Journal of Cellular Biochemistry 96:1137–1148 (2005)

Histone Modifying Enzymes and Cancer: Going beyond Histones

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Abstract Mutations in the molecular pathways that regulate cell proliferation, differentiation, and cell death all contribute to cancer formation. Enzymes that covalently modify histones affect these pathways by controlling the dynamic remodeling of chromatin structure. This article reviews several connections between histone modifying enzymes and cancer that are likely mediated via both histone and non-histone substrates. We propose that multiple protein modifications, including phosphorylation, methylation, and acetylation, cross regulate one another to coordinate intermolecular signaling, and that miscues in this regulation can lead to oncogenesis. J. Cell. Biochem. 96: 1137–1148, 2005. © 2005 Wiley-Liss, Inc.

Key words: chromatin; histone acetyltransferase (HAT)/deacetylase (HDAC); histone methyltransferase (HMT); Aurora kinase; cancer

For a long time, cancer research has focused on the identification of genetic mutations that promote oncogenesis. In many cases, such mutations affect patterns of gene expression in the cancer cell, which in turn affect cell identity and/or cell growth controls. Recently, epigenetic factors that affect these processes have also been implicated in oncogenesis. Heritable alterations in chromatin structure or DNA methylation that alter the expression of tumor suppressor genes or oncogenes are associated with particular types of cancer [Hake et al., 2004]. Many excellent reviews have been written on the role of DNA methylation in cancer [Issa, 2004]. Here we will focus on the role of chromatin alterations and histone modifying enzymes in oncogenesis. First we will review the elements of chromatin and mechanisms of chromatin remodeling. Then, we will describe several examples of alterations in histone modifying enzymes in specific types of cancer. We will next discuss how modification of non-histone substrates by these enzymes might contribute to cancer formation. Finally, we propose that cross regulation of multiple modifications within a given protein provide a means for fine-tuning protein functions and discuss the implications of such regulation on cancer formation and treatment.

CHROMATIN STRUCTURE

The eukaryotic nucleus is only about 6 μM in diameter, but nearly 2 m of DNA are packaged into chromatin within this small space [Alberts, 2002]. The basic building blocks of chromatin are nucleosomes, which contain 146 bp of DNA wrapped nearly twice around an octamer composed of two copies of each core histone protein, H2A, H2B, H3, and H4 [Strahl and Allis, 2000; Jenuwein and Allis, 2001]. Linker histones (e.g., H1) and other non-histone proteins further pack nucleosomes and intervening linker DNA into higher order chromatin structures [Gregory and Shiekhattar, 2004]. Interactions between the globular domains of each of the core histones form the histone octamer. The histone NH_2-terminal tails, which lie outside of the nucleosome, are not important for octamer formation but influence nucleosome-nucleosome

Grant sponsor: NIH; Grant number: GM067718; Grant sponsor: Robert A. Welch Foundation; Grant number: G1371.

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Received 22 July 2005; Accepted 25 July 2005

DOI 10.1002/jcb.20615

interactions as well as interactions between nucleosomes and regulatory factors.

Chromatin can be remodeled by a variety of mechanisms. Large, multisubunit complexes typified by SWI/SNF use the energy of ATP to alter histone-DNA contacts, ultimately causing nucleosome relocation. Substitution of histone variants for canonical histones can alter nucleosome structure and also provides a means for resetting histone modifications. Finally, the histone NH2 tail domains are subject to multiple covalent post-translational modifications, including acetylation of lysines (K), methylation of lysines and arginines (R), phosphorylation of serines (S) and threonines (T), ubiquitylation and sumoylation of lysines, as well as ADP-ribosylation [Strahl and Allis, 2000; Jenuwein and Allis, 2001]. These modifications alter histone-DNA interactions, nucleosome-nucleosome interactions, and the interactions of non-histone regulatory proteins with chromatin. Enzymes that add or remove histone modifications regulate chromatin organization and affect a wide range of DNA-based events including transcription, replication, recombination, and repair, as well as chromosome condensation and nuclear organization [Hake et al., 2004].

Not surprisingly, aberrations in chromatin remodeling are associated with cancer formation. For example, the mammalian homologues of the SNF2 ATP-dependent chromatin remodeling subfamily, BRM and BRG1, help to control cell-cycle progression through their interactions with the Rb tumor suppressor. Mutations in BRG1 have been found in multiple human cancer cell lines [Gregory and Shiekhattar, 2004]. DNA damage-induced phosphorylation of H2A variant, H2A.X, is important to repair of double-strand breaks and to the maintenance of genomic stability [Hake et al., 2004]. The human genomic locus for H2A.X has been mapped to 11q23.3, a region that is often mutated in human cancers [Hake et al., 2004]. As discussed in detail below, aberrant activity or mistargeting of histone modifying enzymes appears to play a direct role in abnormal cell proliferation and cancer development [Gregory and Shiekhattar, 2004; Hake et al., 2004].

HISTONE ACETYLTRANSFERASES (HATS)/ DEACETYLASES (HDACS) AND CANCER

One of the major functions of histone acetylation is to open chromatin to allow transcription factors to gain access to regulatory elements in the DNA [Fischle et al., 2003]. Acetylation neutralizes the positive charge of the epsilon amino group of lysine residues, loosening interaction between histones and the negatively charged DNA. The acetyl moiety can also provide a specific binding site for bromodomain containing proteins, which help to recruit other transcription proteins [Strahl and Allis, 2000]. Interestingly, recent studies have shown that acetylation is not always limited to the NH2 tail regions of histones. Lysine 56 of H3, which is located at the edge of the globular domain of this histone, can be acetylated [Xu et al., 2005], and K56 acetylation facilitates recruitment of the SWI/SNF remodeling complex [Xu et al., 2005].

