Epigenetic Inactivation of a Cluster of Genes Flanking *MLH1* in Microsatellite-Unstable Colorectal Cancer

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Abstract

Biallelic promoter methylation and transcriptional silencing of the *MLH1* gene occurs in the majority of sporadic colorectal cancers exhibiting microsatellite instability due to defective DNA mismatch repair. Long-range epigenetic silencing of contiguous genes has been found on chromosome 2q14 in colorectal cancer. We hypothesized that epigenetic silencing of *MLH1* could occur on a regional scale affecting additional genes within 3p22, rather than as a focal event. We studied the levels of CpG island methylation and expression of multiple contiguous genes across a 4 Mb segment of 3p22 including *MLH1* in microsatellite-unstable and -stable cancers, and their paired normal colonic mucosa. We found concordant CpG island hypermethylation, H3-K9 dimethylation and transcriptional silencing of *MLH1* and multiple flanking genes spanning up to 2.4 Mb in microsatellite-unstable colorectal cancers. This region was interspersed with unmethylated genes, which were also transcriptionally repressed. Expression of both methylated and unmethylated genes was reactivated by methyltransferase and histone deacetylase inhibitors in a microsatellite-unstable colorectal carcinoma cell line. Two genes at the telomeric end of the region were also hypermethylated in microsatellite-stable cancers, adenomas, and at low levels in normal colonic mucosa from older individuals. Thus, the cluster of genes flanking *MLH1* that was specifically methylated in the microsatellite-unstable group of cancers extended across 1.1 Mb. Our results show that coordinate epigenetic silencing extends across a large chromosomal region encompassing *MLH1* in microsatellite-unstable colorectal cancers. Simultaneous epigenetic silencing of this cluster of 3p22 genes may contribute to the development or progression of this type of cancer. [Cancer Res 2007;67(19):9107–16]

Introduction

CpG islands (defined as >200 bp, CpG:GpC >0.6) span the promoters of ~60% of genes, and in normal somatic cells, are usually unmethylated, allowing active transcription from the associated genes. In contrast, cancer cells often exhibit hypermethylation of CpG islands, and commonly affect tumor suppressor genes (1). CpG methylation acts synergistically with repressive histone modifications, such as dimethylation or trimethylation of the histone 3 lysine 9 (H3-K9) residue, to consolidate transcriptional silencing (2). CpG island methylation is a common epigenetic event in colorectal neoplasia, with *MLH1* promoter methylation representing a classic example of this phenomenon. It is well established that biallelic somatic methylation of *MLH1* is seen in ~15% of sporadic colorectal cancers, and these tumors display alterations at microsatellite repeat sequences due to loss of DNA mismatch repair function (3, 4). Curiously, colorectal tumors demonstrating this microsatellite instability (MSI) phenotype occur most frequently in elderly women, and have a distinctive pathologic appearance (5, 6).

Transcriptional silencing of *MLH1* is associated with dense methylation in the proximal "C-region" of the promoter, which lies just upstream of the transcription start site and harbors the CBF transcription factor-binding motif (7). Somatic methylation of the "A-region" further upstream within the CpG island does not affect transcriptional activity of *MLH1* (8), and is likely to represent an age-related phenomenon (9). Treatment with the methyltransferase inhibitor 5-aza-2′-deoxycytidine (Aza), which reduces CpG methylation following DNA replication, restores *MLH1* expression and mismatch repair activity in cell lines with biallelic *MLH1* methylation (4, 8). *MLH1* reactivation is further enhanced by the addition of the histone deacetylase inhibitor trichostatin A (TSA) although treatment with TSA alone is insufficient to induce gene expression, despite the acquisition of H3-K9 acetylation (10, 11). Thus, although acting in concert with additional heterochromatin-related factors, CpG methylation plays a key role in *MLH1* inactivation (4, 8, 10).

In cancer, aberrant CpG island methylation has traditionally been viewed as focal, affecting individual genes (such as *MLH1*) in an isolated manner to cause localized gene inactivation (12). A CpG island methylator phenotype has been proposed in which multiple distinct loci become simultaneously methylated in sporadic colorectal cancers (13), and these tumors show a strong correlation with MSI and the activating V600E mutation of BRAF (14). However, recent studies provide evidence that epigenetic silencing could span extensive chromosomal regions resulting in long-range epigenetic suppression of multiple contiguous genes (15, 16). In colorectal cancers, epigenetic silencing affecting neighboring blocks of genes is found within a 4 Mb region of chromosome 2q14 (16). To date, the functional significance of this finding remains uncertain as the genes in this region have no known association with tumorigenesis. In contrast, the 3p22 region centromeric to *MLH1* contains a number of genes implicated in tumorigenesis (Fig. 1). In particular, *CTDSPL* (*HYA22*), encoding a phosphatase that regulates cell growth and differentiation (17), and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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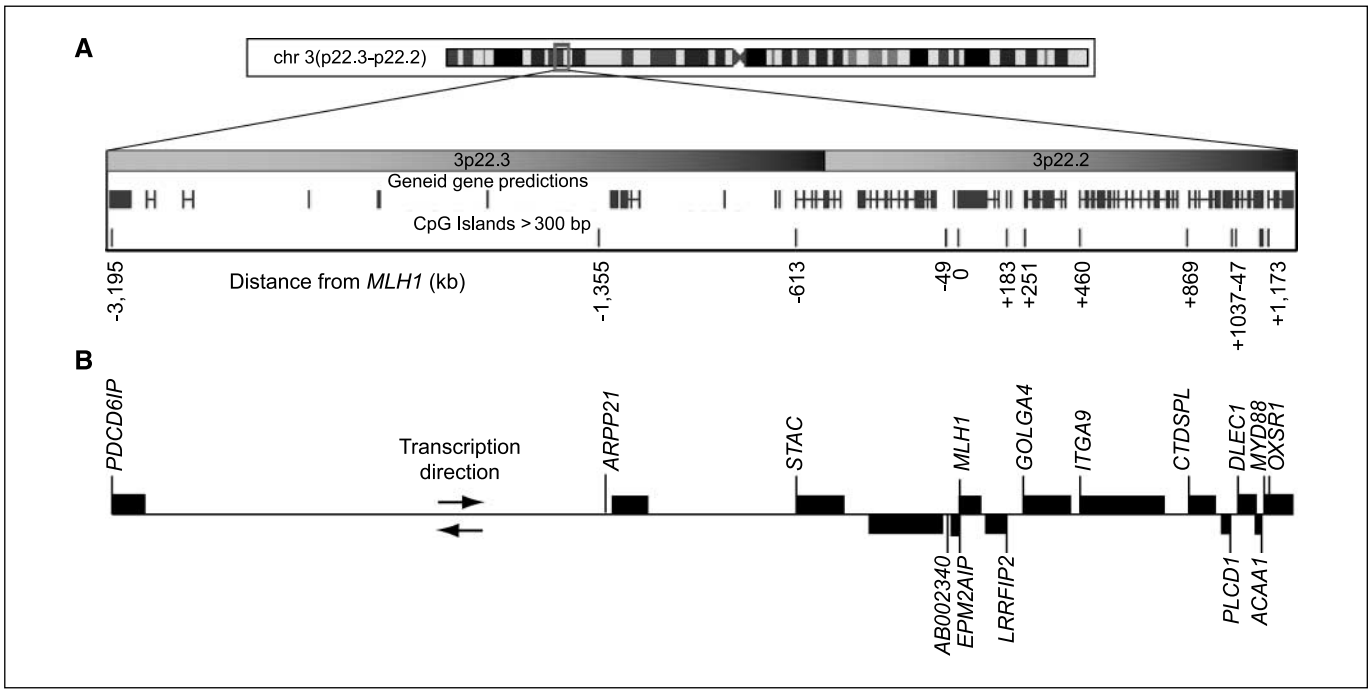


