Silenced Tumor Suppressor Genes Reactivated by DNA Demethylation Do Not Return to a Fully Euchromatic Chromatin State

Kelly M. McGarvey, Jill A. Fahrner, Eriko Greene, Joost Martens, Thomas Jenuwein, and Stephen B. Baylin

Abstract
Histone H3 lysine 9 (H3K9) and lysine 27 (H3K27) trimethylation are properties of stably silenced heterochromatin whereas H3K9 dimethylation (H3K9me2) is important for euchromatic gene repression. In colorectal cancer cells, all of these marks, as well as the key enzymes which establish them, surround the hMLH1 promoter when it is DNA hypermethylated and aberrantly silenced, but are absent when the gene is unmethylated and fully expressed in a euchromatic state. When the aberrantly silenced gene is DNA demethylated and reexpressed following 5-aza-2'-deoxycytidine treatment, H3K9me1 and H3K9me2 are the only silencing marks that are lost. A series of other silenced and DNA hypermethylated gene promoters behave identically even when the genes are chronically DNA demethylated and reexpressed after genetic knockout of DNA methyltransferases. Our data indicate that when transcription of DNA hypermethylated genes is activated in cancer cells, their promoters remain in an environment with certain heterochromatic characteristics. This finding has important implications for the translational goal of reactivating aberrantly silenced cancer genes as a therapeutic maneuver. (Cancer Res 2006; 66(7): 3541-9)

Introduction
One key mechanism by which tumor suppressor gene function is lost in cancer is in association with aberrant promoter DNA hypermethylation–mediated gene silencing (1–3). This transcriptional silencing is known to be additionally associated with multiple promoter region chromatin modifications, including dimethylation of histone H3 at lysine 9, deacetylation at this residue, and loss of the transcriptional activating mark H3K4me2 (4–7). In addition to these findings, it is now appreciated that even further complexity exists for histone modifications. The presence of mono-, di-, and tri- forms of H3K9 methylation, as well as these same modifications at other critical residues such as H3K27, mark the most stably silenced regions of the mammalian genome (8–11). These degrees and sites of modification differ in their functional consequences, further expanding the complex nature of gene modulation by the histone code.

In RKO colorectal cancer cells, hMLH1 is transcriptionally silenced in association with DNA hypermethylation, and the gene promoter has histone modifications characteristic of transcriptional repression, including deacetylation and dimethylation of H3K9 (4). Loss of this gene in colorectal cancer cells via epigenetic silencing produces loss of mismatch repair and the microsatellite instability phenotype (12). In contrast, SW480 colorectal cancer cells do not have the microsatellite instability phenotype. The hMLH1 promoter is not DNA methylated and is transcriptionally active in a euchromatic chromatin state consisting of acetylation at H3K9, lack of methylation at this residue, and presence of methylation at H3K4 (4). When demethylated and reactivated by 5-aza-2'-deoxycytidine (5-aza-dC), the silenced RKO hMLH1 gene seems to return to a euchromatic state as characterized by loss of H3K9me2, acetylation of this residue, and acquisition of H3K4me2 (4). We present here a more extensive characterization of the histone code at hMLH1 in RKO versus SW480 cells and extend our findings to include CDH1, which is silenced with aberrant DNA hypermethylation in MDA-MB-231 breast cancer cells but expressed and unmethylated in MCF-7 breast cancer cells. We also show that the CpG island–containing promoter regions of hMLH1, as well as SFRP1, SFRP2, SFRP5, GATA4, and GATA5, other genes silenced in colorectal cancer (13–15), maintain several repressive histone modification marks even after reexpression of these silenced genes. Thus, DNA demethylation and gene reexpression, induced by either 5-aza-dC treatment or genetic deletion of DNA methylation catalyzing enzymes, can revert only a subset of repressive chromatin marks and may not protect the reactivated tumor suppressor genes from recurring gene silencing. Our results reveal a detailed molecular anatomy of DNA hypermethylated genes in cancer and have important implications for the goal of targeting reversal of aberrant gene silencing for therapeutic purposes.