Two classes of enzymes reversibly control the acetylation level of histones: histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, transcriptional activators recruit HATs, whereas transcriptional repressors and co-repressors associate with HDACs [Di Gennaro et al., 2004].

Translocation, amplification, overexpression, or mutation of *HAT* genes occurs in a variety of cancers, especially those of hematological and epithelial origin [Gibbons, 2005]. For example, Rubenstein–Taybi syndrome (RTS) is a developmental disease connected with the loss of CBP, a HAT that acts as a trancriptional transactivator (for review, see [Gibbons, 2005]). RTS patients have a dramatically increased susceptibility of cancer [Gibbons, 2005]. Mice heterozygous for a CBP deletion have a phenotype similar to RTS and ultimately develop leukemia [Gibbons, 2005], supporting the idea that loss of CBP function is associated with cancer formation. Loss of heterozygosity of p300, which is highly related to CBP, is correlated with formation of colorectal and gastric tumors in humans [Gibbons, 2005]. CBP, p300, and other HATs such as MOZ and MORF, are subject to chromosomal translocations linked with hematological malignancy [Gibbons, 2005]. Together, these findings suggest that these HATs are tumor suppressors [Gibbons, 2005].

HDACs facilitate chromatin folding and also affect the binding of regulatory factors to nucleosomal targets. These enzymes are classified into three different groups (I, II, III) on the basis of their sequence homology to the yeast HDACs [Kouzarides, 1999]. Class I HDACs are similar to yeast Rpd3 and associate with transcriptional repressors and co-factors in the

nucleus [Di Gennaro et al., 2004]. Class II HDACs are similar to yeast Hda1 and are large proteins that shuttle between the cytoplasm and nucleus. Both Class I and Class II HDACs are sensitive to the inhibitor trichostatin A (TSA) [Di Gennaro et al., 2004]. Class III HDACs are not sensitive to TSA, and like their yeast counterpart, Sir2, require nicotinamine adenine dinucleotide (NAD^+) as a cofactor [Di Gennaro et al., 2004].

Aberrant targeting of HDACs is associated with transcriptional silencing of tumor-suppressor genes such as p21. p21 is a cyclin-dependent kinase inhibitor that blocks cell-cycle progression from G_1 into S phase. p21 expression is defective in many different tumors, allowing uncontrolled cell divisions. HDAC inhibitors can reactivate p21 expression and thereby prevent tumor cell proliferation [Gibbons, 2005].

HDAC complexes can be targeted to specific genomic regions not only by interaction with sequence-specific DNA binding factors but also by methyl-DNA binding proteins [Gibbons, 2005]. For example, repression of the tumor suppressor $p16^{ink4a}$ gene in melanomas and solid tumors is often associated with DNA methylation and with the recruitment of a multifactor repressor complex that contains a DNA methyltransferase as well as HDACs to reinforce the silent state [Di Gennaro et al., 2004]. HDACs act as corepressors for oncogenic translocation product fusion proteins such as PML-RAR and AML:ETO in specific forms of leukemia and lymphoma [Di Gennaro et al., 2004].

HDAC inhibitors are currently in clinical trials as anticancer drugs [Di Gennaro et al., 2004]. Treatment of cells with inhibitors such as TSA and oxamflatin promotes cellular differentiation, cell-cycle arrest and apoptosis [Di Gennaro et al., 2004]. Only class I and II HDACs are sensitive to these drugs. These agents change the transcription of fewer than 2% of expressed genes [Di Gennaro et al., 2004], and the affected genes are predominantly involved in cell cycle, apoptosis, or DNA synthesis. HDAC inhibitors also show promise in combination with other drugs such as 5-azacytidine (5Aza) [Di Gennaro et al., 2004]. Combination therapy with both drugs can synergistically activate methylated genes such as $p16^{ink4a}$ in cultured cell lines [Di Gennaro et al., 2004].

Both HATs and HDACs target non-histone protein substrates, which are involved in transcription, nuclear transport, cytoskeleton, and signal transduction [Di Gennaro et al., 2004]. Therefore, the effects of HDAC inhibitors in cancer therapies may not be mediated solely through changes in histone acetylation, as discussed further below.

HISTONE METHYLTRANSFERASES (HMTS)/ DEMETHYLASE AND CANCER

Histone methylation is more complex than acetylation. Both lysines and arginines can be methylated. Lysines can be mono-, di-, or tri-methylated. Arginines can be either mono- or di-methylated, and arginine dimethylation may be asymmetric or symmetric [Noma et al., 2001]. The positions of many methylated residues in histones H3 and H4 have been mapped (Fig. 1). The complexities in the types and levels of methylation provide much regulatory potential as each event may have specific effects on chromatin structure and on the interactions of regulatory proteins with chromatin.

Histone methyltransferases transfer the methyl group from S-adenosylmethionine (SAM) to the side-chain nitrogen atoms of lysine or arginine residues. Based on the sequence and structure of their catalytic domains, these enzymes can be grouped into three classes, arginine methyltransferases, SET domain containing lysine methyltransferases, and Dot1-like lysine methyltransferases [Kim and Huang, 2003]. The arginine methyltransferases are important for transcriptional activation by nuclear hormone receptors [McBride and Silver, 2001]. Lysine methyltransferases are involved in both gene activation and repression, as well as the process of transcription.