Figure 1. Map of genes and CpG islands neighboring *MLH1* within 3p22. *A,* UCSC human genome map of chromosome bands 3p22.3–3p22.2. This is expanded to show genes identified by the GeneID database (*gray boxes* or *connected vertical lines,* exons) and CpG islands spanning the promoters of genes (*vertical lines underneath*) contained within. *Bottom,* the distances in kilobases of CpG islands from *MLH1. B,* map of the genes (*black boxes*) and corresponding CpG islands (*vertical lines*) analyzed in this study. Genes transcribed from the sense strand (*above the horizontal bar*), and genes transcribed from the antisense strand (*beneath the horizontal bar*).

DLEC1 (18), are both tumor suppressor genes. Conversely, *LRRFIP2* is an activator of the canonical Wnt signaling pathway, which serves to increase the abundance of cytoplasmic β-catenin through its interaction with Dishevelled (19). The α-integrin gene *ITGA9* heterodimerizes to form a membrane receptor unit which binds vascular endothelial growth factors, thereby mediating cell adhesion and migration in angiogenesis (20). The genes centromeric to *MLH1*, including *ITGA9, CTDSPL, PLCD1*, and *DLEC1* are frequently deleted in epithelial cancers (21–24), or are hypermethylated in various types of cancers and leukemias (14, 25).

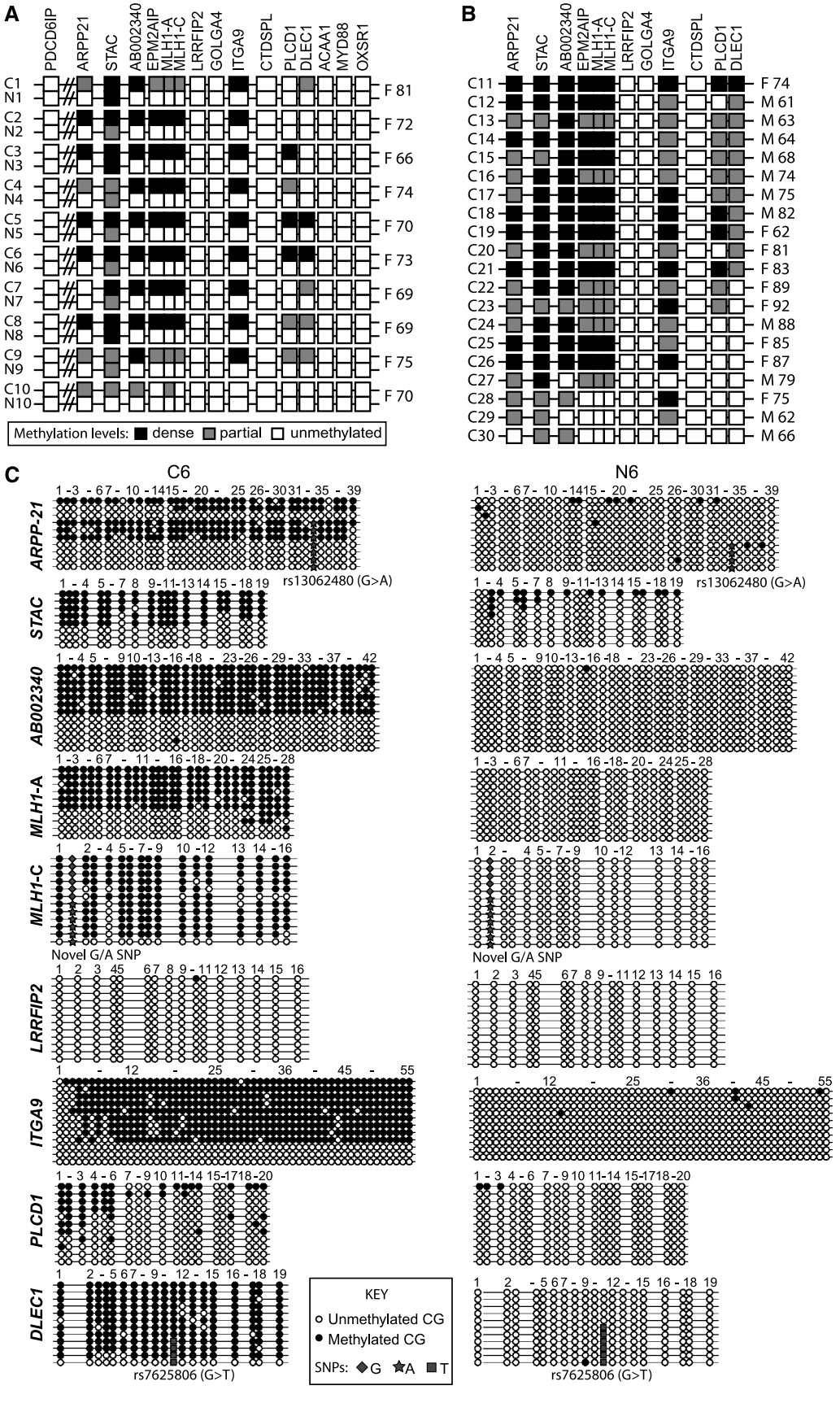
On this basis, we hypothesized that epigenetic silencing of the *MLH1* gene in microsatellite-unstable colorectal cancer is not an isolated event as previously envisaged, but rather, it occurs in the context of long-range epigenetic silencing of the chromosome 3p22 region. Concomitant epigenetic dysregulation of this block of genes may contribute to the progression of these colorectal cancers.

