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Emerging connections between DNA methylation and histone acetylation

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Abstract. Modifications of both DNA and chromatin can affect gene expression and lead to gene silencing. Evidence of links between DNA methylation and histone hypoacetylation is accumulating. Several proteins that specifically bind to methylated DNA are associated with complexes that include histone deacetylases (HDACs). In addition, DNA methyltransferases of mammals appear to interact with HDACs. Experiments with animal cells have shown that HDACs are responsible for part of the repressive effect of DNA methylation. Evidence was found in *Neurospora* that protein acetylation can in some cases affect DNA methylation. The available data suggest that the roles of DNA methylation and histone hypoacetylation, and their relationship with each other, can vary, even within an organism. Some open questions in this emerging field that should be answered in the near future are discussed.

Key words. DNA methylation; acetylation; DNA methyltransferase; histone deacetylase; methyl-binding proteins; chromatin remodeling; trichostatin A; 5-azacytidine.

Introduction

What do a parked car and a silenced gene have in common? For both, the basis of their inactivity is not immediately obvious. The inactivity of a car parked on a hill may result from engaged gears, a hand brake, some other obstruction or a combination of factors. A bit of investigation is needed to distinguish among the different possibilities. Of course it is vastly simpler to determine the basis of automotive inactivity than it is to determine the basis of gene inactivity. Silence of a gene may result from an absence of activators or the presence of sequence-specific repressors. Other possibilities have come to light, however. For example, it is clear that methylation of cytosines in and around genes can result in silencing. It is also becoming clear that modifications of the nucleoprotein 'packaging' of eukaryotic genes influence gene expression. Chromatin comes in numerous 'flavors' resulting from variation in histone composition (e.g. presence or absence of histone H1), variation in histone modifications (acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation) and regional variation in the complement of nonhistone chromosomal proteins. Although there is evidence that some of these variables impact gene expression, we are at an early stage of understanding. An important question at this point in time is, to what extent are the various emerging silencing mechanisms independent and therefore potentially redundant? DNA methylation and histone acetylation provide a good case in point. Both DNA methylation and hypoacetylation of histones H3 and H4 are frequently associated with silent genes. Moreover, as summarized below, mechanistic connections have recently been discovered between these modifications. Curiously, the limited available information suggests that the relative importance of DNA methylation and protein hypoacetylation is variable, even in cases in which they occur together. Considering methylation and hypoacetylation as the handbrake and the foot brake in the automotive analogy, we can rationalize some observations on gene silencing by remembering that there are situations in which a handbrake is sufficient to prevent movement of a car (e.g. while parked in a garage) but there are other situations in which additional measures are required (e.g. when on a very steep hill or when the engine is engaged).

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DNA methylation meets histone acetylation

Although evidence for involvement of histone hypoacetylation and/or DNA methylation in gene silencing has accumulated for nearly a quarter century, the first strong clue to a connection between these processes did not come until 1990. Tazi and Bird compared the composition of bulk chromatin with that of chromatin associated with unmethylated human DNA in the CpG islands that are associated with most genes and showed that chromatin from the unmethylated fraction was depleted in histone H1 and enriched in hyperacetylated histones H3 and H4 [1]. Exploration of the basis for this correlation awaited other developments, including: (i) identification of histone deacetylases (HDACs), the enzymes responsible for removing acetyl groups from histones, (ii) characterization of components of the methylation machinery, especially DNA methyltransferases (e.g. DNMT1 in mammals) and methyl-CpG binding proteins (e.g. MeCP2) and (iii) identification of specific inhibitors of HDACs, most notably trichostatin A (TSA). Once the basics were in place, complementary approaches reinforced each other to give a glimpse of the connections between DNA methylation and histone acetylation. On the one hand, biochemical studies revealed physical associations between HDACs and components of the methylation machinery. On the other hand, characterization of effects of inhibitors of HDACs and DNA methyltransferases demonstrated the significance of these associations.