Several SET domain containing proteins have been implicated in cancer [Lund and van Lohuizen, 2004]. The SET domain is an evolutionarily conserved motif of 130 amino acids first recognized as a common element in a number of *Drosophila* genes encoding chromatin-related transcription factors, including *Su(var)3-9*, Enhancer of *Zeste(E(Z))*, and *Trithorax(trx)* [Jenuwein et al., 1998]. Based on similarities between human SET domains, and their relationship to yeast SET domain proteins, four SET domain sub-families have been identified: the SUV39 family, the SET1 family, the SET2 family, and RIZ family [Kouzarides, 2002].

The SUV39 protein was the first histone methyltransferase to be identified [Rea et al.,

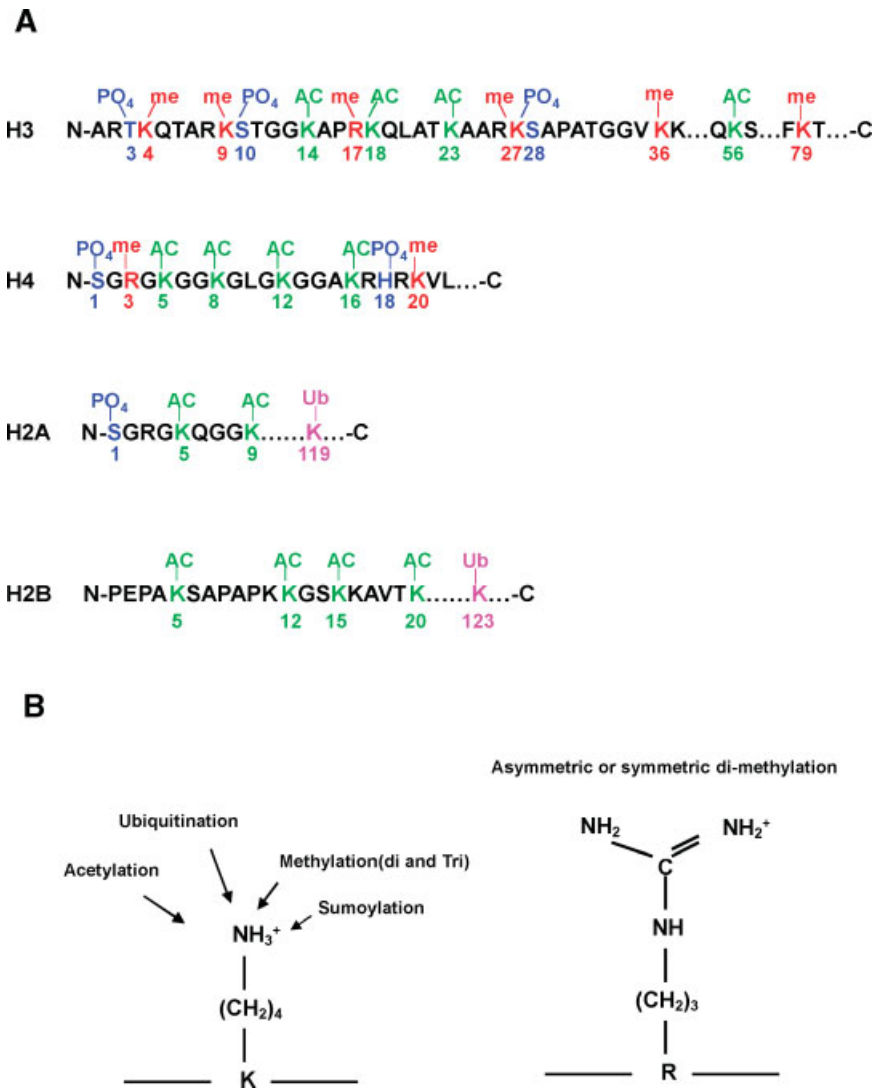

Fig. 1. Post-translational covalent modification of histones. **A**: Presently known covalent modifications of histones H3, H4, H2A, and H2B. For simplicity, only mammalian sequences are shown. Methylation is represented in red. Acetylation, phosphorylation, and ubiquitination are shown in green, blue, and purple, respectively. In budding yeast H2B S10 is phosphorylated. The corresponding residue in human H2B, S14, is a potential phosphorylation site. **B**: Multiple, competing modifications can occur at lysine and arginine residues. Lysines can be acetylated, ubiquitylated, sumoylated, or mono-, di-, or trimethylated. Arginines can be mono- or di-methylated, and dimethylation can be asymmetric or symmetric.

2000], and it catalyzes the methylation of K9 in H3 [Peters et al., 2001]. Different methylation states (mono, di, tri) of H3 K9 are enriched in different regions of the genome, indicating they may serve distinct functions [Wu et al., 2005]. Mice carrying deletions of two highly related SUV39 family members, Suv39h1 and h2 suffer dramatic genome instability, associated with a substantital loss of H3 K9 methylation [Peters et al., 2001]. Approximately 30% of Suv39h1/h2 null mice develop B cell lymphomas, and cells from these tumors contain non-segregated, "butterfly" chromosomes and elongated telomeres [Peters et al., 2001]. Human SUV39 h1/2 methyltransferases associate with the Rb tumor suppressor. The interaction between Rb and Suv39H1 leads to H3 K9 methylation at the promoters of Rb target genes such as E2F, and is associated with their repression [Hake et al., 2004]. Rb cooperates with multiple chromatin remodeling activities including HDACs, HMTs, DNMTs, and ATP-dependent chromatin remodeling factors to regulate gene expression. Aberrations in any of these activities can contribute to defects in RB pathway-related carcinogenesis [Hake et al., 2004].

