Cancer Research

Aberrant Silencing of Cancer-Related Genes by CpG Hypermethylation Occurs Independently of Their Spatial Organization in the Nucleus

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Abstract

Aberrant promoter DNA-hypermethylation and repressive chromatin constitutes a frequent mechanism of gene inactivation in cancer. There is great interest in dissecting the mechanisms underlying this abnormal silencing. Studies have shown changes in the nuclear organization of chromatin in tumor cells as well as the association of aberrant methylation with long-range silencing of neighboring genes. Furthermore, certain tumors show a high incidence of promoter methylation termed as the CpG island methylator phenotype. Here, we have analyzed the role of nuclear chromatin architecture for genes in hypermethylated inactive versus nonmethylated active states and its relation with long-range silencing and CpG island methylator phenotype. Using combined immunostaining for active/repressive chromatin marks and fluorescence *in situ* hybridization in colorectal cancer cell lines, we show that aberrant silencing of these genes occurs without requirement for their being positioned at heterochromatic domains. Importantly, hypermethylation, even when associated with long-range epigenetic silencing of neighboring genes, occurs independent of their euchromatic or heterochromatic location. Together, these results indicate that, in cancer, extensive changes around promoter chromatin of individual genes or gene clusters could potentially occur locally without preference for nuclear position and/or causing repositioning. These findings have important implications for understanding relationships between nuclear organization and gene expression patterns in cancer. *Cancer Res; 70(20); 8015–24. ©2010 AACR.*

Introduction

Epigenetic abnormalities, especially aberrant DNA methylation of promoter CpG islands of cancer-related (CR) genes, are common and early events contributing to gene inactivation during tumorigenesis (1). In addition to DNA methylation, our studies analyzing selected hypermethylated genes as well as global analysis of hypermethylated genes in cultured colorectal cancer (CRC) cell lines have shown that these repressed promoters are marked by trimethylation of H3K27 residues (H3K27Me3) concomitant with decreased levels of the activating mark, dimethylation of H3K4 (H3K4Me2; refs. 2, 3).

The mechanisms underlying CpG hypermethylation in cancer are unknown. It has been shown that aberrantly silenced CR genes could be reactivated by the DNA-methyltransferase (DNMT) inhibitor, 5-aza-2′-deoxycytidine (5-aza-CdR). However, re-expression in response to 5-aza-CdR is transient and the genes get resilenced on drug removal (4, 5). Genetic knockout, as well as 5-aza-CdR–mediated inhibition of the DNMTs, results in the loss of promoter CpG methylation and derepression of the CR genes. However, in CRCs, the levels of the inactive H3K27Me3 mark increases and coexists with increases in the active H3K4Me2 mark indicating that the promoters may still reside in a H3K27Me3-marked heterochromatic environment (2, 3). Another interesting feature associated with aberrant hypermethylation is the long-range epigenetic silencing (LRES) in which a cluster of adjacent genes across a large chromosomal segment undergoes coordinated silencing and shows synergistic reactivation by a combination treatment with 5-aza-CdR and the histone deacetylase inhibitor, trichostatin A (6, 7). This suggests that the entire chromosomal segment is under a common control mechanism involving DNA methylation and heterochromatic histone modification.

A little explored facet of epigenetic regulation in cancer cells concerns the increasing evidence for the role of spatial

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arrangement of chromosomes and genes in transcriptional regulation (8, 9). Gene position has been shown to vary during development and disease states wherein genes reposition to heterochromatic compartments (the nuclear periphery or centromeric heterochromatin) when inactivated and relocate to the interior of the nucleus when activated (9). Furthermore, genes artificially tethered to the heterochromatic environment in the inner nuclear membrane undergo variable degrees of silencing (10–12). Physical association with heterochromatin accompanied by DNA methylation has been observed in a transgene induced to undergo stable silencing by transient, corepressor-mediated targeting (13). Thus, the nucleus can be viewed to have domains of gene activity (euchromatin) and inactivity (heterochromatin) which are proposed to optimize and regulate gene expression (8, 14–16).

In cancer, changes in the spatial organization of chromosome territories, centromeres, telomeres, and specific genes have been observed (17–20). The functional significance of these changes is not well understood. It has been proposed that changes in nuclear structure in cancer cells could influence gene expression (21). It is possible that the nuclear position of genes might play a role in aberrant hypermethylation in cancer cells, especially during LRES where repositioning to a heterochromatic domain might coordinately silence the entire chromosomal segment.

Herein, we address whether or not higher order nuclear positioning of genes has a role in aberrant methylation or if aberrant methylation is associated only with local promoter changes. We have analyzed the relationship between the position of CR gene loci that undergo hypermethylation individually or in the context of LRES, and their nuclear microenvironment (euchromatin/heterochromatin) by immunostaining and fluorescence *in situ* hybridization (FISH) in CRC cell lines. We analyzed the position of the *MLH1, SFRP4, SFRP5*, and *ICAM1* genes, which are frequently DNA-hypermethylated and silenced in CRC lines. We show that hypermethylation mediated aberrant silencing of individual genes or in the context of LRES can occur both in a euchromatic or heterochromatic environment. We observe that aberrant silencing involves local chromatin changes in the absence of a requirement for global positioning to a heterochromatic compartment. These studies have important implications in the understanding of aberrant CpG hypermethylation and the role of nuclear positioning in gene regulation.

Materials and Methods

Combined immunostaining, FISH, and microscopy

Cell lines used in this study were obtained from American Type Culture Collection and were authenticated on June 9, 2010 by short tandem repeat profiling and by matching with the profile published in American Type Culture Collection. Cells grown on coverslips were processed for immuno-FISH using modifications of previously described protocols (22, 23). Immunostained cells were fixed in 50 mmol/L of ethylene glycolbis(succinimidylsuccinate) from Pierce Biochemicals followed by FISH. See Supplementary Methods for protocol/microscopy details.