The first connection: MeCP2 and Sin3

Although not the first methyl-CpG binding protein detected, MeCP2 was the first such protein to be purified and characterized [2–11]. MeCP2 is an approximately 500-amino acid protein, including an 85-amino acid methyl binding domain (MBD) that renders the protein capable of binding to single, fully methylated CpG dinucleotides in arbitrary sequences. MeCP2 is relatively abundant in some cell types (e.g. >100,000/nucleus in brain cells) and is found concentrated in centromeric heterochromatin [4, 5]. Results of in vitro experiments indicated that MeCP2 can bind to nucleosomal DNA and displace histone H1 [6, 7] and suggested that MeCP2 has inherent repressive activity, consistent with the possibility that it blocks transcription directly [6]. Nevertheless, it remained possible that the repressive effect of MeCP2 in the cell was due to a more complicated mechanism. One possibility, raised by discoveries that the yeast repressor RPD3 is an HDAC and that a number of mammalian corepressors recruit HDACs, was that MeCP2 recruits an HDAC, which then causes repression. This would neatly account for the known correlation between methylation of DNA and hypoacetylation of histones. Thus Nan and colleagues, and Jones and colleagues, explored the possibility that MeCP2, from mouse and *Xenopus,* respectively, can recruit HDACs. Both groups found compelling evidence that this is so [12, 13]. MeCP2 was found in complexes with homologues of RPD3 (HDAC1 and HDAC2 in mouse) as well as with a homologue of the yeast corepressor, SIN3, and several other proteins (at least RbAp46, RbAp48, Sap18 and Sap30; see table 1) [14–16]. Experiments with segments of mouse MeCP2 fused to Glutathione-*S*-transferase (GST) demonstrated that a region overlapping with the previously defined transcriptional repressor domain (TRD) could 'pull down' an mSin3A/HDAC complex, and this finding was supported by results of additional coimmunoprecipitation experiments [12]. To assess the significance of the MeCP2/HDAC complex in vivo, mouse fibroblasts were cotransfected with a construct with the MeCP2 TRD fused to the binding domain of GAL4 plus a reporter gene with GAL4 binding sites. The level of repression was measured in the presence or absence of TSA. The HDAC inhibitor substantially relieved repression, suggesting

Table 1. Known components of protein complexes connecting DNA methylation and histone acetylation.

Sin3 complex[a]	MBD1[b] complex	MeCP1 complex	DNMT1 complex	Mi2/NuRD complex	Rb/E2F1complex
Sin3	MBD1	MBD2	DNMT1	Mi-2	DNMT1
RbAp48/p46	HDAC[b]	HDAC1	HDAC1	MBD2	RB
		HDAC2	HDAC2		
HDAC1		RbAp48/p46	MBD2	HDAC1	HDAC1
HDAC2			MBD3	HDAC2	
SAP30			TSG101	MTA2	E2F1
SAP18			PCNA	MBD3	
MeCP2			DMAP1	RbAp48/p46	

[a] Complexes have been characterized in both mammalian and *Xenopus* cells, and some differences have been observed; for simplicity members of complexes defined in mammalian systems are shown. The scientific basis for the various entries is highly variable, and the contents of this table should be regarded as tentative (see text for details and references). Several complexes are known to vary from species to species in number of Retinoblastoma associated proteins and HDACs. The deacetylases found are always Class I.

[b] The MBD1 complex contains repressive activity that can be relieved by TSA, suggesting that an HDAC is present, but which one has not been determined.

that repression by the TRD was at least largely due to recruitment of one or more HDACs. Equivalent results were obtained from similar experiments in *Xenopus* [13]. It is noteworthy that TSA did not fully relieve repression in either system. This is consistent with other evidence that MeCP2 can cause both HDAC-dependent and HDAC-independent repression [6, 8, 11, 17] and suggests that this single methyl-binding protein can induce at least two 'layers' of silencing.

Mi2/NuRD connects DNA methylation, chromatin remodeling and histone deacetylation

