DNA Methylation Does Not Stably Lock Gene Expression but Instead Serves as a Molecular Mark for Gene Silencing Memory

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Abstract

DNA methylation is commonly thought of as a "molecular lock" that leads to permanent gene silencing. To investigate this notion, we tested 24 different histone deacetylase inhibitors (HDACi) on colon cancer cells that harbor a GFP locus stably integrated and silenced by a hypermethylated cytomegalovirus (CMV) promoter. We found that HDACi efficiently reactivated expression of GFP and many other endogenous genes silenced by DNA hypermethylation. After treatment, all promoters were marked with active chromatin, yet DNA hypermethylation did not change. Thus, DNA methylation could not prevent gene reactivation by drug-induced resetting of the chromatin state. In evaluating the relative contribution of DNA methylation and histone modifications to stable gene silencing, we followed expression levels of GFP and other genes silenced by DNA hypermethylation over time after treatment with HDACi or DNA-demethylating drugs. Reactivation of methylated loci by HDACi was detectable for only 2 weeks, whereas DNA-demethylating drugs induced permanent epigenetic reprogramming. Therefore, DNA methylation cannot be considered as a lock for gene expression but rather as a memory signal for long-term maintenance of gene silencing. These findings define chromatin as an important druggable target for cancer epigenetic therapy and suggest that removal of DNA methylation signals is required to achieve long-term gene reactivation. Cancer Res; 72(5); 1170–81. ©2012 AACR.

Introduction

Epigenetic marks such as histone modifications and DNA methylation are involved in cell memory expression patterns which are transmitted through cell division (1). Chromatin modifications are required in all organisms whereas DNA methylation is not present in some lower organisms such as worms and flies, suggesting that chromatin has a broader epigenetic function in gene regulation (1). Histone acetylation is associated with open chromatin and gene expression whereas removal of acetyl groups by histone deacetylases is observed in inactive chromatin. In higher organisms, DNA methylation plays an important role in several physiologic processes, including X chromosome inactivation, genomic imprinting, and silencing of germ cell–specific genes and repetitive elements (2). In cancer, tumor suppressor genes (TSG) are silenced by both DNA hypermethylation and chromatin-repressive marks (2, 3). A common hypothesis is that DNA methylation serves as a "molecular lock" that prevents reprogramming and is responsible for stable gene silencing (1, 4, 5). This concept was built on indirect observations whereby hypermethylated genes in cancer cells could be reactivated only after removal of promoter DNA hypermethylation using hypomethylating drugs such as decitabine (5-aza-2'-deoxycytidine, 5-aza-CdR). In contrast, chromatin acetylation induced by histone deacetylase inhibitors (HDACi), such as trichostatin A (TSA), could not reactivate hypermethylated genes in cancer cells (6–8). However, more recently, some reports have shown that HDACi, such as TSA and depsipeptide, produce gene reactivation from hypermethylated promoters without any changes in DNA methylation at the promoter level (9–12). Because these reports were against the current paradigm, a more detailed look at this issue was needed.

One of the problems in studying DNA methylation–associated silencing of TSG is that reactivation of these genes can impair cellular growth and be difficult to detect and quantitate. A selectable system was recently described to overcome this issue. YB5 cells are derived from the SW48 colon cancer cell line transfected with a GFP driven by a hypermethylated cytomegalovirus (CMV) promoter packaged into inactive chromatin. YB5 carries a single copy of CMV/GFP stably integrated in chromosome 1. GFP can be
reactivated in YB5 cells by treatment with 5-aza-CdR when its promoter region is demethylated and also marked by active chromatin signals characterized by H3K9-acetylation, low level of H3K27 trimethylation, and low nucleosome density (13).

In this article, we use YB5 cells (and 5 other cancer cell lines) to show that the vast majority of HDACi tested can reactivate genes silenced by promoter hypermethylation without detectable changes in DNA methylation. We further show that while DNA methylation cannot prevent gene activation by chromatin reprogramming, it is essential for long-term gene silencing.