Chromatin immunoprecipitation-chip

Chromatin immunoprecipitation (ChIP) on chip analyses was from single experiments and was performed on RKO and SW480 cells as previously described (3). Additionally, previously published ChIP-chip data from HCT116 and DKO were analyzed (3).

Reverse transcription-PCR

Reverse transcription-PCR was done as previously described (2).

Gene density calculations

HGNC genes in 2- and 6-Mb regions around the genes of interest were obtained from BioMart (Ensembl), and gene densities were calculated as the number of genes per megabase.

Analysis of neighborhood expression

Agilent microarray data were used to analyze neighborhood expression. Details of the procedure are given in Supplementary Methods. The data discussed in this publication are accessible through National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23693.

Infinium methylation array

DNA methylation was assessed using the Illumina Infinium platform as previously described (24, 25).

Results

DNA methylation is independent of gene association with chromatin domains

The relationship between the nuclear positions of aberrantly methylated CR genes relative to the chromatin environment was explored by immunostaining for H3K4Me2 or H3K27Me3 domains and DNA-FISH in RKO, SW480, and HCT116 cells. H3K4Me2 and H3K27Me3 mark active euchromatin and facultative heterochromatin, respectively, which are visible as distinct subnuclear domains (26–28). Technical artifacts could arise during the FISH procedure compromising the distribution of chromatin domains; interestingly, the H3K27Me3 mark in SW480 nuclei was especially sensitive to FISH whereas the H3K4Me2 mark was resilient to immuno-FISH (Supplementary Fig. S1). To overcome this, we evaluated various FISH protocols and employed a modified protocol that preserves the chromatin pattern after FISH (see Supplementary Methods; Supplementary Fig. S2A). To show that the H3K27Me3 patterns are maintained before and after FISH, cells were fixed and immunostained and the same nuclei were imaged before and after FISH. Supplementary Fig. S2B is a typical image of a SW480 nucleus stained for H3K27Me3 before and after FISH. Although there is a 15% to 20% reduction in the immunostaining signal following FISH, the different *z*-stacks analyzed show that the histone staining pattern is robustly maintained.

Previous studies have shown that euchromatin and heterochromatin are marked by low and high DNA staining, respectively (29). Consistent with this, H3K4Me2 and H3K27Me3

domains showed weak and dense DNA staining, respectively, indicating that these marks differentiate euchromatin from heterochromatin (Fig. 1A and B). As a control, we first studied the position of the ubiquitously active housekeeping gene, *ACTB*, with respect to euchromatin/heterochromatin. In SW480 and RKO cells, *ACTB* associated with H3K4Me2-marked euchromatin (Supplementary Fig. S3A and B, G and H). Similarly, we used the β-globin gene (*HBB*), which is not expressed in the CRC lines, as a control for an inactive gene. Previous studies have shown that *HBB* localization is developmentally regulated and that it is positioned close to heterochromatin in lineages in which this gene is not expressed (30, 31). In both SW480 and RKO cells, *HBB* associated with H3K27Me3 domains or conversely was excluded from H3K4Me2 domains (Supplementary Fig. S3E and F, K and L).

We then tested whether CR genes are subject to changes in their association with heterochromatic/euchromatic domains in response to hypermethylation. We first studied *MLH1* and *SFRP4*, which are both active and non–DNA-methylated in SW480 cells (Supplementary Fig. S4), and their promoters are enriched for the H3K4Me2 mark and have