Chromatin remodeling factors facilitate movement of nucleosomes on DNA in chromatin. It is thought that such factors make chromatin more fluid and thereby increase the accessibility of sequences involved in a variety of processes, including gene regulation [18]. Mi2/NuRD (nucleosome remodeling histone deacetylase complex) is a large complex with both chromatin-remodeling and histone deacetylase activity [19–23]. This complex, which was isolated in similar form from both human and *Xenopus* cells, includes most of the Rpd3-like HDAC of the cell (HDAC1 and HDAC2 in human cells) and may serve multiple functions in transcriptional repression. For example, the nucleosome remodeling activity of Mi2/NuRD may both facilitate histone deacetylation and help repressors reach targets hidden by nucleosomes. Like the Sin3 complex, the Mi2/NuRD complex contains Retinoblastoma A-associated proteins RbAp46 and RbAp48 (or RbAp46/48 in *Xenopus*) [19–22, 24, 25] (see table 1). Central to the Mi-2/NuRD complex is Mi-2, a member of the SWI2/SNF2 family of ATP-dependent chromatin-remodeling proteins. The complex also contains two proteins that may target it to methylated DNA [21, 24]. One is called MTA-1-like in *Xenopus*, or MTA2 in human cells, because of its similarity to human MTA1 (metastasis associated protein 1) and its close relative MTA1L1 [24]. *Xenopus* MTA1-like protein was found to bind to a methylated sequence, but it also showed binding to a an unmethylated sequence. The other component of Mi-2/NuRD that may bring the complex to methylated DNA is MBD3, a member of a family of proteins that show similarity to the MBD of MeCP2. Two forms of MBD3 were identified in *Xenopus*, resulting from alternative splicing in the putative MBD. The larger version is unable to bind to methylated DNA, but the smaller version shows specific binding to methylated sequences [24]. Whether or not mammalian MBD3 shows significant binding to methylated sequences has been somewhat controversial [24, 26–28]. It is clear, however, that MBD3 and the full Mi2/NuRD complex interacts with MBD2, which is known to bind methylated DNA and is the only member of the MBD family that shows sequence similarity to other members of the family outside of the MBD. Thus it is thought that this chromatin remodeling/deacetylase complex is recruited to methylated DNA directly and/or indirectly [29]. Interestingly, a recent report demonstrates that an MBD2-MBD3 complex uniquely binds hemimethylated DNA, raising the possibility that these proteins play a role in the propagation of epigenetic states through replication [28].

Perplexing methyl-binding proteins become complexed: MBD1 and MBD2

The discovery of multiple MBD-like sequences in public databases [26] raised hopes of finally identifying the components of the first identified methyl-CpG-binding activity, MeCP1 [30, 31]. MeCP1 is easily distinguishable from MeCP2 by bandshift and Southwestern assays [2]. Whereas MeCP2 requires only one fully methylated CpG/GpC to bind, MeCP1 requires at least 12 such symmetrically methylated CpGs, making it more sensitive to density of methylation. MeCP1 is also much less abundant and less tightly bound in the nucleus than MeCP2 [2]. Initially it was thought that the methyl-CpG binding protein MBD1 is a component of the MeCP1 complex [32], but additional studies indicated that this is not the case [33]. Rather, MBD2 appears to be responsible for the methyl-CpG binding activity of MeCP1 [34]. A highly specific anti-MBD2 antibody was found to supershift the MeCP1-methyl-DNA complex and to specifically deplete MeCP1 from a HeLa cell extract. Curiously, MBD2 was originally reported to have DNA demethylase activity [35], but attempts to verify this claim have failed [24, 34]. In addition to MBD2, known components of the MeCP1 complex include histone deacetylases HDAC1 and HDAC2 and RbAp48 [34]. Inhibitor studies with HeLa cells, which lack detectable MeCP2 but are still able to repress methylated genes [31], indicated that repression of transfected methylated sequences is HDAC dependent, consistent with the new findings that MeCP1 represents yet another protein complex with both histone deacetylase and methyl-CpG binding activities. Moreover, transfection experiments with MBD2 fused to the binding domain of GAL4 verified that MBD2 can mediate HDAC-dependent repression [34].

Investigations of the mammalian methyl-CpG binding protein MBD1 demonstrated that it is part of an additional MBD-HDAC complex [33]. Like MeCP2, MBD1 requires only one symmetrically methylated CpG to bind DNA and is found preferentially in heterochromatic foci. In addition, both proteins include powerful repression domains [36, 37] that are HDAC dependent [33]. The MBD1 complex remains undefined, but it apparently does not include either HDAC1 or MBD2. Thus, mammalian cells have at least four distinct complexes that include dif-

ferent methyl-CpG binding proteins and histone deacetylases. Considering that there may be additional methyl-DNA binding proteins and that some of the known MBD proteins, including MBD1 [36, 37], are synthesized in two or more forms, we should not be surprised if the variety of MBD-HDAC complexes continues to grow.