Materials and Methods

Cell culture and drug treatments
All cell lines were obtained from American Type Culture Collection. Cell lines were authenticated at MD Anderson Cancer Center with short tandem repeat PCR method. YB5 cell line is a colon cancer cell line generated from SW48 as previously described (13). YB5 cell line was cultured in L-15 medium, whereas MCF-7, K562, MDA-MB-231, and PC-3 cells were cultured in RPMI-1640. Both cell culture media contained 10% FBS and 100 μg/mL penicillin/streptomyacin solution. Cells growing in log phase were treated with decitabine (5-aza-CdR) at 50 nmol/L for 72 hours. Drug and medium were replaced every day. Cells were cultured an additional 24 hours without drug prior to analysis. HDACi were dissolved in dimethyl sulfoxide, ethanol, or PBS according the manufacturers’ recommendations. HDACi were added for 24 hours at various concentrations prior to analysis.

Histone extraction and Western blot analyses
Total protein extracts were prepared using radioimmuno-precipitation assay (RIPA) buffer as described previously (14) in the presence of sodium butyrate (5 mmol/L) to avoid in vitro histone deacetylation and resolved on 15% SDS-PAGE. Antibodies used were H3K9-acetylation (07-352; Millipore), H4K16-acetylation (39168; Active motif), and total H3 (ab1791 Abcam).

Fluorescence-activated cell-sorting analysis and cell sorting
YB5 cells were trypsinized and stained with propidium iodide. GFP and propidium iodide fluorescence were measured by Gallios flow cytometer (Beckman Coulter). Data were analyzed using Kaluza software. GFP cell sorting was conducted using BD FACS/AriaII. GFP fluorescence of samples was analyzed post-sorting to assess the purity of the sorted cells.

RNA extraction, cDNA synthesis, quantitative real-time PCR
Total RNA (2 μg) was extracted using TRIzol (Invitrogen) and cDNA was synthesized using High-Capacity cDNA Kit (Applied Biosystems). Quantification of cDNA was done by quantitative PCR (qPCR) with the Universal PCR Master Mix (Bio-Rad) using ABI Prism 7900HT. Results were obtained from at least 3 independent experiments where each sample was analyzed in duplicate. J85 was used as a reference gene. cDNA synthesis used the same amount of RNA after treatment with different drugs. All primers, except GFP primers that were described previously (13), are listed in Supplementary Table S1. 5’-Rapid amplification of cDNA ends (5’-RACE) was done as previously described (13).

DNA extraction and DNA methylation analysis
DNA extraction and bisulfite conversion, pyrosequencing, and bisulfite cloning/sequencing were carried out as previously described (13). All primers are listed in Supplementary Table S1, except for all GFP primers that were described previously (13).

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was conducted as described previously (14). Antibodies used were anti-histone H3 (ab1791; Abcam), anti–histone H3K9-acetylation (07–352; Millipore), anti–histone H3K27 trimethylation (07–449; Millipore), anti-histone H3K4 trimethylation (17–614; Millipore), anti-histone H3K36 trimethylation (ab9050; Abcam), and anti-IgG (ab46640; Abcam). Quantification of ChIP DNA was done by qPCR and primers/probes are listed in Supplementary Table S1. Each ChIP assay was validated using targets for the various modifications (ACTB for active histone mark and LINE-1 for inactive histone mark). The value of each histone modification was determined by H3 and IgG normalization using the equation fold enrichment = 2^{[-Ct(ΔΔCt)-Ct(H3)]} – 2^{[-Ct(ΔΔCt)-Ct(H3)]}

Gene expression and DNA methylation microarrays
Gene expression analysis was conducted using the Agilent Whole Genome Array (G4112F) that was scanned using the Agilent G2505B Scanner. Data represent the average expression level of 2 independent experiments. DNA methylation analysis using high-throughput methylation profiling by MCA coupled to CpG island microarray (MCAM) was conducted as described previously (15). After analysis, genes with M values more than 1.3 were considered methylated. Microarray data sets were deposited in the Gene Expression Omnibus (GEO) database with the accession number GSE34429.

Statistical analysis
Differences between groups were assessed using the Student t test. A 2-sided P value of 0.05 was considered statistically significant.

Results
HDACi induce GFP reactivation from a DNA hypermethylated promoter
To examine the effects of HDACi on gene silencing by DNA methylation, YB5 cells were treated with 24 different HDACi that belong to 8 different chemical classes in a wide range of concentrations (Table 1) and 17 of them reactivated GFP. Although GFP reactivation was detected in a wide range of concentrations (even at high doses, Table 1), the subsequent experiments were carried out at doses where the percentage of dead cells after treatment was less than 30% and where GFP synthesis used the same amount of RNA after treatment with different drugs. All primers, except GFP primers that were described previously (13), are listed in Supplementary Table S1. 5’-Rapid amplification of cDNA ends (5’-RACE) was done as previously described (13).
HDACi induced gene reactivation of endogenous genes silenced by promoter DNA hypermethylation.