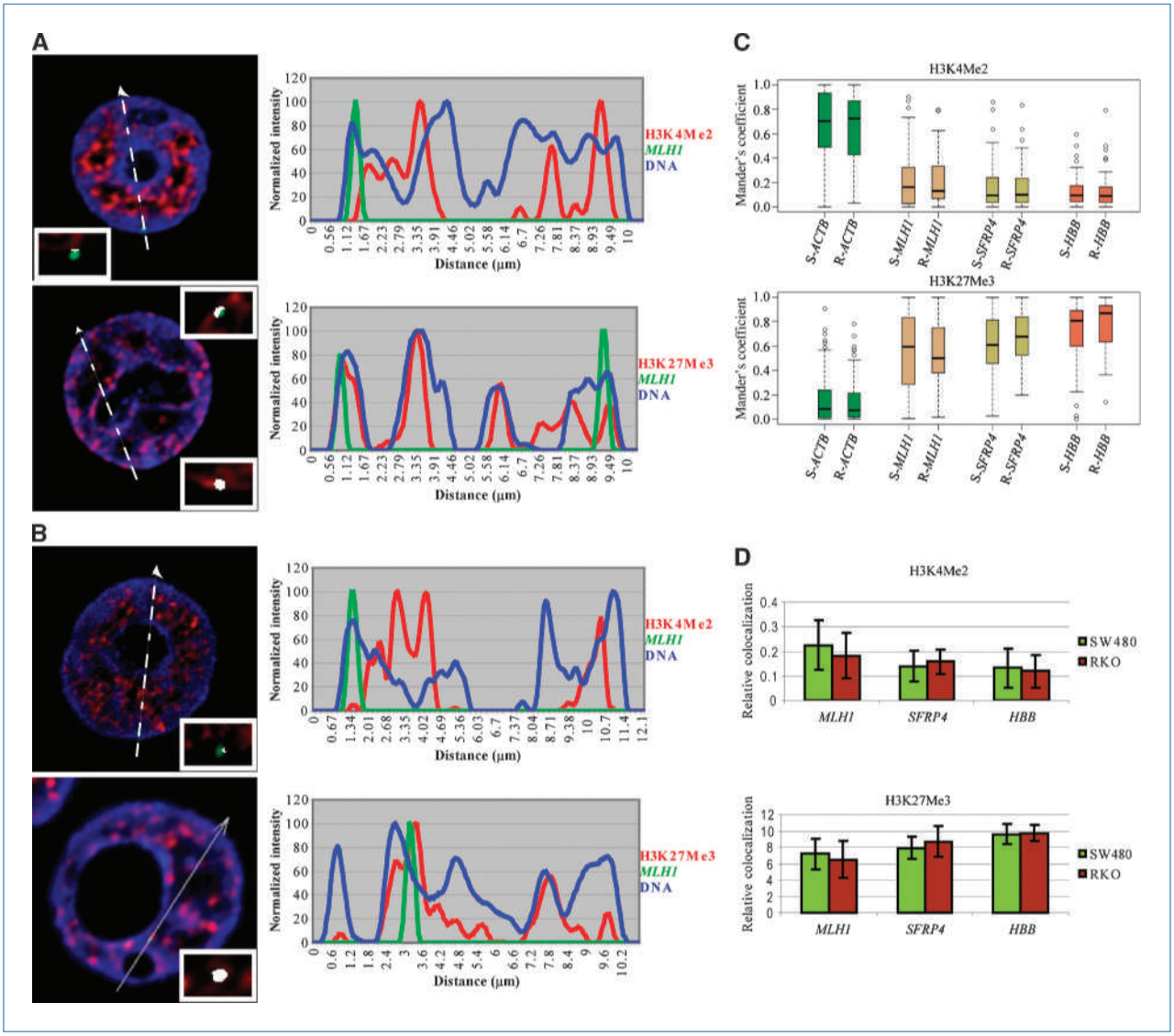

Figure 1. Association of *MLH1* with the H3K27Me3 domains. RKO (A) and SW480 cells (B) immunostained for H3K4Me2 (top) or H3K27Me3 (bottom) and *MLH1* and *ACTB* (shown in Supplementary Fig. S3A and B, G and H) by DNA-FISH. Line scan plots the intensities of the modified histone (red), gene signal (green), and DNA (blue) along the line. Insets, the degree of colocalization of the FISH and modified histone signal shown as white pixels. Only allele(s) in a single *z*-slice are shown here (all alleles are shown in Supplementary Fig. S3). C, boxplots show colocalization (Manders' coefficient) between the FISH and modified histone signal from a single experiment (n = 25 nuclei). *X*-axis labels show the genes analyzed in SW480 (prefix S) and RKO (prefix R). D, colocalization values normalized to *ACTB*. The median Manders' coefficients of *MLH1, SFRP4*, and *HBB* colocalization with the modified histones from three experiments (n = 25, 10, and 10 nuclei) were normalized to that of *ACTB* and plotted as relative colocalization (*Y*-axis). Error bars indicate SD.

reduced H3K27Me3 upstream of the transcription start site (refs. 2, 3, 32; Supplementary Fig. S5A and B). Both genes are DNA-methylated, silenced and have reduced H3K4Me2 in RKO cells (refs. 2, 3, 32; Supplementary Figs. S4 and S5A and B). Although in RKO cells, H3K27Me3 showed increased enrichment at the *SFRP4* promoter, *MLH1* showed only a moderate enrichment of H3K27Me3 upstream of the transcription start site (Supplementary Fig. S5A). ChIP-PCR analysis has shown that the *MLH1* promoter in RKO cells is enriched for H3K27Me3 (ref. 2; data not shown). In both cell types, *MLH1* and *SFRP4* showed an increased association with H3K27Me3 staining similar to *HBB* and in contrast to *ACTB* (*MLH1* shown in Fig. 1A and B; all alleles of *MLH1* and *SFRP4* in both cell types shown in Supplementary Fig. S3A–D and G–J). Quantitation of colocalization between the gene signal and the modified histone signal reveal that most alleles of *MLH1* and *SFRP4* show high association with H3K27Me3 domains in both cell lines (Fig. 1C), with no significant differences between the two cell lines ($P > 0.1$). To enable direct comparison of the colocalization values across cell lines, multicolored FISH was performed for the genes of interest and *ACTB* and the median colocalization was normalized to this latter gene. This normalization, in independent experiments, verified that most alleles of *MLH1* and *SFRP4* associate with the H3K27Me3 mark and fewer with the H3K4Me2 mark in both cell lines (Fig. 1D).

Previous studies have shown that H3K27Me3 domains are enriched at the perinuclear and perinucleolar regions (29). In concordance with the above results showing a high degree of association with the H3K27Me3 domains, *MLH1, SFRP4*, and *HBB* alleles are preferentially located at the perinuclear or perinucleolar regions (Fig. 2A and B; Supplementary Fig. S6A–C), with a median distance from these regions of <0.5 μm. There are three aneuploid alleles of the *SFRP4* and *HBB* loci in SW480 cells, and interestingly, like the diploid alleles of RKO (data not shown), these are all positioned either at the perinuclear or perinucleolar regions indicating that extra gene copies consistently tend to associate with the same chromatin domains.