Materials and Methods

Cell culture. SW480, HCT 116, and DKO cells were maintained in McCoy's 5A modified medium, RKO cells in MEM, and MDA-MB-231 and MCF-7 cells in DMEM. All media (Invitrogen, Carlsbad, CA) were supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) and 1% penicillin/streptomycin (Invitrogen) and grown at 37°C in 5% CO2 atmosphere.

5-Aza-dC treatments. Cells were treated with mock or 1 μmol/L 5-aza-dC (Sigma, St, Louis, MO) for 12 hours to 5 days as previously described (4).

Reverse transcription-PCR and methylation-specific PCR. Reverse transcription-PCR (RT-PCR) and methylation-specific PCR were done as previously described (4).

Chromatin immunoprecipitation. Proteins were cross-linked as previously described (4). For each chromatin immunoprecipitation
However, the presence of H3K9me3 additionally suggests characteristics of a transiently silenced gene with the unmethylated active promoter in SW480 cells (Fig. 1C). These findings have been associated with the stable silencing of genes embedded in facultative heterochromatin, such as those silenced on the inactive X chromosome (18, 19). For the inactive X chromosome, H3K27 methylation is established earlier than the appearance of DNA methylation and could be key for targeting the DNA modification to these specialized chromatin regions (18, 19). We additionally find that the heterochromatin protein HP1α, which is classically considered to be targeted by H3K9me3 (20), is preferentially localized to the silent promoter in RKO cells (Fig. 1D).

The above degrees and sites of histone modifications are catalyzed by different histone methyltransferases. We find enrichment of three key histone methyltransferases at the silenced versus the basally expressed hMLH1 promoter (Fig. 1D). The first, EZH2, is responsible for trimethylation of H3K27 at the inactive X chromosome (18, 19). This enzyme functions in a complex with its partner, the polycomb protein EED, and is known to be overexpressed in several cancer types (21–23). Although this complex has recently been localized to a silenced gene in breast cancer cells (24), the predicted histone modification was not examined and relationships to DNA hypermethylated tumor suppressor genes have not previously been shown. The two other histone methyltransferases, G9a and EuHMTagase1, which catalyze dimethylation of H3K9 in euchromatic regions (8, 17, 25–28), are also enriched at the DNA hypermethylated and silenced hMLH1 promoter (Fig. 1D).