Because these data are not in agreement with other studies on gene reactivation induced by HDACi (6, 7), we asked whether this effect was specific to the GFP locus or could be observed in other methylated genes in various cancer cell lines. First, we analyzed in YB5 cells gene reactivation of other genes in response to depsipeptide (Fig. 3A) and other HDACi (Supplementary Fig. S2A and S2B). For this, reactivation was the highest as detected by fluorescence-activated cell-sorting (FACS) analysis. Following treatment with depsipeptide (for 24 hours at 20 nmol/L), GFP expression was detectable in about 50% of cells as seen by fluorescence and mRNA levels. These results do not support the lock hypothesis and are in agreement with more recent findings showing that HDACi can reactivate hypermethylated genes.

| Table 1. Effects of various HDACi on GFP expression in YB5 cells detected by FACS analysis after a 24-hour treatment |
|---|---|---|---|---|
| Drugs    | Structural class | Target HDAC | Concentration tested | GFP expression |
| SAHA     | Hydroxamic acid   | I, IIa, Ib, IV | 0.3–20 μmol/L | Yes          |
| TSA      | Hydroxamic acid   | I, II        | 50–200 nmol/L | Yes          |
| Oxamflatin | Hydroxamic acid   | HDAC 3, 6    | 100–400 nmol/L | Yes          |
| APHA8    | Hydroxamic acid   | HDAC 1       | 1–25 μmol/L | Yes          |
| Cay-10398 | Hydroxamic acid   | HDAC 1       | 10–40 μmol/L | Yes          |
| M344     | Hydroxamic acid   | HDAC 1, 6    | 1–4 μmol/L | Yes          |
| CHAHA    | Hydroxamic acid   | HDAC 6       | 0.2–1.5 μmol/L | Yes          |
| LBH-589  | Hydroxamic acid   | Pan-HDACi    | 0.02–20 μmol/L | Yes          |
| Depudecine | TSA-like but nonhydroxamate | HDAC 1 | 5–25 μmol/L | Yes          |
| PTACH    | TSA-like but nonhydroxamate | —            | 1–10 μmol/L | Yes          |
| VPA      | Short-chain fatty acid | HDAC 2    | 0.25–10 μmol/L | Yes          |
| Phenylbutyrate | Short-chain fatty acid | I            | 1 mmol/L | Yes          |
| Sodium butyrate | Short-chain fatty acid | I, Ila     | 5–20 mmol/L | Yes          |
| Depsipeptide | Cyclic peptide     | HDAC 1, 2    | 1 mmol/L | Yes          |
| Apicidin | Cyclic peptide     | I, II        | 0.4–40 μmol/L | Yes          |
| HC-Toxin | Cyclic peptide     | HDAC 1, 2, 3, 8 | 25–100 nmol/L | Yes          |
| Sirtinol | Hydroxy naphthaldehyde | Sirt 2 | 1.25–100 μmol/L | Yes          |
| Splitomicin | Napthalenes        | Sirt 2       | 0.1–75 μmol/L | No           |
| Ex527    | Tetrahydrocarbazoles | Sirt 1      | 20–100 μmol/L | No           |
| CHIC-35  | ND                 | Sirt 1       | 0.1–1 μmol/L | No           |
| Camboinol | β-Naphthol         | Sirt1, 2     | 2.5–25 μmol/L | No           |
| PCI-34051 | Hydroxamic acid    | HDAC 8       | 1–10 μmol/L | No           |
| BATCP    | ND                 | HDAC 6       | 1–10 μmol/L | No           |
| Scriptaid | Hydroxamic acid    | HDAC 1, 2, 8 | 0.05–1 μmol/L | No           |

NOTE: HDACi targets (specific isofoms or entire HDAC class) and concentration range tested on the YB5 system are indicated in the table.

