progression. Increasing evidence suggests that epigenetic dysfunction is associated with several types of cancer [72]. Unlike genetic information, epigenetic marks are potentially reversible and therefore represent an opportunity to ameliorate the disease phenotype.

Aberrant DNA methylation can be manifested in two ways: genome-wide hypomethylation and promoter-specific hypermethylation. Genomic hypomethylation is associated with instability as well as the formation of abnormal chromosome structures [1]. In contrast, promoter-specific hypermethylation leads to transcriptional inactivation of genes (notably tumor suppressors). Examples of genes inactivated by inappropriate DNA methylation include those of the cell cycle inhibitor, $p16^{INK4a}$, and the DNA repair proteins BRCA1 and hMLH1 [73]. Because aberrant DNA hypermethylation is often associated with cancer, there have been significant efforts to develop therapeutic agents to target DNMTs. Small molecule inhibitors of DNMTs fall into two classes: nucleoside analogs and non-nucleoside analogs (Figure 1.5). Nucleoside analogs are cytidine mimics that become phosphorylated and incorporated into DNA and/or RNA once inside the cell. These molecules function as irreversible inhibitors in which a covalent DNMT intermediate is trapped

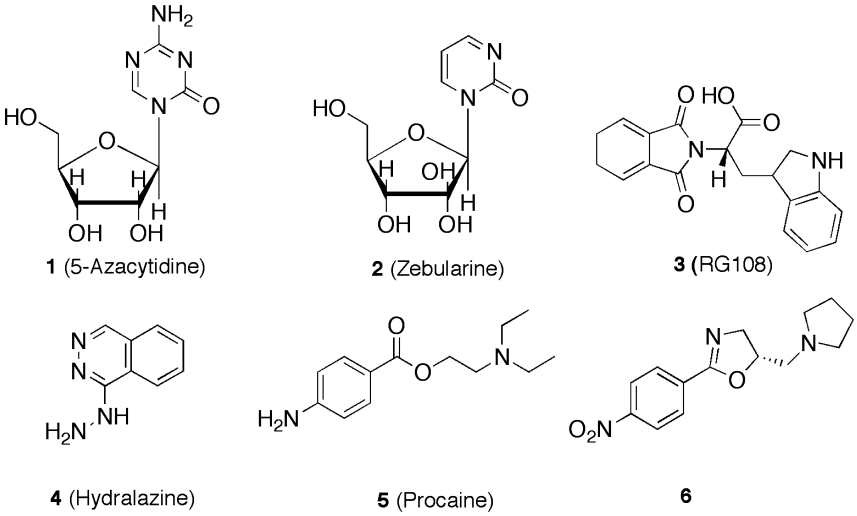

Figure 1.5 Selected small molecule inhibitors of DNMTs. **1** and **2** are irreversible nucleoside inhibitors while **3**, **4**, **5** and **6** are reversible small molecule inhibitors.

at the 6-position of the cytosine ring. In the case of 5-azacytidine (5-Aza-CR; **1**), β-elimination cannot release the DNMT intermediate due to the absence of an acidic hydrogen atom at the 5-position. Zebularine (**2**), which lacks an exocyclic amino group at the 4-position of the ring, does not undergo β-elimination for reasons that are not completely understood. Unfortunately, nucleoside analogs are frequently pleiotropic, often exhibit significant toxicity and have limited stability in aqueous solution. Although zebularine is stable in aqueous solution, it has poor bioavailability [74]. It is unclear whether the effects of nucleoside analog inhibitors are due to decreasing DNA methylation or DNMT depletion [72].