Materials and Methods

Clinical specimens. Patients were drawn from a prospective series of 1,040 individuals who had undergone curative resection of colorectal cancer at St. Vincent's Hospital from January 1993 to June 2006. Written informed consent was obtained from all individuals, and the study was approved by the St. Vincent's Campus Human Research Ethics Committee (approval number H07/002). The clinical, pathologic, and molecular characteristics of these patient samples have previously been extensively documented (6, 26). All tumors in this series had been examined for MSI and for the presence of the mismatch repair proteins (MLH1, PMS2, MSH6, MSH2). The MSI cancers used in this study were selected because they showed loss of expression of MLH1 and PMS2 proteins by immunohistochemistry. The microsatellite-stable (MSS) cancer group was selected from cases in the original cohort which were known to have a cancer that retained MLH1 and PMS2 expression. For each sporadic MSI case, matched MSS controls were selected if they were of the same sex, age (± 5 years), and tumor site. Tumors were analyzed for the presence of a BRAF V600E mutation by allele-specific real-time PCR as previously described (27), on the MyiQ real-time PCR detection system (Bio-Rad). For the purposes of the current study, colonic mucosa were also collected from individuals who had no history or finding of colorectal neoplasia at colonoscopy, whereas adenomas were collected from individuals without synchronous colorectal cancer.

Methylation analyses. Genomic DNA was extracted from tissues using the standard phenol-chloroform technique. Up to 2 μg of DNA were converted with sodium bisulfite as previously described (28). Combined bisulfite restriction analyses (COBRA) were done as previously described

Figure 2. Methylation of an extended region of 3p22 encompassing *MLH1* in microsatellite-unstable colorectal tumors. *A* and *B,* COBRA methylation profiles of CpG islands (*boxes*) corresponding to genes within 3p22 genes, in primary MSI colorectal carcinomas (*C1–30*) demonstrating the absence of the MLH1 protein and paired normal colonic mucosa (*N1–10*). Methylation levels were scored by visually comparing the intensity of the bands representing methylated and unmethylated templates following gel electrophoresis. The sex (*F,* female; *M,* male) and age in years of the individuals studied are listed on the right. *A,* COBRA of genes spanning a 4 Mb region in MSI tumors and paired normal colonic mucosa samples from 10 females aged >65 years (mean, 72.1 ± 4). A cluster of genes (*ARPP-21, STAC, AB002340, EPM2AIP, MLH1, ITGA9, PLCD1*, and *DLEC1*) spanning 2.4 Mb was methylated in the tumors. This was interspersed with unmethylated genes (*LRRFIP2, GOLGA4*, and *CTDSPL*). *B,* COBRA of genes within the affected 2.4 Mb region in an additional 20 MSI tumors from 11 males and 9 females, with ages ranging from 61 to 92 years (mean, 75.9 ± 10.2). *C,* allelic bisulfite sequences of COBRA fragments for a representative MSI tumor (*C6*) and paired normal colonic mucosa (*N6*). *Horizontal lines,* individual alleles with individual CpG sites (*circles*) spaced according to position within the sequenced fragment, and numbered above. Different genetic alleles were distinguishable by the presence of heterozygous SNPs (*gray symbols*) within the sequenced fragments. Dense methylation of both genetic alleles was observed at the informative loci (*ARPP-21, MLH1-C*, and *DLEC1*).


A
PDCD6IP
ARPP21
STAC
AB002340
EPM2AIP
MLH1-A
MLH1-C
LRRFIP2
GOLGA4
ITGA9
CTDSPL
PLCD1
DLEC1
ACAA1
MYD88
OXSR1
C1 N1 F 81
C2 N2 F 72
C3 N3 F 66
C4 N4 F 74
C5 N5 F 70
C6 N6 F 73
C7 N7 F 69
C8 N8 F 69
C9 N9 F 75
C10 N10 F 70
Methylation levels: dense partial unmethylated
B
ARPP21
STAC
AB002340
EPM2AIP
MLH1-A
MLH1-C
LRRFIP2
GOLGA4
ITGA9
CTDSPL
PLCD1
DLEC1
C11 F 74
C12 M 61
C13 M 63
C14 M 64
C15 M 68
C16 M 74
C17 M 75
C18 M 82
C19 F 62
C20 F 81
C21 F 83
C22 F 89
C23 F 92
C24 M 88
C25 F 85
C26 F 87
C27 M 79
C28 F 75
C29 M 62
C30 M 66
C
C6
N6
ARPP-21
rs13062480 (G>A)
STAC
AB002340
MLH1-A
MLH1-C
Novel G/A SNP
LRRFIP2
ITGA9
PLCD1
DLEC1
rs7625806 (G>T)
KEY
Unmethylated CG
Methylated CG
SNPs: G A T

using primers specific to bisulfite-converted DNA that were capable of amplifying fragments within the CpG islands from both unmethylated and methylated templates equally (29). COBRA primers and restriction enzymes for each 3p22 gene are listed in Supplementary Table S1. For allelic bisulfite sequencing, amplified products were cloned in the pGEMT easy vector (Promega) and individual clones were fluorescently sequenced using vector primers. The level of methylation within the amplified promoter fragments was scored as the percentage of methylated CpG sites over the total number sequenced from a minimum of eight sequenced alleles.

Cell culture and treatment with Aza and TSA. The RKO colorectal carcinoma cell line was obtained from the American Type Culture Collection and cultured in RPMI supplemented with 10% FCS and 2 mmol/L of L-glutamine at 37°C under 5% CO_2. Cells were treated at 50% confluency with either 5 μmol/L of Aza or 300 nmol/L of TSA for 48 h, or a sequential treatment of Aza followed by TSA for 24 h each. A mock treatment was done in which the active chemicals were omitted. The cells were then allowed to recover for 72 h prior to harvesting.

Expression analyses. Total RNA was extracted from tissue samples and cultured cells using the PureLink total RNA purification kit (Invitrogen) and treated with DNase I to degrade any contaminating genomic DNA. Complementary DNAs were synthesized from 6 μg total RNA with oligo-dT primers in 50-μL volumes using the Superscript III first-strand cDNA synthesis system (Invitrogen). Duplicate RNA samples were processed with reverse transcriptase omitted for the detection of genomic DNA. Quantitative real-time PCR was done using primers and annealing conditions for 3p22 genes as listed in Supplementary Table S2. Real-time PCR reactions were done in triplicate using the iQSYBR Green Supermix (Bio-Rad) on the MyiQ single-color real-time detection system (Bio-Rad). A melt curve was done following cycling to ensure product homogeneity. Transcript levels were quantified at the cycle threshold with reference to a standard plot generated from a dilution series of 10^8 to 10^2 plasmid copies containing the corresponding transcript insert. Levels of test transcripts were normalized for RNA input and integrity against the *HPRT* and *GAPDH* (30) housekeeping genes.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were done on 2×10^6 RKO cells using the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Upstate Biotechnology), according to the manufacturer's instructions, as previously described (31). The fixed chromatin complexes were immunoprecipitated using either an anti–dimethyl-histone H3-K9 antibody or an anti–diacetyl-histone H3-K9 antibody (Upstate Biotechnology). Controls with antibody excluded were also done, and showed negligible levels of background precipitation. For comparison, ChIP assays were done for selected 3p22 genes, as well as for genes lying outside the region of interest. *CDKN2A* (*P16*^{INK4A}), which is hypermethylated in RKO, represents a positive control for dimethylated H3-K9. The unmethylated and constitutionally active *GAPDH* gene served as a positive control for diacetylated H3-K9, respectively. Semiquantitative real-time PCR was done in triplicate on immunoprecipitated target and input DNA using the primers and annealing conditions listed in Supplementary Table S3. A melt curve was done after 40 cycles of amplification to confirm product homogeneity. For each sample, the C_T values were obtained from the immunoprecipitated target and input chromatin and the difference between the two values (ΔC_T) were calculated, as previously described (31). The relative amount of immunoprecipitated to input material was calculated as $2^{(-\Delta CT)}$, and any background [$2^{(-\Delta CT)}$ values for no-antibody control] were subtracted. The mean and SD was calculated from the three $2^{(-\Delta CT)}$ values generated for each sample.