Double trouble: DNA methyltransferases and histone deacetylases join forces

The various connections between HDACs and methyl-DNA binding proteins are consistent with the idea that silencing by DNA methylation results from hypoacetylation of histones and/or other chromosomal proteins. Evidence that DNA methylation can cause repression not mediated by acetylation suggests that this is an oversimplification, however. As discussed more fully below, DNA methylation and histone hypoacetylation appear to be distinct but connected silencing mechanisms. Recent discoveries of direct connections between HDAC(s) and DNA methyltransferase(s) provide evidence of yet another level of collaboration between these silencing mechanisms.

Starting with the isolation of the first mammalian DNA methyltransferase (MTase) from mouse, DNMT1, about a dozen years ago [38], it has become increasing clear that eukaryotic MTases are multifaceted proteins. In addition to their catalytic domains, all known eukaryotic MTases have large N-terminal domains that apparently connect the MTases to other nuclear proteins. Recent work on DNMT1 has provided evidence that segments of the N-terminal domain interact with proliferating cell nuclear antigen (PCNA) [39], HDAC1 [40, 41], HDAC2 [42], the tumor suppressor protein Retinoblastoma (Rb) [41], the transcriptional activator E2F1 [41], a previously unrecognized corepressor, DNA methyltransferase-associated protein (DMAP1) [42], and MBD3 [28]. Results of transfection experiments suggest that DNMT1 has at least two transcriptional repressor domains, one of which apparently acts by recruitment of a deacetylase since repression is partially relieved by the HDAC inhibitor TSA [40–42]. Results of in vitro and yeast two-hybrid experiments imply that HDAC1 [40] and HDAC2 [42], respectively, interact directly with DNMT1. The observed association of Rb and DNMT1 is suggestive of additional connections between DNA methylation and acetylation, however, since Rb itself can recruit HDACs [41]. On a side note, E2F1, another associate of DNMT1, is subject to acetylation by the histone acetyltransferase PCAF, and deacetylation by a Rb-associated HDAC [43], highlighting the complexity of the acetylation network.

The observed connections between HDACs and DNA MTases raise interesting possibilities. Rountree and colleagues point out that the DNMT1/HDAC complex should be ideally suited to perpetuate the silent state of late-replicating heterochromatic chromosomal regions, which are characterized by hypoacetylated histones and heavily methylated DNA [42]. It was previously known that DNMT1 is recruited to replication foci during S phase [39, 44, 45]. Immunofluorescence imaging of tagged DMAP1 coexpressed with wild-type or deletion DNMT1 constructs demonstrated that DMAP1 is recruited to sites of DNA replication by the 120-amino acid segment at the N terminus of DNMT1 [42]. Interestingly, interaction between this complex and HDAC2 occurs only during late S phase, the same stage that MBD2 and MBD3 apparently associate with DNMT1 [28]. This seems reasonable since methylated DNA replicates late and MBD2 and MBD3 together are thought to bind to hemi methylated DNA, which would result from replication of methylated sequences.

Significance of all the complexity?

As summarized above, biochemical studies over the last few years have revealed multiple physical connections between elements of DNA methylation and histone acetylation machines. Although the biological significance of the various connections has not yet been well established, several in vivo studies carried out in the same time period have provided clues of functional connections between these two biochemical processes. An early indication of a connection between DNA methylation and histone acetylation came from studies of nucleolar dominance in the plant genus *Brassica*. Chen and Pikaard found that silenced ribosomal RNA (rRNA) genes in interspecific hybrids could be activated by treatment of the plants with either the methyltransferase inhibitor 5-azacytidine or histone deacetylase inhibitors (butyrate or TSA), suggesting that DNA methylation and hypoacetylation collaborate to silence the genes [46]. No synergism or marked additivity was detected. These results are consistent with operation of methylation and acetylation in a common pathway that directly or indirectly acts on the ribosomal DNA (rDNA).