RIZ1 is another H3 K9 methyltransferase. This enzyme was originally identified as an Rb-binding protein, but it also serves as a coactivator for the estrogen receptor [Lund and van Lohuizen, 2004]. Since H3 K9 methylation is usually involved in gene repression, this observation suggests that RIZ1 may have another target important for gene activation. The *RIZ1* gene maps to chromosome 1p36, a region that is frequently deleted in human cancers [Gibbons, 2005]. Reduced expression of RIZ1 is observed in several types of cancer, including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma [Gibbons, 2005]. Furthermore, RIZ1 knock out mice develop B cell lymphomas [Gibbons, 2005], indicating that loss of RIZ1 function leads to cancer formation.

The SET1 family of methyltransferases is involved in both gene activation and repression [Strahl and Allis, 2000].This family includes yeast Set1 (the founding member), hSET1A/B, Mll proteins, human polycomb group (PcG) proteins, enhancer of zeste (EZH), and trithorax (TRX) proteins, as well as the TRX-related protein ALR [Schneider et al., 2002]. Recent studies show that hSET1 and MLL proteins are part of complexes that contain the WD-40-repeat proteinWDR5 [Wysocka et al., 2005]. WDR5 binds directly to methyl-K4 in histone H3 and is important for the transition of dimethyl lysine to trimethyl lysine [Wysocka et al., 2005]. WDR5 plays a critical role in *HOX* gene activation and vertebrate development [Wysocka et al., 2005]. MLL proteins have also been extensively studied as a result of their linkage to cancer. The *MLL* gene is translocated in mixed lineage leukemias. Interestingly, the SET domain is usually absent from the oncogenic fusion proteins, so loss of HMT activity might contribute leukemogenesis [Kouzarides, 2002]. Alternatively, the MLL translocation products may provide a gain-of-function for other domains of MLL that inhibits hematopoiesis and facilitates transformation.

EZH2 is an H3 K27 methyltransferase that is a component of the PcG complexes PRC2 and PRC3 [Gibbons, 2005]. Another component of these complexes, SUZ12, cooperates with DNA binding factors to recruit the PcG complexes to specific sites [Gibbons, 2005]. EZH2 is required for cell proliferation, and upregulation of EZH2 has been observed in prostate cancer, lymphomas, and breast cancer [Gibbons, 2005]. SUZ12 is also upregulated in a variety of cancers [Gibbons, 2005]. However, since EZH2 functions in cell proliferation, it is not clear whether over expression of this enzyme is a cause of malignancy or a secondary effect of enhanced proliferation in cancer cells.

Another methylation event related to gene activity is methylation of K36 in H3, which is mediated by Set2 [Schneider et al., 2002]. The putative mammalian homolog of Set2, NSD1, has not yet been shown to possess methyltransferase activity [Schneider et al., 2002]. NSD1 is a co-regulator of certain nuclear hormone receptors [Schneider et al., 2002]. Interestingly, the *NSD1* gene is fused with the *NUP98* gene in a recurrent cryptic translocation, t (5:11)(q35; p15.5), found in some acute myeloid leukemias [Schneider et al., 2002].

The human homolog of yeast Dot1, hDOT1L, is also a H3 K79 specific methyltransferase, and recent studies relate this protein to cancer [Okada et al., 2005]. hDOT1L physically interacts with AF10, which is an MLL fusion partner in certain leukemias [Okada et al., 2005], suggesting that hDOT1L may interact with MLL-AF10 fusion proteins. Interestingly, an artificial MLL-hDOT1L fusion protein is capable of transforming murine myeloid progenitor cells, and the methyltransferase activity of hDOT1L is required for maintenance of this transformation [Okada et al., 2005]. MLL regulates *Hox* genes relevant to leukemia, and elevated expression of Hox7 and Hox9 is observed in both AF10-MLL and MLL-hDOT1L transformed cells [Okada et al., 2005]. Methylation of H3 lysine 79 is increased in AF10-MLL transformed cells [Okada et al., 2005], further indicating that hDOT1L may be recruited by this oncogenic fusion protein. Knocking down the expression of hDOT1L by RNAi inhibits cell proliferation [Okada et al., 2005], suggesting that hDOT1L might function as an oncogene. If so, it may be a useful target for cancer therapies [Okada et al., 2005].

PATHWAYS FOR HISTONE DEMETHYLATION

Histone methylation is generally more prevalent than acetylation. In addition, until recently, no demethylase activities had been identified. These observations led to the idea that histone methylation may be more permanent mark than acetylation, which is dynamically governed by HATs and HDACs. H3 K4

methylation has even been suggested to provide a "memory" of gene transcription [Turner, 2002; Ng et al., 2003]. However, turnover of methyl groups on histones might be achieved by proteolytic clipping to remove the entire histone tail [Allis et al., 1980] or by replacing the methylated histone with a histone variant [Ahmad and Henikoff, 2002]. Several recent findings also suggest enzymatic alternatives for the removal of methyl groups from lysine or arginine. For example, a human arginine deiminase, PADI4/PAD4, opposes the function of arginine methylation by converting arginine to citrulline [Cuthbert et al., 2004]. Also, a nuclear homolog of amine oxidases, LSD1, directly demethylates H3 K4 [Shi et al., 2004]. LSD1 functions as a transcriptional repressor, consistent with its role in reversing H3 K4 methylation [Shi et al., 2004]. The identification of more histone demethylases and the elucidation of their functions is likely to be an active area in future chromatin research.