Results

A cluster of genes within the 3p22 region shows dense biallelic methylation in MSI cancers. To determine whether long-range epigenetic silencing occurs within the region flanking the *MLH1* gene, the methylation profile at the CpG islands of multiple genes within a 4 Mb region of 3p22 (Fig. 1) was examined using COBRA in sporadic MSI colorectal cancers that showed loss of expression of MLH1 by immunohistochemistry. In MSI cancers from females older than 65 years (n = 10), CpG island methylation was found to extend across 2.4 Mb to include the *ARPP-21, STAC, AB002340, EPM2AIP/MLH1, ITGA9, PLCD1*, and *DLEC1* genes in the majority of tumors (C1–9; Fig. 2*A*). In contrast, the paired normal colonic mucosa from each individual was unmethylated in this region, except at *STAC*, indicating that the regional methylation profile was cancer related. A regional pattern of methylation was also found when 20 additional MSI colorectal cancers from males and females ages 61 to 92 years old were analyzed by COBRA (Fig. 2*B*). In total, 25 of the 30 sporadic MSI cancers showed concomitant methylation of *MLH1* and multiple neighboring genes. In 22 of these tumors (C1–9, C11–23), the region of methylation involved *ARPP-21, STAC, AB002340, EPM2AIP/MLH1, ITGA9, PLCD1*, and/or *DLEC1* (Fig. 2). In the other three tumors (C2, C25, and C26), methylation failed to reach the *PLCD1* and *DLEC1* genes (Fig. 2). In just one tumor, *MLH1* was methylated in a focal manner (C27). In four other MSI tumors (C10, C28–30), the "C" region of *MLH1* was unmethylated, suggesting that loss of MLH1 expression had arisen as a result of genetic alterations. For all of the 30 MSI tumors, three of the genes interspersed within the affected region (*LRRFIP2, GOLGA4*, and *CTDSPL*) were consistently unmethylated (Fig. 2).

Bisulfite allelic sequencing was used to confirm the COBRA findings in each of the 3p22 genes in seven of the MSI tumors that showed regional methylation (C1–C7), as well as in their matched normal colonic mucosa. Sequencing showed dense methylation of individual alleles in each tumor, with methylation occurring at the majority of CpG sites within individually cloned fragments (Fig. 2*C*). Although unmethylated alleles were detected in these tumor samples, it is likely that they were derived from contaminating normal cells. Using common single nucleotide polymorphisms (SNP) within the sequenced fragments of *ARPP-21* (rs13062480, rs6786082), *AB002340* (novel G/A SNP), *MLH1-C* (rs1800734 and a novel G/A SNP), and *DLEC1* (rs7625806), we showed in three informative tumors that methylation of the gene cluster affected both genetic alleles (as illustrated for one representative tumor in Fig. 2*C*). Of the 30 MSI tumors, 26 showing regional patterns of methylation that included *MLH1* were positive for the BRAF V600E mutation. The exceptions were four tumors that either lacked methylation of *MLH1* or of flanking genes (C27–C30).

These findings show that the vast majority of MSI tumors which arise as a consequence of biallelic somatic methylation of the *MLH1* promoter also display dense and biallelic methylation across the chromosomal subregion flanking *MLH1*.

Regional 3p22 methylation is not found in MSS cancers or other tissues. The finding of methylation at *ARPP-21* and *STAC* in the MSI cancers lacking methylation of *MLH1-C* or proximal genes (C10, C28–30) suggested that the epigenetic changes at these two loci can occur independently of those at *MLH1* and genes further centromeric. To further examine this possibility, the methylation status of genes in the 4 Mb 3p22 region was examined in MSS cancers (n = 18) and paired normal mucosa (n = 17) using COBRA (Fig. 3*A*). In this group, just one tumor (C42) was positive for the BRAF V600E mutation. Allelic bisulfite sequencing was done for selected samples, and one example is shown in Fig. 3*B*. The *ARPP-21* and *STAC* genes were frequently methylated in MSS cancers. Notably, the level of methylation at these two genes did not differ between the MSI and MSS tumor groups (Fig. 4). Furthermore, their methylation levels were consistently higher in all tumors compared with paired normal mucosa. In contrast to *ARPP-21* and *STAC*, the