In our previous studies, we determined that DNA demethylation and reactivation of the silenced hMLH1 gene induced by treatment with 5-aza-dC in RKO cells was associated with acetylation of H3K9, enrichment of H3K4me2, and loss of H3K9me2 at the promoter (4). In the current study, we conducted a more in-depth examination by analyzing the changes for H3K9 and H3K27 mono-, di-, and trimethylation on gene reactivation. We did time-course studies of 5-aza-dC treatment similar to those previously described (4). As before, DNA demethylation could be observed by 12 hours and gene reexpression by 24 hours (Fig. 2A), and both key gene activation marks, H3K9ac and H3K4me2, sharply increased by 48 hours of treatment (data not shown). After 4 days of 5-aza-dC, at a time when at least 50% of the treated cells robustly express nuclear MLH1 protein (data not shown), of all six histone modifications examined, only H3K9me1 and H3K9me2 were significantly depleted (Fig. 2B and C). For H3K9me2, this decrease was dramatic across the critical region of the hMLH1 promoter, with undetectable levels at one point along the region examined. Notably, G9a and EuHMTagase1, the histone methyltransferases that catalyze dimethylation of H3K9, were also depleted on 5-aza-dC–mediated demethylation (Fig. 2D). However, the actively transcribing gene promoter still possessed the repressive H3K9me3 modification, as well as all forms of H3K27 methylation. At some sites, the H3K9me3 and H3K27me3 marks actually increased at the active promoter. Interestingly, the retention of the H3K27me3 mark persisted over the 5-day period despite the fact that EZH2 was dramatically decreased at the promoter after 5-aza-dC treatment (Fig. 2C and D). Also of note, the key interpreter for gene silencing and spreading of repressive chromatin, HP1α, was also lost at the promoter even as H3K9me3 remained (Fig. 2D).
The above failure of multiple repressive transcriptional chromatin modifications to be reversed during gene reexpression following 5-aza-dC treatment could be due to the short time course of such experiments and/or the fact that not all cells reexpress the genes during the time interval. To investigate this possibility, we turned to a unique setting where DNA demethylation and reactivation of gene transcription are continuous. In these HCT116 cells, termed DKO cells, DNA methyltransferase 1 and DNA methyltransferase 3b have been genetically disrupted (13, 29). For all genes in HCT116 wild-type cells that are DNA hypermethylated and silenced, which we and others have examined, the methylation is abolished and the genes are reexpressed in DKO cells. Importantly, this DKO setting seems to be a homogeneous one in terms of a complete allelic switch from the methylated to unmethylated state and for gene reexpression (29). Notably, the SFRP genes and the GATA4 and GATA5 genes are completely DNA demethylated in all sequenced alleles and reexpressed (13–15). Furthermore, in parallel to reexpression of the anti-Wnt SFRP genes in the DKO cells, the Wnt pathway seems silenced in all cells as evidenced by complete loss of β-catenin from the nucleus (13). In this study, we find further evidence supporting homogeneity of gene expression by examining cells for two genes studied below, SFRP1 and GATA4.

**Figure 1.** Various degrees of H3K9 and H3K27 methylation, as well as HP1α and the histone methyltransferases EZH2, G9a, and EuHMTase1, are increased along a DNA-hypermethylated and silenced versus an unmethylated and active hMLH1 promoter. A, RT-PCR (left) and methylation-specific PCR (right) for hMLH1 in RKO and SW480 cells. B and C, occupancy of hMLH1 promoter by H3K9 and H3K27 mono-, di-, and trimethylation. D, occupancy of hMLH1 promoter by EZH2, G9a, EuHMTase1, and HP1α. Chromatin immunoprecipitation data from the corresponding cell line immunoprecipitated with the corresponding antibody and amplified by PCR. Points, mean; bars, SE. Representative PCR analyses done on the immunoprecipitated, mock (Ab), and a 1:100 dilution of nonimmunoprecipitated (Input) DNA from both RKO and SW480 using the most 3′ primer set are shown beside each graph.
As shown in Supplementary Fig. S1, every cell in the DKO population expresses the protein products of these genes whereas the proteins are not detected in any cells in the wild-type HCT116 population.

In the above setting, we examined the SFRP1 gene in wild-type HCT116 colon cancer cells versus DKO cells. This gene is silenced in association with promoter DNA hypermethylation in HCT116 cells but expressed and unmethylated in DKO cells (Fig. 3A). Similar to the methods used to examine the hMLH1 gene, we employed PCR primer sets that span a region of the SFRP1 promoter upstream and encompassing the transcription start site. We confirm that, as expected, gene reactivation in the DKO cells is associated with an increase in H3K9/K14ac and H3K4me2 (Fig. 3B) and similar decreases in H3K9me1 and H3K9me2 (Fig. 3C). However, as for the hMLH1 gene after 5-aza-dC treatment discussed above, the remaining repressive methylation marks do not significantly decrease, with H3K9me3 actually increasing when SFRP1 is expressed in the DKO cells (Fig. 3C and D).

To examine the potential universality of the above findings, we studied several additional tumor suppressor gene promoters. We focused these studies on the modifications that exhibited the greatest enrichment changes in the previous experiments (i.e., the activation mark H3K4me2, as well as the di- and trimethylation of silencing marks H3K9 and H3K27). Even by examining a very few examples, we can conclude that the changes we observe generally hold true for other tumor suppressor genes with silenced promoters.