Abbreviation: ND, not determined; SAHA, suberoylanilide hydroxamic acid.
we selected 7 TSGs silenced by DNA hypermethylation in YB5 cells (85%–100% methylation as detected by bisulfite pyrosequencing). These play roles in mediating cell adhesion (CDH13), metastasis (TIMP-3), cell cycle (P16), DNA mismatch repair (MLH1), Wnt pathway signaling (WIF-1), mitogen—activated protein (MAP) kinase signaling (DOK5), and cell differentiation (RAR-β). Among these, all but one (RAR-β) are driven by promoter CpG islands (CGI; ref. 20). These genes are epigenetically inactivated in many cancers. Twenty-four hours of treatment with depsipeptide and other HDACi reactivated all these hypermethylated genes as detected by qPCR (Fig. 3A and Supplementary Fig. S2A and S2B), whereas DNA methylation levels did not change as compared with untreated cells (Fig. 3B). These results were extended to 4 other cancer cell lines [K562, a chronic myelogenous leukemia (CML) cell line; MCF-7 and MDA-MB-231, breast cancer cell lines; and PC-3, a prostate cancer cell line] with 6 different genes (CDH1, MGMT, NPM2, RASSF1A, DOK5, and PDLIM4; Supplementary Fig. S3A–S3D) whose promoter methylation levels vary between 65% and 100% methylation as detected by pyrosequencing. Most of them showed reactivation after HDACi treatment. The induction of p21, a cyclin-dependent kinase inhibitor, by HDACi was used as a positive control for HDACi activity as it is considered as a hallmark of the effect of HDACi on gene expression. Our data are in agreement with previous reports on single genes that HDACi, such as TSA, phenylbutyrate, and LBH589 (9, 11, 12, 21, 22), or SIRT1 inhibition (10) can induce gene reactivation of hypermethylated genes without alteration in DNA methylation. Our findings on numerous genes and in different cancer cell lines show that chromatin acetylation...
induced by HDACi overcomes DNA hypermethylation silencing and induces gene reactivation. Altogether, these data show that chromatin remodeling allows a subset of TSGs silenced by DNA hypermethylation to be reactivated in response to HDACi.

Chromatin remodeling despite DNA hypermethylation

Because HDACi-induced gene reactivation was not associated with DNA demethylation, we investigated the effect of the treatment on chromatin modifications at the promoter regions of these hypermethylated genes. We conducted ChIP assays coupled with qPCR analysis for H3K9-Ac, H3K4-me3, H3K36-me3 (marks associated with gene activity), and H3K27-me3 (modification associated with gene repression) in YB5 cells untreated and treated with depsipeptide for 24 hours at 20 nmol/L. Interestingly, we recently reported that regardless of DNA methylation status, gene reactivation is associated with a promoter region marked by active chromatin marks and low nucleosome density (13). Following depsipeptide treatment, all promoter regions of GFP, CDH13, MLH1, and WIF-1 showed an increase in activating marks (1.5- to 24.5-fold).
such as H3K9-Ac (Fig. 4A), H3K4-me3 (Fig. 4B), and H3K36-me3 (Fig. 4C). In contrast, H3K27-me3, a surrogate for chromatin inactivated by polycomb, was reduced after depsipeptide treatment by 1.4- to 4-fold (Fig. 4D). Interestingly, depsipeptide treatment did not seem to have modified nucleosome density (measured by ChIP-PCR of H3/input) on the promoter region of these genes (data not shown). To rule out indirect effects through other chromatin regulators, we measured the expression of DNMT1, DNMT3a, DNMT3b, DNMT3L, DROSHA, DICER, TET1, TET2, and EZH2. We found that most did not change significantly after depsipeptide treatment (Fig. 3A). These data show that HDACi directly induced chromatin remodeling and this was associated with gene reactivation from DNA hypermethylated promoters.

**Genome-wide effects of depsipeptide on hypermethylated genes**

We next investigated on a genomic scale the effect of depsipeptide on gene reactivation of hypermethylated genes. We combined the data of 2 independent gene expression microarrays of untreated and depsipeptide-treated YB5 cells (Supplementary Fig. S4A) with whole genome DNA methylation data using MCAM (Supplementary Fig. S4B; ref. 15). As previously reported by other groups (23, 24), we found that HDACi increased the expression of 11% of the genes with the same amount of genes being repressed (11%; Supplementary Fig. S4D). Whole genome methylation data showed that this colon cancer cell line has more than 330 detectable hypermethylated promoter CGI (i.e., 6% of the gene promoters on the array; Supplementary Fig. S4B). When combining the data