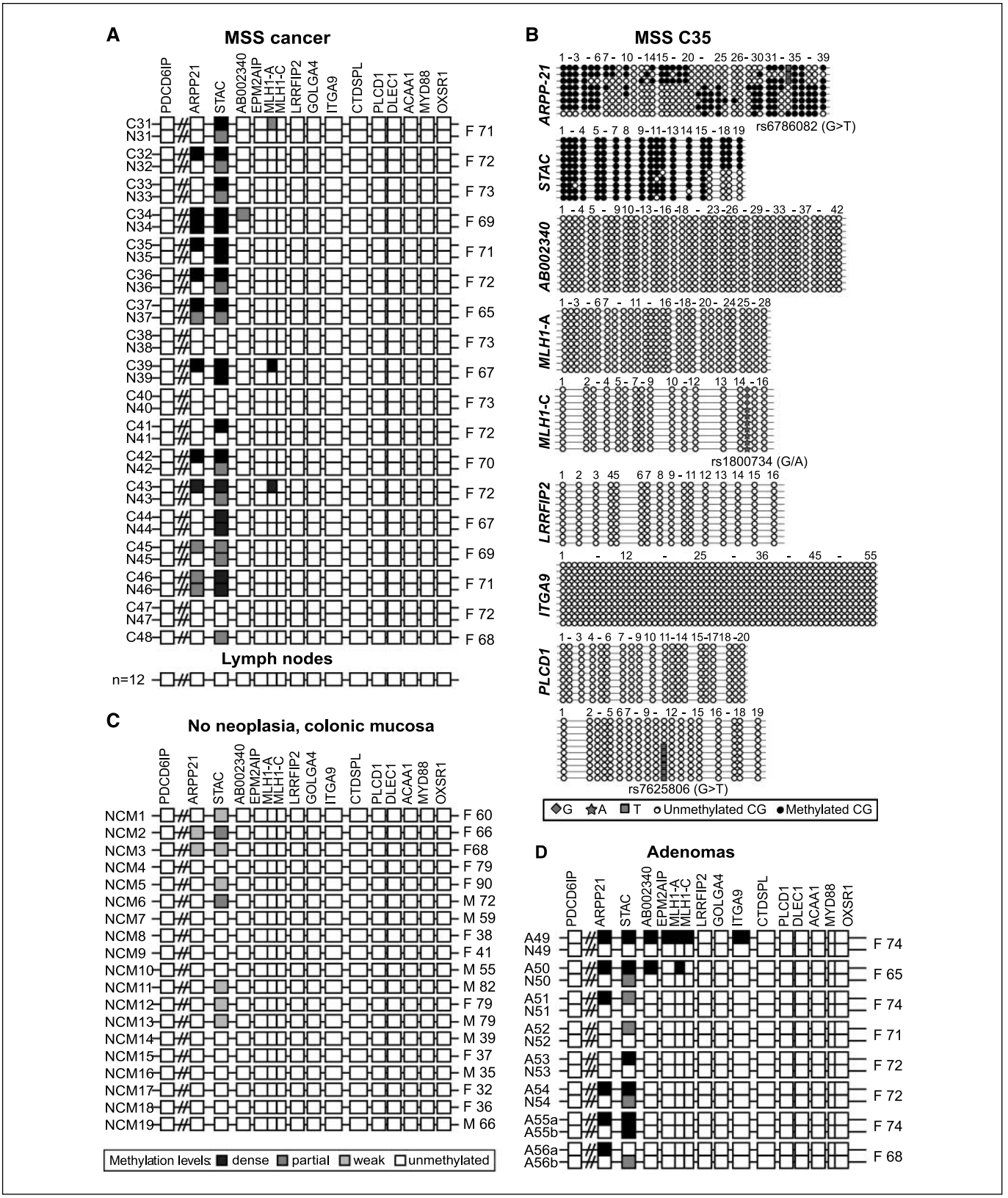


Figure 3. Methylation of distal 3p22 genes in MSS cancers, adenomas and normal tissues. Methylation patterns seen across the 4 Mb region of 3p22 by COBRA (*A, C*, and *D*) and bisulfite sequencing (*B*). *Boxes,* CpG islands. The sex (*M,* male; *F,* female) and age in years of individuals are shown alongside the methylation profiles. *A,* MSS colorectal cancers (*C31–48*), paired normal mucosa (*N31–47*) and 12 pericolic lymph nodes from females aged >65 y (mean, 70.3 ± 2.3). *B,* allelic bisulfite sequencing pattern of a representative MSS cancer (*C35*). Each line represents a single allele, with CpG sites (*circles*) positioned within the fragments. Heterozygous SNPs (gray symbols). *C,* normal colonic mucosa (*NCM1–19*) from individuals without neoplasia, including 8 males and 11 females of varying age range (mean, 58.6 ± 19.0 y). *D,* adenomas (*A49–56*) and paired normal mucosa (*N49–54*) from females (mean age, 71.5 ± 3.2 y).

methylation densities of *AB002340, EPM2AIP, ITGA9*, and *PLCD1* were significantly higher in MSI than MSS cancers, implicating these latter genes in the same epigenetic process affecting *MLH1* (Fig. 4).

To examine the 3p22 CpG island methylation profiles in normal tissues and during early tumor formation, we did COBRA across the 4 Mb region in the normal colonic mucosa from individuals without colorectal neoplasia (n = 19), as well as adenomas (n = 10) and paired normal mucosa (n = 6) from eight individuals (Fig. 3). Bisulfite sequencing was also done on selected samples (Supplemental Figs. S2 and S3). Methylation of *ARPP-21* and *STAC* was detected at low levels in the normal colonic mucosa of individuals without neoplasia aged >60 years (Figs. 3*B* and 4; Supplemental Fig. S2). Methylation of these two genes was frequently observed in adenomas (Fig. 3*C*). Moreover, one adenoma (A49), which was unstable at one of five microsatellite loci, showed a regional profile of biallelic methylation similar to MSI carcinomas, including methylation of *ARPP-21, STAC, AB002340, MLH1/EPM2AIP*, and *ITGA9*. This suggests that the spread of epigenetic change may occur early in tumorigenesis (Fig. 3*D*; Supplemental Fig. S3).

Transcriptional suppression of 3p22 genes in MSI colorectal cancers. Genes in the 3p22 region which are normally active in colonic epithelia include *PDCD6IP, AB002340, EPM2AIP, MLH1, LRRFIP2, GOLGA4, ITGA9*, and *PLCD1*. To determine whether the extensive pattern of methylation observed in MSI tumors correlated with transcriptional silencing of contiguous genes in the region, levels of gene expression were compared between individual MSI carcinomas demonstrating regional 3p22 methylation and MSS tumors, as well as their paired normal colonic mucosa, using quantitative real-time reverse transcription-PCR (Supplemental Fig. S4). Using pooled values, MSI tumors showed a significant reduction in *MLH1/EPM2AIP* expression compared with their paired normal colonic mucosa and MSS tumors, as expected (Fig. 5). Each of the 3p22 genes tested also showed significant transcriptional repression in the MSI tumors compared with the paired normal colonic mucosa, irrespective of the methylation status of the gene promoters themselves, indicating epigenetic suppression on a regional scale in the cancers (Fig. 5). Furthermore, the levels of expression of *PDCD6IP, LRRFIP2*, and *ITGA9* in MSI tumors were also significantly lower than in the MSS tumors, further supporting epigenetic silencing of these genes in MSI carcinomas. However, the levels of some genes (*AB002340, ITGA9, LRRFIP2, PLCD1*) were also reduced in MSS tumors compared with their normal colonic mucosa (albeit to a lesser extent than MSI tumors), suggesting that these genes were also down-regulated in MSS colorectal cancers either by genetic mechanisms or epigenetic alteration less apparent than CpG island methylation. These data show that long-range epigenetic suppression affects multiple genes across the 3p22 region in primary MSI cancers, including those that escape direct promoter methylation.