Figure 2. Gene reexpression after 5-aza-dC treatment leads to loss of H3K9me2, HP1α, and the histone methyltransferases EZH2, G9a, and EuHMTase1 at the hMLH1 gene promoter in RKO cells. A, RT-PCR (top) and methylation-specific PCR (bottom) for hMLH1 DNA in RKO cells either mock or drug-treated with 1 μmol/L 5-aza-dC. B and C, occupancy of hMLH1 promoter DNA by H3K9 and H3K27 mono-, di-, and trimethylation after 3 days of drug treatment. D, occupancy of hMLH1 promoter DNA by EZH2, G9a, EuHMTase1, and HP1α after 3 days of drug treatment. Chromatin immunoprecipitation data from the corresponding treatment group immunoprecipitated with the corresponding antibody and amplified by PCR. Points, mean; bars, SE. Representative PCR analyses done on the immunoprecipitated, mock, and a 1:100 dilution of nonimmunoprecipitated DNA from both mock and 5-aza-dC–treated cells using the most 3′ primer set are shown beside each graph.
limited and randomly chosen region in each gene with chromatin immunoprecipitation analysis, we have readily confirmed the universality of the findings for hMLH1. We first examined the CDH1 promoter in a DNA-methylated and silenced state in MDA-MB-231 cells versus an unmethylated and active state in the MCF-7 breast cancer cell line (Fig. 4A). As seen for hMLH1, all forms of H3K9 and H3K27 methylation were enriched at the silent versus the active promoter whereas the active H3K4me2 and H3K9ac marks were simultaneously diminished (Fig. 4B). We then examined multiple other DNA-hypermethylated and silenced genes in RKO colorectal cancer cells after mock or 5-aza-dC treatment, including SFRP2, SFRP5, and GATA5 (Fig. 4C). We found that, as for the reexpression response of hMLH1 to 5-aza-dC treatment, while the activation mark H3K4me2 increased, the only silencing mark reduced on drug treatment was H3K9me2 (Fig. 4D). The other silencing marks showed no change or increased modestly. These data allow consideration of the important possibility that several repressive histone modifications associated with heterochromatin remain enriched at previously DNA hypermethylated and silent genes that have been reactivated by complete DNA demethylation.

Discussion

Our detailed map examining all possible methylation states for H3K9 and H3K27 methylation at the DNA hypermethylated and silenced hMLH1 gene in colorectal cancer cells reveals the potential
molecular anatomy of many abnormally silenced and DNA-hypermethylated genes in cancer cells. In Fig. 6, we provide a model for hypothesizing how the data might be interpreted for the states of chromatin that might be represented. There are several biological and translational implications for our findings. First, it is clear that for DNA hypermethylated hMLH1, the silencing involves an aberrant accumulation of repressive chromatin marks surrounding the promoter, the particular combination of which is not classically envisioned to occur together at one genomic region. The histone methylation patterns are characteristic of marks present not only at transiently silenced genes in euchromatin, as evidenced by H3K9me2, but also of more stable imprints that are typically associated with facultative heterochromatin and pericentromeric heterochromatin, such as H3K27 methylation and H3K9me3, respectively (8–11, 30, 31). Evidence that constitutively expressed hMLH1 resides in full euchromatin is shown by the virtual depletion of all of the above transcriptional silencing marks in the SW480 cells. This may well explain why we have previously found open chromatin surrounding this gene in the SW480 cells but persistence of closed chromatin in cells where the gene is DNA hypermethylated even after gene reexpression was induced by 5-aza-dC and trichostatin A treatment (40). Retention of repressive methylation marks also characterizes the activated SFRP1, SFRP2, SFRP5, GATA4, and GATA5 genes in a semiheterochromatic state that may facilitate recurring gene silencing. These scenarios suggest that only certain distinct chromatin marks need to be associated with transcriptional reactivation, such as H3K4me2, H3K9ac, and depletion of H3K9me2. By contrast, the other methylation marks, such as H3K9me3, H3K27me2, and H3K27me3, apparently do not directly impair transcriptional reactivation but may serve to index the promoter region for additional epigenetic control. Possibly, these latter marks may be involved in discriminating compromised promoter function and provide imprints to facilitate subsequent silencing if the activating signals decay.