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**Figure 3.** HDACi reactivate endogenous hypermethylated genes in YB5 cell line. A, gene expression was detected by qPCR of untreated (white bars) and treated YB5 cells with depsipeptide at 20 nmol/L for 24 hours (black bars). Tested genes were divided in 3 categories: control genes, hypermethylated genes, and epigenetic regulators. *a*, a significant difference between untreated and treated cells (P < 0.05). Each experiment was carried out 3 separate times; shown is the mean ± SEM. B, DNA methylation analysis of TIMP-3, WIF-1, and CDH13 promoters analyzed by pyrosequencing. YB5 cells were treated with hypomethylating drug 5-aza-CdR (DAC) or with HDACi depsipeptide (n > 3).
of both gene expression and DNA methylation microarrays, we could analyze gene expression and DNA methylation of more than 4,300 genes (Supplementary Fig. S4C). In this gene list, 258 genes showed promoter DNA hypermethylation and 8% (21 genes) of these were reactivated following HDACi treatment (Supplementary Fig. S4E and S4F). Considering that 80% to 90% of hypermethylated genes in cancer are not expressed in normal tissues and thus lack the appropriate transcription factor for activation, our data suggest that the majority of "inducible" genes are actually induced by HDACi (25). In contrast, 14% of the unmethylated genes could be reactivated by treatment with depsipeptide.

DNA methylation is a long-term memory signal for gene silencing

The data described so far show that rapid activation of a DNA hypermethylated promoter is possible with strong drug-induced chromatin acetylation. These results raise the question of the importance of DNA methylation in gene silencing. To study the relative contribution of DNA methylation versus chromatin modifications in gene silencing, we compared the long-term effects of depsipeptide and 5-aza-CdR treatment on gene expression and DNA methylation. YB5 cells were treated with depsipeptide (24 hours) or 5-aza-CdR (72 hours) and were then subjected to cell sorting to obtain enriched GFP-positive cell populations (purity ~70% of GFP+ cells for each treatment conditions). GFP-positive cells were cultured post-sorting without drugs and GFP expression was followed for more than 3 months by FACS analysis (Fig. 5A). During the first week post-sorting, the population of depsipeptide-treated YB5 cells was mostly GFP-positive. Ten days post-sorting, approximately 60% of the cells treated with depsipeptide lost GFP expression and 2 weeks posttreatment, GFP expression was rare among these cells. These data were confirmed with other HDACi such as valproic acid (VPA), apicidin, Cay-10398, and TSA (data not shown). GFP expression was undetectable 25 days following depsipeptide treatment and was similar to untreated cells for the rest of the experiment.

Figure 4. Depsipeptide induces chromatin modifications on promoters of DNA hypermethylated genes. ChIP analysis of (A) H3K9-Ac, (B) H3K4-me3, (C) H3K36-me3, and (D) H3K27-me3 was conducted in YB5 cells untreated (white bars) and treated with depsipeptide (Depsi) at 20 nmol/L for 24 hours (black bars). Chromatin status at the promoter of GFP, CDH13, MLH1, P16, and WIF-1 was quantified by qPCR and expressed as fold enrichment, using the following equation: 2^(-ΔΔCt) = 2^(-ΔCt(IgG) - ΔCt(H3)). Histone marks at ACTB and LINE-1 promoters were used as controls for active and inactive genes, respectively. Each experiment was carried out 3 separate times; shown is the mean ± SEM. *, a significant difference between untreated and treated cells (P < 0.05).
Results obtained with YB5 cells treated with 5-aza-CdR exhibited a different pattern (Fig. 5A). For the first week, the vast majority of the cells exhibited GFP fluorescence. Then, the percentage of cells showing GFP fluorescence decreased to 50% and 35% after 10 days and 25 days posttreatment, respectively. The number of GFP-positive cells decreased slowly thereafter, and after 3 months, 3% of YB5 cells treated with 5-aza-CdR still exhibited GFP fluorescence (Fig. 5A). We similarly investigated the RNA expression of several hypermethylated genes including GFP (Fig. 5B), MLH1 (Fig. 5C), CDH13 (Supplementary Fig. S5A), WIF-1 (Supplementary Fig. S5B), and TIMP-3 (Supplementary Fig. S5C) and found that gene expression was activated immediately by treatment with either epigenetic drug, but expression was silenced 2 weeks after depsipeptide treatment whereas it was maintained following treatment with 5-aza-CdR for up to 9 weeks. Interestingly, other chromatin modifiers, such as histone methyltransferase inhibitors, were previously shown to induce transient gene activation which returned to the original state upon drug removal (26). As previously mentioned, after depsipeptide treatment, DNA methylation in the promoter regions of these hypermethylated genes did not change. In contrast, after 5-aza-CdR treatment, methylation levels decreased significantly at GFP (Fig. 5D), MLH1 (Fig. 5E), CDH13 (Supplementary Fig. S5D), WIF-1 (Supplementary Fig. S5E), TIMP-3 (Supplementary Fig. S5F), and LINE-1 (Supplementary Fig. S5G).