Due to the limitations of nucleoside-mimic DNMT inhibitors, there is considerable interest in developing non-nucleoside inhibitors. For example, RG108 (**3**), a phthaloyl tryptophan derivative was identified in a small molecule screen as a DNMT1 active site inhibitor [75]. RG108 inhibits methyltransferase activity *in vitro* and reduces global methylation levels in human cancer cells [76]. Hydralazine (**4**), a vasodilating drug, is reported to inhibit DNA methylation [77]. Surprisingly, in a recent Phase I study, hydralazine reactivated tumor suppressor genes in cervical cancer patients without altering global methylation levels [78]. Treatment with procaine (**5**), an anesthetic drug, afforded a 40% decrease in cystosine methylation in MCF-7 breast cancer cells [79]. However, *in vitro*, procaine does not exhibit DNMT inhibitory activity [76]. Recently, constrained analogs of the procaine scaffold were developed to increase potency [80]. One pyrrolidine derivative of procaine (**6**) exhibited modest inhibitory activity against DNMT1, but did not appear to decrease global DNA methylation levels in human myeloid leukemia cells relative to treatment with procaine [80]. Natural products such as psammaplin A and

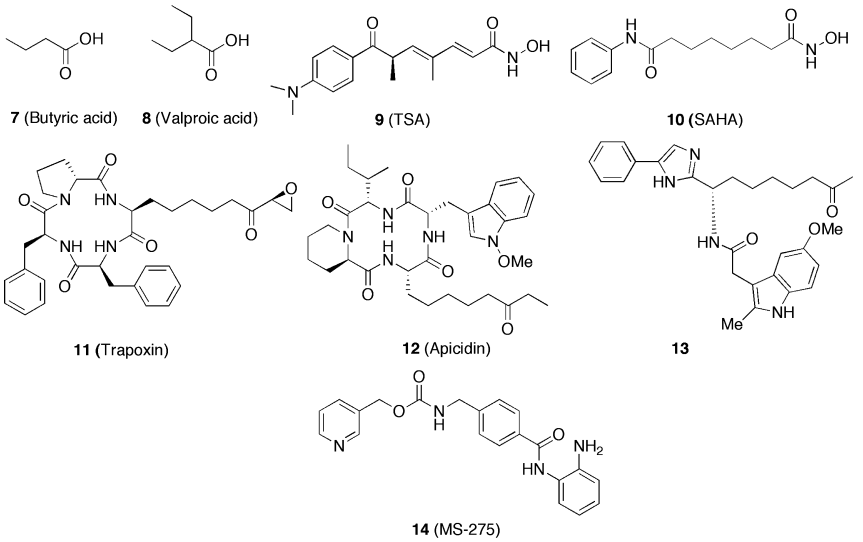

Figure 1.6 Selected small molecule inhibitors of HDACs. Compounds **7** and **8** are short-chain fatty acids, compounds **9** and **10** are hydroxamic acids, compounds **11** and **12** are cyclic tetrapeptides, compound **13** is cyclic tetrapeptide analog and compound **14** is a benzamide.

(−)-epigallocatechin-3-gallate (EGCG) are being evaluated as DNMT inhibitors [1]. Finally, MG98, an antisense oligonucleotide designed to repress expression of *DNMT1* has shown mixed results in clinical trials [72].

HDACs represent another class of epigenetic therapeutic targets. As with aberrant DNA methylation, inappropriate HDAC activity is linked to transcriptional misregulation. At the cellular level, HDAC inhibitors facilitate apoptosis of tumor cells and may control host immune responses and tumor vasculature [32]. To date, the Zn^{2+}-dependent Class I and II HDACs have received the most attention. Inhibitors of Class I and II HDACs include short-chain fatty acids, hydroxamic acids, benzamides and cyclic tetrapeptides [1] (Figure 1.6). Short-chain fatty acids such as butyrate (**7**) and valproic acid (**8**) were the first HDAC inhibitors to be identified. However, these compounds elicit inhibition in the millimolar range, suffer from poor bioavailability and are non-specific. Hydroxamic acids such as the natural product, trichostatin A (TSA) (**9**) and suberoyl anilide hydroxamic acid (SAHA; also known as Vorinostat and Zolinza) (**10**) are among the most successful HDAC inhibitors. TSA is a low-nanomolar Class I and II HDAC inhibitor that antagonizes the growth of non-small-cell lung cancer (NSCLC) at micromolar concentrations [81]. While SAHA is ∼30-fold less potent than TSA as an HDAC inhibitor, it has had considerable therapeutic success [72]. SAHA has shown anticancer activity in Phase I studies of refractory hematologic and solid tumors and a partial response in Phase II refractory cutaneous T cell lymphoma (CTCL) studies. Recently, SAHA received FDA approval for the treatment of CTCL-induced skin lesions [72]. The therapeutic success of