Transcriptional reactivation of 3p22 genes following treatment with methyltransferase and histone deacetylase inhibitors. COBRA and allelic bisulfite sequencing showed the pattern of methylation in the prototypical MSI colorectal carcinoma cell line, RKO, was identical to that found in the majority of primary MSI cancers showing *MLH1* methylation. Dense regional hypermethylation of 100% of alleles extended from *ARPP-21* to *DLEC1*, whereas the interspersed *LRRFIP2, GOLGA4*, and *CTDSPL* genes remained unmethylated (Fig. 6*A*). Analysis of gene expression revealed undetectable or negligible transcription from the genes associated with the hypermethylated promoters (*AB002340, EPM2AIP, MLH1, ITGA9, PLCD1*, and *DLEC1*; Fig. 6*B*). Reactivation of the expression

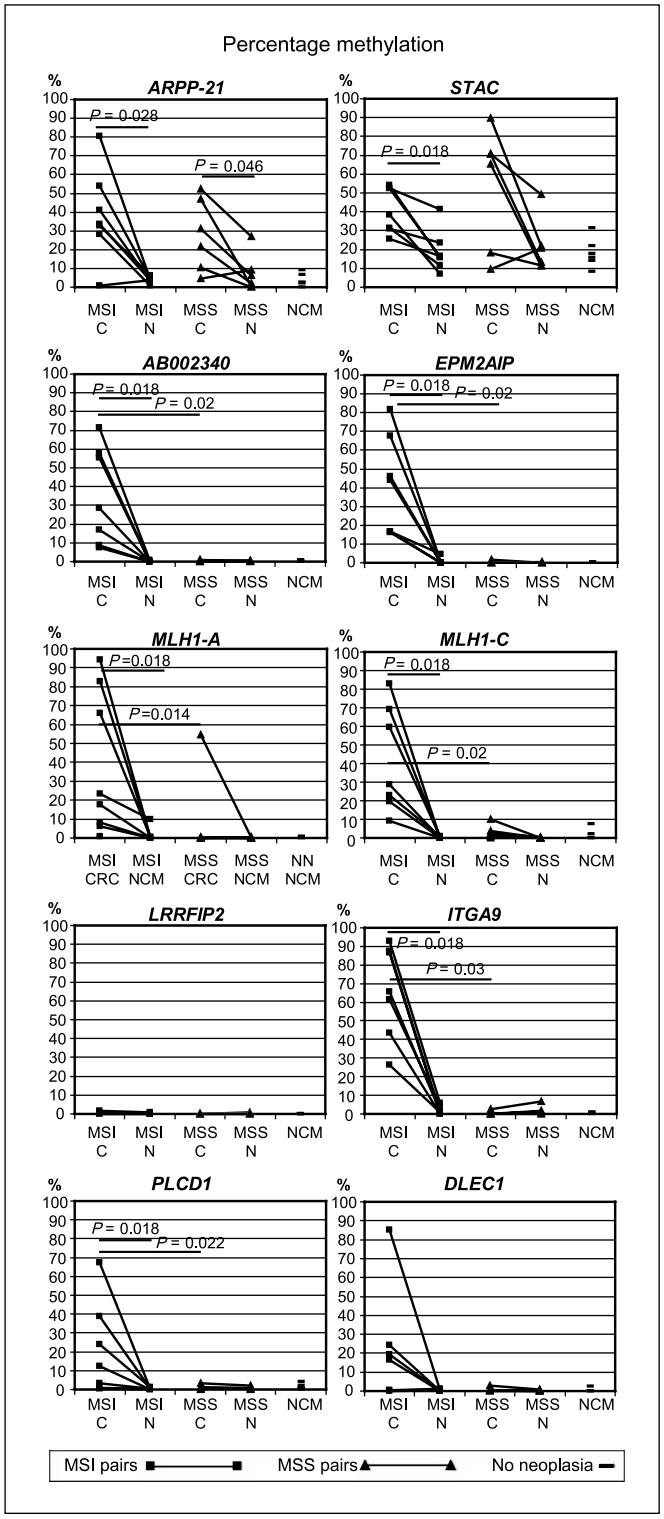


Figure 4. Methylation levels within 3p22 genes in colorectal cancers and paired normal colonic mucosa. The percentage of methylated CpG sites calculated following allelic bisulfite sequencing of 3p22 genes from selected pairs of MSI and MSS colorectal cancers (*C*), their paired normal mucosa (*N*), and the normal colonic mucosa from individuals without cancer (*NCM*) are plotted in on the *Y*-axes. Paired samples are linked by lines. The Wilcoxon rank-sum test was used to compare the independent groups of MSI and MSS cancer, and the paired test was used to compare the methylation levels between the tumors and their matched normal mucosa samples. *P* values for those comparisons that showed a significant difference between groups are superimposed on the plots.

of these genes was observed following treatment with Aza alone, or in combination with TSA. In contrast to *MLH1/EPM2A* and *DLEC1*, treatment with TSA alone also resulted in elevated expression of *AB002340, ITGA9*, and *PLCD1*, although to a lesser degree than with Aza, suggesting that acquisition of acetylation at H3-K9 might be sufficient to alleviate transcriptional repression in some of the flanking genes. The unmethylated genes located within the 3p22 cluster, *LRRFIP2, GOLGA4*, and *CTDSPL*, also showed increased levels of expression following the various treatments, with *CTDSPL* in particular demonstrating a similar pattern of up-regulation as the methylated genes within the region (Fig. 6*B*). The release of a suppressive effect on these genes may be an indirect result of reduced CpG methylation within the broader region. *PDCD6IP*, which lies just upstream of the hypermethylated region, showed only a modest increase in expression following the various treatments (Fig. 6*B*). These data provide further evidence that transcriptional repression of gene activity is associated with suppressive epigenetic alteration across the entire 3p22 region, as it is for *MLH1*. This is tightly associated with methylation, although unmethylated genes are affected to a lesser extent.

Chromatin modifications within the epigenetically repressed 3p22 cluster. To determine if epigenetic silencing across the 1.1 Mb hypermethylated region in MSI colorectal cancer was associated with chromatin state, we assessed the levels of H3-K9 dimethylation and diacetylation, which are marks of repressed and active chromatin, respectively, at selected genes in RKO cells using ChIP (Fig. 6*C*). As previously shown, the hypermethylated *MLH1* promoter was associated with high levels of H3-K9 dimethylation, and was essentially devoid of H3-K9 acetylation (11). The promoters of the other 3p22 genes that were hypermethylated, specifically in MSI cancers (including *AB002340, ITGA9*, and *PLCD1*), were associated with high levels of H3-K9 dimethylation, comparable with *MLH1* and the *CDKN2A* ($P16^{INK4A}$) gene on 9p21, which is also hypermethylated in RKO cells. Conversely, H3-K9 acetylation was undetectable at the promoters of these genes, clearly indicating that CpG island methylation correlated closely with dimethylation of the H3-K9 residue across the entire 3p22 gene cluster. Genes interspersed within the suppressed 3p22 region (*LRRFIP2, GOLGA4*, and *CTDSPL*) and the *PDCD6IP* gene upstream, the CpG islands of which are unmethylated in RKO, showed slightly elevated levels of H3-K9 dimethylation, above those of the unmethylated *MSH2* gene and the constitutively active *GAPDH* control genes, both located on other chromosomes. However, these genes also showed high levels of H3-K9 diacetylation at their promoters, comparable with *MSH2* and *GAPDH*. Thus, the unmethylated genes within 3p22 seem to have a dual occupancy of H3-K9, although diacetylation is the primary modification of this residue. These findings are consistent with the derepression noted for the unmethylated genes in RKO cells treated with TSA, in which H3-K9 acetylation is further increased. However, a more significant increase in expression of the unmethylated genes following Aza treatment may reflect a reduction in CpG methylation of neighboring hypermethylated genes as opposed to the direct histone modifications of their own promoters.