HISTONE PHOSPHORYLATION AND CANCER

Several histones and histone variants are subject to phosphorylation, including H1, H3, H2B, and H2A.X. These modifications are associated with large scale chromatin reorganization during processes such as mitosis, apoptosis, and DNA repair. As aberrant execution of these processes facilitates cancer formation, improper regulation, or function of the kinases that mediate histone phosphorylation can be oncogenic.

Phosphorylation of the linker histone H1 is associated with cell-cycle progression, and H1 phosphorylation has long been a marker for mitotic cells [Boulikas, 1995]. H1 is phosphorylated by numerous kinases in vitro, but in vivo, CDK2 is the major mitotic H1 phosphorylating activity. H1 phosphorylation peaks during metaphase, when chromosomes are maximally condensed. Elevated H1 phosphorylation is observed in many cancer cell cultures and tumors, but this may reflect the enhanced proliferation of these cells [Deshpande et al., 2005]. Phosphorylation of H3 at T3, S10, and S28 also peaks at mitosis [Nowak and Corces, 2004; Dai et al., 2005]. S10 and S28 phosphorylation is mediated by Aurora kinases [Andrews et al., 2003], but T3 phosphorylation is mediated by the Haspin kinase [Dai et al., 2005]. The Aurora kinases are highly conserved and control a number of mitotic events, including chromosome condensation, spindle dynamics, kinetochore-microtubule interactions, chromosome orientation, the establishment of the metaphase plate, and completion of cytokinesis [Andrews et al., 2003]. Although H3 S10 phosphorylation is conserved from yeast to mammals and provides a strong mitotic marker, its function in mitosis is not clear [Andrews et al., 2003]. H3 S10A mutations in *Tetrahymena* cause defects in chromosome condensation and segregation [Wei et al., 1999], but the same mutation in budding yeast has no obvious phenotype [Hsu et al., 2000]. Aurora B, one of three Aurora kinases in mammalian cells, catalyzes the phosphorylation of both S10 and S28 in H3 from prophase to metaphase [Nowak and Corces, 2004], but like S10 mutations, S28 mutations, or S10 S28 double mutations do not yield a mitotic phenotype [Hsu et al., 2000]. Phosphorylation of other substrates by these kinases may be more important to mitotic progression. Several kinetochore proteins and the histone H3 variant CENP-A, which associates with the centromere, are Aurora substrates [Andrews et al., 2003].

All three mammalian Aurora kinase family members (Aurora A, B, and C) are over expressed in many aggressive human cancers [Andrews et al., 2003; Katayama et al., 2003]. Aurora kinase over expression leads to defective chromosome segregation and aneuploidy, which are thought to be early steps in oncogenesis. INCENP and Survivin are required for Aurora B activation and accurate localization on chromosomes and the spindle midzone [Andrews et al., 2003]. Interestingly, expression of INCENP and Survivin is also elevated in many cancers [Adams et al., 2001].

Phosphorylation of an H2A variant, H2AX, is related to maintenance of genomic integrity [Sedelnikova et al., 2003; Bassing and Alt, 2004]. The phospho-H2AX isoform is referred to as γ-H2AX, and nuclear foci containing H2AX become evident soon after cells are exposed to agents that cause double strand DNA breaks (DSBs) or during natural DNA rearrangements, such as VDJ recombination. These foci contain several DNA repair and checkpoint factors, and γ-H2AX facilitates the loading of repair factors to the DNA break site (see [Bassing and Alt, 2004] for review). Three such factors bind specifically to the phospho-SQE motif in γ-H2AX, MDC1/NFBD1, 53BP1, and

NBS1 [Bassing and Alt, 2004]. The SQE motif is a consensus site for phosphorylation mediated by ATM and the related ATR and DNA PK_{cs} kinases [Bassing and Alt, 2004]. Mutations in all of these enzymes are related to human disease, including Ataxia telangiectasia (AT), Blooms syndrome (BS), and severe combined immunodeficiency (SCID). AT and BS patients exhibit high levels of chromosome rearrangements and are susceptible to cancer. Interestingly, H2AX p53 double mutant mice develop lymphomas associated with inappropriate recombination in B cells [Bassing and Alt, 2004]. γH2A X h as been proposed to directly participate in the DSB repair process by holding the broken ends in proximity to one another [Bassing and Alt, 2004]. Separation of the broken ends in the absence of γH2AX may underlie the enhanced occurrence of chromosome translocations seen AT patients. Recent studies indicate that γH2Ax also recruits the INO80 chromatin remodeling complex [Cairns, 2004] and the NuA4 HAT [Downs et al., 2004] to double strand breaks in yeast. Thus, this phosphorylated histone likely stimulates chromatin remodeling to allow repair.

H2B is phosphorylated by the Ste20 kinase in yeast and its homolog, Mst1, in humans [Ahn et al., 2005]. Loss of Mst1 leads to tissue overgrowth in *Drosophila* [Jia et al., 2003]. Interestingly, H2B phosphorylation is elevated during apoptosis and may be sufficient to trigger chromatin aggregation in cells programmed to die [Ahn et al., 2005]. Loss of H2B phosphorylation may inhibit apoptosis, contributing to the tissue overgrowth in Mst1 mutants [Jia et al., 2003]. In addition, *Drosophila* Mst1 phosphorylates an inhibitor of, DIAPI and regulates the expression of both DIAPI and cyclin E [Jia et al., 2003]. Thus, these enzymes control both cell division and cell death, indicating they function as tumor supressors. dMst1 physically associates with two other proteins, Salvador and Warts, which also serve as tumor suppressors in *Drosophila* [Udan et al., 2003].