SAHA may be due to its ability to inhibit multiple HDACs with moderate efficiency. Cocrystal structures of the HDAC core with TSA and SAHA have been useful in dissecting the molecular details that confer HDAC inhibition [82]. In these structures, the inhibitors coordinate to an active site zinc and engage in a series of hydrogen bonds via their hydroxamic acid moieties. The hydroxamic acids are tethered to an aromatic capping group by an aliphatic linker that participates in multiple van der Waals contacts throughout a tubular pocket. The identity of the capping group appears to confer specificity. For example, HDAC6 is not inhibited by compounds with cyclic tetrapeptide capping groups [30].

Cyclic tetrapeptide natural products such as trapoxin (**11**) and apicidin (**12**) are potent HDAC inhibitors. Trapoxin is an irreversible inhibitor that features an electrophilic α-epoxyketone linked to a peptide capping group. Apicidin, in contrast, exploits an ethyl ketone as its zinc-binding group. Apicidin shows antiproliferative activity against a variety of cancer cell lines by a mechanism that appears to involve induction of the $p21^{WAF1/Cip1}$ gene [72]. Recently, nonpeptidic analogs of apicidin were developed to improve potency and selectivity in HDAC inhibition [83, 84]. A 4-phenylimidazole derivative (**13**) with a methyl ketone zinc-binding group selectively inhibits HDACs 1, 2, 3 (IC_{50} values ∼100 nM) and HDAC 6 (IC_{50} ∼300 nM). HDACs 4, 5, 7 are not inhibited by **13** at levels as high as 10 μM. The 4-phenylimidazole derivative displayed submicromolar IC_{50} antiproliferation activity against cervical, colon and kidney cell lines [84]. This compound also inhibits tumor growth in a xenograft model.

Benzamides constitute a fourth class of HDAC inhibitors. One example, MS-275, is a phenylenediamine derivative that exhibits robust HDAC inhibition in patients with advanced myeloid leukemia as well as refractory solid tumors or lymphoma in Phase I studies [72]. MS-275 is currently in Phase II trials. In a recent study aimed at optimizing the benzamide scaffold, several bis-(aryl) type analogs were synthesized and evaluated for their activity against a panel of HDACs [85]. Moradei *et al.* found that a thienyl substitution para to the free amino group in the phenylenediamine core rendered inhibitors specific for HDACs1, 2 with potency superior to that of MS-275. Isoform-specific inhibitors should aid in dissecting the roles of HDACs in normal cellular functioning and cancer.

What does the future hold for pharmacological epigenetic modulators? While there will undoubtedly be improvements in HDAC and DNMT inhibitor design, there is increasing interest in using epigenetic modulators in combination therapy (e.g. to sensitize tumors to cytotoxic agents or radiation) [72]. HDAC and DNMT inhibitors might be used together to achieve gene reactivation. Combination therapy has had some success in the case of patients with myelodysplastic syndrome and acute myeloid leukemia (AML) [86]. Another direction in epigenetic drug discovery might be to target interactions between histone binding modules and their cognate binding partners. There have already been reports of small molecules that block the interaction between bromodomains and nonhistone proteins such as HIV-1 Tat [87, 88] and p53 [89]. Further study of the biological modes of action of epigenetic modulators and the continued identification of new epigenetic biomarkers will be essential.

1.7 Conclusions

In this postgenomic era, the ultimate step in understanding how genotype translates to phenotype will require unraveling the language of epigenetics. This pursuit will involve the development and implementation of new technologies to map the epigenome with increasing resolution as well as improving our understanding of the dynamic interplay that represents the histone code. Deciphering the molecular basis for epigenetic regulation will facilitate the development of new drugs and therapies and provide a fertile area of research for years to come.

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