Discussion

In this study, we have shown that a cluster of genes within 3p22 is epigenetically silenced in the majority of MSI colorectal cancers which display *MLH1* promoter hypermethylation. This finding lends support to a model of tumorigenesis based on long-range epigenetic silencing (12, 16). The findings further raise the possibility that different mechanisms underlie focal and regional epigenetic silencing. In most of the MSI cancers examined, the region of hypermethylation extended over a 2.4 Mb region from *ARPP-21* to *PLCD1* and/or *DLEC1*. In a smaller proportion of tumors (15%), the regional methylation extended only as far as *ITGA9*, spanning 1.9 Mb. However, the affected region that was specific to the MSI tumor group spanned 1.1 Mb encompassing the *AB002340, EPM2AIP, MLH1, ITGA9*, and *PLCD1*, and/or *DLEC1*. The promoters of these hypermethylated genes were also associated with H3-K9 dimethylation and were devoid of H3-K9 acetylation in RKO cells, showing a close correlation between these two key repressive chromatin modifications. In both the microsatellite-unstable RKO cell line and in the primary MSI cancers, the pattern of CpG island methylation closely correlated with transcriptional inactivation. Furthermore, removal of repressive epigenetic modifications from RKO cells resulted in concordant up-regulation of the entire cluster of methylated 3p22 genes that are normally expressed in colonic mucosa (*AB002340, EPM2AIP, MLH1, ITGA9,*

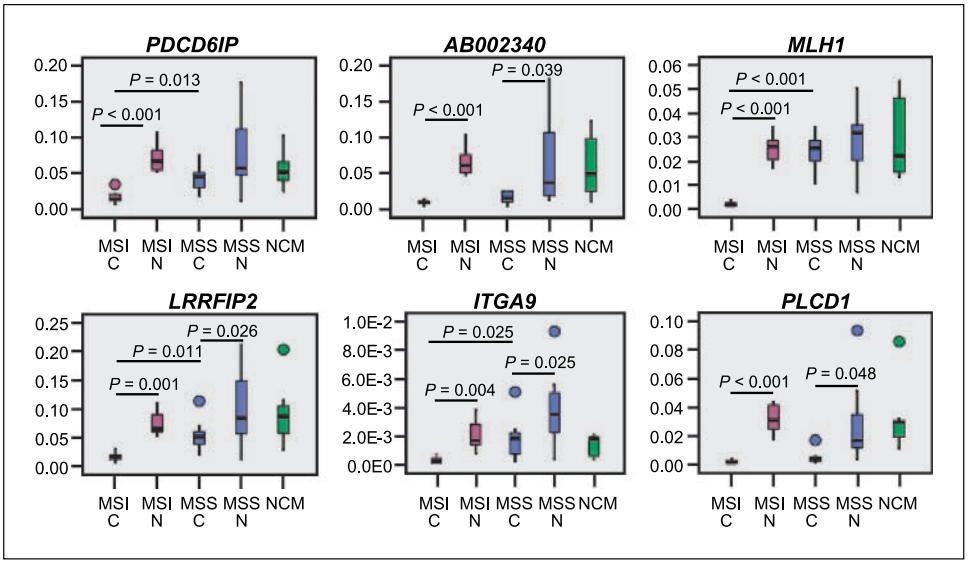


Figure 5. Suppression of gene activity within 3p22 in colorectal tumors. *Box plots,* the pooled levels of expression of 3p22 genes normalized to the means of the housekeeping genes *HPRT* and *GAPDH* on the *Y*-axes for primary MSI ($n = 7$) and MSS ($n = 9$) colorectal cancers, their paired normal mucosa, and the normal colonic mucosa of individuals without neoplasia ($n = 11$). The paired *t* test was used to compare gene expression levels between groups of paired tumors and normal mucosa, and the independent *t* test was used between the unrelated groups of cancers and normal colonic mucosae. *P* values are shown (above the horizontal bars indicating the compared groups) only where the difference in expression levels was statistically significant ($P < 0.05$).