The above analyses show that *MLH1* and *SFRP4* are positioned close to heterochromatin independent of their silencing status. However, such positioning could predispose the genes to permanent silencing by DNA methylation. To test if abnormally silenced CR genes generally tend to position close to heterochromatin, two additional genes silenced in CRC lines were analyzed. *SFRP5* was analyzed in RKO cells where it is DNA-hypermethylated and silenced versus in SW480 cells where it is unmethylated and active (ref. 33; Supplementary Fig. S4). To put these studies into perspective, we first analyzed the local promoter marks from the ChIP-chip data which showed that *SFRP5* is enriched for H3K4Me2 in SW480 whereas it lacks this mark in RKO (Supplementary Fig. S5C). Interestingly, the silenced SFRP5 promoter did not show any enrichment of H3K27Me3. The other gene, *ICAM1*, is unmethylated and active in both RKO and SW480 cells, but in HCT116 cells it is DNA-hypermethylated and silenced (ref. 33; Supplementary Fig. S4). In both SW480 and RKO cells, *ICAM1* is enriched for H3K4Me2 around the transcription start site consistent with its active state. Using previous data (3), we compared the *ICAM1* promoter between HCT116 and its isogenic partner, DKO cells, which have genetic disruption of the major DNA methyltransferases—DNMT1 and DNMT3B (34). In HCT116, the silenced *ICAM1* promoter showed a modest decrease in H3K4Me2 along with a slight enrichment of H3K27Me3 compared with the reactivated promoter in DKO cells (Supplementary Fig. S5D and E).

In all the cell lines, regardless of the above methylation and expression status, most alleles of *ICAM1* and *SFRP5*, like *ACTB*, in contrast with *MLH1* and *SFRP4*, exhibit a preference for the H3K4Me2-labeled euchromatin and are excluded from the H3K27Me3-marked heterochromatin (Fig. 3A and B; Supplementary Fig. S7). Colocalization analysis showed that the majority of *ICAM1* and *SFRP5* alleles associate with the euchromatic mark with little difference between their active and inactive states in SW480 and HCT116/RKO cells ($P > 0.1$; Fig. 3C and D). Thus, these data indicate that there is no general requirement for aberrantly DNA-methylated and silenced genes to be positioned close to heterochromatin. The data here also show that a gene can be active in a heterochromatic environment and still be enriched for euchromatic marks locally at the promoter, as is the case with *MLH1* and *SFRP4* in SW480 cells. These results, in the aggregate, again emphasize that the position of CR genes relative to euchromatin/heterochromatin in CRC lines is independent of their promoter CpG island methylation status, and local epigenetic alterations could exist in the absence of global changes in positioning.

Relation between nuclear position and LRES

The data above reveal that *MLH1, SFRP4*, and *HBB* show association with heterochromatin whereas *ICAM1, SFRP5*, and *ACTB* reside in euchromatin. The factors that could dictate nuclear positions of these gene loci include their relationships to the gene density of the regions in which they reside or the activity of neighboring genes. Previous studies have shown that gene-rich loci reside in euchromatic domains (27). Furthermore, it has been shown in colon cancers that aberrant methylation of *MLH1* is accompanied by LRES of a cluster of neighboring genes in the locus (7). Because the data above showed that *MLH1* associates with heterochromatic compartments, we explored the relationships between nuclear position and LRES. We first analyzed the expression pattern of neighboring genes in a 1 Mb domain around the genes of interest (Fig. 4A). To determine the dependency on DNA methylation–mediated silencing of neighboring genes, reverse transcription-PCR was performed on RNA from SW480 and RKO cells that were mock-treated or treated with 5-aza-CdR. In the case of HCT116, its isogenic line, DKO, was used for reverse transcription-PCR analysis. Figure 4 shows that *MLH1, SFRP4*, and *SFRP5* reside in areas of local spreading of silencing to neighboring genes in a cell type–specific manner—with RKO showing the highest degree of spreading of silencing, HCT116 an intermediate degree, and SW480 the least silencing. *ICAM1* and *ACTB* loci did not show any spreading of silencing to neighboring genes. Thus, the activity of neighboring genes in these analyzed loci does not