The gradual loss of GFP expression after 5-aza-CdR withdrawal coincided with gradual remethylation of the
CMV promoter. Although it has been reported that the p16^INK4A/CDKN2A locus was remethylated after 5-aza-CdR removal, it has been suggested that the apparent remethylation was due to clonal replacement by a subset of cancer cells that were not affected by 5-aza-CdR (19, 27). Cells treated with hypomethylating agents tend to have a longer cell cycle because of the reexpression of growth regulatory signals. Therefore, hypomethylated cell populations can be easily replaced by more rapidly growing methylated populations, which can bias the measurement of DNA methylation (19). To address this issue, we carried out 2 series of cell-sorting experiments using YB5 cells cultured 9 weeks without drug following initial 5-aza-CdR treatment (Fig. 6A). The purity of the sorted GFP-positive cells exceeded 90% whereas the GFP-negative cells contained only 0.2% GFP-positive cells. Gene expression and DNA methylation analysis of GFP-positive and GFP-negative cells at 9 weeks posttreatment revealed that gene reactivation in GFP-positive cells (Fig. 6B) was at this late time point associated with an unmethylated promoter region with an average methylation of 15% (Fig. 6C). GFP-negative cells exhibited 1,000 times less GFP mRNA and a promoter closer to the methylation level of untreated cells with an average methylation of 66% (Fig. 6B and C). This gene expression pattern was also observed for other TSG such as TIMP-3, CDH13, and MLH1 whereas their DNA methylation level was reduced in the GFP-positive and GFP-negative cells (Supplementary Fig. S6A–S6F). Global DNA methylation measured by the LINE-1 assay did not change significantly between untreated cells, GFP-positive, and GFP-negative cells (Supplementary Fig. S6G).

To eliminate the effect of clonal replacement, we carried out cell-sorting and single cloning experiments (Fig. 6D). After clonal expansion of these single clones to obtain enough cells (~2 weeks), we monitored their GFP fluorescence over time (Fig. 6D, black bars) as compared with sorted cells obtained after 5-aza-CdR treatment when the purity was about 70% (Fig. 6D, white bars) and 9 weeks after treatment when the purity exceeded 90% (Fig. 6D, gray bars). Single-cell clones of these GFP-positive YB5 cells obtained 9 weeks after 5-aza-CdR without drugs revealed that 92% to 97% stably express GFP for up to 6 months posttreatment showing stable epigenetic reprogramming (Fig. 6D). Interestingly, the GFP promoter region was completely demethylated in these GFP-positive clones (Fig. 6C). These results clearly show that DNA methylation is the molecular mechanism responsible for long-term gene silencing. Thus, full epigenetic reprogramming and switching from the silent to the expressed state can be accomplished by complete promoter demethylation which is correlated with RNA pol II occupancy (28).

**Discussion**

Early studies have reported that TSG silenced by promoter DNA hypermethylation could be reactivated only after the removal of methylation marks. In these studies, treatment with TSA, an HDACi, could not produce gene reactivation of genes silenced by promoter DNA hypermethylation (6, 7). On the basis of these indirect observations, the function of promoter DNA methylation, as a signal for gene silencing, has been considered as a 'lock' for gene expression. However, other studies have reported that hypermethylated genes can be reactivated by TSA and other HDACi without any loss in promoter DNA methylation (9–12). These reports put in jeopardy the lock hypothesis which has been the paradigm for more than a decade. Hence, we chose to investigate this issue by looking at the effects of more than 20 different HDACi on reactivation of genes silenced by promoter DNA methylation.