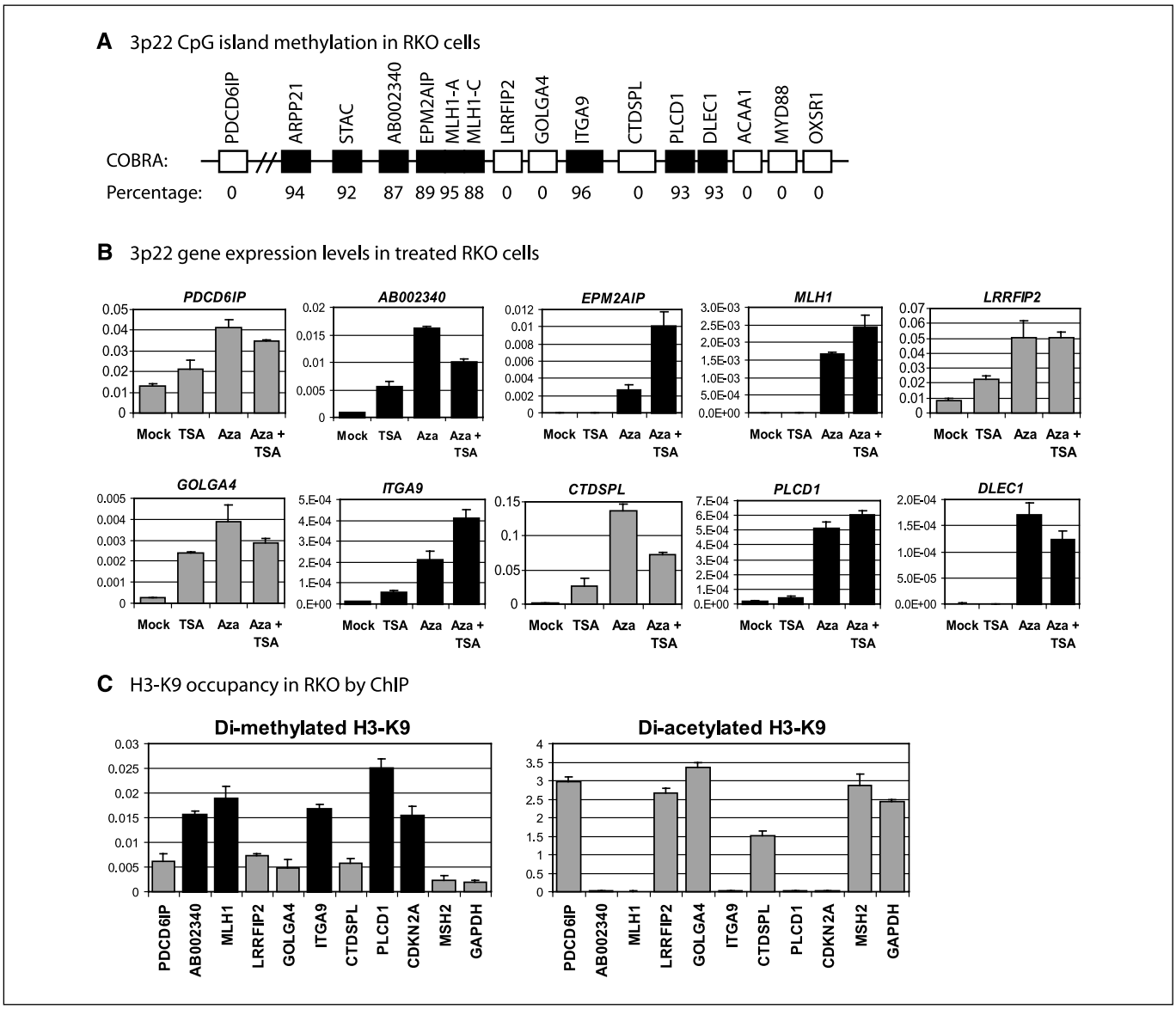


Figure 6. Epigenetic silencing of the 3p22 gene cluster in the RKO carcinoma cell line. *A,* methylation pattern at CpG islands corresponding to genes within 3p22 in RKO cells show the typical MSI pattern of regional methylation (key according to Figs. 1 and 2). The COBRA pattern (*top*), with the percentage of methylated CpG sites within the fragments calculated following allelic bisulfite sequencing (*bottom*). *B,* mean levels (+1 SD) of expression of 3p22 genes normalized to the mean values of housekeeping genes *HPRT* and *GAPDH* in RKO cells following treatments with TSA (*TSA*), Aza (*Aza*), or both (*Aza + TSA*). Treatment without active agent (*Mock*). *Black columns,* genes that were hypermethylated in RKO cells; *light gray columns,* genes with unmethylated CpG islands. *C,* ChIP assays across the 3p22 region in RKO cells using an anti-dimethylated H3-K9 antibody (*left*) and an anti-diacetylated H3-K9 antibody (*right*) showing the levels of immunoprecipitated target. The mean ratio (+1 SD) of immunoprecipitated DNA to input DNA is plotted for each CpG island. *Black columns,* genes with hypermethylated CpG islands in RKO cells; *gray columns,* unmethylated CpG islands in RKO cells. The *CDKN2A* (hypermethylated in RKO), and *MSH2* and *GAPDH* (unmethylated in RKO) represent controls located outside the 3p22 region.

PLCD1, and *DLEC1*). Thus, it seems that the epigenetic state of *MLH1* and many of its neighboring genes is regulated in synchrony. Our finding of epigenetic inactivation of a 3p22 gene cluster encompassing *MLH1* is consistent with the previous finding in RKO cells that the *MLH1* promoter is occupied by chromatin factors normally associated with facultative heterochromatin (11).

Curiously, genes within the cluster that displayed resistance to CpG island methylation (*LRRFIP2, GOLGA4*, and *CTDSPL*) were also transcriptionally suppressed in both primary MSI tumors and in the RKO cell line, although not to the same extent as the hypermethylated genes. However, although modest levels of H3-K9 dimethylation were also observed at these promoter sites, the primary modification was H3-K9 acetylation, which is usually associated with an active chromatin state. This suggests that the elevation of expression of the unmethylated genes in RKO cells following treatment with TSA and Aza may depend more on the release of CpG and H3-K9 methylation from neighboring genes, than the direct modification of their own promoters. Frigola and colleagues also reported the down-regulation of unmethylated CpG islands within the large epigenetically silenced region of 2q14.2 in colorectal cancer, and found this to be associated with H3-K9 dimethylation (16). Coordinate down-regulation of a 3p22.2 cluster (*VILL, PLCD1, DLEC1*, and *ACAA1*) has recently been identified in bladder carcinomas, where it was associated with H3-K9

trimethylation in the absence of CpG methylation (32). This illustrates the additional role of chromatin modification in the regional gene suppression evident in neoplasia. Our results indicate that CpG and H3-K9 methylation occurs in multiple genes within the affected 3p22 region, but fails to encompass all contiguous genes that are subject to long-range epigenetic silencing. Although these intervening genes largely escape direct modification with repressive epigenetic moieties, their activity is nevertheless suppressed, presumably due to the effect exerted by epigenetic modifications at neighboring loci or modifications other than CpG methylation or significant H3-K9 dimethylation.

The broader implication of our findings is that silencing of multiple contiguous genes in the 3p22 region may contribute to the development of MSI colorectal cancer. Supporting this notion is the observation that several genes proximal to *MLH1* have a putative role in tumor progression, and these genes are frequently deleted or methylated in various types of cancer, while *MLH1* is retained (21–24, 33). The *CTDSPL* and *DLEC1* tumor suppressor genes were both transcriptionally repressed in the afflicted 3p22 region in MSI cancers. Simultaneous loss of function of these genes may contribute to the oncogenic role of the microsatellite mutator phenotype in MSI cancers.

We also found dense methylation of *ARPP-21* and *STAC* in both MSI and MSS cancers, as well as in adenomas. Because their methylation status did not differ between the MSI and MSS tumors, epigenetic modification of these genes could occur independently of the proximal gene cluster from *AB002340* to *DLEC1*. Interestingly, low levels of methylation of *ARPP-21* and *STAC* were found in the normal colonic epithelia of older individuals without neoplasia, as well as those with cancer. Although it is not clear why the CpG islands of *ARPP-21* and *STAC* are susceptible to methylation, it is notable that both genes are expressed specifically in neuronal tissue and not in the adult colon (34, 35). It is therefore plausible that this pattern is related to tissue type and the lack of transcriptional activity of these genes permits the accumulation of somatic methylation in colonic epithelia. Furthermore, some CpG islands accumulate methylation with aging, and this process seems to be accelerated during cancer progression (9, 36). The *ARPP-21* and *STAC* genes may represent two further examples of this phenomenon.

Our findings clearly show that epigenetic remodeling spreads across a defined region of 3p22, perhaps through the recruitment of specific heterochromatin-related factors, resulting in concordant gene suppression (11, 37). Long-range epigenetic silencing spanning subregions of 1 to 3 Mb have now been found at 2q14.2 and 3p22, indicating that epigenetic silencing on a regional scale may be commonplace in neoplasia. This has important implications, both in terms of disease mechanisms and in the use of methyltransferase and histone deacetylase inhibitors in cancer therapy. Regional epigenetic suppression at 3p22 and other positions in the genome produce coordinated inactivation of a number of genes, which may act in concert to drive neoplastic progression. The temporal onset of methylation within a region may prove useful in designing screening tests for cancer and may influence the pathway of neoplastic progression. However, the importance of avoiding undue reliance on gene methylation profiles is highlighted by the finding of transcriptional silencing of unmethylated genes.

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