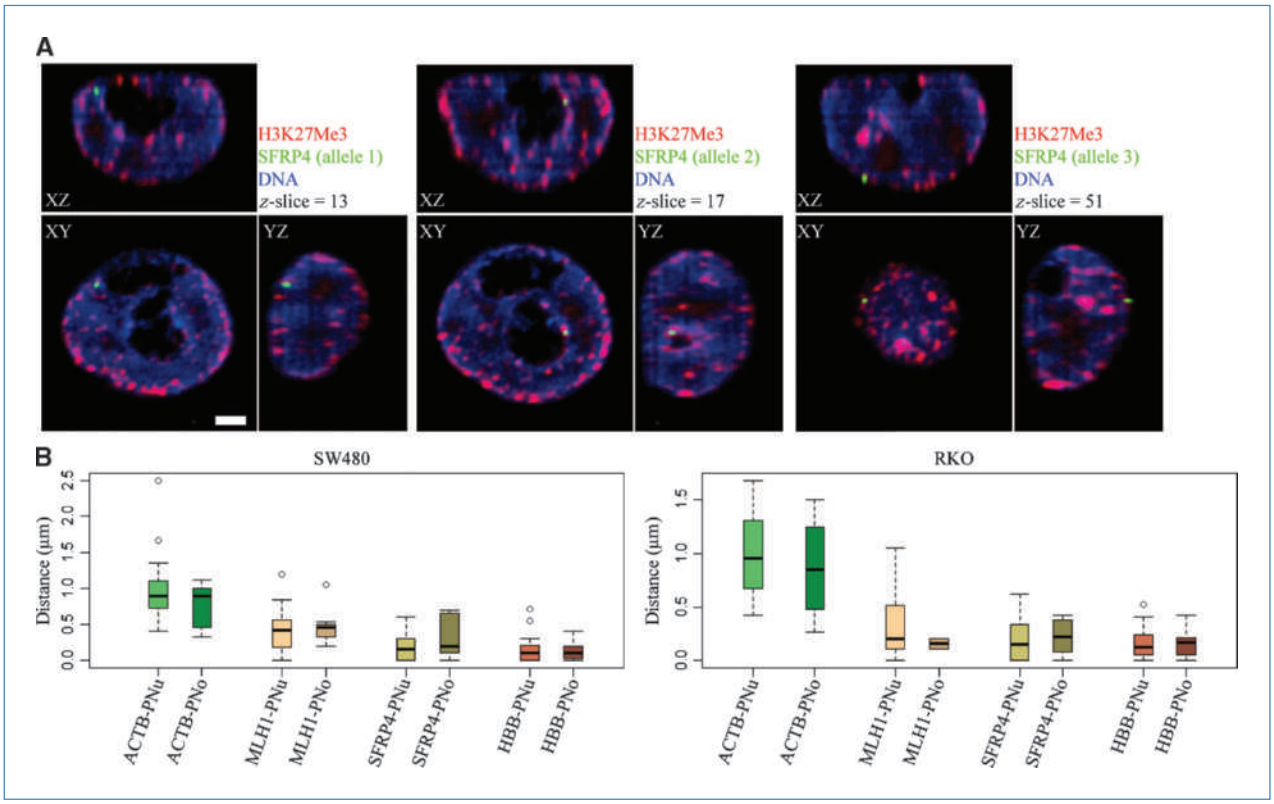

Figure 2. Position of *MLH1, SFRP4, HBB*, and *ACTB* relative to the perinuclear and perinucleolar domains. A, orthogonal sections passing through the three aneuploid alleles of *SFRP4* locus (green) in SW480 cells showing proximity to the perinuclear or perinucleolar regions, which are stained with H3K27Me3 (red). Nucleoli are devoid of DNA staining (blue). Scale bar, 2 μm. B, quantitation of gene position relative to perinuclear (PNu) or perinucleolar (PNo) regions in SW480 and RKO cells. The nearest distances to the perinuclear or perinucleolus for 30 to 45 alleles are plotted (*Y*-axis).

always render genes predisposed to abnormal silencing, and LRES is independent of the nuclear position of involved genes because (*a*) *ICAM1* can be silenced even though it resides in a region where neighboring genes are always active; (*b*) although *MLH1* and *SFRP4* are positioned in heterochromatin in both RKO and SW480, these loci show differential spreading of silencing in different cell lines; and (*c*) even though *SFRP5* is positioned in euchromatin, it is involved in differential, local spreading of silencing to neighboring genes in different cell lines.

It can also be noted from Fig. 4A that the gene densities in these loci differ widely. Previous studies have shown that gene density might be an integral determinant and/or marker of the radial positioning of genomic elements in the nucleus (35, 36) and that the gene density in a window of 2 Mb is a good predictor of radial gene position (37). We compared the gene densities of our genes of interest in a window of 2 and 6 Mb (Fig. 4B), which revealed that the *MLH1* and *SFRP4* loci, which are positioned in heterochromatin, are in gene-poor regions. In contrast, *SFRP5, ICAM1*, and *ACTB* loci, which are positioned in euchromatin, are gene-rich. These data agree with a previous study showing the correlation between high gene density (>16 genes/Mb) and H3K4Me3 domains (27). Thus, we observe a correlation between high gene density and positioning at euchromatic domains, and low gene density and heterochromatic domains.

One exception to the relationships between gene density and gene position is the *HBB* gene, which has a very high gene density but was observed to associate with H3K27Me3-marked heterochromatin. This could be because the majority of genes present in the *HBB* locus belong to the olfactory receptor family of genes. Analysis of whole transcriptome expression arrays (Agilent) of all the cell lines in this study show that the olfactory genes and other genes in a >1 Mb domain around *HBB* are not expressed in any of the CRC lines (data not shown). Furthermore, analysis of ChIP-chip data from all three cell lines revealed H3K27Me3 enrichment at the promoters of 27 of 30 genes in the 2 Mb domain around *HBB* (data not shown), confirming the silent status of genes in this neighborhood. Thus, the fact that the *HBB* locus is an exception to the relationship between gene density and chromatin domains might reflect the unusual situation wherein a high proportion of genes in a locus are under regional control in a tissue-specific manner and indicates that other variables might play an important role in nuclear organization of gene loci.

Relation between DNA methylation, activity of neighboring genes, and CpG island methylator phenotype

Figure 4A indicates that *MLH1, SFRP4*, and *SFRP5* loci in RKO and HCT116 show silencing of more neighboring genes

compared with SW480, especially in the *SFRP5* loci. An important feature of these cells is that RKO has typical features of the CpG island methylator phenotype–positive ($CIMP^+$) phenotype including having a *BRAF* mutation, whereas SW480 is $CIMP^-$ and has wild-type *BRAF*. Analysis of methylation of a set of CIMP markers shows that the majority of these markers are methylated in RKO compared with SW480 indicating the differential CIMP status of SW480 and RKO (Supplementary Fig. S8). A previous study has suggested a link between LRES and CIMP in colon cancer (38). To test if CIMP-dependent long-range silencing at methylated gene loci is a widespread phenomenon, the activity of genes residing near methylated genes was analyzed on a genome-wide scale in RKO and SW480. Agilent Whole Genome expression data were processed to determine the median expression level of probes in a window of seven genes centered around every gene, hereafter referred to as the neighborhood expression score (NES; see Supplementary Methods). In essence, a low NES score for a gene indicates that the gene is present in a genomic neighborhood with low gene expression whereas a high score means that the gene resides in a region with high gene expression. Methylation status of genes in SW480 and RKO was determined using the Infinium methylation array (24).