One aspect of our findings presents a particular conundrum that must be addressed. Classically, the presence of acetylation at the H3K9 residue is thought to block methylation at this site and needed to establish and/or maintain this chromatin are also targeted to the silenced hMLH1 promoter in a steady-state manner. We additionally show localization of HP1α, which is a key protein in the interpretation and spreading of the H3K9 methylation mark. Interestingly, with the exception of the H3K9me1 and H3K9me2 marks, the target transcriptional repression marks are stable over several days of induced gene transcription, although in the case of EZH2, the histone methyltransferase is depleted at the promoter. A similar stability of modifications, and even an increase, is observed at the SFRP1 and other gene promoters examined in the DKO cells, where silenced tumor suppressor genes have been stably reexpressed and completely DNA demethylated. This stability, as well as the loss of H3K9me2, may result from either of two mechanisms, histone replacement or active demethylation of H3K9me2. The histone composition of nucleosomes varies between active transcriptional states and for transcriptional repression. In the repressed transcription state, histone H3 is thought to be replaced by replication-dependent mechanisms only (34–37). However, during transcription (e.g., as induced by 5-aza-dC for the silenced RKO hMLH1 gene), H3 may be replaced by the variant histone H3.3. This transcription-coupled replacement can even provide for continued enrichment of the H3.3 variant as gene activity continues (34–37). H3.3 is enriched for H3K4me2 and H3K9ac, as opposed to H3K9me2, differing little in the other histone modifications (37). Because our PCR-based chromatin immunoprecipitation approach probably detects chromatin modifications across several nucleosomes, it is possible that H3K9me2 is exchanged by H3.3 in some nucleosomes while leaving the H3K9 and H3K27 trimethyl marks in other promoter-proximal nucleosomes. Alternatively, a putative histone lysine demethylase could actively remove H3K9me2 by being recruited with activating protein complexes to the hMLH1 and SFRP1 promoters. This would be consistent with the recently identified LSD1 (38), originally stipulated as an H3K4 demethylase and recently shown to have activity toward H3K9 (39).

Third, our studies with 5-aza-dC and the DKO cells provide a newly described intermediate transcription state for an abnormally silenced gene in cancer cells (see model, Fig. 6). In this setting, despite induction of active transcription sufficient to provide functional protein (12), hMLH1 does not return to the full euchromatic state observed in SW480 cells. This may well explain why we have previously found open chromatin surrounding this gene in the SW480 cells but persistence of closed chromatin in cells where the gene is DNA hypermethylated even after gene reexpression was induced by 5-aza-dC and trichostatin A treatment (40). Retention of repressive methylation marks also characterizes the activated SFRP1, SFRP2, SFRP5, GATA4, and GATA5 genes in a semi-heterochromatic state that may facilitate recurring gene silencing. These scenarios suggest that only certain distinct chromatin marks need to be associated with transcriptional reactivation, such as H3K4me2, H3K9ac, and depletion of H3K9me2. By contrast, the other methylation marks, such as H3K9me3, H3K27me2, and H3K27me3, apparently do not directly impair transcriptional reactivation but may serve to index the promoter region for additional epigenetic control. Possibly, these latter marks may be involved in discriminating compromised promoter function and provide imprints to facilitate subsequent silencing if the activating signals decay.