HISTONE CODE HYPOTHESIS

Multiple histone modifications occur within a short stretch of amino acids on a given histone tail, and several studies indicate that the occurrence of one modification may affect the subsequent addition or function of other modifications [Jenuwein and Allis, 2001; Zhang and Reinberg, 2001]. This cross regulation usually occurs in *cis*. For example, phosphorylation of S10 can enhance acetylation of K14 in H3 [Cheung et al., 2000] and inhibit methylation of K9 [Rea et al., 2000]. Methylation of H3 K9, in turn, inhibits phosphorylation of S10 [Rea et al., 2000]. One case of trans-regulation of histone modifications has been reported to date. Ubiquitylation of histone H2B at K123 in yeast is required for methylation of K4 in H3 [Sun and Allis, 2002]. Particular modifications, or combinations of modifications, can control the binding of regulatory proteins to histones. Bromodomain proteins, for example, bind to acetylated-lysines [Strahl and Allis, 2000] and chromodomain proteins bind to methylated lysines [Jenuwein and Allis, 2001]. However, phosphorylation of S10 in H3 blocks binding of the chromodomain in HP1 to K9, indicating that modifications regulate not only the occurrence of other modifications but also their functions [Fischle et al., 2003]. Collectively, such studies indicate that histone modifications provide a highly regulated "code" that is read by histone binding proteins [Jenuwein and Allis, 2001]. This code provides an important epigenetic mechanism for the regulation and inheritance of gene expression states. As such, the pathology associated with mutations in histone modifying enzymes as described above may well reflect cascading alterations in multiple histone modifications that in turn affect several different transcription programs.

HISTONE MODIFYING ENZYMES, THEIR NON-HISTONE SUBSTRATES AND CANCER

Many histone modifying enzymes also use non-histone proteins as substrates (summarized in Table I). The activities of HATs, HDACs, and histone arginine methyltransferases on non-histone substrates are well documented [Kouzarides, 1999; McBride and Silver, 2001; Katayama et al., 2003], but only a few examples of lysine methylation within non-histone substrates have yet been reported [Chuikov et al., 2004; Kouskouti et al., 2004]. Most histone kinases have many other substrates, which may be as, if not more, important to their functions than the histones. Therefore, these enzymes are not listed in Table I.

The majority of the non-histone substrates identified for HATs, HDACs, and HMTs are

TABLE I. Non-Histone Substrates of Histone Modifying Enzymes

Enzymes	Non-histone substrates	Function
HATS		
CBP/p300	p53, p73, ELKF, HMGI(Y), TCF, NF-kB, MyoD, E2F1, GATA1, GATA2, GATA4, HNF4, MEF2, KLF5, STAT3, SP1, RUNX3, AML1, BETA2, NF-E2, ER81, Cart1, KLF13, IRF-2, SREBPS, BCL6, IRF-7, CREB-2, EST1, B-Myb, C-Jun, Fen-1, TAT, RelA, NF-Y, ER81, AML1/MDS1/EVI1	Transcription factors
	HMG1, HMG14	High mobility group proteins
	ACTR, SRC-1, TIF2, β-catenin, RB, RIP140	Transcription co-regulators
	TFIIE, TFIIF	General transcription factors
	Flap endonuclease-1, thymine DNA glycosylase, Werner DNA helicase	DNA metabolic enzymes
	Importin-α, Rch-1	Nuclear import factor
	α-Tubulin	Cytoskeletal protein
	Smad7	TGFβ signaling regulator
PCAF	MyoD, E2F, HIV TAT, p53, C-Myc, Ku70, GATA2, ER81, KLF13, NF-E4, NF-kB, IRF7, TAL1/SCL, HMGI(Y), AML1/MDS1/EVI1	Transcription factors
	CTIIA	Transcription co-regulators
	HMG17	High mobility group proteins
	TFIIE, TFIIF, TAF(I)68	General transcription factors
	IRF-2, IRF-7	Interferon regulatory factor
	PCAF (autoacetylation)	HAT
Gcn5	Sin1(yeast)	Transcription regulator
	C-Myb, C-Myc, p53	Transcription factor
TFIIB	TFIIB (autoacetylation)	General transcription factor
ESA1	ESA1 (autoacetylation)	HAT
TIP60	C-myc	Transcription factor
$TAF_{II}250$	TFIIE	General transcription factor
HDACs		
HDAC1	p53, KCF5, MyoD, RB, CIITA	Transcription factor
HDAC3	SRY, RelA, NF-kB	Transcription factor
HDAC6	HSP90	Chaperon protein
SIRT2	α-tubulin	Cytoskeletal protein
SIRT1	TAF(I)68, p53	Transcription factor
	p300	HAT
Histone Arginine methyltransferase		
PRMT1	hnRNPs	Heterogeneous nuclear ribonucleoproteins
	Sam68	Src mitotic substrate
	Stat1	Signal transducer and transcription regulator
Hmt1/Pmt1 (yeast)	Npl3/Hrp1	Yeast hnRNPs
PRMT5	snRNPs	Spliceosomal nuclear ribonucleoproteins
CARM1	p300	HAT
Histone Lysine methyltransferase		
Set9	p53	Transcription factor
	TAF10	General transcription factor
Set1 (yeast)	Dam1	Kinetochore protein

involved in gene regulation. In part, this observation may reflect the focus of the chromatin field on transcription. No doubt as the role of histone modifying enzymes in DNA replication, recombination, and repair becomes better defined, more non-histone substrates involved in these processes will also be identified. In addition, a few substrates have been identified that are not directly connected to DNA-templated processes. For example, Dam1, a yeast kinetochore protein, is methylated by Set1 [Zhang et al., 2005], and the nuclear import factor Importin α is acetylated by CBP/p300 [Di Gennaro et al., 2004].