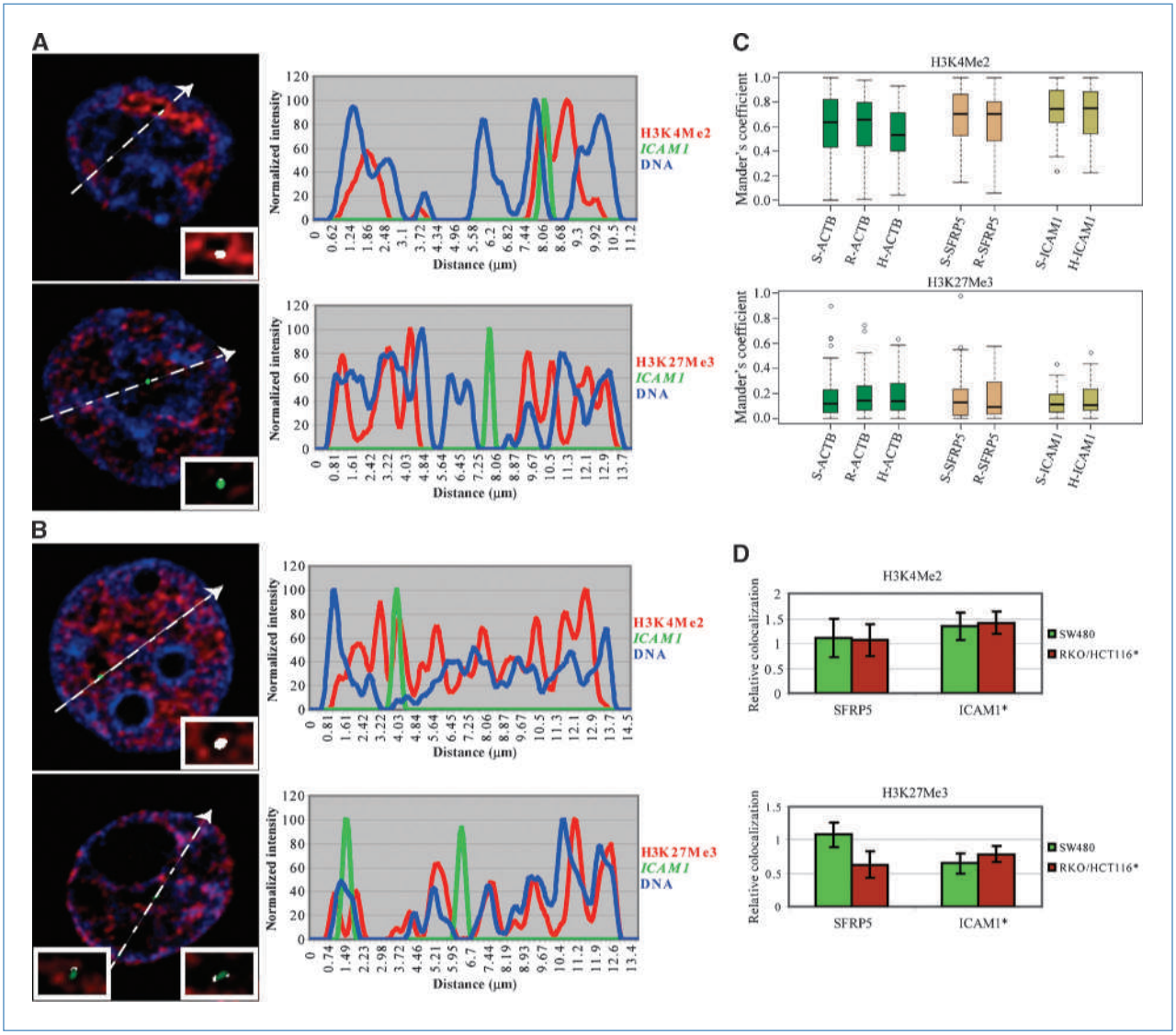

Figure 3. Association of *SFRP5* and *ICAM1* with the H3K4Me2 domains. HCT116 (A) and SW480 cells (B) immunostained for H3K4Me2 (top) or H3K27Me3 (bottom) and *ICAM1* and *ACTB* (data not shown) loci by DNA-FISH. Figure details same as Fig. 1A and B. All alleles are shown in Supplementary Fig. S6. C, quantitation of colocalization from a single experiment (n = 20 nuclei) as in Fig. 1C. SW480, RKO, and HCT116 are labeled with prefix S, R, and H, respectively. D, colocalization values of *SFRP5* and *ICAM1* normalized to *ACTB* as in Fig. 1D (n = 20, 10, and 10 nuclei) plotted as relative colocalization (Y-axis). Asterisk indicates that *ICAM1* localization was compared between SW480 and HCT116 cells.