A study in the filamentous fungus *Neurospora crassa* provided additional evidence of functional connections between DNA methylation and protein acetylation and revealed an unexpected indication that acetylation can influence DNA methylation [47]. Previous work in *Neurospora* had shown that heavy methylation, such as that resulting from repeat induced point mutation (RIP), inhibits transcription elongation by an undefined indirect mechanism [48]. As a test of the possibility that hypoacetylation is involved, a strain harboring a methylated copy of the bacterial *hph* gene, which normally causes resistance to hygromycin, was challenged with TSA and/or 5-AC. TSA, like 5-AC, derepressed *hph,* and when used together, the drugs resulted in even greater derepression.

Similarly, both drugs caused repression of an allele of *am* whose expression depends on methylation of an adjacent transposon [47]. Surprisingly, TSA resulted in dramatic loss of methylation of the *hph* and *am* sequences, suggesting that acetylation of histones or other proteins can directly or indirectly influence DNA methylation. The mechanism of this TSA-mediated hypomethylation remains to be discovered. It is noteworthy that most methylated sequences in the Neurospora genome appeared unaffected by TSA, and no similar effect of the drug has been yet reported in other systems.

Studies with human cells have produced evidence of separable 'layers' of silencing by DNA methylation and histone hypoacetylation. In a study of several genes that became inactivated and hypermethylated in tumor cells (*MLH1, TIMP3, CDKN2B* and *CDKN2A*), TSA treatment alone was unable to cause reactivation [49]. Nevertheless, TSA could induce reactivation after a pretreatment with a low dose of 5-AC that itself produced little or no reactivation and caused only slight reduction in methylation overall. Similar evidence of synergistic action of inhibitors of HDACs and DNA methylation was reported for a silent allele of the human *FMR1* gene [50], and other cases of 'dominant' repression by methylation have been observed [51]. These results reinforce the notion that the inhibitory effect of DNA methylation is not simply a result of recruitment of HDACs.

Although it is too early to formulate a full model of the interrelationships between DNA methylation and protein acetylation, it is not too early to consider the question, Why all the complexes? For example, why are HDACs associated with multiple methyl-DNA binding proteins? This may be related to the more basic question of why there are multiple methyl-DNA binding proteins. Several possibilities have been raised in this regard. First, it is clear that the various MBD proteins have different specificities, for example with respect to density of methylation [6, 37]. Density of methylation is known to influence gene repression [6, 51–54], which could account for the observations that heavily methylated genes in animal cells cannot be derepressed by TSA without pretreatment with 5-AC [49, 50, 51]. In addition to differences in specificity of methyl-DNA binding proteins, these and other HDAC-associated proteins show variation in their distribution within the cell, among different cells and among different stages of the cell cycle. Other factors that may play a role in whether a particular HDAC complex is responsible for a particular case of silencing include genomic location and chromatin type, i.e. the constellation and state of associated histone and nonhistone chromosomal proteins.

Concluding thoughts

The recent discoveries of connections between DNA methylation and protein acetylation have brought together two fascinating processes that are still poorly understood. The recent rate of revelations relating methylation and acetylation has been invigorating, but there are still more questions than answers. Some open questions are, Do the associations between HDACs and elements of the methylation machinery (MBDs and MTases) simply reflect a preferred way that DNA methylation represses genes, or do the different complexes serve qualitatively different functions? Does methylation serve as a 'lock' on silencing by hypoacetylation in some cases but not in others? To what extent, and how, does protein acetylation feed back on DNA methylation? Do the various HDAC complexes operate primarily on histones, and if so, how specific are they for particular histones and/or particular lysines? How big is the 'sphere of influence' of the HDAC complexes, i.e. are they tightly tethered? Does hypoacetylation extend beyond methylation or vice versa? How sensitive are the complexes to local variation in chromatin, such as resulting from other modifications of histones (e.g. phosphorylation and methylation), presence or absence of heterochromatin proteins, availability of histone acetyltransferases, and so forth? To what extent are the various complexes associated with DNA replication and/or sensitive to replication timing? These and other questions will be answered in the coming years by exploitation of an expanding arsenal of research techniques including sophisticated immunological methods such as chromatin immunoprecipitation. We also expect that genetic data from model organisms such as mice, *Arabidopsis* and *Neurospora* will be invaluable, for example to assess the significance of the connections detected biochemically. It seems safe to predict that our understanding of the relationships between DNA methylation and protein acetylation will improve rapidly in the next few years. Eventually we may even come to regard complexed complex complexes as 'simple'.

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