Collectively, these observations indicate that histone modifying enzymes affect cell proliferation and differentiation in multiple ways in addition to chromatin remodeling. In particular, acetylation and methylation of several transcription factors, such as C-Jun, NF-kB, and E2F, impact key signal transduction pathways connected to carcinogenesis [Kouzarides, 1999].

IS THE HISTONE CODE LIMITED TO HISTONES?

Like histones, many non-histone proteins are subject to multiple, clustered post-translational

modifications. For example, the p53 tumor suppressor can be phosphorylated, acetylated, sumoylated, ubiquitylated, and methylated [Bode and Dong, 2004], and several of these modifications are located close to one another. Although little information is available regarding cross-regulation of post-translational modifications in non-histone proteins, "cross-talk" between different modifications provides a wealth of regulatory potential. In part, our lack of knowledge is due to difficulties in both predicting and detecting modifications such as acetylation or methylation. No consensus sites are yet available for substrate recognition by protein acetyltransferases or methyltransferases, and it is much more difficult to detect incorporation of ^{14}C- or ^{3}H-labeled acetyl- or methyl- moieties into proteins than it is to detect incorporation of ^{32}P. Although "pan" acetyl-K or methyl-K antibodies are commercially available, these often have limited sensitivity. Thus, detection of individual acetylation or methylation sites in a given protein usually requires protein purification and mass spectrometry and/or the development of antibodies specific for modified isoforms of the protein.

Phosphorylation and methylation in H3 at S10 and K9, respectively, have been proposed to act as a binary switch that controls the binding of HP1 and possibly other chromodomain proteins [Fischle et al., 2003]. Phosphorylation of S10 inhibits HP1 binding, and also inhibits methylation of K9 [Zhang and Reinberg, 2001]. Methylation of K9, in turn, inhibits phosphorylation of S10. Thus, this cassette may be more of a regulatory loop than a switch [Zhang and Reinberg, 2001].

So far, only three non-histone proteins, TAF10, p53, and Dam1, have been defined as substrates for SET-domain HMTs (Table I). p53 and TAF10 are both methylated by Set9, which also methylates K4 in H3. Dam1 is methylated by yeast Set1, another H3 K4 methyltransferase. Comparing the sequences of these proteins proximal to the methylation site does not reveal a consensus site, but strikingly, in every case the methylated lysine is adjacent to a serine or threonine (Fig. 2A). In most cases, this residue is phosphorylated, raising the possibility of cross-regulation between phosphorylation and methylation as seen at K9 and S10 in H3. Indeed, such regulation is observed in Dam1 [Zhang et al., 2005]. Methylation of K233 in this protein inhibits phosphorylation of one or more of the flanking serines at positions 232, 234, and 235 [Zhang et al., 2005]. These sites are phosphorylated by the Ipl1 Aurora kinase in vitro and S235 fits a consensus for Ipl1 phosphorylation in vivo [Zhang et al., 2005]. Loss of Set1 suppresses chromosome segregation defects that occur in *ipl1* mutant cells, consistent with the inhibition of Ipl1 functions by Set1. These data provide the first example of cross-regulation of serine phosphorylation and lysine methylation in a non-histone protein. It will be interesting to determine if such regulation also occurs in p53 (at S371 and K372) and TAF10 (at S188 and K189), as well as in H3 (at T3 and K4). It will also be interesting to determine whether these modifications affect the functions of p53, Dam1, and TAF10 through changes in the conformations of the proteins and/or by altering their interactions with other proteins. Methylation stabilizes p53, indicating it may alter p53 structure and interactions with Mdm2, which targets p53 for proteolysis [Chuikov et al., 2004]. Methylation of TAF10 increases its affinity for interactions with RNA polymerase [Kouskouti et al., 2004], again indicating that methylation might alter the conformation of the protein. It will be interesting to determine whether certain chromodomain proteins (or other methyl-binding proteins) bind to methylated p53, TAF10, and Dam1, and if so, whether phosphorylation of the neighboring serines affect this binding. Moreover, it is intriguing to note that two proteins have been reported to recognize methyl-K4 in H3, Chd1 [Pray-Grant et al., 2005], and WDR5 [Dou et al., 2005; Wysocka et al., 2005]. We predict that phosphorylation of T3 will directly influence these interactions. Interestingly, the Chd1 protein is part of the SAGA HAT complex in yeast, and the WDR5 protein is part of a complex that contains the MLL HMT (which methylates H3 K4) and the MOF HAT (which acetylates H4 K16) [Dou et al., 2005]. Thus, phosphorylation, methylation, and acetylation may all be cross-regulated, in *cis* and in *trans*. p53 is acetylated at K373, immediately adjacent to the methylation site at K372, consistent with this idea.

CONCLUSIONS AND PERSPECTIVES

We propose that such interplay between post-translational modifications is not limited to the histones and the few proteins listed above.