One aspect of our findings presents a particular conundrum that must be addressed. Classically, the presence of acetylation at the H3K9 residue is thought to block methylation at this site and
Figure 5. Multiple hypermethylated tumor suppressor genes retain repressive chromatin marks after DNA demethylation and gene reexpression. A, RT-PCR (left) and methylation-specific PCR (right) for SFRP2, SFRP5, and GATA5 in RKO cells either mock or drug treated with 1 μmol/L 5-aza-dC. B, occupancy of SFRP2, SFRP5, and GATA5 promoter DNA by H3K4 dimethylation, and H3K9 and H3K27 di- and trimethylation in untreated RKO cells versus RKO cells treated with 5-aza-dC for 3 days. C, RT-PCR (left) and methylation-specific PCR (right) for SFRP2, SFRP5, GATA4, and GATA5 in HCT116 and DKO cells. D, occupancy of SFRP2, SFRP5, GATA4, and GATA5 promoter DNA by H3K4 dimethylation, and H3K9 and H3K27 di- and trimethylation in HCT 116 colon cancer cells versus DKO cells. Representative PCR analyses done on the immunoprecipitated, mock, and a 1:100 dilution of nonimmunoprecipitated DNA from either mock and 5-aza-dC-treated RKO cells, or HCT 116 and DKO cells are shown. Chromatin immunoprecipitation data quantitated from the corresponding cell line/treatment group immunoprecipitated with the corresponding antibody and amplified by PCR. Columns, mean; bars, SE.
the growing clinical use of 5-aza-dC, especially in the treatment of
many cancers (8–11, 20).

One family of proteins which are critical in the recognition of the
silenced state (42). One intriguing possibility for gene expression in
this state is seen in transcription of genes that the genes never leave an area enriched in heterochromatin. Theoretically, this could only reflect the presence of a mixed population of nucleosomes with their attendant H3K9 residues accompanying the active transcription state of the gene. One possibility for such a scenario is that even the DKO cells, which have long-standing gene reexpression, may have one allele transcribing the genes and one remaining silent. Although immunofluorescence data in Supplementary Fig. S1 support the notion that all DKO cells are uniformly expressing protein, we certainly cannot rule out this possibility of monoallelic expression. However, we favor another explanation as we do not see DNA methylation patterns suggesting this is the case. The methylation-specific PCRs in Figs. 3A and 3C show no retained methylation signal in the DKO cells for all genes examined. Furthermore, when these genes have been examined by bisulfite sequencing in the DKO cells, all alleles are observed to have lost DNA methylation for each CpG site in the promotor CpG island (13). We then favor the important probability that the genes never leave an area enriched in heterochromatin.

Precedent for such a possibility is seen in transcription of genes embedded in heterochromatin and has been described in Drosophila (41) and during experimental activation of a reporter gene construct that had integrated in heterochromatin in its basal, silenced state (42). One intriguing possibility for gene expression in a heterochromatic environment might be our observed loss of the protein HP1α with 5-aza-C treatment. This protein is a member of a family of proteins which are critical in the recognition of the H3K9me3 mark, gene silencing, and spreading of the H3K9me3 mark across regions (8–11, 20).

Finally, there is an important implication of our findings about the growing clinical use of 5-aza-dC, especially in the treatment of the pre-leukemic disease myelodysplasia and of leukemias (43–45). Our findings may likely explain why DNA-hypermethylated genes, after demethylation and activation by 5-aza-dC treatment of cells, readily reaccumulate DNA methylation and return to a gene silencing state once the drug is removed (46). In this regard, our current observations suggest that retention of all the H3K27, and some H3K9 methylation marks, which have been associated with recruitment of DNA methylation (47–49), may render the promoter primed to reassume a DNA-hypermethylated and resilenced state associated with gain of the critical H3K9me2 mark. Indeed, when RKO cells are released from 5-aza-dC treatment, the hMLH1 gene becomes resilenced (Supplementary Fig. S2). This scenario will constitute a molecular barrier that must be overcome, probably through continuous and/or repeated drug administration, in an attempt to continue maximizing the efficacy of therapeutic strategies targeting reversal of tumor suppressor gene silencing.

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References


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