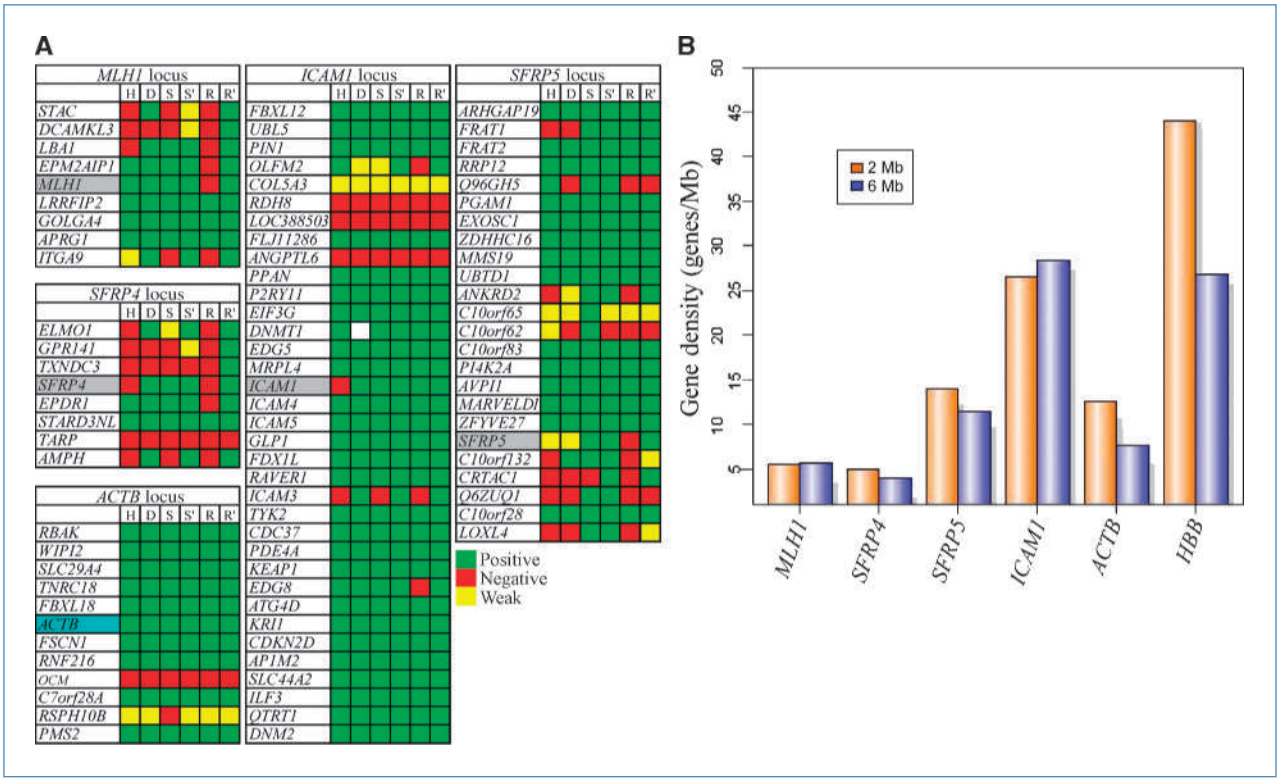

Figure 4. Relationship between nuclear position, LRES, and gene density. A, expression of genes in a 1 Mb domain around the genes studied here in HCT116 (H), DKO (D), SW480 (S), SW480 + 5-aza-CdR (S′), RKO (R), and RKO + 5-aza-CdR (R′). The CR genes are highlighted in gray and *ACTB* control is highlighted in blue; genes are listed top to bottom in the order of their 5′ to 3′ position in the genome (UCSC). Positive expression (green), no expression (red), and weak expression (yellow) are shown. White box in DKO in the *ICAM1* locus indicates the absence of DNMT1 gene due to genetic knockout. *MLH1, SFRP4*, and *SFRP5* loci tend to show variable degrees of silencing of adjacent genes in RKO, HCT116, and SW480, in that order. B, gene densities (*Y*-axis) in a 2 and 6 Mb window centered on the listed genes plotted as genes/Mb.

Previous studies have shown that the genome tends to be organized into regions of high and low gene expression (39). To test if the NES captures the activity-dependent organization of the genome, the distribution of NES values of low and high expressing genes was compared with that of all genes. Supplementary Fig. S9A and B show that low and high expressing genes tend to have low and high NES values, respectively ($P < 10^{-16}$), indicating that the NES is a good measure of the neighborhood expression status. This is further supported by the observation that randomization of the genomic positions of the probes abolishes these gene activity–dependent NES values (Supplementary Fig. S9C and D).

To test if long-range silencing at methylated gene loci is a general phenomenon in CIMP$^+$ cells, the NES score distributions of genes methylated in both SW480 and RKO (285 genes) were analyzed (Fig. 5A). In both SW480 and RKO, methylated genes reside in a continuum of low to high expressing regions. However, there are no significant differences in the distribution of NES values between the two cell types ($P > 0.2$). Similarly, genes methylated either only in SW480 (27 genes) or only in RKO (438 genes) show similar NES values in both cell types (Fig. 5B). Furthermore, based on the Infinium array data, SW480 cells have 312 methylated genes of which only two genes (*NMBR* and *RASGRF1*) have 2-fold lower NES values in SW480 compared with RKO. RKO has 723 methylated genes of which only 10 genes (*SFRP5, ALK, CYP1B1, GBX2, CLSTN2, SHOX2, ADAMTS14, FGF4, BARX2*, and *CHODL*) have 2-fold lower NES values in RKO compared with SW480. Thus, except for a few loci, the excess methylated genes in CIMP$^+$ RKO do not show a general tendency to be in loci with low neighborhood gene expression.

Discussion

Our data indicate that stable silencing by aberrant DNA methylation of the CR genes analyzed is independent of their position within the nuclear microenvironment or nuclear subcompartments, that is, the perinuclear or perinucleolar domains. These results are, perhaps, contrary to what might have been expected based on work from others, using development/differentiation systems. These previous studies have suggested a general model in which genes reposition away from the heterochromatin (perinuclear or pericentromeric) when activated and gravitate to heterochromatin when silenced (40, 41). In these models, the perinuclear and pericentric heterochromatin is purported to play a role in establishing/maintaining domains of transcriptionally inactive