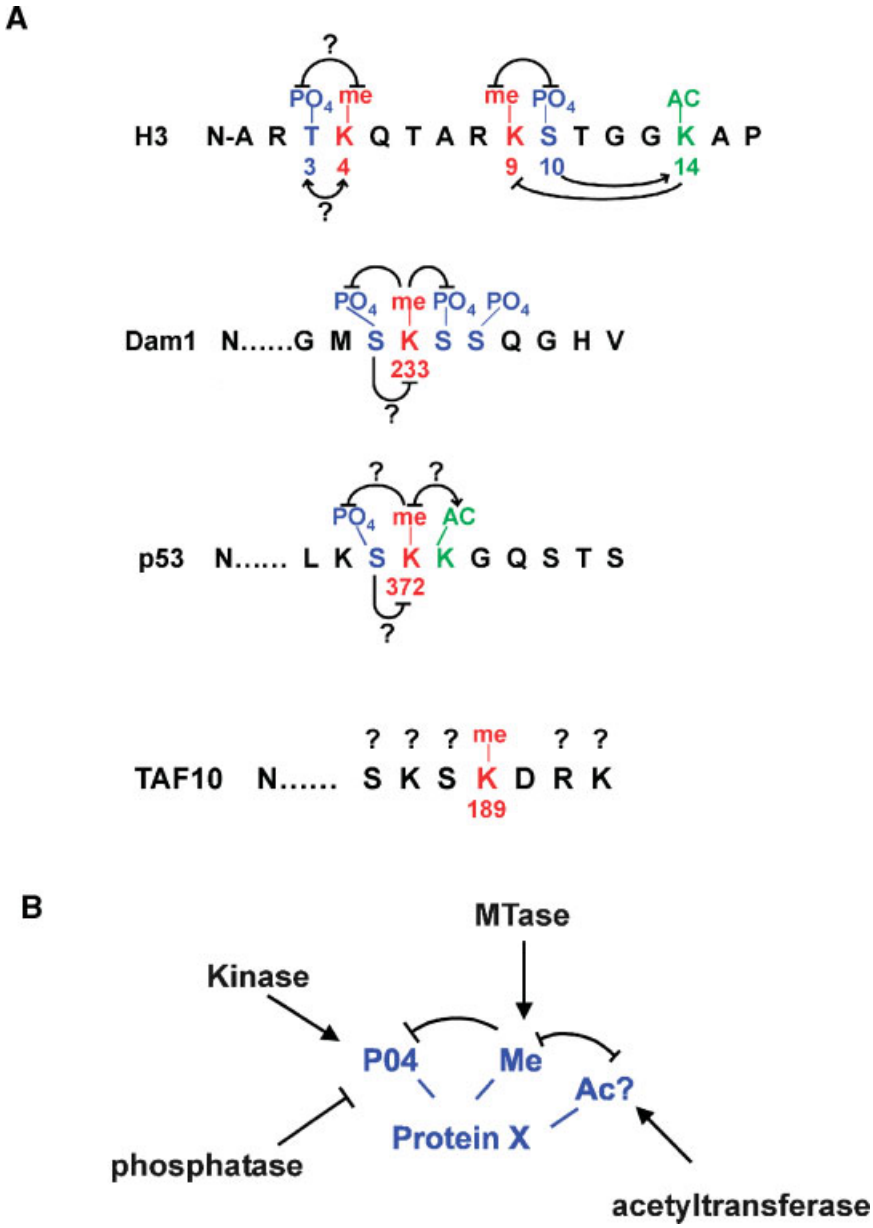

Fig. 2. Cross regulation of protein modifications. **A**: Shown are residues 1–16 of histone H3, 230–239 of Dam1, 369–378 of p53, and 186–192 of TAF10, along with modifications known to occur in these regions. Arrows indicate positive regulation of one modification by another; blunted lines indicate inhibition. Question marks indicate sites of predicted cross-regulation that have not yet been experimentally demonstrated. **B**: A general model of cross talk between protein modifications. We propose that relationships between different post-translational modifications defined in the ''histone code'' are not likely to be restricted to histones. For example, methylation of a protein may influence the phosphorylation of adjacent serines or threonines and may either stimulate or inhibit acetylation of neighboring lysines. Alterations in levels or activities of modifying enzymes in cancer cells, then, may lead to cascades of changes in protein modifications.

Rather, cross-regulation of post-translational modifications is likely to be a fundamental mechanism for regulating protein function (Fig. 2B). A quick search of the pubmed database revealed that more than 10,000 papers were published in 2004–2005 on protein phosphorylation, whereas less than 800 studies dealt with protein acetylation, and less than 200 dealt with lysine methylation. Very few dealt with interactions between these modifications, and those were mostly in regard to histones. The importance of protein phosphorylation to cell growth, differentiation, signaling pathways, and transformation into cancer is

unquestionable. However, phosphorylation is likely only to be the tip of the iceberg in regulation of protein function by post-translational modifications. Defining nodes of cross-regulation of phosphorylation, methylation, and acetylation in specific proteins has the potential to add critical new insights into all of the above processes. These nodes will be regulated by the enzymes that govern the modifications. Thus the several connections between mutations in these enzymes and cancer formation reviewed here will likely not only foreshadow the discovery of additional links between chromatin remodeling and oncogenesis but also links between multiple signaling pathways that are fine tuned via protein methylation and acetylation. Defining these connections will provide new insights to cancer formation and will provide new avenues for development of therapies.

ACKNOWLEDGMENTS

We thank J. Latham, G. Srajer, and S. Stratton for critical comments and discussion. This work was supported by grants from the NIH (GM067718) and the Robert A. Welch Foundation (G1371) to S.Y.R.D. We apologize to colleagues whose work has not been cited in this article due to space limitation.

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