Using the well-characterized YB5 system and other cancer cell lines, we discovered that most of the HDACi tested could reactivate hypermethylated genes in a dose-dependent pattern regarding their chemical class and HDAC affinity. DNA methylation analysis revealed that gene reactivation was generated without any loss of promoter DNA methylation. Methylation levels were carefully assessed before and after treatment by pyrosequencing and bisulfite cloning sequencing as it was reported that HDACi could potentially reduce DNA methylation levels by nonspecific mechanisms (16–18). However, our study, as well as others (19), show that methylation levels did not change 24 hours after HDACi exposure or several days posttreatment. Therefore, these data confirm that HDACi can reactivate gene expression through hypermethylated promoters, which shows that DNA methylation does not lock gene expression in that it does not prevent reactivation by chromatin remodeling. It is not clear why previous studies reported that HDACi do not reactivate the expression of hypermethylated genes, although it may relate to the use of low doses of HDACi for short periods of time and the use of insensitive methods for gene expression analysis. Alternately, it is possible that some genes/cell lines are resistant to this effect, although we observed it for most genes and most cell lines tested.

The fact that DNA methylation does not lock gene expression raises the question of the relative contribution of DNA methylation and chromatin modifications to gene silencing. The YB5 system was particularly suitable to investigate this issue as after treatment with either depsipeptide or with 5-aza-CdR, we were able to sort the GFP-expressing cells and monitor GFP fluorescence for several months. We discovered that a treatment with HDACi can transiently reactivate hypermethylated genes (GFP and other TSG) for up to 2 weeks without any changes in DNA methylation level in their promoter regions. On the other hand, treatment with 5-aza-CdR leads to gene reactivation of GFP and other TSG for several months. Moreover, the decline in GFP-expressing cells after 5-aza-CdR, thought largely to be due to remethylation, is in fact attributable, in part, to clonal replacement by YB5 cells that are methylated and do not express GFP. Indeed, cell sorting and single-cell cloning 9 weeks after drug removal led to clones where the promoter region was completely demethylated and expression permanently on. Thus, efficient demethylation leads to permanent gene reactivation, showing that DNA methylation provides a memory signal for the silent state.

Our data show that the respective roles of DNA methylation and chromatin remodeling can be completely separated using the YB5 selectable system. The chromatin state determines the immediate gene expression potential, whereas DNA methylation provides a long-term memory for gene silencing. Thus, DNA methylation does not provide a "lock" function as...
previously thought because gene expression can be restored by drug-induced chromatin modifications without any DNA demethylation (i.e., without breaking the lock). Rather, DNA methylation provides a “spring” function, which does not suppress gene expression but brings back silencing, presumably through the previously defined order of events: methyl-binding protein recruitment, histone deacetylation, histone methylation, HP1 binding, and so on (3). This explains why physiologically, DNA methylation at promoter CGIs is only involved when very long-term silencing is required and why it provides such a selective advantage to cancer cells when TSGs are silenced by this mechanism (3). Interestingly,
after treatment with HDACi, gene expression is not sufficient to lead to permanent expression and DNA demethylation. It is possible that gene reactivation induced by HDACi may be caused by either (i) bypassing transcription factors, whereby histone acetylation will directly trigger RNA pol II activation leading to reactivation or (ii) transient binding of transcription factors to promoter regions, with gene silencing rapidly restored by repressive signals arising from DNA methylation. Restoring a silenced state is likely when histones are replaced during cell divisions in the face of persistent DNA methylation. Importantly, after the treatment with hypomethylating drugs, it has been previously shown that removal of DNA methylation marks will allow the binding of transcription factors leading to permanent epigenetic resetting promoting the emergence of stably reactivated clones. This was shown by 5-aza-Cdr–induced DNA demethylation in YB5 cells where the CREB transcription factor bound only the CMV promoter only when it was hypomethylated (13).

These data have implications for therapeutic intervention. We show that genes silenced by DNA hypermethylation in cancer can be significantly but transiently reactivated through chromatin remodeling without any changes in DNA methylation and this may be part of the clinical mechanisms of action of HDACi. Moreover, our results provide a molecular explanation for the synergy between decitabine and HDACi (6, 29) in which the combination induces more complete epigenetic reprogramming. Finally, while these findings validate chromatin as a key target for therapeutic intervention in cancer, they also suggest that stable reprogramming may require the removal of DNA methylation signals.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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References

7. Suzuki H, Gabrelian E, Chen W, Anbazhagan R, van Engeland M, Meng CF, Zhu XJ, Peng G, Dai DQ. Promoter histone H3 lysine 9 di-methylation and this may be part of the clinical mechanisms of HDACi. Moreover, our results provide a molecular explanation for the synergy between decitabine and HDACi (6, 29) in which the combination induces more complete epigenetic reprogramming. Finally, while these findings validate chromatin as a key target for therapeutic intervention in cancer, they also suggest that stable reprogramming may require the removal of DNA methylation signals.

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References


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