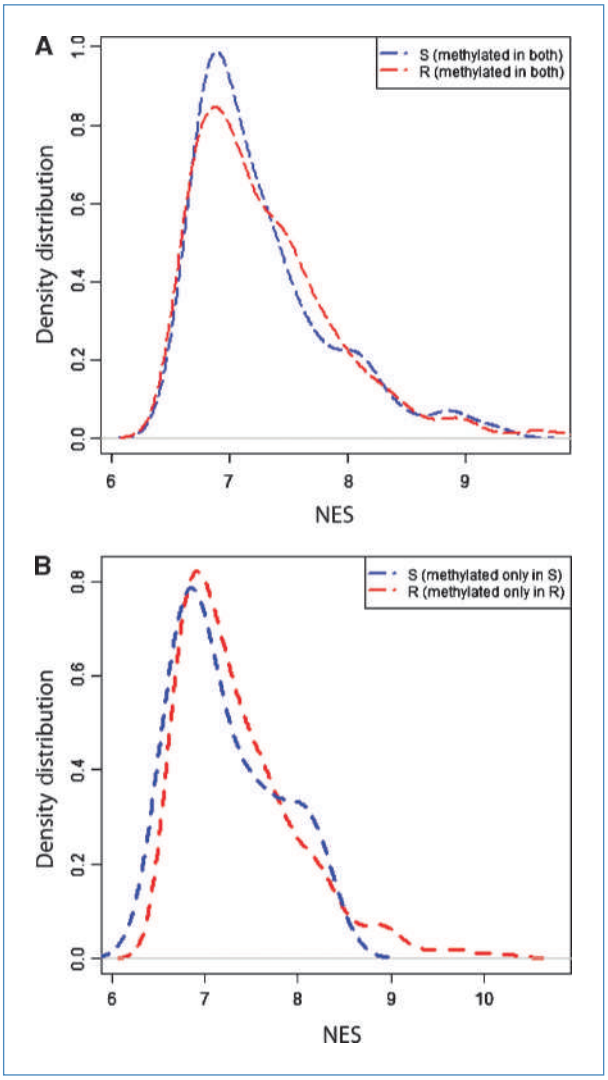

Figure 5. Relationship between LRES and CIMP. A and B, NES for every gene was calculated as the median expression values of three upstream and three downstream genes. The NES was used as a measure of LRES. A and B, distribution of the NES values for genes methylated in both SW480 and RKO (A) and genes methylated only in SW480 or only in RKO (B). S and R in legend denote SW480 and RKO.

regions wherein genes get recruited for stable silencing during differentiation (42).

Despite the above general models of development, other recent studies might help explain the lack of an obligatory requirement for hypermethylated CR genes to be positioned in heterochromatic domains to maintain the stability of their expression patterns. It has been observed that active genes dynamically shuttle in and out of transcription hubs wherein the active phase of the gene is characterized by association with the transcription hub (16). Similarly, the active genes observed to be present in heterochromatic domains in a majority of the cells in this study might be expressed by dynamic and temporally short association with neighboring euchromatic domains. Furthermore, recent reports analyzing the influence of the heterochromatic environment at the nuclear periphery on gene expression have shown that although artificial tethering of a gene to the periphery could downregulate the expression of some genes, other neighboring genes relocated to this new environment remain transcriptionally active (10–12). Thus, the response elicited from a gene by the environment is gene-specific and our results support the idea that genes could reside in euchromatin and be silenced and vice versa. This is highlighted in the observation that the active *MLH1* and *SFRP4* genes in SW480 cells are enriched for H3K4Me2 locally around the transcription start site but a majority of the alleles are in a H3K27Me3-labeled microenvironment.

In the last few years, LRES has been reported in colon, bladder, and lung cancers (6, 7, 43, 44). The mechanism underlying LRES is not known and could possibly involve long-range interactions between genomic elements and chromatin remodeling (45). If so, our data indicate that the mechanisms responsible for LRES could encompass loci that reside in either euchromatin or heterochromatic domain and exclude a major role for gene position with respect to chromatin environment in this process. Our data in this regard, again, support the notion that aberrant promoter CpG hypermethylation and its association with CR gene silencing are independent of nuclear position of the affected genes.

Tumors differ greatly in the incidence of gene methylation resulting in the $CIMP^+$ and $CIMP^-$ phenotypes. In a recent study, Karpinski and colleagues observed that LRES at the 2q14.2 loci correlated with the CIMP phenotype in a panel of colon tumor samples (38). In the current study, gene expression analysis by PCR showed that *MLH1, SFRP4*, and *SFRP5* reside in a genomic region that shows long-range silencing of neighboring genes in a $CIMP^+$ cell type. However, our global analyses of the direct relationship between gene methylation and long-range silencing as a function of CIMP show that, except for a few loci, the majority of methylated gene loci in SW480 and RKO display similar levels of neighborhood gene expression. Thus, it seems that CIMP-dependent long-range silencing of methylated genes occurs only at a few loci and that the majority of methylated genes do not show CIMP-dependent long-range silencing. A caveat in the current analysis of CIMP-dependent long-range silencing is that cancer cell lines were compared. Further understanding of the relation between CIMP and long-range silencing will require direct comparison of matched tumor and normal colonic epithelium.

To our knowledge, our data are the first to show the relationships among nuclear position of genes under epigenetic regulation individually or in clusters (LRES), chromatin domains, and nuclear compartments (periphery and perinucleolar) in the cancer cell model. It is clearly established that the organization of genes and chromosomes is very different in tumor cells compared with normal cells (17, 20). Based on these reports, it is plausible that the position of the CR genes analyzed here may differ from the normal colonic epithelia. Also, it is possible that large-scale changes in nuclear organization might be an early event in

tumorigenesis and might play a role in the initial establishment of methylation patterns. However, our data here establish that silencing of CR genes by aberrant CpG methylation does not require positioning of the genes at heterochromatic compartments. A previous study in a breast cancer model system showed that the changes in position of a panel of gene loci are independent of gene expression changes (20). It is not clear what causes the changes in nuclear organization in cancer cells and its effect on cancer progression. In future work, it will be interesting to understand the significance of the nuclear reorganization that accompanies tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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